

# Assessment of oxygen profile of fungal pellets considering micromorphology

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UNIVERSITY OF ZAGREB  
FACULTY OF FOOD TECHNOLOGY AND BIOTECHNOLOGY

# GRADUATE THESIS

Zagreb, November, 2022.

Karla Miličević

**ASSESSMENT OF OXYGEN  
PROFILE OF FUNGAL PELLETS  
CONSIDERING  
MICROMORPHOLOGY**

Experimental work for this graduate thesis was done at the Technical University of Munich (TUM), School of Life Sciences, Freising, Germany. The thesis was made under the mentorship of professor Heiko Briesen and professor Božidar Šantek, and with the help of PhD student Charlotte Deffur.

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### ASSESSMENT OF OXYGEN PROFILE OF FUNGAL PELLETS CONSIDERING MICROMORPHOLOGY

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**Abstract:** With the progress of science and development of knowledge in mathematics, engineers try to find better ways of understanding the functioning of the metabolism of microorganism in order to use that knowledge to optimize processes, obtain valuable products, etc. Filamentous fungi, along with bacteria, are main producers of proteins, organic acids, antibiotics and other pharmaceutical agents. The aim of this work was to estimate parameters that affect oxygen consumption in fungal pellets. Along that route, oxygen profiles and a relationship between micromorphology and oxygen concentration profiles were shown. After that, consumption parameters were estimated in a single pellet model that can be used to predict the oxygen profile in pellets of which micromorphological information is available and to identify critical diameters.

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### PROCJENA PROFILA KISIKA U FUNGALNIM PELETIMA UZIMAJUĆI U OBZIR MIKROSTRUKTURU

*Karla Miličević, univ. bacc. ing. tech. 0058211270*

**Sažetak:** S napretkom znanosti i razvojem znanja u matematici, inženjeri pokušavaju pronaći bolje načine razumijevanja funkcioniranja metabolizma mikroorganizama kako bi to znanje iskoristili za optimizaciju procesa, dobivanje vrijednih produkata i sl. Filamentozne gljive, uz bakterije, su glavni proizvođači proteina, organskih kiselina, antibiotika i drugih farmaceutskih sredstava. Cilj ovog rada bio je procijeniti parametre koji utječu na potrošnju kisika u fungalnim peletima. Na tom putu prikazani su profili kisika i odnos između mikromorfologije i profila koncentracije kisika. Nakon toga, parametri potrošnje procijenjeni su u modelu jednog peleta koji se može koristiti za predviđanje profila kisika u peletama o kojima su dostupni mikromorfološki podaci i za određivanje kritičnih promjera.

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# 1. INTRODUCTION

The development of science as we know it today has led to numerous important discoveries. It helped to expand the knowledge about microorganisms that are all around us and in us.

In addition to the fact that our life depends on microorganisms, they can also be used as sources of various products that benefit mankind. Engineers and scientists, thanks to the development of knowledge in mathematics, find new and better ways of understanding the functioning of the metabolism of microorganisms on daily basis in order to use this knowledge for greater use of everything that can be obtained from them.

Fungi are incredibly diverse eucaryotic organisms, with commonly encountered forms including yeast, molds, truffles and mushrooms. They are recyclers, decomposers of plant debris and are used in production of a large quantity of industrially important products. Therefore, the goal is to gain as much knowledge as possible about their overall functioning and thus improve the utilization of the production process of various important products.

Filamentous fungi cultivated as biopellets are well established in biotechnology industries. Thereby, hyphal growth and fungal morphology affect product titers and required process conditions. Inside the pellet, mass transfer, substrate consumption and biomass formation are strongly dependent on local hyphae density and pellet size.

There are already many mathematical models that describe different fungal processes. Each of these models takes into an account some features and ignores others in order to simplify it. The aim of this study was to estimate parameters in oxygen consumption therm. Along that way oxygen profiles and a relationship between micromorphology and oxygen concentration profiles were shown. After that, we were able to estimate consumption parameters in a single pellet model that can be used to predict the oxygen profile in pellets of which micromorphological information is available and to identify critical diameters.

## **2. THEORETICAL PART**

### **2.1. FILAMENTOUS MICROORGANISMS**

As for filamentous microorganisms, one can talk about bacteria and fungi. They are main producers of different pharmaceutical agents, such as proteins, organic acids and antibiotics. Filamentous fungi are mainly used to produce industrial enzymes, while filamentous bacteria are mostly used in the production of secondary metabolites. These microorganisms are two different groups of microorganisms, but with many similarities between them. Firstly, as the name says, both have filamentous growth and can form pellets in submerged cultures. Secondly, the main substrates, that also influence growth and morphology of these microorganisms are glucose, sucrose and oxygen. Furthermore, their growth kinetics can often be expressed with the same mathematical models and equations (Nielsen, 1996).

This master thesis concentrates on filamentous fungi. They are eucaryotes, a large clade of microorganisms that occupy a wide range of ecological niches. On one side, they are recyclers, they form symbiosis with 93% of all flowering plant families, and, as stated earlier, they produce a large quantity of industrially important products. On the other hand fungi are also a threat to the ecosystem, human health and food security (Steinberg et al., 2017). The reason for their successful growth and survival is largely due to a chain of cells separated from each other by septa, called elongate hypha (Harris, 2001).

#### **2.1.1. Biotechnological application of filamentous fungi**

The reason for today's great interest in increasing knowledge about filamentous fungi lies in their application to obtain important biotechnological products. They produce many organic acids, enzymes, pigments, mycotoxins, polysaccharides, alkaloids and antibiotics. Different types of industrially important filamentous fungal products are shown in Table 1.

**Table 1.** Different types of industrially important filamentous fungal products (El-Enshasy, 2007)

| Product                                  | Microorganism                      |
|--|------------------------------------|
| <b>Antibiotics</b>                       |                                    |
| Penicillin G and V                       | <i>Penicillium chrysogenum</i>     |
| Cephalosporin C                          | <i>Cephalosporium acremonium</i>   |
| Griseofulvin                             | <i>Penicillium patulum</i>         |
| Penicillin N                             | <i>Emericellopsis sp.</i>          |
| Pleuromutilin                            | <i>Pleurotus mutilus</i>           |
| Cyclosporin A                            | <i>Tolpocladium inflatum</i>       |
| Cyclosporin A and B                      | <i>Cylindrocarpum lucidum</i>      |
| <b>Enzymes</b>                           |                                    |
| Glucose oxidase, pectinase and phytase   | <i>Aspergillus niger</i>           |
| Xylanase and invertase                   | <i>Aspergillus awamori</i>         |
| Amylase and glucoamylase                 | <i>Aspergillus oryzae</i>          |
| Cellulase and hemicellulase              | <i>Trichoderma reesei</i>          |
| <b>Mycotoxins</b>                        |                                    |
| Aflatoxins, citrinin and ochratoxin      | <i>Aspergillus sp. Fusarium</i>    |
| Trichothecenes and zearalanone           | <i>sp. Penicillium sp.</i>         |
| Citrinin, ochratoxin                     |                                    |
| <b>Other native fungal products</b>      |                                    |
| Riboflavin                               | <i>Ashbya gossypii Aspergillus</i> |
| Citric and gluconic acid                 | <i>niger Aspergillus oryzae</i>    |
| Kojic acid and biotin                    | <i>Aspergillus terreus</i>         |
| Itaconic acid                            | <i>Aureobasidium pullulans</i>     |
| Pullulan                                 | <i>Fusarium culmorum</i>           |
| Biotin                                   | <i>Claviceps purpurea</i>          |
| Ergot alkaloid                           | <i>Gibberella fujikuroi</i>        |
| Gibberellic acid                         | <i>Martierella isabellina</i>      |
| Linoleic acid-carotene                   | <i>Phycomyces blakesleanus</i>     |
| <b>Recombinant heterologous proteins</b> |                                    |
| Human interleukin-6                      | <i>Aspergillus niger</i>           |
| Tissue plasminogen activator (tPA)       | <i>Aspergillus niger</i>           |
| Human interleukin-6                      | <i>Aspergillus nidulans</i>        |

Additionally, fungal cells are also used for development and commercializations of new products derived from genetic engineering. Enzymes produced by fungi are mostly involved in the decomposition and recycling of complex biopolymers from animal and plant tissues. The majority of these enzymes are hydrolytic and play an important role in fungal nutrition, releasing carbon and nitrogen locked in insoluble macromolecules obtained from the metabolic

activities of other organisms (El Enshasy, 2007).

Today, species of the genus *Aspergillus* are increasingly used. Organisms such as *Aspergillus niger* and *Aspergillus oryzae* are Generally Regarded As Safe (GRAS), thus the development of an expression system in these microorganisms is desirable. Microorganisms such as *Aspergillus* and *Trichoderma* species are known for their abilities to produce and secrete very high levels of proteins, with *A. niger* being capable of producing 25–30 g/L of glucoamylase and *Trichoderma reesei* reported to be capable of producing 100 g/L of extracellular protein (Demain and Vaishnav, 2009).

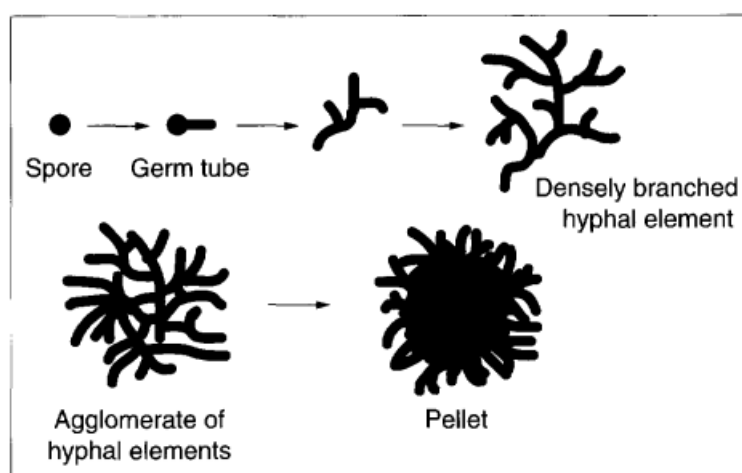
Additionally, according to Iwashita, 2002, filamentous fungi are exceptional candidate host for the production of recombinant proteins. Accordingly, there were many reviews related to the topic of production of recombinant proteins by these organisms. Different authors used different methods, not just DNA-based methods, but also RNA-based methods such as antisense RNA, hammerhead ribozymes and RNA interference approaches that were found to be very useful for silencing particular genes in filamentous fungi (Fulci and Macino, 2007; Yamada et al., 2007).

#### 2.1.2. Fungal morphology

Vegetative cells of filamentous fungi are connected in multicellular structures called hyphal elements, which make up mycelium. A hyphal element arises from the outgrowth of a single spore. Upon germination, the growth of the spore rapidly becomes polarized, resulting in the formation of a germ tube which extends its length by growth at the tip. Eventually, the germ tube develops into a hypha, and when the hypha has exceeded certain length new tips are formed along the hypha, resulting in a hyphal element consisting of a branched network of hyphae (Nielsen, 1996).

In more detail, fungi grow from spores that are produced due to different biotic and abiotic stresses or as a part of their sexual and asexual life cycles. Besides the genetic regulation of sporulation, there are many environmental conditions such as pH, light, temperature, C/N ratio, humidity, type and concentration of carbon sources, oxygen concentration and others, that can induce sporulation (El Enshasy, 2021). The robustness of spore structure is based on their high dense and compact structure with high protection by different surrounding layers of special structures. The spore internal features are characterized by the accumulation of different ingredients, like trehalose, heat stress proteins, mannitol and dehydrins, that act as preservatives during dormancy (Baltussen, 2020). On the other hand, the outer layer of most spores is made out of polysaccharides chitin,  $\beta$ - and  $\alpha$ -glucans, again surrounded by another layer that consists

of melanin and hydrophobin. Melanin, dark colored protein complex, acts like first defense against inconvenient environmental conditions, while hydrophobins form hydrophobic coating on the surface of spore and in that way manage the spore surface tension and spore-spore aggregation. Differentiation process involves different cell types leading from conidia over germ tubes to hyphae. Biomass growth is highly polarized, instead, occurring only on the hyphal tip. Tip growth and branching eventually result in different macroscopic appearances of the culture, from dispersed hyphae to pellets, as earlier stated (Grimm, 2005). Development of hyphal element from spore is shown in Figure 1.



**Figure 1.** Development of a hyphal element from a spore (Nielsen, 1996)

Hyphal growth takes place only at the tip and several cells behind it supply necessary cellular material and therefore are involved in the tip extension process. The hyphae of most fungi are divided into cells by internal walls called septa (singular would be septum). The part of the hyphal element between the apex and the first septum is referred to as the apical compartment (Pazouki and Panda, 2000). Septa generally have pores that are large enough for ribosomes, mitochondria, and even nuclei to flow through. On the other hand, it is important to mention that there are some fungi that lack septa (Dang et al., 2011).

#### 2.1.2.1. Microscopic morphology

In general, when it comes to morphology, one can talk about microscopic and macroscopic morphology. Parameters that are used to describe microscopic morphology are hyphal length and number of tips. In addition, micromorphological descriptors for dispersed hyphae exist. However, due to a lack of suitable methods of analysis, these descriptors have not yet been studied for pellets (Schmideder et al., 2020). An overview of morphological properties and their corresponding measurement techniques is given in Table 2.

**Table 2:** Morphological properties determined for pellets and dispersed hyphae (Schmideder, 2022)

|                  | Class  | Property  | Technique   | References   |
|------------------|--|---|---|--|
| Pellet           | Size   | Projected area<br><br>Diameter Perimeter<br>Chord length Signal<br>length Size distribu-<br>tion  | Microscopy<br>Microscopy<br>Microscopy<br>FBRM<br>Flow cytometry<br>Laser diffraction | Barry et al. (2015); Cairns et al. (2019a); Schrinner et al. (2020); Walisko et al. (2017); Wucherpennig et al. (2011)<br>Cairns et al. (2019a); Schrinner et al. (2020); Walisko et al. (2017); Willemseet al. (2018); Wucherpennig et al. (2011)<br>Cairns et al. (2019a); Schrinner et al. (2020); Walisko et al. (2017); Wucherpennig et al. (2011)<br>Grimm et al. (2004); Kelly et al. (2006); Pearson et al. (2003, 2004) Ehgartner et al. (2017); Schrinner et al. (2020); Veiter and Herwig (2019)<br>Lin et al. (2010); Petersen et al. (2008); Quintanilla et al. (2018); Rønnest et al. (2012); Wucherpennig et al. (2011)   |
|                  | Shape  | Circularity<br><br>Aspect ratio   | Microscopy<br><br>Microscopy  | Barry et al. (2015); Schrinner et al. (2020); Walisko et al. (2017); Wucherpennig et al. (2011)<br>Cairns et al. (2019a); Wucherpennig et al. (2011)   |
|                  | Compactness                                  | Solidity of periphery<br>Relative annular diameter<br>Compactness of core   | Microscopy<br>Flow cytometry<br>Flow cytometry  | Cairns et al. (2019a); Wucherpennig et al. (2011)<br>Ehgartner et al. (2017)<br>Ehgartner et al. (2017); Schrinner et al. (2020); Veiter and Herwig (2019)   |
|                  | Combination of size, shape, and compact-ness | Morphology number   | Microscopy  | Cairns et al. (2019a); Wucherpennig et al. (2011)  |
|                  | Spatial distribution of hyphal material      | Hyphal fraction   | Microscopy of slices  | Hille et al. (2005, 2009)  |
| Dispersed hyphae | Micromorphology of dispersed hyphae          | Total hyphal length<br><br>Number of tips Number of branches<br>Hyphal growth unit<br>Branch angle Internodal length<br>Hyphal diameter | Microscopy<br><br>Microscopy<br>Microscopy<br>Microscopy<br>Microscopy<br>Microscopy  | Barry et al. (2015); Bocking et al. (1999); Cardini et al. (2020); Kwon et al. (2013); Lecault et al. (2007); Sachs et al. (2019); Schmideder et al. (2018); Vidal-Diez de Ulzurrun et al. (2019)<br>Barry et al. (2015); Lecault et al. (2007); Sachs et al. (2019); Schmideder et al. (2018); Vidal-Diez de Ulzurrun et al. (2019)<br>Barry et al. (2015); Lecault et al. (2007); Sachs et al. (2019); Schmideder et al. (2018)<br>Barry et al. (2015); Bocking et al. (1999); Choy et al. (2011); Colin et al. (2013); Kwon et al. (2013); Sachs et al. (2019)<br>Du et al. (2016); Lehmann et al. (2019); Yang et al. (1992b) Du et al. (2016); Lehmann et al. (2019); Sachs et al. (2019)<br>Choy et al. (2011); Colin et al. (2013); Lehmann et al. (2019) |

#### 2.1.2.2. Macroscopic morphology

Macroscopic morphology refers mainly to the size of the pellet itself. To describe pellet size, one often uses equivalent diameter, pellet perimeter and core circularity (Nielsen, 1996).

The most important processes that influence the macroscopic morphology are pellet formation, pellet growth, pellet break-up and pellet erosion. Pellet formation is different with different fungal strains. It is often divided into three different types (Nielsen, 1996). First is the spore coagulative type, where the spores coagulate and upon germination of the agglomerated spores' pellets are formed. The second is the non-coagulative type where pellet is formed from a single spore. Finally, the third type is the hyphal element agglomerating type where hyphal elements agglomerate and form a clump that later develops into a pellet.

Second process that influences the macroscopic morphology is pellet growth. According to Emerson (1950), and more other early years research, pellet growth is not exponential, but may be described by cube root law in Equation 1:

$$m^{1/3} = k_p t + m_0^{1/3} \quad [1]$$

where  $m$  is the pellet mass,  $m_0$  pellet mass at time  $t=0$ ,  $t$  time and  $k_p$  kinetic parameter that presents a function of the thickness of this shell, the density of the pellet and the specific growth rate of the cells within the pellet. This kind of growth is a result of mass transfer limitations that result in growth only in an outer shell of the pellet.

At all times there is hydrodynamic force that affects size of the pellet by fragmentation of the hyphae at the surface of pellet. This mechanism is called erosion and influences pellet size and shape. Other than pellet erosion, there is also pellet break-up. It is mostly a result of mass transfer limitation, that causes a non-growing zone at the center of the pellet which, eventually, leads to structural destabilization and break-up of the pellet (Nielsen, 1996).

#### 2.1.3. Cultivation of filamentous fungi

Fungi are heterotrophic microorganisms and therefore require organic matter for growth and energy. They are usually isolated and grown on a Petri dish with a rich medium such is potato dextrose agar. Except for nutrients in medium, other factors that affect the fungal growth are C/N resource and ratio, pH, temperature, humidity and light (Nevalainen et al., 2014). Fungi can be grown in liquid cultures for various purposes, for instance production of fungal biomass, production of enzymes and other. For industrial purposes, they can be grown either in liquid cultures or in bioreactors as so-called solid-state fermentation where the process can be automated, and parameters controlled. One of the applications of solid culture is production of

fungus mycelia and spores for biological control (Brand, 2010).

There are different media, cultivation methods and bioreactor designs that can be used for isolation and cultivation of filamentous fungi.

Therefore, three models of cultivations can be considered (Mussoni et al., 2015):

- submerged fermentation that consists of two morphological types: pellets and free mycelium,
- semi-solid fermentation where the mycelium develops in the interior and on the surface of a solid substrate in the absence of free water and
- biofilm formation where mycelium develops on an inert support integrated in an adapted reactor.

There are different factors that affect which bioreactor will be used. For instance, the solid media, compared to submerged fermentation, contains less water, but has an important gas phase between the particles, which is very important to know because of the poor thermal conductivity of air compared to water. On the other hand, difficulties with submerged fermentation are related to oxygen transfer limitations that depend upon the shape, size and the aeration system of the reactor. Finally, biofilm cultures should represent a good objective due to the fact that aerial growth with a flowing medium, which seems to be similar to their natural development, can result in continuous high-density production of secondary metabolites and conidia (Mussoni et al., 2015).

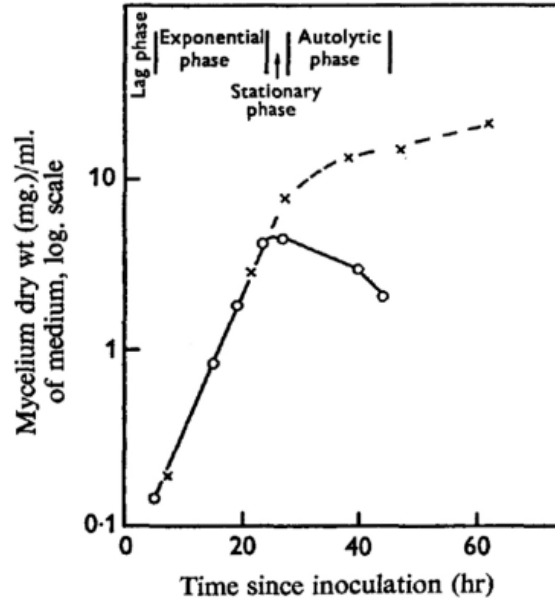
#### 2.1.4. Growth kinetics

Nowadays, different authors have explained the growth kinetics and those studies often require analysis of branching patterns; and that is now facilitated thanks to development of computerized image analysis technique (Prosser and Tough, 1991).

Hyphae can extend either by tip growth or by intercalary growth, that only occurs in aerial structures, for example conidiophores and sporangiophores. Conidiophores are septate hyphae that bear conidia, non-motile spores, at its top. Sporangioophores are specialized aerial hyphae that bear sporangia, specialized sacs, which contain non-motile sporangiospores. Conidia are not enclosed in an enclosure, while sporangiospores are enclosed in sporangia. During normal, unrestricted hyphal growth, the extension zone has constant diameter, length and shape, and differs from subapical regions in having a thinner wall and greater susceptibility to lysis by osmotic shock or changes in enzyme activity. In growing hyphae, vesicles are concentrated in a Spitzenkörper which disappears when extension stops (Gooday, 1983).

There are four growth phases: the lag phase, the exponential phase, the stationary phase and the autolytic (death) phase. These phases are shown in Figure 2.





**Figure 2.** Growth of *Aspergillus nidulans* in submerged culture with 10 g l<sup>-1</sup> glucose (° — °) and 50 g l<sup>-1</sup> glucose (× - - ×) (Trinci, 1969)

The lag phase represents the time of adaptation of microorganism to the newly created conditions in which it produces required enzymes. Then comes the log (exponential) phase. This phase, provided that all substrates and no inhibitors are present, for dispersed growth of filamentous fungi can be described by Equation 2. Integrated form is shown in Equation 3.

$$\frac{dX}{dt} = \mu_{\max} \cdot X \quad [2]$$

$$X_t = X_0 \cdot e^{\mu_{\max} \cdot t} \quad [3]$$

$X$  [g L<sup>-1</sup>] is the fungal biomass,  $\mu_{\max}$  [h<sup>-1</sup>] the maximum growth rate and  $t$  [h] is the time.

Growth kinetics of filamentous microorganisms in pellet form was earlier showed in Equation 1 as the cube root law due to the mass transfer limitations.

As the exponential phase is coming to an end, the stationary phase follows. This is the phase when cells remain metabolically active, but ceases due to glucose depletion, limitation of other required nutrient, accumulation of the inhibitory products and adverse pH value.

Monod Equation (Equation 4) is often used to describe dependence of the growth rate on the substrate concentration:

$$\frac{dX}{dt} = \mu_{\max} \cdot \left( \frac{cs}{K_s + cs} \right) \cdot X \quad [4]$$

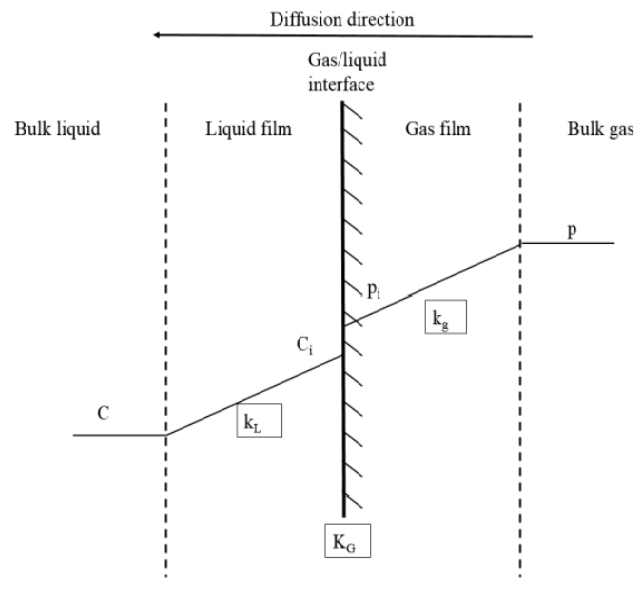
where  $K_s$  [g L<sup>-1</sup>] is the half saturation constant for uptake of the growth limiting substrate (Vesýck and Van Impe, 1997).

### 2.1.5. Oxygen transfer

In addition to carbon and nitrogen sources, oxygen also plays an important role and has been identified as the prime limiting substrate (Veiter et al., 2018).

Filamentous fungi are usually cultivated under submerged condition. Therefore, for oxygen to reach the pellet itself, there must be a source of oxygen that is mostly gaseous, so several processes must be considered. First, the transfer of oxygen from gas bubbles to the liquid phase in which the fungal culture is grown, then the transfer of oxygen through the liquid phase to the surface of the pellet and finally the transfer of oxygen from the surface to the center of the pellet. The simplest theory on gas–liquid mass transfer is the two-film model (Whitman, 1923) shown in Figure 3. This model assumes that the conditions are stationary, flow in the gas and water films is laminar and that the current balance is established in the interface.

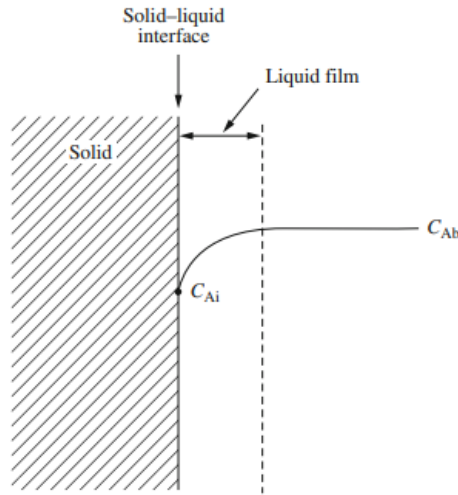
Mass transfer of solute from one phase to another involves transport from the bulk of one phase to the phase boundary or interface, then movement from the interface into the bulk of the second phase. The film theory is based on the idea that a fluid film or mass transfer boundary layer forms wherever there is contact between two phases. Turbulence in each fluid dies out at the phase boundary. A thin film of relatively stagnant fluid exists on either side of the interface; mass transfer through this film is affected solely by molecular diffusion (Doran, 2013). Using this theory transfer of oxygen from gas bubbles to liquid phase can be explained.



**Figure 3.** Mass transfer through the film separating the gas and liquid phases (Wang et al., 2018)

Considering the fact that this is a multiple phase system, after explaining how the oxygen is transferred from gas to liquid phase, it should also be explained how oxygen is transferred from

the liquid to the solid phase. So, if the bulk fluid is moving, the overall rate of mass transfer will be higher than molecular diffusion due to the contribution of convective currents. Therefore, the mass transfer between a moving liquid and a solid happens because of concentration gradient between those two phases that is shown in Figure 4.



**Figure 4.** Concentration gradient for liquid-solid mass transfer (Doran, 2013)

$C_{Ab}$  is oxygen concentration in the bulk liquid outside the film and  $C_{Ai}$  is oxygen concentration at the phase boundary. That boundary layer develops near the interface due to the reduced fluid velocity. As oxygen is consumed by reaction at the surface, the local concentration of it decreases and a concentration gradient is established through the film. The concentration difference between the bulk liquid and the phase interface drives mass transfer of oxygen from the liquid to the solid, allowing the reaction to continue. In Equation 5 the volumetric rate of mass transfer is shown.  $K_L$  is the liquid-phase mass transfer coefficient and  $a$  is the liquid-solid interfacial area per unit volume.

$$N_A = k_L a \cdot (C_{Ab} - C_{Ai}) \quad [5]$$

The overall resistance to oxygen transfer in a submerged culture system can be examined in terms of a series of resistances involving a gas film and a liquid film for the gas bubbles, a liquid film around the microorganisms aggregated in the form of a pellet, and intraparticle diffusion, but it was shown that the gas-film resistance is negligibly small compared to the liquid-film resistance for air bubbles (Kobayashi et al., 1973).

As mentioned, the oxygen transfer is measured by  $k_L a$  [ $\text{cm h}^{-1}$ ], volumetric mass transfer coefficient.  $k_L$  [ $\text{cm h}^{-1}$ ] is the mass transfer coefficient in the liquid film and  $a$  [ $\text{cm}^2 \text{cm}^{-3}$ ] the

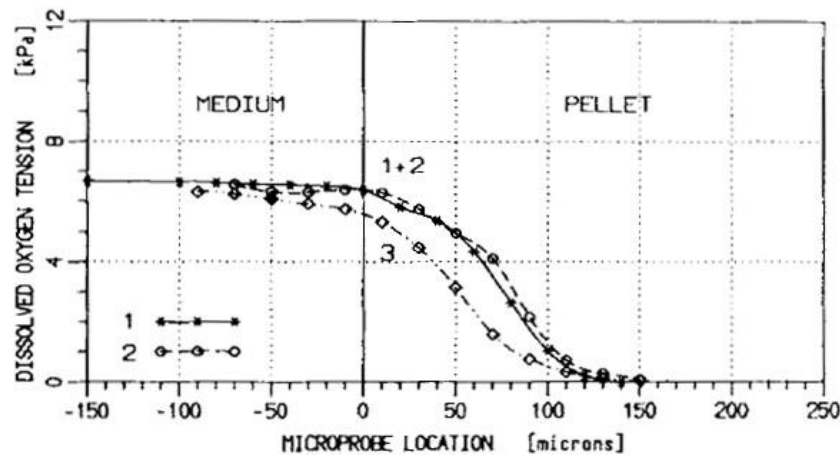
specific interfacial area for air-liquid mass transfer. This coefficient depends on many factors such as the diffusivity of the solute and the hydrodynamics of the phase. Dissolved oxygen concentrations are changed by varying the agitation speed, the volumetric flow rate of air, the broth temperature, the operating back pressure, and the oxygen enrichment of the inlet-air stream (Finkelstein and Ball, 1992). One way to calculate  $k_La$  is with the Equation 6.

$$k_La = K \cdot \left(\frac{P_g}{V_r}\right)^\alpha \cdot (V_s)^\beta \cdot (\mu_a)^{-1.86} \quad [6]$$

where  $P_g$  [hp] the gasses powered input,  $V_r$  [L] is the volume of the reactor,  $V_s$  [ $\text{cm min}^{-1}$ ] is the superficial gas velocity,  $\alpha$  and  $\beta$  are exponents,  $K$  [-] is a proportionality constant and  $\mu_a$  is an apparent viscosity term. Furthermore, to describe the oxygen concentration in reactors with filamentous fungi, OTR (Oxygen Transfer Rate) is used, as it is shown in Equation 7.

$$\text{OTR} = k_La \cdot (c_{o,s} - c_{s,r}) \quad [7]$$

Every change in fungal morphology during growth, affects oxygen uptake rate in submerged fungal culture. As long as there is enough oxygen to all cells within the pellet, it grows in size and density. As time passes, the pellet grows, nutrients are consumed and the oxygen concentration in the center of the pellet itself drops to zero. That happens because dense hyphae structure prevents further oxygen from entering and therefore is the diffusion of the oxygen slower than the consumption rate. This results in growth only on the pellet surface, while autolysis occurs in the center which leads to changes in metabolism and fungal production of enzymes. In short, oxygen concentration decreases within the pellet. At the surface, concentration is almost the same as the concentration in medium. Changes in the oxygen concentration within the pellet are shown in Figure 5. according to Wittier at al.,1986.

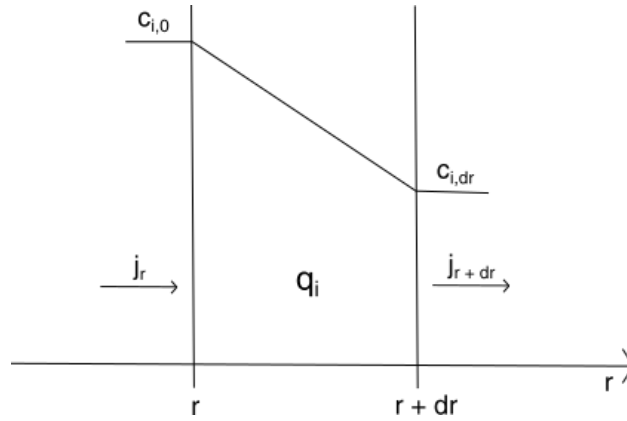


**Figure 5.** Dissolved oxygen tension over the location in the medium and pellet, as determined with a microprobe. Curve 1: oxygen tension measured by the microprobe on the way into the

pellet (with aerator B), Curve 2 on the way out of the pellet (with aerator B) and Curve 3 into the pellet (with aerator A) (Wittier et al., 1986)

Diffusion is described by Fick's laws of diffusion. Fick's first law (Equation 8) describes the diffusion without convection where  $D$  [ $\text{m}^2 \text{h}^{-1}$ ] is the location dependent diffusion coefficient,  $j$  [ $\text{kg s}^{-1} \text{m}^{-2}$ ] diffusion flux,  $c_i$  [ $\text{g L}^{-1}$ ] the concentration of diffusive substance  $i$  and  $r$  [ $\text{m}$ ] the radius.

$$j_r = -D \cdot \frac{dc_i}{dr} \quad [8]$$



**Figure 6.** Diffusion across a thin film (Cussler, 2009)

Figure 6 shows steady diffusion through a thin film for spherical coordinates. On each side of the film with thickness  $dr$  there is a well mixed solution with concentrations  $c_{i,0}$  and  $c_{i,dr}$  where the solute diffuses from the higher ( $c_{i,0}$ ) to the lower concentration ( $c_{i,dr}$ ). This is achieved by a mass balance of the sphere as shown in Equation 9 where  $V$  [ $\text{L}$ ] is the volume of the thin film.

$$\frac{\partial(V \cdot c_i)}{\partial t} = 4 \cdot \pi \cdot r^2 \cdot j_{i,r} - 4 \cdot \pi \cdot (r + dr)^2 \cdot j_{i,r+dr} + V \quad [9]$$

Taylor expansion of Fick's law (used to describe  $j_{i,r+dr}$ ) and the differential volume of one sphere layer are included in this equation. For the condition  $dr \rightarrow 0$ , a very thin film, Equation 9 can be approximated with Equation 10 (Cussler, 2009).

$$\frac{\partial c_i}{\partial t} = D_i \cdot \left( \frac{\partial^2 c_i}{\partial r^2} + \frac{2}{r} \cdot \frac{\partial c_i}{\partial r} \right) + \frac{\partial D_i}{\partial r} \cdot \frac{\partial c_i}{\partial r} + q_i \quad [10]$$

The changes in concentration within the pellet due to diffusion are described by Fick's second law (Equation 11).

$$\frac{\partial c}{\partial t} = D \cdot \frac{\partial^2 c}{\partial x^2} \quad [11]$$

This law is described by a partial differential equation and predicts how diffusion causes the concentration to change with time.  $C$  [ $\text{mol m}^{-3}$ ] is the concentration,  $t$  [ $\text{h}$ ] is time,  $D$  [ $\text{m}^2 \text{h}^{-1}$ ] is

the diffusion coefficient and  $x$  [m] is the position.

The limitation of either oxygen or nutrient transport can cause a reduction in growth and ultimate autolysis of the pellet core. In less dense pellets, however, molecular diffusion is free to operate throughout the pellet with additional transfer occurring via turbulent diffusion and convective flow (Whittler et al., 1986; Huang and Bungay, 1973). Dense pellets with large diameter often face oxygen and nutrient limitations in pellet core where the oxygen diffusion is hindered when a critical diameter is exceeded (Hille et al., 2005); while in very porous pellets with small diameters the uptake of the growth-limiting oxygen substrate is not limited in transport. In a highly viscous broth, the mass transfer and mixing performance are impeded. To ensure a sufficient supply of nutrient and oxygen, it is necessary to increase power input, but carefully, because it leads to morphological changes due to high mechanical stress.

## **2.2. MODELS FOR FILAMENTOUS FUNGI**

A model is an informative representation of an object, person or system that shows analogous behavior in the important properties, and that allows within a limited region a prediction of the behavior of the original system. A model replaces experimental studies and has economical and cost related advantages, because its system is simpler, smaller, cheaper, or faster than the original system. For research and development, a model has the advantage of less complexity than reality. Additionally, in a model certain effect can be emphasized or suppressed (Schügerl and Bellgardt, 2000).

To date, many authors have developed mathematical models for filamentous fungi. In the beginning, these were simpler models with many approximations, while today scientists are trying to develop more accurate models that would consider many other features.

As for the modeling process, there are different modeling approaches at different levels. For instance, Yang et al. (1992) introduced stochastic modeling approaches that allow monitoring micromorphological evolution. Some similar approaches were also recently applied to generate random hyphal structures by Schmideder et al. (2020). Furthermore, for prediction of bead concentration profiles, continuum transport models like those proposed by Buschulte (1992) can also be used.

Heterogeneity in morphology of mycelial populations is affected by growth, aggregation, breakage, formation of dispersed fungal entities over time. Population Balance Modeling (PBM) describes how heterogeneity of hyphal entities changes within cultivation (Schmideder 2020). Nielsen and Krabben (1995) formulated a bivariate PBM, where characterizing variables are the total length of hyphal elements and the number of growth tips and since then, several

different models have been proposed.

Nowadays there are models that describe morphology using just few variables, such as number of tips and a total hyphal length. Those models can describe an influence of different cellular mechanisms on the morphology and therefore it is possible to set up a population balance equation which describes the distribution of morphologies. But, on the other hand, those models are still quite hard to solve. Thus, in literature, there are simpler models, that are easier to solve. They give a good description of the average properties of the microscopic morphology. But it must be considered that events at the average level are not identical to those at the individual level. That is why these simplified models will not often give a credible representation of growth kinetics. To conclude, while using a model to understand growth kinetics, it is important to ensure that all processes influencing the overall microscopic morphology are considered, because, for instance, pellet formation by agglomeration of hyphal elements results in a significant change in the microscopic morphology; and if that is not included in the model, a large deviation from the actual situation can be obtained (Nielsen, 1996).

In order to set up a well-structured mathematical model it is necessary to have wide knowledge of the biochemical mechanisms underlying the growth process, interactions between different macromolecules and cytological organelles, and about the bioreactor system used for production. There are models on molecule and enzyme level (enzyme synthesis models), on intracellular component level (structured cell model), on cellular level (kinetic model), on cellular environmental level (unstructured reactor model) and on dynamic cellular environmental level (structured reactor model) (Syddall et al., 1998).

In this thesis the goal is to develop a mathematical model that would be able to give a good description of oxygen transfer and consumption within the pellet considering micromorphological features. The model base will be Buschulte's pellet model.

#### 2.2.1. Buschulte's pellet model

As it was stated earlier, the base for this model is Buschulte's pellet model for *Streptomyces*. As every other model, Buschulte's model was also based on different assumptions. He assumed the following (Stoll, 2017):

- the pellet is spherical, all state variables are spherical symmetric and only one radial coordinate is modeled,
- the pellet is homogeneous, and it consists of a hyphae network,
- in the voids inside the pellet is media,
- outside of the pellet is homogeneous fluid and the boundary layer is spherical symmetric,

- the diffusing substances have low concentrations and the influence of the convective stream on the substance transport can be neglected,
- the substance streams of higher concentrated substances are limited to small values because of the limited metabolism,
- due to the low density difference between the biomass and the fluid the pellet is not flown through (this is necessary for the condition of spherical symmetry),
- the only substrates that influence the activity of the metabolism are the substrate concentrations considered in the balance equations and kinetics.

Model is calculating the biomass on the change of hyphal length and number of tips. The length growth of hyphae and the change in number of tips are dependent on the availability of oxygen. The oxygen concentration is dependent on the location in the pellet ( $r$  [m]) and the process time ( $t$  [h]). Equation 12 describes the hyphal length growth where  $c_h$  [ $m^{-2}$ ] is the hyphal length per volume.

$$\frac{\partial c_h(r,t)}{\partial t} = q_h(c_h, c_t, c_o) \quad [12]$$

The source term  $q_h$  [ $m^{-2} h^{-1}$ ] describes the growth of the hyphae without lysis. The growth is described in Equation 13 where it is shown that it depends on the oxygen concentration, the maximum hyphal growth rate and the number of tips.

$$q_h(c_h, c_t, c_o) = \alpha_{max} \cdot \psi_x(c_o) \cdot c_t(r, t); \psi_x > 0 \quad [13]$$

$\psi_x(c_o)$  is the activity and  $\alpha_{max}$  [ $m h^{-1}$ ] is the maximum growth rate of the hyphal length. As the hyphae only grows at the tips, the number of tips is essential for growth and not the hyphal length itself.

The hyphal tip grows similarly to the hyphae, but an additional diffusion term through the mycelium is necessary, as described in Equation 14.

$$\frac{\partial c_t(r,t)}{\partial t} = \frac{1}{r^2} \cdot \frac{\partial [D_x \cdot r^2 \cdot (\psi_x \cdot \frac{\partial c_t(r,t)}{\partial r} + c_t(r,t) \cdot \frac{\partial \psi_x}{\partial r})]}{\partial r} + q_t(c_h, c_t, c_o) \quad [14]$$

$D_x$  [ $m^2 h^{-1}$ ] is the diffusion coefficient for the movement of the tips and  $c_t$  [ $m^{-3}$ ] is the number of tips per volume. It includes a term for the global movement of the tips (diffusion term) and a second term to produce new tips due to branching. As a result of the growth in length, the tips change their location, which is modeled with a diffusion term. The diffusion coefficient is constant in the pellet and is calculated as shown in Equation 15.

$$D_x = k_{Dx,max} \cdot \alpha_{max}; \psi_x(c_o) > 0 \quad [15]$$



where  $k_{Dx,max}$  [m] is a model parameter. The diffusion term is valid for positive activity only. As soon as growth stops, the diffusion stops as well. The source term for the tips  $q_t$  without lysis is described in Equation 16.

$$q_t(c_h, c_t, c_0) = \beta_{max} \cdot \psi_x(c_0) \cdot c_h(r, t) \psi_x > 0 \quad [16]$$

where  $\beta_{max}$  [ $m^{-1} h^{-1}$ ] is the maximum branching rate. The equation shows the dependency of the branching from the hyphal length ( $c_h$ ). The boundary condition for the tip equation in the middle of the pellet, given by sphere symmetry, is a constant tip concentration as shown in Equation 17.

$$\frac{\partial c_t}{\partial r} |_{r=0} = 0 \quad [17]$$

On the surface of the pellet Equation 16 is valid, as the flow of hyphae over the radius (left side of equation 18) equals the newly build pellet volume (right side of Equation 16).

$$-\left(4 \cdot \pi \cdot r^2 \cdot D_x \cdot \frac{\partial(c_t \cdot \psi_x)}{\partial r}\right) |_{r=r_m} = 4 \cdot \pi \cdot r_m^2 \cdot \frac{dr_m}{dt} \cdot c_t(r_m) \quad [18]$$

$r_m$  [m] is the maximum pellet radius. Due to this boundary condition all tips are within the pellet radius.

For oxygen the balance equation consists of a diffusion term and a consumption term  $q_o$  [g/Lh], as shown in Equation 19.

$$\frac{\partial c_o(r, t)}{\partial t} = \frac{1}{r^2} \cdot \frac{\partial[D_{o,eff} \cdot c_h(r, t) \cdot r^2 \cdot \frac{\partial c_o(r, t)}{\partial r}]}{\partial r} + q_o(c_h, c_t, c_0) \quad [19]$$

$D_{o,eff}$  [ $m^2 h^{-1}$ ] is the effective diffusion coefficient of oxygen. It includes the change of the hyphal density in the pellet and it described in Equation 20.

$$D_{o,eff} = D_o \cdot (1 - c_h)^{1.76} \quad [20]$$

With the effective diffusion coefficient, the density of hyphae in the pellet is considered and it changes within the pellet.

The oxygen consumption term (source term) includes two parts, the consumption of oxygen for growth and for maintenance metabolism. This is modeled without lysis as shown in Equation 21.

$$q_o = -\frac{\sigma_h(gm^{-1})}{Y_{XO(-)}} \cdot \alpha_{max}(mh^{-1}) \cdot \psi_x(c_o) \cdot c_t(m^{-3}) - m_{o,max}(h^{-1}) \cdot \sigma_h(gm^{-1}) \cdot \psi_m(c_o) \cdot c_h(m^{-2}) \quad [21]$$

$\sigma_h$  [g  $m^{-1}$ ] is the length related dry mass,  $Y_{x,o}$  [g biomass g  $O_2^{-1}$ ] the yield of the amount of biomass produced per gram of oxygen, and  $\psi_m$  the activity for maintenance. The length related dry mass

is used to convert the hyphal length and its growth rate into biomass in order to calculate the oxygen consumption for growth and maintenance. It is calculated from the hyphal density  $\rho_h$  [g L<sup>-1</sup>] and the hyphal diameter  $d_h$  [m] as shown in Equation 22.

$$\sigma_h = \rho_h \cdot \frac{\pi \cdot d_h^2}{4} \quad [22]$$

The boundary condition for oxygen in the middle of the pellet is given by sphere symmetry as for the tips in Equation 15. For the pellet surface, the boundary condition is modeled by a boundary layer model. As the time constant of the diffusion is several ratios smaller than the growth, a quasi-stationary balance for oxygen in the boundary layer is applied. For an ideally stirred tank reactor the boundary layer is towards zero, as the concentration in the reactor is always ideal mixed. This case is shown in Equation 23.

$$c_{0,m} = c_{0,s} \quad [23]$$

where  $c_{0,m}$  [g L<sup>-1</sup>] is the oxygen concentration in the medium. For non-stirred conditions the boundary layer is very thick, and Equation 24 is valid.

$$\frac{dc_0}{dr} \big|_{r=r_m} = \frac{1}{r_m} \cdot (c_{0,s} - c_{0,m}) \quad [24]$$

The boundary condition for non-stirred conditions is later used for the pellet model. The kinetics of the substrate consumption are given by the activities  $\psi_x$  for the biomass and  $\psi_m$  for the maintenance and they are shown in Equation 25 and Equation 26.

$$\psi_x = \frac{c_O(r,t)(gL^{-1}) - c_{O,crit}(gL^{-1})}{K_{x,O}(gL^{-1}) + c_O(r,t)(gL^{-1})} \quad [25]$$

$$\psi_m = \frac{c_O(r,t)(gL^{-1})}{K_{m,O}(gL^{-1}) + c_O(r,t)(gL^{-1})} \quad [26]$$

where  $K_{x,o}$  [g L<sup>-1</sup>] is the Michaelis constant for biomass formation from oxygen and  $K_{m,o}$  [g L<sup>-1</sup>] the Michaelis constant for maintenance from oxygen. Additionally, to the growth of hyphae and tips, the pellet grows in total radius. This is modeled with a ODE dependent on the substrate availability ( $\psi_x$ ) and the growth of hyphae and a proportion factor  $\gamma$  [-] to consider the different angle of the tips growth direction at the surface of the pellet. Equation 27 shows the change in pellet radius.

$$\frac{dr_m}{dt} = \gamma \cdot \alpha_{max} \cdot \psi_x \cdot (c_0(r = r_m, t)) \quad [27]$$

### 3. EXPERIMENTAL PART

#### 3.1. WORK PLAN

In this chapter work plan of the thesis will be presented. Cultivation, as well as the microelectrode measurement, that will be shown later, was carried out by M. Sc. Charlotte Deffur with the cooperation with the Institute of biochemical engineering, Technical University of Braunschweig.

##### 3.1.1. Strain and cultivation

*A. niger* SKAn 1015 was used as a model strain. Cultivation was carried out in shake flasks. In each flask there was 50 mL of liquid medium and target population of *A. niger* was  $10^6$ . Sampling time points are shown in Table 3.

**Table 3.** Sampling of *A. niger* SKAn 1015

| Cultivation time | Number of measured pellets |
|------------------|----------------------------|
| 42 h             | 1 (P1.1.)                  |
| 46 h             | 1 (P2.1.)                  |
| 50 h             | 1 (P3.1.)                  |
| 54 h             | 2 (P4.1., P4.2.)           |
| 64 h             | 1 (P5.1.)                  |

##### 3.1.2. MATLAB

The program used for the calculations and stimulations was MATLAB version R2021b including Optimization Toolbox. It is widely used software all around the world for solving mathematical problems and displaying the results graphically. MATLAB was installed on a personal computer with windows 10.

This work was about a single fungal pellet. The pellet model is a one-dimensional problem and in MATLAB can be implemented and solved by using mathematical equations for PDEs (Partial Differential Equations) and ODEs (Ordinary Differential Equations)

##### 3.1.3. VGSTUDIO

VGSTUDIO is a software used for visual quality inspection in industrial applications and for the visualization of data in different academic fields. It can be used in different stages of work, from precise reconstruction of three-dimensional volume data sets using the images taken with CT (computed tomography) scanners to visualization and creation of animations.

### 3.2. METHODS

In this work, the relationship between micromorphology and oxygen concentration profiles by generating three-dimensional microtomography images of fungal pellets from different cultivation times in which the oxygen profile was previously determined with microelectrode measurements was investigated.

#### 3.2.1. Microelectrode measurement

The data used in this work were obtained by microelectrode measurements by M. Sc. Charlotte Deffur. Measurements of spatially separated oxygen profiles were performed in a flow cell outside the bioreactor, where different, well-defined hydrodynamic conditions could have been adjusted. A pellet is fixed in a loop of a human hair, connected to a gel loader tip and placed in the middle of the cross-sectional area of the glass tube. The microelectrode was mounted on the micromanipulator and driven into the pellet in steps of 10 mm. Profiles were measured at the equator of the pellet, perpendicularly to the flow. This proved to be necessary to prevent the pellet from being compressed by the shaft beyond a certain penetration depth. (Hille, 2005). The pellet, that was attached to the microelectrode system by human hair, is shown in Figure 7.



**Figure 7.** A photograph of a pellet attached to a microelectrode system using human hair

#### 3.2.2. Optimization of O<sub>2</sub> curves and determination of boundary layer

As already explained in section 2.1.5., oxygen transfer goes from gas bubbles, through the liquid phase to the surface of the pellet and from the surface to the center of the pellet. Thus, the concentration of oxygen used for aeration of the liquid medium will not be the same as the

oxygen concentration that reaches the pellet surface and the pellet center.

Therefore, to know where the pellet starts, what data relate to the pellet and what to the bulk phase and boundary layer, it was necessary to find appropriate method that could have been used to optimize those data. The data and optimized O<sub>2</sub> profiles are later going to be shown in results.

In this work, optimization of O<sub>2</sub> curves was carried out in MATLAB. Firstly, it was necessary to show how the oxygen concentration changes per pellet radius. In the same graph in MATLAB, the first derivative was then displayed and it was searched for where its highest value is. The first derivative was obtained with MATLAB function  $Y=\text{diff}(X)/h$  that approximates partial derivatives, where  $f$  is a vector of function values evaluated over domain  $X$ , and  $h$  is an appropriate step size. The point, that represents particle surface, was then obtained by projection of the highest value of the first derivative onto the curve that shows oxygen concentration per pellet radius. Continuing, the data above that point up to the aligned part of the curve refer to the boundary layer, that is, part of the fluid where flow is affected by the solid. Such optimized O<sub>2</sub> curves and determined boundary layer are later shown in the chapter 4.2.

### 3.2.3. Parameter estimation

Finally, the aim of this work, an estimation of the parameters that influence oxygen consumption in the fungal pellet, was made. An important aspect of mathematical modeling is the verification of formulated models using experimental data. Thus, the experimentally obtained values must be compared with the simulated values and an appropriate selection of model parameters is crucial (Buschulte, 1992).

To reiterate, the term for oxygen consumption consists of two parts. One part refers to the consumption of oxygen for growth, and the other part refers to the consumption of oxygen to maintain metabolism. Therefore, it was necessary to estimate the following parameters: yield constant of mass of biomass produced per mass of oxygen used,  $Y_{x,o}$  (-); Michaelis constant for biomass formation from oxygen,  $K_{x,o}$  (kg m<sup>-3</sup>); critical oxygen concentration,  $c_{o,crit}$  (kg m<sup>-3</sup>); maximum maintenance rate for oxygen,  $m_{o,max}$  (h<sup>-1</sup>) and Michaelis constant for maintenance from oxygen,  $K_{m,o}$  (kg m<sup>-3</sup>).

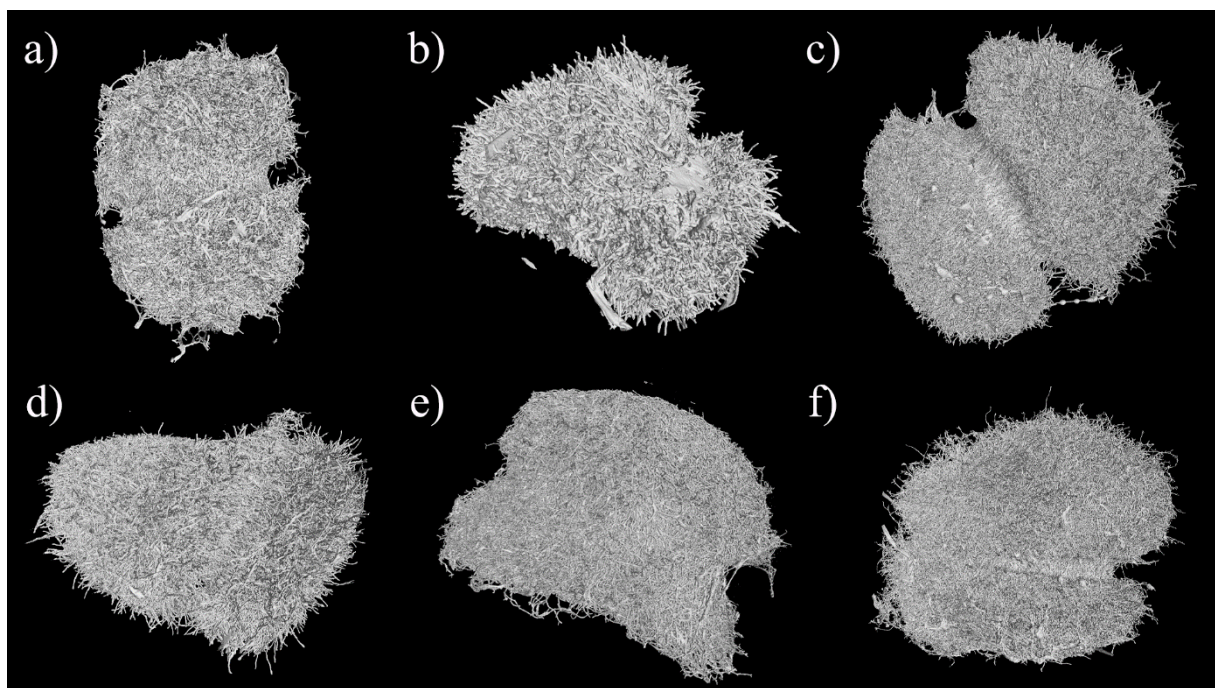
Optimization was done in MATLAB using an optimization software package called Optimization Toolbox which is an add-on product on MATLAB. Therefore, for parameter estimation in this work a nonlinear least-squares solver, i.e., `lsqnonlin` function, was used. A more detailed description of parameter estimation and the result itself is presented in the chapter 4.4.

## 4. RESULTS AND DISCUSSION

The goal of this work was to analyze the oxygen profiles inside the fungal pellets, estimate the parameters that influence oxygen consumption, and determine which of these parameters have the greatest influence on it. Thus, this chapter shows the determination of the diameter of the pellet and the boundary layer, which were used to find the center of the pellet. This is followed by a display of optimized oxygen profiles and changes in micromorphological parameters. Finally, the results of the parameter estimation itself are presented and comments and discussion are given.

### 4.1. DETERMINATION OF PELLET DIAMETER

For 6 pellets, for which an oxygen profile was determined, three-dimensional volume data was constructed using software VGSTUDIO. These 3D volume data are shown in Figure 9.

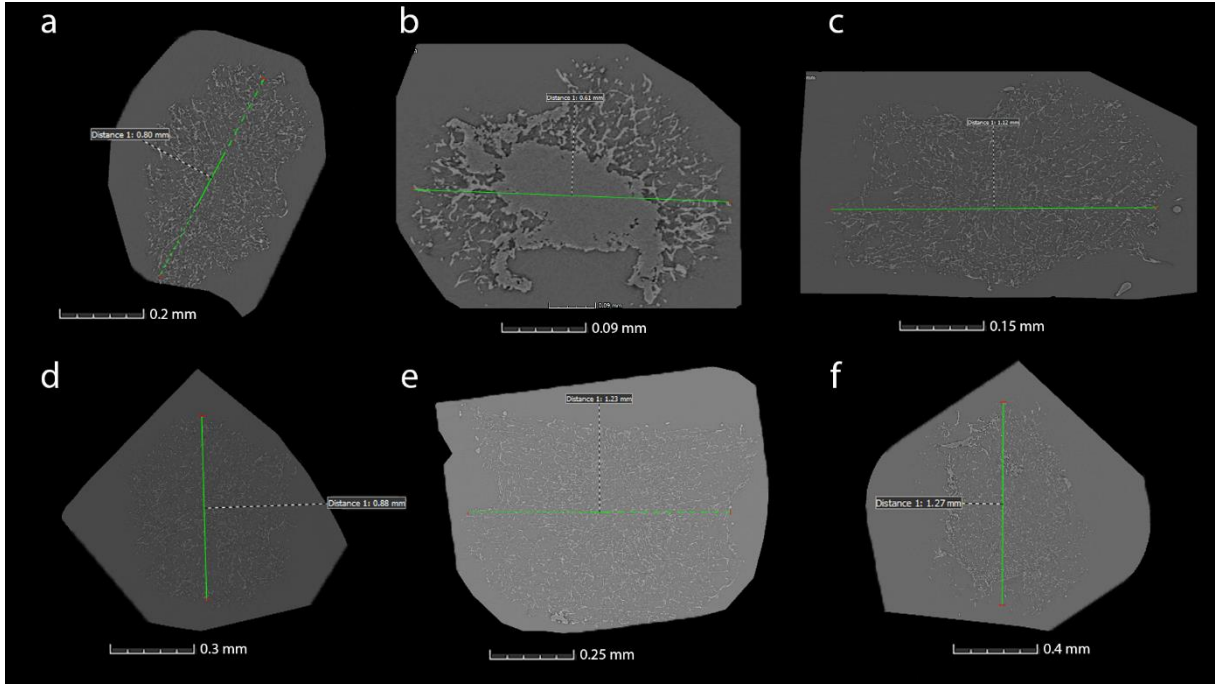


**Figure 9.** Three-dimensional volume data for pellet a) P1.1., b) P2.1., c) P3.1., d) P4.1., e) P4.2. and f) P5.1.

The constrictions visible on the pellets were formed when determining the oxygen concentration using microelectrodes, since the pellets were attached to the electrode using human hair.

To be able to determine the center of the pellet, it was necessary to determine where the boundary layer is and what is the diameter of the pellet itself.

The pellet diameter was also determined using VGSTUDIO software and the results are shown in Figure 10.



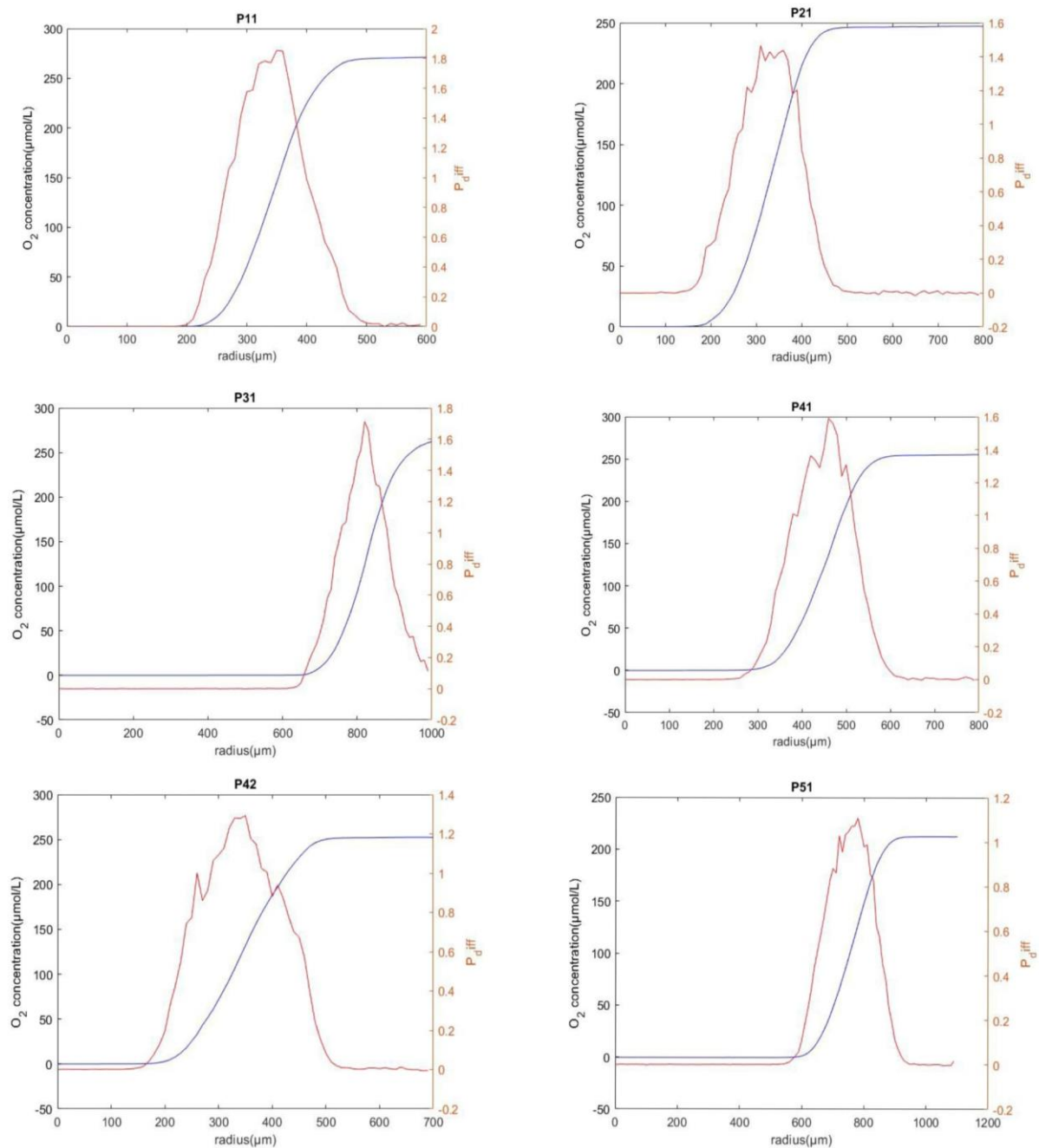
**Figure 10.** Diameter determination for pellet a) d (P1.1.) = 0.80mm, b) d (P2.1.) =0.61mm, c) d (P3.1.) =1.12mm, d) d (P4.1.) =0.88mm, e) d (P4.2.) =1,23mm and f) d (P5.1.) =1,27mm

As expected, the size of the pellets increased with time. This was visible from the diameter values shown in Figure 10. Visible deviations can be explained by the fact that some smaller pellets were probably taken during sampling in later times of cultivation. Furthermore, the deviations in the shape of the pellets and the measured diameter can also be affected by damage caused during the freeze-drying phase.

#### 4.2. DISPLAY OF OPTIMIZED OXYGEN PROFILES AND CALCULATION OF BOUNDARY LAYER

In Figure 11. it is shown how the oxygen concentration per pellet radius changes. The blue curve shows the change in oxygen concentration through the pellet, and the red curve the first derivation. By projecting the maximum of the first derivation (red curve) onto the blue curve (oxygen concentration per pellet radius), a point representing the particle surface was obtained. Finally, as stated earlier, the data above that point up to the aligned part of the curve refer to the boundary layer.

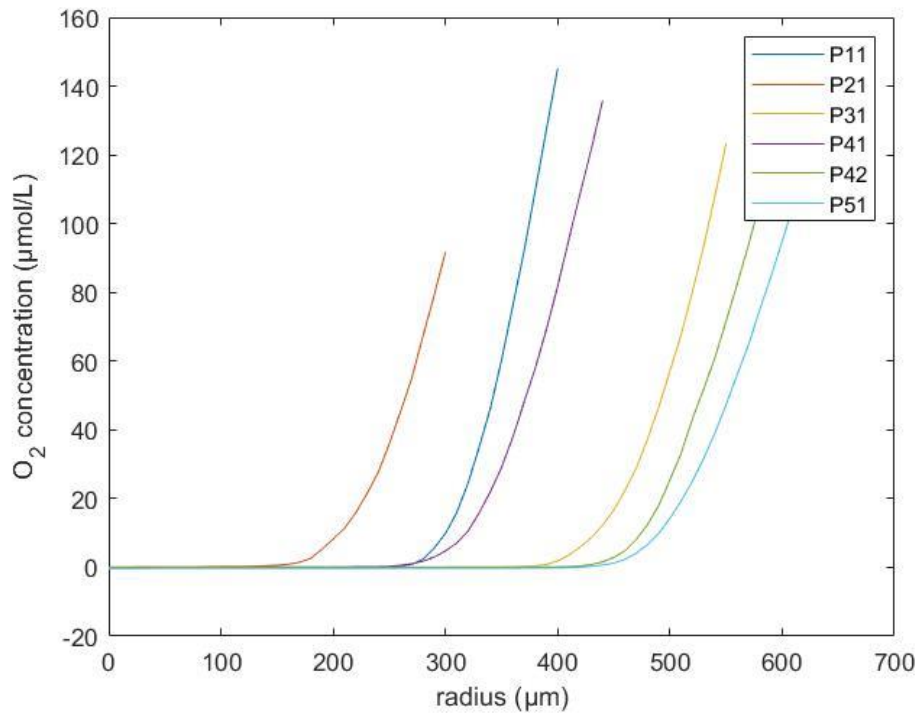




**Figure 11.** Change in oxygen concentration per radius for pellets P1.1., P2.1., P3.1., P4.1., P4.2. and P5.1.

Considering the diameter of the pellets obtained with the VGSTUDIO software and the particle surface, the center of the pellets can be determined. In this way, the  $O_2$  curves were optimized and shown on the same graph for all 6 pellets in Figure 12. Thus, this graph shows the data relating to the pellets themselves, from the center to the surface, while all other data are discarded.



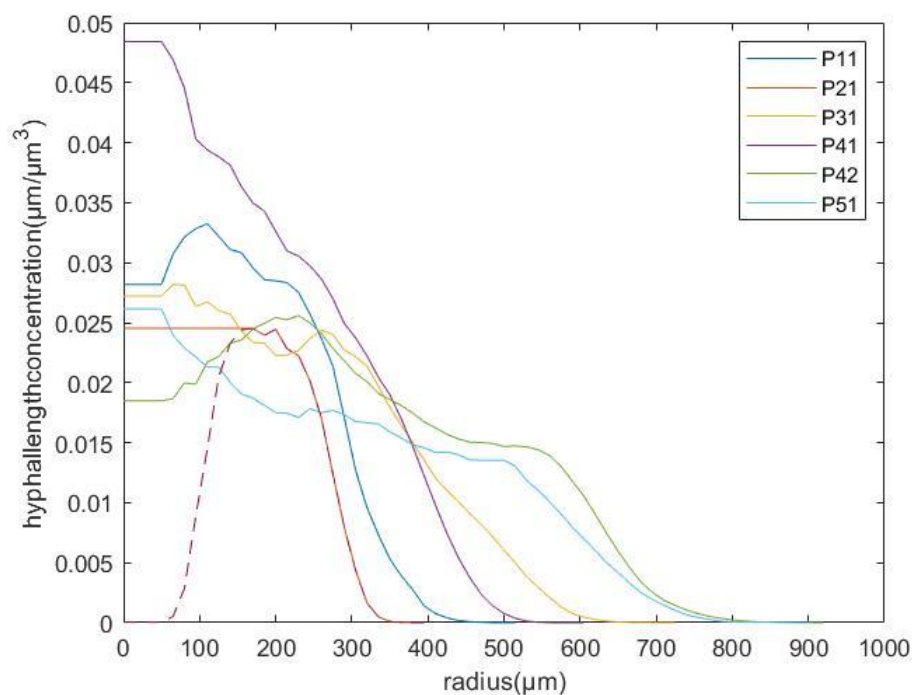


**Figure 12.** Optimized oxygen profile for pellets P1.1., P2.1., P3.1., P4.1., P4.2. and P5.1.

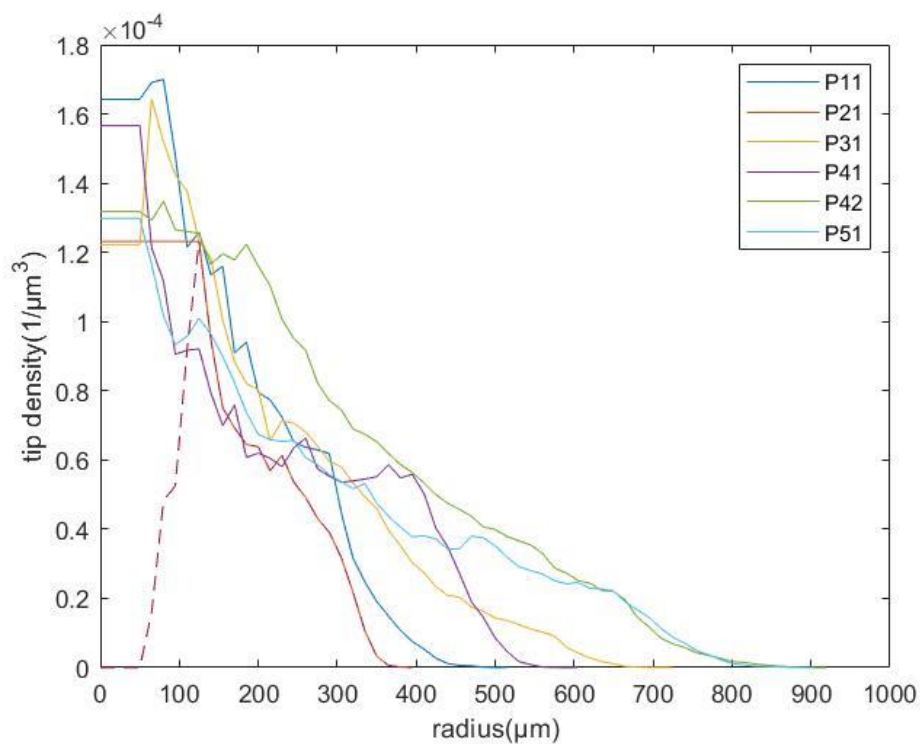
In Figure 12. it is visible that the drop of the oxygen is steep. Furthermore, the oxygen penetration depth ranged from a minimum value of approximately 150  $\mu\text{m}$  for pellet P2.1. up to a maximum value of approximately 450  $\mu\text{m}$  for pellet P5.1.

It would be good if, in future work, the oxygen concentration was measured throughout the entire pellet, in contrast to the measurement carried out in this work, where the concentration was measured from the surface to the center of the pellet. In this way, the S-curve as shown in Figure 11. would not be obtained, but the S-curve will be directed in both directions, that is, the curve will first rise and then fall again. This way of carrying out oxygen concentration measurements would make it easier to accurately find the center of the pellet.

### 4.3. DISPLAY OF CHANGES IN MICROMORPHOLOGICAL PARAMETERS PER RADIUS



**Figure 13.** Change in hyphal length concentration per radius



**Figure 14.** Change in tip density per radius

Figure 13. and Figure 14. show changes in hyphal length concentration and tip density per radius. In these figures the origin of the axis represents the center of the pellet, therefore the larger the radius, the closer to the surface of the pellet.

In both figures, for pellet P2.1., dashed line represents raw data and full line is modified curve. The reason why the raw data curve looks like this is probably that during the microelectrode measurements, while taking out the pellet from the liquid phase and freeze-drying of the pellet in the nitrogen, there was somehow a breakdown in the center of the pellet. In Figure 13. it is also visible that the pellet P5.1., in the outer area, was not as dense as other pellets. In these figures it is shown that the center of the pellet was the densest, and as the pellet grew, that is, by moving away from the center of the pellet, hyphal length concentration and tip density were decreasing. When compared with changes in oxygen concentration per radius (Figure 12.) the increase in hyphal length concentration and tip density is inversely proportional to the oxygen concentration. So, the more hyphae, the higher the oxygen consumption. The oxygen concentration was high at the surface and decreased towards the middle of the pellet.

Oxygen measurements are very time consuming and can only be performed in low throughput. However, information on the supply of the cells inside the pellet is very valuable. Another very important fact is that the characteristic time for the pellet to grow is much longer than the characteristic time for the transfer of oxygen throughout the pellet, so it can be said that the oxygen profile in the pellet is in a pseudo-stationary state (Cui et al., 1998).

#### 4.4. NORMALIZATION AND PARAMETER ESTIMATION

For *A. niger* SKAn 1015, that was used as a model strain, parameters from different literature sources were used and shown in Table 4.

**Table 4.** Parameters for *Aspergillus niger* SKAn 1015 used in the model

| parameter                              | literature value    | literature source  |
|--|---------------------|--|
| $c_{o,crit}$<br>( $\text{kg m}^{-3}$ ) | $6 \cdot 10^{-4}$   | Buschulte, T.K. Mathematische Modellbildung und Simulation von Zellwachstum, Stofftransport und Stoffwechsel in Pellets aus Streptomyceten. PhD thesis, Fakultät Verfahrenstechnik der Universität Stuttgart, 1992 |
| $K_{x,o}$<br>( $\text{kg m}^{-3}$ )    | $1 \cdot 10^{-4}$   | Cui, Y.Q., Okkerse, W.J., Lans, R.G.J.M. van d., Luyben, K.C.A.M.: Modeling and measurements of fungal growth and morphology in submerged fermentations, 1998  |
| $K_{m,o}$<br>( $\text{kg m}^{-3}$ )    | $1,6 \cdot 10^{-5}$ | Buschulte, T.K. Mathematische Modellbildung und Simulation von Zellwachstum, Stofftransport und Stoffwechsel in Pellets aus Streptomyceten. PhD thesis, Fakultät Verfahrenstechnik der Universität Stuttgart, 1992 |
| $Y_{x,o}$<br>(-)                       | 1                   | Cui, Y.Q., Okkerse, W.J., Lans, R.G.J.M. van d., Luyben, K.C.A.M.: Modeling and measurements of fungal growth and morphology in submerged fermentations, 1998  |
| $m_{o,max}$<br>( $\text{h}^{-1}$ )     | $7,2 \cdot 10^{-3}$ | Cui, Y.Q., Okkerse, W.J., Lans, R.G.J.M. van d., Luyben, K.C.A.M.: Modeling and measurements of fungal growth and morphology in submerged fermentations, 1998  |

Before estimation, data were normalized due to computational accuracy.

To be able to assess the oxygen consumption in the fungal pellet, an appropriate equation was required which is shown in Equation 21.

The aim of this work was to estimate parameters in the previous equation, determine which of these parameters have the greatest influence on oxygen consumption and to what extent. Therefore, the numerical calculation of the model was implemented in MATLAB where estimation was done. As an addition to this master thesis, there is a MATLAB script with all the codes used to obtain results in this work.

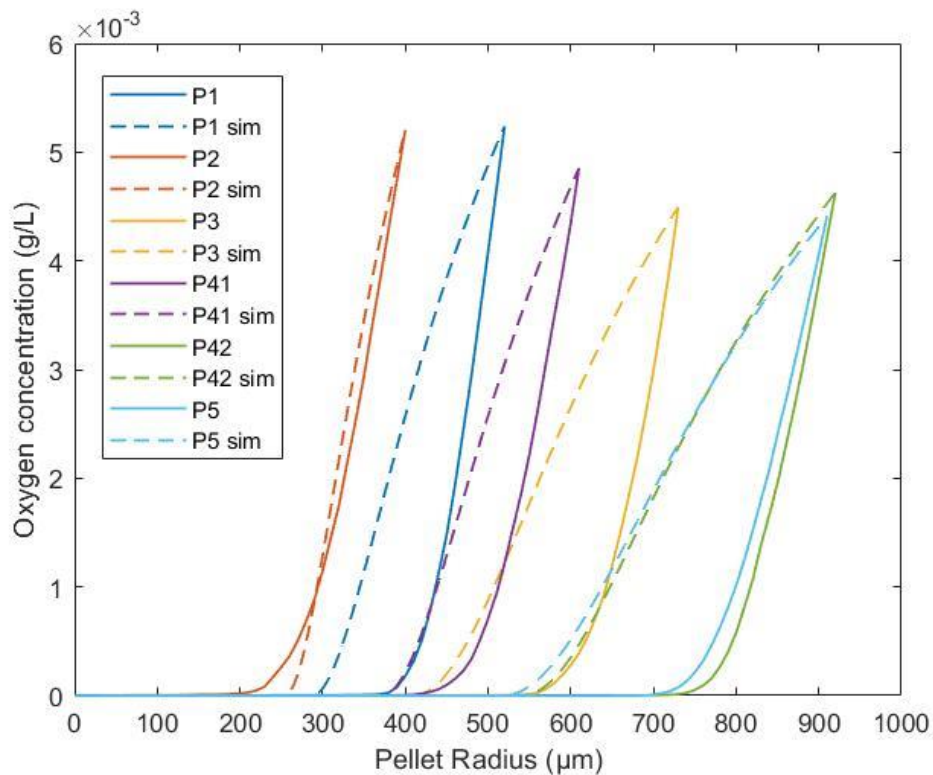
As previously stated, parameters were estimated using a nonlinear least-squares solver,

i.e., lsqnonlin function. After that, Jacobian for 6 different pellets used in this work was obtained. The Jacobian matrix is the coordinate-based matrix representation of the derivative of a vector-valued or multivariable function when the derivative of that function exists.

Table 5. shows literature values and values obtained by optimization in MATLAB for oxygen consumption parameters, while Figure 15. shows curves of oxygen concentration change for experimental and simulated values and thus shows how well the model corresponds to reality.

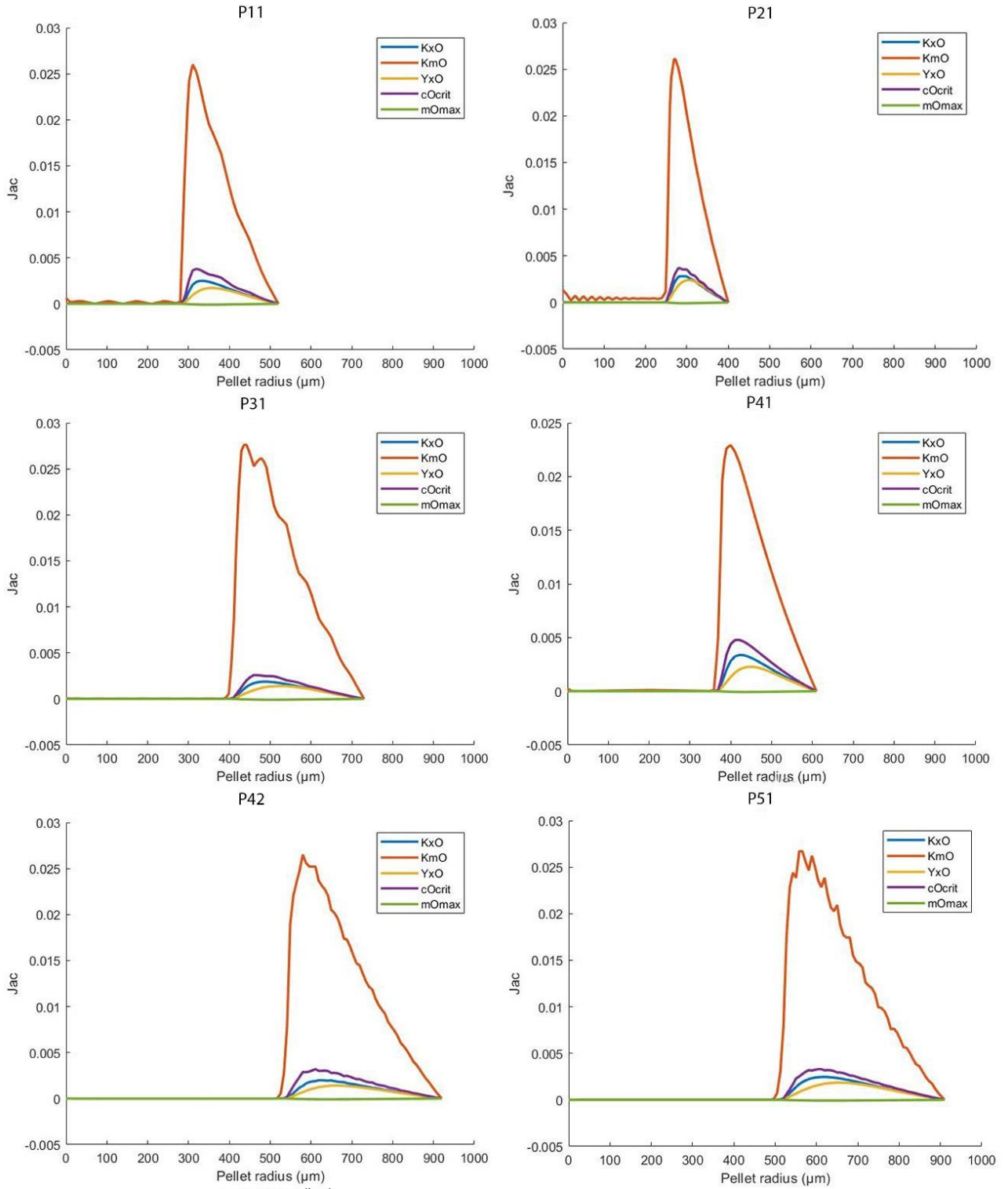
**Table 5.** Display of parameter values optimized in MATLAB compared to literature values

| parameter                               | literature value    | optimized value     |
|---|---------------------|---------------------|
| $c_{o,crit} \text{ (kg m}^{-3}\text{)}$ | $6 \cdot 10^{-4}$   | $5,9 \cdot 10^{-5}$ |
| $K_{x,o} \text{ (kg m}^{-3}\text{)}$    | $1 \cdot 10^{-4}$   | $7,3 \cdot 10^{-6}$ |
| $K_{m,o} \text{ (kg m}^{-3}\text{)}$    | $1,6 \cdot 10^{-5}$ | $1,6 \cdot 10^{-6}$ |
| $Y_{x,o} \text{ (-)}$                   | 1                   | $1,4 \cdot 10^{-1}$ |
| $m_{o,max} \text{ (h}^{-1}\text{)}$     | $7,2 \cdot 10^{-3}$ | $2,8 \cdot 10^{-1}$ |



**Figure 15.** Change in oxygen concentration per radius for pellets P1.1., P2.1., P3.1., P4.1., P4.2. and P5.1. for experimental and simulated values

In Figure 15. there is a visible deviation of the simulated curves from the experimental ones for all pellets except for pellet P2.1. Possible reasons for these deviations are discussed a little further down in the paper.



**Figure 16.** An influence of parameter change as a function of pellet radius for pellets P1.1., P2.1., P3.1., P4.1., P4.2. and P5.1.

Figure 16. shows Jacobian (Jac) function for all 6 pellets, where each graph in this figure separately shows an impact of all five parameters for every pellet. While obtaining these graphs absolute values were taken.

Cui et al. (1998) have assumed that the yield of *A. niger* on oxygen is 1. In this model, yield constant of mass of biomass produced per mass of oxygen used,  $Y_{x,o}$  (-), is  $1,4 \cdot 10^{-1}$ . This value is a little smaller than the literature value. Partly, the reason for that could be that the strain used in this work is less efficient in the oxygen consumption. Metz and Kosen (1977) and Kobayashi et al. (1973) have reported  $K_{x,o}$  ( $\text{kg m}^{-3}$ ) Michaelis constant for biomass formation from oxygen, to be in the order of  $10^{-4} \text{ kg m}^{-3}$  for *A. niger*. The simulated value in this model is  $7,3 \cdot 10^{-6} \text{ kg m}^{-3}$  which is also a little less than the literature value. Furthermore, literature value of Michaelis constant for maintenance from oxygen,  $K_{m,o}$  ( $\text{kg m}^{-3}$ ) is  $1,6 \cdot 10^{-5}$  (Buschulte, 1992) and the value obtained with this model is  $1,6 \cdot 10^{-6}$ . Dissolved oxygen concentration in the bulk is often much higher than the saturation constant. Thus, in case of filamentous fungi, dissolved oxygen limitation under the studied conditions hardly occurs, and pellet growth is mainly controlled by oxygen transfer from the bulk into the pellets (Cui et al., 1998). The literature value for  $c_{o,crit}$ , critical oxygen concentration, according to Buschulte (1992) is  $6 \cdot 10^{-4} \text{ kg m}^{-3}$  while the simulated value obtained in this model amounts to  $5,9 \cdot 10^{-5} \text{ kg m}^{-3}$ . There is a certain difference between the literature and the simulated value. Both, Buschulte's model and the model from this work, refer to the single pellet. Therefore, the comparison of parameter values is accepted. Estimated value of critical oxygen concentration, which was obtained by simulation in MATLAB, coincides quite well with the literature value. The fact that Buschulte's single pellet model was related to *Streptomyces*, while the model in this work refers to *A. niger*, probably has a certain influence on the present difference in values. So far, the parameters that describe how microorganisms consume substrates and oxygen for growth have been mentioned, but since they also consume them to maintain metabolism, there are also other parameters. An important parameter for oxygen consumption is maximum maintenance rate for oxygen,  $m_{o,max}$  ( $\text{h}^{-1}$ ). Cui et al. (1998) reported that the value of  $m_{o,max}$  for *A. awamori* was found to be  $7,2 \cdot 10^{-3} \text{ h}^{-1}$ . The estimated value in this model amounts to  $2,8 \cdot 10^{-1} \text{ h}^{-1}$  which is approximately 100 times higher than literature value. As was said for the critical oxygen concentration, the literature value of maximum maintenance rate for oxygen refers to a different strain than the one used in this paper, and this certainly has an effect on the fact that the simulated value differs from the literature value.

The difference in oxygen consumption, and thus the parameters of the model, is influenced by the used strain and cultivation conditions, so it is to be expected that the simulated values will

not ideally match the literature values. Cui et al. (1998) conducted an experiment with *A. awamori* grown as loose mycelia in a stirred bioreactor, while in this work, *A. niger* SKAn 1015 was used as a model strain, and a single pellet extracted from the liquid phase from a shaker flask was used for modeling.

It can be seen in Figure 16. that the greatest influence on the oxygen consumption has Michaelis constant for maintenance from oxygen. Other four parameters have significantly less influence. Furthermore, the values of the Jacobian go to zero at the pellet border, because less hyphae means less oxygen consumption. Thus, the consumption parameters also have less influence.

This was modeled dependent on the diffusion and consumption of oxygen in the pellet. As the pellet grew, diffusion could be a reason for the limiting conditions in the center of the pellet that could explain why growth stops there. As shown in Equation 12. and 14., the growth of hyphae and tips is dependent on oxygen concentration over the activity that is dependent on radius (Equation 25.). Since they are interdependent, with decreasing oxygen concentration, the activity is also decreased. Therefore, when oxygen concentration decreases below the critical value, the activity is below zero.

Accordingly, looking at Equation 21., we conclude that the  $\psi_m$ , activity of maintenance, has the greatest influence on oxygen consumption. Already looking at the oxygen concentration profile, it can be seen that the oxygen concentration was already quite low, almost zero, around the center of the pellet. That could explain why the second part, that refers to the oxygen consumption for maintenance of metabolism, has a greater influence. Because, if the concentration of oxygen is already low, there will not be any significant activity for growth. Therefore, in future experiments, if the estimation of other parameters, that are growth related, is the goal, the advice would be to use earlier time points where oxygen concentration would be higher.

Model predictions are in reasonable agreement with the literature. However, there are certain deviations between model simulation and literature. To begin with, it would be good if the literature data and the simulated model were related to the same microorganism that would be cultivated under similar conditions. For example, if the fermentation was carried out in air lift bioreactors, instead of in stirred bioreactors, the forces would be less and therefore the fungal pellets would be less damaged and whole experimental work would be more accurate (Cui et al., 1998). Besides, a common mistaken assumption is that the input and output variables are linearly related, which is generally not the case. Another factor, which could have a great influence on the result, is that during the oxygen measurement, a lower concentration was



measured than it actually is. Therefore, to ensure that parameter estimation was conducted well, in future work, it would be good to do additional sensitivity analysis that show, according to Saltelli (2002), how uncertainty in the output of a model (numerical or otherwise) can be apportioned to different sources of uncertainty in the model input. It would show what is the effect of change in absolute terms of an input variables on the absolute amount of an output variable and thus increase the credibility of this estimate.

## 5. CONCLUSIONS

Based on the conducted research, experiments and the obtained results, it can be concluded the following:

1. Filamentous fungi are a very important group of microorganisms because of their application to obtain important biotechnological products.
2. Oxygen, as a key substrate for growth, production and maintenance activities, has been identified as the prime limiting substrate. Moreover, micromorphological and macromorphological structure of filamentous fungi has a great impact on oxygen consumption.
3. Mathematical models are used to understand growth kinetics. Therefore, in order to set up a well-structured model it is necessarily important to have a wide knowledge of everything that affects growth kinetics and to implement that knowledge in the model.
4. Model in this work is based on Buschulte's single pellet model. Its numerical calculation was implemented in MATLAB where parameter estimation was done.
5. Oxygen consumption is influenced by several different parameters. Some of these parameters describe growth of the pellet while others describe the maintenance of metabolism.
6. The parameter that have the greatest impact on oxygen consumption in this work is Michaelis constant for maintenance from oxygen, while other test parameters have a significantly smaller influence. The reason for this could be that the measurement was carried out at later time points where the oxygen concentration was already quite low, so the activity for maintaining the metabolism, and not for growth, mainly prevailed.
7. The model predictions are mostly in line with the literature. However, the existing deviations could be explained by the fact that the literature data and the model do not refer to the same strain of microorganism and the cultivation conditions are different.
8. For future work, it would be good to carry out uncertainty and sensitivity analysis, which would contribute to the credibility of the model, because they allow monitoring the influence of the values of different input parameters on the output parameter. In addition, to improve this model, other initial conditions and literature parameter values could be found and included in it.
9. Above all, the implemented model shows well how the oxygen concentration inside the pellets changes, which parameters have the greatest influence on it, and might be a possible basis for future work.

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## DECLARATION OF ORIGINALITY

I Karla Miličević declare that this master's thesis is an original result of my own work and it has been generated by me using no other resources than the ones listed in it.

Karla Miličević

Signature