# 1 Introduction and aim of work

The filamentous fungus *Aspergillus niger* is a widely applied strain mainly due to its metabolic versatility and secretion efficiency for industrial biotechnological production, such as citric acid, glucoamylase and antibiotics production [Meyer et al., 2011; Papagianni et al., 1998; Papagianni, 2004; Paul et al., 1999; Punt et al., 2002]. Depending on the product targeted, *A. niger* is grown either as freely dispersed mycelia (e.g. pectic enzyme production or production of penicillin) [Calam, 1976; Vecht-Lifshitz et al., 1990] or as pellets of aggregated biomass (e.g. citric acid production) [Gómez et al., 1988; Metz et al., 1979] in submerged cultivations. Advantages of pellet cultivations are the significant decrease of the viscosity and the easier separation of the biomass from the cultivation broth [van Suijdam et al., 1980; Wucherpfennig et al., 2010]. Nevertheless, in the pellet cultivations, there is an entangled relationship among the operating environmental conditions, pellet morphology, transport phenomena within the pellets and the related product formation [Hille et al., 2005; Krull et al., 2010; Pazouki and Panda, 2000; Withers et al., 1998]. Selected studies on morphology and productivity have included the variation of operating parameters, such as inoculum level, pH value, osmolality, supplementation with microparticles as well as aeration- and agitation-induced mechanical stress [Driouch et al., 2010a, 2010b, 2011 and 2012; McIntyre et al., 2001; Lin et al., 2010; Wucherpfennig et al., 2011]. Properly controlled fungal morphology can improve the production yield significantly [El-Enshasy et al., 1999; Pluschkell et al., 1996].

Pellet growth of filamentous fungi is always connected with the morphological developments. The morphological developments and the associated impact on the product formation play an essential role, particularly regarding the mass transport

in the biopellets. The biomass dry weight concentration alone is insufficient to describe the dependence of the productivity on the morphological characteristics adequately [Krull et al., 2010]. Besides the pellet growth, the pellet damage mechanisms are to be considered, such as erosion processes, which have the main effect on the pellet surface structure. In a given stirring system, the intensity of mechanical stress is proportional to the volumetric power input. It was above all necessary to describe the impact of the fluid dynamics on the pellet morphological characteristics.

Aeration and agitation are of prime importance in aerobic cultivations. Higher aeration and agitation rates have two main effects on the cultivation of filamentous fungi in a stirred tank reactor: on the one hand, enhanced oxygen supply and better mixing as well as mass and heat transfer. On the other hand, higher aeration and agitation intensities lead to higher energy input connected with higher mechanical stress on the fungal pellets, causing morphological changes [El-Enshasy et al., 2006; Žmak et al., 2006] and variations in biomass growth as well as in product formation [Amanullah et al., 2000; Li et al., 2000; Mantzouridou and Kotzekidou, 2002; Nielsen et al., 1995; Rosa et al., 2005]. Due to higher mechanical stress, smaller pellets and a higher pellet concentration can occur. The more favorable form for production is smaller pellets, since problems relating to nutrient and oxygen limitations within the pellets will be proportionately acuter in larger than in smaller pellets [Pazouki and Panda, 2000].

A major aspect of interest in aerobic cultivations is the energy input, which is consumed in aeration, agitation and refrigeration. For a given set of process conditions, e.g. fixed bioreactor geometry, stirrer and cultivation temperature, energy optimization is still possible, namely through optimal operation of aeration and agitation [Alves and Vasconcelos, 1996]. With respect to the pellet growth, a clear dependence of the pellet morphology on the volumetric power input by agitation and aeration can be observed. An increase in the volumetric power input by agitation or aeration leads to smaller pellets with a higher pellet concentration. A detailed description of the pellet structure in dependence on the volumetric power input by agitation and aeration has to be considered as well.

An operating window diagram as a complex example for cultivation optimization regarding agitation and aeration is exemplified in Figure 1.1 [Lilly, 1997]. A bounded region, within which operation is considered most favourable, indicates the effect of two key parameters on a process operation, here agitation and aeration, which are in turn constrained by six factors: 1) operating costs, 2) foaming, 3) mixing, 4) oxygen supply, 5) carbon dioxide production and 6) shear levels. Knowledge of the precise relationships between the two key parameters and the six factors would enable the detailed shape and position of the window [Woodley and Titchener-Hooker, 1996].

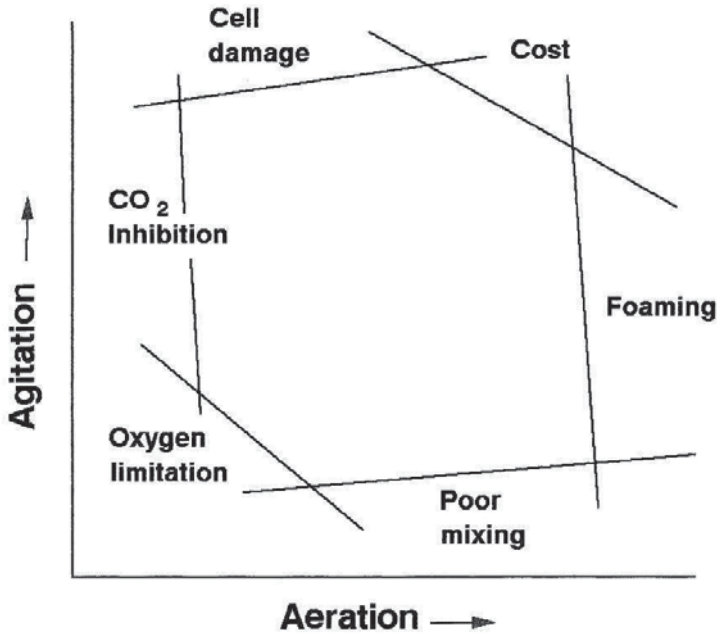


**Figure 1.1:** Operating window diagram showing the boundaries for cultivation scale up (redrawn from Buckland and Lilly, 1993) [Lilly, 1997]

In this work, the impacts of the volumetric power input by agitation and aeration on the pellet morphology and the resulting product formation were investigated. The aim of this study was to characterize the fungal pellet morphology of *A. niger* under different volumetric power inputs by agitation and aeration and thus to determine the impact of aeration and agitation on the pellet morphology, including pellet size, pellet concentration, pellet surface and internal structure, and its consequential influence on production of the model enzyme glucoamylase.

This work focused on the following main tasks:

- Investigations of the effect of the volumetric power input by agitation on the pellet growth, the pellet size and concentration, whereby additional efforts were made to analyze the heterogeneity within a pellet size distribution with the analysis software MATLAB and a self-written script (see chapter 4.1).

- Further investigations of the influence of the volumetric power input by agitation with the aid of biopellet preculture suspensions on the pellet growth, the glucoamylase (GA) product formation, the pellet macromorphology (pellet size and concentration) and the pellet micromorphology (pellet internal structure) (see chapter 4.2).

- Investigations of the different impact of the agitation- and aeration-induced power inputs on the pellet growth, the GA production and the pellet macromorphology (size and concentration) as well as micromorphology (internal and surface structure), whereby additional efforts were made to reach comparable initial oxygen conditions to decouple the growth factor from the GA production (see chapter 4.3).

# 2 Theoretical background

## 2.1 Submerged cultivation of filamentous fungi

### 2.1.1 Importance of filamentous fungi

Filamentous microorganisms, understood with both prokaryotes, such as streptomycetes, as well as eukaryotes, mostly filamentous fungi, have the connectional characteristics of apical growth. Growth of the hyphae occurs highly polarized, with a linear extension rate at the hyphal apex [Harris et al., 2009; Wucherpfennig et al., 2010]. Very different organisms of this kind have developed similar phenotypes, mostly due to the adaptation to certain environmental conditions. Therefore, the filamentous fungi, with respect to their position in the nutrient cycle, generally belong to the degrading, saprophile organisms [Abarca et al., 2004]. Saprophile microorganisms have a versatile metabolism. Many of their degradation enzymes, including proteases, amylases and pectinases, are technically produced and used in industrial processes [Finkelstein, 1987].

In industrial cultivation processes, filamentous fungi are widely applied [Papagianni, 2004; Punt et al., 2002.]. In the competition for nutrients with other microorganisms, filamentous fungi have developed other interesting properties. The acidification of the medium, for example, is the basis for the technical production of citric or gluconic acid, both organic acids, which are obtained in a scale of tons from filamentous fungi such as *Aspergillus niger* [Magnuson and Lasure, 2004]. *A. niger* has a long tradition in the production of citric acid. The worldwide

annual production of citric acid was about 800,000 tons in 1998 and is mainly realized by using biotechnological methods [Magnuson and Lasure, 2004].

Filamentous fungi are characterized by their excellent secretion systems, so that further product processing in downstream processing is facilitated considerably. In addition, filamentous fungi as eukaryotes are capable of complex post-translational modifications, such as glycosylation of proteins [Punt et al., 2002]. This is particularly important with regard to the production of pharmaceutical proteins because therapeutically effective proteins can remain in human blood circulation over a longer time only in the presence of glycosyl residues [Gerngross, 2004; Maras et al., 1999]. By using genetic engineering methods, it is possible to stably introduce genes of interesting heterologous proteins into the genome of filamentous fungi with subsequent expression. In the case of *A. niger*, due to its safety status of GRAS (generally regarded as safe) from the U.S. Food and Drug Administration (FDA), it can be used particularly well as a host and production organism in the biotechnological industry [Abarca et al., 2004; Li et al., 2000]. The most common method to improve the product yield of the desired heterologous protein is the fusion of a target heterologous gene with the homologous gene coding for glucoamylase in *A. niger* and the utilization of the well-functioning secretion system of the glucoamylase as well [Archer et al., 1995; Ward et al., 1990; Wiebe et al., 2001]. Furthermore, by using the protease deficient mutants, the degradation of target proteins by the host's own proteases can be prevented [Conesa et al., 2000; Meyer et al., 2011].

The morphology of filamentous microorganisms plays an important role in terms of biomass productivity. The morphology influences both the synthesis of proteins and their secretion as well. There are known processes, in which the dispersed mycelium is advantageous, such as the production of penicillin by *Penicillium chrysogenum*. On the contrary, for example, the production of citric acid by *A. niger* is carried out by cultivating small pellets [Amanullah et al., 2000; Nielsen et al., 1995; Paul et al., 1999].

### 2.1.2 Aggregation behavior and pellet formation

The interaction of spores plays an important role in the pellet formation of filamentous microorganisms. Filamentous microorganisms are in general classified into coagulating and non-coagulating types according to their mechanism of pellet formation. For non-coagulating filamentous microorganisms, there is no aggregation of spores, so that a pellet can develop from a single spore. The concentration of the resulting pellets corresponds to the starting concentration of the spores in the inoculum. For coagulating filamentous microorganisms, such as *A. niger*, spores aggregate in the early stage of morphologic development before germination and hyphal growth take place, so that the resulting pellet concentration is significantly lower than the initial spore concentration [Metz and Kossen, 1977; Kossen, 2000]. The formed pellets of coagulating filamentous microorganisms have thus adsorbed spores on the hyphal surfaces in the pellet interior, which mostly do not germinate due to substrate limitation or steric effects.

Studies of Grimm et al. (2004) have shown that the pellet formation in *A. niger* proceeds in different stages. Based on the time-dependent development of particle concentration, a kinetic model proposed for conidial aggregation of *A. niger* is exemplified in Figure 2.1. Two distinct steps and mechanisms of aggregation can be distinguished. The first step of conidial aggregation takes place immediately after the inoculation of the medium with spores and leads very rapidly to a dynamic equilibrium among the individual conidia and the conidial aggregates. The particle concentration remains constant through two inverse reactions: aggregation of conidia to conidial aggregates and disintegration of these aggregates. Both the rate constants of formation and disintegration of aggregates have been determined by measuring the concentration of conidia at the beginning of the cultivation ($N_0$) and the concentration of particles at steady state ($N_{SS1}$) during the first hours of cultivation. In contrast to the first aggregation step, where the collision of conidia is presumed to be responsible for the process, the second aggregation step is thought to be initiated by germination of conidia about eight hours after starting

the cultivation. Germination and hyphal growth of germ tubes provide additional surface for the attachment of non-germinated conidia, which leads to a strong decrease in particle concentration. This second aggregation step has a much larger share in the time space of the entire aggregation than the first aggregation. Depending on the spore concentration in the inoculum, only about 20 % of the spores germinate during the second aggregation. The germination time of *A. niger* was shown to increase with raised osmolality [Wucherpfennig et al., 2010]. After several hours at the end of the second aggregation step a second steady state is reached at a lower particle concentration ($N_{SS2}$). The specific hyphal length growth rate and the ratio of particle concentration to the growing adhesion hyphal surface are decisive matters of the second aggregation step.

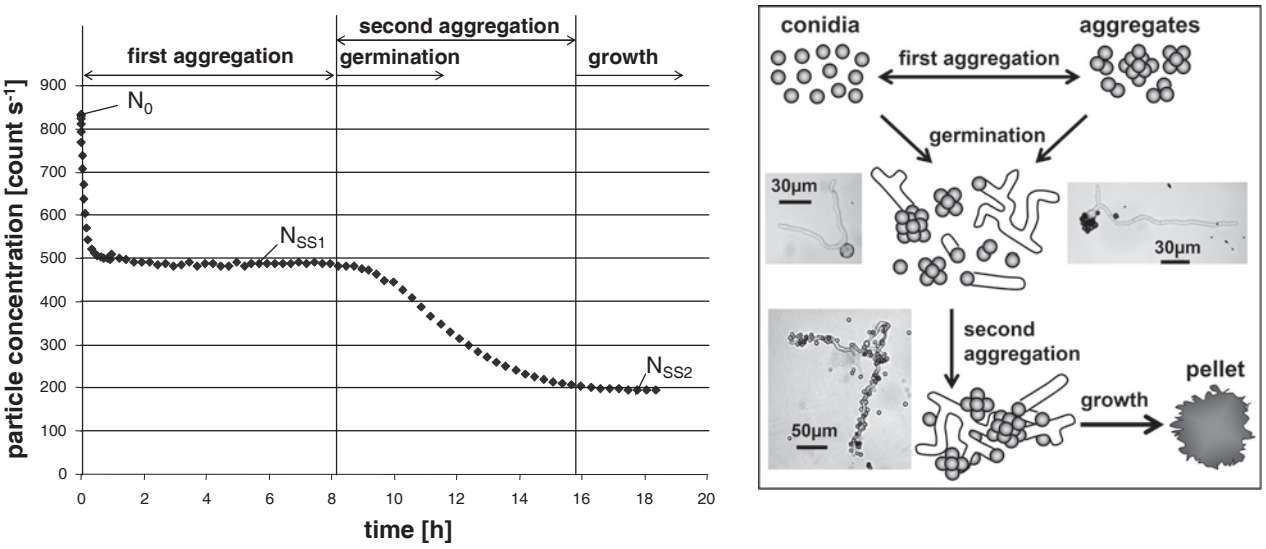


**Figure 2.1:** Kinetic model of conidial aggregation of *A. niger* AB1.13 based on experimental results; left: characteristic time course of particle concentration; right: aggregation model derived from kinetic data [Grimm et al., 2004; Kelly et al., 2006; Lin et al., 2008]

The pellet formation mechanism of *A. niger* shown in Figure 2.1 is confirmed by the measurements of the aggregate chord length with the aid of an in-line particle size analyzer (FBRM D600L, Lasentec) and the microscopic image analysis [Grimm et al., 2004; Kelly et al., 2006]. Both aggregation steps can be described by population dynamics and simulated by using the program package PARSIVAL (PARticle SIze eVALution, Computing in Technology GmbH (CiT), Rastede) for

the treatment of general particle population balances [Grimm, 2006; Lin et al., 2008]. From the conidial and hyphal aggregates, biopellets can be formed in the process of further cultivation [Grimm et al., 2004; Kelly et al., 2006].

According to DIN (German Institute for Standardization) 53206, an aggregate is a collection of primary particles that have tightly intergrown together, whose surface is smaller than the sum of the surfaces of the primary particles. An agglomerate, in contrast, is only a loose combination of primary particles. In this sense, the first step of conidial aggregation is more precisely a conidial agglomeration step. However, through hyphal growth and branching an aggregate is formed out of an agglomerate according to DIN 53206. To avoid misunderstandings, the term ”aggregation” is uniformly used for the first and second step.

An increase in spore aggregation, however, can increase production of pellets only to a limited extent. The formation of pellets is not only effected by strain-specific properties, but also, for example, by the fluid dynamic conditions in the reactor [Amanullah et al., 2002; Grimm et al., 2005]. The fluid dynamic conditions take part not only in the process of spore aggregation but also in hyphal break, hyphal growth and hyphal branching in various degrees. Further studies of Grimm et al. (2005) with respect to the aggregation behavior of spores in the early stage of cultivation have shown that the first aggregation is influenced by the fluid dynamics only to a small extent. The diameter of the flow eddies in a given reaction system is much larger than the resulting size of conidial aggregates. On the contrary, the fluid dynamic effects, besides the influence of surface properties, are responsible for the second aggregation. The aggregates of spores and hyphae reach sizes that can be influenced by the flow eddies, so that the exposed hyphae of the aggregates are subjected to mechanical stress and shaved off from the surface. This means that at this stage of cultivation, the aggregates are increasingly influenced by the fluid dynamic conditions. An increase in mechanical stress resulting from a higher power input leads to smaller and more compact pellets [Grimm et al., 2004 and 2005].