
1 Introduction

1.1 The concept of morphology and its use as a bioprocess parameter

The concept of morphology was first introduced by Johann Wolfgang von Goethe for description and differentiation of natural entities such as leaves [1]. The word originates from the Greek word “morphe” meaning *form* and “logos” translating to *word* or *research*. Transitional morphological forms were used by Goethe as a means of tracing identity, particularly in botany where morphologic appearance is most important for identification and naming [2]. Since then, morphology has been accepted as a general term for description of the outward appearance of an object and is considered to have two major aspects, shape and surface texture [3]. Color, pattern and size are also often used morphological characteristics. Shape is the expression of external morphology and for some is synonymous with form. However, these termini are regularly confused with each other [4]. Shape is a fundamental property of all objects, but it remains one of the most difficult to characterize and quantify for all but the very simplest of shapes [3]. Outward appearance can seldom be described by discrete morphological attributes; many of the morphological descriptors used routinely represent imprecise variables, such as long, short, large or irregular, which are often used in an ambiguous manner [5]. Techniques of geometric morphometrics, in contrast, are able to describe shapes accurately by mathematical geometry in terms of form, roundness, irregularity and sphericity. A more accurate characterization of shape also leads to more potential sources of morphological variation available for analysis [5].

In biotechnological processes the morphology of cells or microorganisms has been often recognized as being important, because general conditions of many organisms can be judged by their appearance. For mammalian cells, for example, cell size has been recognized as a major determinant of productivity [6]. For plant cells in suspension aggregate size has been acknowledged as an important process parameter [7], whereas the coloration of cells was shown to provide an indication on cell age and viability [8]. For morphologically very complex filamentous microorganisms like *Aspergillus niger*, specific morphologic phenotypes have been revealed to correlate with maximum process performance [9]. Most data, however, is based on observation and appearance. Since an accurate quantification of morphology is mostly non-existent, it is hard to incorporate morphological data into existing process models. To be of value as a process parameter, an accurate quantification has to be established. Quantified morphological information can be used to build morphologically structured models of predictive value [9].



The use of computer-assisted automatic image analytic systems combined with geometric morphometrics has already been shown to be an invaluable tool for characterization of complex relationships between morphology and productivity. New models of morphology and process performance will lead to improved design and operation for eukaryotic cultivations, and will help to transfer laboratory models into industrial practice.

1.2 Objectives

One of the main aims of this thesis was to elucidate the relationship between morphology and process performance for two industrially relevant eukaryotic model processes: the production of fructofuranosidase with the filamentous fungus *Aspergillus niger* and the synthesis of paclitaxel with *Taxus chinensis* plant cell cultures. The first and foremost goal was the development and evaluation of novel methods for characterization and quantification of fungal morphology, based on microscopy and automatic image analytic techniques. A further objective was the assessment of additional tools for adjustment and customization of *A. niger* morphology, besides the already established method of micro particle supplementation [10-14].

Using established parameters for detailed morphologic description and new tools for morphologic adjustment, the investigation of the relationship between fungal morphology and productivity was a major focus. Furthermore, fungal morphology was to be correlated with rheological culture broth characteristics. All these findings were to be used to demonstrate the connection between morphology, rheology and productivity.

For the industrially established process of paclitaxel production by submerge cultivation of *Taxus chinensis* plant cells, a reliable and applicable way to measure aggregate size was to be determined and evaluated. Moreover, the connection between aggregate color and cell viability was an aspect for investigation. To achieve this goal, a reliable molecular assay for viability estimation had to be introduced first.

2 Theoretical Background

2.1 Cultivation of filamentous Microorganisms

Due to their metabolic diversity, high production capacity, secretion efficiency, and capability of carrying out post-translational modifications, filamentous fungi are widely exploited as efficient cell-factories in the production of metabolites, bioactive substances and native or heterologous proteins, respectively. The commercial use of fungal microorganisms is reported for multiple sectors such as detergent industry, food and beverage industry, and pharmaceutical industry [9, 15-17]. However, one of the outstanding, and unfortunately, often problematic characteristics of filamentous fungi is their morphology, which is much more complex than that of unicellular bacteria and yeasts in submerged culture [13]. Depending on the desired product, the optimal morphology for a given bioprocess varies [18]. Optimal productivity correlates with a specific morphological form [14, 19, 20].

2.1.1 *Filamentous fungus Aspergillus niger and the model product fructofuranosidase*

The filamentous fungus *Aspergillus niger*, the black mold, belongs to the division Ascomycota, defined by the ascus (from the greek word “sac”), which is formed as microscopical sexual structure by spores by some of its members. The Ascomycota are the largest phylum of fungi, with over 64,000 species, which may be either single-celled (yeasts), filamentous (hyphal) or both (dimorphic) [21]. Most ascomycetes grow as mycelia and can form conidospores; they are able to reproduce in sexual and non-sexual form. Some molds, like *A. niger*, can only reproduce asexually, do not have a sexual cycle, and do not form asci [21]. For *A. niger*, asexual reproduction occurs through the dispersal of conidia, produced from fruiting bodies termed conidiophores, the morphology of which can vary extensively from species to species [21].

The genus *Aspergillus* comprises about 250 species [22], including important industrially used species (*A. niger*, *A. oryzae*, *A. awamori*, *A. sojae*, *A. terreus*) and pathogenic and potentially harmful species (e.g. *A. fumigatus*, *A. parasiticus*, *A. flavus*) [23]. In their natural habitat, the soil, *Aspergilli* form a mycelial network of hyphae. They degrade an abundance of organic material, which is broken down into low-molecular-weight compounds which can be used as nutrients. The degradation is achieved by secretion of several hydrolytic enzymes, which are able to break down macromolecules like sucrose, starch, pectin, cellulose or even lignin [24].



Aspergilli can grow at a wide range of temperatures (10–50 °C), pH (2.0–11.0), and osmolarity (from nearly pure water up to 34% salt) [25], and are able to excrete large amounts of metabolites, e.g. up to 200 g L⁻¹ of citric acid into the culture medium [26]. As eukaryotic organisms, *Aspergilli* offer valuable advantages for enzyme secretion, such as facilitated proteolytic processing and protein folding as well as posttranslational modifications [16]. Furthermore, the genus can be used for solid-state or submerged fermentations and respective fermentation protocols have been established for large-scale industrial processes [23]. *Aspergilli* have been used for food production and beverage processes for more than 1,500 years [27]. *A. niger*, especially, stands out as a very attractive host for the biotechnology industry, partly due to its GRAS status (generally regarded as safe) issued by the American Food and Drug Administration (FDA). Industrial strains can secrete large quantities of many economically desired products, e.g. 25 g/L cephalosporin, 20 g/L glucoamylase, 40 g/L cellulase and 50 g/L penicillin [9, 28, 29]. Because of the progressing development of genetic engineering and efficient expression systems, *Aspergillus* species are also starting to receive attention as a host for the production of heterologous proteins [30].

In the current study the enzyme β -fructofuranosidase (EC 3.2.1.26) was produced. Fructofuranosidase is used industrially in the confectionery and food industry for the production of inverted sugar. The substrate sucrose is converted by specific cleavage of the β -1,2-glycosidic bond between the monosaccharides glucose and fructose [31, 32]. The enzyme also has a fructosyltransferase function through which higher molecular sugars, so called fructooligosaccharides (FOS) can be formed [32-34]. All oligosaccharides from D-fructose molecules with β -(2-1)-glycosidic bonds and a terminal glucose residue are called FOS [34]. For the food industry, FOS are of interest mainly as nutritional supplements and alternative sweeteners [34, 35]. They are only a third as sweet as sucrose and cannot be digested by human digestive enzymes, making them calorie-free [34-36]. FOS also promote the growth and activity of symbiotic gut bacteria, reduce levels of cholesterol, phospholipids and triglycerides in the blood, strengthen the immune system and thus have a positive impact on general health [33-37]. Fructooligosaccharides have also been proven to reduce risk of colorectal cancer, thus a future application of FOS as a complementary strategy in the prevention and treatment of colorectal cancer is feasible [37-39]. These functional properties make FOS interesting for the pharmaceutical industry, as prebiotic food components, for the production of functional foods or as therapeutics [33, 35].

Currently, the market value of fructooligosaccharides is at U.S. \$ 200 per kilogram, with applications in prebiotic products which are already commercially available, in the pharmaceutical



and diagnostic sector [35, 37, 40]. In the future, custom made FOSs might even increase the application potential. These could be enzymatically-produced sucrose analogues in which, for example in place of the glucose, the sugars mannose, galactose, or xylose are linked to fructose. The resulting novel, customized FOS may be of interest due to their properties for various industries and applications [37]. Compared to other strains, the *A. niger* strain SKAn 1015, used in this study, is sufficiently more productive to be interesting for industrial applications [37].

2.1.2 Growth and morphology of *A. niger*

In submerged cultivation two distinct growth forms of *A. niger* can be observed, the mycelial and the pelleted form [41-43]. Pellets are characterized by the mycelia developing into stable, spherical aggregates consisting of a more or less dense, branched and partially intertwined network of hyphae [43-45]. The morphological form which is adopted is a subject of considerable interest, due to the direct and indirect impact of morphology on produced metabolites [9, 46]. Moreover, process characteristics will often vary most significantly between the myriad morphological growth forms which are formed by the filamentous microorganism during submerged cultivation (Figure 2.1).

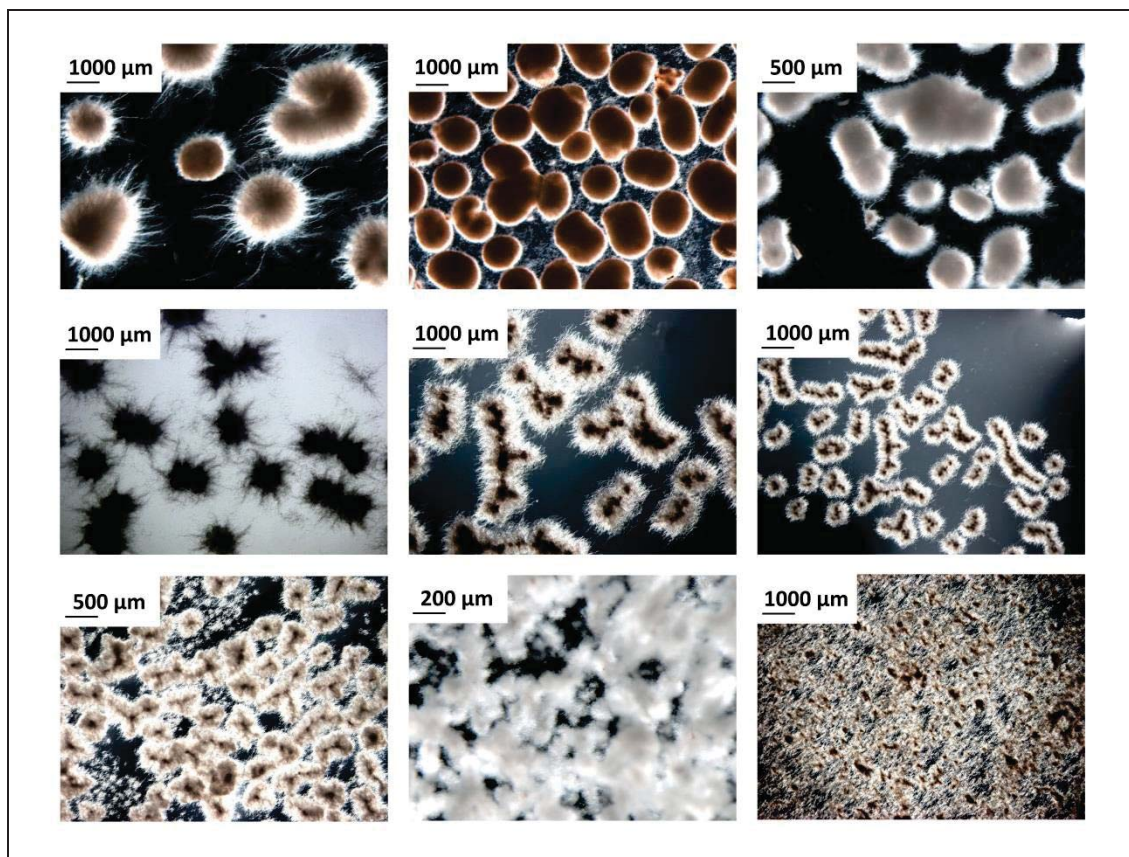


Figure 2.1: Morphologic diversity of filamentous fungus *A. niger* SKAn 1015 in submerged bioreactor cultivation.