

Fungal Morphology in Industrial Enzyme Production—Modelling and Monitoring

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Abstract Filamentous fungi are widely used in the biotechnology industry for the production of industrial enzymes. Thus, considerable work has been done with the purpose of characterizing these processes. The ultimate goal of these efforts is to be able to control and predict fermentation performance on the basis of “standardized” measurements in terms of morphology, rheology, viscosity, mass transfer and productivity. However, because the variables are connected or dependent on each other, this task is not trivial. The aim of this review article is to gather available information in order to explain the interconnectivity between the different variables in submerged fermentations. An additional factor which makes the characterization of a fermentation broth even more challenging is that the data obtained are also dependent on the way they have been collected—meaning which technologies or probes have been used, and on the way the data is interpreted—i.e. which models were applied. The main filamentous fungi used in industrial fermentation are introduced, ranging from *Trichoderma reesei* to *Aspergillus* species. Due to the fact that secondary metabolites, like antibiotics, are not to be considered bulk products, organisms like e.g. *Penicillium chrysogenum* are just briefly touched upon for the description of some characterization techniques. The potential for development of different morphological phenotypes is discussed as well, also in view of what this could mean to productivity and—equally important—the collection of the data. An overview of the state of the art techniques for morphology characterization is provided, discussing methods that finally can be employed as the computational power has grown sufficiently in the recent years. Image analysis (IA) clearly benefits most but it also means that methods like near infrared measurement (NIR), capacitance and on-line viscosity now provide potential alternatives as powerful tools for characterizing morphology.

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These measuring techniques, and to some extent their combination, allow obtaining the data necessary for supporting the creation of mathematical models describing the fermentation process. An important part of this article will indeed focus on describing the different models, and on discussing their importance to fermentations of filamentous fungi in general. The main conclusion is that it has not yet been attempted to develop an overarching model that spans across strains and scales, as most studies indeed conclude that their respective results might be strain specific and not necessarily valid across scales.

Keywords Filamentous fungi • Industrial enzymes • Morphology • Rheology • Mass transfer • Productivity • Submerged fermentations • Modelling • Monitoring • Optimizing

List of Abbreviations

AMG	Glucoamylase
CM	Capacitance Measurement
EDCF	Energy Dissipation Circulation Function
FDA	Food and Drug Administration
GMP	Good Manufacturing Practices
GRAS	Generally Recognized as Safe
IA	Image Analysis
NIR	Near Infrared Measurements
OTR	Oxygen Transfer Rate
OUR	Oxygen Uptake Rate
PAT	Process Analytical Technology
PLS	Partial Least Squares
VSC	Vesicle Supply Center

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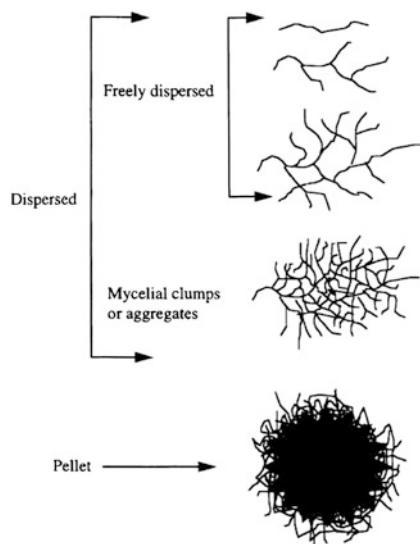
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1 Introduction

Filamentous fungi are widely used in the biotechnology industry for the production of different compounds like organic acids, industrial enzymes, antibiotics, etc., for an extensive list see Papagianni [1]. The widespread use of filamentous fungi as production host is due to three main advantages which the fungi possess: (1) Filamentous fungi have an exceptional ability of secreting large amounts of proteins [2]; (2) They possess a special posttranscriptional modification machinery which allows for glycosylation, correct protein folding, etc. [3]; and, (3) A large number of species are approved by the regulatory authorities and generally recognized as safe (GRAS). Nevertheless, operating a process with filamentous fungi also has a few major disadvantages due to the unavoidable oxygen transfer and mixing limitations that occur as a consequence of the high viscosity of the medium, which is due to the combination of the high biomass concentration and the fungal morphology [4].

Filamentous microorganisms manifest two main types of morphology in submerged fermentations, usually classified as dispersed and pelletized morphology. The first category is characterized by biomass that grows in the form of freely dispersed hyphae or mycelial clumps, see Fig. 1. In the second category, pellets are highly entangled and dense spherical agglomerates of hyphae which can have diameters varying between a couple of micrometers up to several millimeters [5]. Depending on the desired product, the optimal morphology—and here optimal is to be understood as yielding the highest productivity—for a given bioprocess varies and cannot be generalized; in some cases both types of morphology are even combined in one process [6]. The pelleted morphology type is often preferred because of the resulting Newtonian fluid behavior of the medium which allows for

Fig. 1 Types of morphology typically found in submerged cultures of filamentous fungi [5]



better mixing and simplifies downstream processing in terms of pumping and separation of the biomass. However, the pelleted morphology results in nutrient concentration gradients within the pellet [7]. This situation is not observed in freely dispersed mycelia allowing for enhanced growth and production (provided sufficient bulk mixing capacity is available), which has been attributed to the fact that the morphology at the microscopic level has an influence on the production kinetics, e.g. on the secretion of enzymes. The latter was reported by Spohr et al. [8] who observed an increase in protein secretion from a more densely branched mutant of *Aspergillus oryzae* in comparison with the wild type. However, on the macroscopic level this type of morphology greatly affects the rheology of the fermentation broth, and therefore the transport processes in the bioreactor, and will thus increase the required power input for broth homogenization (mixing). So, the morphology of filamentous fungi is double edged, as the productivity as well as the fermentation conditions can be affected by the outer appearance of the fungus. The challenge is to separate these effects to be able to connect productivity gains to the correct phenomenon causing it. If this challenge could be overcome, the process knowledge of the fermentation scientist would be enriched tremendously, and would undoubtedly result in productivity gains.

The aim of this paper is to give a review of the research work that has been done in order to elucidate the relation between morphology and productivity and all the related variables in filamentous fungi fermentations, specifically for the production of industrial enzymes. In order to do this, an introduction to the main industrial strains is given, followed by a brief review of the morphology and physiology of filamentous fungi. A short description of the complex interaction of the different variables involved in submerged fermentations is presented. Also, this review will give an overview of the technologies that are available for morphology characterization and will discuss the latest technologies. In the final section, the paper links the capacity to characterize and model morphology to potential applications for influencing or controlling morphology as a tool for future process optimization.

2 Filamentous Fungi for Enzyme Production

2.1 Important Strains and Products

Due to their exceptionally high capacity to express and secrete proteins, filamentous fungi have become indispensable for the production of enzymes of fungal and non-fungal origin. Currently, native or recombinant industrial enzymes are mainly produced by *Aspergillus niger*, *A. oryzae* and *Trichoderma reesei* [9, 10].

The *Aspergillus* genus is one of the favorite expression systems in the production of industrial enzymes, and in particular the species *A. niger* and *A. oryzae* have been frequently used, due to their high titers of native hydrolytic enzymes, especially amylases and proteases [11]. Glucoamylase (AMG) is a homologous protein of

A. niger used for the conversion of starch to sweeteners and in the production of first generation ethanol [12]. Amylases are also added to detergents to assist in stain removal [13]. Other enzymes produced by these microorganisms include glucose oxidases, catalases, pectinases, lipases, phytases and xylanases, which are usually used in the food, detergent, textile, pulp and paper industry [14].

T. reesei is mainly known for producing cellulases, which are enzymes capable of degrading cellulose into simple sugars. They are widely used in the pulp and paper industry for the reuse of waste paper [15]. In addition, cellulases are also used within the textile industry for cotton softening and denim finishing. Another important application of these enzymes is within detergents, where they are used for color care, cleaning and anti-redeposition in washing powders [13]. Also, an enormous interest in these enzymes has arisen in the biofuel industry, as they are used in the saccharification of lignocellulosic materials which will be converted to bioethanol later on [16, 17].

2.2 Introduction to Morphology of Filamentous Fungi

Filamentous fungi are complex microorganisms constituted by complicated hyphae. A hypha is formed by one or more cells surrounded by a tubular cell wall. A hyphal element is formed by a main hypha that emerges from one spore; this main hypha is typically branched, and these branches have their own sub-branches and so on [18], as displayed in Fig. 1. *Ascomycota*, the group of organisms in which the fungi covered in this review are included, have hyphae that are divided into compartments by internal cross-walls called septa. Each septum possesses a pore large enough to allow cell organelles to flow between compartments. The collective term for the mass of hyphae is mycelium, Fig. 1. Furthermore, a hyphal element can entangle with another hyphal element and form more complex structures. The morphology of filamentous fungi is usually characterized by four variables: the length of the main hyphae (L_c), the total length of all the hyphae (L_t), the number of tips (n) and the length of a hyphal growth unit (L_{hgu}) [18]. The reader is referred to Table 1 for the definitions of additional morphological terms. The hyphal cell wall is formed by polymeric microfibrils of various biochemical composition arranged in a series of layers [19]. The microfibrils forming the hyphal wall usually consist of chitin, a polymer of *N*-acetyl-glucosamine [19].

One of the most important and interesting things to recognize in filamentous fungi is their apical extension just at the hyphae tips. This theory was established in the nineteenth century, when Reinhardt [20] proposed that fungal growth takes place by enlargement of the hyphae only at the apices. The elongation occurs by means of wall expanding according to a gradient, maximally at the extreme tip, and the materials necessary for cell wall expansion are provided by the cytoplasm [20]. Several growth models aiming to describe the exact mechanisms of how this process takes place have been proposed, and the most important ones are the steady state model [21, 22] and the hyphoid model [23]. Some other models have also

Table 1 Common morphological terms [92]

Area or projected area	—area of the projection of a three-dimensional object into a two-dimensional image
Main hyphal length	—length of the main hypha in a mycelium, which might be taken as the longest connected path though a mycelial tree
Branch length	—length of an individual hyphal branch
Total hyphal length	—sum of main hyphal length and all branch lengths in a mycelial tree
Branching frequency	—number of branches (and sub-branches) in a mycelial tree
Number of tips	—number of branches plus two (for the main hypha. Some tips might be extending (growing); others not
Hyphal growth unit	—total hyphal length divided by the number of tips

been developed but these should rather be considered as combinations of the two former models [24, 25]. This is probably how the process is indeed carried out, since the main two theories are not self-exclusive given that they describe different features of the wall building process during tip growth [24].

In general, the theories describe hyphal growth as a consequence of a combination of wall biogenesis and turgor pressure. Wall biogenesis is ultimately an activity of the cytoplasm and that is where the building materials and necessary enzymes are synthesized; they are then later on transported in vesicles to the hyphal tip. These vesicles are accumulated in the apical dome forming a moving vesicle supply center (VSC). The VSC is an organelle from which vesicles move radially to the hyphal surface in all directions at random, and the forward migration of this pseudo-organelle is what generates the hyphoid shape [25]. Then, in the hyphal tip there are two main processes taking place, softening and hardening of the apical cell wall caused by the enzymes carried out in the vesicles. This process makes the hyphae tip more plastic and that is precisely where protein secretion takes place, carried out by a bulk flow from the cytoplasmic side to the wall. Wösten et al. showed with immunological techniques that secretion of glucoamylase in *A. niger* was carried out at the hyphal tips [26]. It is important to have this fact in mind, since it gave direction to the different research projects that were done in the area, as further described below.

2.3 Complexity of the Subject

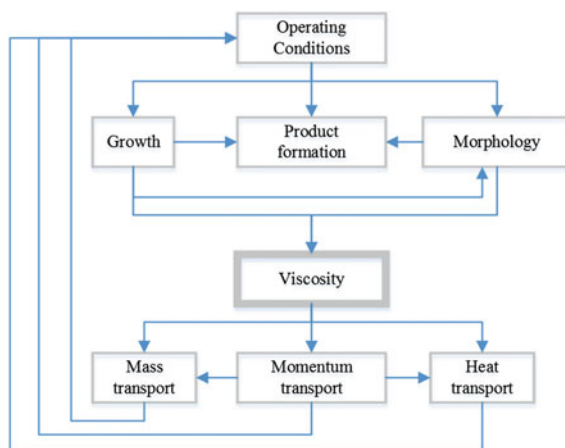
In terms of mass transfer and rheology, filamentous fungi are very challenging hosts for the production of proteins in submerged fermentation, since their morphology is connected to these two variables, both at the microscopic and macroscopic level. Therefore, studying the relationship between fungal morphology and productivity

in submerged fermentations is not an easy task due to the abundance of interrelated factors, which affect directly and indirectly the microorganism's morphology and product formation.

The particular morphology of a filamentous fungus leads to entanglements of the hyphae at high biomass concentrations (20–50 g/L); this phenomenon causes very high viscosities with non-Newtonian fluid behavior [27]. Considering a typical stirred tank reactor, it is well-known that the shear rate is at its maximum at the agitator tip, and it decreases when approaching the vessel walls. Therefore the viscosity will be low close to the impeller, and will increase towards the vessels walls. This results in a lot of problems with respect to mass transfer, momentum transfer and heat transfer [27]. Metz et al. describe the viscosity as the center of the multi-directional and circular interrelation of all the factors, see Fig. 2. Thus, the broth viscosity has a major effect on the transport phenomena in the bioreactor which then will affect the process conditions [27]. Metz et al. considered the process conditions at the top of the complex interactions. These process conditions include the medium composition, mode of operation, temperature, pH, etc. The process conditions will directly affect the morphology, product formation and growth; all these variables are correlated with each other [27].

In addition to all the above-mentioned factors, there is also the fungal physiology and metabolism, which will affect morphology. For example, there is a continuous discussion with respect to shear damage of filamentous fungi; i.e. damage and fragmentation of filaments can be caused by shear forces from the impeller and by aeration. However, the aging factor of the microorganisms also plays a role making the cell walls weaker when cells grow older, and thus more susceptible to fragmentation [4]. Autolysis is another phenomenon observed in filamentous fungi which in addition contributes to hyphal fragmentation [28]. Also strain optimization plays a role, especially classical mutagenesis, since selected strains might increasingly form more single cell like structures. This results in lower viscosities, and thus better oxygen transfer, rather than higher titers [29].

Fig. 2 Representation of all the interrelated variables in submerged fermentation. Adapted from [27]



3 Technologies for Morphology Characterization

The focus in this review paper is on on-line measurements as they have the advantage that sampling is not required. Thus, the complete content of the bioreactor is eventually measured, and there is no reduction in data quality due to sample handling or storage. Recent developments, especially in data handling, have led to the development of several approaches for rapid data generation. In the following, some methods will be discussed which benefitted greatly from the increase in calculation power to run complex algorithms which allow to interpret sensor signals with the aim of predicting biomass behavior. The latest development is Laser Scattering which in theory is able to measure crystal slurries or the shape of cells in fermentations. In practice, there is a question mark on how this technique can be employed in fermentation. Current literature describes that there might be challenges with regards to turbidity of the fermentation broth, cell density and air bubbles [30]. Despite the big potential, more mature methods will be discussed.

Image analysis

Image analysis (IA) would be the natural choice to analyze morphology as biomass literally can be seen. By taking pictures, the different morphologies (macro or micro, depending on the magnification) are digitally frozen and the obtained picture has to be processed to provide numerical characteristics of the broth sample. Therefore, IA as a method has to be divided into two phases: (1) acquiring the image; and, (2) evaluating the image. Both phases have their specific challenges.

Probes with the purpose of taking images in situ started to be described in the mid-90s. A good description of the state of the art from that time is given by Suhr et al. [31]. The challenges in *S. cerevisiae* cultivation were the resolution of the resulting picture, obtaining the correct focus and the proper illumination triggered with the correct timing. Almost 20 years later, Suhr also co-authored an article in which in situ microscopy was proved adequate for measuring/monitoring animal cell cultures in perfused bioreactors [32]. The above-mentioned start-up difficulties (resolution, illumination, focus) could be overcome. Systems are described with mechanical [33] or optical definition of sample volume [34]. The latter could suffer greatly from turbid liquids and probe fouling. Some mechanical samplers/in situ microscopes feature a retractable sample chamber that could be steam cleaned and hence biomass attached to the lens could be removed [35]. Still, problems with air bubbles, turbid media and fouling of the probe, especially when dealing with filamentous organisms, have not been solved completely yet.

IA in the 90s was mainly manual meaning that tips, hyphae and/or particle diameter had to be annotated by the user (e.g. [36]). Since then, the digital processing power increased greatly and in 2011, Barry and William attested that on-line calculations of total hyphal length and number of hyphal tips amongst others now are possible [6]. There are many parameters that could be acquired and described with IA. In the case of pelleted growth, it could be the projected area of the particle, diameter, shape and circularity. Lately, also calculations via fractals have been included [37, 38]. As long as manual adjustments have to be made to acquired

images, it might be faster to employ other measurement principles, i.e. laser diffraction as well/instead [39]. Else, considerable achievements were made within dedicated algorithms for IA. Please refer to Papagianni (2014) for the latest technology, which provides an overview of methods and processes, respectively [40].

Near infrared measurement (NIR)

The on-line NIR suits the regulatory requirement for process analytical technology (PAT) even for pharmaceutical purposes as it is a fast measurement, the sensors can be built in accordance to GMP regulations, and it allows running a well-defined process. The measurement principle is the absorbance of near infrared wavelength by the medium leading to specific spectra for each compound present in the media [41]. To be able to interpret the spectra correctly, deep knowledge about the process is required [42], as well as proper calibration [43] which leaves out the determination of most complex ingredients by means of NIR. It is furthermore not possible to measure reliable NIR spectra in turbid media, which includes aerated media to some extent [44].

Usually, there are disturbances in the measurement, and while the trends look similar, details of the spectra might vary and thus, pre-processing of data is applied which removes assumed artefacts of the measurement [45]. To even out some of the peaks in the measurement and to pronounce some minor effects, the second derivative of the wavelength spectrogram is the basis for further analysis [43]. The data handling can be reduced in complexity by restricting the analysis to just one major compound for just two frequencies and by use of a Fourier transformed signal [46]. In fact, the data handling is the most important part of the NIR measurement as the signal can be correlated to other parameters like biomass, product or substrate concentration. A challenge though is that the NIR spectrum has to be interpreted, and sometimes (especially when in lack of deep process knowledge), several explanations could be attributed to the same spectrum.

There currently is no literature present about the use of NIR to characterize or control fermentations with filamentous organisms. It has been successfully demonstrated that *S. cerevisiae* fermentations can be controlled and the respective responses can be predicted using a soft sensor based on the input of a PLS (Partial Least Squares) modelled NIR signal [47].

Capacitance

Starting in the early 90s, the capacitance measurement (CM) or dielectric spectroscopy [48] became increasingly popular and was tested for different biotechnological processes [49]. While originally more used for yeast fermentations [50], the attention very soon also turned to other microorganisms as well as fungi [51]. The experiments reported in the work of Krairak et al. [51] were characterized by pellet formation of the fungus—thus not really filamentous growth—and considerable disturbances by air bubbles and agitation. Even though the low pass filter for removing noise from the signal was described before [52], its technical application was first possible with the availability of increased miniaturized calculation power.

This measurement technique is extremely interesting for online bioprocess monitoring because the measurement principle differentiates between live and dead

biomass: “Cells behave as capacitors due to the presence of charged molecules both inside (cytoplasm) and outside (culture broth), separated by a plasma membrane. Capacitance, measured by application of an electric field, is directly proportional to the cell concentration—Dead cells without intact membranes do not contribute to charge polarization” [53]. The same source marks this technique as “promising” for pharmaceutical GMP purposes which means that there might be good potential also for industrial fermentations aiming at production of bulk products. It is advised though to take the same approach to process control as with NIR: Verifying the CM with other, maybe even off-line, methods [54], in order to reduce the interferences to the signal from stirring and agitation.

In the special case of filamentous organisms, the publications are rather scarce. Posch et al. concluded that the currently best use of the CM would be a good building block for a soft sensor that calculates parameters related to broth rheology and organism morphology [55]. In contrast, Rønnest et al. demonstrated that good correlations between the capacitance signal and off-line dry matter measurements could be achieved for a filamentous fermentation broth [44]. With a scan through different frequencies, it was also possible to retrieve additional data about the relative size and the distribution of cells. In the case of the (non-filamentous) organism *E. coli*, a combination of a CM and a soft sensor based on first principles elemental balances successfully detected cell changes at the time of induction with subsequent predictions of the final titer [56]. Also, CM is considered to be an important tool in view of the FDA PAT initiative that was launched 2004: It is a stable technique able to provide real-time process-related information through non-destructive and non-invasive measurements [42].

The often expressed limitation of CM is the conductivity of the medium, if a medium with high salt concentrations is employed (above 50 mSiemens). The status in 2014 is though that it is possible to go as high as 100 mSiemens while still producing a signal of acceptable quality (personal communication, Bent Svanholm, Svanholm.com).

On-line Viscosity

It is often reported that different morphologies cause different fluid behavior of the broth, which is expressed in the broth viscosity. Several publications came to the conclusion that the viscosity is mainly affected by biomass concentration and morphology, stating that filamentous growth leads to an entangled network of hyphae and thus to higher viscosity [27]. Later publications confirmed this trend [36, 55, 57] and therefore, it could be concluded that an on-line viscosity measurement certainly can provide real-time information that is useful in view of controlling fermentations. The challenge is, though, that most on-line rheology measurements in industrial fermenters create a non-uniform shear field, and that parts of the broth may not undergo the same forces. This adds on to the empirical approach to calculate the shear rate based on the torque of the impeller [58]. The lack of precision makes the on-line viscosity measurement a rarely described tool for generating data that can be used in view of fermentation process control. There are works though which correlate the viscosity of the broth with the morphology,

and consequently with productivity. A non-protein product example was described by Dhillon et al., namely the case of citric acid production with *A. niger* growing on apple pomace sludge, where rheology was altered by adding methanol leading to a less pseudoplastic fluid behavior [59]. In this special case, morphology is not the only factor with an influence on viscosity because also the viability of the cells is reduced by adding methanol. An interesting approach to reduce viscosity was demonstrated by Cai et al. [60]: *A. glaucus* was genetically modified so that it lacked the polarized growth of hyphae. As a result, hyphae grew curved leading to a much denser pellet and less viscous broth, and a more than 80 % increase in the production of aspergiolide A, a secondary metabolite. Most of the latter is attributed to the fact that a better oxygen transfer rate (OTR) could be achieved. This finding shows the importance of being able to monitor the broth characteristics in order to control fermentation. For the moment, however, not that many efforts are put in applying online viscosity to control (industrial) fermentation.

4 Modelling the Morphology

In an effort to achieve process improvements within the biotechnology industry, considerable focus has been put in the developing and maturing of engineering tools which facilitate process optimization. One of these tools is mathematical modelling, including both empirical and first-principles models. For a review of more engineering tools see [61]. Different research projects have been carried out with the objective to model and understand the different phenomena taking place in submerged fermentation, and to develop an improved understanding of the interactions between the variables illustrated in Fig. 2. These attempts have mainly been focused on modeling micromorphology, hyphal fragmentation and rheology, and on developing an understanding on how this affects productivity.

4.1 Micromorphology and Productivity

Since the early 90s, it has been demonstrated that protein secretion in *A. niger* was carried out mainly in the tips of fungal hyphae [26]. Therefore, several studies have been conducted aiming at correlating the number of hyphal tips with enzyme production. As an example, *A. oryzae* producing α -amylase was further investigated by comparing three different strains in batch cultivations [8]. The strains were: a wild-type (A1560), a transformant with extra copies of the coding gene and a morphological mutant (made from the transformant). By comparing the α -amylase concentration at the end of the batch and the specific branching frequency of the two recombinant strains, it was concluded that a more densely branched strain is superior in protein production (Table 2). This higher productivity might be attributed to the fact that the limiting step in high yielding protein strains is the secretion process [8].

Table 2 Final amylase concentration and specific branching frequency of the three strains of *Aspergillus oryzae*

Strain	Final concentration α -amylase (FAU/ml)	Branching frequency— k_{bran} (tip/ $\mu\text{m/h}$)
Wild-type	0.63	0.0023
Transformant	2.22	0.0010
Morphological mutant	3.09	0.0021

Adapted from [8]

Bocking et al. [62] investigated the topic in the same strain (A1560). In addition, they studied a transformant strain able to produce glucoamylase along with α -amylase (AMG#13). Nine morphological mutants were generated from these two strains: 4 from A1560 and 5 from AMG#13. The mutants were screened such as to keep the same maximum specific growth rate and a lower hyphal growth unit length (more branched strains). All the strains were studied in batch, continuous and fed-batch cultivations. No clear correlation between branch frequency and ability of secreting protein was observed for the highly branched mutants in the fed-batch and continuous fermentations. They, however, found a correlation between higher branch frequency and viscosity reduction. This could lead to the conclusion that the observed productivity increase by Spohr et al. [8] was achieved due to a better OTR rather than a higher productivity. However, one should be aware that the comparisons are done in different operating modes (batch vs. fed-batch); thus different physiological state due to different grow rates might have an effect on the rate of fragmentation resulting in systems with different viscosity. Though, in the batch experiments for AMG#13 and its highly branched mutant, the latter did have an increase in glucoamylase compared with AMG#13. This might suggest that under maximum growth rate conditions there is a correlation between branch frequency and secretion, as suggested by Spohr et al. [8].

For the first time, Haack et al. reported the swelling of the hyphal tips as a consequence of high productivity in a recombinant strain of *A. oryzae* producing lipase [63]. It was suggested that tip swelling and productivity are linked, since the hyphae tips return to normal shape after the production stops due to oxygen limitations. It was indicated that these findings could help to identify the fraction of productive cells in industrial fermentation, since it is known that production heterogeneities occur in full scale due to poor mixing [61].

Most of these models are purely empirical without any structured background, and have been mainly developed using IA. Nonetheless, they have been used as practical tools for comparison. Agger et al. developed a morphologically structured model able to describe growth and product formation in batch, continuous and fed-batch cultivations for *A. oryzae* (A1560) [64]. By dividing the fungal hyphae into three different regions—extension zone representing the tips of the hyphae, active zone which is responsible for growth and product formation and an inactive hyphal region, Fig. 3—a model able to predict product formation as a function of morphology was developed. Different to the previous models, it was verified by IA

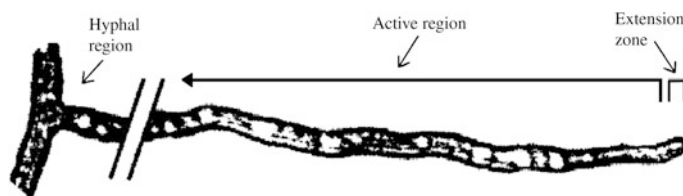


Fig. 3 Illustration of the structuring of the hyphal elements in the model of Agger et al. [64]

combined with fluorescence microscopy. The model performed well in batch and continuous cultivation. However, there seemed to be an under-prediction of product formation for a fed-batch fermentation. This difference was attributed to rheological changes or hyphae fragmentation not accounted for in the model.

As seen in Fig. 2, due to the complex interactions between the variables in a fermentation process it is also possible to affect morphology by changing the process conditions (media composition). Ahamed and Vermette [65] conducted a study where they indirectly affected morphology by varying the carbon source in the batch phase of a fed-batch process. The study was performed in *T. reesei* in the strain Rut-C30 producing cellulases. They observed a more branched morphology in the medium which also presented the highest enzyme titers. A non-linear correlation for the volumetric enzyme productivity was developed as a function of the average projected area of the total mycelia (entangled mycelia plus branched mycelia) and the number of hyphae tips. This study confirms the relation between enzyme productivity and number of tips.

4.2 Shear Stress and Morphology

One of the problems with filamentous microorganisms relies in the oxygen mass transfer limitations in the culture broth due to the high viscosities and the non-Newtonian behavior. An obvious strategy to overcome this problem is to increase agitation power. However, the question of shear damage due to fragmentation or morphological changes arises. Early works indicated that there is shear damage to the cells caused by the impeller which usually leads to reduced productivity. Nevertheless, these studies were conducted in *Penicillium chrysogenum* for penicillin production [66, 67] and are therefore out of the scope of this review. In this section, the research that has been done in order to understand the relation of shear stress caused by the impeller on morphology for filamentous fungi producing industrial enzymes will be summarized, and only for the cases where dispersed mycelium is observed (i.e. not pellets). The other focus will be the work of Jüsten et al. who developed a function capable of correlating mycelial fragmentation and power inputs at different scales and agitator types, the so called energy dissipation circulation function (EDCF) [68]. The EDCF is defined as the ratio of energy dissipation in the dispersion zone to the liquid circulation time. This model

considers the specific energy dissipation and the amount of time that the cells are subject to the shear caused by the impeller (circulation frequency). The theory is an extension of the work done by Smith et al. which managed to account for the circulation frequency [67]. However, Jüsten et al. accounted for different impellers types. Even though the function was developed for *P. chrysogenum*, its applicability was later tested in *A. oryzae* managing to successfully correlate fragmentation [69]; therefore, in this review the EDCF is considered as the standard tool for comparison of the different works.

Amanullah et al. studied the effect of agitation on mycelial morphology and enzyme productivity in continuous cultures of a recombinant strain of *A. oryzae* producing α -amylase and amyloglucosidase. They found that agitation intensity has an effect on morphology. When the agitation speed was reduced by half, the mean projected area increased almost threefold. Productivity, however, was not affected [70]. A later study of the same group carried out with the same strain in fed-batch fermentations confirmed that mycelial morphology (measured as a mean projected area) is dependent on agitation intensity but that there is no correlation with enzyme production assuming a non O_2 limited process for the fed-batch phase. However, in the batch phase there was a positive correlation with agitation intensity and productivity, which could be attributed to higher biomass concentration and growth rates at higher agitation speeds [71]. An important conclusion of these two studies was that given a specific growth rate, it is possible to correlate EDCF with morphology measured as the mean projected area [71].

Fragmentation caused by different specific power inputs measured as EDCF was investigated by Li et al. for full scale fed-batch fermentations [72]. The shear forces caused by the impeller on *A. oryzae* resulted in mycelial fragmentation; however, this fragmentation was not correlated to the specific power input since a similar morphology was observed at the two levels of EDCF. Hence, fungal morphology does not seem to be dependent on mixing intensity at production scale. Effects of changes on impeller power input on productivity were not studied. In a later study this correlation was investigated. Yet again, two specific power inputs measured as EDCF were tested, and neither the morphology, productivity nor rheology differ at these two levels of EDCF under non-oxygen limited conditions [73].

Albæk et al. developed a correlation able to predict viscosity based on the mentioned EDCF and biomass measurements in fed-batch fermentation with *A. oryzae* [74]. The correlation does not take into consideration morphological data; however, the accuracy of the general model prediction demonstrates the ability of EDCF to correlate morphological data as a function of viscosity. They also observed a positive correlation with increasing agitation speed and product formation. This might be correlated to a better OTR, indicating that morphology actually does not necessarily play a major role in productivity [74].

4.3 Morphology and Rheology

The study of the rheology of culture broths has a crucial importance in the design of fermentation processes; viscosity will affect the transport phenomena taking place in a bioreactor. Fluid viscosity appears in several correlations involved in mixing and mass transfer, e.g. the Reynolds number and correlations for the volumetric mass transfer coefficient. Also, the yield stress appears in relationships used to estimate cavern sizes [75]. Therefore, it is important to study the rheology of fermentation broths, and if possible to predict it. The traditional approach for modelling the rheology of filamentous fermentations broths is based on attempts to correlate the parameters for different viscosity models (e.g. power law model) to the biomass concentration [76] (for a review of rheology see [27, 57]). However, these correlations are of a limited value [36]. Viscosity is a property that is not only dependent on biomass concentration, but is affected by the structure and extent of physical interlacing of hyphal networks [77]. Thus, several other studies have focus on correlating viscosity not only to the biomass concentration, but also to the different morphological parameters, i.e. projected area, hyphal growth unit, etc., typically characterized by IA. Other authors have characterized the morphology by other methods in order to correlate it to rheology. These attempts are further described in this section.

The direct relation between the consistency index parameter of the power law model and different morphological parameters was studied for continuous cultivations of *A. niger* [78]. The dissolved oxygen and growth rate were varied at two different biomass concentrations. It was found that at constant biomass concentration the consistency index could be linearly correlated to the roughness. By including a biomass term in a linear model, a good correlation was obtained. In this work, the correlation was not further tested for different levels of biomass concentration or for batch or fed-batch fermentations. It has to be kept in mind that this is one of the first attempts to quantitatively model the rheology of filamentous fungi based on morphology parameters. As a continuation of this work, Riley et al. developed a model to predict the consistency index based on biomass concentration and the mean mycelial maximum dimension (the longest feret across the convex area of a mycelial particle) for *P. chrysogenum*. The model was able to predict the parameter with an average root-mean-squared deviation below 30 %, which despite all the sources of errors is considered as a good result [79]. This microorganism is not included within the strains considered in this review paper. However, as an extension of this work, Riley and Thomas checked the relevance of the correlation to other fungi [80]. They reformulated the model at a different magnification for *P. chrysogenum* and tested its applicability to predict the values of the consistency index for fermentation broth of *A. niger* and *A. oryzae*. The biomass concentration and the mean mycelial maximum dimension are also used in this model as the morphological parameters. The correlation performed well for fermentation broths of *A. oryzae*, but failed to describe the consistency index for *A. niger*. No simple model was found for the flow behavior index. In fact, it was considered as a

constant value, which is not always the case. Similarly, Malouf aimed at correlating the rheology properties of *T. reesei* fermentation broths to morphological parameters [81]. The Herschel-Bulkley consistency index was successfully correlated to the mean roundness by two separate correlations for the batch and fed-batch phases.

Wucherpennig et al. studied the rheology of culture broths from *A. niger* in shake flask fermentations [37]. IA was used for morphological characterization, which was described by conventional and fractal morphological parameters. Two different fractal parameters were good at predicting rheological properties; nevertheless they were not superior to the parameters developed using conventional particle shape analysis (e.g. Morphology number [82]).

Other authors have used laser diffraction and multivariate data analysis as a tool to model the rheology of fermentation broths. Petersen et al. correlated the rheological properties of commercially relevant *A. oryzae* fermentations with respect to particle size distribution data [75]. The study was conducted in fed-batch fermentations where different feed strategies were applied, similar to the work of Bhargava et al. [83]. A partial least squares regression model was able to predict viscosity by using particle size distribution, biomass concentration and process information. In terms of practical applicability, this model is superior to the models that have been developed using IA due to the simplicity of the measurements; however, a limitation is that the model was not able to predict the rheological properties of fermentation broths of different strains and/or scales.

Another aspect which is important to consider when dealing with rheology of filamentous fungi, is how to evaluate the shear rate in the tank. Until now, it has not been possible to estimate a reliable shear rate in the fermentation tank itself. As mentioned before, in an STR it is well-known that the shear rate is at its maximum at the agitator tip and decreases towards the vessel walls. Calculating shear rate can therefore be expressed as the maximum or the average shear rate. Hitherto, it is not clear which shear rate is governing the mass transfer processes, and the way of calculating this shear rate is limited to the Metzner and Otto correlation [84]. According to Stocks [85], it should not be forgotten that this empirical correlation was developed for Reynolds numbers in the laminar and transitional regime and not for turbulent conditions where it is also frequently used in practice. Thus its applicability is limited to laboratory and pilot-scale fermenters, even though it has been typically employed for calculating shear rates in full-scale fermenters also [85]. A special challenge emerges when a reliable shear rate to evaluate the viscosity across different scales has to be estimated. This might be the reason why the developed models were not able to make predictions that apply across scales; therefore, the applicability of other correlations should be explored [86]. Adding on to that challenge is the use of different instruments to measure rheology [36]. Developing models that could predict performance of fermentations with filamentous fungi across scales with a (relatively) high accuracy is therefore still considered to be one of the major scientific challenges in this field.

4.4 Process Conditions and Morphology

Bhargava et al. [83] performed a study where they investigated the effect of different feeding strategies on morphology, protein expression and viscosity during a fed-batch fermentation. The work was done with *A. oryzae* (A1560). Tests with varying the feeding profile were carried out, keeping the same total amount of glucose, but fed in cycles (pulsed feeding). These experiments were compared with a continuously fed fermentation. The biomass, oxygen uptake rate (OUR) and total base added for pH control showed no significant difference indicating that pulsed feeding during fed-batch operation has no apparent effect on cellular metabolic activity. Neither was there a significant difference for the different cultivations in the measured extracellular protein content. Nevertheless, a considerable effect on fungal morphology (measured as average projected area) from the start of the fed-batch phase was observed. The pulsed feeding resulted in smaller hyphal elements in comparison to the elements resulting from continuous feeding. The smaller elements lead to a significant decrease in viscosity. As a consequence, the effect of the cycle time on morphology, rheology and productivity on the same strain was tested in a later work [87]. As before, no effect on biomass was observed, while the mean projected area and the viscosity decreased with the increase in cycle time. Shorter cycles resulted in constant productivity and OUR while longer cycles caused a decrease in productivity at a higher OUR—it appeared that the fungus was forced to form conidia due to starvation. This yet again shows the double sided effect of morphology, viscosity and product formation.

Other authors have also studied the effect of agitation intensity on other process variables, rather than just on morphology. Marten et al. [88] studied the relation between rheology, mass transfer and mixing for *T. reesei* in batch fermentations. They concluded that the Casson model and the Herschel-Buckley model are better in describing the rheological behavior of *T. reesei* broths in comparison with the power law model. However, a practical correlation was not obtained with respect to the effect of agitation intensity on rheology and biomass concentration.

In an attempt to understand the effect of shear stress on morphology and rheology also in *T. reesei*, Patel et al. studied the effect of agitation intensity in fed-batch fermentations [89]. With respect to shear stress and productivity, no clear correlation was observed. With respect to morphology, in the batch phase of the fermentation, no effect on agitation intensity was observed either. However, for carbon source limiting growth, there seemed to be an effect of agitation intensity on morphology, since a higher degree of fragmentation was observed as the fed-batch phase proceeded. It is not clear whether this degree of fragmentation is caused by the agitation or by self-fragmentation of the microorganisms. This however, resulted in a lower viscosity towards the end of the fermentations. A higher apparent viscosity was observed in the experiment with the highest agitation speed, which might be attributed to the higher biomass concentration. The apparent viscosity of all the experiments was evaluated at a constant shear rate and not at the shear rate in the tank, making the comparison difficult.

Table 3 Effects of morphology alteration on productivity, biomass and rheology

Authors	Microorganism	Operation mode	Growth rate (1/h)	Affected morphology parameter	Effect on productivity	Effect on growth rate/biomass	Effect on rheology	Notes
Spohr et al. [8]	<i>Aspergillus oryzae</i> (A1560)	Batch	0.18–0.27	Morphology-branching frequency	Yes. Higher productivity in the more branched strain	Yes. Lower growth rate for the more branched strain	N/A	Comparison at a specific biomass concentration
	<i>Aspergillus oryzae</i> (A1560)	Batch	0.28	Morphology-branching frequency	No	No	N/A	
Bocking et al. [62]	<i>Aspergillus oryzae</i> (AMG#13)	Batch	0.30	Morphology-branching frequency	Yes. Higher productivity for the more branched mutants	No	Yes. Lower viscosity for the highly branched mutants	
	<i>Aspergillus oryzae</i> (AMG#13)	Fed-batch	–	Morphology-branching frequency	Yes. Lower productivity for the more branched mutants	Yes. Lower biomass for the more branched mutant	N/A	Constant feed rate
	<i>Aspergillus oryzae</i> (AMG#13)	Fed-batch	–	Morphology-branching frequency	Yes. Slightly more productivity for one of the more branched mutants	Yes. Slightly less biomass for one of the more branched mutants	Yes. Lower viscosity for the highly branched mutants	DOT controlled feed rate

Table 4 Effects of shear stress on morphology, productivity, biomass and rheology

Authors	Microorganism	Operation mode	Growth rate (1/h)	Conditions	Effect on morphology	Effect on productivity	Effect on growth rate/biomass	Effect on rheology	Notes
Amanullah et al. [70]	<i>Aspergillus oryzae</i> (A1560)	Continuous	0.05	Agitation intensity reduction 1000–550 min ⁻¹	Yes. Increase in mean projected area. Increase in hyphae length. Increase in number of tips	No	No	N/A	
Amanullah et al. [71]	<i>Aspergillus oryzae</i> (A1560)	Batch	Max	Agitation intensity 825, 675, 525 min ⁻¹	Yes. Higher mean projected area at lower agitation speed	Yes. Higher productivity at higher agitation speed	Yes. Higher growth rates and biomass at higher agitation speed	N/A	
Li et al. [72]	<i>Aspergillus oryzae</i> (A1560)	Fed-batch	<0.02	Agitation intensity 825, 675, 525 min ⁻¹	Yes. Higher mean projected area at lower agitation speed	No	No	N/A	These under no oxygen limited conditions
Li et al. [73]	<i>Aspergillus oryzae</i> (A1560)	Fed-batch	<0.03	Power input	No	N/A	No	N/A	Change in morphology as a function of time
Albaek et al. [74]	<i>Aspergillus oryzae</i> (A1560)	Fed-batch	<0.03	Power input	No	No	No	No	These under no oxygen limited conditions
Albaek et al. [74]	<i>Aspergillus oryzae</i> (property strain)	Fed-batch	Function of the feed flow rate	Agitation power	N/A	Yes. Higher productivity at higher agitation power	Yes. Higher biomass at higher agitation power	No significant	For the same impeller choice
Albaek et al. [74]	<i>Aspergillus oryzae</i> (property strain)	Fed-batch	Function of the feed flow rate	Impeller type	N/A	No	Yes. At some conditions higher biomass for the axial impeller	Yes. Lower viscosity for the axial impeller	At the same conditions

(continued)

Table 4 (continued)

Authors	Microorganism	Operation mode	Growth rate (1/h)	Conditions	Effect on morphology	Effect on productivity	Effect on growth rate/biomass	Effect on rheology	Notes
Marten et al. [88]	<i>Trichoderma reesei</i> Rut-C30	Batch	–	Agitation intensity 250, 400, 500 min ⁻¹	N/A	Yes, Higher productivity at the higher agitation speed	No	Yes, Higher viscosity at higher agitation speed	
Patel et al. [89]	<i>Trichoderma reesei</i> Rut-C30	Batch	–	Agitation intensity	No	Not clear	Yes, Lowest growth rate at the lower agitation speed	Yes, Lower viscosity at the lowest agitation	
	<i>Trichoderma reesei</i> Rut-C30	Fed-batch	–	Agitation intensity	Yes, Lower clump fragmentation at the lower agitation speed	Not clear	Yes, Higher biomass at the higher agitation speed	Yes, But not clear correlation	

5 Conclusion

The key issue relies on how to incorporate all the work that has been done for the past years in order to optimize the filamentous fermentation process. As illustrated in Fig. 2, there are many variables which will influence the performance of fermentation processes, and in order to study all of them, an extensive (up to now impossible) design of experiments needs to be performed. The complex inter-correlation renders it impossible to affect a variable while keeping the rest constant, as seen in Tables 3 and 4. Thus, until now the best way of dealing with this appears to be data reconciliation for the work that has been done, which is not an easy task, due to the different set-ups used. For example, Li et al. [72, 73, 90] found no effect on the different variables from changes in specific power input; these results at full scale production differ significantly from the findings at bench scale [70, 71], which again makes it very difficult to draw a practical conclusion about the relation and interaction of all the variables involved in fermentations processes with filamentous fungi.

In addition to this, one of the biggest challenges with respect to the study of filamentous fungi in the production of enzymes is the lack of relevant industrial data. The difference between data generated by academia and the industry is enormous. For example processes studied in this review, and typically reported as a result of a study that has taken place at a university department, deal with titers of barely a couple of grams per liter of extracellular protein. In industry there are reports of titers up to hundreds of grams per liter [13]. The question remains on whether it is possible for industry to apply the results of the model studies developed by academia. It is to expect that the behavior of the industrial microorganisms would be completely different due to the stress on the host organism that is caused by such high expression levels. Therefore, if the aim is to produce results with both academic value and industrial relevance, then it is important to have a proper collaboration between industry and academia in order to overcome this issue. So, there is a need for the definition of one or more well-defined case studies that should be available publicly, with limited but sufficient industrial value to be of practical use. The case studies should allow academia to work in concentration ranges which are relevant for industry. The same is valid for the scale: Bench reactors are just much smaller and not all effects, especially regarding mixing and bubble interference to new probes like NIR, can be studied properly with respect to industrial challenges. However, this is not an easy task, since very different—often competing—interests might be involved: indeed, a problem is that a case study with practical value does not automatically have sufficient academic value, and the other way around.

The reader might also notice that many of the articles analyzed in this review date back to the nineties. It seems like many groups stopped their work in relation to fungal morphology in submerged cultivation around the year 2000. There are some groups continuing research, also employing new technologies/measurement systems. It can just be assumed that the costs for the sensors are too high and/or the probes themselves are too bulky for bench scale reactors. In this case, the above

stated/desired case promoting industry—academia collaboration would become even more important. The trade-off for companies would be releasing (confidential) process data in order to obtain e.g. new process strategies based on the potential use of the new sensor types described in Sect. 3.

Another consideration is that the development of “omics” technologies made it possible to study filamentous fungi from a different perspective [3, 91]. It might be that the solution leading to a more complete understanding of the microorganisms is to be found in an integration of the studies described in this review, and those related to the “omics” field. However, before such integration can be realized and applied in practice on industrially relevant case studies, it is quite clear that considerable additional research work will be needed in this area.

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