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review

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The Morphology of Filamentous Fungi in Submerged Cultivations as a Bioprocess Parameter

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Dedicated to the memory of Professor Vera Johanides

Summary

Morphological characteristics of mycelial submerged cultures have been established as one of the key bioprocess parameters. The morphological type and the related physiology strongly depend on environmental conditions in the bioreactor, and in turn affect the rheological properties of the broth and thereby bioreactor performance. Accordingly, the productivity and energy consumption of the process are functions of the morphology. This review treats fungal morphology with related rheological properties and metabolic activity by emphasizing the influence of engineering variables on biomass growth. Besides, the literature concerning interrelation between the morphology and bioreactor performance including some aspects of bioprocess design is reviewed.

Key words: filamentous fungi, morphology, pellets, bioprocess design

Introduction

Industrial bioprocesses with filamentous fungi embrace the production of a majority of commercially important products of biotechnology, in the sense of quantity as well as the diversity of metabolites. These are mainly the submerged culture processes, where a dynamic relationship exists between environmental conditions and the growth pattern of these modular microorganisms. Distinct cultivation conditions result in different morphological and physico-chemical characteristics of fungal hyphal elements and thereby their tendency to aggregate. Consequently, on a macroscopic level, one can distinguish between the filamentous growth form, where the hyphae are freely dispersed in the medium, and the pellet form, where mycelium develops spherical aggregates, consisting of highly entangled networks of hyphae. Besides, some intermediate forms can be recognized such as flocculent or granular growth (*1*), while the term »clump« has been used either for the description of small loose mycelial aggregates, present in a dispersed growth form (*2,3*), or for big and heavy masses of aggregated mycelium (*4,5*). Fig. 1 presents different macro-morphological forms of filamentous fungus *Rhizopus nigricans*, obtained under different cultivation conditions.

The importance of morphological type for the production of specific metabolites and for the energy consumption related with adequate mixing and oxygen supply has now long been established (*6,7*). Primarily this knowledge was focused on the bulk citric acid (*6*) and penicillin production (*7,8*), while in the last few decades it gained much broader attention and a more theoretical approach, summarized in several review papers (*1,9–14*).

The outstanding progress in methods, enabling quantitative characterization of mycelial morphology, has given new opportunities for better understanding and control of bioprocesses (*3*). The characteristics of hyphal elements, *e.g.* hyphal length and diameter, the

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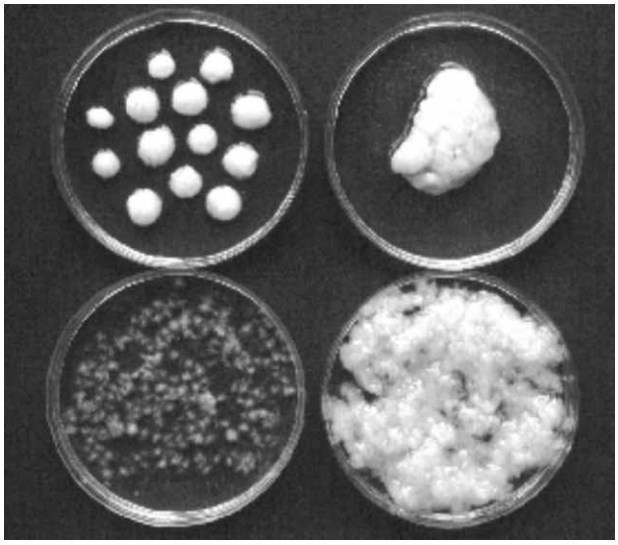

Fig. 1. Different submerged cultivation conditions result in various growth forms of steroid-transforming filamentous fungus *Rhizopus nigricans*

number of tips and branching frequency, were in the last decade introduced into morphologically structured models of mycelial lifecycle and physiology (*11*,*15–17*). Besides, quantitative characterization of cellular differentiation and the related physiology of some industrially important filamentous microorganisms has been reported (*3*,*18–21*).

However, as recently shown on one of the most studied processes with filamentous microorganisms – penicillin G production with fungi of genus *Penicillium* – the complex models of cellular differentiation have so far had a limited potential for the application in automatic control systems for industrial bioprocesses (*22*). For this purpose the study of engineering aspects, such as mixing, aeration and reactor design in relation with fungal growth, and of morphology and productivity is necessary. Biochemical engineers are trying to elucidate the complex interdependence of mycelial morphology, physiology, rheological properties of bioprocess broths and transport phenomena related to bioreactor performance, in order to optimize operating conditions in bioreactors for process improvement. This review paper deals primarily with fungal morphology as related to other bioprocess parameters, although some data concerning filamentous prokaryotes, *i.e.* actinomycetes, are also included. Some problems associated with the behaviour of highly viscous filamentous broths, as well as the characteristics of pellet cultures, are discussed. The bioprocess design parameters, which may be chosen to obtain appropriate morphology and the desired effective productivity, are also summarized.

Mechanisms of Submerged Growth of Filamentous Fungi

The main common feature of filamentous microorganisms is their polarized growth pattern in the form of filaments, called hyphae. A typical fungal hypha grows out of a single cell – spore as a multinucleate tube containing cytoplasm, which moves within a hypha towards the hyphal tip, where it grows. New tips are grown out of the main hyphae and produce branches, which result in a network of hyphae, called mycelium (Fig. 2). The hyphae of higher fungi, *i.e. Ascomycetes* and *Basidiomycetes*, have numerous cross walls or septa and are able to fuse adjacent hyphae and thereby establish protoplasmic continuity in a reticulate mycelium. On the contrary, in lower fungi such as *Zygomycetes* and *Oomycetes* septa and hyphal fusions are relatively uncommon (*23*).

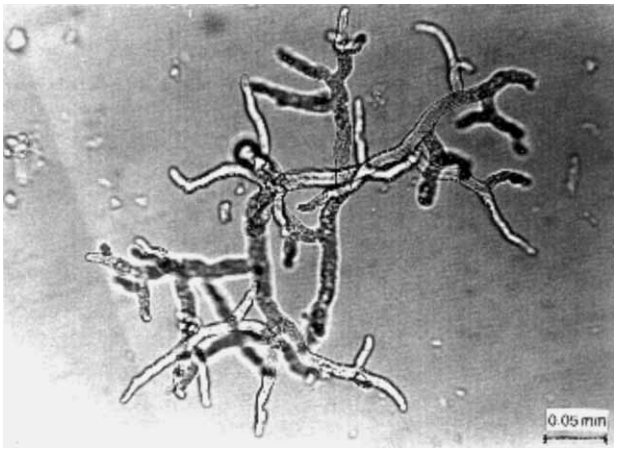

Fig. 2. Mycelium of *Rhizopus nigricans* at an early stage of submerged cultivation

The life cycle of filamentous fungi starts and ends in the form of spores, the dormant state of the microorganism, providing the dispersal or the survival of the species. The time and needs for spore germination depend on the type of dormancy: at exogenous dormancy spores usually germinate readily in suitable conditions, while in constitutive (endogenous) dormancy spores need a resting period before germination or germinate only after the application of some specific stimulus or counter inhibitor (*24*,*25*). At the required physical and chemical conditions, spores enlarge (swell) due to the intensive formation and accumulation of new active cell material, *e.g.* mitochondria, endoplasmatic reticulum *etc*. After a specific period, polarization of growth occurs, resulting in germ-tube formation. The fraction of spores that will germinate and the dynamics of swelling and germ-tube formation in submerged culture mainly depend on the state of inoculum and medium composition and have been suggested to be regulated separately (*16*,*26*).

The morphology of a mycelium is determined by the mechanisms, which regulate the polarity and the direction of growth of hyphae and the frequency with which they branch. It has been indicated that phosphoinositides, calcium, calmodulin and cyclic nucleotides are involved in the mechanisms that regulate hyphal extension and branching but the precise nature of these mechanisms has yet to be elucidated (*27*). The principal participants of the hyphal tip extension are vesicles at the apex supplying wall subunits, lytic and synthetic enzymes for cell wall synthesis, the cytoskeleton that

transports them, and the cell wall, which determines the shape of the cell (*28,29*). A mathematical model has been developed (*30*), relating hyphal tip growth with the position and movement of the vesicle supply center (VSC), which moves the vesicles in a random direction to the cell surface, where they fuse with the protoplasmic membrane and thereby expand the cell surface. Recently, the role of glucose concentration on mitosis and hyphal extension was studied in the filamentous fungi *Aspergillus oryzae* and *A. niger*. When performing the chemostat cultivation procedure with homogenous morphology it was found that the increase in substrate concentration and the related specific growth rate resulted in an increased average number of nuclei in the apical compartment for both species. This suggests that multinucleate microorganisms have the ability of coordinating nuclear division, cell structure and size with a specific growth rate (*31*).

The total length of a mycelium increases exponentially under unconstricted conditions with the increasing number of actively growing tips by branching. Considering the tendency of the systems to channel energy along paths of least resistance we may regard branching as increase of the distributive surface in the case of excess of uptake over throughput (carrying) capacity to the existing release points on the boundary. A computer simulation based on an uptake-throughput model of mycelial development has been shown, where the angle of branching with regard to the main hypha depended on whether the region of maximum boundary deformation was symmetrical or not around the point of origin (*32*). In the presence of septa, the throughput is impeded, which may account for the correlation between septation and anastomosis and also between septation and branching. Considering the last mentioned correlation, Yang *et al.* (*33*) have derived a mathematical model, based on fundamental cellular and physical mechanisms of hyphal tip extension and septation, and on the stochastic nature of the branching process, *i.e.* tip growth directions and outgrowth orientations of branches. By the experimental determination of model parameters, the simulation of the mycelial growth of filamentous fungi and bacteria up to the formation of pellets has been performed (*33*).

High local shearing forces, present especially in the regions near impellers, may prevail over the tensile strength of hyphae, which results in hyphal fragmentation. The newly formed hyphal tips do not grow and therefore must be distinguished from the actively growing tips, formed by branching (*11*). The fragmentation is mainly a function of the shearing forces (see the subsequent discussion on mixing and aeration), although cell structure and substrate availability may also be of importance. The flexibility of hyphae can vary due to changes in cell wall composition, the branching pattern and turgor pressure, which are influenced by the osmotic pressure of the culture medium. A result of increased osmotic pressure in the medium is lower cell turgor pressure and consequently more flexible hyphae, which reduces broth viscosity (*34*). The physiological state of the hyphae, characterized by the proportions of vacuoles, has been found to have a significant influence on the breakage of mycelial hyphae. The fragmentation of the submerged culture of *Penicillium chrysogenum* was greater when the hyphae became heavily vacuolated due to nutrient limitation (*35*).

Sporulation as the end of the development cycle of fungi is rarely achieved during submerged cultivation, partially due to relatively good nutrient availability and partly due to the physical nature of hyphal wall. The asexual sporulation of lower fungi is typically induced by nutrient depletion (*36*). On the other hand, the mechanisms controlling the asexual spore formation, *i.e.* conidiation of higher fungi, differ among species and are mostly still not known (*37*). Chemostat experiments have indicated that the main trigger for fungal sporulation is nitrogen limitation and has mainly been observed at low specific growth rates (*38*). The limited transport of substrate into the interior of mycelial aggregates may also result in asexual sporulation (*10*). Aerial conidiophore formation has been observed during submerged cultivation of *Aspergillus oryzae*, presumably as a consequence of stress induced by the transition from batch to chemostat cultivation (*31*).

Pelleted growth form

Pellets may be classified as stable spherical or oval agglomerates, consisting of branched and intertwined networks of hyphae (*10*). From the process economics point of view pelleted morphology exhibits some advantages over the filamentous growth form. Namely, substantial improvement in culture rheology may be obtained, resulting in lower power consumption for sufficient bulk heat and mass transfer. Besides, it offers the possibility of biomass reuse and thereby continuous operation of the process. Also the problems associated with the tendency of the filamentous form of fungi to grow on fermenter walls, wrap around the impeller and foul the agitator blades or to block the gas distributors, are consequently overcome (*1,9,10*).

On the other hand, mycelial pellets are very nonuniform cell aggregates, where the limitation of transport causes biochemical and structural differentiation, leading to a variety of specific metabolic patterns. Accordingly, for some product formation, the pellet morphology is a prerequisite, which will be discussed later (*9*).

The characteristics used in describing macroscopic morphology are the size of pellets and the shape of the outer surface, which vary from fluffy or hairy to smooth. For the quantitative description of pellet shape, core circularity ratio based on the relation between pellet perimeter and the dense core area of the pellet has been introduced, which is 1 for circular objects and greater than 1 for others (*2*). The pellets may be of loose or compact structure, which defines their stability on shear forces and transport facilities.

Pellet formation

Traditionally we can distinguish between coagulative and non-coagulative pellet formation (*39*). In the first case, spores conglomerate at an early stage of development, while in the later, the pellet may arise from a single spore. Among the coagulative type one can include *Aspergillus nidulans* (*40*), *A. niger* (*12*) and *A. oryzae* (*17*) and basidiomycete fungus *Phanerochaete chrysospo-*

rium (*41*). The medium pH value has been shown to have a decisive influence on spore coagulation, which is therefore thought to be in relation with the pH-dependent surface properties of spores (*17,42*). Besides, strong agitation has been found to be unfavourable for coagulative pellet formation (*9*). In addition, the inoculum in the form of dispersed filaments cannot develop pelleted morphology (*12*).

The non-coagulative type of pellet formation is characteristic of some actinomycetes from the genus *Streptomyces* (*33,43*) and fungi belonging to *Rhizopus* spp. and *Mucor* spp. (*9,44*). Also the well-known antibiotic-producing *Penicillium chrysogenum* was traditionally thought to be of the non-coagulative type (*39*), but recent studies have revealed pellet formation as a consequence of hyphal element agglomeration (*12,45*).

The size and type of the inoculum, agitation, medium composition, temperature and pH are the main factors influencing the formation of pellets. Some common approaches for inducing mycelial pellet formation in submerged cultures have been suggested, such as low inoculum concentration, low temperature and a nitrogen-limited medium. Due to the numerous factors affecting pellet formation and growth, attempts to obtain uniform pellets of a desired size, composition and productivity are still a matter of study for each particular strain (*1,9,10,12*)

Pellet growth

The growth of pellets is restricted to the surface, while in the center the limitation of nutrients, due to the limited intraparticle diffusion, cause cell lysis. Therefore a typical pellet consists of three regions: the growing region at the outer shell, the non-growing region of mycelial biomass and the hollow center (*46*). The differentiation of biomass results in different biochemical compositions of fungal cells and their walls (*47–49*).

Numerous mathematical models concerning mycelial growth in the form of pellets have been proposed to describe the complex interaction between hyphal growth and substrate consumption, including the diffusion limitation problem on macro scale. Well-known is the »cube root« correlation between total pellet biomass and cultivation time (*50,51*). A similar relation has been obtained also with single pellets of *Aspergillus nidulans*, as long as the pellet radius increased at a constant rate (*40*). The problem of oxygen diffusion and limitation inside the pellets of *P. chrysogenum* and *A. niger* were also theoretically and experimentally studied (*52,53*), and direct measurements of oxygen profiles inside the pellet of *P. chrysogenum* by a needle sensor (*46*) were of the utmost importance. The investigations have been expanded also to glucose as a substrate and the result is the very frequently cited as an unstructured model for the growth of mycelial pellets of *P. chrysogenum* in submerged cultures (*54*). Another unstructured kinetic model for mycelial pellet cultures of *Phanerochaete chrysosporium* describes the culture dry weight, respiration, glucose consumption, nutrient nitrogen uptake, pellet size and oxygen effectiveness factor during the lag, primary growth, secondary metabolic and death phases. An oxygen microelectrode has also been used in this investigation (*55*).

Intraparticle convection can contribute significantly to the total intraparticle transport rate. In the work by Stephanopoulos and Tsiveriotis (*56*), the intraparticle diffusion-convection-reaction problem is formulated and solved analytically for a zero-th order nutrient uptake rate. The general relation between Peclet number and Thiele modulus is derived for the estimation of nutrient depletion in the pellet.

A mathematical model for the growth of mycelial pellet populations which considers pellet growth and death with fragmentation, providing new centers of growth, and predicts the effects of shear forces on pellet growth and size distribution has been developed (*57*). In a simple morphologically structured model, apical cells, subapical cells and hyphal cells are the compartments of a branched hyphal element, which is combined with a simple population model to describe the growth of *Geotrichum candidum*, *Streptomyces hygroscopicus* and *Penicillium chrysogenum* (*58*). The previous model has been extended with expressions for spore germination, tip extension, branching and hyphal break up. It has been found that the tip extension rate and the branching frequency increase with dilution rate in a steady state chemostat (*16*). These two models can be applied to describe the growth of pellets on a microscale.

The microscopic (total hyphal length and total number of tips) and macroscopic (shape and size of pellets) morphology during cultivations of *Aspergillus oryzae* have been studied. The specific growth rate of an average hyphal element corresponded to the specific growth rate of microbial biomass in the form of free hyphal elements. The cube-root law was again used to describe pellet growth. A substantial fraction of biomass inside the pellets was found to be limited by oxygen (*17*). In the study of growth characteristics of *Rhizopus nigricans* pellets, the synergistic effect of microbial age, physiological state and adaptive control of metabolism during starvation, as well as improved effective diffusion of nutrients through the texture of mycelium on pellet size, has been pointed out (*59*). The size of *Rhizopus nigricans* pellets was mainly influenced by the inoculum concentration, presence of baffles and Ca^{++}. The smooth structure resulted in cultivations at lower temperature, high agitation rates and high nitrogen concentrations, while fluffier pellets were obtained at higher temperatures (*5*).

Pellet fragmentation and breakdown

Agitation can damage mycelial pellets just as it damages particular hyphae. The fragmentation of pieces of hyphae from the outer pellet surface may result in hyphal fragments, which serve as new centers for biomass growth, or it may cause hyphal damage accompanied with cytoplasm release. On the other hand, total break-up of pellets can occur, presumably, in the case of hollow, centrally autolysed aggregates.

The fragmentation and break-up of pellets, caused by shear forces, therefore influence the pellet size and the quantitative relation between pellets and free mycelium in the culture (*12*). Besides, the shape of a pellet – whether it is smooth and compact or a fluffy, loose structure – strongly depends on the energy dissipation rate, which will be discussed in the subsequent chapter.

Operating conditions have a significant effect on the size distribution of pellets and their structural density, which varies along its radius (*60*). Furthermore, the effective internal diffusivity coefficient varies with the density of the pellet. There is enhanced transport by convection through the sparse outer regions of the pellet and hindrance of transport in the inner dense regions (*61*). A mathematical model to describe this problem was first presented by Edelstein and Hadar (*62*) and improved by Prosser and Tough (*63*). However, the instability of these two models was suppressed in a revised finite element model by Tough *et al.*, which considers pellet growth and death with fragmentation providing new centers for growth and predicts the effects of shear forces on the rate of pellet break-up (*57*). The size distribution of *Penicillium chrysogenum* pellets was strongly influenced by shear forces (*64*). There have also been some attempts to correlate the mean pellet diameter and the number of non-disrupted pellets with mixing. Semi-empirical equations correlating time-dependent mean pellet diameter and the number of non-disrupted pellets with impeller diameter and speed have been proposed and applied to *P. chrysogenum* fermentation (*65*,*66*).

Morphological characterization by image analysis

Image analysis has been developed as a fast and accurate method for quantitative morphological characterization, since parameters of an observed object such as size, count, shape, position and brightness can easily be obtained in this way (*68*). Therefore, also the germination of spores, the length and diameter of hyphae, the number of branches, as well as differentiation (on a microscale) and smoothness or hairiness, and the presence and size of void core for pelleted growth (on a macroscale) may be evaluated (*13*,*67*,*68*).

Image analysis of fermentation broth begins with sample preparation, such as dilution and shaking to disperse the mycelia, pellets or spores, and staining to enhance the contrast of microscopic images. Slides have to be prepared carefully to avoid dirt and gas bubbles. Two major components, hardware and software, are necessary for an image analysis system. The hardware of a typical image analysis system can be divided into four groups of components: 1) primary image sources such as a microscope, a macroviewer or a photograph, 2) acquisition devices such as a scanner or a monochrome or color video camera, 3) optional gain, stage, lamp and focus controllers and 4) image processing system such as a personal computer or work station and image processing software with optional image acquisition and image processing hardware. A vital element of an image processing system is the software, which consists of programs controlling the hardware in carrying out various operations, measurements and analyses. The following distinct steps are involved in image analysis applications: image acquisition, gray scale processing, object detection, binary image processing, image editing, measurements and calculations, and data analysis (*68*).

Pioneer work in this field was done by Metz *et al.* (*69*), who described a semi-automatic method for a quantitative representation of the morphology of *P. chrysogenum*. A successful application of the method instead of the digitizing-table method was done by Adams and Thomas (*70*), to characterize mycelial morphology of *Streptomyces clavuligerus*. A fully automatic system for morphological characterization, which gained in speed but lost in accuracy in some cases, was developed and tested on *S. clavuligerus* and *P. chrysogenum* (*45*). An automated image analysis method has been presented also by Cox and Thomas (*2*) who classified and measured the shape and the size of *Aspergillus niger* pellets.

Image analysis has become a necessary tool for characterizing and developing mathematical models that describe morphology and growth of filamentous microorganisms. For example, characterization of pellet morphology (*71*), measurement and simulation of morphological development (*72*) and a mathematical model of apical growth, septation and branching (*33*) for *Streptomyces tendae* have been verified by means of image analysis. In addition, a morphologically structured model of the growth and product formation by *Aspergillus oryzae* was developed and verified in the same way. The model enabled accurate simulations of various cultivating conditions (*73*). Also the characterization of the physiology of *P. chrysogenum* in submerged culture has been done by color and monochrome image analysis. Among the six identified physiological states of biomass, growth and differentiation, the states with high respiratory activity were identified as the most important for the production process (*19*). Furthermore, image analysis can be used in localizing respiration sites (*21*) and cellular differentiation and its relation to the production of secondary metabolites, as has been demonstrated in *Streptomyces ambofaciens* (*20*).

Since image analysis is a powerful tool for the characterization of morphology and simple differentiation of filamentous microorganisms, it is of considerable significance for bioprocess control. The reducing cost of the equipment, both in terms of hardware (image analysis can nowadays be performed on a PC) and software packages gives image analysis the opportunity to become a routine laboratory tool (*3*).

Characterization of rheological properties of fermentation broths

A number of process parameters, such as inoculum conditions, growth rate, medium composition, dissolved oxygen level in the broth and mixing intensity affect cell morphology and consequently have a profound influence on broth rheology, which, in turn, is responsible for a successful momentum and mass and heat transfer during fermentation processes in a bioreactor. Mixing and aeration (which supplies oxygen to the organisms and removes carbon dioxide) and cooling (which leads away the metabolic heat) have a strong influence on the productivity and efficiency of the entire fermentation process (*74*,*75*).

Dispersed filamentous broths mostly show non-Newtonian behaviour and the apparent viscosity is normally quite high. They generally exhibit a decrease in viscosity with increasing shear rate. The Power law is the most widely used model for describing the rheological properties of fermentation broths (*34*,*74*–*77*). However, yield stress is often observed in fermentation fluids and a number of authors have taken into account

this important phenomenon by using the Bingham model (*77–79*). Sometimes, especially at low shear rates, experimental data obey neither the Power law nor the Bingham model. In this case, the Casson model can be successfully applied (*34,80*).

When the fermentation medium contains a polymeric substrate such as starch, apparent viscosity will decrease during batch fermentation due to its enzymatic degradation and consumption. Also a change from non-Newtonian to Newtonian behaviour can occur. On the other hand, when a polymer is produced during batch fermentation, its increasing concentration raises the viscosity of the broth, in some cases up to 24.0 Pas (*74*). Although experimental results have shown time--dependent behaviour of microbial broths, no rheological model was found in the available literature to describe this complex phenomenon. Basic rheological models are presented in Table 1.

Table 1. The most common rheological models used for fermentation broths; τ is the shear stress (N m^{-2}), η is the viscosity (N m^{-2} s), $\dot{\gamma}$ is the shear rate (s^{-1}), τ_o is the yield stress (N m^{-2}), K is the consistency index (N m^{-2} s^n), n is the flow behaviour index and K_c is the Casson viscosity (N m^{-2} s)$^{1/2}$

Newton's law model	$\tau = \eta\dot{\gamma}$
Bingham model	$\tau = \tau_o + \eta\dot{\gamma}$
Power law model	$\tau = K\dot{\gamma}^n$
Casson model	$\tau^{1/2} = \tau_o^{1/2} + K_c\dot{\gamma}^{1/2}$

Measurement of rheological properties of filamentous fermentation broths and interpretation of results is therefore an important area in the field of biochemical engineering. There are several difficulties involved in measurements with conventional rheological measurement devices such as concentric cylinder, cone and plate or capillary viscometer. Undesired phenomena such as settling of biomass, phase separation at the rotating cylinder surface, inhomogeneity of the broth in the form of pellets and friction in a narrow gap cylinder configuration, as well as slip velocity near the wall of the pipeline viscometer, are often the cause of inadequate results. To avoid these problems, torque measurement of a rotating turbine was used to study the rheology of penicillin broths. The morphology factor was introduced for the quantitative description of mycelial morphology and data were interpreted with the Casson equation (*80*). Technical aspects of the rheological properties of microbial cultures, together with numerous useful data about instruments and culture fluids, have been given by Charles (*74*). Rheological models, which can be used to describe mold suspensions, and measurement techniques, are described also in the work presented by Metz *et al.* (*34*).

An impeller measuring system was developed to measure the rheological properties of *Aureobasidium pullulans* (*81*). A systematic study of the rheological properties of filamentous microorganisms *A. niger*, *P. chrysogenum and Streptomyces levoris* was done with three pipeline viscometers, two rotating cylinder viscometers, as well as helical ribbon and turbine viscometer. The results were compared, critically discussed and viscometer configurations for a particular application were suggested (*76*). The review of static and dynamic measurement of rheological properties and various types of instruments was presented by Olsvik and Kristiansen (*75*). A tube viscometer and a rotating cylinder viscometer were used to measure rheological properties and to estimate heat transfer coefficients during the cooling of a filamentous fermentation broth (*82*). In these works, the concept of average shear rate, originally presented by Metzner and Otto (*83*), was suggested to be used also in the calculations of rheological properties of fermentation broths in reactor vessels.

Mycelial Morphology and Metabolite Production

Since morphological types influence the rheological properties of submerged cultures and the related mass and heat transfer through the bulk medium, changes in macro-morphology will also strongly affect transport feasibility of nutrients and metabolites into and out of the hyphae, *i.e.* on a microscopic scale. The result is cell differentiation in terms of biochemical specialization and structural changes (*e.g.* vacuolation). Growth of filamentous microorganisms in the form of aggregates can even cause asexual sporulation, rarely achieved in submerged cultivation of fungi, differentiation into pycnidia or formation of arthrosporoid-like cells (*10*). If we consider the transition from primary to secondary metabolism as a consequence of cell differentiation, then close interrelation between morphological type and metabolite production may be expected.

It has been stated that for the formation of some (especially secondary) metabolites specific morphology is necessary: pelleted growth is preferred and industrially applied for itaconic acid production by *Aspergillus terreus*, for citric acid production by *A. niger* and for some fungal enzymes such as cellulolytic enzymes of *Trichoderma reesei*, polygalacturonidase of *A. niger* or α-galactosidase of *Mortierella vinaceae*, while filamentous growth is better for pectic enzyme synthesis by *A. niger* and fumaric acid production by *Rhizopus arrhizus* (*1,10,13, 84*).

Appropriate morphology is also a prerequisite for the synthesis of ergot alkaloids in the filamentous fungus *Claviceps purpurea*. Alkaloid synthesis is linked to a specific stage in the fungal life cycle: in nature, alkaloids are synthesized in the course of developing sclerotia, while in submerged cultures, lacking sexual reproduction, alkaloid synthesis proceeds in morphologically and biochemically similar sclerotia-like cells (*85*). Differentiation, as well as alkaloid biosynthesis, are strongly influenced by aeration rate and agitation, defining the air/water interface, which was found to define the uptake of oxygen from the gaseous or the liquid phase. The differentiation into productive sclerotial growth was stimulated by the shift of respiration from dissolved oxygen to the gaseous form, reached by increased air input and by the use of higher biomass concentrations in the production phase (*86*). Besides, the immobilization of *Claviceps paspali* resulted in morphological differentiation into swollen, arthrosporoid-like cells, connected with improved, prolonged vitality and alkaloid biosynthesis

(*87*). For the mathematical description of such morphologically complex fermentations, a model based on microbial lifetime, introducing the relativity concept, was developed (*88*).

Current research on protein secretion in filamentous fungi suggests that it is intimately associated with the process of growth at the hyphal tip, which implies the importance of the morphology in the production of numerous, commercially attractive, extracellular enzymes and recombinant proteins (*89*). The ability to control the growth of microorganisms in order to develop more tips, *e.g.* by enhanced branching frequency, would therefore increase the productivity of mycelium.

Also for penicillin G production by *P. chrysogenum*, which during submerged cultivation is in the form of dispersed and aggregated mycelia, the special role of the hyphal tips has been clearly established. Several structured models have been developed, where the productive and the non-productive cells have been distinguished in correlation with cytoplasmic content (*22*). Besides, the cell growth models distinguish between different regions along hyphae which plays a specific role in hyphal entanglement, tips generation and consequently product formation (*15,58*). However, much of the research and the resulting models have been done on somewhat ideal laboratory-scale conditions. Few studies were made on incomplete and changing mixing patterns during large-scale operations, on the effect of oxygen and carbon dioxide on rheology, mass transfer and metabolism, and on the role of reactor and agitator design. Studies of the influence of energy dissipation rate, gassed flow number, impeller type and diameter and reactor dimensions on micro and macromorphological characteristics revealed some useful data for penicillin synthesis, recently reviewed by Patnaik (*22*).

Studies on conditions leading to high yields of gamma-linolenic acid (GLA) by *Mortierella ramanniana* mutant revealed better accumulation of a lipid with high GLA content in pellets than in the filamentous growth form (*90*).

Besides the correlation between the main morphological types and metabolite production, there are several reports on the relation between the structure and size of pellets and their activity. This feature is related to the diffusion and reaction in mycelial aggregates of varying characteristics, although mainly discrepancy with the models describing inorganic catalysts, where the specific interface area is proportional to their efficiency, is found. This reflects the complex interrelation between transport limitations (related to the size and structure of mycelial networks) and shifts in metabolism as a response to the changes in microenvironment.

The production of extracellular cellulolytic enzymes by *Trichoderma reesei* was strongly affected by agitation, resulting in different characteristics of pellets (*91*). The shaken-cultures studies on the aflatoxin production, a secondary metabolite of *Aspergillus flavus* and *A. parasiticus*, revealed the highest production of toxin by compact, smooth pellets of 0.6 mm in diameter, obtained by addition of some polymers or by using appropriate inoculum concentration (*92*).

The production of β-fructofuranosidase by the pelleted growth form of *Aspergillus japonicus* acheived the best yields with fluffy, loose pellets with diameters below 3 mm, obtained by addition of some polymers or surfactants, although variations of enzyme expression could also be a result of changes of cell physiology and transport phenomena in the broth in the presence of these additives. In addition, a shift from filamentous to pellet morphology was observed when some inorganic salts were added, resulting in different enzyme production (*93*). Also Mn(II) peroxidase production by *Phanerochaete chrysosporium* was proved to be influenced by pellet size and varied with different agitation conditions and inoculum concentrations. Again, optimum was achieved with intermediate pellet diameters (2–3 mm), while larger and smaller pellets exhibited lower protein excretion capacity (*41*).

The yield of steroid biotransformation by different *Rhizopus* spp. was proved to depend on fungal macromorphological characteristics (*44,48*). With pelleted growth form, the possibility for biomass reuse was exposed (*44*). *Rhizopus oryzae* pellets of different sizes were used in the study of heavy metal removal from aqueous solutions. Again, the intermediate optimal diameter of pellets was found for an effective biosorption process (*94*). Fumaric acid production with the same species was correlated with the morphological changes, induced by the variation of initial pH value of the medium and trace metal concentration. Pellets of 1–1.5 mm diameter have been found to give the highest fumaric acid yield (*95*).

The morphology and the related production of xylanase by *Aspergillus awamori* in a pilot plant stirred tank and an airlift loop reactor were studied. In the stirred tank reactor, pellets smaller than 1 mm were formed, while in the airlift loop reactor the size of the pellets was 4–7 mm due to a lower specific power input and low shear rate. However, the highest xylanase productivity was obtained in the stirred tank bioreactor at intermediate stirrer speed (*96*).

Bioprocess Design Considering Morphological Characteristics

An efficient large-scale bioprocess relies on optimization of both the abiotic phase, *i.e.* bioreactor design and performance, and the biotic phase, *i.e.* the genetic structure the microorganism, as well as its morphology and differentiation. As already mentioned, understanding the complex interrelation between morphology and bioreactor performance is the basis for successful bioprocess design. From the engineering point of view, the influence of fungal morphology on culture rheology is the most important. Recurrently, the effect of environmental conditions on fungal growth and morphology must be considered in bioprocess design, *e.g.* mixing and aeration conditions, the mode of cultivation and the growth kinetics, influenced by temperature, dilution rate or medium composition. Furthermore, the choice of a suitable bioreactor type and proper scale-up should ensure growth in appropriate morphology.

Rheological properties of fermentation broths

The rheology of a fermentation broth, which usually changes in the course of batch cultivation, is a result of very complex and synergistic interactions between several parameters. It is often very difficult to experimentally study the effect of only one of them, for example: biomass concentration, cell morphology (including size, shape and mass), cell surface structure, cell flexibility and deformability, mixing intensity, concentration of substrate and concentration of polymeric product. Investigations on the influence of only biomass concentrations on broth rheology are rather old and scarce and were abandoned more than two decades ago because of the already mentioned complexity of the problem. One simple correlation has been proposed, in which the viscosity of the mold suspension is proportional to the biomass dry weight to exponents which range from 1.1 (*97*), through 2.0 (*98*) to 2.65 (*78*). Another simple equation correlates yield stress and mycelial concentration to exponents 2.3 to 2.5 (*99*) and 2.5 (*80*).

Micromorphological parameters and biomass concentration were correlated with rheology of the broth. For example, Metz *et al.* (*34*) suggested a correlation between Casson parameters τ_0, K_c and the dry weight, the dimensionless hyphal length and the length of hyphal growth unit for filamentous suspensions of *Penicillium chrysogenum.* The Power law consistency index was correlated with the *Aspergillus niger* biomass concentration and the roughness factor of the clumps, which describes their hairiness (*100*). Similarly, biomass concentration, mean clump compactness and mean clump roughness were taken into account in the rheological characterization of *Penicillium chrysogenum* fermentation broths (*101*). Broth rheologies of three studied actinomycetes showed pseudoplastic behaviour as soon as growth occurred (*102*). Correlations between rheological parameters, morphological parameters and biomass concentration for different mold suspensions were proposed also by several other authors (*103–106*).

Biomass in the form of pellets causes more Newtonian properties of the fermentation broth. An interesting study with a mold suspension of *Absidia corymbifera* in two different growth forms, filamentous and pelleted, was done by Kim *et al.* (*107*). Filamentous mycelial suspensions showed marked non-Newtonian behaviour and were correlated by a pseudoplastic model. On the other hand, almost Newtonian behaviour was observed with pelleted mycelial suspensions. However, at higher concentrations of pellets, the rheological behaviour of broths became pseudoplastic.

Dissolved oxygen concentration (DOC) affects the specific growth rate and consequently broth rheology. An interesting effect of DOC on rheological properties during continuous cultivations of *Aspergillus niger* was observed. Namely, at DOC above 10 % saturation, the consistency index K increased with the dilution rate and the related specific growth rate, while the reduction of DOC below 10 % saturation decreased K with increasing dilution rate (*75*).

Mixing and aeration

Mixing is one of the most important and necessary operations, used in bioprocessing to achieve uniform concentration, temperature and other properties of the environment for fermentation in a bioreactor. Breaking up the bulk flow into smaller and smaller fluid eddies under turbulent conditions is usually carried out with an impeller in a stirred tank bioreactor, with a recirculation pump in jet and loop bioreactors or simply by introducing gas into the reactor vessel through several types of aeration devices in bubble columns and airlift bioreactors. Mixing involves blending substrates, dispersing air, maintaining suspension of cells, dispersing immiscible liquids and promoting heat transfer. Interactions between fluid eddies and microbial cells have the potential to influence the cell morphology or even cause mechanical damage and fragmentation to cells and consequently affect broth rheology and product formation (*13,14*)

Van Suijdam and Metz (*60*) developed a model describing the influence of shear stress on fungal micromorphology. It was found that the mean main hyphal length of free filamentous mycelia decreases with increasing energy dissipation rate during mixing. The morphologically structured model for submerged growth of *Penicillium chrysogenum,* developed by Nielsen and Krabben (*16*), considered the linear relation between the rate of fragmentation and energy input to the bioreactor.

The influence of mixing and aeration on macromorphology (pellets) has also been intensively studied. The pellet size and the pellet porosity of *Aspergillus awamori* were found to be inversely proportional to the specific energy dissipation rate. The hyphal chip-off from the pellet outer zone, which reseeded the pellet growth, was observed during a submerged cultivation (*108*).

Specific growth rates and the overall biomass concentrations of *Penicillium chrysogenum* increased with agitation intensity, while the specific product formation was generally higher at lower impeller speeds (*109*). Agitation intensity was reported to affect also mycelial micromorphology of recombinant *Aspergillus oryzae*, but did not directly influence product formation (*110*).

Agitation conditions and shear stress in stirred tank bioreactors are associated with the type of impellers and have a strong influence on microbial morphology, which controls the rheological characteristics of fermentation broth. The influence of agitation conditions on the morphology of *Penicillium chrysogenum* (freely dispersed and aggregated forms) was examined using radial, axial and counterflow impellers in laboratory and pilot plant reactors of different sizes. To characterize the damage caused by different impellers, the mean total hyphal length and the mean projected area were determined by image analysis. The energy dissipation/circulation function was a better correlating parameter than the tip speed and the specific energy dissipation rate (*111*).

Mixing time as a function of agitation rate was studied and average viscosity was simultaneously measured during *P. chrysogenum* fermentations. During the first 50 h, pellet morphology prevailed; the average viscosity

was low but increased. After 50 h, dispersed growth started to prevail. As a result, viscosity and consequently mixing times increased, the latter being 100–300 times higher than those determined in pure water (*112*).

Excellent circulation and mixing were achieved in a laboratory airlift bioreactor during pelletized growth of *Neurospora crassa* for tyrosinase biosynthesis. At aeration rate 1 ($L \cdot L^{-1} min^{-1}$), oxygen transport was not a limiting factor and good biomass yield and tyrosinase activity were achieved (*113*). On the other hand, gentle mixing in a laboratory bubble column was not effective for the production of lignin peroxidases with pelleted morphological form of *Phanerochaete chrysosporium*. Namely, ligninolytic activity was 70 % lower and occurred two days later compared to shake flasks experiments, probably due to inadequate mixing and oxygen transfer (*114*).

The oxygen level alone, which is controlled by mixing and aeration conditions in different types of bioreactors, affects the type of morphology. The response of cells to the oxygen partial pressure is a complex phenomenon. The specific growth rate, which is strongly dependent on oxygen concentration, can influence the cell wall properties, the length of hyphae, and the roughness of the clumps, and consequently the viscosity of the culture (*75*).

Morphological differences of *Aspergillus niger* were caused by different shear fields and distinct oxygen concentration zones in the pilot plant stirred-tank and bubble column bioreactor. It was found that low shear rate resulted in the formation of looser pellets with larger diameter and conversely, higher shear field caused formation of compact spherical pellets (*115*).

Power consumption

Extensive research about the power characteristics of aerated and agitated fermenters has been done in the last few decades. Calculation of power consumption of the aerated system is based on the nonaerated one (*116*). The fall in power consumption under aerated conditions using the standard Rushton turbine for example is a complex interaction of impeller action, aeration and gas recirculation as well as the bulk flow pattern. Generally, the increase of aeration rate decreases power consumption and 50 % power reduction is not uncommon. The estimation of power consumption for low viscosity and Newtonian broths can be done reasonably well with the Michel and Miller correlation (*117*):

$$P_g = 0.7 \cdot [(P^2 \cdot N \cdot D^3) / Q_v^{0.56}]^{0.45}$$

where P_g is power consumption of the aerated system (W) and P of the nonaerated one (W), N is impeller speed (s^{-1}), D impeller diameter (m) and Q_v gas volumetric flow rate ($m^3 s^{-1}$). Other correlations are also available in the literature. However, the situation is much more complex for high viscosity and non-Newtonian broths. Here, the ratio between gassed power and ungassed power consumption passes through a minimum with increasing agitator speed and the values lower than 0.5 were obtained. Agitator speeds at or above this minimum should be used for good gas dispersion. The power consumption drop under aerated conditions could be reduced by using some new types of impellers (*118*, *119*).

The total power consumption into the bubble column and the air-lift reactor can be calculated on the basis of two contributions: the isothermal expansion of the gas moving up the reactor and the kinetic energy transferred to the fluid by the jet of gas entering the reactor:

$$P_g = Q_m \cdot [(R \cdot T/M) \cdot \ln(p_o / p) + v^2 / 2]$$

The symbols are: Q_m – gas mass flow rate (kg s^{-1}), R – ideal gas constant (J $mol^{-1} K^{-1}$), M – molecular mass (g mol^{-1}), p_o – absolute pressure at the sparger orifice (N m^{-2}), p – absolute pressure at the top (N m^{-2}) and v – gas velocity at the sparger hole (m s^{-1}).

Oxygen transfer

Oxygen transfer rate from air to the fermentation broth is proportional to the volumetric oxygen mass transfer coefficient k_La (h^{-1}) and average oxygen concentration difference between equilibrium dissolved concentration C^* (kg m^{-3}) and dissolved oxygen concentration C (kg m^{-3}). Under steady state, it should be equal to the oxygen consumption rate $R \cdot X$ (kg $m^{-3} h^{-1}$) by a microorganism with biomass concentration X (kg m^{-3}). For optimal operating conditions, oxygen concentration in the liquid should be above some critical oxygen concentration (C_{cr}), under which microbial activities are considerably repressed. The following equation can be used for calculations (*120*):

$$k_La \cdot (C^* - C_{cr}) = R \cdot X$$

During antibiotic fermentations with filamentous microorganisms in dispersed growth form, relatively high biomass concentrations, pseudoplastic behaviour of the broth and high oxygen consumption rates are reported. The values for oxygen transfer rate can be in the range of 0.6–2.5 kg $m^{-3} h^{-1}$ and therefore also high values of k_La (70–250 h^{-1}) should be ensured. This is not easily achieved because the volumetric oxygen mass transfer coefficient is very much dependent on the viscosity of the broth and increased mixing and aeration in stirred tank fermenters can damage microbial cells and repress the product synthesis. During fermentations with pellets, the problem of oxygen transfer is not so pronounced (*120*).

Numerous articles have focused on oxygen volumetric mass transfer coefficient. However, only a few correlations can be used for the estimation of this parameter in real fermentation broth on industrial scale, where effects of antifoam agents, electrolytes and coalescence are evident. The problems are even more pronounced with high viscosity of mycelial suspensions, where mass transport from the gas to the liquid phase decreases as a result of strongly enlarged coalescence of bubbles (*60*).

Probably the most useful type of correlation for stirred tank reactors has been proposed by Taguchi *et al.* (*121*):

$$k_La = A \cdot (P_g/V)^b \cdot v_g^c$$

Van't Riet (*122*) has correlated experimental data of numerous independent studies with this equation and determined the following coefficient and exponents: A=0.026, b=0.4 and c=0.5 for water and A=0.002, b=0.7 and c=0.2 for the electrolyte solution. The effect of electrolytes was studied also by Zlokarnik (*123*). However, it was found that exponents b and c are lower at large scale; namely, the contribution of surface aeration to the total oxygen transfer could be significant at small scale while the effect of coalescence is more pronounced at large scale (*124*). According to investigations in viscous and non-Newtonian systems, k_La is proportional to effective viscosity with exponents from –0.4 to –0.8 (*125–127*). However, on the basis of some industrial experiments with filamentous broths, some authors claim that the range of those exponents is too high (*128,129*). The effective viscosity can be estimated using the correlation of Metzner and Otto (*83*).

For bubble columns, the following empirical correlation was suggested by Deckwer (*130*):

$$k_La = A \cdot v_g^{\ b}$$

Coefficient A depends on the gas distributor system and medium properties and is in the range 0.4–1.4, while exponent b=0.8 for water and electrolyte solutions. The effect of the non-Newtonian properties of the media can be taken into account as suggested by Deckwer (*131*). He found that in CMC solutions k_La is proportional to the effective viscosity to the power of –0.8, which can be estimated with the correlation proposed by Nishikawa *et al.* (*132*).

Heat transfer

Aerobic fermentation processes are usually exothermic and the metabolic heat as well as heat introduced to the system by mixing has to be drawn away through the heat exchange system. An external jacket, an internal coil and an external surface heat exchanger are used for this purpose. For steady state operation, the required heat transfer area A (m^2) can be estimated from the equation:

$$A = (\dot{Q}_{gr} + \dot{Q}_m)/U \cdot \Delta T$$

Here, $\dot{Q}_{gr}$ and $\dot{Q}_m$ (W) are the rate of metabolic heat generation and the rate of heat generation by mixing, U ($W\ m^{-2}\ K^{-1}$) is the average overall heat transfer coefficient and ΔT (°C) is the average temperature difference between the fermentation broth and the cooling media. Metabolic heat generation is proportional to the oxygen uptake rate and a relatively good estimate can be obtained by laboratory experiments. Heat generation varies during the batch fermentation and it can reach values between 5–15 $kW\ m^{-3}$.

In viscous systems, the major heat transfer resistance is usually in the liquid film on the broth side of the cooling surface and as a first approximation it can be assumed that the overall heat transfer coefficient is similar to the individual heat transfer coefficient on the broth side $U \cong h$. However, for more accurate calculations, thermal resistance through the heat exchanger wall and on the side of the cooling liquid should be taken into account (*128,133*). For filamentous fermentations, the value of U is in the range 300–700 $W\ m^{-2}\ K^{-1}$, while during fermentations of polymeric products, U is in the range 100–200 $W\ m^{-2}\ K^{-1}$. In these processes, 1.5 $m^2\ m^{-3}$ or even higher specific heat transfer areas are required. The problem becomes even more pronounced when the average temperature difference for cooling is less than 10 °C and the volume of the fermenter exceeds 50 m^3, since the specific geometric area available in the fermenter is inversely proportional to its size. In this case, an additional heat exchange surface in the form of internal coils or external loops or a special cooling liquid system is necessary.

Numerous articles report on studies of individual heat transfer coefficients in CMC systems, various suspensions and different types of fermentation broths. The film heat transfer coefficient depends on viscosity, which in turn is strongly dependent on characteristic morphology, biomass concentration and the presence of high molecular extracellular components. The following correlation is recommended for a single Rushton turbine in a jacketed baffled vessel of standard configuration without aeration (*134*):

$$h = 0.74 \cdot (\lambda/T) \cdot Re^{2/3} \cdot Pr^{1/3} \cdot (\eta/\eta_w)^{0.14},$$

where the symbols are: λ – thermal conductivity ($W\ m^{-1}\ K^{-1}$), T-vessel diameter (m), Re – Reynolds number, Pr – Prandtl number, η – effective viscosity of the broth (Pa s), η_w – viscosity at the wall temperature (Pa s). However, aeration causes a decrease of the heat transfer coefficient (*133,135*).

For pneumatically mixed systems, the equation below can be used (*130*):

$$h = 0.1 \cdot \rho \cdot c_p \cdot v_g \cdot [(\rho \cdot v_g^{\ 3}/\mu \cdot g) \cdot Pr^2]^{-0.25},$$

where ρ is broth density ($kg\ m^{-3}$), c_p – specific heat capacity ($J\ kg^{-1}\ K^{-1}$), v_g – superficial gas velocity ($m\ s^{-1}$), μ broth viscosity (Pas), g – gravitational acceleration ($m\ s^{-2}$) and Pr – Prandtl number. For the calculations of effective viscosity, correlations suggested by Metzner and Otto (*83*) and Nishikawa *et al.* (*136*) can be used.

Growth rate and cultivation mode

The growth rate of filamentous fungi depends both on physical and chemical conditions. Since hyphae grow only at the tip, the total biomass concentration in submerged cultures increases by the number of branches and by the formation of new fragments, caused by shear stress as discussed above.

Optimal temperature, initial pH and high concentrations of limiting nutrients in batch cultures or high dilution rates in chemostat cultures increase the biomass growth rate. It was found in several fungal strains that conditions leading to an increased specific growth rate reduced the length of the hyphal growth unit and hence increased the branching frequency (*11,23*). A review made by Nielsen (*11*) revealed that the hyphal growth unit volume, *i.e.* the total volume of a hyphal element divided by the number of tips, denoting the average volume of cytoplasm involved in the growth of each tip, was fairly independent of the growth rate, when the effect of fragmentation was negligible. Besides, the hyphal growth unit length decreases and the hypha diameter

increases with the specific growth rate. It was suggested that the increase in the specific vesicle production must therefore increase with dilution rate and consequently with growth rate (*11*).

On the other hand, the formation of different macromorphological types strongly depends on the growth rate. Changes in specific growth rate due to low glucose concentration effect branching frequency and hyphae growth rate of *A. niger* (*137*). The stimulative effect of dilution rate on the formation of *Penicillium chrysogenum* pellets in continuous cultures was reported, which may be explained by the more profound agglomeration of long and highly branched hyphal elements, developed in such conditions (*138*). Contradictorily, submerged cultivations of actinomycetes *Streptomyces tendae* proved that the pellet formation was induced at oxygen sufficiency in shaken cultures, observed at a low biomass growth rate. It was suggested that the hydrophobic interactions of the cell wall, which were controlled by dissolved oxygen concentration, are the main forces inducing cellular aggregation (*43*).

The mode of cultivation also affects the morphological form of a specific fungus. In a typical batch process with distinctive non-stationary conditions, the microorganism passes through different growth phases, resulting in changing micro- and macromorphological characteristics of the mycelium. The submerged cultivation of *Aspergillus oryzae* with a typical coagulating type of pellet formation revealed fairly constant pellet concentration for the first 30 h and a simultaneous linear increase in pellet diameter, followed by a rapid increase in pellet concentration due to the break-up of pellets accompanied by a decrease in average pellet diameter (*17*). On the other hand, the batch cultivation of *Penicillium chrysogenum* revealed an exponential increase of total hyphal length after spore germination and a population of freely dispersed hyphal elements. After 32 h of cultivation, the agglomeration of large hyphal elements lead to a rapid decrease in the average total hyphal length and a linear increase in pellet concentration (*139*). The model describing hyphal growth and fragmentation in a batch culture of *P. chrysogenum* was developed, based on the kinetics for spore germination, tip extension, branching and hyphal break-up (*16*).

The growth of *Penicillium chrysogenum* in a repeated fed batch cultivation, inoculated with biomass mainly in the form of pellets, was followed by biomass and pellet concentration analysis, and determination of pellet size and circularity (*139*). In each of the three growth periods there was a rapid growth phase resulting in increased biomass concentration accompanied by increased equivalent pellet diameter, while the structure of the mycelium changed from the initial increase in pellet concentration towards a decreased fraction of pellets, presenting less than 5 % of total biomass at the end of cultivation. Thus during the repeated fed-batch process an increasing fraction of the biomass was present as dispersed hyphal elements. This indicated fragmentation of pellets, shown also by a decrease in core diameter and core circularity during each growth period (*139*).

Chemostat culture studies are the best experimental tools to determine values for growth parameters and physiology of filamentous fungi, while continuous operation is not widely used in large-scale industrial applications (*11*). The ability to control the specific growth rate by varying the dilution rate and the concentration of a single substrate has provided additional information on the kinetics of branch formation and hyphal extension. The current knowledge indicates that hyphal diameter increases linearly with the specific growth rate and that the branching frequency changes with the specific growth rate (*11,31*). A linear correlation between the dilution rate and the tip extension rate was found in the chemostat cultivation of filamentous fungus *P. chrysogenum* (*16*).

Carbon dioxide evolution

During aerobic fermentations, the production of carbon dioxide occurs as a consequence of microbial respiration. Its presence in the fermentation broth influences the morphology of several filamentous microorganisms (*140*). For *P. chrysogenum* cultivations it was found that CO_2 stimulates chitin synthesis, a component of cell wall which increases its plasticity and consequently the rheology of the fermentation broth (*141*). The elevated partial pressure of CO_2 increased the mean hyphal length and the mean branch length during the batch growth of *A. niger* (*142*). Higher partial pressure of CO_2 can also severely inhibit penicillin production, although it was found that it causes lower apparent viscosity and hence better oxygen transfer in suspensions of *P. chrysogenum* (*143*). Therefore, aeration is necessary not only to provide oxygen to the microorganism, but also to remove carbon dioxide and to avoid its undesired effects on the cultivation.

Choice of bioreactor

The viscosity of the culture broth, oxygen demand and therefore the potential oxygen supply, as well as heat evolution and the necessary cooling, are the main parameters which dictate the choice of the bioreactor. However, product price, productivity, reactor size, the necessary control of process parameters and the mode of operation also influence this choice. Generally, the stirred tank bioreactor is better for viscous broths, but is impractical at volumes greater than 500 m^3 because the agitation power becomes too high. The problem of a necessarily large size can be solved with the bubble column or airlift bioreactors, which can reach volumes of several thousand of cubic meters, but are not suitable for viscous broths. In some rare cases also other types of bioreactors are reported (*14*). In the last few decades numerous articles covering various aspects of design and operation have been devoted to various types of bioreactors. However, data obtained from industrial fermenters with real viscous and filamentous broths are not so frequent. For example, a critical discussion with numerous examples of calculations in stirred tank bioreactors, was written by Charles (*118*). Similarly, bubble columns, as well as airlift bioreactors were presented by Schugerl (*144*) and Lubbert (*145*), respectively.

The shear rate in the stirred tank bioreactor varies from very high values close to the impeller or aeration device, to low values some distance away. It is therefore difficult to define an average shear rate in the broth and

consequently estimate its viscosity for non-Newtonian broths for the whole bioreactor. The equation of Metzner and Otto (*83*) is often used to calculate the average shear rate. Shear stress is also a function of the size of bioreactor. If standard scale up methods, for example constant tip speed or constant power input per unit volume, are used, the resulting agitation speed is different for each scale-up criteria (*146*). Oxygen and heat transfer rates, which are a function of rheological properties of the fermentation broth and the power input, also depend on the type and size of reactor. Generally, high power inputs are required to obtain high transfer rates and the more viscous and non-Newtonian the fluid becomes, the higher power input is necessary. This is one of the reasons why the stirred tank bioreactor is the most frequently used for submerged cultivations of mycelial microorganisms. However, the quality of bulk mixing in viscous mycelial fermentations, especially in large bioreactors (> 500 litres) is an important design and scale-up parameter. This can be improved by choosing an efficient type of impeller, for example Prochem Hydrofoil, or by retrofitting the standard Rushton turbine. Improved bulk mixing also improves the oxygen transfer (*147,148*).

The major alternatives to standard stirred tank bioreactors are bubble column bioreactors and airlift bioreactors (*149–151*). Here, a significant reduction of energy consumption and consequently of operating costs can be achieved. Since only gas sparging provides the agitation, the mechanical complexity of the system and the risk of contamination is reduced. Compared to stirred tanks, airlift bioreactors are considered low-shear systems with more evenly distributed energy dissipation and shear stress throughout the reactor. In these reactors, also the efficiency of oxygen transfer is comparable with stirred tanks. Taking these advantages into account, there are numerous successful industrial applications of these reactors. Gluconic acid production with *Aspergillus niger* was equivalent in airlift and stirred tank reactors (*152*), while cellulase production by *A. fumigatus* was better in an airlift bioreactor (*153*). The production of myco-protein from filamentous fungal fermentation with *Fusarium graminearum* in a 40 000-liter pressure cycle fermenter is also a good example of its applicability (*154*). However, high broth viscosity may cause problems due to reduced oxygen mass transfer and consequently decrease in product yield, for example during cephalosporin C production with *Cephalosporium acremonium* (*155*) or during the cultivation of *C. cellulolyticum* (*156*). Optimization of airflow rate during cultivation of *Sclerotium glucanicum* has been done to solve this problem (*157*). Induced pelletization has also been successfully used in the production of penicillin (*158*).

The basic types of airlift bioreactors can be modified to improve their performance. For example, mixing and oxygen mass transfer rates in airlift reactors during the production of cephalosporin C were substantially improved by incorporating static mixers within the airlift vessel (*159*). Similarly, much better mixing and oxygen mass transfer were achieved by inserting impellers in the draught tube of an airlift bioreactor during polysaccharide production (*160,161*). The performance of a laboratory external loop bioreactor with a centrifugal pump called »cyclone bioreactor« for the cultivation of *Aspergillus niger* was investigated. In the absence of recirculation the cell morphology was highly branched with several main hyphae. Increasing the centrifugal pump speed resulted in significantly shorter cells with a distinct main hypha and several short branches, as well as in a prolonged lag phase of the cell growth (*162*).

Scale up

One of the main engineering tasks in the field of biotechnology is an effective transfer of a biological process from the laboratory to the production scale. This is not easy and involves several compromises. In general, it is not possible to provide the same environment in large-scale and small-scale vessels because impeller tip speed, superficial air velocity and the corresponding flooding tendency, the quality of mixing and heat transfer surface to volume ratio change with size. Scale-up criteria, originally developed for chemical processes can be used for this purpose, for example constant power input per unit volume, oxygen transfer coefficient or tip speed. Scale up is associated with the rheological characteristics of the broth: it is easier with low-viscosity and Newtonian broths, to some extent also with pellets. The problem is more pronounced when the fluid is viscous, non-Newtonian and when the viscosity changes during fermentation. Therefore it is not surprising that the usual scale-up methods involve empiricism (*118,124, 146*). From the biological point of view, physiological similarity must be taken into account (*163*). This is actually seen in the majority of scale-up examples, so that the conditions to ensure the same morphological characteristics in large-scale production are usually investigated.

Scale up criteria for a dispersed growth form of *Penicillium chrysogenum* was studied in 10- and 100-liter stirred tank bioreactors at various aeration rates and stirrer speeds in the transitional region of Reynolds numbers. It was found that equal energy dissipation rate, as well as equal impeller tip speed scale-up criteria, were not valid as a measure of hyphal damage and production rate. However, the results were successfully correlated with a correlation based on the circulation rate through the impeller zone (*164*). Similar results for fermenters of different sizes with different types of impellers were obtained also by Justen *et al.* (*111*). Scale up of γ-linolenic acid production with *Mortierella ramanniana* in the pelleted morphological form was studied in a 30 liter jar and a 10 000-liter stirred tank bioreactor. It was found that the impeller tip speed velocity may be used as a scale-up parameter (*90*). To avoid the problem of oxygen transfer in a highly viscous broth during penicillin production with filamentous fungus *P. chrysogenum* in a stirred tank bioreactor, this mold was grown in pelleted form in a tower-loop reactor. Under these conditions the specific productivities were higher than in the stirred tank bioreactor and the product recovery was much simpler (*158*). Therefore, this can be the strategy for more economical large-scale production.

During a scale-up process, both physical and biological processes should be taken into account. A relatively simple technique, regime analysis that identifies the important reactions during the process and estimates the

rate limiting step can be used for this purpose. In this procedure, scale-down by volume and estimation of rate constants is an essential part of the procedure. After this step, the process can be scaled-up by control of the critical parameter. The principle of scale-down and optimization studies was successfully applied in the case of the gluconic acid fermentation (*165*) and oxytetracycline biosynthesis (*166*).

Conclusions

The literature review confirms that the morphological type and the related physiology are the key variables, necessary to explain and predict the bioreactor performance and to design a bioprocess. They strongly depend on the environmental conditions and affect the rheological behaviour of the broth, which in turn affect transport phenomena in a bioreactor. In the case of filamentous growth form, the broth usually exhibits non-Newtonian and viscous behaviour, where bulk mixing, aeration and cooling might not be successful. Rheological properties of filamentous fermentation broths have been correlated by the concentration of the biomass, its growth rate and morphology. Especially in the last decade, the introduction of image analysis into morphological studies enabled better understanding and modelling of rheological behaviour of filamentous fermentation broths.

Pellets have become an interesting growth form of filamentous microorganisms due to better culture rheology which causes lower power consumption for sufficient bulk heat and mass transfer, offers the possibility of biomass reuse and thereby continuous operation of the process and facilitates the separation process. In addition, pellets include several physiological states of the microorganism with the specific metabolite production, which may not occur in the filamentous form.

Image analysis is now a well-established method for the characterization of complex mycelial morphology, while in physiological studies it has provided better understanding and determination of microbial activities. Therefore it has become an important instrument for the research and design of filamentous fermentations and in the future it presents a great engineering challenge to be routinely used for fermentation control on the production scale.

Most of the research studies which correlate morphology and engineering parameters such as quality of mixing, oxygen transfer rate, volumetric oxygen and heat transfer coefficients during filamentous fermentations, have been done in laboratory and pilot-plant bioreactors. These parameters change with scale and therefore scale-up is still an art and not exact science, especially for the mycelial submerged cultures.

Mathematical modelling has become a powerful engineering tool in a complex system such as growth of filamentous microorganisms. Besides simple unstructured models, also numerous morphologically structured models have been proposed, considering different morphological forms of mycelial culture as a consequence of metamorphosis reactions. However, they may contain a large number of parameters, which makes these models too complex and not usable for process automation. Therefore, the main goal of mathematical modelling in the future should be to develop reliable mathematical models of a bioprocess with minimal complexity and necessary data to control mycelial fermentations on industrial scale.

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Morfologija filamentoznih gljivica pri submerznom uzgoju kao pokazatelj bioprocesa

Sažetak

Utvrdeno je da su morfološke značajke micelijskog submerznog uzgoja glavni pokazatelji bioprocesa. Morfološki tip i njemu svojstvena fiziologija jako ovise o reakcijskim uvjetima u bioreaktoru, a ujedno utječu na reološka svojstva podloge, a time i na učinak bioreaktora. Stoga proizvodnost i utrošak energije u procesu ovise o morfološkim osobinama gljivica. U radu je obrađena morfologija gljivica s odgovarajućim reološkim svojstvima i metabolizamskom aktivnošću, ističući utjecaj proizvodnih varijabla na rast biomase. Osim toga dan je pregled literature o međusobnim odnosima morfologije i učinkovitosti bioreaktora obuhvaćajući ih s različitih gledišta provedbe bioprocesa.