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Hydrodynamics, Fungal Physiology, and Morphology

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Abstract Filamentous cultures, such as fungi and actinomycetes, contribute substantially to the pharmaceutical industry and to enzyme production, with an annual market of about 6 billion dollars. In mechanically stirred reactors, most frequently used in fermentation industry, microbial growth and metabolite productivity depend on complex interactions between hydrodynamics, oxygen transfer, and mycelial morphology. The dissipation of energy through mechanically stirring devices, either flasks or tanks, impacts both microbial growth through shearing forces on the cells and the transfer of mass and energy, improving the contact between phases (i.e., air bubbles and microorganisms) but also causing damage to the cells at high energy dissipation rates. Mechanical-induced signaling in the cells triggers the molecular responses to shear stress; however, the complete mechanism is not known. Volumetric power input and, more importantly, the energy dissipation/circulation function are the main parameters determining mycelial size, a phenomenon that can be explained by the interaction of mycelial aggregates and Kolmogorov eddies. The use of microparticles in fungal cultures is also a strategy to increase process productivity and reproducibility by controlling fungal morphology. In order to rigorously study the effects of hydrodynamics on the physiology of fungal microorganisms, it is necessary to rule out the possible associated effects of dissolved oxygen, something which has been reported scarcely. At the other hand, the processes of phase dispersion (including the suspended solid that is the filamentous biomass) are crucial in order to get an integral knowledge about biological and physicochemical interactions within the bioreactor. Digital image analysis is a powerful tool for getting relevant information in order to establish the mechanisms of mass transfer as well as to evaluate the viability of the mycelia. This review

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focuses on (a) the main characteristics of the two most common morphologies exhibited by filamentous microorganisms; (b) how hydrodynamic conditions affect morphology and physiology in filamentous cultures; and (c) techniques using digital image analysis to characterize the viability of filamentous microorganisms and mass transfer in multiphase dispersions. Representative case studies of fungi (*Trichoderma harzianum* and *Pleurotus ostreatus*) exhibiting different typical morphologies (disperse mycelia and pellets) are discussed.

Keywords Hydrodynamics · Mass transfer · Morphology · Physiology · Image analysis

Abbreviations and Symbols

C_{O_2}	Concentration of dissolved oxygen in the liquid (kg O_2 m^{-3})
d_{32}	Sauter mean diameter (μm)
D	Diameter of the impeller (m)
D_{crit}	Critical diameter (m)
D_{eff}	Diffusion diameter (m^2 s^{-1})
d_{eq}	Equilibrium diameter (μm)
d_h/d_r	Hyphal gradient in the pellet periphery (% μm^{-1})
d_i	Size of the drops/bubbles (μm)
D_{O_2}	Molecular diffusion coefficient (m^2 s^{-1})
EDCF	Energy dissipation/circulation function (kW m^{-3} s^{-1})
F_{lG}	Gaseous flow (–)
i	Volume unit
k	Constant that depends on the geometry of the impeller (–)
k_b	Number of volumes sampled (–)
k_La	Volumetric oxygen transfer coefficient (h^{-1})
L	Hyphal length (μm)
N	Stirring speed (s^{-1})
n_i	Number of drops/bubbles per volume i
P	Power supplied (kW)
P_p	Porosity of the pellet (–)
P/V_L	Volume power drawn (kW m^{-3})
q_{O_2}	Specific rate of oxygen consumption (kg O_2 kg^{-1} s^{-1})
r	Aggregate density (kg m^{-3})
R_{O_2}	Rate of oxygen consumption per unit volume (kg O_2 m^{-3} s^{-1})
t_c	Circulation time (s)
V_L	Volume of liquid (m^3)

Greek Letters

λ	Size of Kolmogorov microscale (μm)
ε	Local energy supplied (W kg^{-1})
ν	Viscosity (Pa s)

Abbreviations

6PP 6-pentyl-α-pyrone
ATP Adenosine triphosphate
CFU Colony-forming units
DNA Deoxyribonucleic acid
FDA Fluorescein diacetate
GFP Green fluorescent protein
RNA Ribonucleic acid
RPB Reciprocating plate bioreactor
rpm Radians per minute (stirring speed)
vvm Volume of gas per volume of liquid per minute

Contents

1 Mycelial Cultures: Basic Aspects

Mycelial microorganisms play a major role in the bioprocessing industry for the production of various primary and secondary metabolites such as enzymes, antibiotics, organic acids, extracellular proteins, and flavoring compounds [1–4].

Filamentous fungi are lower eukaryotic heterotrophic microorganisms that depend on external sources of carbon and have both sexual and asexual reproduction. The mycelium is broadly composed of vegetative branched filaments (hyphal) with rigid and thick cell walls. These cell walls are composed of fibrous polysaccharides (chitin and β-glucan) and glycoproteins that determine the fibrillar structure of the wall. The hyphal growth takes place in the apical region (tips). When a new tip is formed, it

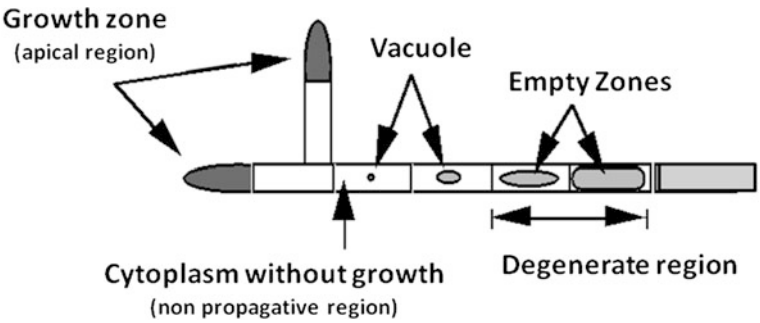

Fig. 1 Schematic diagram of the regions of cell growth and differentiation of the fungus *P. chrysogenum* (adapted from Ref. [5])

grows to a certain length, and a septum with a pore is created at the rear of the tip, creating a non-growing cell. The net result of the apical extension is the formation of non-propagative cells which, in turn, produce vacuoles. Vacuoles grow as a function of their distance from the apical region. Given that the mycelial structure is complex and multicellular, the distribution of cell ages is wide compared to unicellular microorganisms. Therefore, hyphae may exist in different physiological states and show different structural features such as growing apical cells, vacuolated regions, and lysated cells which are metabolically inactive (Fig. 1).

Mycelial cultures have different physical characteristics to unicellular cultures (bacillococci or yeast) primarily due to their large morphological and physiological diversity that develops during growth. The morphology of filamentous fungi in submerged culture depends on the environmental conditions in the bioreactor, the concentration of the inoculum, spore viability, pH, temperature, dissolved oxygen concentration, and mechanical stress [6–12]. In submerged culture, fungi develop two types of extreme macroscopic morphologies: pellets and filamentous mycelium. Pellets are compact hemispherical aggregates of hyphae in which the diffusional limitations to the center of the pellet represent the principal growth restriction. At the other hand, filamentous or sparse mycelium increases the viscosity of the broth and can drastically reduce the oxygen transfer capacity of the bioreactor [13, 14].

1.1 Cultures with Dispersed "Filamentous" Morphology (Dispersed Mycelium)

Fermentation broths are generally heterogeneous suspensions of microorganisms, culture medium components (nutrients), and various metabolites or products dispersed in a liquid phase, generally water. Unlike other broths, the rheological properties of the predominantly filamentous mycelial cultures change with time.

These changes are mainly due to the increase in cell mass, the morphological changes of the hyphae, and the interactions between hyphae [15–18].

The hyphae are usually long, thin, and branched: They are also interlocked due to their high length to diameter ratio, thereby forming a three-dimensional network. These organisms grow in a highly dispersed manner and consequently generate highly viscous and rheologically complex suspensions [19]. Most mycelial broths behave similar to pseudo-plastic fluids. Their viscosity decreases when the deformation gradient increases. Thus, in cultures carried out in mechanically stirred bioreactors, the flow characteristics change around the impeller (high-speed deformation region). This implies that the fluid surrounding the impeller has a low viscosity due to the high deformation gradient in this zone of the reactor [16, 20]. However, in areas near the walls of the bioreactor, the deformation rate applied to the fluid is lower, causing a noticeable increase in viscosity. This causes serious problems in the mixing, creating "dead" or poorly mixed areas, in which there is a reduction in the mass transfer rate and the formation of concentration gradients of nutrients and dissolved oxygen. These differences are stronger as the process progresses and the organism grows [15, 21–25]. Filamentous growth has been reported to be favorable for the production of various antibiotics or enzymes [4, 26–28].

1.2 Cultures with an Aggregate Morphology or "Pellets"

It has been reported that pellets favor the production of some metabolites as glucoamylase in *Aspergillus niger* [29]; lovastatin in *Aspergillus terreus* [30]; fumaric acid in *Rhizopus delemar* [31]; and ergothioneine in *Lentinula edodes* [32]. However, the differentiation and physiological state of the cells that carry out the synthesis of these metabolites are not yet well understood.

For practical purposes, the pellet-type morphology is more convenient in fermenters because the culture media in which pellets develop have a low viscosity and behave as Newtonian fluids, facilitating the mixing and homogenization of culture broth. For example, Cai et al. [33] obtained a mutant for a gene that affected the polarized growth of *Aspergillus glaucus*. Unlike the native strain that had a dispersed filamentous growth, the mutant grew into pellets and produced 82 % more aspergiolide A, than the native strain, and exhibiting low-viscosity broths reaching high dissolved oxygen tension during culture.

Despite the positive effects of pellet morphology on viscosity and oxygen transfer, limitations in nutrient diffusion from the broth to the center of the pellet can arise as the pellet size increases throughout fermentation. This phenomenon causes significant physiological changes in the organism. In cultures with a predominant pellet morphology (i.e., basidiomycetes), it is likely that nutritional or oxygen limitations inside the aggregates adversely affect the growth and productivity of these fungi. For this reason, even when high concentrations of dissolved oxygen are maintained in the medium, the oxygen concentration can be limited within the mycelial pellet when the aggregates grow above a critical diameter [23].

Therefore, it is essential to characterize mycelial morphology to understand the effect of process variables on the physiology of filamentous cultures. For example, Cui et al. [23] proposed an equation (Eq. 1) to determine the maximal diameter that can be reached by an aggregate (D_{crit}) without diffusional limitations at the center of the aggregate.

$$D_{crit} = \sqrt{\frac{24 \times C_{O_2} \times D_{eff}}{R_{O_2}}} \tag{1}$$

In this equation, D_{crit} is the critical diameter of the aggregates (m), C_{O_2} is the concentration of dissolved oxygen in the liquid (kg O_2 m^{-3}), D_{eff} is the effective diffusion of the oxygen in the aggregates (m^{-2} s), and R_{O_2} is the rate of oxygen consumption per unit volume (kg O_2 $m^{-3}s^{-1}$). R_{O_2} equals the product of the specific rate of oxygen consumption (q_{O_2}) and aggregate density (r). D_{eff} is the product of the molecular diffusion coefficient (D_{O_2}) and the porosity of the pellet (P_p) (Eq. 2).

$$D_{eff} = D_{o_2} \times P_p \tag{2}$$

With equations such as those proposed by Cui et al. [23], it is possible to estimate whether there is a limitation in oxygen diffusion into the pellet according to the morphological characteristics of the aggregates (porosity and diameter) and other factors such as dissolved oxygen concentration, rate of oxygen consumption, and density of the aggregates.

A negative correlation between hyphal gradient (d_h/d_r)—a morphological parameter describing the pellet periphery—and the effective diffusion coefficient has been reported [34]. The authors showed that while diffusion limitation in pellets is mainly a function of its size, the influence of the pellet periphery over the diffusion is rather high.

Furthermore, using mutant strains (GFP), the intraparticle metabolic activity can be determined and correlated it with the productivity of the process. Driouch et al. [35] demonstrated with this tool that the fructofuranosidase productivity of *A. niger* increases when titanate microparticles were used in the culture to decrease the size of the pellets. The diameter decreased from 1,700 μm (control) to 300 μm (microparticles). It was shown that, in the control culture pellets, only the surface layer (200 μm) showed metabolic activity associated with the production of fructofuranosidase. Furthermore, lavendamycin production by *Streptomyces flocculus* increased by 600 % when agitation was increased and was correlated with a decrease in the size of the pellets [36]. Pellet growth is required for efficient glucoamylase production by *A. niger*. Cultures carried out at low energy dissipation (0.063 W/kg) presented high pellet concentration and glucoamylase activity [29].

Given that morphology influences both growth and metabolite productivity in fungi, it is necessary to understand the mechanism of pellet formation and their behavior as a result of agitation at different intensities [13]. They have been generally categorized as a coagulant type or a non-coagulant type [37]. The coagulant

type forms aggregates that grow to form pellets composed of spores and hyphal aggregates formed in the early stage of culture. For the non-coagulant type, a single spore grows into a pellet.

This phenomenon has been clearly described for *A. niger* in which aggregation takes place in two steps. The first one is related to surface interactions between conidia before germination, and the second one to hyphal growth [10, 38]. Aggregation of *A. niger* conidia can be manipulated by pH, which has an important influence in both first and second aggregation steps. Power input has a significant effect on pellet concentration during the second step of aggregation. Cultures carried out at pH 4 showed an increased number of pellets and specific glucoamylase productivity than those performed at pH 7. A clear correlation between product formation and the number of pellets suspended was found [10]. Furthermore, it has been shown that increasing the volumetric power input by aeration yields smaller pellets, higher pellet number, and improved glucoamylase production [39]. Production of glucoamylase seems to occur in the outer layer of the pellets, a region that is rich in single-stranded RNA, explaining the higher productivity of such cultures where small and numerous pellets are present [40].

A. niger also exhibits macro and micro colonies, which are highly heterogeneous with respect to growth, secretion, and gene expression [41]. Gene expression of 7-day colonies of *A. niger* shows that mycelia is highly differentiated, as more than 25 % of the active genes showed significant differences in expression between the inner and the outer zones of the colony [42]. More recently, the study of the heterogeneity of *A. niger* microcolonies has been reported [43]. This work shows that the gene expression of glucoamylase and ferulic acid esterase was higher in the small colonies than in the larger ones. Furthermore, RNA content per hypha was 45 times higher at the periphery than in the center of the microcolony.

Operational and culture conditions strategies as ultrasound, agitation intensity, inoculum density, substrate concentration, and manipulation of broth viscosity have been studied in order to control fungal morphology and process productivity. The manipulation of viscosity, by adding Xanthan gum, surfactants, or glass beds on the broth can be used to increase pellet count, biomass concentration and decrease pellet volume of *Streptomyces hygroscopicus*. In these cultures, specific production of geldanamycin was inversely correlated with the mean pellet diameter [44, 45].

Inoculum density also has shown to determine morphology, broth rheology, and erythromycin production by *Saccharopolyspora erythracea*. Low inoculum densities yield pellet morphology and Newtonian rheology. At the other hand, clumps yield non-Newtonian rheology and high erythromycin and were obtained at high inoculum densities [46].

The agitation intensity is also an important parameter that affects the morphology of filamentous microorganisms; and in some cases, the production of metabolites, for example, intracellular content of ergothioneine in *L. edodes* pellets is almost 3 times of that found in free filaments or clumps. In this case, the pellet size increased as agitation is reduced, favoring the highest productivity of ergothioneine [32].

Ultrasound applied during *A. terreus* cultivation influences morphology and lovastatin production; however, it does not affect fungal growth. Sonication

negatively affects lovastatin production, and this was associated with the change from pellet to disperse hyphal morphology occurred at high power input values [30]. Fumiglaclavine production by *Aspergillus fumigatus* is also increased by ultrasound exposition, which produces smaller and looser pellets as compared with untreated control [47].

Initial substrate concentration influences growth morphology of *R. delemar*. At the best initial concentrations, small pellets, high biomass growth, and an increased fumaric acid production are obtained [31]. Osmolality can also affect fungal morphology and productivity. High specific productivity of fructoranosidase by *A. niger* can be achieved by the addition of sodium chloride to reach an osmolality of up to 3.2 osmol/kg. In such conditions, fructofuranosidase production is about 3 times that obtained in the control cultures [27]. In that work, a significant correlation between specific fructofuranosidase productivity and the switch between pellets to filamentous morphology were reported.

2 Hydrodynamic Stress in Mycelial Cultures

2.1 Factors Determining Fungal Morphology and Metabolite Production

The hydrodynamic conditions generated during cell culture in bioreactors can affect both shape (morphology) and cellular metabolism. When mechanical stress is applied to the wall or cell membrane, various specific receptors (such as ion channels) deform and stimulate a series of cascade reactions, which appear to affect the synthesis of proteins and RNA [48, 49]. G-protein-coupled receptors (GPCR) have been implicated in cellular responses to shear stress and morphogenesis in several fungal species. However, it is unknown how mechanical stress modulates GPCR function [50]. This phenomenon, defined as the change in the physiology of a culture depending on the shear rate gradients caused by the movement of a fluid, is commonly called hydrodynamic stress and is particularly important for mechanically stirred tanks. Specific oxygen uptake has been recently proposed as a shear stress indicator in fungal cultures [51].

There are several examples where shear stress seems to enhance fungal metabolite productivity. However, most of the reports fails to evaluate whether the effects observed are only related to shear stress and/or to an increase in the oxygen transfer rate. High lipase production by *Rhizopus chinensis* is enhanced at low inoculum density and high shear stress, which leads to a predominant pelleted morphology [52]. At the other hand, xylanase production by *Aspergillus oryzae* was not affected by agitation [53]. Impeller choice does not affect enzyme production in fed-batch cultures of *A. oryzae*; however, growth and enzyme production were enhanced at high aeration and volumetric power inputs [54]. Production of pristinamycins by *Streptomyces pristinaespiralis* is influenced by agitation. At low power input, oxygen limitation was observed and no pristinamycin production was

observed. After oxygen limitation is overcome, product formation was related to power input in a bell-shaped way with a maximum at 8 W/L [55]. When this fungus was grown in the range of 0.5–6 W/L, a direct correlation between growth and maximal specific growth rate was found [56]. More recently, the same authors have shown that pristinamycins production occurred only when $k_L a$ was higher than 100 h^{-1}, while final product concentration and bacterial pellet diameter were correlated to *P*/*V*. A strong correlation between pristinamycins concentration and pellet interfacial area was found [57].

Negative effects of extreme shear stress on fungal productivity can also be found. High oxygen concentration and low shear stress are necessary for lovastatin production by *A. terreus* [58, 59]. Production of exopolysaccharides (EPS) by *Paecilomyces tenuipes* in airlift and stirred reactors does not differ significantly when loose aggregates are the dominant mycelial morphology. However, molecular weight and chemical composition of the product is affected by agitation in bioreactors [60]. On other hand, production of EPS by *Tremella fuciformis* is higher in airlift reactors than in stirred reactors and related to a dominant population with yeast-like morphology [61]. An increase of 40 % in the production of manganese peroxidase, between immobilized and stirred cultures of *Nematoloma frowardii*, has been reported [62]. Xylanase production by *A. terricola* seems to be affected by shear stress as enzyme production was significantly lower in stirred tanks than in airlift reactors [63]. Production of arpergiloide-A by *A. glaucus* can be increased by 322 % by using a combination of a pitched blade (upper), a disk blade turbine (lower), and n-dodecane as oxygen carrier, if compared with cultures carried out with two disk turbines [64]. Chitinases production by *Lecanicillium muscarium* is strongly affected by aeration and agitation. At low agitation and aeration, an increase on both parameters yields an increase in enzyme production, likely due to an increase in oxygen availability. Here again, enzyme production decreased once a certain threshold of shear stress is attained [65]. The use of rocking agitated disposable reactors has been reported for shear stress sensitive microorganisms. In a bag reactor, fungal cultures of *Pleurotus sapidus* and *Flammulina velutipes* grew in form of small pellets, while in stirred tanks cultures, an intense aggregation was observed [66].

Microorganism sensitivity to mechanical stress is primarily determined by the structure (presence and/or absence of a cell wall) and size of the cells [67, 68]. Cell size plays a major role in cell sensitivity to mechanical stress (Table 1). In general,

Table 1 Size and sensitivity of microorganisms to shear (adapted from Ref. [67, 77])

Microorganism	Size (μm)	Sensitivity
Bacteria	1–10	–
Filamentous fungi	>100	++
Animal cells	20	++
Animal cells in microcarriers	150	+++
Disperse plant cells	100	+
Aggregated plant cells	>1	++

the larger the cell, the greater the damage because the degree of interaction between cells and small eddies is greater. Eddies are formed from the turbulence generated in the mixing tank. Eddies are the result of turbulent flow (see further below) and are responsible for causing mechanical damage to cells [69–73]. It is hypothesized that most of the damage to biological particles is caused by eddies that are the same size as or smaller than the cell. Larger eddies carry the particles in a convective motion. Eddies of comparable or smaller sizes can act simultaneously and in opposite rotational directions on the cell surface, subjecting them to deformation gradients and therefore to shearing forces [48, 74]. As a result, the intensity in the interaction between particles and eddies determines cellular damage or mycelial aggregation during cultivation. For example, growth and pellet morphology of *A. niger* was studied under different fluid dynamic conditions. Higher shear stress negatively affected fungal growth and leads to small and dense pellets. Results showed that maximal shear stress, occurring at the vicinity of the impellers, is the main factor affecting pellet growth [75].

In mechanically stirred bioreactors, the size of terminal eddies (minimum scale with a turbulent flow) can be calculated according to the Kolmogorov microscale [76] equation (Eq. 3). In this equation, ε is the local energy supplied by the liquid mass (W kg^{-1}), λ is the size of the microscale (μm), and ν is the viscosity (Pa s). According to the Kolmogorov microscale equation, if the power supplied is reduced (i.e., by decreasing the stirring speed or the diameter of the impeller) and/or the viscosity of the medium increases, the damage to the biological particles will be reduced because the eddies increase in size. λ values oscillating between 10 and 50 μm are typical in bioreactors with an energy dissipation between 0.1 and 100 W kg^{-1} and water as the fluid [77, 78]. Therefore, the most susceptible systems are those with sizes equal or superior to this range (Table 1). These systems include filamentous fungi, plant, and animal cells (>20 μm).

$$\lambda = \left(\frac{\nu^3}{\varepsilon}\right)^{1/4} \tag{3}$$

In mycelial cultures, based on the theory of isotropic turbulence [41], van Suijdam and Metz [79] proposed for the first time that the hyphal length of *P. chrysogenum* may be associated with the size of the eddies generated in a reactor and, therefore, with the operating conditions. Similarly, Ayazi-Shamlou et al. [70] and Cui et al. [77] reported that the fragmentation of the hyphae of the fungus *P. chrysogenum* was determined by the ratio of the average hyphal length (L) and smaller eddies whose size (i.e., Kolmogorov microscale, λ) depended on the energy supplied to the system. However, unlike observations in studies with animal cells, no association between L and λ was reported. This might be explained by the fact that initial physical properties were used to estimate λ. The above assumption may be valid at the beginning of the cultures or when the values of viscosity and/or density do not change significantly. However, when the rheological properties of the medium change (as in filamentous cultures), it is necessary to include any changes in viscosity in the

calculation of λ. At the same time, and as a result of an increased viscosity, it is likely that the supply of energy (ε) decreases due to the higher power drop caused by the formation of gas cavities behind the impellers [80].

The importance of including the actual viscosity value and that of the power supplied (i.e., in situ measurement) in λ was shown by Li et al. [72] in mycelial cultures of *A. oryzae*. As a direct result of increased viscosity (approximately 0.1 to 1.3 Pa s), Li et al. [72] estimated significant increases in λ, which reached values of up to 900 μm. Similarly, Large et al. [81] reported significant increases in λ (100 μm at 0 h to 350 μm at 70 h) due to the increased viscosity of the medium during the filamentous cultivation of *Streptomyces clavuligerus*.

Given the important differences between typical λ values and the average hyphal length (10–50 vs. 100–300 μm, respectively [70, 77]), the shearing forces interact intensively with the surface of the hyphae and fragment them. The size of the mycelial aggregates will therefore be the final result of the balance between the fragmentation (product of hydrodynamic stress) and the apical growth of the fungus. As a result, an equilibrium diameter (d_{eq}) is established when the local shearing forces are equal to the tension forces of the hyphae. According to Li et al. [72], these forces depend on the physiological state and composition of the cell wall of the fungus. Therefore, the use and development of image analysis techniques in mycelial cultures allow us to observe in an objective and rigorous manner the morphological characteristics generated by mechanical stresses.

In mechanically stirred tanks, the morphological properties depend on the stirring speed, the number, diameter and geometry of the impellers, and the fluid characteristics. These conditions determine the hydrodynamic and mechanical forces present in the bioreactor. For instance, *T. harzianum* clump diameter showed a strong dependence on Kolmogorov microscale (λ), calculated using the specific energy dissipation rate in the impeller swept volume and the measured apparent broth viscosities. The mean clump diameter of *T. harzianum* aggregates was about 0.5 λ, which means that fungal morphology of this culture was determined by the size of eddies [73].

In general, increasing the energy supplied reduces the size of the mycelium, which favors the fragmentation of the hyphae. Amanullah et al. [82] reported a reduction in the average total length of *A. oryzae* when the mixing speed was increased (and therefore also the energy supplied) from 550 to 1,000 rpm (from 2.2 to 12.6 kW m^{-3}) in a 6-L bioreactor equipped with two Rushton turbines. Similarly, Papagianni et al. [83] noted a decrease in the average diameter and length of the hyphae by increasing the agitation speed from 200 to 600 rpm in submerged 8 L cultures of *A. niger*. Johansen et al. [84] reported the reduction of hyphal average total length by increasing the agitation speed of a Rushton turbine from 400 to 1,100 rpm (from 0.6 to 13 kW m^{-3}) in 6 L cultures of *A. awamori.* Ayazi-Shamlou et al. [71] also observed a decrease in the size (average length) of the hyphae when the power supplied increased (1.7–33 kW m^{-3}) in submerged cultures (7 and 150 L) of *P. chrysogenum*. Furthermore, the size of the aggregates and/or pellets is also significantly influenced by the energy delivered. Paul et al. [85] observed a decrease in the equivalent diameter and the area of *A. niger* aggregates when the agitation

rate increased (500–800 rpm) after 17 h of culture. Meanwhile, Cui et al. [78] reported that the reduction in the average diameter of *A. awamori* pellets in 3 L cultures (grown in bioreactors with two Rushton turbines) increased with the agitation speed (from 340 to 1,050 rpm).

The morphological characteristics of the mycelial aggregates (average projected area, diameter, length, porosity, circularity, etc.) correlate with various operating parameters of a bioreactor. Tip speed has been used as a scale-up criterion for EPS production by *Ganoderma lucidum* in stirred tanks [86]. However, the most widely used parameter is the supplied volumetric power, or *P*/*V*, which has also been used as a scaling criterion in various bioprocesses [87, 90]. Volumetric power (*P*/*V*) assumes a homogeneous and constant energy dissipation throughout the tank. This factor integrates into a single parameter the speed of stirring, workload, geometry, diameter, and number of impellers, in the bioreactor. Therefore, when *P*/*V* increases (consequently causing mechanical stresses on the mycelium), the size of the aggregate decreases. This phenomenon has been reported by several authors in cultures of *P. chrysogenum* [21, 26, 70, 89], *A. oryzae* [82, 90], *A. awamori* [84, 77], and *A. niger* [83, 85].

However, Jüsten et al. [91] showed that the association between morphology and P/V is valid only for the scale studied. In their work, Jüsten et al. [91] evaluated the effect of the energy supplied to the average projected area of *P. chrysogenum* using three scales of work (1.4, 20, and 180 L) and a Rushton turbine. According to their results (Fig. 2), it was clear that there was a correlation between projected area and the delivered energy (P/V_L) for each scale of work. This suggests that there is a possible effect of tank size (volume) on energy dissipation and, therefore, on morphological characteristics. Similar results were reported by Makagiansar et al. [26] in

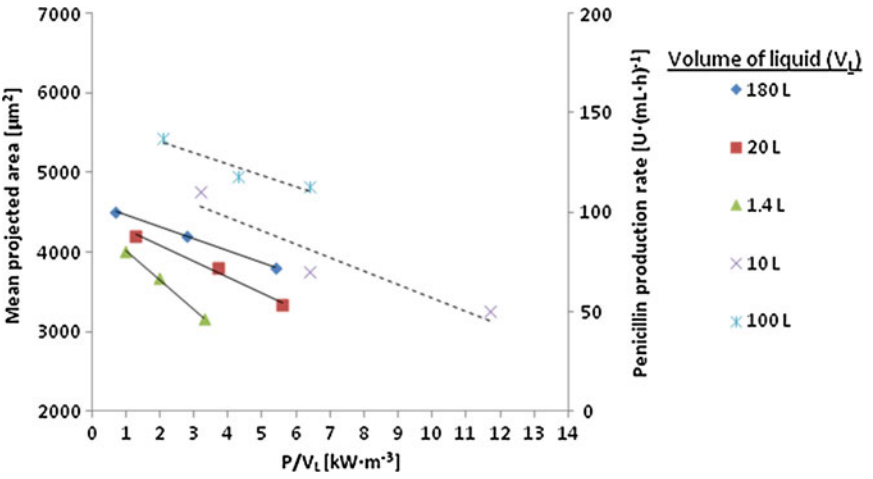

Fig. 2 Effect of the volumetric power supplied (P/V_L) on the average projected area and the rate of penicillin production in cultures of *P. chrysogenum* at different scales (adapted from Ref. [21, 91])

P. chrysogenum cultures in bioreactors of 5, 100 and 1,000 L equipped with three Rushton turbines.

Recently, Marín-Palacio et al. [92] showed that the parameter P/V does not determine the microbial growth, morphology, and recombinant protein production in cultures of *Streptomyces lividans*. These authors concluded that the transfer of oxygen in the center of the pellets could explain the changes observed between the different hydrodynamic conditions evaluated.

2.2 The Concept of "Energy Dissipation/Circulation Function" (EDCF)

Given the limited relationship between volumetric power and morphology observed in different scales of work, various reports suggest the use of the energy dissipation rate or the EDCF ("energy dissipation/circulation" function) to correlate hydrodynamic morphological characteristics with the operating variables of a bioreactor [21, 24, 26, 82, 89–91]. Smith et al. [21] developed the concept of the energy dissipation/circulation function (EDCF) based on the previous work of van Suijdam and Metz [79] and Reuss [93]. These authors proposed that fragmentation (and therefore morphological characteristics) depends on the energy dissipated in the region of the impellers (i.e., energy dispersion area) and the frequency of the passage of mycelium through the area. However, the function given by Smith et al. [21] did not allow for evaluating the effect of impellers other than the Rushton turbines. In subsequent studies, Jüsten et al. [89, 91] modified the EDCF to incorporate an additional geometric factor that was able to evaluate the hydrodynamic effect of different types of impellers (axial and radial). These authors also included the negative effect of viscosity on the power supply (power drop) in the calculation of circulation frequency. The energy dissipation rate (EDCF) is given by Eq. 4:

$$\text{EDCF} = \left(\frac{P}{k \times D^3}\right) \times \left(\frac{1}{t_c}\right) \tag{4}$$

In Eq. 4, $P/(k \cdot D^3)$ is the specific energy dissipation in the impeller sweeping area (kW/m^3), P is the power supplied (kW), D is the diameter of the impeller (m), k is a constant that depends on the geometry of the impeller (dimensionless), and $1/t_c$ is the frequency of circulation of the microorganism in the area of the impeller. This variable is defined by Eq. 5:

$$\frac{1}{t_c} = \frac{Fl_G \times N \times D^3}{V_L} \tag{5}$$

Here, Fl_G is the gaseous flow (dimensionless), N is the stirring speed (s^{-1}), and V_L is the volume of liquid (m^3). Therefore, EDCF quantifies the energy delivered to the fluid in the area of the impellers at a given time. The correlation observed

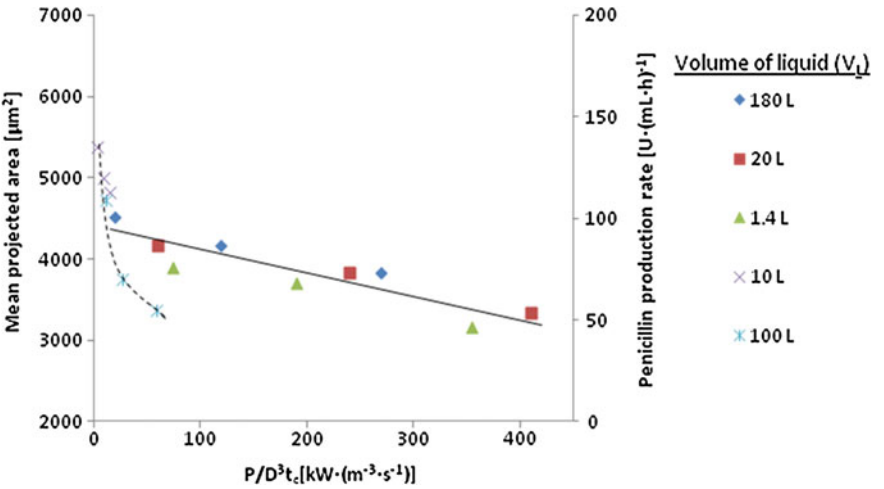

Fig. 3 Effect of the energy dissipation rate (EDCF) on the average projected area and the rate of penicillin production in cultures of *P. chrysogenum* at different scales (adapted from Ref. [21, 91])

between mycelial size and EDCF suggests that the fragmentation of the hyphae depends not only on the energy supplied but also on the frequency at which the mycelium is exposed to high levels of mechanical stress (Fig. 3).

In a very interesting study, Jüsten et al. [91] demonstrated that the mycelial morphology of *P. chrysogenum* (average projected area for the aggregates and total average length of disperse mycelia) depended on the EDCF, regardless of the scale used (1.4, 20 and 180 L). Figure 3 shows the effect of the EDCF on the average projected area. As expected, the projected area decreased when the energy supplied increased. However, unlike P/V, the EDCF correlated well with all the experimental data (Fig. 2 vs. Fig. 3). The fact that the aggregate size decreased when the EDCF increased suggests that increased mechanical damage occurs when the energy supplied increases or when the circulation time decreases (t_c). In both cases, movement of the mycelium through the impeller region (area of high mechanical stress) was favored. Thus, the mycelium was exposed to different levels of shearing stress, and the intensity depended on its position inside the tank and the fluid circulation rate. In a similar study, Makagiansar et al. [26] observed a reduction of the average hyphal length with an increase in the power supplied and/or the frequency of circulation. Therefore, fragmentation and, consequently, the size of the mycelia of *P. chrysogenum* depended on the EDCF in the three working scales used (5, 100, and 1,000 L).

Given the strong interdependence between morphology, physiology and metabolite productivity, and the notorious influence of hydrodynamic stress on morphology, different groups have used the EDCF to find associations between other biological parameters in addition to mycelial morphology and the operational conditions of a bioreactor.

In cultures of *P. chrysogenum*, Smith et al. [21] showed that the rate of penicillin production depended on the EDCF, regardless of the working scale used (10 and 100 L). However, the rate also depended on the *P*/*V* (Figs. 2 and 3). Similar results were reported by Makagiansar et al. [26] in cultures of *P. chrysogenum* in which the average hyphal length and the specific productivity of penicillin produced in bioreactors of 5, 100, and 1,000 L depended on the EDCF. Meanwhile, Jüsten et al. [89] observed that, in addition to the average projected area, the specific growth rate (μ) and the specific productivity of penicillin, depended on the EDCF function in *P. chrysogenum* cultures at different stirring rates and with three different shapes of impellers (flat blade, Rushton turbine, and pitched blade).

Rocha-Valadez et al. [73] reported that in cultures of *T. harzianum*, the energy dissipated (EDCF) has effects on the mycelial diameter. When the energy increases from 9.5 to 95.9 kWm^{-3} s^{-1}, the diameter decreases from $\sim$400 to 200 μm in cultures in which the circulation time is greater than or equal to 3 s. Furthermore, such *T. harzianum* cultures were conducted at equivalent yielding P/kD^3 conditions and developed using two different Rushton turbines diameter sets in order to study the effect of circulation time on fungal morphology. For the studied conditions, $1/t_c$ had a greater effect over mycelial clump size and growth rate than P/kD^3. Consequently, broth viscosity, and hence Kolmogorov microscale, was a function of impeller diameter, even among cultures operated at equivalent specific energy dissipation rates [73]. These effects have an impact on both, hydrodynamic and 6PP productivity, because larger cell aggregates increase viscosity by altering the rate of energy dissipation and therefore the growth rate. These effects are also reflected in the dispersion of the phases present in the fermentation (see further below), having a direct effect on the process of breaking bubbles and drops of other physical phases present in multiphase systems. Although the EDCF has been used to correlate changes in morphology and the metabolism of hydrodynamic sensitive microorganisms, this parameter allows the analysis of the shearing energy intensity only in regions close to the impeller, because this function is derived from Kolmogorov's theory of isotropic turbulence [94].

It should be pointed out that, however, the use of the EDCF to correlate metabolite productivity with hydrodynamic conditions is not universal. This was demonstrated by Amanullah et al. [82, 90] in continuously fed cultures of *A. oryzae* for the production of recombinant proteins (α-amylases and amyloglucosidases). This was probably due to fact that the fed-batch cultures were carried out under conditions of substrate limitation. As in batch cultures, without substrate limitation, biomass concentration and AMG secretion increased with increasing agitation intensity.

Although it is difficult to draw general conclusions, due to (a) the complexity of the systems and the different specific characteristics of the mycelial cultures that have been studied, (b) the somehow limited experimental strategies, which in the majority of the cases are not able to rigorously discriminate between mechanical (stress) effects and those associated with dissolved oxygen, and (c) the not clearly defined term "hydrodynamic stress," the review of the literature revealed some general points:

(a) The overall mycelial size is inversely proportional to the global energy supplied to the bioreactor.
(b) More particularly, the overall mycelial size is an inverse function of the energy dissipated in the vicinity of the impellers, as well as of the time that the biomass is present in that zone.
(c) In general, within a range of low dissipated energy, the increase in power improves the production of fungal metabolites; however, the effect could be a dissolved oxygen one.
(d) In the range of high dissipated energy, an increase in power leads to saturation or decreasing profiles in terms of metabolite production.
(e) The mechanism of mycelial damage has been shown to be associated with the length of Kolmogorov eddy size; however, very few works have reported estimated values based on experimental determination of power drawn.

2.3 *The Use of Microparticles to Manipulate Fungal Morphology (and Metabolite Productivity)*

The use of microparticles in stirred tanks is a useful strategy to enhance the enzyme [19, 95–100] and secondary metabolites production [101–103] by different fungal strains [98], by controlling the morphology of fungal microorganisms.

In a pioneer work [95] using talc microparticles (≤42 μm), Kaup et al. were able to control fungal morphology of *Caldariomyces fumago* from pellet to single hyphal growth, which yielded a fivefold increase in the maximum specific productivity of chloroperoxidase. In another work, glass beads of 4 mm were successfully used to produce filamentous growth and to scale up from microtiter plates to bench-scale cultures [101].

Addition of titanate microparticles (8 μm) to *A. niger* cultures yielded a sevenfold increase on the production of fructofuranosidase and glucoamylase [99]. Interestingly, microparticles were found to be part of the inner core of fungal pellets with hyphae occupying the external shell of the pellets. Using a recombinant strain expressing GFP, it was possible to demonstrate that active mycelia of control cultures is restricted to the external 200 μm surface of the pellets, while pellets obtained in microparticle containing cultures were fully active.

Lipid accumulation by *Mortierella isabellina* is enhanced by controlling fungal morphology, from pellets to dispersed mycelia, using different concentrations of magnesium silicate microparticles. Lipid cell content increased with the increase of microparticle concentration. At 10 g/L, lipid production was 2.5-fold higher than in the control experiment [102]. In another work, the production of the fungal volatiles 2-phenylethanol and 6-pentyl-α-pyrone by *A. niger* and *Trichoderma atroviride*, respectively, was also enhanced by the addition microparticles [103].

Although results obtained with the use of microparticles in fungal cultures are strain and particle specific, two are the main factors involved in its positive effects

on process productivity. First, the presence of microparticles favors the reduction of aggregates size, in which the nutrient limitations occur in pellets with a diameter above 200 μm. With microparticles, fungal morphology can be tailored; therefore, mass transfer limitations are reduced when dispersed cells are obtained instead to large pellets. The second main factor affecting morphogenesis and metabolic responses of filamentous microorganisms is the mechano-sensitivity of fungal cells to shear stress. G-protein-coupled receptors (GPCR) have been implicated in cellular responses to shear stress and morphogenesis in several fungal species. However, it is unknown how mechanical stress modulates GPCR function [50].

2.4 Filamentous Growth: The Case of Trichoderma Harzianum

Trichoderma is a filamentous fungus that is commonly found in soil and belongs to the group *Deuteromycetes*. The fungi from this genus have the ability to synthesize different products of interest [4, 104–109] such as enzymes (i.e., cellulases, chitinases, glucanases, glucosidases, and xylanases), antibiotics (cyclosporine and trichodermine), aromatic compounds (6-pentyl-α-pyrone and γ-decalactone), organic acids (citric acid), or extracellular proteins. Additionally, they have been used as biological control agents. The growth of filamentous fungi in liquid cultures broth may generate serious deficiencies in mixing and affect the productivity of the respective bioprocesses (as already discussed in previous sections).

Trichoderma harzianum has been a good model of study because it produces metabolites of industrial interest, and the biomass itself has applications as biological control agent.

Hydrodynamics and mass transfer are particularly important in the submerged culture of *Trichoderma* sp. because mycelial growth (Fig. 4), morphology, and the

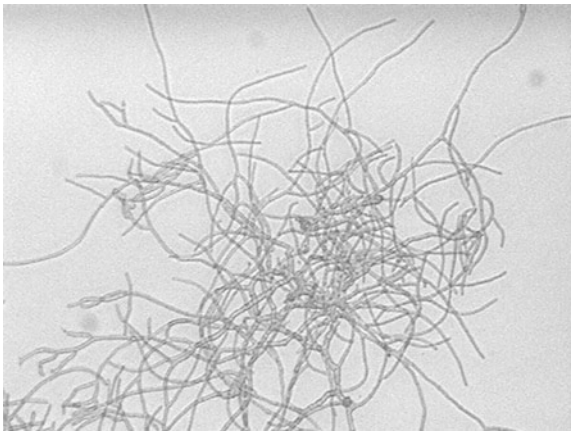

Fig. 4 Filamentous growth of *Trichoderma harzianuam* cultivated in a stirred tank (photograph obtained with a magnifier 10×)

production of various metabolites depend on the environmental conditions prevailing in the bioreactor. Several authors (see below) have observed a similar bell-shaped association between metabolite productivity (i.e., cellulases, glucanases, xylanases, or chitinases) and power or energy supplied usually manipulated through changes in stirring speed. The general explanation for this phenomenon is that the increase in stirring speed reduces oxygen limitation problems often present inside the aggregates or pellets. However, the positive effect caused by increasing the agitation rate is limited and is mainly determined by the sensitivity of fungi to hydrodynamic stress. The mechanical forces damage the mycelium and thus adversely affect the production of the metabolite of interest.

Mukataka et al. [104] observed that in 2 L cultures of *T. reesei* QM 9414 using five shaking speeds (from 100 to 500 rpm) for the production of enzymes and extracellular proteins, there was an optimal stirring condition for each metabolite. Therefore, the speed for the production of cellulases, glucosidases, and biomass was 200, 400, and 300 rpm, respectively. Meanwhile, the extracellular protein reached a similar (and maximum) concentration between 300 and 400 rpm. The authors suggest that the low production of metabolites observed in cultures grown under conditions of lower agitation was due to limited oxygen, because the pellet size was relatively large (ranging between 1 and 2 mm). However, very high agitation speeds created significant morphological changes (short and loose mycelium) and a reduction in biomass and product concentration. According to the authors, this phenomenon was the result of mycelial damage generated by an increase in shearing stress in the bioreactor. Lejeune and Baron [105], Marten et al. [110], and Felse and Panda [111] made similar observations in cultures of *T. reesei* QM 9414, *T. reesei* RUT-C30, and *T. harzianum* NCIM 1185 for the production of cellulases, xylanases, chitinases, and extracellular proteins in bioreactors of 15, 10, and 2 L, respectively. Furthermore, according to Felse and Panda [111], the energy supplied could induce cellular differentiation of *T. harzianum* because the fungi sporulated (cell differentiation in fungi that occurs when environmental conditions are not favorable for development) in cultures grown in the highest stirring conditions (>270 rpm).

The work of Godoy-Silva et al. [112] reported that, in addition to the energy supplied, the hydrodynamic conditions generated by the propellers markedly influenced the growth, morphology, and production of γ-decalactone (peach aroma) by *T. harzianum* in 10 L cultures with similar initial volumetric power drawn (0.056 kW m^{-3}). Low mechanical stress conditions (using a helical impeller) originated a higher mycelial growth in the form of pellets, compared to a system of high hydrodynamic stress (three Rushton turbines) that generated dispersed mycelium. Additionally, production of γ-decalactone was inversely proportional to the cell concentration because the highest productivities were achieved using the Rushton turbine system, where the biomass concentration was lower. Furthermore, Galindo et al. [113] observed a bell-shaped association in *T. harzianum* 500-mL flask cultures between the energy supplied and the specific productivity of 6-pentyl-α-pyrone (coconut flavor), similar to what was observed in bioreactors. Maximum productivity [0.72 mg6PP (g^{-1} h^{-1})] was obtained at a P/V of 0.9 kW m^{-3} (250

rpm, using 80 mL of medium), while the highest mycelial growth was reported at 1.13 kW m^{-3} (300 rpm). This condition presented aggregates exhibiting the smallest diameter.

Rocha-Valadez et al. [114] reported the effect of EDCF (Eq. 3) in a range between 3 and 96 kW m^{-3} s^{-1} on the morphology, mycelial growth, biomass viability, and production of 6-pentyl-α-pyrone in *T. harzianum* cultures under non-limiting dissolved oxygen conditions. It was observed that the average maximum diameter of aggregates depended on the EDCF. An increase in the EDCF [3–96 kW m^{-3} s^{-1}] reduced the diameter of the mycelial aggregates from 0.39 to 0.19 mm because fragmentation was favored. It is thought that fragmentation of the mycelium was the result of hydrodynamic stress because the average diameter of the aggregates was directly related to eddy size experimentally estimated according to the Kolmogorov microscale. The highest degree of interaction between particles and eddies was observed in the region of the impellers because the frequency of passing of the mycelium through that zone determined the aggregate size.

An increase in the EDCF caused significant changes in the metabolism of *T. harzianum* [114]. The hydrodynamic conditions resulting in higher stress reduced the specific growth rate (from 0.052 to 0.033 h^{-1}) and increased the specific C_{O_2} production, although the glucose consumption rates (0.05 ± 0.009 g g^{-1} h^{-1}) remained constant. The hydrodynamic conditions also influenced the viability and cell differentiation (i.e., sporulation) of the organism because they, respectively, decreased and increased as the EDCF increased.

The production of 6-pentyl-α-pyrone (6PP, coconut aroma) was stimulated between 7 and 10 kW m^{-3} s^{-1}) [113]. The maximum specific productivity showed a bell-shaped association with EDCF in cultures carried out at ≥20 % of dissolved oxygen. The reduction in 6PP biosynthesis [after 20 kW m^{-3} s^{-1}] was due to the effect of hydrodynamic stress that generated the largest mycelial damage (fragmentation) and cell differentiation (sporulation) of the fungus. Furthermore, it was proposed that the biosynthesis of 6PP increased due to metabolic changes seen when increasing the EDCF between 3 and 10 kW m^{-3} s^{-1}, when possible nutrient limitations (oxygen and/or glucose) were not present.

Overall, metabolite production by *Trichoderma* spp. shows a strong dependence on hydrodynamic conditions and cellular differentiation (i.e., sporulation) that occurred at high power inputs. It has been shown that metabolite productivity can be increased by the use of microparticles [103] which leads to a disperse morphology. However, there is a generalized lack of information regarding the relationships between hydrodynamics, oxygen transfer, morphology, cell differentiation, and metabolite productivity. Indeed, most of the literature does not differentiate between hydrodynamic and oxygen effects and characterization of fungal morphology as a function of these factors is scarce. Furthermore, it is crucial to understand cell differentiation within fungal aggregates in order to understand the complex relationships between physicochemical conditions prevailing in the bioreactor and metabolite productivity of the process.

2.5 Pellet Growth: The Case of Laccase Production

The production of laccases by ligninolytic basidiomycetes, also called white rot fungi, has been widely studied due to the ability of these microorganisms to grow on inexpensive substrates, excrete enzymes, and oxidize xenobiotics. *Trametes*, *Pleurotus*, *Lentinula*, *Pycnoporus*, *Botryosphaeria,* and *Phanerochaete* have been the most studied genera in recent years [115] because they can be easily cultivated in vitro and their enzymes can be easily purified.

Pleurotus is the genus of white rot fungi. In these fungi, laccases are the most important components of the extracellular lignin degradation systems [115]. The production of laccases has previously been reported for various species of the genus *Pleurotus*, including *P. pulmonarius* [116], *P. sajor-caju* [117], and *P. eryngii* [118, 119].

Pleurotus ostreatus produces laccases and manganese peroxidases but no lignin peroxidases [120]. This basidiomycete fungus is able to grow and degrade large amounts of agricultural and forestry wastes (lignocellulosic) by attacking different polymers of wood. This activity is made possible by the secretion of several laccase isoenzymes with unique physicochemical catalytic characteristics. This variety of laccase isoenzymes is related to different functions such as the synthesis and degradation of lignin, the development of fruiting bodies, pigment production, and cellular detoxification [121].

Laccase isoenzymes synthesis and secretion are strongly influenced by nutrient levels, growth conditions, stages of development, and the addition of inducers to the medium. It has been shown that most of these factors act at the transcriptional level to produce different responses between different isoforms of the same strain and in different fungal species [122].

The efficient production of laccases involves a combination of factors such as a highly productive strain, a culture medium specifically designed, the use of inductors, and the design of the bioreactor.

The production of laccases has been reported in solid state and submerged cultures, as well as in immobilized cultures [123]. However, the highest yield reported so far (300 U/mL) has been achieved in mechanically stirred fed-batch submerged cultures of *Trametes pubescens* induced with copper [124].

Several authors have reported that agitation and aeration influence the production of laccase by different fungal strains. However, the evaluation of the effect of agitation has resulted in contrasting findings, which did not clarify the role of this parameter in the production of the enzyme. On the one hand, some authors argued that high shear stresses (generated by agitation) are responsible for the low production of laccases [125–127]. On the other hand, it has been reported that the production of laccase is not affected by agitation [128, 129]. In addition, other authors have shown that laccase production is affected by agitation following a Gaussian bell-shaped distribution. This indicates that there is a stimulation effect at low agitation and a negative effect at high agitation [130].

Fenice et al. [126] evaluated the effects of agitation and aeration in submerged fermentation cultures of the fungus *Panus tigrinus* CBS 577-79. The wastewater from olive milling was used as the growth medium for the production of laccases. The study of the effect of stirring was conducted in 2 L reactors using two Rushton impellers and stirring speeds varying from 250 to 750 rpm with an airflow of 1 vvm. The effect of aeration was studied by varying the speed of aeration from 0.5 to 1.5 vvm and maintaining the impeller speed at 500 rpm. In this work, it was observed that lower enzymatic activities were achieved by increasing the stirring rate. Although these results could point out to a negative effect of the shear stress on the organism, it was also observed that the increase in flow aeration from 1 to 1.5 vvm adversely affected the specific activity of the laccases.

In 2004, Tavares et al. [128] conducted an experimental design that applied response surface methodology to optimize the production of laccases from *Trametes versicolor* in bioreactors. The rates of agitation (100–180 rpm), pH (3 and 5), and glucose concentration (0 and 9 g/L) were evaluated. In this study, the agitation speed had no effect on the maximum laccase activity, while the pH of the medium was the most important factor, followed by glucose concentration.

In a study using cultures of *Trametes versicolor* ATCC 200801, Birhanli et al. [130] described that the stirring speed had a bell-like effect on the production of laccase. Stirring speeds ranging from 0 to 250 rpm were evaluated. The highest laccase activity was obtained at 150 rpm, while lower activities were obtained at lower (0, 50 and 100 rpm) and higher (200–250 rpm) stirring speeds. The decrease in laccase activity above 150 rpm was attributed to shear stress on the pellets by high-speed stirring.

Overall, it is accepted that the shear generated by impellers negatively affects the production of laccases by superior fungi [123]. However, in the studies described above, it was not possible to separate the influence of the agitation from the effects of dissolved oxygen concentration.

A throughout study aimed at understanding the roles of hydrodynamic effects and dissolved oxygen on the mycelia growth and laccase production by *P. ostreatus* CP50, grown in a mechanically agitated bioreactor, was recently reported [131]. This study evaluated the particular effect (and possible interactions) of the initial EDCF, aeration rate, and copper induction, using a factorial experimental design. Under the experimental conditions tested (EDCF 0.9 to 5.9 kW m^{-3} s^{-1}), there was no evidence of hydrodynamic stress on the growth of *P. ostreatus*. Actually, the growth of the fungus was stimulated with increasing EDCF. However, it was found that the specific production of laccases decreased with increasing EDCF and aeration. The results show that laccase production by *P. ostreatus* CP50 is favored at a low EDCF. However, preliminary evidence indicates that the negative effect of the agitation could be related to the stimulation of protease production (decreasing enzyme activity) and may not be a direct result of the hydrodynamic stress.

2.6 Physiological Characterization of Fungal Aggregates by Image Analysis

From the previous sections, it is clear the lack of systematic information on the size of the pellets (Fig. 5) obtained during cultivation. However, the understanding of the hydrodynamic and dissolved oxygen effects on the growth and productivity of these cultures requires the accurate and quantitative characterization of fungal morphology.

The amount of viable biomass, in addition to morphology, is an important parameter that affects metabolite productivity of filamentous fungi in fermentation systems. The biomass determines the oxygen demand of the system during fermentation. The quantification of dry weight and turbidity is the most commonly used techniques to detect increases in biomass. Methods have also been used to measure the cultivability of cells by counting colony-forming units (CFUs) [132, 133]. However, these techniques do not provide information regarding the biocatalytic activity or the viability of cells. Furthermore, many of these techniques can lead to significant errors due to the effect of dilution.

Accurate viability assessment can significantly help in improving fermentation process design as well as in physiological studies and those involving monitoring and control of these bioprocesses. Fluorescence and image analysis are used jointly to analyze fungal physiology [134] and actinomycete differentiation [135, 136] and to evaluate viability through the spore germination percentage [137] or the percentage of intact membranes in the hyphal area (considered viable regions) relative to the total mycelial area [138].

Hassan et al. [139] correlated the cultivability (as the best indicator of cell viability) with the percentage of active biomass (measured by the percent of fluorescein diacetate-stained area in the hyphae) of the biological control agent *T. harzianum*. A fluorescence method based on the fact that metabolically active cells are able to hydrolyze intracellular fluorescein diacetate (FDA) producing fluorescein was used for the quantification of fungal viability. Fluorescein fluoresces at

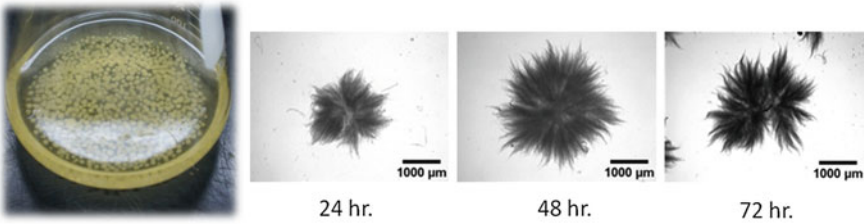

Fig. 5 Pellet growth of *Pleurotus ostreatus* in a flask and morphology at different growth time in stirred tank

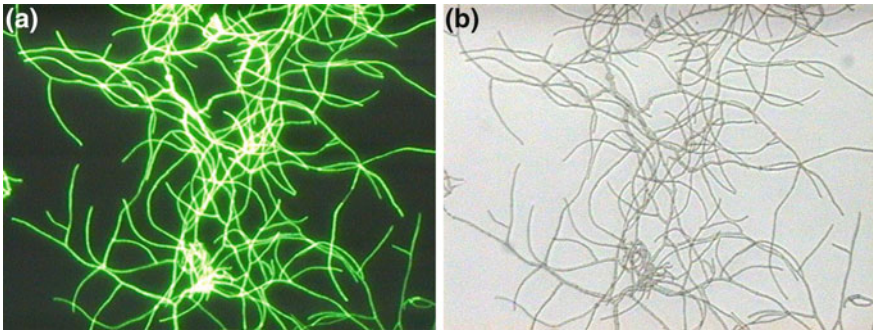

Fig. 6 Photomicrographs (10×) of *Trichoderma harzianum* with fluorescein diacetate staining. **a** Only metabolically active regions can be detected in the fluorescent image. **b** The total area of the aggregate is detected in white light microscopy [139]

490 nm and can be used to quantify the percentage of viable cells in cell clumps. The percentage of active biomass (viability) was calculated as the ratio of the stained area (in the fluorescent image) to the total area detected in the image with white light. An example of the images (before processing) is shown in Fig. 6. Pinto et al. [140] also used this last principle. Wei et al. [141] used dark-field illumination with an in situ probe inside a bioreactor to estimate the viability of the brewer's yeast *Saccharomyces cerevisiae*. They implemented two support vector machine-based classifiers for separating the cells from the background and for distinguishing live from dead cells. Their results showed very good accuracy compared with other standard methods. The method is not directly applicable to filamentous fungi, but the principles used in that work [140] may constitute a good basis to be adapted to this purpose. Lecault et al. [142] proposed a semiautomatic image analysis protocol allowing the detailed analysis of morphological parameters in addition to the assessment of the percentage viability of biomass using FDA. Their method was tested on *T. reesei* during fed-batch fermentation in a reciprocating plate bioreactor (RPB). Viability was assessed by measuring the area ratio of the fluorescent regions to the dark regions (a mask of the bright field image was subtracted from the fluorescent image to isolate the objects being analyzed). From the total area and the viable area, the percentage of viability was calculated. Choy et al. [143] used a similar method for a *Trichoderma reesei* viability estimation and found satisfactory results. Although reliable overall viability assessment of fungal cultures is now possible, it is crucial to develop techniques aimed to evaluate fungal differentiation within the mycelial aggregates. Such information has been proven to be very useful to the understanding of the effect of physicochemical environment on the growth and metabolite productivity of fungal cultures [35, 40–43, 99].

3 Phase Dispersion and Mass Transfer Characterization in Cultures Containing Fungal Biomass

A common limiting step for achieving high productivity of fungal products (the biomass itself and/or metabolites) resides in the transfer of oxygen (usually from air bubbles) to the bulk liquid in which the active biomass is suspended. There are various strategies to improve the oxygen transfer to the filamentous fermentations as well as to minimize cell damage (reducing the effect of mechanical stress by agitation), such as the design of new-generation agitators [144], using different combinations of axial and radial impellers [145, 146] and by reducing mechanical agitation by the use of ultrasound [147]. All these approaches have proven effective in a variety of models of study; however, despite the variety of studies in a wide range of operational conditions, it has not been possible—so far—to establish a general model that could describe the relationships between oxygen transfer and parameters such as scale, mycelial morphology, and operational parameters of the process [148].

The k_La, OTR, and DOT are some of most studied parameters for analyzing the problems of oxygen transfer to the broths containing filamentous microorganisms [17, 51, 149–152]. However, these parameters are global ones and do not provide information regarding the mechanisms of mass transfer occurring at the level of single bubbles or populations of them. Furthermore, it is very limited the available information describing what happens (in terms of the size distribution of air bubbles and mass transfer area) when biomass—a suspended solid of complex morphology—is present. In addition, oils are commonly used in fungal fermentations as carbon sources, oxygen vectors, and extractive agents [64, 153–156]. However, the literature has only scarcely documented the effects of these insoluble oils in terms of the dispersion of the phases and oxygen transfer. Here, we review the relative few papers available on the field.

The presence of an organic phase in fungal cultures results in a fermentation system that requires the mixing of four phases: the culture medium (liquid phase), solvent (organic phase), mycelium (solid phase), and air (gas phase) (Fig. 7). The dispersion of these four phases occurs by continuous breaking and coalescence of air bubbles or oil droplets, which depend on the conditions of turbulence and the physical properties of the medium. Solvents have been successfully used to increase the production of aroma compounds such as 6PP, which is a coconut-like aroma compound and also a potent fungicide [155, 156].

Galindo et al. [153] published the first paper in which the distribution of air bubbles and oil droplets were characterized in a four-phase system. The Sauter mean diameter of the bubbles was smaller for increasing concentrations of castor oil and *T. harzianum* biomass. This work also reported by the first time the phenomenon of air bubbles embedded within oil drops, which has important implications in terms of the mechanism of mass transfer in such systems. Morphology of the biomass also affected the dispersion. Lucatero et al. [157] reported the effect of mycelial morphology on the dispersion of oil and air in simulated fermentation systems of *T. harzianum*. They observed that the number of bubbles trapped in

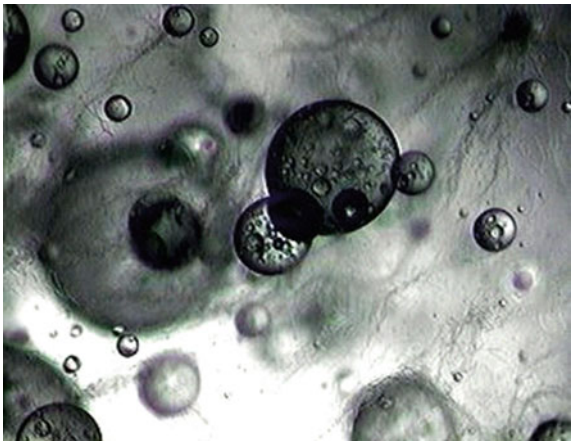

Fig. 7 Dispersion of four phases (filamentous biomass, liquid mineral media, castor oil, air) in the *Trichoderma harzianum* extractive fermentation

castor oil drops and the number of free bubbles depended mainly on the presence and concentration of dispersed mycelium. Moreover, Corkidi et al. [158] were able to document the bubble inclusion phenomenon in castor oil drops. Pulido-Mayoral and Galindo [159] conducted a study on the effects of protein concentration on the dispersion of droplets and bubbles and on the volumetric oxygen transfer coefficient (k_La) in a simulated culture medium of *T. harzianum* with three phases: aqueous medium, castor oil, and air. It was observed that as the protein concentration increased, the Sauter mean diameter of the castor oil droplets decreased compared to the experiments without proteins.

In order to calculate the area of mass transfer to characterize the phases dispersions, the distribution diameter of air bubbles and oil drops must be known, which requires the measurement of a large quantity of objects. The characterization of these dispersions (partly, in terms of the size distribution of drops and bubbles in the dispersion) is crucial and necessary for understanding and optimizing these processes [160]. The bubble's size directly influences gas holdup and the interfacial area; therefore, the size distribution of bubbles is a good criterion for evaluating the efficiency of a gas–liquid contactor [161]. However, this characterization is time-consuming and tedious when manual methods are used. Furthermore, the manual analysis is influenced by subjective criteria that can change between analysts; therefore, the bubble- and drop-size distributions obtained can be biased. This situation emphasized the need for automated methods for characterizing multiphase systems. Bubble sizes in stirred dispersions have been measured by different techniques [162–164], and the results obtained have improved the understanding of important aspects of the stirred tank applications, such as mass transfer in chemical reactors. However, most of the techniques have been implemented in systems of two or three phases with low global holdup (≤1 %). There is little information for systems with four phases that include the biomass of filamentous fungi.

In a stirred tank, the formation of drops and bubbles depends on the balance between the process of breakage and coalescence of the phases mixed. The diameter

of droplets and bubbles is usually expressed as an average diameter, such as the statistical mean diameter, volume mean diameter, or the Sauter diameter. The Sauter diameter is the most widely used measure in mass transfer studies because it links the volume of the dispersed phase with the area for transfer [165], and it is defined as:

$$d_{32} = \frac{\sum_{i=1}^{k_b} n_i d_i^3}{\sum_{i=1}^{k_b} n_i d_i^2} \tag{6}$$

where k_b is the number of volumes sampled, n_i is the number of drops/bubbles per volume i, and d_i is the size of the drops/bubbles i (Eq. 6).

Various techniques have reported the measurement of the diameter of drops and bubbles. Raman et al. [166] have reviewed the field. For the case of multiphase dispersions containing solids (i.e., biomass), in situ techniques measuring drop and bubble sizes with digital images and object recognition techniques are the only ones that are suitable [167].

In systems that include solid phases, the prediction of the mass transfer rate between gas–liquid phases is particularly complex due to the interactions between the solid particles and the other dispersed phases. Not surprisingly, the phenomena of phase dispersion and mass transfer in these systems are poorly understood at the micro level.

Some of the techniques used and reported in the literature in order to determine the diameter of droplets and bubbles are based on the acquisition and analysis of digital images. For techniques using video and digital photography, the use of cameras with high resolution and short exposure times compared to the timescale of the dynamics of the process is essential to avoid blurred images [168]. Pacek et al. [165] reported the use of advanced noninvasive video techniques in which the videos were taken from the outside of the stirred tank and were analyzed a posteriori. However, this configuration only allows for taking pictures of the processes occurring near the tank wall, giving limited information on the dynamics of the other regions within the tank.

The techniques of digital image analysis are very useful because they allow to distinguish between different phases present in the mixture. The main limitation of the techniques using digital imaging is to count with proper illumination to allow the acquisition of clear images that facilitate subsequent manual or automatic analysis. Lighting that gives depth, gives a uniform image, and allows an analysis of different regions within the stirred tank (such as near the discharge areas of the impellers) is required. Junker et al. [169] used a light source with a wavelength of 700 nm (red light), which allowed the acquisition of clear images even in high-turbidity systems.

On the other hand, several authors have described image analysis techniques that automatically recognize the circles that correspond to oil droplets or gas bubbles in the different multiphase systems (two-phase systems) [167, 170–176]. Those reports are mainly based on three techniques: (a) pattern detection using the Hough transform, (b) the use of templates, and (c) the detection of changes in a grayscale gradient. However, only Taboada et al. [177] have reported a semi-automatic image analysis method for the segmentation of bubbles and drops that was tested in two, three, and

four-phase simulated fermentation model systems. The method was based on a windowed Hough transform. The method was able to reduce the total processing time up to half compared with a totally manual procedure, and the manual intervention time for the segmentation procedure was reduced to a minimum.

4 Conclusions and Perspectives

Mycelial cultures are characterized by complex interactions between the physicochemical parameters of the process (hydrodynamics, energy supply, and oxygen transfer) and growth of the biomass, morphology, and metabolite productivity. The knowledge of the effects of operational and culture conditions over the fungal growth and morphology are crucial. Deeper knowledge is required on the fundamentals of mechanosensitivity of fungal strains and its transcriptional effects on fungal morphology. On the physicochemical side, the concept of the energy dissipation circulation function (EDCF) is one of the crucial developments in the field for the understanding and predicting the effects of mechanical stress in fungal cultures. The concept of EDCF, although not universal, has been successfully applied in a wide variety of microorganisms, products, and culture conditions. Because there are continuous changes in the morphology, physiology, and mass transfer conditions of the microorganisms during fermentation, it is critical to establish an *on line* and in situ monitoring of microbial behavior and phase dispersion to control the process and to discriminate between hydrodynamic effects and those due to dissolved oxygen tension. Digital image analysis is a technique that has proven useful for characterizing multiphase dispersions containing filamentous microorganisms and for determining culture viability. Overall, what is required is to promote and to strengthen the multidisciplinary research between chemical and biochemical engineering with aspects of science and engineering of image processing in order to establish the link between these disciplines with the biological topics covered in this chapter (morphology and physiology of fungal microorganisms).

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