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Morphological Control in Filamentous Fungal Fermentation

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Significance of filamentous fungal fermentation

The use of filamentous fungi for the production of commercially important products has increased rapidly due to an ability of fungi to produce and secrete extracellular enzymes to break down agricultural products, residues and plant biomass. In addition, filamentous fungi are competent to secrete a large amount of the heterologous protein. Due to the similar patterns of glycosylation and disulfide forming to the mammalian cell, filamentous fungi carry correct posttranslational modification processes for recombinant protein. However, filamentous fungal fermentation is widely distinguished as a complicated process due to the undesirable change in morphology during the cultivation. The change in morphology directly affects fungal growth and metabolism. Besides the genotype of the fungal strain, the environmental factors such as temperature, pH, nutrient, shear rate and oxygen transfer influence the fungal growth pattern and morphology. In this review article, we have attempted to comprehend these significant factors influencing fungal morphology and production rate under different cultivation methods.

Fungal morphology

Fungal morphology plays an important role in the metabolism during the fermentation process. Diverse morphology is required for an optimal product yield in different process. [1] For instance, pellet morphology provides a higher production rate in xylanase production by Aspergillus awamori and in exo-biopolymer production by Paecilomyces japonica. [2-3] On the other hand the filamentous morphology is preferred in lactic acid production by Rhizopus arrhizus and β-carotene synthesis by Blakeslea trispora. [4-5] But high production rate of swainsonine by Metarhizium anisopliae in stirred tank bioreactor is obtained by mixed hyphal and pellet morphology. [6]

In submerged cultivation, filamentous fungi exhibit distinctive morphological forms dependent on

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the operating condition. Fungal morphology is generally classified into two different groups: dispersed mycelia and pellets (Figure 1). Dispersed mycelia can be further divided into freely dispersed mycelia and mycelial clumps. Freely dispersed mycelia include mycelia with up to three hyphal overlaps which result in hyphal loops whereas mycelial clumps contain more than three loops. Mycelia with a few overlaps are called entanglements. [7] Freely dispersed mycelia can grow when dissolved oxygen and substrate concentration are sufficient in the culture medium. However, the strong mechanical forces can deactivate loose mycelia at some level of magnitude.

Cui et al. [8] investigated A. awamori CBS 115.52 growth and constructed the mathematical model. They found that the mechanical forces deactivated cells and decreased specific growth rate. The inactivation is proportional to the specific energy dissipation rate with an exponent of 0.25 (1).

$$\mu_f = \mu(1 - k_d \varepsilon^{0.25}) \quad (1)$$

Where μ_f is the deactivated specific growth rate of loose mycelia, μ is the specific growth rate without mechanical damage, k_d is the inactivation coefficient of hyphae due to the mechanical damage and ε is the specific energy dissipation rate (W/kg). The results showed that such an

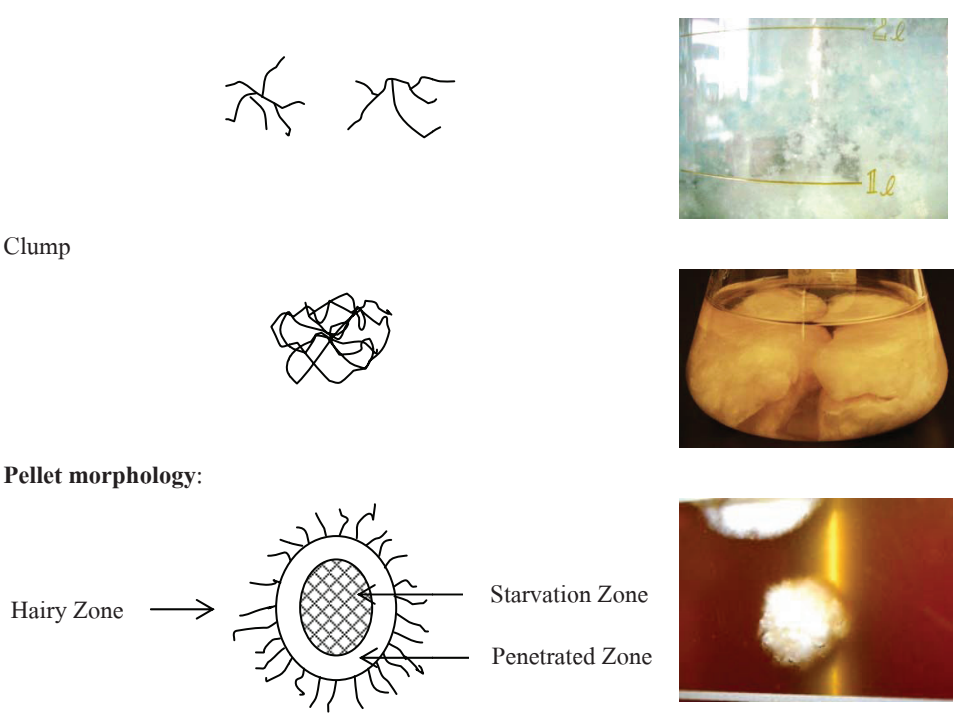

Figure 1 Morphology usually found when growing fungi in submerged culture

inactivation mainly occurred on loose mycelia because they were more susceptible to mechanical force. In addition, the size of vacuoles formed in fungal hyphae, which are increasing with the fermentation time, leads to the reduction of hyphal activity per mass and the subsequent decrease in specific growth rate. The outer hyphae in mycelial clumps can be also shaved off by the hydrodynamic forces present in the fermentor resulting in small pieces of freely dispersed mycelia and the reduction of clump roughness. Li et al. [9] suggested that shaving off by the hydrodynamic forces was the major mean by which mycelial clumps broke apart and the fragments found during agitation were primarily originated from mycelial clumps.

Considering the pellet structure, it can be divided into three distinctive zones (Figure 1). The outer zone or the hairy part, growing to the outside environment can be shaved off by hydrodynamic forces. The severity of pelet shaving is a function of hydrodynamic forces whereas the breakage of pellets occurs less often when substrate and oxygen are available. In the penetrated zone, oxygen penetrates into this zone and the fungal cells in this region are active. The depth of this zone depends on the pellet density, growth rate and bulk dissolved oxygen tension in the culture medium. In the starvation zone, oxygen and nutrients cannot penetrate into this zone so dissolved oxygen is limited and autolysis occurs due to oxygen starvation. [10] Therefore, pellet growth is mostly controlled by oxygen transfer before the substrate is depleted in the bulk. Cui et al. [11] reported that the shaving intensity was proportional to the specific energy dissipation rate (2).

$$R_{sha} = K_{sha} \cdot \varepsilon \cdot R_{PG} \qquad (2)$$

Where R_{sha} is the shaving rate of hyphae from pellet surface (kg/m^3·s), R_{PG} is the pellet growth rate (kg/m^3·s), K_{sha} is the shaving coefficient (kg/W) and is the specific energy dissipation rate (W/kg). The shaved off hyphae from the hairy part reseed the loose mycelia mass fraction.

In filamentous fungal fermentation, the rheological properties appear to be linked to mycelial morphology. Growing filamentous fungi in freely dispersed morphology leads to a highly viscous and pseudoplastic fermentation broth. This causes the difficulty in mixing, which in turn affects mass and heat transfer, leading to the decrease in productivity and the production of undesirable metabolites. Thus, rigorous mixing is required for adequate gas dispersion and homogeneity. [12] As the result, high power input needs to be provided. Although stronger mechanical force can result in a higher dissolved oxygen tension and more branching of hyphae which leads to the higher productivity, the excess mechanical intensity can damage the loose mycelia and create dense pellets. [10] Riley et al. [7] reported the influence of fungal concentration and morphology including clump roughness and compactness on the properties of fermentation broth. The correlations found with freely dispersed morphology constructed in their study and other related studies are summarized in Table 1, where C is compactness, C_m is dry fungal concentration (g/L), K is consistency index index (Pa·s^n), L_e^* is dimensionless length, L_{hgu} is hyphal growth unit (μm), R is roughness, and τ_y is yield stress (Pa).

Table 1 Correlations for rheological behavior prediction of various filamentous fungi, measured by different rheometric techniques [7]

Correlation	Organism	Rheological measurement technique	Fermentation system	References
$K = C_m^{2.8} \times R^{0.7} \times C^{1.2} \times const^{a}$	*Penicillium chrysogenum*	Disk turbine rheometer	Batch	Tucker and Thomas (1993)
$K = C_m^{2.3} \times R^{-0.96} \times C^{0.79} \times 6.6 \times 10^{-5}$	*P. chrysogenum*	Disk turbine rheometer	Batch	Tucker (1994)
$K = -0.56 + 0.0018 \times R \times C_m^{1.7}$	*Aspergillus niger*	On-line impeller rheometer	Continuous and fed batch	Olsvik et al. (1993)
$K = 0.38 + 4.8 \times 10^{-5} \times R \times C_m^{2.9}$	*A. niger*	On-line impeller rheometer	Batch	Olsvik and Kristiansen (1994)
$\tau_y = 4.2 \times 10^{-6} (C_m)^{2.6} (L_e^*)^{2.2}$, $\tau_y = 7.2 \times 10^{-3} (C_m)^{2.2} (L_{hgu})^{0.65}$, $\tau_y = 4.8 \times 10^{-7} (R)^{3.2} (C_m)^{2.5}$	*Streptomyces levoris and A. niger*	Rotating vane technique	Batch (fermentor and shake flask)	Mohseni and Allen (1995)

[a]**Value of constant not given in the literature**

On the other hand, when growing filamentous fungi in pellet morphology, the fermentation broth has Newtonian-like behavior with low viscosity. However, increase in the pellet size during the fermentation creates the problems in nutrient transport into the pellet cores, thus reducing fungal activity at the pellet center. As a result, the production rate decreases. Change in the pellet size during the fermentation is a function of growth rate, oxygen penetration depth in the pellets, shaving intensity, autolysis rate and rate of change in pellet density. The mathematical descriptive model proposed by Cui et al. [8] is shown below (3).

$$4\pi R_p \rho_p \frac{dR_p}{dt} = \frac{4\pi}{3}\left[\mu(1-K_{sha})(R_P^3 - R_{OP}^3)\rho_p - K_{aut} R_{OP}^3 \rho_p - \frac{d\rho_p}{dt} R_p^3\right] \quad (3)$$

Where R_p is pellet radius (m), ρ_p is the pellet density (kg/m^3), R_{OP} is the radius where dissolved oxygen in the pellet is 0 (m), K_{sha} is the shaving coefficient (kg/W), and K_{aut} is the autolysis coefficient (s^{-1}). The left handed side term represents the rate of fungal cell accumulation in a pellet. At the right side of the equation, the first term in the bracket is the rate of pellet growth minus shaving. The growth only occurs in the region where dissolved oxygen is available. Shaving occurs at the outer part of the pellet due to the hydrodynamic forces. The second term refers to the rate of autolysis due to lacking dissolved oxygen or nutrients. The last term expresses fungal accumulation rate due to the change in pellet density.

Many studies indicate that environmental conditions and the genotype of fungal strain influence the growth pattern of filamentous fungi by affecting the morphology, growth rate, and product formation. This is because the change in fungal morphology during fermentation affects nutrient consumption as well as oxygen uptake rate. [1, 13-14] Many researchers have attempted to control and maintain the optimal fungal morphology during the fermentation. Generally, there are four major factors strongly affect fungal morphology and metabolism. Those include:

Substrate: Nutrient uptake governs growth rate. Nutrient supplementation during the fermentation controls fungal fragmentation and vacuolation involving in morphological alteration. [15] Sinha et al. [3] observed the effect of substrate concentration on fungal morphology during exo-biopolymer production by *P. japonica* in a batch bioreactor. They reported that pellet morphology varied significantly with sucrose concentration. Pellet roughness and hairiness increased as sucrose concentration increased from 20 to 60 g/L. However, insignificant difference in the circularity index showed that sucrose concentration had no effect on the shape of the pellets. Not only carbon sources are important in cell growth, nitrogen sources also involve in the biosynthetic pathways of the organism. Du et al. [16] studied the effect of nitrogen sources on fungal morphology and antibiotic production. They indicated that in the medium containing different nitrogen compounds, the morphology of *Rhizopus chinesis* 12 varied significantly. In addition, various inorganic salts have influenced fungal morphology. Gerlach et al. [17] observed the effect of phosphate concentration on *A. awamori* morphology in an airlift tower loop reactor. The results indicated that with 0.3 g/L initial KH_2PO_4, loose pellets of 6 mm diameter with hairy surface and low density at the center were formed. At 1.05 g/L initial KH_2PO_4, dense pellets of 4 mm diameter with uniform pellet density were found. Hollow pellets of 7 mm diameter with dense surface were observed in the culture containing 2.1 g/L initial KH_2PO_4. As previously discussed nutritional source and concentration strongly influence fungal morphology and production rate. Many studies have been conducted to determine the optimal nutritional source and concentration for a particular production. Fungal morphology accordingly varies from one process to another different process depending on the process parameters.

pH: Fungal growth and metabolism inevitably lead to the change in hydrogen ion balance and therefore, pH of the culture medium.[5,18] The pH of the culture medium is one of several parameters affecting fungal morphology. Change in cell size and shape as well as the metabolic rate are the consequence for the response of cell to the change in pH.[6,19-20] Kim et al. [21] optimized the culture conditions for exo-polysaccharide (EPS) production by *Cordyceps militaris* C738 in submerged culture. It was found that pH strongly influenced

fungal morphology and EPS production. They compared morphological properties of the cultures with and without pH control by characterizing pellet roughness and compactness and reported that the larger and more compact pellets formed at pH 6.0 were favorable for EPS production (0.91 g/g cell/day). Additionally, the intracellular pH controls the internal concentrations of various species of CO_2. This implies that CO_2 diffusion is influenced by pH of the culture medium. Many studies indicate that when CO_2 concentration increases, the degree of hyphal swelling increases corresponding with the morphological change and product synthesis. McIntyre and McNeil [22,23] used computerized image analysis to quantify the change in morphology of A. niger when CO_2 level increased. They found that short stubby hyphae, which was preferred in citric acid production, drastically reduced when CO2 level was raised. In particular, the hyphal growth unit (HGU) increased with the exposure to CO_2 level of 5% or higher. At the high level of CO_2 (12% and 15%), HGU values increased more than twice compared to the values obtained from the standard batch process. This result at high CO_2 level represented mycelial particles with fewer branches per unit length which caused the reduction of citric acid production.

Shear rate: Shear force in filamentous fungal fermentation markedly influences fungal morphology particularly under submerged condition. Strong mechanical force results in a higher dissolved oxygen tension and more branching of hyphae. The pellet formed under high shear rate may become dense and strong with high tensile strength. [24] Amanullah et al. [25] reported that the increase in agitation speed to 550 rpm resulted in a rapid increase in the freely dispersed fraction to 42%. In their study, it was likely that the fragmentation of aggregates at the higher speed increased the proportion of biomass in the freely dispersed form. The interaction between the fungal cells and the vortices formed by mixing is the principle mechanism of mycelial damage due to excessive shear forces. [26] Many works reveal that mixing by mechanical force causes the dramatic changes in morphology and productivity. Fungal mycelia undergo the complete transformation when the mechanical force changes. As the fermentation proceeds, increased vacuolation weakens the filaments and makes them more susceptible to damage. Vacuolation is a time related process; as a result, the distribution of vacuoles in fungal hyphae is proportional to the age of the compartments. Papagianni et al. [27] found that increasing agitation controlled the morphology of *A. niger* by altering the specific growth rate and branching initially, following by fragmentation and regrowth. It is also believed that change in cell wall compositions and certain cell wall structures participate in the mechanism of resistance to shear force. Many works report the degree of morphological damage by shear force in term of the specific energy dissipation rate. Three damaging mechanisms may occur, i.e. the interaction between fungal cell and vortices generated by the turbulent intensity and convective flow, the impact between fungal cell and impellers or baffles in the mechanically stirred tank and the flow collision between fungal cell and fungal cell. [11]

Dissolved oxygen tension: Undoubtedly, oxygen transfer is the most important phenomenon to maintain the aerobic fungal fermentation. [28] The effect of dissolved oxygen tension on fungal morphology has been observed in many studies but inconsistent results have been reported. In some documents, it was indicated that the morphology of free filamentous mycelia hardly changed with the large variation of dissolved oxygen tension whereas some of them reported a remarkable difference in the mycelial appearance found when supplying the bio-

reactor with oxygen instead of air. Several studies investigated the effect of oxygen tension along with shear rate and broth rheology and found that these three parameters were closely related to morphology alteration with high complexity. [29] In submerged culture, oxygen transfer process involves the transfer from the air bubble into the solution, dissolved oxygen transfer through the culture broth to the surface of the fungal cell and the diffusion into the cell. The oxygen transfer rate from the gas phase into the culture broth is indicated by the volumetric oxygen transfer coefficient, $K_L a$. Many experimental results show that $K_L a$ is influenced by the air flow rate or superficial air velocity, agitation, air pressure, temperature, vessel geometry, fluid characteristics (density, viscosity, surface tension), the presence of antifoam agents, concentration and physical properties of immobilized materials (density, particle size). [20, 30-32] The major problem in oxygen transfer from the gas phase into the liquid phase is due to the low oxygen solubility in the liquid medium. In the fermentation with the presence of freely dispersed mycelia, the highly viscous culture medium causes the limitation of gas-liquid oxygen transfer and the homogeneity in the bioreactor. [10] On the other hand, when growing the fungal cells in the pellet form, oxygen depletion in the pellet occurs even at the high bulk oxygen tension. Many works have been proposed to improve the oxygen transfer rate in submerged fermentation. The simple technique to improve oxygen transfer is to increase agitation intensity and air flow rate. However, turbulence and shear rate, which are often associated with high agitation intensity and air flow rate cause the damage on the fragile cells like filamentous fungi. Oxygen vector is applied in many fungal fermentation processes to improve oxygen transfer. It is believed that oxygen vector forms the new interfacial area between gas and liquid; hence, enhances oxygen transfer in bulk liquid. Some studies revealed that oxygen vector helps enhance the production rate by altering fungal morphology. In many studies, high dissolved oxygen can be maintained by a mixture of air and oxygen, pure oxygen or an increase in partial pressure of oxygen in the fermentor. It is believed that high pressure applied into the fermentor helps increase the oxygen solubility. Also using a mixture of oxygen and air or pure oxygen will enhance $K_L a$ value. [33] Immobilization in inert support provides many advantages on morphological control and high product yield compared to free cell cultivation. However, a rigorous study on mass transfer limitation is still essential for immobilization process.

Conclusion Remark

Production of a particular product economically involves the uses of bioreactors. Reactor performance has a profound effect on the productivity. Understanding the nature of the fermentation system of such a particular product is helpful for bioreactor selection and design. For filamentous fungal fermentation, morphology plays a significant role relating to product formation and secretion as well as the characteristic of the culture broth, mixing, heat and mass transfer. Table 2 represents the examples of culturing *Aspergillus niger* at the certain conditions to obtain various desired products. As discussed in this paper, it can be concluded that fungal morphology is closely related to the changes in environmental conditions such as temperature, pH, shear force, oxygen and nutrition although somehow such the correlation is not directly apparent. Therefore, to develop the high performance bioreactor to favor the fungal growth, one should be able to understand and control the fungal morphology during cultivation.

Table 2 Preferred fungal morphology found in selected different fermentation processes of *Aspergillus niger* operated at the optimal conditions

Substrate	Product	Production rate	Fermentation condition	Morphology	Reference
Molasses based medium containing 2.0×10^{-5} M $CuSO_4 \cdot 5H_2O$	Citric acid	92.0 g/L	Batch, fermentor, 6 days	Mixed pellets	Haq et al., 2002
Semi-synthetic medium containing wheat bran	Phytase	8090 U/L	Shake flask culture, 144 hours	Pulp and pellets	Papagianni et al., 1999
Fermentation medium containing a mixture of 1.5% w/w olive oil and 0.5% w/w sucrose	Lipase	29.6 triolein U/mL	Shake flask culture, 8 days	Mycelia	Macris et al., 1996
Sugarcane molasses-based medium with 0.5 g $CaCl_2$/L	Citric acid	0.37 g/h	Jar fermentor, 15 days	Loose pellet	Pera and Callieri, 1999
Fermentation medium containing high glucose concentration (18%)	Gluconic acid	~175 g/L	Shake flask culture or modified batch recirculation fermentor	Immobilized cell on a highly porous cellulose support	Sankpal and Kulkarni, 2002

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