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# Fungal morphology: a challenge in bioprocess engineering industries for product development

Hesham A El Enshasy[1,2,3]

Fungal biofactories are well established in bioprocess industries. They have been used for the production of essential biomolecules in chemical, food, and pharmaceutical industries. The high growth rate and the ability of fungal cells to hydrolyze wide range of complex and economic substrates make them among the superior microorganism for large scale production. In addition, they have high capacity for product excretion in high concentration which reduces the overall throughput of the production process and reduces the downstream cost as well. However, growth morphology in submerged culture is one of the greatest challenges in bioprocess industries. The same strain can exhibit extremely different morphologies with any minor alteration in cultivation conditions or medium composition, and thus affect the product yield. In this review, we present the fungal morphology in a complete and full-scale approach from spore induction in hyphal cells up to complete biopellet formation.

**Addresses**
[1] Institute of Bioproduct Development (IBD), Universiti Teknologi Malaysia (UTM), Skudai, Johor, Malaysia

[2] School of Chemical and Energy Engineering, Faculty of Engineering, Universiti Teknologi Malaysia (UTM), Skudai, Johor, Malaysia
[3] City of Scientific Research and Technology Applications (SRTA), New Burg Al Arab, Alexandria, Egypt

Corresponding author: El Enshasy, Hesham A (henshasy@ibd.utm.my)

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## Introduction

Filamentous microorganisms have a history in biotechnology industries for the production of many essential metabolites for pharmaceuticals, cosmetics, food, feed, and chemical industries [1••,2,3]. Fungi are characterized by their high capacity for the efficient production of a wide range of hydrolytic enzymes by converting complex materials to metabolizable low molecular weight compounds such as fermentable sugars, amino acids, and low molecular weight compounds [1••]. Nowadays, several fungal strains belonging to *Aspergillus* and *Penicillium* species are considered as GRAS (Generally Regarded As Safe) according to the Food and Drug Administration (FDA). Therefore, they are highly recognized in biotechnology industries as major biofactories for different types of biological products [4–6]. However, unlike unicellular microorganisms, fungi are characterized by their highly complex morphological features in submerged cultures. The growth morphology can range from fully dispersed mycelium to compact pellet structure. Minor changes in fungal morphology leads to significant chenges in production yield with an impact on mass transfer and mixing in the bioreactor [7,8,9•,10•]. To achieve maximal production of the targeted metabolites, specific morphological structure should be maintained. Table 1 provides a comprehensive overview about some industrial fungal products preferred morphology and major production companies. However, it is not easy to achieve the desired morphology as it can be affected by many variables related to the type of strain used, cultivation conditions, and medium composition. Recent research reported also that fungal morphology can be genetically manipulated to optimized the desired morphological structure [11,12].

In general, controlling the morphological structure in submerged cultivation system is considered one of the main industrial challenges in biotechnology industries, especially during scaling up processes. Growth morphology not only affect the mixing characteristics and controlling of the process, but also reflects directly on overall volumetric and specific product yields. Therefore, for an efficient production of fungal metabolites, controlling the growth morphology became of ultimate importance. This was also driven by industrial demand to improve the overall process performance for both upstream and downstream. Fungal morphology in submerged culture can be in filamentous growth, pelleted growth, or a mixture of both shapes. However, the rheological properties of fermentation broth are highly linked to fungal growth morphology [33]. In filamentous growth, cells are exposed to oxygen and nutrients for better mixing, but the main drawback is the increase in culture viscosity due to the non-Newtonian fluid characteristics [34]. On the other hand, pelleted growth can facilitate the bioprocess control. However, besides the advantages of pelleted growth in upstream process, it helps in the reduction of downstream cost and pellets can be easily separated. On the other hand, pelleted growth has also disadvantages

**Table 1**

**Fungal biofactories for the production of different metabolites in fermentationindustries (modified from Refs. [10•,11,13•,14])**

| Fungal biofactory | Metabolite/(preferred morphology) | Applications/ Industry | Manufacturing companies |
|---|---|---|---|
| *Acremonium chrysogenum* | Antibiotics: *Cephalosporins* (Filamentous/ fragmented hyphal structure [15] | Pharmaceutical | Novartis (Switzerland), Lupin Ltd. (India) |
| *Aspergillus awamori* | Enzymes: *Amylase, Glucoamylase* (Filamentous growth [16]), (Small pellet with mycelial network [17]) | Food | Novozymes A/S (Denmark) |
| *Aspergillus niger* | Organic acids: *Citric acid* (Small pellet not exceed 2.2 mm [4,18]<br>Enzymes: *Glucose oxidase* (Small pellet not exceed 0.4 mm [19], *Phytases*, *Pectinase*, *Proteases*, *Glucoamylase*, *Chymosin*) | Food, Feed, Pharmaceutical industries | DSM (The Netherlands), Novozymes A/S (Denmark), Zymergen (USA), COFCO, RZBC Group (China), Cargill (USA), Christian Hansen (Denmark), Mitsubishi Foods Co. Ltd. (Japan). Jiangsu Boli Bioproducts Co., Ltd (China), BASF (Germany) |
| *Aspergillus oryzae* | Organic acids: *Kojic acid* (Pelleted growth [20]). *Malic acid* (small hairy pellet [21,22]). *Lactic acid* (Pelleted growth [23]<br>Enzymes: *Amylase* (filamentous growth [24]), *Lipases*, *Invertase*, *Protease*, *Laccase* | Food, Feed | Novozymes A/S (Denmark), Amano Enzymes Inc. (Japan), Gekkeikan Sake (Ltd. (Japan), Jiangsu Boli Bioproducts Co., Ltd (China) |
| *Aspergillus terreus* | Organic acids: *Itaconic acid* (hairy less dense pellet (clump structure, of 0.4–0.5 mm [25])<br>Secondary metabolites: *lovastatin* (pellet of 2.5 mm diameter [26] | Food, Pharmaceutical | Alpha Chemika (India), Chengdu Jinkai Biology Engineering Co., Ltd. (China), Pfizer (USA), Merck (USA) |
| *Blakeslea trispora* | *Vitamins, Astaxanthin β-carotene*: (Filamentous growth with long hyphae [27] | Food, Pharmaceutical | DSM (The Netherlands), Universal Foods Corp. (Japan), Pharmacia & Upjohn (USA), Lycored (Israel) |
| *Cordyceps militaris* | *Cordycepin* (Filamentous and small pellet) Polysaccharides | Food, Pharmaceutical | Zhenjiang Wanfeng Medicines Group Co. Ltd. (China), Bioalpha (Malaysia) |
| *Fusidium coccineum* | Antibiotic: *Fusidic acid* | Pharmaceutical | Leo Pharma A/S (Denmark) |
| *Ganoderma lucidum* | *Polysaccharides, biomass* (small pellet) | Food, pharmaceutical | |
| *Lentinus edodes* | *Eritadenine* (filamentous [28]) | Pharmaceutical | New product |
| *Penicillium chrysogenum* | Antibiotic: *Penicillin* (small loose pellet structure with low mycelia density [29,30] | Pharmaceutical | DSMZ (The Netherlands), Merck (USA), Pharmacia & Upjohn (USA) |
| *Rhizopus oryzae* | Organic acid: *Lactic acid*, *Fumaric acid* (Small pellet 2.0–2.5 mm [31]) | Chemical, Food, Pharmaceutical | Corbion (The Netherlands) |
| *Tolypocladium inflatum* | Antibiotic: *Cyclosporin A* (small pellet less than 0.25 mm, [32]) | Pharmaceutical | Novartis (Switzerland) |

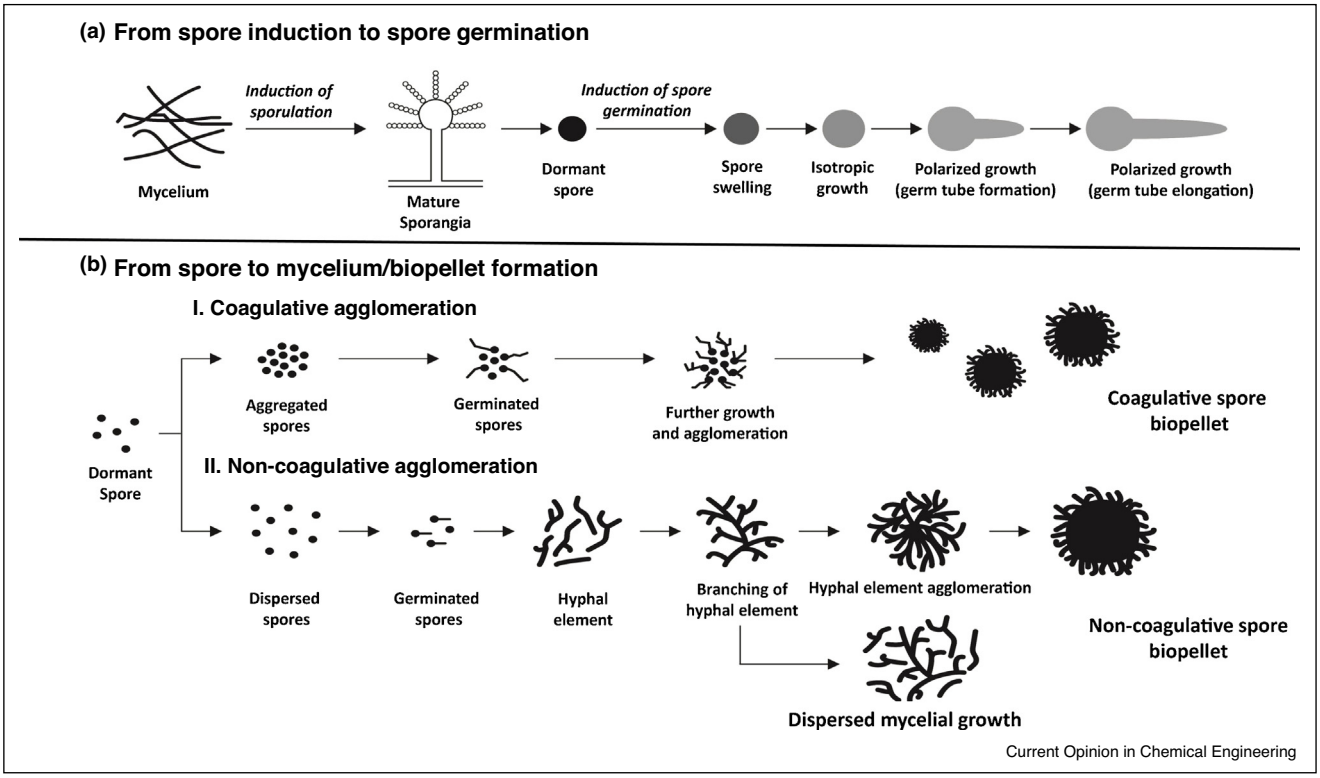
related to mass transfer limitation inside the pellet structure, and only outer layers exposed to oxygen and nutrient remain viable and active [35]. The aim of this review is to provide a comprehensive overview about all the steps affecting the fungal morphology development from spore induction to the development of fungal macromorphological development as shown in Figure 1

## From fungal spore induction to spore germination

Spores are considered as the dormant stage in microorganisms life cycle. They are either produced naturally or under induction of different biotic or abiotic stresses. Generally, they are produced as part of their asexual and sexual life cycles. Besides the genetic regulations of sporulation process, environmental conditions such as light, pH, temperature, mycelial injury, type and concentration of carbon and nitrogen sources, C/N ratio, humidity, oxygen, and osmotic stress can contribute largely to spore induction. In addition, some exogenous chemical addition such as calcium, volatile organic compounds, and cyclic adenosine monophosphate (cAMP) can play regulatory role in sporulation process [36]. However, light (wavelength and exposure time) is usually considered as one of the key factors to induce sporulation in species such as *Trichoderma*, and to a lesser extent for other fungal strains belonging to the species of *Aspergillus*, and *Penicillium* [36–39]. However, the light stimulatory effect on spore induction is age and metabolic stage dependent of the exposed hyphae [40]. Light can also mediate its effect through the regulatory Velvet proteins which play essential roles in sporulation and metabolite production as well [41,42].

Gnerally, the factors affecting sporulation are belived to be strain dependent. Moreover, these factors not only stimulate hyphal differentiation for sporangiophone and spore formation, but also influence the quality of developed spores in terms of size and germinability. Recent study reported that the spore size distribution and spore resistance can be affected by temperature in *Penicillium roqueforti* [43]. It has been also reported that the heat

**Figure 1**




Different stages of morphological development of sporulating fungi. **(a)** From spore induction to spore germination, **(b)** from spore to the development of mycelium/biopellet formation (modified from Veiter *et al.* [10•]).

resistance of *Paecilomyces variotii* spores is highly dependent on size, shape, and size distribution in spore population [44]. In addition, spore structure and morphology play significant role in the future growth of fungus in submerged culture system.

## Fungal spore structure

Spores are characterized by their high tolerance to the environmental condition compared to hyphae/vegetative cells. Therefore, they are the preferred structures for cell banking and long-term preservation. The robustness of spore structure is based on their high dense and compact structure with high protection by different surrounding layers of special structures. The spore internal features are characterized by the accumulation of different ingredients which support spore protection and act as preservatives during dormancy [45•]. These include the following:

- Trehalose: to protect against dehydration, oxidative-stress, and thermal-stress, and cell wall stability
- Heat stress proteins (HSPs): To prevent desiccation, and provide protection against pH stress, thermal stress, and osmotic stress.
- Mannitol: To provide protection against high temperature and oxidative stress.
- Dehydrins: To protect against spore dehydration, thermal-stresses, osmotic-stresses, and pH-stresses.

The outer layer of most fungal asexual spores is made of polysaccharides (chitin and combination of α-glucans and β-glucans), and is surrounded by a rodlet layer of complex structure composed of a phenolic compound (melanin) and hydrophobic protein (hydrophin).

Melanin is a dark colored protein complex which confers the black or dark color of fungal spores [46,47]. Fungal melanin plays an essential role in the protection of spore against environmental stresses including physical stress (osmotic stress, photo-inactivation, and radiation), and chemical stresses (pH, heavy metals, and free radicals). Therefore, it acts as the first defense layer against harsh environmental conditions [48–50]. Beside melanin, a special group of surface-active amphiphilic protein (hydrophobins), a protein structured layer composed of

special molecular arrangement of a combination of neutral, hydrophilic, and hydrophobic amino acids are also exist in outer layer of spore [51]. This protein is necessary for conidial hydrophobicity, increasing wet-resistance, and thus it protects spores during dormancy. Hydrophins also support the resistance against environmental stresses such as temperature as well as physical damage [52,53]. In addition to their roles in spore protection, they have also applications in food industries such as foam enhancer, emulsifier, and biosurfactant [54,55]. However, hydrophobins play critical role in fungal morphology in submerged culture system as they govern the spore surface tension and spore-spore aggregation.

## Factors affecting spore germination (swelling and germ tube formation)

Different internal and external factors are contributing to the kinetics of spore swelling, germination, and germ tube emergence, and elongation. Initially, spore moves from dormant to non-dormant phase. Spore dormancy can be classified into two types: endogenous (constitutive or strain dependent) and exogenous (superficial or environmental dependent). The constitutive dormancy is gene regulated and can be broken by exogenous addition of activators of cAMP pathway. It was reported that the spore germination and germ tube formation process are governed by a mitogen-activated protein kinase (MAP kinase) *mpkA* and protein kinase C *pkcA* genes in *Aspergillus nidulans* [56]. On the other hand, the exogenous dormancy is initiated by either physical (heat or radiation) or chemical induction. Recent data reported that non-thermal plasma can initiate germination in *Aspergillus oryzae* spores by increasing intracellular calcium levels, and activation of *mpkA*, and other genes involving in germination process [57]. The optimal conditions (environmental and nutritional signals) for spore swelling initiation could be different from those optimum for microbial growth [58,59].

In general, the process of spore germination involves three main steps: (1) spore swelling, (2) germ tube development, and (3) germ tube elongation. The swelling step is initiated by water uptake and associated by a volume increase up to threefold with isotropic growth.

After reaching a critical size, growth is switched from isotropic to polarized growth for germ tube formation under the control of nutrient availability [58,59]. Recent study reported that the minimal medium for spore germination of *Aspergillus niger* should include a mixed nutrients of suitable carbon source for induction with either inorganic nitrogen or magnesium sulphate [60•].

The rate of spore swelling can be increased in the presence of glucose under acidic environment which can help to increase the water uptake under the control of osmotic water permeability coefficient regulatory genes or aquaporin-encoding gene [61]. During this fast growth phase, many metabolic pathways are switched on such as those related to carbohydrate metabolism and protein synthesis. It is also noteworthy to mention that the spore swelling rate and subsequent metabolic activities of spores of the same age exhibited intrapopulation variation which is mainly related to the spore age and outer layer structure [62].

The swelling phase is characterized by high growth rate and usually following exponential mode, while the germ tube elongation (polarized growth) is following linear growth rate. Extensive branching facilitates the faster growth rate and can reach exponential growth mode. It has been reported also that several genes related to cell wall synthesis and cytoskeleton structure are upregulated during the switch from isotropic to polarized growth phase [63,64].

## Monitoring of spore germination and branching

Several techniques have been developed for germination process monitoring. Most of research have been carried out to investigate individual spore using morphological approach. This was achieved by using different types of microscopy (light, fluorescence, confocal laser, and electron microscope) combined with image analysis system [65]. In addition, atomic force microscopy (AFM), and force spectroscopy (FS) have been applied to study the germination of *A. nidulans* spores [66]. This real time imaging method allowed to monitor the growth under the effect of different physical and chemical stimulants [67]. Florescence staining methods using acridine orange (AO) to differentiate between germinated and non-germinated spores have been also applied. This based on that the DNA rich spores (which considered as non-germinated), will be take green fluorescence while the RNA rich spores (physiologically active and germinated) will be stained with red color [9•]. Spore staining using Alamar blue (resazurin, 7-hydroxy-3H-phenoxazin-3-one 10-oxide) have been applied to differentiate between viable and non-viable spores. The metabolically active or germinated spores have the capacity to convert the dark blue of resazurin to pink color (resorufin) [68].

A new approach for spore germination assessment using both morphological and metabolomic data have been also proposed [69]. This based on the microscopic and metabolic profile analysis using high performance liquid chromatography-mass spectrometry (HPLC-MS). This approach provided a strong correlation between morphological and physiological changes during germination process by tracking of all the intracellular metabolites (36 compounds in spores and 28 compounds in mycelium) using metabolomic approach [69].

Ehgartner *et al.* [70] have developed an at-line methods for spore viability staining monitored by flow cytometry

(FCM). This method allowed a real-time monitoring for a spore viability and physiological status during bioprocess operation. The differentiation between non-germinated and germinated spores have been carried out using logistic regression using large number of data generated by FCM analysis [71].

## Biopellet formation and engineering

The initial classification of fungal macromorphology in submerged culture divided the growth features into five classes [72]. These include: (1) dispersed mycelia with short filaments, (2) fluffy mycelia and diffuse mycelial network, (3) mycelial aggregates with lock-like mycelia, (4) pelleted growth with compact center and hairy surface, and (5) compact pellet with clear spherical structure and smooth outer surface. On the other hand, microbial biopellet has been classified according to the mechanism of pellet formation into two main types (Figure 1b):

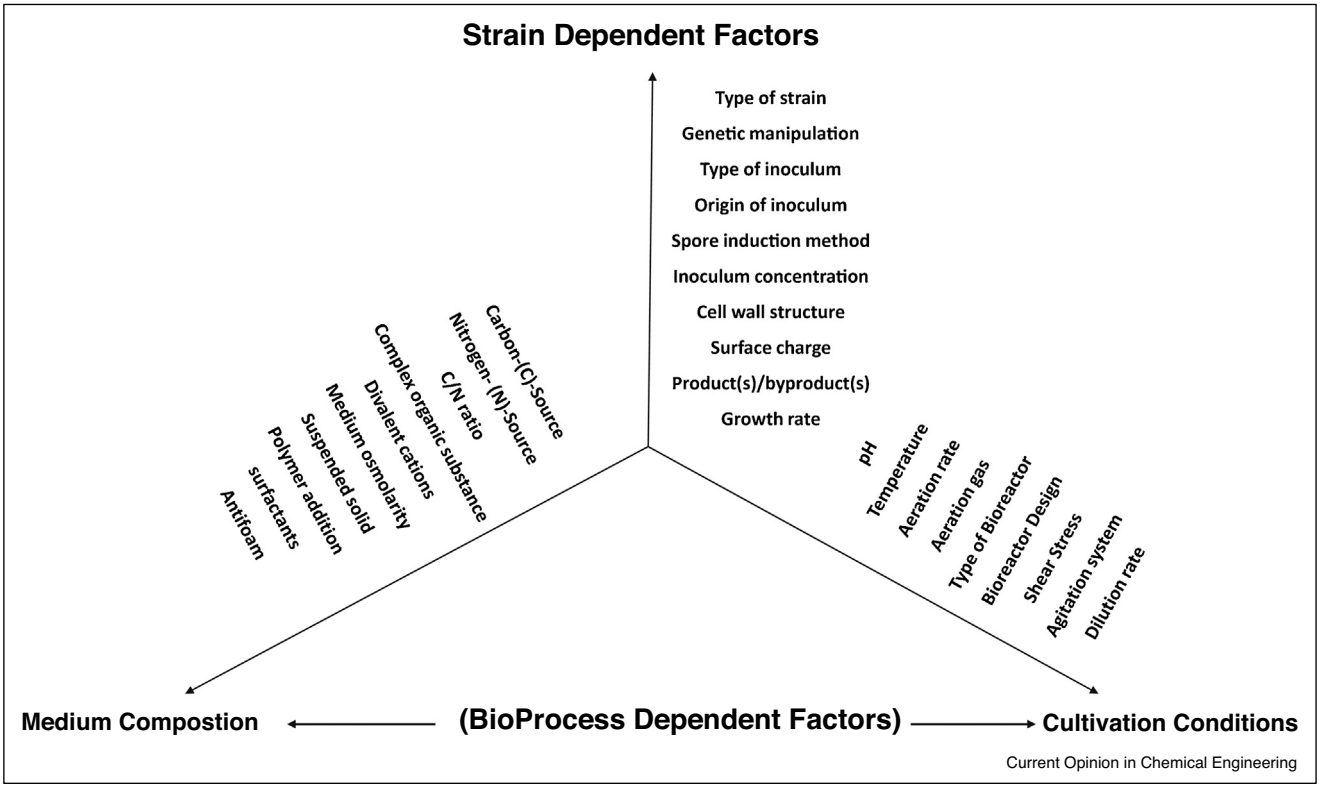
(1) Coagulative or spore agglomerate: Spores coagulate before or during swelling/germination phase to develop biopellet of intertwined hyphae.
(2) Hyphal element agglomeration type: spores germinate first to produce hyphae which agglomerate later to form hyphal clump which develop pellet structure.

For each production process, specific morphological features are required to achieve maximal productivity (Table 1). For example, the maximal penicillin yield is associated with a hairy large viable outer layer of lose pellet [73,74]. To achieve maximal recombinant protein production in *A. niger*, hairy small pellet less than 400 μm diameter with dense structure is desired [19,35,75].

## Factors affecting biopellet formation and structure

Different factors affecting growth morphology and biopellet formation have been extensively studied and reviewed [9•,10•,76,77]. These factors can be summarized into three categories: (1) strain, (2) medium composition, and (3) cultivation conditions Figure 2 shows all the factors. The strain dependent factors are considered as the key factors in this process which are not only dependent on the type of strain but also related to the method of sporulation induction which affect inoculum quality, spore hydrophobicity, and spore structure. Recently, different molecular approaches have been also applied to manipulate growth morphology. This based on the data of gene sequence analysis which shows that more than 2000 genes encoded proteins can participate in fungal

**Figure 2**




Factors affecting fungal macromorphology and biopellet engineering (size, structure, and density) during cultivation in submerged culture (Modified from El Enshasy [9•]).

growth and development. The manipulation of these genes can lead to significant change in growth morphology [11]. Cai *et al.* [78] reported that the deletion of polarized growth gene in *Aspergillus glaucus* mutant Δ*AgkipA* leads to small pellet formation, and increase the anticancer polyketide aspergiolide A by 82%. Deletion of *racA* gene (a gene regulates the polymerization of actin in apical tip) can generate a hyperbranching mutant which can increase enzyme production. This method was applied to increase glucoamylase secretion in *A. niger* by fourfold [79], and cellulase production in *Trichoderma reesei* by threefold compared the wild type strains [80]. Other study reported also that silencing of chitin synthase genes (*chs*C) expression resulted in an increase in citric acid productivity in *A. niger* through the formation of small and dense pellet structure which is desired morphology in this process [12]. Therefore, genetic approach of fungal morphology engineering become a very powerful tool to switch mycelial growth to the desired morphology in many industrial strains.

On the other hand, medium composition, is easily manipulated and that includes basic nutrients (C-sources, N-sources, P-sources, and minerals), and exogenous compounds such as alcohols, polymers, antifoam, and surfactants. Other non-essential medium components such as solid microparticles can act as an agglomeration center of spore and can help to shift growth morphology to smaller pellet [81•]. It was also reported that addition of microparticles such as talc powder and aluminum oxide enhanced the production of multienzyme (phytase, cellulase, amylase, xylanse) in *A. oryzae* culture through the reduction of biopellet size by almost 90% [82].

Cultivation conditions include temperature, pH, other variables related to bioreactor design (type, size, agitator type and size), and bioprocess operation conditions (aeration, agitation, mixing). These factors are of special interest as manipulations of these variables are achievable. In addition, the data generated are very useful in design scaling up strategy for fungal bioproducts development [9•,19,81•,83]. However, an alteration in one factor can affect other factors. For example, change in type or concentration of carbon source can affect, growth rate, C/N ratio and it can enhance the production of biometabolites which can affect medium viscosity and pH.

In many processes, increasing agitation rate supports the production of large number of pellets of smaller size with compact and dense structure. This step helps to achieve the maximal active biomass, and allows for good mixing of Newtonian fluid of culture based on lower viscosity compared to mycelial growth.

## Scaling up of fungal culture of pelleted growth morphology

Scaling up of fungal cultures with controlling the desired morphology is a challenge. Scaling up involves significant changes in main variables which affect growth morphology in great extent. These include bioreactor design (type of bioreactor, size and dimensions, internal structure design, stirrer design and dimension, type of stirrer, agitation rate, and aeration rate) [83]. Each of these individual factors have shown significant effect on biopellet morphology. Therefore, besides the generic scaling up criteria in bioprocess such as: keeping the same power input, mixing time, and oxygen transfer rate, scaling up based on maintaining the same growth morphology become one of the new challenges to achieve maximal yield. For example: higher shear stress by agitation can convert growth morphology from large to small dense pellet with smooth outer surface and further increase in agitation can break fungal pellet and shift the growth to disperse fragmented hyphae. Therefore, replacing the standard Rushton-turbine (a 90° angled flat blade impeller of strong radial flow mixing) with low shear impeller such pitched-blade can reduce the pellet destruction during high mixing [84].

## Morpho-physiological characterization of biopellet

Different morphological and physiological approaches have been used to study the biopellet structure to link biopellet morphology/structure and physiological status. Studies focused on direct microscopic analysis with image analysis system for full characterization of fungal bioparticle [85–87].

However, for a better understanding of the relation between biopellet size/structure, cell viability, and productivity, different techniques have been developed. Cell viability inside fungal pellet was determined using a combination between fluorescein diacetate (FDA) and ethidium bromide (EB) or propidium iodine (PI) [88]. Acridine Orange (AO) staining followed by biopellet visualization under fluorescence microscope was also useful approach to differentiate between the growing and non-growing cells. DNA rich and dsRNA parts of biopellet emit green fluorescence, where ssRNA rich part emit orange/red fluorescence. Using this method, the productive and the non-productive parts were differentiated [9•,19] Figure 3 describe the concept of AO straining method for quantitative determination of bioactive fractions in biopellet population. For aerobic microorganism such as *A. niger*, the outer layer of biopellet of maximal of 200 μm is the most physiologically active layer. Recent research also confirmed the potential use of cell sorting method (flowcytometry) for rapid and automated analysis of large sample of pellet after using different fluorescence staining. In addition, the development of new generation of cell sorting machines COPAS (Complex object

parametric analyzer and sorter) extends the applications of this method to study larger pellet up to 1500 μm diameter [74,89,90].

Witter *et al.* [91•], were among the first groups who reported the quantitative measures of the oxygen transfer in *Penicillium chrysogenum* biopellet using micro dissolved oxygen electrode. Other studies used a combination technique involving confocal laser scanning microscopy (CLSM) and oxygen microelectrode system [92]. Other method was also developed to study in depth the productive and non-productive zones in biopellet by measuring oxygen, substrate uptake, and metabolite production. This method was based on using time-of-flight secondary ion mass spectrometry (ToF-SIMS) with $^{18}O$ and D-[1-$^{18}C$] glucose as tracer substrate [93]. A recent study of Schmideder *et al.* [94•] enabled the development of a universal model to estimate the oxygen diffusion, substrate and metabolites transfer within fungal biopellet using X-ray microtomography (μCT) combined with 3D-image analysis and computational software [95].

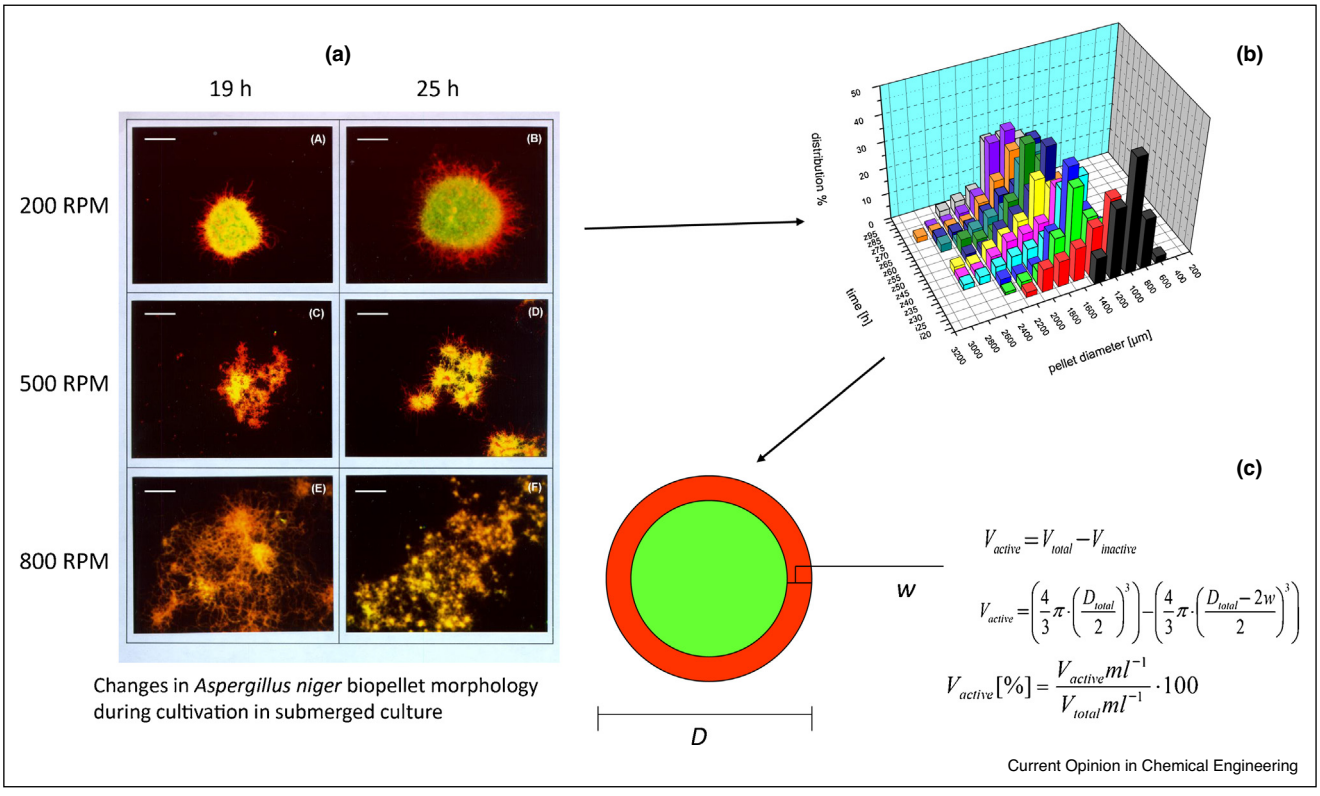
As shown, most of studies came with almost the same conclusion that pellets of less hyphal density facilitate oxygen and substrate/product transfer. Whereas, in large compact pellets, only the outer layer exposed to oxygen and substrates represents the active fraction of biopellet [91•,96].

## Biopellet in co-culture system

Compared to the extensive data presented on the biopellet formation of different type of pure cultures, little information is available on the biopellet engineering in mixed culture system.

Fungal pellet can act as natural self-immobilization matrix for other organisms. This approach will reduce the immobilization cost in many processes. Moreover, the advantage of this system will be more significant with co-pelletization of microbes of different oxygen requirements. This helps to develop a well-designed self-engineered biopellet structure of microbial consortium (aerobic/facultative aerobic/anaerobic organisms) depending on the distance from the biopellet surface. However, most of mixed culture biopellet is composed of (fungal/algal) biosystem reported on the potential use of mixed biopellet of *A. niger* and *Chlorella vulgaris* for cadmium removal in wastewater [97–99]. This method facilitates

**Figure 3**




Determination of biopellet active fraction of *Aspergillus niger* during cultivation in submerged culture using acridine orange (AO) staining method **(a)**, Biopellet produced at different agitation speed visualized under fluorescence microscope after AO staining. **(b)** Determination of biopellet size distribution during cultivation using minimal sample size of 100 pellets, **(c)** Calculation of bioactive fraction of the total biomass based on the red/orange colored part of the biopellet (Modified from El Enshasy *et al.* [19]).

the harvesting of microalgae biomass from wastewater which contributes for large portion of treatment cost up to 20–30% when using traditional separation techniques [100,101]. This mixed culture biopellet concept was also useful for the removal of different types of pharmaceuticals from wastewater [99]. To apply this concept, the microbe-microbe interaction in the consortium should highly considered. They should be able to co-exist in a mutualistic (the interacting species/strains gain benefit) or in commensalistic interaction (one species benefit from others without benefit/harm effect to other) [102]. Therefore, the microbial consortium in mixed biopellet should be well selected. The main drawbacks of the applicability of mixed biopellet are the potential antagonistic effect within the microbial consortium especially during substrate limitation, the difficult of contamination detection, the difficulties of controlling the optimum balance among the microbial system involved

In general, the environmental and industrial applications of the mixed culture biopelletare still in the development stage. The well-engineered biopellets (composed of symbiotic biosystems act as cascaded biofactory to perform complementary biochemical reactions in bioprocess industries) will provide a competitive solution to reduce the cost of production in both upstream and downstream applications.

## Conclusion and future perspectives

Although the filamentous microorganisms have long history in fermentation industries, only limited information is available about controlling growth morphology during cultivation at a large scale standards. This poor knowledge can be attributed to the industrial nature and the competitive behaviors of different companies. Published data focused on individual factor (one factor at time: OFAT) approach which lacks the in depth knowledge about the interactions between the different factors. In addition, studies on biopellet formation did not take in account the pre-spore formation phase (spore induction) stage which can greatly influence spore structure, hydrophobicity, resistance, and germinability. Studies of biopellet formation need to consider the whole process from spore induction up to biopellet formation. This needs to include all factors (genetic, environmental, nutritional, and engineering variables) which can affect each step and how these factors act together at the end of the biopellet development. This can help to design well customized process to control growth morphology in submerged culture. In addition, molecular biology techniques to regulate gene expression through gene silencing and induction gained popularity, to customize the preferred morphology for each particular process. In addition, more research is also required for on-line determination of biopellet viability during cultivation. This will assist for on-time change of cultivation parameters to shift the pellet morphology toward the desired structure to achieve the maximal production yield. Furthermore, understanding of co-culture system biopellet engineering will be also a useful tool to improve many industrial processes.

## Conflict of interest statement

Nothing declared.

## Declaration of Competing Interest

The authors report no declarations of interest.

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Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest


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