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FORMATION, MORPHOLOGY AND BIOTECHNOLOGICAL APPLICATIONS OF FILAMENTOUS FUNGAL PELLETS: A REVIEW

FORMACIÓN, MORFOLOGÍA Y APLICACIONES BIOTECNOLÓGICAS DE GRÁNULOS DE HONGOS FILAMENTOSOS: REVISIÓN

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Abstract

Bioprocesses with fungal biomass have been the subject of a growing interest in biotechnology industry because they represent a rich source of enzymes, antibiotics, vitamins, and complex metabolites, which have a great potential for pharmaceutical, agricultural, and environmental applications, among others. Filamentous fungi have different growth patterns (as mycelia, clumps, and pellets) in industrial processes. Therefore, the study of the morphology presented by this group of organisms is necessary to establish the conditions of production of new products or to improve the existing processes, even at the laboratory scale. Among the various forms of fungal growth, the formation of pellets represents many advantages in bioprocesses, and thus it is interesting to determine the factors that induce it. This manuscript describes the state of the art of fungal pellets, provides information on conditions of both inoculation and incubation that favor the formation of pellets at laboratory scale, as well as the advantages, applications and perspectives that pellets have in the field of biotechnology.

Keywords: fungal biomass, bioprocess, metabolites, hairy region, filamentus fungi.

Resumen

Los bioprocesos con biomasa fúngica han sido objeto de un creciente interés en la industria biotecnológica, ya que representan una rica fuente de enzimas, antibióticos, vitaminas y metabolitos complejos, que tienen un gran potencial para aplicaciones farmacéuticas, agrícolas y ambientales, entre otras. Los hongos filamentosos tienen diferentes patrones de crecimiento (como micelios, grumos y gránulos) en los procesos industriales. Por lo tanto, el estudio de la morfología presentada por este grupo de organismos es importante para establecer las condiciones de producción de nuevos productos o para mejorar los procesos existentes, incluso a escala de laboratorio. Entre las diversas formas de crecimiento fúngico, la formación de gránulos representa una serie de ventajas en los bioprocesos, por lo que es necesario conocer más acerca de los factores que la inducen. Este artículo describe el estado del arte de los gránulos fúngicos, proporciona información sobre las condiciones de inoculación e incubación que favorecen su formación a escala de laboratorio, así como las ventajas, aplicaciones y perspectivas que tienen los gránulos en el campo de la biotecnología.

Palabras clave: biomasa fúngica, bioprocesos, metabolitos, región de hifas, hongos filamentosos.

1 Introduction

Biotechnology of fungi has allowed the industrial exploitation of their intrinsic biochemical activities. The most attractive group for biotechnological processes are the so-called filamentous fungi or molds, which impact our lives in many ways (Torres-Barajas and Aguilar-Osorio, 2013; Ward, 2012; Meyer, 2008). This term designates the group of species that produces filaments in the form of hyphae, which is more common for the Fungi kingdom. The term is also used in contrast to yeasts, which are essentially unicellular fungi with the ability to reproduce repeatedly (Evans, 1988).

Filamentous fungi are heterotrophic eukaryotic microorganisms that depend on external sources of carbon and can reproduce sexually or asexually

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(Piepenbring *et al*., 2015). The mycelium is composed of hyphae, which are the basic units of filamentous fungi with a strong cell wall (Steinberg, 2007). This cell wall is composed of polysaccharides (chitin and β-glucan) and glycoproteins that determine the fibrillar structure of the wall (Preechasuth *et al*., 2015; Serrano-Carreón *et al*., 2015).

The growth of the hypha takes place in the apical region. When a new limb is formed, it grows to a determined length and a septum with a nucleus is created in the posterior part to the tip. These septa are invaginations of the cellular wall that limit the plasma membrane and contain a central pore of 50-500 nm that allows the transmission of cytosol and organelles as in Ascomycota and Basidiomycota (Voisey, 2010; Patnaik, 2000). The result of the apical extension is the formation of non-propagating cells, which eventually produce vacuoles. These, in turn, grow as a function of their distance to the apical region (Figure 1). Since the mycelial region is complex and multicellular, the age distribution of the cells is extensive compared with the unicellular microorganisms. In turn, the growth of hyphae allows the fungus to explore a wide area in search of nutrients (Sietsma and Wessels, 2006). Therefore, hyphae may exist in several physiological states. Their structure and characteristics may be different for each group, such as apical growth cells, regions with vacuoles, and lysed cells, the last being metabolically inactive (Serrano-Carreón *et al*., 2015).

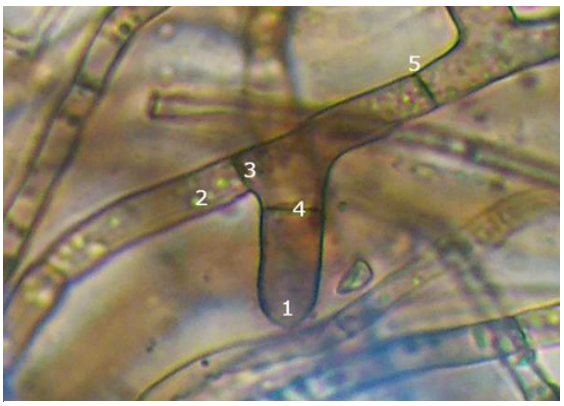

Fig. 1. Regions of cell differentiation of *Cladosporium cladosporiodes*: 1) apical region, 2) nucleos, 3) pore, 4) septum and 5) no propagative region.

The bacterial biomass has filaments too. However, concerning the mechanism of cell growth, these two groups show large differences. Eukaryotic fungi are organisms that present a complex morphology with cell organelles and a cytoskeleton directly linked to growth, whereas filamentous bacteria or Actinobacteria do not have such a cellular organization (Driouch *et al*., 2010).

In spite of these differences, the two groups show similarities in their morphology, growth patterns, growth forms, and kinetic growth of hyphae and mycelia in both solid and liquid media. Yet, fungal biomass presents a number of advantages over bacteria from the industrial point of view, namely, the larger number of enzymes produced, the removal of complex contaminants of both organic and inorganic origin, their endurance to fluctuations in pollutant loads, and their resistance to low pH and nutrient levels (Espinosa-Ortiz *et al*., 2016; Moreira *et al*., 2003).

2 Morphologies of filamentous fungi

The morphology of fungal biomass in liquid cultures has been established as an important parameter in bioprocesses. In the growth of filamentous fungi, diverse forms can be recognized. The common pattern of growth of the filamentous fungi is from a hypha that forms a criss-cross network, which we refer to as mycelium. Finally, the pellets are aggregates of strongly-interlaced, spherical or semi-spherical, compact hyphae (Figure 2) (Cai *et al*., 2014; Liu and Wu, 2012; Žnidaršič and Pavko, 2001; Gibbs *et al*., 2000; Cui *et al*., 1997). In addition, the term "clump" is often used to describe small aggregates of mycelium or large and heavy masses of aggregated mycelium.

Between 80,000 and 120,000 species of fungi have been identified with different morphologies. Even the same species can give rise to different forms depending on the conditions of cultivation to which they are submitted (Zhang and Zhang, 2016).

Due to the problem of controlling the morphology of fungi in bioprocesses, the automation of their production processes has been difficult. This is the case for the production of Penicillin G from species of the genus *Penicillium* (Žnidaršič and Pavko, 2001). Submerged fungal cultures normally result in excessive growth of mycelium, which hinders mass transfer and the production and excretion rates

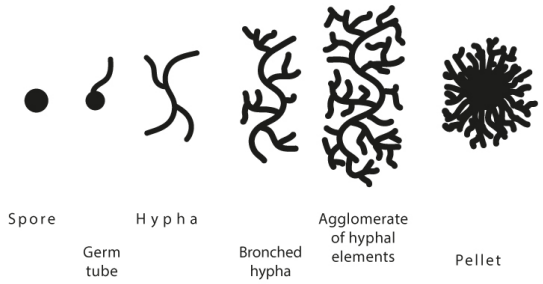

Fig. 2. Formation of a fungal pellet from a spore.

of metabolites (Wucherpfennig *et al*., 2010). The excessive growth of fungal mycelium can obstruct the sampling channels in bioreactors, as well as in the nutrient addition lines and mixers, resulting in the increase in the viscosity of the medium and limitations in mass and oxygen transfer, among other disadvantages. These obstacles in the use of fungal biomass restrict the operation time of the bioreactor, whereby batch cultures are preferred, and in which the fungi are grown for short periods of time, and the culture medium is then discarded to start a new batch (Moreira *et al*., 2003).

3 Fungal immobilization

This technique can be defined as the adhesion of the biomass to a solid or porous matrix that limits the free migration of cells. This technique represents a solution to many common problems in bioprocesses. In general, there are two types of immobilization: (1) fixation, in which the microorganisms adhere to the surface by themselves or by chemical bonding and (2) entrapment, which involves the retention of microorganisms at the intersections or pores of fibrous materials or are physically trapped within a solid or porous matrix (Figure 3) (Sun *et al*., 2010; Moreira *et al*., 2003; Khoo and Ting, 2001).

Filamentous fungi show a natural inclination to adhere to surfaces and thus to be immobilized. Research on adhesion mechanisms is still limited, but macromolecules located on the surface of filamentous fungi, such as polysaccharides, seem to act as a kind of "glue" to initiate surface microbial interactions (Moreira *et al*., 2003).

Fig. 3. Immobilized mycelium of *Cladosporium cladosporioides* in *Luffa cylindrica*, before and after the secretion of a pigment.

In industrial bioprocesses, the fungal immobilized biomass has been the solution to different problems, such as the difficulty of separation of microbial cells from medium after biosorption, mass losses during separation and low mechanical strength and small particle size, which make problematic the use of fungal biomass in batch and continuous systems. Further, the immobilization may extend the life of biocatalyst and facilitate its recovery and reuse. Finally, the separation of products from immobilized cells is easier than that from suspended cells (Velasco-Bucheli *et al*., 2017; Arica *et al*., 2004).

The fungal immobilization is achieved using natural polymers (i.e., alginate, chitosan, chitin, *luffa* and cellulose derivates) or synthetic polymers (i.e. polyurethane foam, among others). Some findings show a correlation between the fungal products and the support used in the immobilization. Velasco-Bucheli *et al*. (2017) found that the production of 1-phenylpropane-1,3-diol by the fungus *Colletotrichum acutatum* was clearly dependent on the support used (polyurethane), which was reused at least 3 or 4 times without significant loss of activity. Additionally, the immobilization technique brings many benefits to fungal bioremediation, such as a higher efficiency of pollutants degradation, multiple uses of the biocatalysts, reduced costs due to the elimination of the cell filtration stage, the ensuring of a stable microenvironment for cells and enzymes, a reduced risk of genetic mutations, resistance to shear forces present in bioreactors, increased biocatalyst survival during storage, and increased tolerance to high pollutants concentrations and adverse environmental conditions (Dzionek *et al*., 2016).

Thus, the formation of pellets can be considered as a form of self-immobilization, resulting in the agglomeration of mycelium. Production and yield of

the metabolites are in function of the morphology of the fungus. An example of this case is a study aimed to compare the production of lactic acid and fumaric acid by *Rhizopus oryzae* in the form of pellets and clumps under the same culture conditions (24 g L^{-1} PDB, 6.0 g L^{-1} peptone of soybean, 6.0 g L^{-1} of $CaCO_3$, inoculum of 1.5×10^9 spores L^{-1}, 27 °C and 190 rpm). When using pellets of *R. oryzae*, the maximum concentrations produced were 65 g L^{-1} of lactic acid after 30 h, and of 31.0 g L^{-1} of fumaric acid after 96 h. In contrast, when biomass in the form of clumps was used, concentrations of 20 g L^{-1} of lactic acid and 21.5 g L^{-1} of fumaric acid were reached after 30 h and 96 h of culture, respectively (Liao *et al*., 2007a).

Industrially, the formation of metabolites, especially secondary metabolites, occurs only when the fungal biomass grows as pellets (Junker *et al*., 2004). This is the case of the production of itaconic acid by *Aspergillus terreus*, citric acid and polygalacturonases by *Aspergillus niger*, cellulolytic enzymes by *Trichoderma reesei*, and α-galactosidases by *Mortierella vinaceae*. This morphology is also necessary for the synthesis of ergot alkaloids by the filamentous fungus *Claviceps purpurea* (Zhang and Zhang, 2016; Liu *et al*., 2008; Žnidaršič and Pavko, 2001).

4 Morphology of fungal pellets

Fungal pellets are aggregates of hyphae that have a circular, ellipsoidal or oval shape, ranging in size from a few micrometers to several millimeters. The history of fungal growth in the form of pellets dates back to the late nineteenth century. Metz and Kossen (1977) carried out a bibliographical review about the morphology of pellets in fungi and found that authors like Ray had reported, in 1897, the formation of spherical colonies of *Aspergillus candidus*. Later, in 1933, Kluyver and Perquin reported the observation of mycelial beads of *Aspergillus flavus*. In a fungal pellet, four main regions are observed (Figure 4): i) The first is a semi-anaerobic compact region (R1) constituting the nucleus of the pellet; (ii) the second region (R2) is a layer surrounding the nucleus, which may differ among fungal pellets because some of them are hollow; iii) in the third region (R3), the hyphae already begin to show signs of autolysis, and iv) the fourth region (R4) is the area of viable hyphae or "hairy region", which is the most metabolically active part of the pellet (Espinosa-Ortiz *et al*., 2016).

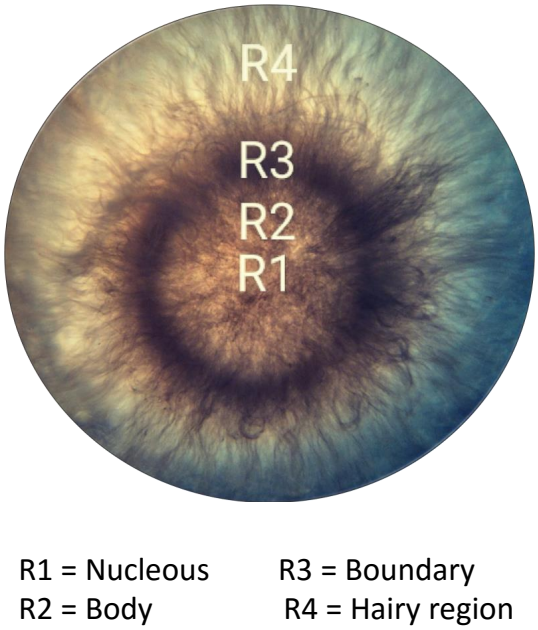

Fig. 4. Regions of a fungal pellet of *Cladosporium cladosporioides*.

The growth in pellet form is an important parameter for all fungi used in industry (Anuradha *et al*., 2014). The following are the main advantages and disadvantages of using fungal pellets (Espinosa-Ortiz *et al*., 2016; FazeliNejad *et al*., 2016; Krull *et al*., 2013; Nyman *et al*., 2013; Pamboukian *et al*., 1998).

Advantages:

- The high productivity of biomass and the easy separation of the medium.
- The improved rheology of the medium (low viscosity, Newtonian fluid behavior).
- The lower energy consumption for maintaining stable temperatures in the process.
- The greater mass transfer.
- It allows the continuous operation and the reuse of the biomass.
- The better transfer of mass, oxygen, and nutrients, due to the contact surface.
- The low obstruction effect.
- The easy process scale-up.
- The high viable cell loading and volumetric productivity.

- The low adhesion of biomass to the surface of the bioreactor.

Disadvantages:

- Limited transport of oxygen and nutrients to the cell nucleus, resulting in zones with different growth and metabolic patterns.
- Some pellets may present autolysis.
- Non-uniform size of pellets.

5 Forces involved in the formation of fungal pellets

Two types of forces are involved in the formation of fungal pellets from spores: non-specific interactions (NSI) and specific interactions in spore cell wall components (SICW).

NSI include electrostatic interactions (van der Waals forces and electrostatic repulsion) and hydrophobicity (Zhang and Zhang, 2016). In general, microbial cells, including fungal spores, are negatively charged, but it has been shown that spores show different charges on their surface with different isoelectric points. For example, *Paecilomyces fumosoroseus* is negatively charged under neutral conditions, with an isoelectric point of 3.4 (Zhang and Zhang, 2016). The negative charges of the spores can be evidenced as electrophoretic mobility (MEF) or zeta potential (Wargenau *et al*., 2013; Wargenau *et al*., 2011), according to colloidal science. The MEF of microorganisms depends on pH and cations (concentration, ionic strength, and oxidation state). An augmentation in the pH value increases MEF in the negative direction and decreases spore aggregation. On the other hand, hydrophobicity consists of specific interactions of the components of the cell wall of the spores, and it is a determining factor in their aggregation and in the adhesion of the microorganisms to the surfaces (Priegnitz *et al*., 2012). Hydrophobicity is produced by active surface proteins, hydrophobins, found in spores and aerial mycelium, but not in the vegetative mycelium. There are also the so-called repellent proteins, present in some species such as *Botrytis cinerea* and *Ustilago maydis*, which have a similar function to that of hydrophobins.

Concerning the SICW, the main components of the cell wall of the spores responsible for this type of interaction are chitin, glucan, hydrophobins, melanin, ions, proteins, and lipids. Different components provide different MEF values because from the spore stage to the formation of the hyphae, they exhibit different charges due to variations in their chemical composition (Gougouli and Koutsoumanis, 2013). The greatest contrast between the wall of the spore and the hyphae is in their content of carbohydrates and proteins. The wall of the spore contains 2.5% of carbohydrates while the hypha contains 64.5%. On the other hand, the spore wall contains 63.4% of protein, while the hypha contains only 6.6%.

Similarly, by their type of formation, the fungal pellets are divided into two types: coagulative and non-coagulative (Grim *et al*., 2005). Coagulative pellets (made by some species of the phyla Ascomycota and Basidiomycota), can be formed in two ways depending on the formation of aggregates when the spores are incubated. On the one hand, some aggregates are formed six to eight hours after the spores are incubated; these aggregates form a nucleus and give rise to the growth of hyphae, which in turn form pellets. The formation of coagulative pellet depends on the concentration of the inoculated spores, and many studies agree that the pellets of the *Aspergillus* species are of this type. On the other hand, coagulative pellets can also be formed when the spore aggregates germinate to form pellets directly. In this case, the rate of spore aggregation is linear to the pellet growth (Zhang and Zhang, 2016; Meyer, 2008).

In non-coagulative pellets (such as those formed by some species of phylum Zygomycota and by others belonging to the phylum Ascomycota), the germination of a single spore results in the formation of the pellet. This type of granulation occurs under strong agitation and aeration regimes.

Table 1. Fungal species forming coagulative and non-coagulative pellets (Zhang and Zhang, 2016).

Coagulative pellet	Non-coagulative pellet
Aspergillus niger	*Rhizopus* spp.
Aspergillus nidulans	*Mucor* spp.
Aspergillus oryzae	*Penicillium chrysogenum*
Phanerochaete chrysosporium	*Mortierella vinacea*
	Aspergillus ochraceus ATCC 3150
	Rhizopus oryzae

An example of this mechanism is exhibited by *Aspergillus ochraceus*. Table 1 shows some examples of the classification of the pellets according to their formation process.

6 Factors that influence the morphological characteristics of fungal pellets

As we have seen, there are many factors affecting the formation and morphology of fungal pellets, such as agitation, nutrient concentration in the medium, pH, temperature, dissolved oxygen concentration, presence of additives or surfactants, viscosity of the medium, inoculum size and inoculation and incubation conditions, among others (Steinberg, 2007; Saraswathy and Hallberg, 2005; Nielsen, 1996; Olsvik and Kristiansen, 1994). These factors affect differently each strain, e.g. *Rhizopus* spp. require vigorous agitation to form pellets, whereas in the case of *Penicillium chrysogenum*, an alkaline pH is necessary to induce their formation. In this way, the study of the formation of pellets must be performed individually for each strain (Liu *et al*., 2008). In general, factors influencing the formation and morphology of fungal pellets can be grouped as shown in Figure 5.

Hereunder, the main factors that influence the morphological characteristics of fungal pellets are presented.

6.1 Inoculation

The ideal inoculum for the formation of fungal pellets depends on the strain, since the cell shape and quantity directly affect the formation, growth, and morphology of the pellets in submerged cultures. The fungal life cycle begins and ends with sporulation (Anuradha *et al*., 2014), to which the formation of pellets belongs, although some studies report the formation of this morphology from a suspension of hyphae or fragments of mycelium (Xin *et al*., 2012; Casas *et al*., 2003; Nielsen, 1996). The production of pellets is traditionally initiated with a spore solution (usually $< 10^8$ spores mL^{-1}) (Espinosa-Ortiz *et al*., 2016). However, inducing a sporulation state in a fungus is usually somewhat complicated for some species, since sporulation is an inactive and survival phase (Kumar and Attri, 2016). Once the conditions for the development of spores have been found, the germination process occurs, with which several physical and chemical factors interfere. Although the germination process is difficult to reach in liquid cultures, the development of aerial conidiophores of *Aspergillus oryzae* in submerged cultures has been reported (Žnidaršič and Pavko, 2001). This was achieved due to the abundance of nutrients and to the natural physiology of the wall of the hyphae. The asexual sporulation of the lower fungi as Oomycetes and Zygomycetes (about 1,500 species: aquatic, amphibious, and terrestrial as *Rhizopus*), is induced by the reduction of nutrients.

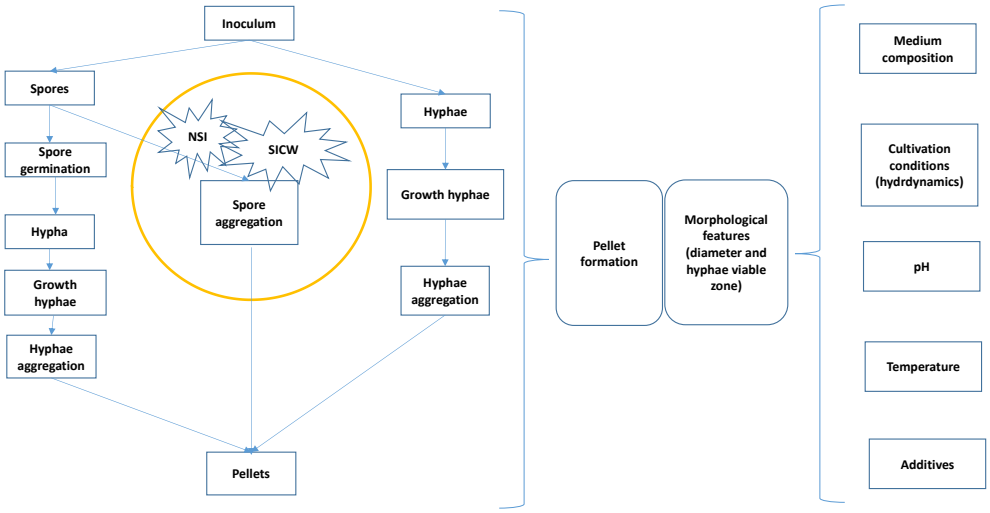

Fig. 5. General diagram of the factors affecting the formation and morphology of fungal pellets. NSI: Non-specific interactions; SICW: Specific interactions in spore cell wall components.

In contrast, the mechanisms that control the sporulation and conidia formation in the high fungi (Ascomycetes and Basidiomycetes) differ between species, although some studies have shown that the major triggers of sporulation in these fungi may be the limitation of both the nitrogen source and the transport of the substrate to the interior of the mycelial aggregates (Žnidaršič and Pavko, 2001). The spores inoculation to form fungal pellets still poses a great obstacle for their industrial popularization and wide application (Xin *et al*., 2010). On another hand, the inoculation technique using filamentous mycelium to produce pellets in a bubble column has proved to be a convenient method, yielding many small pellets with a fairly homogeneous size distribution (Gabriel *et al*., 1996).

6.2 Composition of the medium

The source of carbon, nutrients and some additives, such as polymers, surfactants, and chelates, are key to the formation of pellets. Different carbon sources, such as sucrose, glucose, fructose and xylose, have been used to evaluate the induction of granulation and their influence on morphology. Lactose has been used as a carbon source for the growth of *Aspergillus niger* as a dispersed mycelium (Espinosa-Ortiz *et al*., 2016). The principal medium used in fungal culture for the production of pellets is the PDB (potato dextrose broth), which contains mainly glucose, some vitamins and low nitrogen concentrations. Liao *et al*. (2007b) compared the production of *R. oryzae* pellets using PDB and glucose, and obtained higher yields with the PDB medium. This was attributed to the effect of the vitamins it contains. In another study, Liu *et al*. (2008) reported that lower concentrations of PDB allowed a higher biomass yield ($Y_{B/S}$ = 0.440 g g^{-1}) of formed pellets. This was also attributed to the effect of its vitamin content. Nair *et al*. (2016) evaluated various carbon sources, i.e., glucose, arabinose, sucrose, and galactose in concentrations of 20 g L^{-1}, and concluded that they did not present a significant effect on the morphology of the pellets of *Neurospora intermedia*. It should be noted that the carbon source, which showed the highest yield in the biomass was arabinose ($Y_{B/S}$ = 0.440 g g^{-1}), compared to the lower yield obtained with galactose ($Y_{B/S}$ = 0.184 g g^{-1}). García-Reyes (2016) compared the formation of *Cladosporium* pellets in PDB medium and in an infusion of *Opuntia* spp., obtaining a greater production of pellets and biomass in the infusion of *Opuntia* spp. (0.0638 g dry biomass, n = 119) than in PDB (0.0142 g dry biomass, n = 22) at pH 5. However, a comprehensive investigation of the effects of medium composition and inoculum on pellet formation and growth is lacking.

6.2.1 Incubation conditions

Another factor affecting the morphology of the pellets is the hydrodynamic conditions inside the bioreactor since the laminar and turbulent regimes affect the pellets by the Reynolds number (Espinosa-Ortiz *et al*., 2016). In a bubble column bioreactor (BCB), the morphology of the pellets of *Rhizopus nigricans* under a laminar regime produced homogeneous pellets without signs of fragmentation. These same pellets, when subjected to a turbulent regime, were fragmented and in some cases destroyed. Several authors have reported that vigorous agitation leads to strong and compact fungal aggregates and that, on the contrary, low agitation regimes produce large and less stable aggregates. Such is the case of the pellets of *Neurospora intermedia*, because a rate of 100 rpm produced pellets with a diameter of 6.54 ± 0.62 mm while a rate of 150 rpm produced pellets of 1.92 ± 0.33 mm (Espinosa-Ortiz *et al*., 2016; Nair *et al*., 2016; Liu *et al*., 2008).

On the other hand, high agitation causes hydrodynamic stress within the reactor, impacting the growth of the regions of the pellet, which leads to a rupture or a kind of "shaving" in the hairy region (case of STR). Some authors have reported that it is possible to obtain as much dispersed mycelium as new pellets from this rupture of hyphae.

Aeration also plays an important role in the morphology and the formation of pellets. In the case of *Aspergillus oryzae*, continuous air flows favor the growth of the pellets, whereas a poor concentration of oxygen can produce pellets with large diameters and hollow nuclei (Cox and Thomas, 1992). Another study shows the use of an oxygen pulse in a fluidized bed reactor to control the size and morphology of the pellets of *Aspergillus niger* and *Phanerochaete chrysosporium*, which resulted in uniform pellets and high yields of the metabolite. This is because the area most impacted by this factor is the hairy region (Zhang and Zhang, 2016; Espinosa-Ortiz *et al*., 2016; Nair *et al*., 2016; Liu *et al*., 2013; Liu *et al*., 2008; Moreira *et al*., 1996).

6.3 pH

Another important factor in the formation of fungal pellets is pH. Like the other variables, its effect is a function of the strain used, since, in several cases, only a change in pH is necessary to induce to induce the pellet form. As examples, the formation of pellets of *Penicillium chrysogenum* needs to be induced by alkaline pH values (pH > 7.4), or *Rhizopus oryzae* , which grows as pellets in an acid environments (pH 3 - 7). *Neurospora intermedia* forms pellets of uniform size (2 - 3 mm) and produces a higher yield of ethanol ($Y_{P/S}$ = 0.367 g g^{-1}) in the pH range of 3 - 4. However, it presents the highest biomass production at pH 9 ($Y_{B/S}$ = 0.452 g^{-1}) (Nair *et al*., 2016; Liu *et al*., 2008; Liao *et al*., 2007b; Metz and Kossen, 1997).

6.4 Temperature

The temperature directly affects the formation and morphology of the pellets. Liu *et al*. (2008) reported that a temperature of 38°C favors the rapid formation of pellets in cultures of *Rhizopus oryzae*. However, this same temperature affects the yield of the biomass, compared to the use of temperatures between 22 and 33°C. Žnidaršič *et al*. (2000) compared the effect of temperature (19 and 23°C) on the morphology of *Rhizopus nigricans* pellets. The pellets obtained at 19°C were aggregates with smaller diameters (1.1 ± 0.3 mm) and with little or no region of viable hyphae, whereas the pellets obtained at 23°C had a larger diameter (1.3 ± 0.4 mm) and pronounced regions of viable hyphae.

6.5 Additives

Several authors have evaluated the effect of various additives on the formation of fungal pellets. The granulation of *Rhizopus oryzae*, using a biodegradable rice polymer at three concentrations (4, 10 and 20 g L^{-1}) and with four different particle sizes (3 mm x 5 mm, 1.4 mm and < 0.6 mm), was evaluated. An increase of 16% in the biomass production was obtained at a concentration of 4 g L^{-1} of the biopolymer, while the particle size did not produce significant differences ($P > 0.05$) in the pellets (Liu *et al*., 2008). Another study evaluated the effect of the addition of glycerol and calcium chloride on the morphology of the *Neurospora* pellets. With the addition of these compounds, small aggregates of the compact nucleus and little growth in the "hairy region" were obtained. However, both the biomass yield and the ethanol production improved (Nair *et al*., 2016). Zhang and Zhang (2016) have reported the use of microparticles (MgO-SiO_2-H_2O, Al_2O_3, $TiSiO_4$) to form pellets. Finally, Liao *et al*. (2007b) have shown the formation of smooth-appearing pellets by adding mineral ions (KH_2PO_4, $MgSO_4$, $ZnSO_4$) and $CaCO_3$ (as charge neutralizer) in the solution.

7 Applications of fungal pellets

The production of large quantities of enzymes (specific and non-specific) and of other metabolites makes fungi interesting from an industrial point of view. Their easy reuse (in the form of living or dead biomass), as well as their adsorption capacity of heavy metals, also allows the use of fungal pellets for the removal of organic and inorganic contaminants from aqueous wastes.

7.1 Production of metabolites

Fungal biomass in granular form is considered a requisite for the successful production of metabolites, such as citric acid, and enzymes, such as glucose oxidase, polygalacturonase, phytase and glucoamylase (Zhang and Zhang, 2016). *Aspergillus niger* is the most used species in the industry due to the variety of enzymes that it is able to produce (Table 2). The production of various acids by this fungus requires the formation of pellets; for example, 99% of citric acid produced globally (about 1.5×10^6 tons per year) is produced by *Aspergillus niger* in granular form (Zhang and Zhang, 2016; Reyes-Ocampo *et al*., 2013).

The production of certain metabolites or enzymes, such as polygalacturonase, is also related to the size of the pellets because a higher production of this enzyme is associated to compact pellets. In many cases, the fungus morphology is directly related to the fermentation productivity (Papagianni, 2004). The following are the main fungal metabolites (enzymes, organic acids, and antibiotics) of industrial interest.

Table 2. Major classes of enzymes, organic acids, and antibiotics of commercial importance produced by filamentous fungi and some of their sources (Papagianni, 2004).

Sources	Enzymes	Organic acid	Antibiotics
Aspergillus awamori	α-Amylase Amyloglucosidase α-Galactosidase β-Galactosidase Glucoamylase α-Glucosidase Invertase		
Aspergillus niger	α-Amylase Amyloglucosidase Catalase Cellulase α-Galactosidase β-Galactosidase β-Glucanase Glucoamylase Glucose aerodeshydrogenase Glucose oxidase α-Glucosidase α-D-glucosidase β-Glucosidase Hemicellulase Hesperidinase Invertase Lipase Pectinase Fitase Protease Tanase	Citric acid Gluconic acid L-Malic acid	Penicillins
Aspergillus oryzae	α-Amylase Amyloglucosidase α-Galactosidase β-Galactosidase β-Glucanase Glucoamylase β-Glucosidase Hemicellulase Invertase Lipase Protease	Gluconic acid, L-Malic acid and Kojic acid	
Aureobasidium pullulans	α-Amylase Amyloglucosidase Glucoamylase		
Rhizopus oryzae	α-Amylase Amyloglucosidase Glucoamylase Pectinase	Fumaric acid, Kojic acid, D-Lactic acid and L-Malic acid	

Trichoderma viride	α-Amylase Cellulase β-Glucanase Hemicellulase	Citric acid	
Rhizopus niveus	β-Amylase Amyloglucosidase Glucoamylase Lipase		
Aspergillus phoenicis	Amyloglucosidase Hemicellulase	Citric acid	
Eupenicillium javanicum	Catalase Glucose oxidase	Gluconic acid	
Penicillium vitae	Catalase		
Aspergillus sojae	Cellulase		
Aspergillus terreus	Cellulase	Itaconic acid	
Penicillium citrinum	Cellulase		
Penicillium funiculosum	Cellulase β-Galactosidase		
Ttrichoderma longibrachiatum	Cellulase Hemicellulase		
Trichoderma reesei	Cellulase β-Glucanase β-Glucosidase Xilanase		
Aspergillus carneus	Dextranase		
Chaetomium gracile	Dextranase		
Penicillium funiculosum	Dextranase β-Galactosidase		
Paecilomyces lilacinus	Dextranase		
Penicillium lilacium	Dextranase		
Penicillium pinophilum	Dextranase		
Mortierella vinaceae	α-Galactosidase		
Penicillium dupontii	α-Galactosidase Protease		
Aspergillus nidulans	β-Galactosidase		Echinocandin B Penicillins
Fusarium oxysporum	β-Galactosidase		
Neurospora crassa	β-Galactosidase Invertase		
Penicillium funiculosum	β-Galactosidase		
Acremonium persicinum	β-Glucanase		Cephalosporins Penicillins
Geotrichum candidum	β-Glucanase Lipase		
Penicillium amagasakiense	Glucose oxidase		
Penicillium simplicissimum	Glucose oxidase	Gluconic acid	
Penicillium vermiculatum	Glucose oxidase		
Aspergillus flavus	α-Glucosidase	Kojic acid L-Malic acid	Penicillins
Aspergillus fumigatus	α-Glucosidase		
Mucor circinelloides	α-Glucosidase		
Humicola sp.	Lipase		

Rhizomucor miechei	Lipase		
Rhizomucor spp.	Lipase		
	Pectinase		
	Protease		
Rhizopus arrhizus	Lipase		
Penicillium roqueforti	Lipase		
Aspergillus alliaceus	Pectinase		
Aspergillus sp.	Pectinase		
Rhizopus sp.	Pectinase		
	Protease		
Aspergullis ficuum	Phytase		
Aspergillus melleus	Protease		
Aspergillus saitoi	Protease		
Penicillium dupontii	Protease		
Penicillium sp.	Protease		
Cryphonectria parasitica	Rennet		
Rhizomucor miehei	Rennet		
Rhizopus pusillus	Rennet		
Aspergillus tamarii	Tanase		
Aspergillus citricus		Citric acid	
		L-Malic acid	
Aspergillus clavatus		Citric acid	
Penicillium decumbens		Citric acid	
Penicillium isariiforme		Citric acid	
Rhizopus stolonifer		Fumaric acid	
		Kojic acid	
Aspergillus carbonarius		Gluconic acid	
Aspergillus wentii		Gluconic acid	
		L-Malic acid	
Penicillium chrysogenum		Gluconic acid	Cephalosporins
		D-Araboascorbic	Penicillins
Penicillium leteum		Gluconic acid	
Aspergillus itaconicus		Itaconic acid	
Aspergillus candidus		Kojic acid	
Aspergillus parasiticus		Kojic acid	
Propionibacterium jensenii		Kojic acid	
Rhizopus microsporum		Kojic acid	
Aspergillus atroviolaceus		L-Malic acid	
Aspergillus ochraceus		L-Malic acid	
Penicillium griseoroseum		Erythorbic acid	
Acremonium chrysogenum			Cephalosporins
			Penicillins
Acremonium kiliense			Cephalosporins
Fusarium solani			Cephalosporins
			Cyclosporines
Nectria lucida			Cephalosporins
Tolypocladium geodes			Cyclosporines
Tolypocladium inflatum			Cyclosporines
Trichoderma polysporum			Cyclosporines
Aspergillus rugulosus			Echinocandin B
Calcarisporium arbuscula			Fusidic acid
Fusarium coccophilum			Fusidic acid

Mortiella ramannianua	Fusidic acid
Penicillium aurantiogriseum	Griseofulvin
Penicillium griseofulvum	Griseofulvin
Penicillium italicum	Griseofulvin
Aspergillus giganteus	Penicillins

7.2 Biosorption

Biosorption is a physicochemical and metabolically independent process carried out by biological material and is based on a variety of mechanisms, such as absorption, adsorption, ion exchange, surface complex formation and precipitation (Fomina and Gadd, 2014; Gadd, 2009). The biosorption processes have used various species of fungi to remove three important groups of organic pollutants:

(i) Synthetic dyes, wich are widely used in the textile industry, as well as in the paper industry and printing services. These organic pollutants are usually associated with high concentrations of COD, BOD, suspended solids, toxicity, and color.

(ii) Phenols: These organic compounds are present mainly in the wastewater of the pharmaceutical, pulp and paper, and pesticide production industries and solvents.

(iii) Finally, drug components (*e.g.*, anti-inflammatory, analgesic, antibiotic, psychiatric, and anticancer drugs): These microactive organic pollutants are a major concern due to the reduced efficiency of the wastewater treatment plants to remove them (Espinosa-Ortiz *et al*., 2016; Michalak *et al*., 2013).

Fungal pellets are also an alternative in biosorption processes intended to remove metal ions in solution, mainly due to the lower generation of toxic waste and the lesser energy needs regarding conventional treatments (*i.e.*, coagulation-flocculation) (Espinosa-Ortiz *et al*., 2015; Bayramoğlu *et al*., 2009; Bayramoğlu and Arica, 2008). Some of the fungi that have been used in the form of pellets for the removal of contaminants from aqueous solutions are shown in Table 3 (Pethkar *et al*., 2001; Filipovic-Kovacevic *et al*., 2000; Gabriel *et al*., 1996).

The maximum adsorption capacity of metal ions by fungal pellets occurs under acidic conditions (at pH values comprised between three and six). The biosorption processes of metal ions decrease above pH six. This is due to the formation of insoluble complexes under alkaline conditions (Filipovic-Kovacevic *et al*., 2000). Some studies have reported that optimum pH values needed to obtain high metal ion removals in biosorption processes are lower than five (Pethkar *et al*., 2001; Filipovic-Kovacevic *et al*., 2000). In the biosorption processes, there are different factors that affect the capacity of the pellets to remove metals, such as the morphology. Besides, when the objective is the removal of several metals from aqueous solutions by using pellets, the biosorption process may require more time, as compared to the removal of a single metal (Michalak *et al*., 2013).

In contrast to the metal ions biosorption process, a strain of *Aspergillus fumigatus* is able to achieve a biodecolorization of 90% of a dye (i.e., methylene blue) in a range of pH of 7-13 (Kabbout and Taha, 2014). Filamentous fungi, mainly white rot fungi and brown rot fungi, employ two mechanisms to carry out the biodecolorization process, namely adsorption and enzymatic degradation (Yang *et al*., 2016; Hiu and Yien, 2015). During the adsorption process, it has been observed that temperature increases in the culture of fungal pellets improve the biodecolorization efficiencies. Likewise, it has been observed that biodecolorization is more effective under aerobic conditions (Sen *et al*., 2016). Concerning enzymatic degradation, lignolytic enzymes that employ filamentous fungi for biodecolorization are manganese peroxidase, laccase, and lignin peroxidase (Sumandono *et al*., 2015). This process depends on the composition of the solution and its characteristics, such as pH, ionic strength, and temperature (Kaushik and Malik, 2009).

7.3 New applications of fungal pellets

Fungi having a high oil content have been identified recently. This opens the door to bioenergy research with filamentous fungi. The most promising species are *Mortierella isabellina* and *Mortierella alpine*, which contain 86% of dry mass of fatty acids (Zhang and Zhang, 2016).

Table 3. Species of filamentous fungi used in the form of pellets for biosorption and biodecolorization of organic compounds.

Specie	Organic compounds	Inorganic compounds
Phanerochaete chrysosporium		Cadmium (II) and Lead (II) (Li *et al*., 2004) Selenium (Espinosa-Ortiz *et al*., 2015)
Aspergillus niger 450 *Aspergillus ustus* 326 *Stachybotrys* spp. 103	Humic Acid-like (Vuković *et al*., 2008)	
Lentinus edodes		Mercury (II), Cadmium (II), and Zinc (II) (Bayramoğlu and Arica, 2008)
Coriolus versicolour NBRC 9791	Azo dye acid orange 7 (Hai *et al*., 2013)	
Funalia trogii ATTC 2008001 *Coriolus versicolour* ATTC 200801 *Phanerochaete chrysosporium* ME446 *Pleurotus florida* *Pleurotus ostreatus* *Pleurotus sajor-caju*	Astrazone Black, Astrazone Blue and Astrazone Red (Yesilada *et al*., 2003)	
Trichoderma spp.	Acid Brilliant Red B (Xin *et al*., 2012)	
Lentinus polychoruous Lév.	Anionic reactive dye mixtures of Reactive Blue 19, 160, and 198 (Wangpradit and Chitprasert, 2014)	
Anthracophyllum discolor	Pentachlorofenol (Bosso *et al*., 2015)	
Funalia trogii	Phenol and Chlorofenol (Bayramoglu *et al*., 2009)	
Penicillium oxalicum		Reactive Blue 21 (Cu)(Xin *et al*., 2010)

Another innovative application of fungal pellets to sewage treatment and biofuel production is their use as immobilizing supports of other microorganisms, such as microalgae and yeasts in a co-culture (Choi *et al*., 2016; Zhang and Zhang, 2016).

7.4 Limitations on the use of fungal pellets in industry

The bioprocesses with fungal pellets present a number of process engineering advantages, such as the separation of liquid and solids in batch or continuous reactors, avoiding the use of the centrifugation or filtration in the process. For the fungal industrial application in biosorption processes, immobilization of biosorbent is necessary for solid/liquid separation. However, more studies are needed to develop industrial applications.

The majority of the studies of fungal pellet formation have been developed in batch reactors at laboratory scale, and a few in column reactors. One of the major technological challenges in the fungal pellet formation is the enhancement of the inoculation

process in continuous reactors. This step would help solve the scaling of the process.

Whereby, it is necessary the study of each individual fungal strain for pellet formation because the change in fungal morphology is influenced by different factors: medium composition, inoculum, pH, medium shear, additives, culture temperature, medium viscosity and others. For example, *Penicillium chrysogenum* requires high pH and some strains such as *Rhizopus* sp. need strong agitation to form pellets (Liao *et al*., 2007; Metz and Kossen, 1977).

Conclusions and perspectives

The formation of fungal pellets and the control of their morphology are complex processes that depend mainly on the inoculum and incubation conditions. These factors vary with each species, and, therefore, it is not possible to establish a general methodology for the formation of fungal pellets. The use of fungal pellets is a subject that is extensively studied due to their wide range of applications.

Many industries such as those of petroleum, mining, solvent, paper and pulp, printing, iron-steel, textile, coke, pesticide, paint, pharmaceutics and wood preserving chemicals, discharge large volumes of wastewater. Biosorption processes are a promising alternative to replace or to enhance the available treatment processes for the removal of pollutants such as metals, dyes, phenolics and pesticides, among others. Despite the success of the application of fungal pellets in the removal of organic and inorganic wastewater, its application has not been carried out on an industrial scale yet.

Currently, the use of fungal pellets in industrial bioprocesses provides several advantages, such as an increased production of metabolites and a low cost of production. For this reason, the study of new fungal species is important since different metabolites and applications are obtained every day for different fields of biotechnology, such as the use of oily species or the immobilization of algae or bacteria adhered in fungal pellets in the area of bioenergies, among others.

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Abbreviations

BCB	bubble column bioreactor
BOD	biochemical oxygen demand
COD	chemical oxygen demand
MEF	electrophoretic mobility
h	hours
g	grams
L	liters
mL	milliliters
mm	millimeters
nm	nanometers
PDB	potato dextrose broth
rpm	revolutions per minute
STR	stirred tank reactor
$Y_{P/S}$	yield of the product relative to the substrate (g g^{-1})
$Y_{B/S}$	yield of biomass to substrate (g g^{-1})

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