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Characterization of Fungal Morphology using Digital Image Analysis Techniques

Maria Papagianni*

Department of Hygiene and Technology of Food of Animal Origin, Faculty of Veterinary Medicine, Aristotle University of Thessaloniki, Greece

Abstract

The use of filamentous fungi for the production of commercially important products is old but keeps increasing during the last decades. New classes of compounds are being added in the list of products of fungal fermentations as a result of progress in methodologies and applications of biotechnology. Fungi are morphologically complex organisms that differ in structure throughout their life cycle. In submerged fermentation fungal morphology may take distinct forms ranging from dispersed filaments to densely interwoven masses of mycelium known as pellets. Each morphological form has each own characteristics that have a critical impact on the overall process outcome. Dispersed growth results in highly viscous broths with pseudoplastic behavior that have a negative impact on mass and energy transfer rates resulting in higher energy input requirements. Due to the high industrial relevance of fungal morphology there has been a substantial development of tools and techniques to characterize morphology and extract quantitative information that can be used in process control and optimization studies. Digital image analysis is the state of the art method to characterize and quantify fungal morphology in the developmental process from spores to filamentous structures to pellets. The progress made in the area since the 1990s, when the first image analysis methods were reported, is discussed in detail throughout the review.

Keywords: Digital image analysis; Fungal morphology; Fermentation

Introduction

Filamentous fungi are exploited in industry for the production of a wide range of compounds of commercial importance. Organic acids, antibiotics, enzymes, proteins (native and heterologous) and vitamins are produced efficiently by filamentous fungi in submerged fermentation systems in long-established processes [1]. Historically, the fermentation of these microorganisms was employed for the production of traditional oriental foods e.g. miso, tempeh, soy sauce and many others in solid state fermentation systems. This practice is still common in countries of the Far East while it attracts increasing industrial interest for the production of fungal metabolites due to simpler facilities and lower costs compared to those of submerged fermentation systems [2].

Filamentous fungi are morphologically diverse and complex organisms. Different morphological forms are found in submerged fermentations ranging from filamentous growth to compact pellets, with a whole array of morphological forms between the two extremes [3]. Their morphology at the sub-cellular level –the micro-morphology- also changes during the course of a submerged fermentation [3-5]. Factors affecting fungal morphology in submerged growth include the nature of the growth medium (presence of solids, type and concentration of the carbon source, levels of nitrogen and phosphate, trace minerals, pH, dissolved oxygen and carbon dioxide), the type of the inoculum (vegetative or spores) and a large number of physical factors such as agitation, rheology, fermenter geometry, as well as the mode of culture itself e.g. batch, fed-batch or continuous culture [3]. It has been demonstrated in a large number of studies that fungal morphology has a strong effect on the productivity of mycelial processes and in many cases a certain morphological type has been associated with increased metabolite production [3]. From the large number of factors that affect morphology and their interrelationships, one can easily understand that control of fungal morphology in order to optimize production is a difficult task. It remains a very difficult task to extract quantitative relationships between process variables, metabolite production and fungal morphology in a fermentation process and therefore even more difficult to deduce general relationships between them. The role of many factors affecting fungal morphology is still not fully understood. However, and despite the difficulties, developing a thorough understanding of the role of process variables in morphological development and its subsequent impact on process productivities remains always a key target in the optimization of fungal fermentation processes.

To evaluate the importance of fungal morphology in a process, quantitative work is prerequisite. Great progress has been made in this area since the 1970s when investigations relied upon manual measurements performed under a microscope or on photographs were reported. Progress was made with the use of simple digitizing tables in the works of Metz et al. [6] and van Suijdam and Metz [7]. Progressively, with the availability of personal computers and digital video cameras, image analysis systems were developed. These were characterized by a low degree of automation [8] but soon highly automated systems were developed [9] with some degree of manual operation always needed for the production of accurate data. The later years have seen a substantial development of tools and techniques to characterize fungal morphology and extract quantitative information on morphological characteristics. Digital image analysis systems run with powerful software packages that permit fully automated work and statistical evaluation of the measured data [10,11]. Recently, confocal

laser scanning microscopy (CLSM) and fluorescence microscopy have been employed successfully in fungal morphology studies offering precise structural analysis of the mycelium and spores [12-18].

This review highlights the progress made in the area of characterization of fungal morphology using image analysis techniques since the 1990s and presents selected relevant examples of applications.

Digital Image Analysis Systems

A typical image analysis system consists of the hardware and software components. The hardware components include the image source, which in the case of fungal morphology studies is a microscope, the image capture device, which is a monochrome or color video camera, a central processing unit (CCP), and the display monitor. Since the early 1990s when the first reports on digital image analysis of fungal morphology appeared, the progress in the area of relevant hardware has been enormous and will not be discussed in detail here. Once the image has been "frozen" and transferred to the computer, the relevant software is required to extract the data from it. Today, dedicated to image analysis software packages are available, ranging widely in abilities and costs. Their common characteristic in most cases is that they have been designed for application developers rather that for final users and the image analysis routines come as a library supported by a common programming language. The required sequence of operations can be encoded in the program to permit automatic repeatable analysis and measurements. The basic stages of the image analysis procedures are shown in Figure 1. Having completed the measurements and calculations, modern software packages offer the option the obtained data to be classified and further analyzed to give the final results in tabular or graphical form, and to be statistically treated making the need for use of statistical software mostly unnecessary.

Digital image analysis is the state of the art method to characterize and quantify fungal morphology in the differentiation process from spores to filamentous structures to pellets. Hyphal growth, septation and branching are commonly quantified in filamentous growth forms, while the number of features can be high enough in pelleted

Figure 1: The basic steps of image analysis.

morphologies to include, apart from the size-relevant, parameters that give a measure of shape such as circularity, compactness, roughness and convexity.

Characterization of Fungal Macro-Morphology

Filamentous structures

The first significant advance in quantification of filamentous structures through image analysis was the work of Adams and Thomas [8] who described a semi-automatic system to perform measurements on filaments. The method was more accurate and faster than the digitizing tables used by Metz et al. [6] and van Suijdam and Metz [7] in the early 1980s although considerable manual intervention was still required, e.g. binary images were manually edited. In 1990, Packer and Thomas [19] presented a software application for use with a general purpose image analyzer that enabled fully automated analysis. The program required the manual setting of several application-specific parameters, e.g. grey- level and circularity thresholds, the specification of a "measuring frame" and a maximum length threshold. Hyphal elements were described in terms of the main hyphal length, number of tips, hyphal growth unit and other parameters, while for the first time the aggregated biomass -defined as "clumps"- was characterized and quantified. The method was tested on *Streptomyces clavuligerus* and *Penicillium chrysogenum* and compared to a manual image processing method (manual selection of mycelia and manual editing of binary images) proved to be only marginally faster due to the slow skeletonization algorithm – a problem that should have been overcome with the use of modern hardware.

Later, Tucker et al. [9] expanded the work of Packer and Thomas [19] by developing a method that could give important measurements on clumps. Clumps were identified by "ultimate skeletonization" which was successive removal of pixels until either a single point or a loop remained. Projected areas and perimeters of clumps were estimated through pixel counts and clumps were characterized in terms of their circularity and compactness. The method also permitted a detailed examination of mycelial structures beyond the classical hyphal growth unit. Having removed the small, "artificial" branches, free hyphal elements were subjected to a "shrink-back" algorithm that involved an iterative pruning of mycelia to identify branch-points and branches were classified as zero-order, first order etc. The method of Tucker et al. [9] was later used by Ammanulah et al. [20] to describe the dynamics of mycelial aggregation in batch and continuous cultures of *Aspergillus oryzae*.

The method of Tucker et al. [9] was further improved in the work of Paul and Thomas [21]. Mycelial trees, "simple" clumps and loose entanglements (clumps containing only 1-3 holes resulting from hyphal crossovers) were quantified in more detail and the morphology of *P. chrysogenum* in submerged culture was evaluated. A more detailed description of clumped morphology was proposed by Papagianni et al. [22] in fermentations of citric acid producing *A. niger*. Clumps were quantified in terms of their outer perimeter (P1), the perimeter of cores (P2) and the total length of filaments (l) that arose from the cores and their branches. The high degree of mycelial entanglement at the periphery of clumps made it impossible to distinguish between main filaments and branches and was suggested that l could serve as an indication of the degree of branching. The hyphal diameter (d) was also determined. The image analysis method described by Papagianni et al. [22] involved significant manual intervention (mainly on the definition of the area of measurements) but a large number of steps
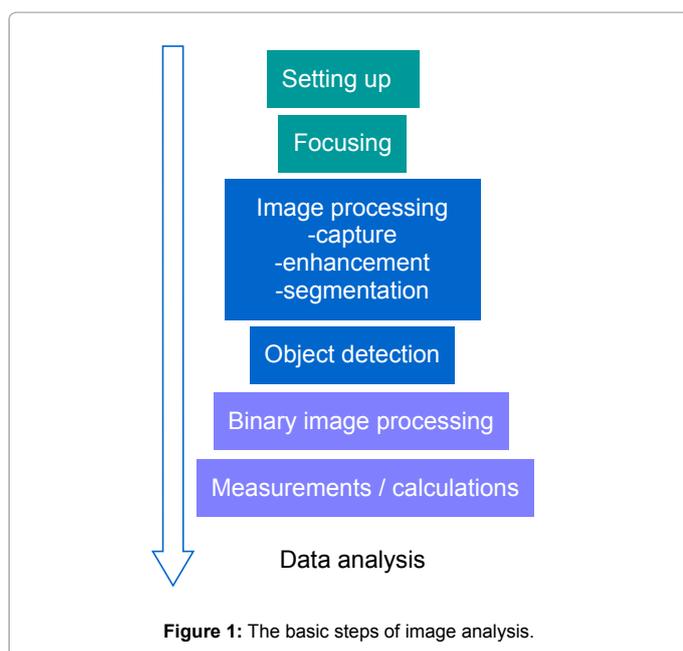
in image processing and measurements were performed automatically. The method was later employed by the same group [23] to quantify the relationship between citric acid production and *A. niger* morphology as functions of the mixing intensity in a stirred tank and a tubular loop bioreactor and a set of empirical relationships were presented for the first time.

Figures 2 and 3 are photographs of filamentous mycelium of citric acid producer *A. niger* grown in submerged culture. Figure 3 shows a typical clump.

Pelleted growth

Pelleted morphologies (Figure 4) are common in submerged fungal fermentations. A number of image analysis methods have been proposed for the morphological characterization of fungal pellets. Reichl et al. [24] described the pellets of *S. tendae* by mean sizes, shape, content and frequency distributions. Pellet classification by means of the shape factor was introduced for the first time in that work. Cox and Thomas [25] proposed an image analysis method to characterize pellets based on the presence of a central core while they further classified them into smooth and "hairy" types. Pellet characterization using image analysis was reported by Durant et al. [26] who also described pellet zones using dyes, while later they improved their method using color image analysis [27].
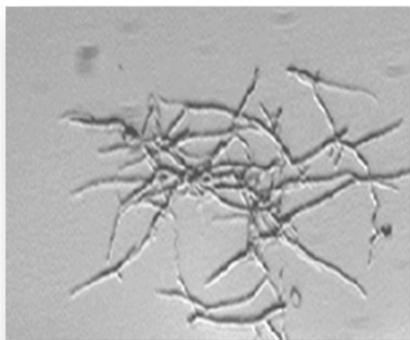
Figure 2: Filamentous growth (dispersed growth) of *A. niger* mycelium in submerged citric acid fermentation (x 200).
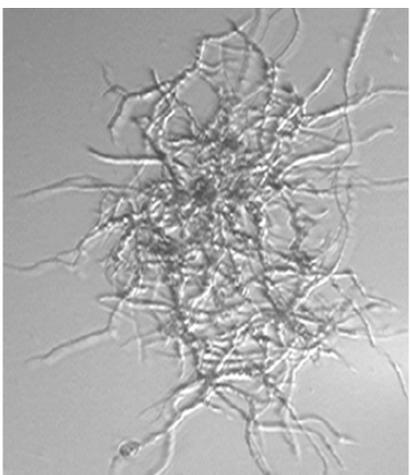
Figure 3: An *A. niger* clump in submerged citric acid fermentation (x 200).
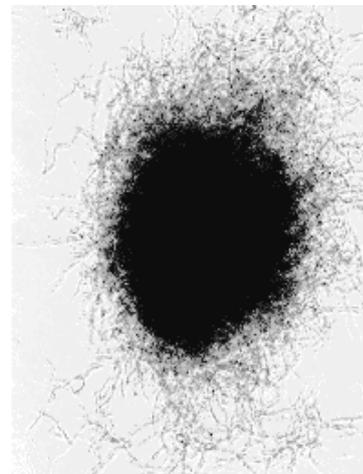
Figure 4: A typical fungal pellet by *Aspergillus ficuum* in submerged fermentation (x 100).

In the case of large pellets and pellets of the "hairy" type a combination of microscopic and macroscopic observations is often needed and different means of image capture are required. Paul and Thomas in their study with *A. niger* [21] suspended pellets in a cavity slide of 1 mm depth, suitable for mounting on the microscope's stage. For pellets of a larger that 0.6 mm diameter, a macro-viewer attached to a camera was required. A macro-viewer was used by Papagianni and Mattey [28] for the characterization of *A. niger* pellets in citric acid fermentation. O'Cleirigh et al. [29] in their work with *S. hygroscopicus* used a flatbed scanner to acquire images of safranin-stained pellets placed in Petri dishes in water suspension. The monochrome images were processed and binarized and the pellets were characterized in terms of their equivalent diameters, number and volume. Bizukojc and Ledakowicz [30] used a similar method with *A. terreus* pellets, although the Petri dishes were photographed rather than scanned. Rühl and Kües [31] placed the pellet suspension on a glass plate which was illuminated from below and acquired images which were further subjected to image analysis studies. It is obvious for the last studies that the set up work prior to image analysis was time consuming resulting in long overall image-processing times.

A number of studies have examined the filamentous region of pellets which vary in extent in various types of pellets e.g. smooth or "hairy" pellets. The filamentous fraction of pellets was separated from the core in the work of Paul and Thomas [21] at the step of the binary image processing. Similarly Park et al. [32], removed the pellet annular region in binary images by repeated opening cycles until only the pellet core remained. The filamentous area was calculated by subtracting the core from the total mycelial area. Also, Müller et al. [33] included in their work studies on the compactness of pellets of *A. oryzae* (estimated as the ratio of the projected area and the projected convex area of particles).

All morphological parameters mentioned above are presented in Table 1 along with their definitions [34].

Characterization of fungal morphology at the level of the fine structure of hyphal elements

The number of studies that focused on the fine structure of hyphal elements during fermentation remained very limited over

Morphological Parameters	Definition
Measures of size	
Area or projected area (A)	The area of projection of a 3-dimentional object into a 2-dimentional image. This is often represented by the sum of pixel values within the region specified by the selected object and given by: $$area = \sum_{(i,j) \in A} \sum fij.$$ A is the selected object, and (i,j) ∈ A, all pixel locations within A. The area is expressed as an actual area by multiplying by a calibration constant squared.
Distance	Distances are line lengths in general, with the simplest of all distance measurements being that between two specified pixels. There are several ways in which distances can be defined or approximated, as in the case of curved lines.
Perimeter (P)	Boundary lengths of objects. Since pixels lie on a rectilinear grid, it is necessary to include diagonal inter-pixel distances where appropriate.
Convex perimeter (Pc)	The length of the perimeter obtained by joining the outer points of an object, i.e. by filling in all the concavities in an object.
Length (L)	The length of a rectangular object having the same area and perimeter as the measured object. It is estimated from area and perimeter by $$L = \frac{P + \sqrt{P^2 - 16A}}{4}.$$ The maximum Feret diameter (Feret diameter = the diameter measured using a pair of calipers) is one definition of the Length of an object, which may alternatively be specified as $\max_{(i,j),(k,l) \in A} \sqrt{(i-k)^2 + (j-l)^2}$.
Width (W)	The width of a rectangular object having the same area and perimeter as the measured object. It is estimated from area and perimeter by $$W = \frac{P + \sqrt{P^2 - 16A}}{4}.$$ Alternatively it can be defined as the range of columns the object covers, i.e. $\max_{(i,j),(k,l) \in A}(j) - \min_{(i,j),(k,l) \in A}(j)$, or as the Feret diameter at angle $\theta = 0°$.
Equivalent circular diameter (D)	This is the diameter of a circle having the same area as the measured feature. It is estimated from area by $D = \sqrt{4A/\pi}$.
Height	This is the range of rows the object covers, i.e. $\max_{(i,j),(k,l) \in A}(j) - \min_{(i,j),(k,l) \in A}(j)$. It is the Feret diameter at angle $\theta = 90°$.
Measures of shape	
Circularity (C)	A shape factor that describes the deviation of an object in an image from a true circle. It is estimated from area and perimeter by $c = \frac{P^2}{4\pi A}$. The value of 1 corresponds to a circle, while larger values to shapes having a higher ratio of perimeter to area.
Compactness or Fullness (F)	Defined as the ratio of the area of an object to the area of a circle with the same perimeter. It is estimated by $compactness = 4\pi \frac{area}{(perimeter)^2}$. It is probably the most commonly used shape statistic and it is used to characterize the structure of mycelial clumps and pellets. A pellet without hairy regions has F ≅ 1 while a loose clump has F<1.
Roughness (R)	A measure of the irregularity of the perimeter of an object obtained from the circularity measurement around an object boundary.
Convexivity	Convexivity can be obtained by forming the ratio of the perimeter of an object's hull to the perimeter of the object itself.
Object count	
Number of objects	Number of objects per field of view and cumulative counts for a sample.
Hyphal tips	Number of tips per hypha, mean number of tips per hypha for a sample.

Table 1: The main morphological parameters of fungal morphology obtained through image analysis and their definitions according to Glasbey and Horgan [34].

the years. The structural complexity of hyphal elements of the filamentous growth form was evaluated for the first time by Paul et al. [35] using a fully automatic image analysis method. The method permitted the discrimination between cells filled with cytoplasmic material, vacuolated cells and degenerated, empty cells. Proportions of the classified objects were quantified. Their work was extended and presented in detail later with another couple of reports [4,36]. The methodology followed in these works involved a series of grey-scale and binary editing to extract vacuoles and present their distributions in volume and shape (circularity). The method was tested by monitoring the morphological parameters throughout fed-batch penicillin fermentation with *P. chrysogenum*. The extracted data was used for the construction of a powerful structured model for hyphal differentiation and penicillin production that was presented later by the same group [37].

In another work, Paul and Thomas [21] described an image analysis system designed to quantify vacuolation and active, growing regions of neutral red-stained *P. chrysogenum* mycelium. Through grey-scale and binary editing operations, valuoles and active hyphae were extracted from the image and measurements were followed. Their results showed

that the vacuolated volume of hyphae increases during the course of a fed-batch fermentation and valuoles become larger and less circular while hyphal width (diameter) increased rapidly up to about 30 hours from inoculation to decline rapidly thereafter. These image analysis techniques permitted the quantified characterization of simple cellular differentiation of filamentous fungi and provide a valuable tool in studies of the relationship between differentiation and metabolite production.

A semi-automatic method for the characterization and quantification of vacuolation in citric acid producer *A. niger* fed-batch fermentations was presented by Papagianni et al. [5]. Images from phase-contrast microscopy (x 400) were processed and vacuoles were segmented through grey-level thresholding. Size and circularity filters were used to discriminate between vacuoles and various vacuole-like artifacts, and vacuoles were quantified automatically in terms of perimeter, diameter, circularity and area, while their volume was estimated assuming the hyphae to be cylindrical. Finally the percentage of vacuolated volume of filaments was calculated. The method was tested throughout citric acid fed-batch fermentations and time profiles of morphological parameters such as mean perimeter of

clumps, length of filaments and vacuoles, along with kinetic parameters such as specific growth and production rates were used to establish a relationship between vacuolation, hyphal fragmentation and product formation under various agitation conditions and glucose levels. The results showed that vacuolation weakened the hyphae and low glucose levels created the conditions that favored fragmentation and made the mycelium susceptible to it when exposed to increased agitation conditions.

Identification of the metabolically active fraction of the mycelium by image analysis studies

In studies on the relationship between morphology and productivity, identification of the metabolically active fraction of the mycelium is often investigated. Fluorescence microscopy has proved a valuable tool for identifying active tips of filaments and active regions in pellets [38]. Fluorescent stains coupled with image analysis have been used in the works of Vanhoutte et al. [39], Agger et al. [40], Wongwicharn et al. [41], Hamanaka et al. [42] and Amanullah et al. [13]. Vanhoutte et al. [39] using a differential staining procedure and color image analysis in studies on the growth and differentiation of *P. chrysogenum* showed and quantified six physiological states: growing material (zone 1), three differentiated states characterized by increased granulation (zones 2,3,4), a highly vacuolated state (zone 5) and dead segments empty of cytoplasm (zone 6). Ager et al. [40] used a double staining procedure (calcofluor and DiOC6), fluorescence microscopy and automatic image analysis in studies on the morphology of *A. oryzae*. Organelles and cell walls were stained and the fraction of active cells within a hypha was determined using separate filter blocks to view the fluorescence produced from each stain. A morphologically structured model was constructed which was confirmed when applied to fed-batch and chemostat experiments. Amanullah et al. [13] used calcofluor white stain to distinguish between active and non-active tips in *A. oryzae* mycelium. Active tips appeared bright while inactive tips did not fluoresce. Their methodology involved a significant amount of manual work as the stained-active- tips had to be "cut" from the image. Calculations were mainly based on the grey-level threshold which was defined by the user and involved an amount of error since the point at which fluorescence was deemed may have varied. Also, tips within mycelia clumps could not be observed and therefore were not considered.

Fluorescent staining coupled with image analysis was also used to identify the active regions in pellets. Hamanaka et al. [42] studied the intracellular product distribution inside the pellets of arachidonic acid producer *M. alpina*. In another study, the same group used fluorescence microscopy and image analysis to localize lipid formation at the edge of *M. alpina* pellets [42]. Microtomed sections of pellets labeled with fluorescein isothiocyanate (FITC) and pellets stained with Nile red were subjected to image analysis using fluorescent microscopy and a cavity ratio was estimated on the average FITC intensity across the section diameter. FICT staining was found to be low at the center of pellets and particularly at the later stages of fermentation, indicating a hollow core (autolyzed mycelium) the size of which correlated with total pellet volume. Nile red was also restricted to the periphery of pellets giving the evidence that intracellular lipids were not present in the core of pellets. A similar methodology was followed by Bizukojc and Ledakowicz [30] to determine the active region in pellets of *A. terreus*. Microtome sections of methyl blue stained pellets were examined with fluorescent microscopy and their active peripheral region appeared reddish-violet while the interior greyish-white. The two regions were segmented and

the volume of active biomass in each pellet was estimated based on the radius of the whole pellet and the radius of the inactive region.

Another work in which fluorescence microscopy was used along with image analysis is that by El-Enshasy et al. [14] who estimated the active fraction of biomass in pellets of *A. niger*. Acridine orange dye, indicative of active protein synthesis, was used to stain heat-fixed on microscope slides samples. The unproductive core of pellets appeared fluoresced green while the active periphery exhibited a strong red-orange color. Images were analyzed manually in that work by drawing of diameters and estimating the depth of the active regions. Finally, the volume of productive mycelium was estimated in a similar way to that of Bizukojc and Ledakowicz [30].

Conclusions

Since the introduction of digital image analysis techniques, the amount of research carried out on the morphology of filamentous fungi has been impressive and therefore, the potential of image analysis has been well documented through these studies. The large efforts in the characterization of fungal morphology made evident the relationship between process parameters, fungal morphology and process productivities in a number of industrially important fermentations and facilitated the way for a further step in fungal biotechnology aiming at targeted engineering of fungal morphology. Quantitative information on mycelial differentiation has been used in the construction of structured models with predictive value and this understanding can be used in process optimization and design. The development of novel techniques in the area of morphological studies coupled with advances.

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