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REVIEW ARTICLE

Image analysis of the morphology of filamentous micro-organisms

P. W. Cox, G. C. Paul and C. R. Thomas

Author for correspondence: C. R. Thomas. Tel: +44 121 414 5355. Fax: +44 121 414 5324.
e-mail: C.R.Thomas@bham.ac.uk

Centre for Bioprocess Engineering, School of Chemical Engineering, The University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

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Overview

Image analysis is a powerful tool for characterizing the gross and internal morphology of filamentous micro-organisms. Methods are available for characterizing both dispersed and pelleted growth forms, and for identifying apices, vacuoles and degenerated regions of hyphae. The technique has also been applied to the morphological characterization of dimorphic yeasts. Such methods can be used to study the complex interactions between morphology, productivity and process conditions in submerged fermentations of filamentous micro-organisms, including the effects of changes in inocula, and to study the growth of such organisms on solid surfaces. Amongst other applications, image analysis has been used to relate fermentation broth rheology to morphology, to study mycelial fragmentation by impellers, and to investigate structural and biochemical differentiation of hyphae both on solid surfaces and in submerged fermentations. This review describes these image analysis methods and their actual and potential applications in the study of filamentous micro-organisms.

Filamentous micro-organisms

Filamentous micro-organisms such as many fungi and actinomycetes present a diverse and complicated range of gross and internal morphologies. In the industrial context, fermentation productivity depends strongly on morphology, although not always directly. Typically, filamentous micro-organisms consist of hyphae which are long relative to their width, are often branched, and form extended structures called mycelia (Fig. 1). A critical aspect of the development of mycelia is that hyphae show strongly polarized growth. This leads to structural and biochemical differentiation between apical and distal regions of the hyphae. This has been discussed fully by Gow (1994).

Because of the complexity of fungal and actinomycete morphology, conventional microscopy and analysis only lead to qualitative data. This problem can be overcome, and quantification achieved, using image analysis.

Image analysis

Image analysis is a powerful technology that allows the assessment and measurement of structure from examination of electronic images. Its importance in research on filamentous micro-organisms is in its capability to characterize size and shape quantitatively (Thomas, 1992; Paul & Thomas, 1998). Images are generally presented as square arrays of pixels (typically 512×512) each of which has a particular 'greyness' or brightness value, usually one of 256 possibilities between black and white. Therefore, objects within an image, such as mycelia, might be distinguishable from each other and the background on the basis of spatial separation and/or greyness. 'Image processing' transforms the original image by mathematical algorithms to enhance desired detail and to give improved contrast between the object of interest and the background or other objects. With sufficient contrast, objects of interest may then be 'detected', i.e. identified by their greyness value, leading to a masking binary image, in which each pixel is either 'on' or 'off'. This data reduction step is usually desirable to quicken subsequent processing, although sometimes the consequential loss of information is not tolerable, and the grey image must remain in use. If a binary mask is created, it may then be modified further by binary image processing operations to give a final image suitable for measurement, i.e. for the actual image analysis. These measurements might be made upon individual objects, e.g. mycelia, or on all of the objects in the image at one time, say to give the proportion of material of a certain type.

Some applications of image analysis to the characterization of filamentous micro-organisms are described below. Very detailed descriptions of the methodologies can be found in Paul & Thomas (1998).

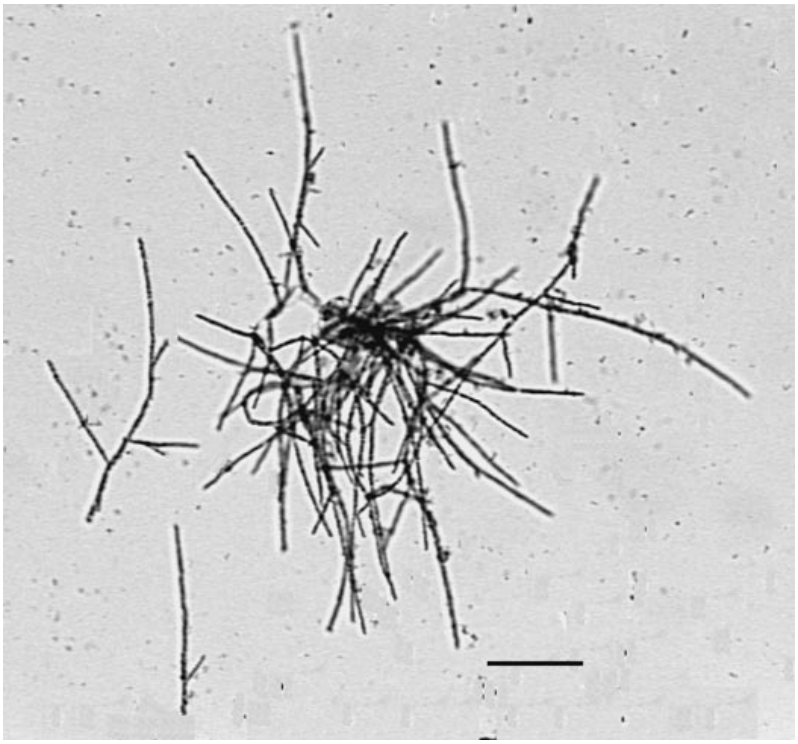

Fig. 1. Dispersed mycelia of *P. chrysogenum* taken from a laboratory-scale agitated fermenter. Bar, 90 μm.

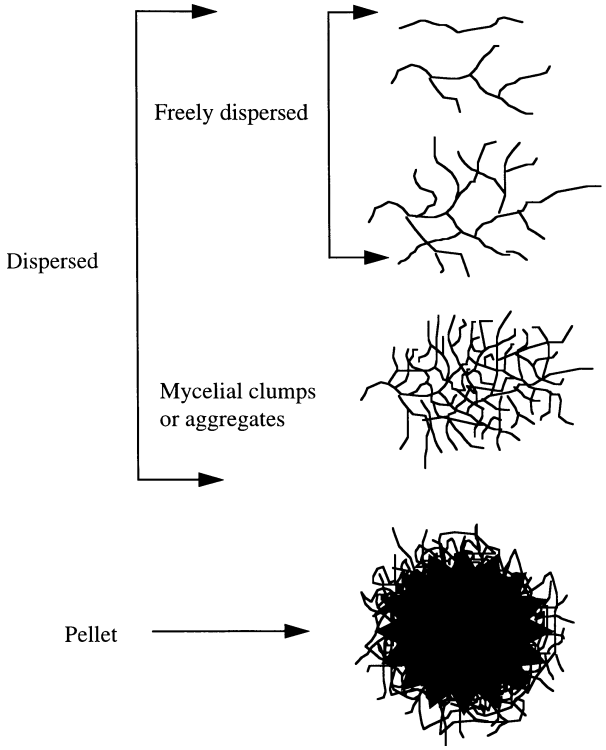

Fig. 2. Forms of (gross) morphology found in typical submerged cultures of filamentous fungi and actinomycetes.

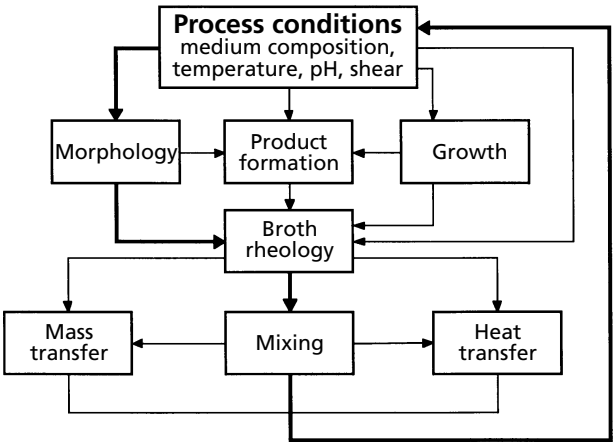

Fig. 3. Complex interactions between morphology, productivity and process conditions in submerged fermentations of filamentous micro-organisms (after Metz, 1976).

Gross morphology

Many industrial fermentations involving submerged cultures of fungi and actinomycetes have been examined extensively, with the intention of relating gross morphology and productivity to process conditions (e.g. Clarke, 1962; Smith & Calam, 1980; Peberdy, 1994). The morphologies encountered range from freely dispersed hyphae to pellets (highly entangled masses of hyphae, possibly as large as a centimetre across). The dispersed forms divide into freely dispersed and aggregated or 'clumped' forms, as shown in Fig. 2. Each of the morphological forms described in Fig. 2 is discussed

in depth below. Fig. 3 shows the complex interactions between the morphology, the productivity and the process conditions in fermentations of filamentous micro-organisms. Clearly, morphology has a central role, and considerable efforts have therefore been expended in the development of rapid and quantitative methods of morphological characterization, such as image analysis. This latter method has provided a powerful advance over manual methods (such as that of Van Suijdam & Metz, 1981), being more rapid and consistent and having potential for automation.

Dispersed morphology

Fig. 1 shows dispersed mycelia of *Penicillium chrysogenum*. This dispersed morphology is typical of many filamentous micro-organisms, whether growing in submerged culture or on solid surfaces. Several workers have developed image analysis methods to study mycelia of this form. In early work, Reichl *et al*. (1990) used image analysis to examine *Streptomyces tendae* growing upon cellophane on top of nutritionally complete solid media. Although effective in measuring growth, the method was restricted to examination of small numbers of hyphae. Septal positions were identified by staining of the cellular compartments with the fluorochrome acridine orange. This gave a well-resolved picture of the arrangement of compartments within the mycelial structure as the hyphae extended over the growth surface. However, the method was not intended for use upon filamentous micro-organisms growing in submerged cultures. Yang *et al*. (1992) used a similar method to examine the extension of hyphae grown out from single spores within growth chambers. The growth rate of the tips and the angles of branch formation were measured until the mycelia became 'too complex' to examine. Meanwhile, Adams & Thomas (1988) had developed a semi-automatic method of determining important morphological parameters for samples from submerged fermentations. For the freely dispersed forms only (see Fig. 2), these included the total length of hyphae in each mycelium, the main hyphal length, and the number of tips. In addition to calculating the mean values of these parameters, the hyphal growth unit, the importance of which was first suggested by Trinci (1974), could be estimated easily. It was clear that the morphology in a typical sample was very variable, and measurements on many fields of view were necessary to obtain good precision of the means. Even with many measurements, estimations of hyphal width have not been found to be reliable with the method of Adams & Thomas (1988) or with the subsequent methods of Packer & Thomas (1990) and Tucker *et al*. (1992). There remains the fundamental difficulty that the magnifications required to measure accurately hyphal width are too large if (at the same time) typical mycelia are to be small with respect to the image dimensions. This is essential if excessive truncation at the edges of the images, leading to serious inaccuracies in all of the measurements, is to be avoided. As the hyphal width is not readily available, the hyphal growth volume cannot be estimated without working at two magnifications.

Belmar-Beiny & Thomas (1991) used the method of Adams & Thomas (1988) to study agitation effects upon *Streptomyces clavuligerus*, showing morphological dependence upon stirring intensity. Packer & Thomas (1990) and Tucker *et al*. (1992) automated the procedure of Adams & Thomas (1988) by use of a scanning microscope stage and automatic image capture from a computer-controlled microscope. With automatic scanning, it was discovered that clumps (Fig. 2) were the predominant form within the fermenter, with this form typically in excess of 90% of the biomass for batch *P. chrysogenum* fermentations (Packer & Thomas, 1990). These clumps, which exist in significant amounts in nearly all submerged fermentations of fungi and streptomycetes, were not artefacts caused, for example, by mycelia overlapping on the slide (Tucker *et al*., 1992; Tucker & Thomas, 1993). Clumps therefore form a fundamental morphological unit of the biomass, certainly with respect to broth rheology and possibly also to micro-organism physiology, and disaggregation of these clumps would require hyphal breakage to disrupt their structure. Recently, Yang *et al*. (1996) also considered clump morphology and its characterization by image analysis. In considering clumps for the first time, Packer & Thomas (1990) demonstrated that automatic image analysis was not only faster and more convenient than earlier methods, but also might provide new and potentially valuable morphological measurements. One possible measurement on a clump is its 'projected area', which is the area of the three-dimensional clump as observed in its two-dimensional image captured by the camera. As dispersed mycelia settle into a relatively thin (nearly two-dimensional) layer on a slide (see Fig. 1), the projected area is a reasonable measure of clump size (Tucker *et al*., 1992). Tucker *et al*. (1992) also measured two other parameters to describe clumps. The 'fullness' measurement gave an estimate of the packing density of the material within the clump, whilst 'roughness' was a shape parameter. On a typical sample, all of these measurements can be completed within 2 h, whilst area measurements alone take substantially less time (about 16 min for 100 mycelia). For fermentation technologists the preponderance of clumps is important, as it has been shown that broth rheology may correlate better with clump morphological parameters than with the morphology of the freely dispersed form (Tucker & Thomas, 1993; Olsvik *et al*., 1993). Very recently, it has been shown that the best correlations for a wide range of *P. chrysogenum* fermentations are with the projected area (G. Riley, unpublished data); fullness and roughness measurements may not be worth the extra time taken in their measurement.

Further refinements to the classification of Tucker *et al*. (1992) have been suggested. Tucker *et al*. (1992) considered that any mycelium with overlapping hyphae should be classified as a clump, but this does not allow for freely dispersed material in which hyphae within a mycelium have overlapped. These 'entanglements' (see

Fig. 2), so-named by Pons *et al*. (1997), can be characterized with the other freely dispersed forms, except the main hypha cannot be identified and its length measured (Paul & Thomas, 1998). The hyphal growth unit can still be estimated.

Although it is true that most submerged fermentations of filamentous micro-organisms contain clumps, some research does continue considering only the freely dispersed forms. There may indeed be some fermentations in which this form predominates, especially immediately after inoculation, and under conditions of severe nutrient limitation, which seems to promote fragmentation (Paul *et al*., 1994a). It is just possible that in some circumstances the mycelial morphology within clumps might be inferred from the more easily characterized freely dispersed form, although this has yet to be demonstrated. Whether this is the case or not, it can be concluded that the existence of clumps should be of concern in any studies involving morphology, and image analysis is needed to make the necessary measurements.

It is not always necessary to use very powerful systems to obtain useful results from image analysis. Wiebe & Trinci (1991), for example, related hyphal morphology to fermentation conditions using a relatively simple system. Micrographic images of *Fusarium graminearum* were presented to a computer monitor via a digital camera. Measurements were achieved by drawing directly over hyphae in the image. Lengths could be determined using a pre-set calibration factor. Although this method did not exploit the full potential of image analysis, being essentially manual, measurements of main hyphal length (longest connected path), total length and hyphal diameter and derived values such as hyphal growth unit could be generated. A similar method using drawn lines on a monitor was used by Merson-Davies & Odds (1992) to study the morphology of *Candida albicans*. Martin & Bushell (1996) used a simple but elegant method to relate antibiotic production to sites of synthesis at known distances from the extending tip in *Saccharopolyspora erythraea*. Photographic images from slides were analysed by inscribing (automatically) a circle around each mycelium. This gave a measure of mycelial diameter, which could then be related to antibiotic production, apparently dependent on hyphal length. These simple systems, whilst giving useful results, are not convenient as automatic methods, and can require subjective decisions from operators. For these reasons automation is preferable.

With good image analysis methods, some of the complex interactions shown in Fig. 3 can be investigated. For example, Jüsten *et al*. (1996) used image analysis to demonstrate that hyphal fragmentation in an agitated fermenter might be correlated with the 'energy dissipation/circulation function' across several scales of operation and with many different impeller types. This function combines the energy dissipation rate local to the impeller with a frequency of passage of the mycelia through this region of high agitation intensity as they circulate around the fermenter. Measurements of distributions of projected area indicated that the size reduction of the clumps was primarily due to erosion rather than clump rupture. An important corollary of this observation was that the class of freely dispersed forms in an agitated fermenter is likely to contain fragments from clumps. This casts yet more doubt on the value of morphological measurements that do not include clump characterization. The success of studies such as that of Jüsten *et al*. (1996) is dependent on an ability to measure mycelial size distributions, for which image analysis is the ideal tool.

It should be noted that morphological analysis on submerged fermentations containing large amounts of solids such as bran is very difficult, even with image analysis. The method of Tucker *et al*. (1992) can handle more discrete solids (e.g. in Pharmamedia) but the hyphae are difficult to distinguish from bran-like materials. It is possible that fluorescent staining of the mycelia might help, but only limited attempts have been made to achieve this. However, the use of calcofluor white gives a detectable difference in staining between fungal cell walls and surrounding cellulosic bran (P. W. Cox, unpublished results).

What might be described as clumped morphologies, but in a different guise, are the mycelial forms found when filamentous micro-organisms grow on solid surfaces. These are also amenable to image analysis. In this context, the use of fractal measurements has aroused interest because of the likely link between colony fractal dimension and micro-organism foraging strategy (Donnelly *et al*., 1995). Jones *et al*. (1995) used image analysis to relate enzyme production from hyphal tips of *Pycnoporus cinnabarinus* to the fractal dimension of colonies of this organism. Donnelly *et al*. (1995) used fractal analysis to identify species differences and their growth parameters, whilst Olsson (1994) demonstrated the uptake of nutrients by fungal colonies growing on agar plates. Following removal of the mature colony, chemical 'development' of the plate highlighted nutrient concentrations which were quantified by grey scale measurements using an image analyser.

Fungal growth on substrates other than the ubiquitous agar has also been quantified using image analysis. Green *et al*. (1994) found image analysis to be useful when observing mycorrhizal extension towards baits, because of its accuracy, reproducibility and ease of use. Schönholzer & Zeyer (1995) and Li *et al*. (1997) quantified fungal structures on phylloplanes with image analysis, whilst Donnelly & Boddy (1997) observed and characterized growth and measured colony fractal dimensions of basidiomycetes extending upon soil. These novel uses of image analysis will provide new insights into surface growth of filamentous micro-organisms and their foraging strategies.

Pelleted morphology

Pellets are highly entangled, dense masses of hyphae which can range in size from several hundred micrometres in diameter to several millimetres. The general structure of fungal pellets is a core of such densely

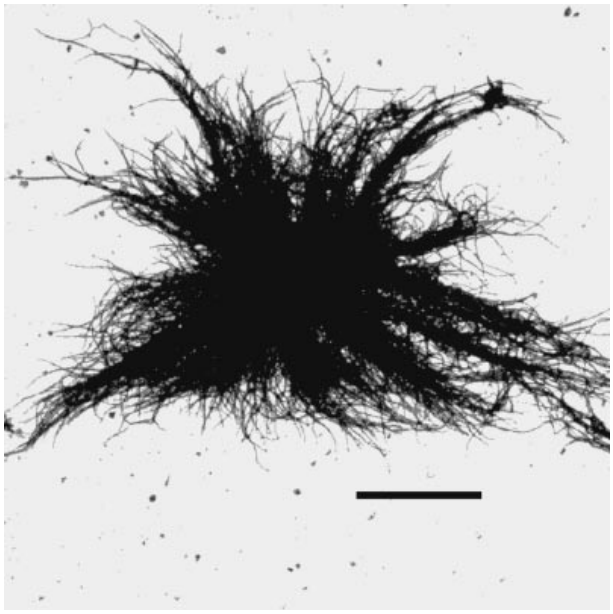

Fig. 4. Typical pellet of *A. niger.* Bar, 1·1 mm.

packed hyphae, surrounded to a greater or lesser extent by a more dispersed annular or 'hairy' region, containing radially growing hyphae. Fig. 4 shows a typical example. It is the presence of a discernible core of greater packing density which might be used to discriminate pellets from clumps, although in reality there is a continuum of behaviour from one form to the other. It should also be noted that diffusional limitations of oxygen or other nutrients through the entangled hyphae may restrict growth at the centre of large pellets, and may lead to autolysis of the core to produce a hollow pellet (Clarke, 1962). Analysis of such objects is complicated by their three-dimensional structure, which is a consideration in morphological studies. It may be necessary to use a chamber on the microscope stage to preserve the pellet shape. In using image analysis to size pellets, it is usually assumed that they are nearly spherical, so that useful information can be obtained from the observable (two-dimensional) projected image.

In an early study, Reichl *et al*. (1992) used image analysis to investigate the shapes and sizes of *S. tendae* pellets at a low magnification at which individual hyphae were not distinct. The chosen morphological parameters were percentage occurrence of pellets by number, the projected area of all morphological forms, the percentage of pellet area, and pellet shape (circularity). This method worked on images of pellets held in a chamber mounted on a microscope, a procedure which maintained the pellet structure. Meanwhile, Cox & Thomas (1992) worked on samples taken from a pelleted *Aspergillus niger* fermentation and applied an automatic image analysis method to separate cores from peripheral hyphae, on a morphological basis. This basis was to assume that no slide background would be visible through a core, and to remove (in the image) the hairy region from each pellet in gradual steps until a solid core was obtained. The size (equivalent diameter) and shape (circularity) of the cores could be measured. In addition, the fullness and size of the hairy region of the pellet could be quantified, in a way similar to clump characterization for the dispersed form. Nielsen *et al*. (1995) developed yet another semi-automatic image analysis method to study pellets, and demonstrated a relationship between the balance of pelleted and freely dispersed forms and penicillin production from *P. chrysogenum*. In a different approach using a type of diffusional criterion to identify pellet cores, Durant *et al*. (1994a, b) identified cores as the parts of pellets remaining stained with crystal violet after decolourization of the annular region by rapid flushing away of stain with ethanol. The cores and the annular regions could be separated by their different colours using image analysis. More recently, it has been shown that the rate of diffusion of protons into the core of alkali-saturated pellets can be tracked by following the decolourization of phenolphthalein (Thomas & Paul, 1996) (Fig. 5). Modelling of the diffusion process may allow assessment of pellet internal structure without the need for sectioning.

It has been mentioned that Nielsen *et al*. (1995) developed a method to characterize all of the forms they observed in *P. chrysogenum* fermentations. It should be noted that Jüsten *et al*. (1996) used projected area to describe all of the dispersed forms in their agitation trials. Clearly, one method for all forms would be valuable. Pichon *et al*. (1993) claimed to have developed such a system, able to identify and measure dispersed forms and clumps, and to measure the sizes of pellets and their degree of hairiness. Unfortunately, to visualize the dispersed forms clearly, the pellets had to be compressed between a slide and coverslip. The resulting distortion of the pellets means that it might be better to analyse dispersed and pelleted forms separately.

Dimorphic yeasts

The shape of yeast cells in their more usual unicellular form has been studied by image analysis. Pons *et al*. (1993) devised a semi-automatic method for the characterization of the size and shape of yeast cells, whilst Suhr *et al*. (1995) have suggested direct control of yeast fermentations using on-line image analysis to provide process information. Dimorphic strains may be induced to produce pseudohyphae from the original yeast form, and the differences in productive capabilities of these differentiated hyphae have aroused considerable interest (O'Shea & Walsh, 1996). However, image analysis studies of dimorphism and its relation to productivity are still lacking. Kron *et al*. (1994) have used time-lapse video microscopy for the examination of cell division in pseudohyphae, but it was O'Shea & Walsh (1996) who developed a semi-automatic method for characterizing cells into six morphological forms, ranging from branched mycelial forms to ovoid yeast unicells. They used this method to identify the size and shape of the various forms and then to quantify the proportion of

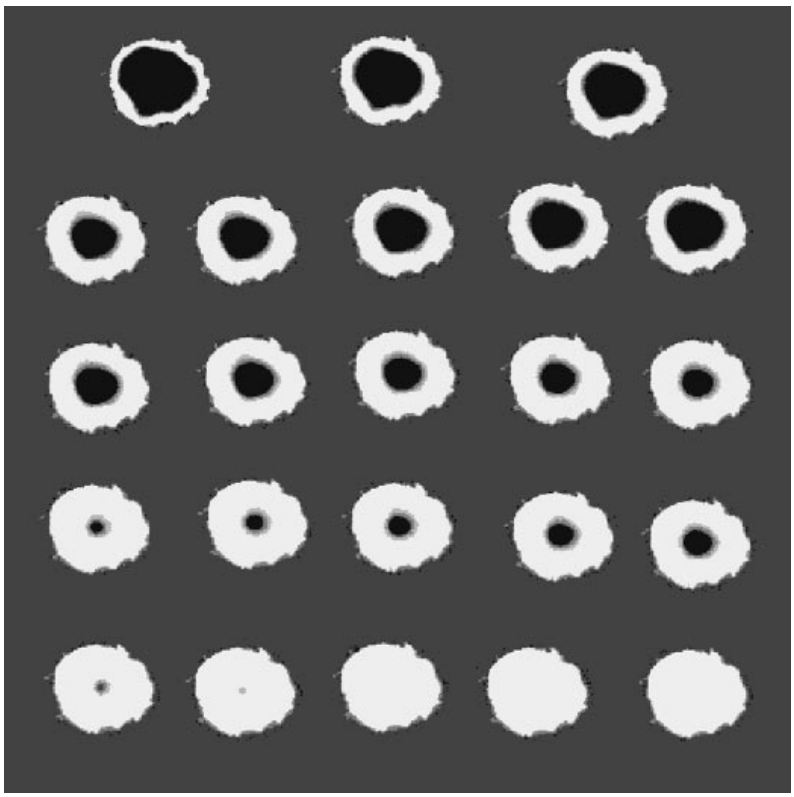

Fig. 5. Composite image showing decolourization of a phenolphthalein-stained *A. niger* pellet by proton diffusion into its structure. Pellet diameter 4 mm; time between images 2·2 s. Figure reproduced from Thomas & Paul (1996) with permission.

each type. As presented, the method was semi-automatic, but it appears that it would lend itself to full automation. This would increase its value in studies of dimorphism in yeasts. It might also be useful to characterize dimorphism using the 'morphological index' of Merson-Davies & Odds (1992). Although Merson-Davies & Odds (1992) used very simple image analysis techniques, the concept of defining with a single number a cell shape in the continuous range of morphologies from spherical cells to true hyphae has clear potential. This might be fulfilled by more automation of the method.

Spores

As the morphology and physiology of filamentous micro-organisms in submerged cultures are dependent on the state of the inoculum (Smith & Calam, 1980; Tucker & Thomas, 1994), studies on the germination of spores in seed cultures are of great importance. Paul *et al*. (1993) developed a powerful method for this purpose and used it to study the germination of *P. chrysogenum* spores. In contrast to traditional methods for assessing the ability of spore preparations to germinate, i.e. counting colonies on spore-inoculated agar plates, the image analysis method examined samples taken directly from a test inoculation fermentation. The dynamics of swelling and germ tube formation could be assessed, in terms of spore size, percentage germination (Fig. 6) being defined as the production of a germ tube of at least half the spore radius in length, following Trinci (1971), and also the number of germ tubes per spore. Distributions as well as means were given for these parameters. The analysis rate was rapid (under 30 min per sample of about 400 spores). Besides the speed, this image analysis method had the advantage over the traditional method of characterizing germination behaviour with the spores held under the intended inoculation conditions. Using their method, Paul *et al*. (1993) showed that the quality of *P. chrysogenum* spore preparations had a large influence on the germination

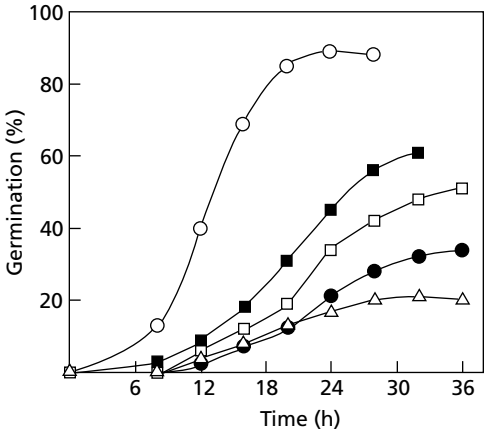

Fig. 6. Percentage germination of *P. chrysogenum* spores with time in different inoculum medium and two ages of spores. S1, fresh spores; S2, old spores; M1 and M2, defined media; M4, complex medium (Paul *et al*., 1993). △, S1M1; □, S1M2; ○, S1M4; ●, S2M2; ■, S2M4.

behaviour (Fig. 6). There were also differences between a defined and a complex medium in both swelling and germ tube formation. It appeared that there was a critical spore size beneath which spores would not produce a germ tube. For the complex medium, most spores produced a germ tube at this critical size (and possibly others later). In the defined medium, however, spores could continue to swell to sizes significantly above the critical size. This suggested that there were separate triggers for swelling and germ tube formation, but so little is known about fungal spore germination it was difficult to draw any strong conclusion. An attempt to model (mathematically) the swelling and germ tube formation failed (G. C. Paul, unpublished work), mainly because this lack of knowledge did not allow a rational choice of model structure. Nevertheless, Nielsen & Krabben (1995) used the data of Paul *et al*. (1993) in a broader model of the penicillin fermentation. In any case, it is clear that image analysis would be a useful tool for investigating fungal spore germination and for improving our understanding of this important aspect of fungal growth.

Tucker & Thomas (1994) showed that the concentration of spores in an inoculum (and presumably their quality) can markedly influence subsequent morphological development, as characterized by image analysis. Using *P. chrysogenum* spore concentrations spanning several orders of magnitude, morphologies ranging from freely dispersed hyphae and clumps to pelleted forms could be obtained. It is quite possible that much of the variability of fungal and actinomycete fermentations arises from the inoculum stage, especially when spores are in use. In this context, the image analysis method of Paul *et al*. (1993) could also be a useful quality control tool.

Oh *et al*. (1996) applied yet another image analysis technique to quantify the effects of antifungal treatments upon spore germination. Samples of test species were immobilized onto microwell plates and allowed to germinate under varying concentrations of the test compounds. Automatic scanning and visualization via a computer-controlled microscope enabled the test samples to be continually re-examined and germination quantified over time. The method was used successfully with a variety of species with disparate spore morphologies, some of which might cause difficulties with the more direct method of Paul *et al*. (1993).

Differentiation

Particularly within fungal fermentations producing secondary metabolites, the production is a consequence of differentiation, both as structural changes, e.g. vacuolation, or as biochemical specialization. Vanhoutte *et al*. (1995) described a staining technique which would subdivide the hyphal structure into six physiologically distinct regions which could be identified by image analysis. The position and relative size of each of the identified regions could be determined, and for *P. chrysogenum* it was suggested that one of these regions (located subapically) was the primary location of penicillin biosynthesis. Unfortunately, the analysis was slow and involved, and was very sensitive to the colour temperature of the microscope lamp illuminating the specimen (and that of any incident light). The method was not robust and did not lend itself to routine fermentation monitoring. Nevertheless, the basic concept was excellent, and alternative ways to achieve similar ends are discussed below.

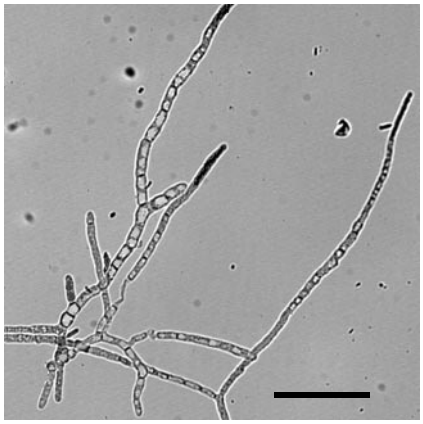

Fig. 7. Mycelium of *P. chrysogenum* showing vacuoles, degenerated regions, and some tips stained with neutral red. From a typical fed-batch fermentation, sampled during the production phase. Bar, 50 μm.

The method of Paul *et al*. (1992, 1994b) and Paul & Thomas (1996) appears to be very suitable for assessment of vacuolation and simple differentiation in fungal hyphae. At magnifications higher than those required for characterizing gross morphology, both vacuoles and degenerated regions of *P. chrysogenum* hyphae could be identified. Degeneration was considered by Paul *et al*. (1994b) to have occurred when vacuolation had left empty compartments. Autolysis might also occur giving further degradation (McNeil *et al*., 1998), as described later. Using the method of Paul *et al*. (1994b), actively growing tips were also identified by neutral red staining (Turian, 1976), which distinguished active tips from non-active ones by the accumulation of a dark stain (Fig. 7). Following the ideas of Megee *et al*. (1970) and Nestaas & Wang (1983), the metabolism of these active tips was considered to be so involved with tip extension that these were not regions of penicillin biosynthesis. Furthermore, although vacuoles may have some role in penicillin biosynthesis (Lendenfeld *et al*., 1993) and in other aspects of metabolism, it is unlikely that the penicillin is produced within vacuoles or degenerated regions. Using the method of Paul *et al*. (1994a), the remaining 'non-growing cytoplasm' could be identified and its proportion by volume of the total biomass could be estimated. It was assumed that this type of material was important in penicillin synthesis, which is supported by the studies of Lendenfeld *et al*. (1993) and Müller *et al*. (1992). Data from the analysis were obtained within 30 min of sampling, which was

relatively rapid when compared with the typically long time courses of industrial penicillin fermentations (in excess of 200 h). Recently, Paul & Thomas (1996) published a mathematical model for the production of penicillin based upon differentiation measurements made by this image analysis method. For a limited range of fermentation conditions, the model had excellent predictive power. Further refinements are in hand, but the basic concept of basing a structured model of the penicillin fermentation on image analysis of simple differentiation must be more satisfactory than treating the biomass as if it were homogeneous.

Unfortunately, some strains of fungi appear not to respond so readily to neutral red staining. As an alternative, calcofluor white may be used to identify active tips. Gull & Trinci (1974) proposed that extending tips of *Botrytis cinerea* 'glowed' appreciably more than non-extending tips when stained with calcofluor white, which demonstrates an affinity for 1–4-β-chitin linkages. Using image analysis it is possible to distinguish the bright fluorescence of an extending tip from the remainder of the hyphae (A. Amanullah & P. W. Cox, unpublished data). As well as providing a basis for determining the numbers of extending/non-extending tips, measurement of tip size and shape might be determined using this stain and image analysis.

Regardless of the tip identification method, it is difficult to estimate more than the proportion by volume of active tips, relative to the total amount of biomass. Because of hyphal overlapping, absolute counts of tips (especially if inactive and unstained) are generally very unreliable. It may be possible to obtain such counts for freely dispersed mycelia, but there is no reason to believe that these are representative of the whole biomass, especially at times of severe clump fragmentation. However, measurements on the freely dispersed material might be the only practical possibility, and such data might (at least) be of qualitative value.

The method of Paul *et al*. (1992) requires fresh samples, as vacuolation rapidly changes after sampling. Jüsten *et al*. (1994) did demonstrate that the vacuolation could be preserved for some 72 h using a glutaraldehyde-based fixative, but unfortunately the tip staining did not survive the fixing process. Modern methods of image capture and transfer (e.g. through the Internet) might alleviate the problem of analysing samples taken in laboratories (or industrial fermentation facilities) distant from the place of analysis.

Latterly, Agger *et al*. (1998) have devised another method for determining active regions and tips of fungi (in this case *Aspergillus oryzae*) in submerged fermentations. Samples were loaded with the fluorescent stain 3,3′-dihexyloxacarbocyanine ($DiOC_6$). This revealed the presence of mitochondria along the length of the hyphae. The use of image analysis and a process model related the occurrence of high numbers of mitochondria to hyphal activity, in particular enzyme production.

An area of increasing interest and detailed study is protein secretion by filamentous fungi (Peberdy, 1994). The ability of the extending hyphal tip to secrete heterologous protein is industrially important. As shown in extensive reviews (Jeenes *et al*., 1991; MacKenzie *et al*., 1993; Wessels, 1993; Peberdy, 1994), conclusive evidence of a link between hyphal tip numbers and activity, and product titres, has not been established. This may well be a question that is amenable to study by image analysis, as many of the necessary techniques seem to be available. For example, simple differentiation can be evaluated rapidly and routinely (Paul *et al*., 1994b) whilst the work of Agger *et al*. (1998) showed how correlations between organelle location and metabolic activity might be found, and biochemical differentiation assessed. With some method to count hyphal tips, it should be possible to establish the link, if any, between secretion and morphology.

The method of Paul *et al*. (1994b) may not work well on samples taken late in fermentations. Studies by McNeil *et al*. (1998) using image analysis have examined the process of hyphal 'death' and subsequent autolysis in *P. chrysogenum*. Image analysis measurements were made upon mycelia to give main and total hyphal lengths, branch lengths and hyphal growth unit. In addition, the lengths of autolysed regions of the mycelia were measured. From such measurements, correlations between observed morphology and degradative enzyme titres were made and related to penicillin production.

Subcellular events in fungi have also been examined using a combination of high-resolution video microscopy and image analysis. Regnaga-Peña *et al*. (1997) examined the elongation and branching processes of hyphae by imaging the Spitzenkörper and have related its organizational activity, via a computer model, to growth and morphogenesis.

As actinomycetes are generally smaller in diameter than filamentous fungi, it is difficult to characterize their differentiation using conventional microscopy. However, Drouin *et al*. (1997) and Mauss *et al*. (1997) have succeeded in quantifying differentiation in *Streptomyces* hyphae using image analysis. Using a low-light, integrating camera, these workers acquired images of sufficient clarity to allow individual hyphal compartments in the mycelia to be distinguished, and septa and empty regions to be identified. From these images, measurements of hyphal length and width could be obtained, and also the number of septa could be determined along with the proportion of empty regions. Mauss *et al*. (1997) also examined the location of oxidative respiration along the length of the *Streptomyces* hyphae. Measurements of the specific respiration activity of the hyphae (estimated by image analysis) were related to the global activity of the culture.

Future developments

Future developments for image analysis applications with filamentous micro-organisms probably lie in extensions to differentiation studies. Fluorescent stains and antibody labelling, along with genetic routes of

identification, may allow more specific characterization of localized differentiation or biosynthetic specificity.

Whilst developing new staining protocols should allow improved analysis of differentiation, recent advances in molecular techniques and visualization technologies may produce even more highly sensitive methods of detection of specific biosynthetic activities. Emerging technologies now make *in vivo* genetic labelling and imaging possible. Fluorescence *in situ* hybridization (FISH) allows the rapid detection of regions of DNA or RNA, e.g. mRNA, *in vivo* by the attachment of specific haptens to target nucleic acid sequences. Attached to the bound hapten is a fluorochrome-producing enzyme which produces a localized fluorescent marker around the bound region, thus allowing identification of active areas. Li *et al*. (1997), for example, have used a combination of image analysis, FISH and confocal microscopy to identify specific fungal species populations from phylloplane samples.

Similarly, luciferase and green fluorescent protein reporter genes allow assessment of metabolic activity by examination of specific gene induction (Suelmann *et al*., 1997). The reporter genes, high-molecular-mass introns, are incorporated into the host genome at probable sites of expression of some target enzyme. In this way, induced expression of the target sequence also induces expression of the reporter gene and the production of the specific fluorescent marker protein, or in the case of luciferase, a bioluminescent marker. More recent developments in such techniques (for example, fluorescent proteins other than green), may allow simultaneous recognition of various expressed products, precursors and biosynthetic machinery in fungi.

Most fluorescent images are subject to blur. Image deconvolution restores image quality by electronically removing out-of-focus information to leave a clean image from a narrow focal plane. However, attempts to apply this method to large sample numbers may not be practical because of the processing time required.

One area that has advanced significantly recently is the availability of improved cameras, improved particularly in terms of resolution and low-light response. The majority of commercially available fluorescent probes are FITC conjugates, which may not be suitable for imaging by older commercial CCD cameras because of the low levels of emitted light. However, a variety of manufacturers now offer low-light imaging systems at acceptable costs. Similarly, high resolution or large image format cameras are increasingly available with imaging systems.

Although now a maturing technology, confocal microscopy is infrequently used in the study of filamentous micro-organisms, primarily because of the high capital expenditure. Potentially, highly detailed physiological data are obtained by use of this technique. Nevertheless, present applications appear to be restricted to qualitative examination of fungal morphology and physiology, e.g. host–parasite interactions, as recently reviewed by Czymmek *et al*. (1994). It is possible that confocal microscopy will eventually become cheap enough for its routine use in microbiological laboratories, adding real power to more conventional image analysis methods.

For many years, the use of image analysis in microbiology has been limited because of the high costs of capable systems. Increases in personal computer power have made it practical to move image analysis from dedicated, purpose-built machines to cheap hardware. Many image analysis software packages are available to use on personal computers. Notable examples are Qwin (Leica UK), Visilog (Noesis) and PC Image (Foster & Finlay Associates), all of which have been used for characterizing filamentous micro-organisms. Image analysis is no longer the preserve of the few but is accessible to most laboratories.

Concluding remarks

Image analysis is a powerful tool for characterizing the morphology and simple differentiation of fungi and actinomycetes. The reducing cost of the equipment means that it should become a routine laboratory tool in research on filamentous micro-organisms. Such routine use is strongly recommended.

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