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Morphological Measurements of Submerged Culture of *Aspergillus niger* by Fully Automatic Image Analysis

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A fully automatic image analysis method was applied to obtain detailed data on morphological parameters of a glucoamylase fermentation broth with *Aspergillus niger* No. PFST-38, a mutant strain for glucoamylase hyperproducer. In the initial stage of fermentation, there was an increase in hyphal length, whereas at the end of the fermentation a decrease in hyphal length and increase in hyphal thickness were observed. The percentage of clumps declined with dilution and the influence of shear stress upon hyphal length was negligible. It was found that the slower the decrease in the main hyphal length and the number of tips with the fermentation time, the higher the glucoamylase production rate was recorded. The production rate of glucoamylase was closely related to the increase in the hyphal thickness.

Most of commercial glucoamylases are produced by fermentation of filamentous microorganisms, for example, *Aspergillus niger*. Mycelia of such microorganisms are generally in either pelleted or filamentous form. In the latter case, hyphal entanglement can cause the suspensions to be highly viscous and non-Newtonian, causing mass and heat transfer problems in fermentation. Characterization of mycelial morphology is therefore important in the designing and operating of fermentations of filamentous microorganisms. Recently, a method using photomicroscopy incombination with an electronic digitizer was developed and application of this instrument for the characterization of mycelial broth was also described (5).

The microscopic morphology was defined by van Suijdam (10) as the actual geometry of the hyphae, and includes hyphal dimensions and hyphal-hyphal interactions in the broth. It is generally considered that an increase in the power input per unit volume will lead to a reduction in the apparent viscosity (3). This may be related to the decrease in hyphal length when the impeller speed in mycelial cultures was increased (9, 11).

Carter and Bull (2) concluded from the works with *Asp. nidulans* that a higher agitation intensity resulted in shorter, more branched hyphae, with smaller septum-to-septum distances. They used the mean hyphal segment length as a quantitative index for the morphology. Katz *et al.* (4) reported increased branching for an *Asp. nidulans* culture when the growth rate was increased.

Zetalaki and Vas (12) observed a reduction in the viscosity of the culture when the oxygen tension in an *Asp. niger* culture was increased, but no difference in morphology parameters could be found.

Photomicroscopic measurements or image analysis of individual mycelium have been employed by several workers to determine the effects of mycelial morphology on broth rheology and productivity (7).

Image analysis software has been developed for rapid characterization of the usual morphological parameters of filamentous microorganisms and also for the proportion of biomass in the form of aggregates of clumps (6). We have explored the use of image analysis for the quantitative measurements that were previously totally impractical with microscopic method.

MATERIALS AND METHODS

Fermentations

Aspergillus niger PFST-38 was grown in a 30-*l*-fermentor (B.E. Marubishi, Japan) at 500 rpm and 1.0 vvm in batch culture on the defined growth medium (Table

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Table 1. Concentrations of components in the medium for the production of glucoamylase with *Asp. niger* No. PFST-38

Component	Concentration (g/l)
Corn meal	200.00
Defatted soybean meal	30.00
Corn steep liquor	50.00
α-Amylase	0.05

1). Samples were taken every 24 hr during a 7-day fermentation.

Specimen Preparation

Samples taken from the fermentation broth were mixed immediately with an equal volume of fixative (13 ml of 40% formaldehyde and 5 ml of glacial acetic acid added to 200 ml of 50% v/v ethanol). The fixed sample was further diluted with the fixative to 10 folds or to 20 folds when solids are present. A duplicate set of specimen was prepared from each sample broth.

A small amount (0.8 ml) of Lactophenol Tryphan Blue stain (0.25 g Tryphan Blue to 100 ml Lactophenol) was added to 9.2 ml of fixed and diluted sample, and a drop of approximately 0.5 ml was spread evenly onto a slide and covered with a 324 mm^2 coverslip. The coverslip was sealed to prevent dehydration of the sample during processing. To reduce the errors caused by the slide preparation, 10 slides were measured at each time, and 1 sample was measured from each of 10 repeated dilutions. The dilution was adjusted in order to separate mycelial particles on the slide until there were only a few appeared in the field of view.

Morphological Measurements

For image analysis, a Magiscan MD image analyzer (Joyce Leobl Ltd., Gateshead, UK) attached to a Nikon Optiphot microscope (Nikon UK Ltd, Telford, UK) was used. An automatic stage with a 20-μm step size was fitted to the microscope, giving automatic X, Y, Z motion. Light control was also made automatically. For image analysis, a magnification of 200 times was used. The circularity parameter for the dust screen was set at 0.04, and the parameter for eliminating branch artifacts was set at 4 pixels. For each sample four measurements were made; the total area of the mycelia, the average hyphal thickness, the distribution of main and total hyphal lengths, and the number of tips.

Mycelial Weight

The amount of biomass was determined by weighing the filter cake after drying it at 105℃ overnight.

Glucoamylase Activity

Glucoamylase activity in a reaction mixture that contained 0.5 ml of 2% soluble starch in 0.2 M sodium acetate buffer (pH 4.8) and 0.5 ml of enzyme solution was measured. After aerobic incubation at 60℃ for 30 min, the reaction was stopped by cooling the mixture on ice, and then the mixture was boiled in a steam bath for 10 min. The released glucose was quantified by using the hexokinase-glucose-6-phosphate dehydrogenase method (1). One unit of glucoamylase activity was defined as the amount of enzyme that liberated 1 μmol of glucose per min under the assay conditions used.

RESULTS

Morphological Variations during the Batch Fermentations for Glucoamylase Production with *Asp. niger* No. PFST-38

Figure 1 shows typical morphologies for the *Asp. niger* No. PFST-38 fermentation. Figure 1 is of the sample at 48 hr of cultivation and it shows a clump. The free microorganism was highly branched and had the largest mean hyphal lengths. Fragmentation resulted in fewer branches and shorter lengths compared to those found in the sample at 72 hr of cultivation. Same regrowth was then observed in the sample at 96 hr and at 120 hr of cultivation, but the microorganisms remained relatively unbranched, as shown in Fig. 1.

Figure 2 shows the time courses of the growth of main hyphal length and the number of tips for the *Asp. niger* No. PFST-38 fermentation. As can be seen from Fig. 2, during the initial stage of a fermentation, there was an increase in hyphal length. Generally at the end of a fermentation, there is usually a decrease in hyphal length. This can be caused mainly by the decaying processes, but the lower growth rate in the later stage of the fermentation could be another explanation. However, looking at Fig. 2, we can see that the hyphal length was constant or even decreasing, while the dry weight was still increasing. Therefore it must be concluded that the hyphae had been broken. The number of tips during fermentation increased until 72 hr of cultivation, and thereafter remained reasonably constant.

Figure 3 shows the percentage of clumps, which is appeared densely in this kind of fermentation despite the fragmentation of free mycelia. Because of clumping, the time taken for measuring all hyphal parameters per microorganism was long , i.e., 2 min/microorganism. And also, this figure shows the time courses of mean hyphal thickness. As can be seen from Fig. 3, the hyphal thickness increased at the end of the fermentation. It is found that the slower the main hyphal length and the number of tips decreased with the fermentation time, the higher the glucoamylase production rate was achieved. Also,

48 hr

The free microorganisms were highly branched and had the largest mean hyphal length

120 hr

The microorganisms retained relatively unbranched and had the increased hyphal thickness

Fig. 1. Typical morphologies of *Aspergillus niger* No. PFST-38 sample at 48 hr and 120 hr of cultivation (One scale indicates 1.0 μm).

*The strain was grown in a 30 l-fermentor at 500 rpm and 1.0 vvm in batch culture on the defined growth medium (Table 1).

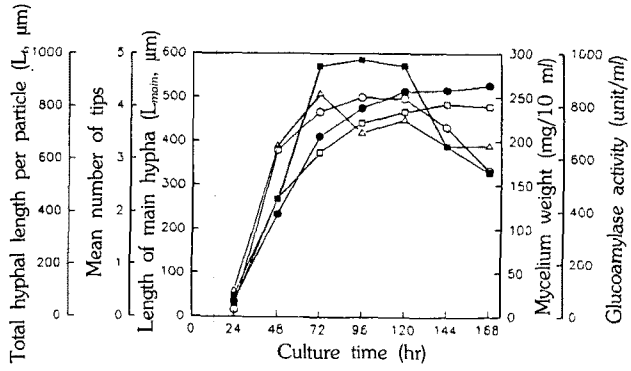

Fig. 2. The time courses of the length of main hypha, total hyphal length, mean number of tips, glucoamylase activity and mycelium weight during a bactch culture with *Asp. niger* No. PFST-38.

Cultivation condition: 500 rpm, 1.0 vvm, 30℃ in 30 *l* jar fermentor.

○−○: Length of main hypha, ●−●: Mycelium weigh, ▲−▲: Mean number of tips, △−△: Total hyphal length, □−□: Glucoamylase activity

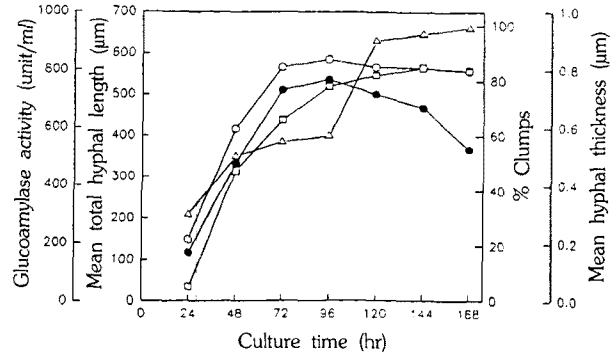

Fig. 3. Time courses of mean total hyphal length, mean hyphal thickness, glucoamylase activity and percent clumps for a batch culture of Asp. niger No. PFST-38 measured by using fully automatic image analysis.

Cultivation condition: 500 rpm, 1.0 vvm, 30℃ in 30 *l* jar fermentor.

●−●: Mean total hyphal length, ○−○: % clumps, △−△: Mean hyphal thickness, □−□: Glucoamylase activity

the production rate of the glucoamylase is closely related to the increase in the hyphal thickness.

Effect of Dilution on Measured Morphological Parameters

Table 2 and Figure 4 show the effect of dilution on the morphological parameters of a sample from each fermentation. Except for low dilutions, which made overlapped view on the slide due to the high cell densities, the morphological parameters seemed fairly insensitive to the extent of dilution. However, the percentage of clumps could be a dilution dependent as shown by Table 2. Table 2 also shows the times taken, the number of fields analyzed, the mean number of microorganisms per field, and the mean time per microorganism.

Influence of Shear Stress upon Morphology

In Table 3 a series of data from batch culture experiments with varying stirrer speeds are given. The values in this table are for the period of constant morphology. The influence of the shear stress upon the hyphal length was apparent. The total length (L_t) and hyphal growth unit (L_{hgu}) increased until the agitation speed became 500 rpm. It may due to the positive effect of agitation to the mycelial growth. On the other hand, L_t and L_{hgu} decreased at 600 rpm of agitation. This shows the shear stress effect to the mycelial disintegration.

Table 2. Effect of dilution on the various morphological and measurement parameters of the sample at 72 hr of cultivation of *Asp. niger* No. PFST-38 by using fully automatic image analysis

Dilution (fold)	10	40	160
Number of microorganisms measured	101	103	102
Mean total hyphal length (μm)	420	455	480
Mean number of tips	3.8	4.2	4.0
Percent clumps (%)	88	67	43
Total time taken (min)	18	19	13
Number of fields analyzed	16	19	11
Mean number of microorganisms per field	6.3	5.4	9.3
Mean time to measure one microorganism (min)	0.18	0.18	0.11

Table 3. Morphological data at different stirrer speeds in batch fermentation of *Asp. niger* No. PFST-38

Experiment No.	N (rpm)	L_t (μm)	L_{hgu} (μm)
1	200	430± 175	65± 11
2	200	490± 213	68± 13
3	200	405± 62	57± 12
4	400	612± 133	92± 18
5	400	580± 145	63± 18
6	400	572± 166	88± 32
7	600	382± 98	41± 16
8	600	276± 132	53± 18
9	600	288± 115	48± 21

L_t: Total hyphal length per particle (μm)
L_{hgu}: Hyphal growth unit (μm) ($L_{hgu}=L_t/(n+2)$)
n: Number of branches (dimensionless)

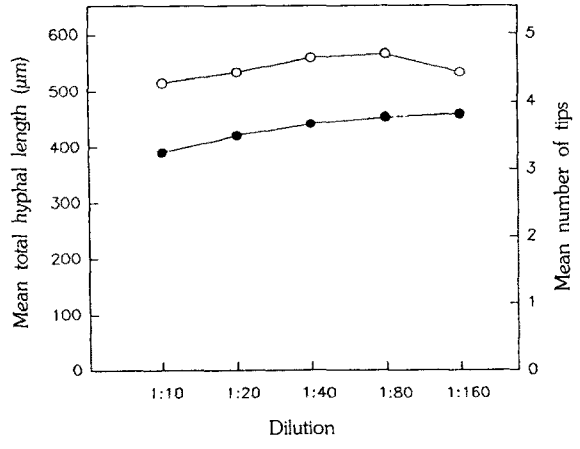

Fig. 4. Effect of dilution on measured morphological parameters of a sample from *Asp. niger* No. PFST-38 fermentation (72 hr).
The fixed sample diluted with fixative.
○−○: Mean number of tips, ●−●: Mean total hyphal length

However, an enormous increase in energy input might be necessary in order to get a substantial decrease in the hyphal length by shear stress during the fermentation.

DISCUSSION

Figure 3 shows clearly that the fully automatic image analysis method can be applied to obtain reasonably precise data on the morphological parameters of a fungal fermentation. The high degree of clumping in *Asp. niger* No. PFST-38 fermentation provides a very stringent test of the image analysis because there are relatively few free microorganisms to measure. Except for low dilutions, the morphological parameters measured by fully automatic image analysis were fairly insensitive to the extent of dilution. At low dilutions, the artifactual crossovers of microorganisms were observed, which could lead to bias against long microorganisms if clumps were formed. Provided that these low dilutions are avoided, the actual dilution to a certain range does not seem to be a critical problem in achieving accuracy, although it affect the time taken for analysis.

It is probable that the analysis time is very much dependent on the skeletonization process. The present implementation of this process is slow, and a nearly empty field takes as long time as for a relatively full one. Analysis times could be significantly shortened by increasing the speed of skeletonization, either by improved software, or by implementing the process with hardware.

It could be presumed that in a filamentous fermentation some materials are in permanent aggregates, i.e., aggregates which could only disintegrate if microorganisms within them were disrupted. Such aggregates might affect mass transfer and broth rheology, and it would be useful to have a method to estimate the proportion of biomass in such a form in a fermentor.

Besides permanent aggregates, loose agglomerations of mycelia could also exist. These would disaggregate easily, for example, on passing through the impeller regions of a stirred tank vessel. In the case of *Asp. niger* No. PFST-38 fermentation (Table 2), the percentage of clumps declined with dilution. This was because of the loose nature of the aggregates, which easily disintegrated by dilution. The image analysis can be used for rapid and , in most cases, accurate characterization of the filamentous microorganisms morphology. The fully automatic method reported here not only estimates the usual morphological parameters, but also the proportion of biomass in clumps, which could be related to permanent aggregates in a fermentor (8). This method is useful for a routine study of the morphological development in

mycelial broth, and facilitates engineering studies for broth rheology, and mixing and mass transfer of the filamentous fermentations.

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