Kinetic Studies on the Aggregation of *Aspergillus niger* conidia

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Abstract: Morphology has a crucial effect on productivity and the supply of substrate for cultures of filamentous fungi. However, cultivation parameters leading to the desired morphology are often chosen empirically as the mechanisms governing the processes involved are usually unknown. For coagulating microorganisms like *Aspergillus niger* the morphological development is considered to start with the aggregation of conidia right after inoculation. To elucidate the mechanism of this process, kinetic studies were carried out using an in-line particle size analyzer. Based on the data obtained from these experiments a model for conidial aggregation is proposed in this article. It consists of two separate aggregation steps. The first one takes place immediately after inoculation, but only leads to a small decrease of total particle concentration. Most suspended conidia aggregate after a second aggregation step triggered by germination and hyphal growth. Aggregation velocity of this second phase is linearly dependent on the particle growth rate. © 2004 Wiley Periodicals, Inc.

Keywords: *Aspergillus niger*; morphology; conidial aggregation

INTRODUCTION

Filamentous fungi are employed in a large scope of biotechnological processes ranging from the production of organic acids (citric acid) to antibiotics (penicillin) and enzymes (glucoamylase). A potential application in heterologous protein production has been described based on their ability for efficient secretion and glycosylation of proteins (Amanullah et al., 2001; Punt et al., 2002). Productivity was found to be closely linked to fungal morphology, which varies from dispersed mycelia to compact spherical pellets of different size and density (Ganzlin, 2000). Morphology is closely linked to the rheological properties of the fermentation broth (Riley et al., 2000). In cultures of dispersed mycelia the viscosity can rise to levels at which appropriate mass and heat transfer cannot be provided for by the mixing system. Rheology has a lower impact on pellet cultures, but pellet size can obstruct mass transfer (Paul et al., 1999). The centers of large pellets can be starved of oxygen and autolyse. In both cases morphological development as dispersed mycelia or as pellets needs to be observed closely. Organisms have been classified in relation to their mechanism of pellet formation: conidia of coagulating microorganisms usually aggregate in the early stage of cultivation and pellets are formed by mycelia growing out of these aggregates, while a pellet of the noncoagulating type grows out of one spore (Metz and Kossen, 1977).

The development of digital image analysis methods has promoted the mathematical description of fungal growth (Paul and Thomas, 1998). Hence, a variety of models describing the swelling of spores, growth of germ tubes, septation, and branching have been proposed (Prosser, 1994). Even complex models covering many of these processes have been established for noncoagulating *Streptomyces* species. These models describe single conidia and pellets, however, omitting the interdependence of coagulating organisms (King, 1995).

Aggregation has been described as the consequence of random encounters of conidia in suspension, but little is known about the aggregation process itself. Strain characteristics, medium composition, mechanical forces, and the concentration of conidia in the inoculum are frequently cited as parameters influencing the morphological development (Amanullah et al., 2001; El-Enshasy et al., 1999; Tronchin et al., 1995). All of these parameters may have an effect on aggregation.

The hydrodynamic conditions in a bioreactor for example have an influence on particle velocities and therefore on collision frequency. Collision frequency will be raised by increasing the impeller speed of a stirred-tank reactor. On the other hand a higher rotation speed also leads to stronger shear forces, counteracting the aggregation of conidia. Indeed, published data suggests that high agitation rates disturb the formation of pellets (Coudhary and Pirt, 1965).

The effect of the concentration of conidia on pellet formation has been described in more detail. Low concentrations in the inoculum result in low amounts of pellets with large diameters, while higher concentrations lead to smaller, but more pellets (Withaker and Long, 1973; Xu

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et al., 2000). This observation agrees with descriptions of crystallization processes, where a large number of small particles are expected from high ion super-saturation and few big particles arise from low ion super-saturation. This phenomenon is explained by a mechanism of two steps consisting of nucleation and growth of existing particles (Schwarzer and Peukert, 2002), but a formal comparison of the aggregation of conidia with precipitation processes should be handled with care.

This is especially true for the particle interaction forces involved in the aggregation process. Literature provides ambiguous information on the importance of intermolecular forces. Gerin et al. (1993) concluded that aggregation is not dependent on hydrophobicity and the electrical properties of *Phanerochaete chrysosporium* conidia. Meanwhile, Ryoo and Choi (1999) argue that pellet formation is driven by cell wall hydrophobicity of *Aspergillus niger*. Mutants of *Aspergillus nidulans* lacking hydrophobic proteins in the cell surface tended to grow more in form of free hyphal elements than the wild-type strain. This finding suggests some influence of cell wall components, yet these mutants still were able to form pellets. Therefore, agglomeration of conidia was not attributed to hydrophobic and electrostatic interactions alone (Dynesen and Nielsen, 2003).

The interpretations of the mechanism of aggregation described are based on the description of pellets grown under different conditions. Parameters influencing the aggregation of conidia may have an influence on cell growth, though. It is therefore difficult to quantify any influence of aggregation on pellet formation and to describe the importance of this first step for fungal growth. A direct insight into conidial aggregation and the influence of different parameters should be possible through kinetic studies of this process. They give evidence for the mechanism of pellet formation. Based on kinetic experiments a model for conidial aggregation of *Aspergillus niger* is presented in this article.

MATERIAL AND METHODS

Strain and Media

Glucoamylase producing *Aspergillus niger* AB 1.13 was used as the model strain in all experiments. The origin of this uridine auxotroph strain is described by Mattern et al. (1992). To obtain conidia, a culture was grown on potato dextrose agar (PDA) with 0.1% (w/v) uridine at 30°C. Conidia were harvested after 5 days by flooding the agar plates with sterile NaCl solution (0.9% w/v) and scraping the aerial mycelium. Cell concentrations were determined by measuring the optical density of the suspension at 600 nm (Fujita et al., 1994). The values proved to correlate well with manual cell counts.

For aggregation studies conidia were inoculated in a defined medium containing (in g/L): xylose, 10.0; uridine, 0.24; $(NH_4)_2SO_4$, 1.65; KH_2PO_4, 2.5; $MgSO_4 \cdot 7\ H_2O$, 0.2; $CaCl_2 \cdot 2\ H_2O$, 0.1, and 0.1 mL/L trace element solution containing (in g/L): $ZnSO_4 \cdot 7\ H_2O$, 50; $Fe(NH_4)_2(SO_4)_2 \cdot 6\ H_2O$, 10; $CuSO_4$, 1.6; $Na_2MoO_4 \cdot 1\ H_2O$, 0.5; $MnSO_4 \cdot 1\ H_2O$, 0.5.

Particle Size and Concentration

The time-dependent particle size distributions and concentrations were measured with an in-line particle size analyzer (FBRM D500; Lasentec, Redmond, WA). The working principle of the analyzer is based on laser scanning microscopy. A laser beam is focused to a spot rotating at constant angular velocity. Laser light is scattered back by particles crossing the beam. The scattered light is collected on a photo detector. Characteristic chord lengths are determined from the reflection time and the rotational speed of the laser spot. In the range of chord lengths from 0 to 500 μm the amount of counts per second corresponds to the concentration of all particles in solution as will be demonstrated. The amount of counts between chord lengths of 0 and 10 μm was tracked as well to compare conidial and small particle concentration with that of all particles. The median value of the chord length distribution between 0 and 500 μm was observed for 20 h to calculate the growth rate of aggregates.

Aggregation Assay

Conidial aggregation was studied with the particle size analyzer in a 400- mL glass beaker (75 mm diameter) tempered at 30°C and stirred with a four-blade turbine impeller (50 mm diameter). Agitation rate was set to 240 min^{-1}. Two hundred milliliters of medium adjusted to pH 5.5 were inoculated with a suspension of harvested conidia to give particle concentrations between $5 \cdot 10^5\ mL^{-1}$ and $5 \cdot 10^6\ mL^{-1}$. A sample of 1 mL was taken immediately after inoculation to determine cell concentration. Several samples were taken during cultivation and characterized by microscopy.

To ensure that the observed phenomena were independent of the experimental set-up and the measuring device 100-mL shake flasks were filled with 20 mL of the same medium and inoculated with conidia to a final concentration of about $10^7\ mL^{-1}$. Samples were taken every hour and particle concentrations determined by microscopy using a Thoma counting chamber. Particle counts were performed using digital image analysis software (KS300, Carl Zeiss Vision, Hallbergmoos, Germany).

RESULTS

Conidial concentration was determined by measuring the optical density of a sample immediately after inoculating the aggregation assay. The initial value of counts per second given by the particle size analyzer was correlated with the conidial concentration determined by the optical

density of the suspension. The linear dependency obtained proved that the number of counts per second is equivalent to the amount of particles in the culture broth.

Figure 1 presents the course of counts per second of particles in the range of 0–10 μm and in the range of 0–500 μm over time. In both cases, particle concentration decreases immediately after inoculation. After reaching about 80–90% of the initial value, the conidial concentration and the entire particle concentration remain relatively constant for the following hours. Eight to nine hours after inoculation a second decrease of particle concentration can be observed. At this time the first germ tubes are visible under the microscope. The growth of germ tubes leads to a decreasing number of particles with sizes below 10 μm. The number of all particles in solution decreases at the same time indicating that a second aggregation process has begun.

The kinetic behavior observed therefore suggests that two separate conidial aggregation processes are involved, the first one taking place right after inoculation and the second one 8–9 hours later. Conidia attached to hyphae were frequently observed after germination started. The second and more prominent decrease in particle concentration is apparently brought about by the germination process and the related increase in surface.

This behavior is independent of the experimental set-up and of the measuring device as shown in Figure 2. Particle concentrations were measured by digital image analysis of samples taken from shake-flask experiments. The values fluctuate more than in Figure 1 due to statistical reasons, but the same trend of particle concentration over time can be observed. Again, a decrease in particle concentration in the beginning is followed by a constant period of several hours and a sharp decrease once germination starts.

The aggregation assay in the glass beaker was carried out with different starting concentrations of conidia.

Different inoculum concentrations resulted in the same time-dependent behavior of particle concentration as depicted in Figure 3. Yet, aggregation velocity seems to

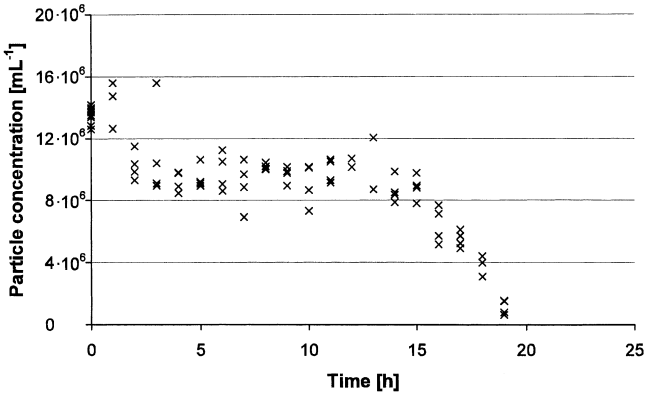

Figure 2. Particle concentration over time measured by digital image analysis of samples taken from 100-mL shake-flask experiments.

increase especially for the second aggregation phase starting about 8 h after inoculation. For the second aggregation process particle concentration decrease is linear over a long period of time. Aggregation velocity is given by this decrease over time. It can therefore be estimated by simple linear regression with correlation coefficients usually over 0.99. The aggregation velocity is given by the absolute value of the slope of this linear decrease.

Figure 4 shows the aggregation velocity calculated from the slope of counts per second in the range of 0–500 μm in the second aggregation step. This aggregation velocity is plotted against particle concentration at the beginning of the second aggregation process. Aggregation velocity increases with particle concentration from less than 40 to 160 counts per second per hour for particle concentrations between $4 \cdot 10^5$ mL^{-1} and $3 \cdot 10^6$ mL^{-1}, respectively. For particle concentrations above $3 \cdot 10^6$ mL^{-1} no increase in aggregation velocity could be observed.

The second aggregation phase was attributed to the appearance of germ tubes and the consequent increase in surface available for attachment of conidia. In that case the aggregation velocity should be linked to the particle growth

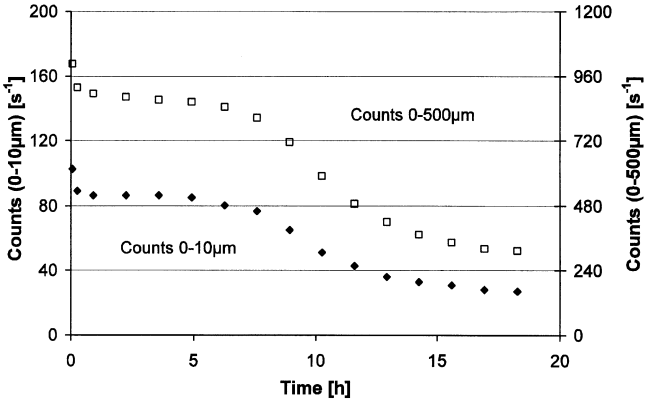

Figure 1. Time-dependent development of the concentration of all particles in solution (0–500 μm) and of the concentration of conidia and small aggregates (0–10 μm).

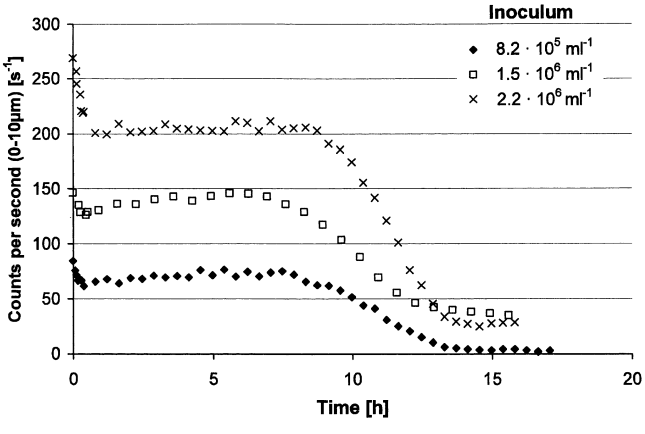

Figure 3. Concentration of conidia and small aggregates (0–10 μm) over time for different inoculum concentrations.

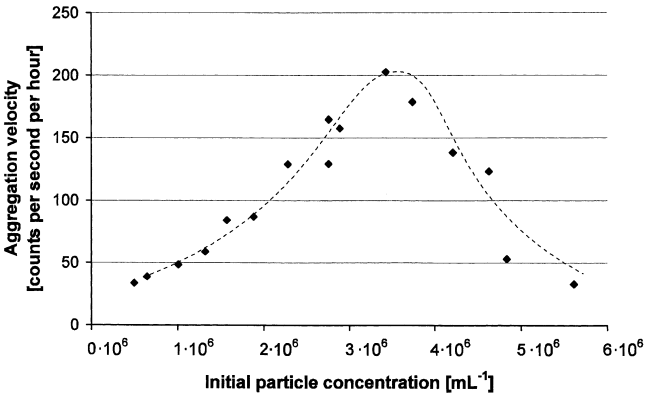

Figure 4. Aggregation velocity of the second aggregation step calculated from the slope of total particle concentration over time plotted against the particle concentration at the beginning of the aggregation process.

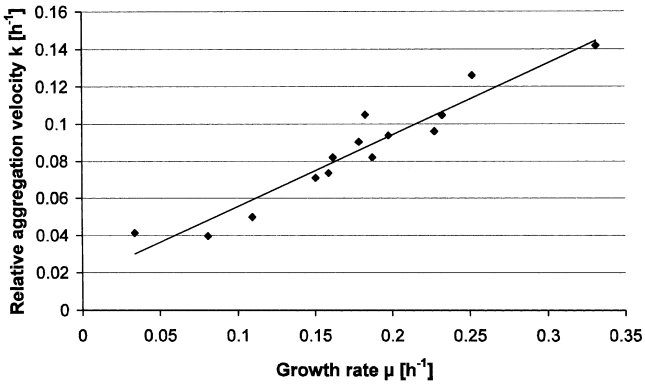

Figure 6. Linear dependency of the relative aggregation velocity calculated from the decrease in total particle concentration and the growth rate determined from the increase of the median value of particle chord length.

rate. Hyphal growth can be described by Eq. (1) with L being the particle length and μ the growth rate (Prosser, 1994). Equation (2) is obtained by integrating Eq. (1). L_0 is the starting length and $L(t)$ the length at the time t

$$\frac{dL}{dt} = \mu \cdot L \tag{1}$$

$$\ln L(t) = \ln L_0 + \mu \cdot t \tag{2}$$

According to Eq. (2) the growth rate was calculated from the increase of the particle median chord length (0–500 μm). This value increases exponentially with time starting about 8–10 h after inoculation (see Fig. 5). The particle growth rate is given by the slope of each curve. It appears to be lower for cultures with higher inoculum concentrations.

It was shown that the aggregation velocity depends on the initial particle concentration. Division of the measured particle concentration by this initial value leads to a relative particle concentration. The relative aggregation velocity k is defined by Eq. (3) as the slope of particle concentration over time divided by the initial particle concentration N_0. It has the same dimension as the growth rate (time^{-1}).

$$\frac{1}{N_0} \cdot \frac{dN}{dt} = k \tag{3}$$

The two parameters k and μ describe the aggregation process and the particle growth rate, respectively. They were measured independently but are correlated by a linear dependency as displayed in Figure 6. It proves that particle growth is related to the second aggregation step and is proportional to the aggregation velocity.

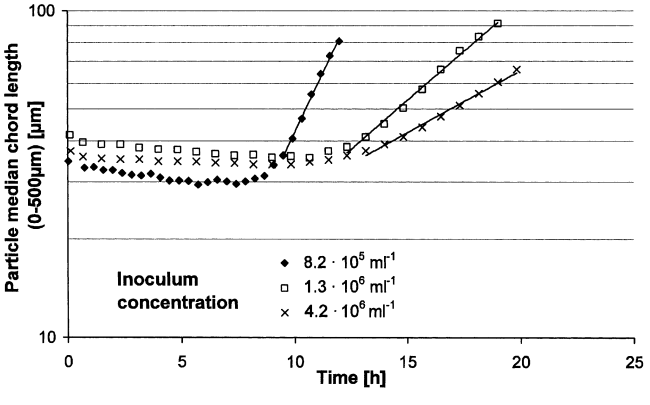

Figure 5. Development of the median value of particle chord lengths over time measured by the particle size analyzer in the aggregation assay with different inoculum concentrations.

DISCUSSION

Kinetic studies provide a new insight into the process of conidial aggregation and pellet formation. Based on the course of particle concentration over time the following mechanism is proposed for conidial aggregation of *Aspergillus niger* AB 1.13. A model is sketched in Figure 7. Aggregation of conidia starts immediately after inoculation of the medium and reaches a steady state during the first hours of cultivation. At this point the same amount of conidia will probably attach to aggregates, as conidia are detached due to shear stress in the reaction system. The location of this steady state would therefore depend on molecular forces between interacting cell surfaces and shear forces depending on the hydrodynamic conditions in the cultivation vessel. The experimental set-up chosen provides relatively low shear forces considering reaction volume (200 mL) and agitation rate (240 min^{-1}). The steady state is reached at relatively high particle concentrations of about 90%.

The germination of conidia brings the steady state to an end. Germ tubes and hyphae provide additional surface for aggregation initiating the second aggregation step, which leads to a strong decrease of particle concentration. This hypothesis based on microscopic observation (cp. Fig. 7b) is supported by the linear dependency of the relative aggregation velocity and the particle growth rate found. The

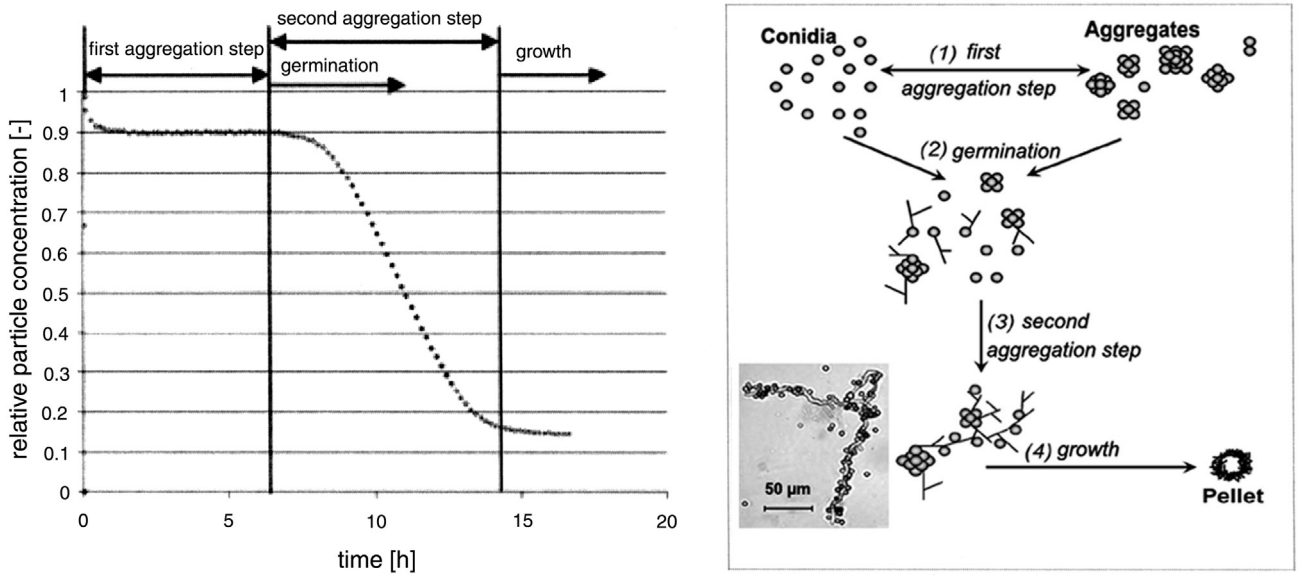

Figure 7. Kinetic model of conidial aggregation of *Aspergillus niger* AB 1.13 based on experimental results. Two separate aggregation steps were observed. The first one takes place immediately after inoculation. The second one is triggered by germination of conidia 8 hours after starting the cultivation.

increase of the median value of chord lengths (see Fig. 5) may be explained by the aggregation of spores and by hyphal growth. Growth rates were measured in the range of 0.1–0.3 h^{-1}, which are characteristic for exponential cell growth. If only aggregation was responsible for the increase of the median chord length, spherical particles would be expected. The volume of such an aggregate composed of circular conidia can be estimated from the conidial volume and the number of conidia per aggregate assuming random package. In a rough estimation each aggregate of 70 µm chord length described in Figure 5 should consist of more than 1600 spherical conidia of approximately 5 µm diameter. At the end of the second aggregation process the measured particle concentration lies between 10^4 mL^{-1} and 10^5 mL^{-1}. This means that much more than 10^7 and up to 10^8 conidia per mL would be required to build these particles if only aggregation was involved, but conidial concentrations in the inoculum never surpassed 6 10^6 mL^{-1}. It is therefore deduced that the measured growth rate is mainly due to hyphal growth.

The influence of particle concentration on the velocity of the second aggregation phase is shown in Figure 4. It gives evidence for at least two counteracting effects on the aggregation process. The increase in aggregation velocity with raising particle concentrations can be explained by the higher probability of collisions taking place. Aggregation velocity does not increase permanently though. It decreases at conidial concentrations beyond 3 $10^6 mL^{-1}$. By raising conidial concentration a slow down of the particle growth rate was observed as well (see Fig. 5). Raising concentrations of conidia may disturb germination or hyphal growth. As a consequence less surface would be available for aggregation, counteracting the increase in aggregation velocity caused by more frequent collisions.

A new steady state is reached after several hours at a very low particle concentration. Conidia are bound to aggregates or germinated at this time and no single conidia are left. Mycelial fragmentation and growth may well lead to further aggregation steps beyond conidial aggregation.

CONCLUSION

The model presented in Figure 7 is based on experimental results and is the first step for a detailed analysis of the conidial aggregation mechanism. Both, the influence of shear forces on the process, caused by agitation and aeration, and the effect of surface forces acting between the particles has not been quantified yet. If the latter influence the aggregation process, information may be had by varying ion concentrations or pH values and observing the kinetic behavior. Furthermore, force and material balances should be formulated. This way the exact decrease of particle concentration due to settlement on the reactor surface could be determined. A balance of all relevant forces should finally allow predictions for conidial aggregation.

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