0273-2289/99/81/0001/$12.75

Fungal Morphology in Submerged Cultures and Its Relation to Glucose Oxidase Excretion by Recombinant *Aspergillus niger*

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Received December 1, 1997; Accepted April 2, 1998

Abstract

The effect of culture conditions such as medium composition and shear stress on the fungal pellet morphology in shake-flask cultures and its relation to glucose oxidase (GOD) excretion by recombinant *Aspergillus niger* NRRL 3 (GOD 3-18) was investigated. It was shown that culture conditions resulting in the formation of smaller fungal pellets with an increased mycelial density result in higher yields of exocellular GOD. The pellets obtained in shake-flask cultures showed distinct layers of mycelial density with only the thin outer layer consisting of a dense mycelial network. The performance of the recombinant strain and the process of pellet formation was also analyzed during batch cultivation in a stirred-tank bioreactor. It was shown that the process of pellet formation occurred in two steps: (1) aggregation of free spores to spore clusters with subsequent germination and formation of small aggregates surrounded by a loose hyphal network, and (2) aggregation of the primary aggregates to the final full-size pellets. The fungal pellets formed during bioreactor cultivation were smaller, did not show large differences in mycelial density, and were more efficient with respect to the production of exocellular GOD. The decreasing pellet size also correlated with an increased mycelial density, indicating an improvement of the transport of nutrients to the inner parts of the pellet.

Index Entries: *Aspergillus niger*; recombinant strain; glucose oxidase; protein excretion; fungal morphology.

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Introduction

Glucose oxidase (GOD) (β-D-glucose:oxygen, 1-oxidoreductase, EC 1.1.3.4) is an enzyme that converts glucose to gluconic acid. In addition to gluconic acid production, GOD has many other industrial applications *(1,2)*. In the food industry, it is used for preservation, e.g., to remove oxygen from beverages and powdered eggs. In the pharmaceutical industry, GOD is used to stabilize ascorbic acid and vitamin B_{12}. In addition, GOD is used for the enzymatic determination of glucose in commercial glucose analysis kits and as immobilized enzyme in biosensors.

GOD is produced by various fungi, with *Aspergillus niger* as the most commonly employed fungus *(3)*. GOD is, in general, present as a cell-bound enzyme in *A. niger (3)*. This cell-bound localization is quite disadvantageous concerning the purification of the enzyme *(4)*. Therefore, efforts have been undertaken to improve the exocellular production of GOD by inserting an efficient secretion signal sequence in front of the *god* structural gene using genetic engineering techniques *(5)*. Studies on the exocellular production of GOD by this recombinant *A. niger* strain revealed a transient cell-bound accumulation of the processed and mature enzyme followed by a slow release into the culture medium *(6)*. This observation and the difference in the performance of this recombinant strain grown under variant conditions *(5)* also affecting the growth morphology suggest that the amount of GOD excreted into the culture medium might be influenced by the fungal morphology.

Filamentous fungi such as *A. niger* exhibit different growth morphologies in submerged cultures that strongly affect the overall cell performance and process parameters. For example, the secretion of heterologous proteins by recombinant *A. niger* has been reported to depend on the fungal morphology *(7)*. The morphology of filamentous fungi can vary from compact pelleted to filamentous growth forms. The most important advantage of the pelleted morphology is the decrease in the viscosity of the culture fluid, resulting in improved mixing and mass transfer properties. In addition, the pelleted growth form facilitates downstream processing by simplifying solid-liquid separation procedures. The major disadvantage of the pelleted morphology is the mass transfer resistance observed in fungal pellets resulting in significant concentration gradients of oxygen and other nutrients from the culture fluid to the inner pellet core *(8)*. The limitation in the transport of oxygen and other nutrients can finally result in autolytic processes within the inner parts of the fungal pellets *(8,9)*.

Two different mechanisms are known to lead to the formation of pellets. Pellets of the noncoagulative type arise from one single spore, and the number of pellets are directly correlated to the number of spores used as inoculum. *A. niger* forms pellets of the coagulative type. These pellets are formed by a one- or two-step procedure: (1) spores aggregate first to form pellet nuclei and the aggregated spores germinate and grow to a single pellet *(10)*, or (2) the aggregated spores germinate and subsequently com-

bine to form larger bioparticles *(11)*. The process of pellet formation is complex and affected by many factors, e.g., the strain used, the growth rate, the medium composition, the presence or absence of surfactants and polymers in the growth medium, and the shear force the cells are exposed to *(12)*.

In this study, the effect of culture conditions, such as medium composition and shear stress on the fungal morphology in shake-flask cultures and its relation to GOD excretion by recombinant *A. niger*, is investigated. In addition, the performance of the recombinant strain and the process of pellet formation is analyzed during batch cultivation in a stirred-tank bioreactor.

Materials and Methods

Microorganism and Culture Conditions

The construction of the recombinant strain *A. niger* NRRL-3 (GOD 3-18) has been described previously *(5)*. The genetically modified strain carries multiple chromosomal copies of the *god* structural gene from wild-type *A. niger* fused to the α-amylase signal sequence of *A. oryzae*. The expression of recombinant *god* is controlled by the *gpdA* promoter of *A. nidulans*.

The basic minimal medium (3.0 g/L $NaNO_3$; 1.0 g/L K_2HPO_4; 0.5 g/L $MgSO_4 \cdot 7H_2O$; 0.5 g/L KCl; 0.01 g/L $FeSO_4 \cdot 7H_2O$; 80.0 g/L glucose) was supplemented with yeast extract (Difco, Heidelberg, Germany) as indicated in the Results and Discussion section. The preparation of the medium and the inoculum was as described previously *(5)*. Baffled or nonbaffled 100-mL Erlenmeyer flasks with a working volume of 20 mL were used for shake-flask cultures. Baffled flasks contained two opposite baffles. Shake-flask cultures were incubated on a rotary shaker (Pilot Shake Rc 6SR, B. Braun Diessel Biotech GmbH, Melsungen, Germany) at 125 rpm and 30°C. In addition, a 2-L stirred-tank bioreactor (Setric SET002; Setric Genie Industriel, Toulouse, France) with a working volume of 1 L was employed. The stirrer speed was adjusted to 200 rpm after inoculation and raised to 400 rpm after 13 h of cultivation. The stirrer speed was increased again 15 h after inoculation to 600 rpm and kept constant for the rest of the cultivation. Temperature and airflow were kept constant at 30°C and 0.5 vvm, respectively. The pH was controlled at 5.5 by the addition of 1 mol/L of NaOH. Other culture conditions were as described previously *(5)*.

Analytical Methods

Sample preparation and analysis of glucose concentration, cell dry mass (CDM), and endo- and exocellular GOD activities were carried out as described previously *(5,6)*. For a single time course data point from a shake-flask culture, the total culture content was used for analysis. All data points correspond to three independent shake-flask experiments.

The number of different bioparticles in the sample (spores, aggregates, and pellets) was determined by microscopy using a Thoma counting chamber (0.02 mm × 0.0025 mm^2) (Omnilab, Braunschweig, Germany), and

their average size was determined from 30 to 40 randomly chosen bioparticles. For calculating the average pellet density, the data of the CDM, the number of pellets per unit volume, and their average size were used. Photographs were taken using a Zeiss Photomicroscope (Axioskop) (Oberkochen, Germany).

Results and Discussion

Effect of Medium Composition and Shear Stress on Fungal Morphology and Exocellular GOD Production in Shake-Flask Cultures

Effect of Yeast Extract Addition on Fungal Morphology and Exocellular GOD Production in Shake-Flask Cultures

The effect of the addition of different yeast extract concentrations to the basic minimal medium on fungal growth, exocellular GOD production, and fungal morphology was studied in shake-flask cultures (Fig. 1). The results showed that the addition of increasing concentrations of yeast extract to the basic minimal medium increased the final biomass concentration concomitantly. However, an increase in the final exocellular GOD activity occurred only when yeast extract was added to the medium in concentrations up to 1 to 2 g/L. A further increase resulted in decreasing exocellular GOD activities that declined to nondetectable levels with the addition of yeast extract in concentrations above 6 g/L.

An investigation of the fungal morphology revealed a change from a filamentous to a pelleted morphology on the addition of yeast extract to the basic minimal medium. Further studies on the effect of yeast extract on the fungal morphology showed that increasing concentrations of yeast extract caused the fungal pellets to increase in size but to decrease in density and number per unit volume (Fig. 1B).

The stimulatory effect of yeast extract in concentrations up to 1 to 2 g/L on the exocellular production of recombinant GOD in shake-flask cultures can be attributed to the presence of amino acids, vitamins, and/or other compounds in this complex organic substrate. However, this effect of the complex organic substrate on the exocellular production of GOD is apparently abolished when the addition of higher concentrations of yeast extract causes the formation of bigger and less dense pellets.

Effect of Baffles on Fungal Morphology and Exocellular GOD Production in Shake-Flask Cultures

To study the relation of exocellular GOD production to pellet properties in more detail, the recombinant fungus was grown on basic minimal medium supplemented with 2 g/L of yeast extract in shake flasks with and without baffles (Fig. 2). The presence of baffles is expected to result in higher shear stress, causing the fungus to grow in pellets of smaller size. These experiments revealed that the final CDM increased by only 20% whereas the maximum exocellular GOD activity increased by 140%, when

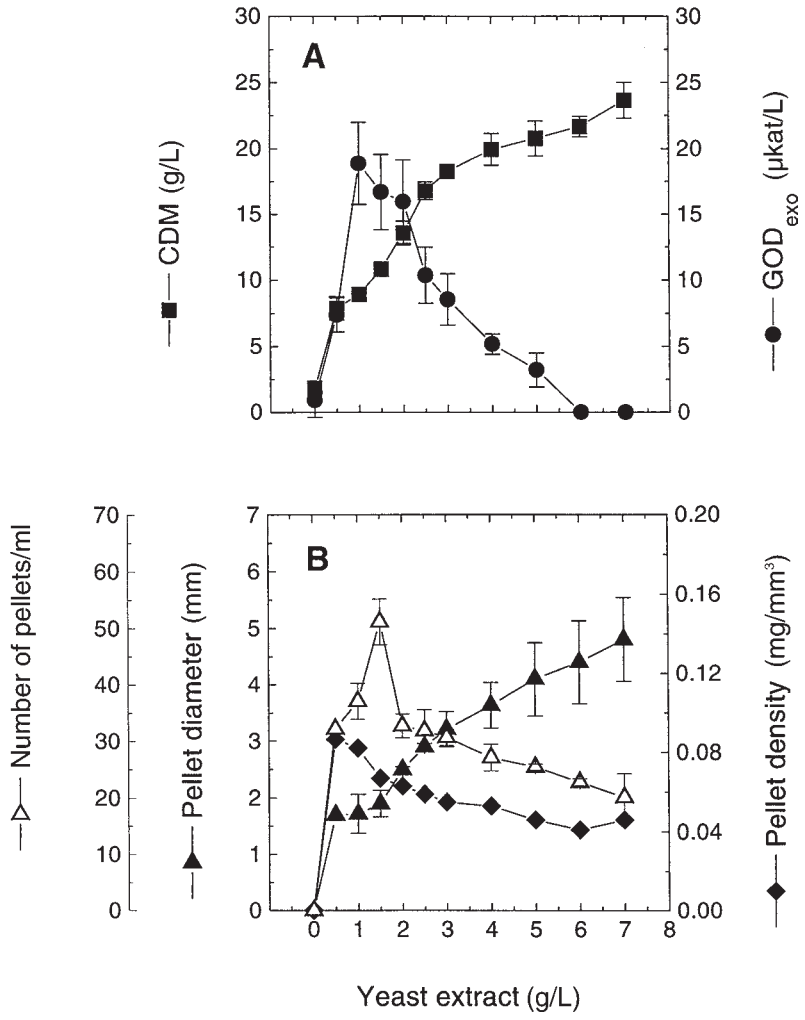

Fig. 1. Effect of yeast extract addition on **(A)** fungal growth and exocellular GOD production, and **(B)** fungal morphology of recombinant *A. niger* NRRL3 (GOD 3-18) grown for 47 h in baffled shake-flask cultures on basic minimal medium supplemented with different concentrations of yeast extract. Error bars correspond to samples taken from three independent shake-flask experiments.

nonbaffled shake flasks were replaced by shake flasks equipped with two baffles (Figs. 2A,B). An analysis of the fungal morphology in these two different types of flasks revealed more and smaller pellets per unit volume with an increased density in baffled compared to nonbaffled shake flasks (Figs. 2C,D). These results clearly demonstrate that in shake-flask cultures, pellets of bigger size and lower density perform less efficiently with respect to the exocellular production of GOD. Microscopic studies revealed that fungal pellets obtained from shake-flask experiments show distinct layers of mycelial density (Fig. 3). The thin outer layer (Layer A) consists of a dense mycelial network. The adjacent and biggest layer (Layer B) shows a

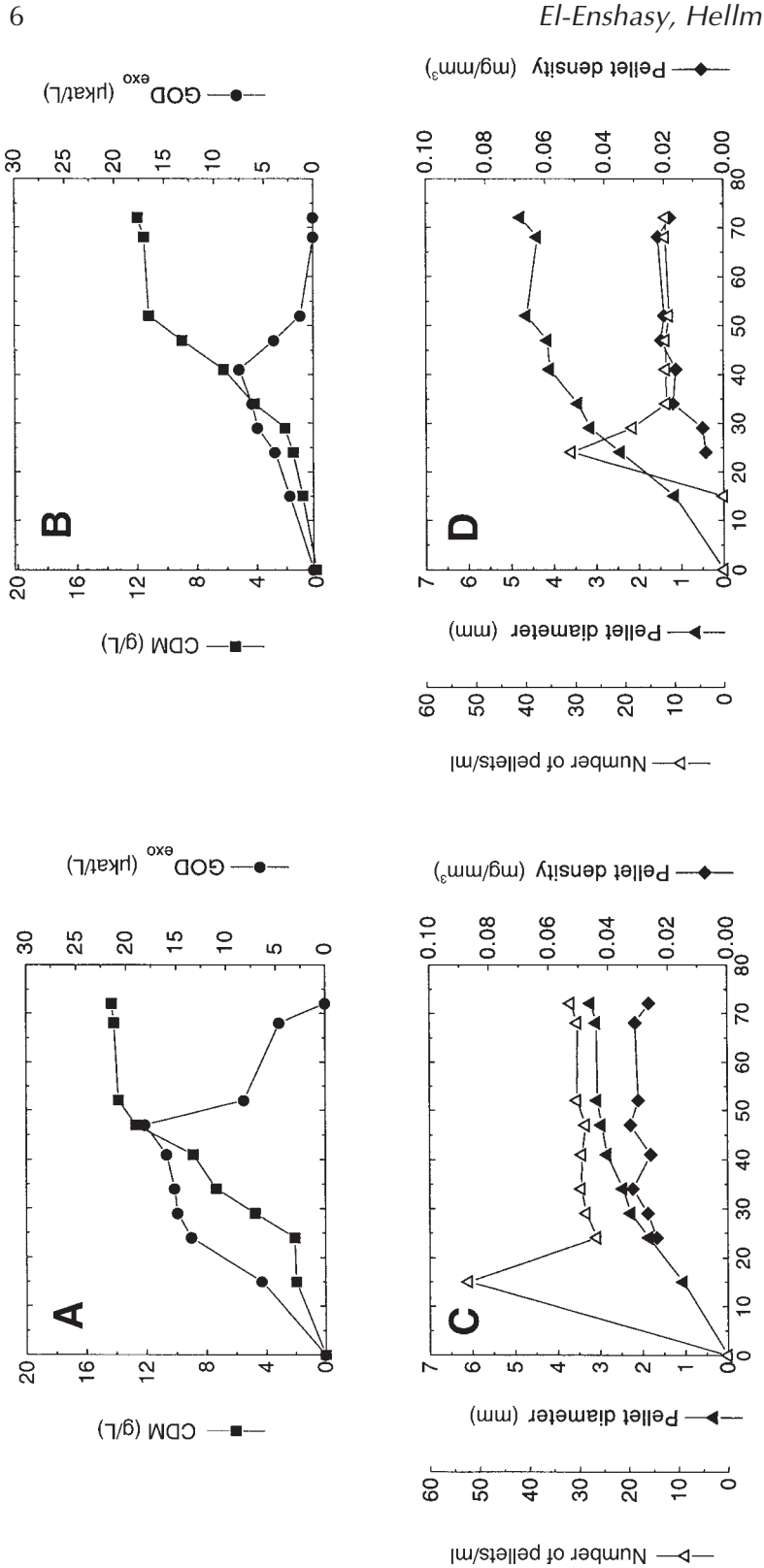

Fig. 2. Effect of shear stress on the performance of recombinant *A. niger* NRRL3 (GOD 3-18) in shake-flask cultures. **(A,B)** Cell growth and exocellular GOD production, and **(C,D)** fungal morphology of cells grown in (A,C) baffled and (B,D) nonbaffled shake flasks on basic minimal medium supplemented with 2 g/L of yeast extract. Each data point corresponds to samples taken from three independent shake-flask experiments. Error bars are not included for more clarity, but are similar to those in Fig. 1.

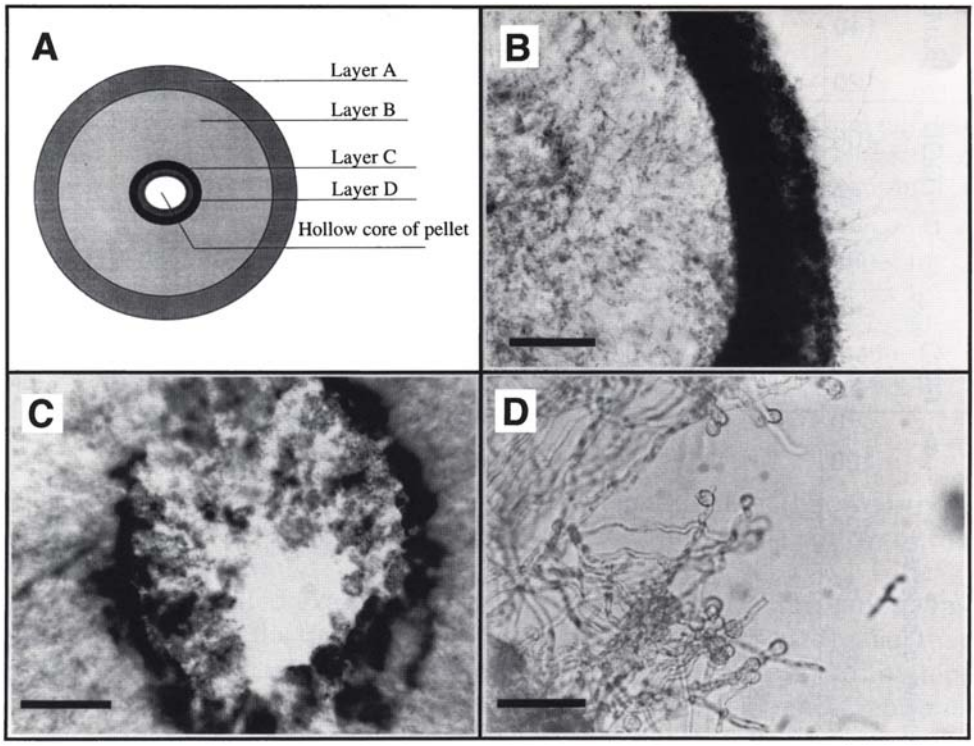

Fig. 3. Microscopic analysis of pellets obtained after cultivation of recombinant *A. niger* NRRL3 (GOD 3-18) for 47 h in nonbaffled shake-flask cultures on basic minimal medium supplemented with 2 g/L of yeast extract. **(A)** Schematic representation of the different pellet layers and microscopic pictures of **(B)** the thin outer layer (Layer A) and the adjacent and biggest layer (Layer B); **(C)** the hollow pellet core surrounded by Layers B–D; and **(D)** details of the hollow pellet center. Bars represent (B,C) 300 and (D) 37.5 μm.

strong decrease in the mycelial density. The neighboring inner layer (Layer C) appears to be of intermediate density and is composed of hyphal cells and nongerminated spores. The next layer (Layer D) contains aggregates of nongerminated spores in addition to germinated spores with short hyphal tips. In larger pellets (>2.5–3.0 mm in diameter), the inner core of the pellet appears hollow.

The structural analysis of fungal pellets obtained from shake-flask cultures and the observation that pellets of larger size and lower density perform less efficiently clearly demonstrate that the outer more dense layer exhibits the highest contribution to the exocellular production of GOD. The inner less dense layers are clearly subjected to substrate-limiting conditions resulting in autolytic processes within the inner parts of the pellet. The conclusion that the inner parts of the pellet are subjected to substrate limitation or even starvation conditions is additionally corroborated by the presence of fungal spores in the pellet core.

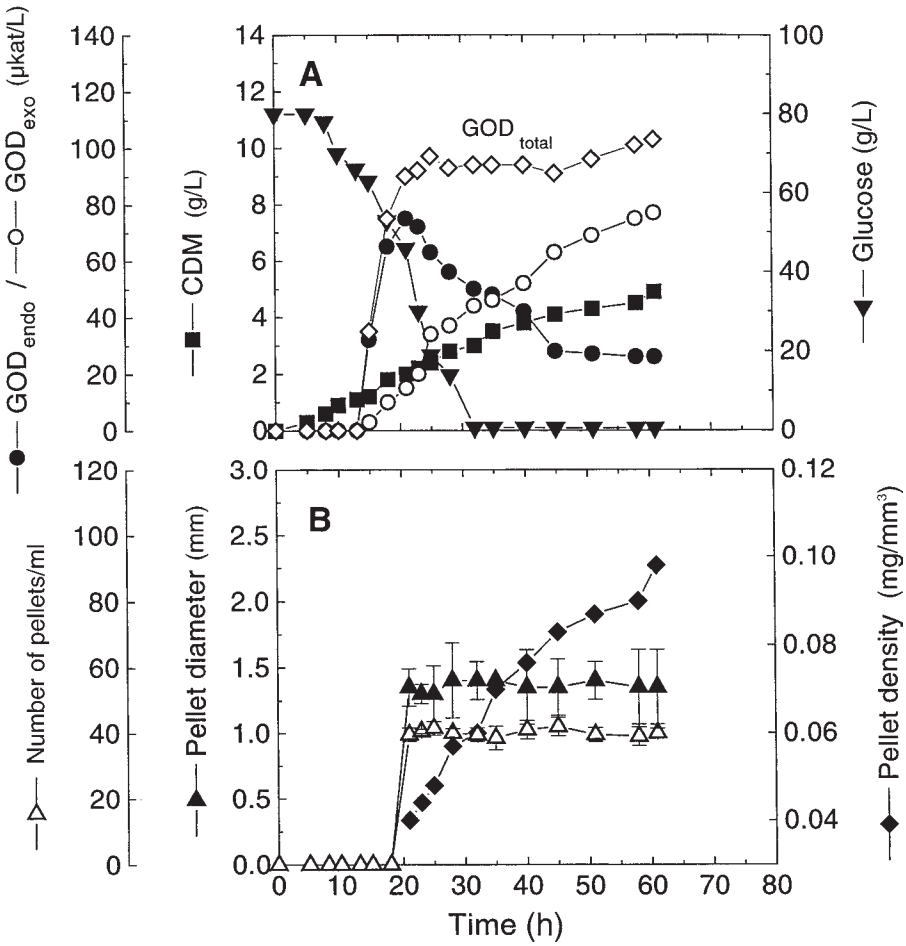

Fig. 4. Cultivation of recombinant *A. niger* NRRL3 (GOD 3-18) in the bioreactor Setric SET002 on basic minimal medium supplemented with 2 g/L of yeast extract. **(A)** Time course of volumetric endo- and exocellular and total GOD activities, CDM, and glucose concentration; **(B)** pellets per unit volume and pellet diameter and density. Bars represent the scattering in pellet size and density.

Production of GOD and the Process of Pellet Formation During Batch Cultivation in a Stirred-Tank Bioreactor

The production of GOD and the process of pellet formation was studied in more detail during batch cultivation in a stirred-tank bioreactor using the same medium composition and inoculum size employed in shake-flask experiments. As described previously, the majority of GOD is produced during the first 20 h of cultivation and, thereafter, is slowly released into the culture medium from its initial cell-bound localization (Fig. 4A; *see also* ref. *6*). A morphological analysis revealed the appearance of defined fungal pellets after 20 h of cultivation (Fig. 4B). Afterward, pellets did not show any further change in numbers per unit volume and size (Fig. 4B). However, the

Table 1
Process of Pellet Formation
During Bioreactor Cultivation of Recombinant *A. niger*

Cultivation time (h)	Number of free spores (per mL)	Number of aggregates (per mL)	Average diameter of aggregates (mm)	Total number of bioparticles (per mL)
0	1.0×10^7	—	—	1.0×10^7
5	2.1×10^4	1.9×10^4	0.045 ± 0.03	4.0×10^4
10	3.0×10^3	1.0×10^3	0.150 ± 0.05	4.0×10^3
15	—	1.2×10^2	0.600 ± 0.20	1.2×10^2
20	—	40	1.300 ± 0.20	40 ± 4
30	—	40	1.300 ± 0.20	40 ± 4

CDM continued to increase after the first 20 h of cultivation (Fig. 4A), indicating that the average pellet density increased during the course of the cultivation (Fig. 4B).

A more detailed examination of the process of pellet formation was carried out during the first 20 h of cultivation. These analyses revealed that pellet formation occurred in two steps (Table 1, Fig. 5). During the first step, free spores aggregated to form spore clusters varying in size and shape and containing different numbers of spores (Table 1, Fig. 5A). The spores in these preliminary aggregates germinated, and the appearance of small flocculates surrounded by a loose hyphal network was observed 10 h after inoculation (Table 1, Fig. 5B). A further decrease in the number of aggregates during the following 10 h of cultivation revealed that these flocculates did not transform into the final pellet but agglomerated with each other to form larger aggregates (Table 1, Fig. 5C). The full-size pellets were obtained 20 h after inoculation (Table 1; *see also* Figs. 4B and 5D). Thereafter, the size of the fungal pellets and their numbers per unit volume did not increase further. A microscopic investigation of pellets obtained after 30 h of cultivation revealed that during the course of the cultivation, the mycelial density of the pellets increased and the pellets developed a less hairy surface (Fig. 5E). This observation is in agreement with the preceding observation that the increase in the cell dry weight with culture time is not reflected in an increase of the pellet size and numbers per unit volume, but in an increase of the pellet density (Fig. 4B).

Conclusion

Our comparative study of the results obtained from shake-flask and bioreactor cultures revealed that fungal pellets with decreasing size perform more efficiently with respect to the exocellular production of GOD (Table 2). The decreasing pellet size correlates with an increased mycelial density, indicating an improvement of the transport of nutrients to the inner parts of the pellet. However, a direct comparison of the performance

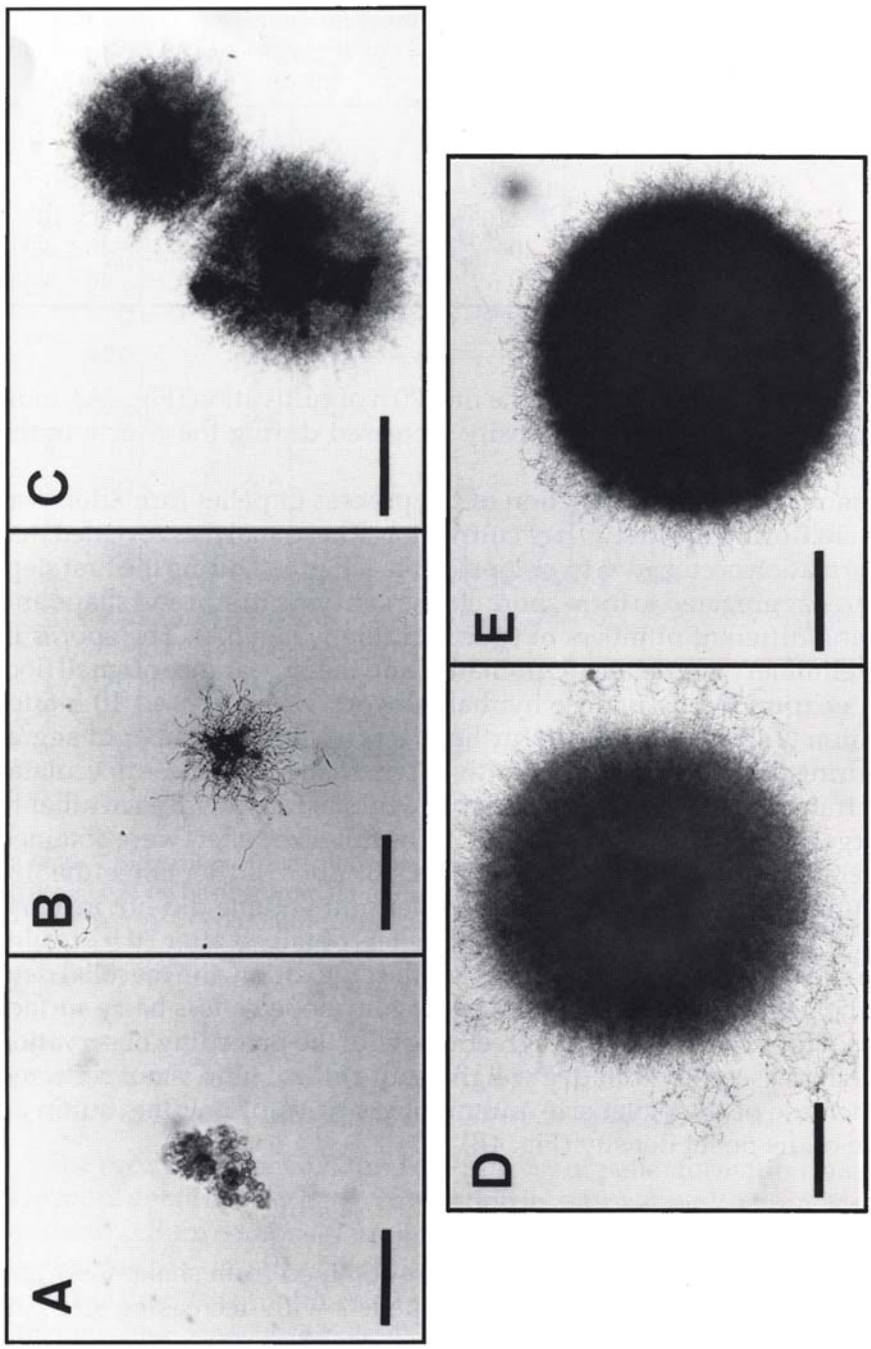

Fig. 5. Microscopic analysis of pellet formation during bioreactor cultivation of recombinant *A. niger* NRRL3 (GOD 3-18). Microscopic pictures of samples taken at **(A)** 5, **(B)** 10, **(C)** 15, **(D)** 20, and **(E)** 30 h postinoculation. Bars represent (A) 37.5, (B) 75, and (C–E) 300 μm.

Table 2
Exocellular Production of GOD and Pellet Morphology
in Shake-Flask and Bioreactor Cultures of Recombinant *A. niger*[a]

	Shake flask		
	Nonbaffled	Baffled	Bioreactor
Volumetric GOD activity (μkat/L)	7.4800	18.1300	77.0000
Specific GOD activity (μkat/g of CDM)	2.6800	6.5100	16.6600
Pellet diameter (mm)	4.7700	3.2100	1.3000
Pellet density (mg/mm^{-3})	0.0220	0.0326	0.0980

[a]Cultures were grown on basic minimal medium supplemented with 2 g/L of yeast extract.

of fungal pellets in shake-flask and bioreactor cultures is not feasible owing to the more efficient aeration and the controlled pH in bioreactor cultures. For example, in shake-flask cultures the formation of gluconic acid causes the pH to decline to 3.0 (data not shown), a pH value that causes the irreversible inactivation of GOD (*[13]*; *see also* Figs. 2A,B).

Acknowledgment

H. El-Enshasy wishes to express his gratitude to the Deutscher Akademischer Austauschdienst (DAAD), Bonn, Germany, for a doctoral fellowship. Also, we would like to acknowledge the continuous support of W.-D. Deckwer.

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