Acknowledgements

We thank D. Cheng, S. Choi and S. Wilcoxen for technical assistance; M. Horvath, H. Liang, P. Schlunk, H. Tager and H. Zabin for helpful discussions; and P. Gardner and D. Steiner for generous gifts of oligodeoxynucleotides. We acknowledge support (to TCT) from Merck Sharp and Dohme Laboratories, the Bristol Myers Company, the Duchossois Foundation, the NIH and from an NSF Presidential Young Investigator Award. WSS was supported by the Medical Scientist Training Program.

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Mycelial morphology and metabolite production

Sergei Braun and Susan E. Vecht-Lifshitz

Mycelial microorganisms are exploited extensively in the commercial production of a wide range of secondary metabolites. They can be cultured as free mycelia, as aggregated forms (pellets/flocs), or as artificially bound/entrapped cells, though problems are associated with the culture of each morphological type. Since the morphological type can strongly influence metabolite production, the methodology for inducing pellet formation, and the type of pellets produced are an important consideration for effective metabolite production.

Filamentous microorganisms, moulds and actinomycetes, account for the majority of industrial fermentations, in terms of bulk as well as the diversity of metabolites produced (see Glossary)[1]. These microorganisms grow as long, thin, branched threads of mycelium. At high biomass concentration, mycelial suspensions constitute non-Newtonian fluids. These are very viscous[2], except in the region near the impeller (Fig. 1). The impeller blades cause high shear stresses. In this region the broth is well aerated, but overheated due to poor heat transfer, whereas at the periphery of the fermentor, the zone adjacent to the cooling surface is stagnant, oxygen-starved and overcooled (Fig. 1). Only a small part of the fermentor, volume, therefore, may be maintained at the optimal production conditions. Increasing the agitation rate improves the overall homogeneity, though this also raises the power consumption and often damages the cells due to high shearing. Hence, these filamentous organisms, with metabolic characteristics of commercial interest, are far more difficult to use industrially than 'well-behaved' (in Newtonian terms, see Glossary) bacterial or yeast cells.

S. Braun and S. E. Vecht-Lifshitz are at the Biotechnology Unit, Institute of Life Sciences, the Hebrew University of Jerusalem, Jerusalem 91904, Israel.

Pellets and immobilized cells

Considerable improvement in the rheology of cultures of filamentous mycelia may be achieved by using either mycelial pellets (see Glossary)[2] or compact beads of immobilized cells.

On a macroscopic scale, a well-stirred fermentor containing mycelial pellets seems fairly homogeneous. On a microscopic scale, however, the heterogeneity imposed by mass-transport limitations within the pellet becomes apparent. Pellets[3] (see Fig. 2) and immobilized cells[4]

are subject to both external and internal mass- and heat-transfer limitations. In these cases, growth occurs only in a peripheral zone (W) where a good oxygen supply is available.

Below the growth zone are layers of biomass subject to various conditions of mass-transfer limitation. Wittler *et al.*[5] used histological staining to discern four different layers in the pellets of *Penicillium chrysogenum*. The outer layer (L1, 60% vol. in 2 mm pellet), two intermediate layers (L2 and L3, 38%) and the central layer (L4, 2%). The outer layer was rich in cytoplasm. Growth occurred at the outer boundary of this layer (W, 25–100 μm)[1,3], but even at high nutrient concentrations, the thickness of the L1 layer never exceeded 0.4 mm. The intermediate layers were partially lysed, while in the centre, the hyphal structure had disintegrated completely. Similar pellet morphology is observed in *Streptomyces tendae* (Fig. 2) and other *Streptomyces* species[6].

Glossary

Arthrospore – a cell formed by fragmentation of hyphae in fungi.

General metabolite – a metabolic intermediate or product found in most living systems, essential to growth and life, synthesized by a limited number of biochemical pathways.

Mycelial pellets – in the pellet form mycelium develops as spherical stable aggregates, consisting of branched and intertwined networks of hyphae (trap-nets).

Newtonian fluid – a fluid, the viscosity of which is independent of the rate of shear.

Pycnidium – an asexual, hollow, flask-shaped fruiting body, lined inside with asexual pycnidiophores.

Secondary metabolite – a metabolic intermediate or product, formed as a differentiation product in the restricted taxonomic groups, not essential to growth and life of the producing organism, and synthesized from several general metabolites by a wider variety of pathways than is available for general metabolism.

Trap-net formation – a stabilization of mycelial aggregates by the mechanical entanglement of filaments.

Factors affecting pellet formation

Application of mycelial aggregates to metabolite production depends upon obtaining uniform pellets of a desired size. This is not easily accomplished, since many factors[7] influence pellet formation (Table 1).

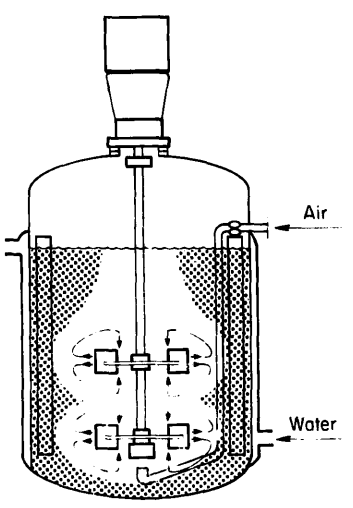
The morphology of a filamentous microorganism developing in any fermentation system may be represented as a final result of competing influences; an equilibrium between forces of cohesion and of disintegration. The factors involved are summarized in Table 1. Shear forces may be unambiguously assigned the role as disintegrating factors. At pH values above 5.5, cell walls of most microorganisms are negatively charged, tending to cause separation of the aggregating cells by electrostatic repulsion. This may be suppressed by an increase in ionic strength, or bridging cells with Ca^{2+} ions. Addition of polycations[8,9] usually induces aggregation, whereas polyanions suppress it[9,10].

Genetic factors influence the cell-wall composition and surface properties, and determine formation and composition of a slime layer. Genetic and environmental factors govern production of surface-active agents, and of lectins. Both of these affect forces of cohesion and/or repulsion between cells. Attachment of growing hyphae to solid substrates may act as a nucleus for forming pellets[7,11]. Surface modification affects the aggregation directly. However, it is difficult to assign some factors (e.g. growth rate, limiting nutrients or inoculum size) as being responsible specifically for cohesion of disintegration of mycelial aggregates.

Assessing the factors presented in Table 1 even for the familiar, industrially important *Aspergillus* and *Penicillium* spp leads to contradicting reports[2,7,12,13]. Attempts to treat pellet formation as a general phenomenon (in contrast to studying pellet formation in a specific strain), are frequently met by industrial microbiologists with considerable mistrust. Remarkably, the effect of various fermentation parameters on pellet formation seems to be quite similar in filamentous systems as genetically remote as fungi and actinomycetes. Thus, in *P. chrysogenum* (B. Metz, PhD thesis, Delft Technical University, the Netherlands, 1976), *A. niger*[11], *Streptomyces tendae*[14], and *S. griseus* (Vecht-Lifshitz and Braun, unpublished), pellets are formed at inoculum below 10^{11} spores m^{-3} (fermentor volume); at higher inocula, filamentous growth prevails.

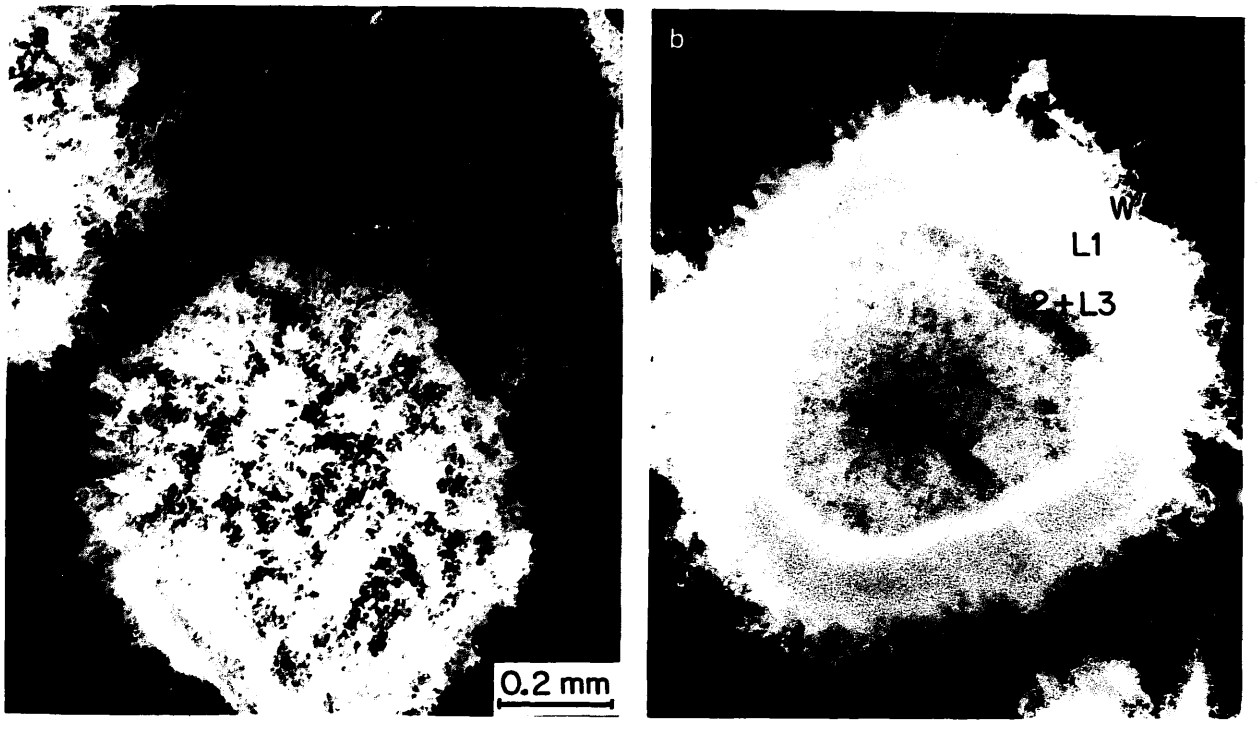
Factors favoring increased growth rate, such as media rich in easily assimilable nutrients, reduce pellet formation in fungi[15] (B. Metz, op. cit.) and in actinomycetes[14]. Such observations led to a limitation hypothesis[15] (B. Metz, op. cit.), which suggested that the lack of any particular nutrient, including oxygen, induces pellet formation. Indeed, increased mycelial aggregation was noted as a consequence of nutrient limitation, especially nitrogen[14,15]. There are, however, quite a few reports contradicting the limitation hypothesis with respect to oxygen. Hockenhull[16] stressed that the pellet morphology predominates in the early life of a culture (where oxygen supply is sufficient), while dense, older cultures tend to be filamentous. We have observed[14] the

Fig. 1



A typical viscous stirred tank fermentation; near the blades the broth is well aerated, but the zone adjacent to the cooling surface (the peripheral, shaded, area) is stagnant and starved of oxygen.

Fig. 2



Mycelial pellet of S. tendae. *(a) SEM micrograph of a 1 mm pellet. (b) Cross section of* S. tendae *pellet (120 μm thick). The outer layer (L1) with its growing boundary (W), partly lysed middle layers (L2+L3), and the lysed central zone (L4) are distinct.*

same phenomenon in *S. tendae*. At high inoculum sizes, aggregated growth morphology was observed early in fermentation. The onset of oxygen limitation coincided with the disruption of pellets and the beginning of a predominantly filamentous growth[14].

Reflecting on the surprising similarity between fungi and actinomycetes in aggregation patterns, Lawton *et al.*[6] recently stated:

> 'At the present time it is not known why an actinomycete should produce a particular kind of morphology in shaken liquid culture. It may be that production of morphological types may be under similar controls to those involved in fungal pelleting... Pellets may originate from spores coagulating together, freshly germinating spores aggregating, or by mycelial entanglement'.

The last sentence refers to coagulative pellet formation mechanism proposed by Takahashi and Yamada[17]. In some fungi, spores coagulate whilst germinating and give rise to a net (trap-net) of intertwining hyphae. Pellet formation in *A. niger* occurs through such a mechanism.

Spore coagulation may play a role in pellet formation, provided that trap-nets develop, though pellets frequently form in the absence of any visible spore agglutination. Even in *A. niger*, the number of pellets equals the number of initial spore clumps only at a low power input; with increased power input, the spore to pellet ratio tends towards unity in *A. niger*[18], whereas in shear sensitive *S. tendae* pellets, this ratio may reach several orders of magnitude below unity[14].

Table 1

Factors affecting aggregation

Microbiological	Physico-chemical
Genetic	Shear forces
Cell-wall composition	Surface-active agents
Inoculum size	pH, temperature
Growth rate	Ca^{2+} ions
Nutrition	Ionic strength
Carbon:nitrogen ratio	Suspended solids

This ratio (one pellet formed from one spore or less) results from new pellets arising through pellet fragmentation. Hence, the cohesive forces underlying the pellet formation mechanism should be sought among the adhesive interactions between hyphal surfaces.

Pellet formation and cell adhesion

A wider view of interactions between microbial surfaces, beyond pellet formation, includes a range of adhesion and aggregation phenomena[7,19]. These occur in most classes of eukaryotes (plant and animal) and prokaryotes.

In cell adhesion and aggregation, there are two distinct stages[19]: (1) 'flocculation of once discrete cells'; and (2) 'flocculent growth without separation of daughter cells from their parents (aggregation and microbial film formation)'. In pellet formation, initial adhesion of spores or hyphae is analogous to the first stage, while trap-net formation is analogous to the second. The distinction between the initial flocculation and subsequent flocculent growth is based on the different energetics of the two stages: two cells

will aggregate when they have sufficient kinetic energy to overcome an energy barrier as they approach each other from an infinite separation, whereas two cells will remain joined when they have insufficient energy to overcome the energy barrier from zero separation[19].

The analogy between the processes of adhesion and pellet formation leads to a more meaningful assignment of dispersive and cohesive forces (Table 2).

One can discriminate between specific and non-specific cell adhesion[20]. The interaction is termed specific if biological recognition of the receptor–ligand type is observed. Thus, in flocculating yeasts, cell–cell aggregation has a lectin–carbohydrate based mechanism. Two mitochondrial genes involved with the mechanism of yeast flocculation have been identified[21]. It is not known whether similar interactions are involved in pellet formation in fungi. However, the lectin–carbohydrate mechanism has been demonstrated to be operational in fungus–plant, fungus–nematode and fungus–fungus interactions[22].

Non-specific cell cohesion phenomena are common to a variety of biological systems, as diverse as formation of bacterial films, platelet adhesion, biological fouling. Such adhesion appears to depend upon surface hydrophobicity, which occurs above the critical surface tensions[23] of 20–30 dynes cm^{-1}. Surface hydrophobicity determines pellet formation by *S. tendae*: modification of surface hydrophobicity by surface-active agents affected both pellet formation in the fermentation system, and the ability of free mycelial to flocculate[14,24]. Natural surfactants have been shown to regulate aggregation of microorganisms[25], and such soluble surfactants may be present in *S. tendae*[14,24].

Once the initial adhesion is achieved, trap-net formation stabilizes the forming pellet in mycelial microorganisms. The trap-net effect may be considerable, especially in fungi, with their sturdy hyphae. The anionic detergent Carbopol dispersed spore aggregates of *A. niger*, but had little effect on preformed pellets, indicating the role of the trap-net effect in pellet stability[8].

Consideration of diverse pellet formation phenomena provides some indication of ways in which pellet formation may be induced in fermentation cultures (Box 1).

Table 2

Forces between microorganisms affecting aggregation/ dispersion in fermentation processes[a]

Factors favoring dispersion
Electrostatic forces
Steric interaction forces
Excretion of material between cells
Hydrodynamic forces (shear stress)
Factors favoring aggregation
Entrapment
Incipient flocculation (interaction between attached or adsorbed molecules)
Charge-mosaic interaction (flocculation by cationic polyelectrolytes)

[a] Data from Ref. 20.

Aggregates as differentiated mycelial tissue

Industrial filamentous microorganisms, both eukaryotic and prokaryotic, belong to the spore-forming species, and possess two types of metabolism – general and secondary (see Glossary). The transition from general to secondary metabolism under a variety of external and internal stimuli is, in fact, a process of cell differentiation. In mycelial aggregates, the high level of cell-to-cell interaction and signalling, resulting from short diffusional distances, leads to a state of differentiation qualitatively different from that of free dispersed mycelia. Mycelial aggregates may be viewed, not merely as mechanical conglomerates, but rather as complex differentiated tissues, phenotypically characterized by a specific set of metabolic activities. The closest analogy to this concept would be the obvious distinction between the unicellular and multicellular forms of a slime mould.

A differentiated phenotype may be characterized by features such as asexual sporulation[11] of moulds in submerged cultures which are rarely achieved in free mycelia, and which frequently require mycelial aggregation. In *Ampelomyces quisqualis*, development of pellet morphology is a prerequisite of mycelial differentiation into pycnidia (see Glossary) (S. Braun, unpublished). Cell immobilization has similar effects on differentiation of moulds. Shifts from general to secondary metabolism in pellets also reflects differentiation – the cumulative alkaloid production by immobilized *Claviceps paspali*[26] mycelium was seven times higher than that produced from free cells. The immobilization shifted fungal metabolic activities toward secondary metabolism. This shift correlated with morphological differentiation into arthrosporoid-like cells[26].

Metabolic activity in pellets

Formation of mycelial pellets is, in some instances, a prerequisite for successful production of secondary metabolites (such as itaconic and citric acids[2,13], and some fungal enzymes[27,28] such as polygalacturon-

Box 1

Possible approaches for inducing mycelial pellet formation in fermentation culture

- Use nitrogen-limited medium.
- Do not use readily assimilated carbon sources.
- Add cationic polyelectrolytes.
- Add mild non-ionic detergents.
- Add Ca^{2+} ions.
- Use small inoculum.
- Reduce shear.
- Reduce temperature.
- Maintain medium at about pH 5–6.
- Increase dissolved oxygen concentration.

Fig. 3

Cross-section (160 μm thick) of a three-day-old pellet of Gibberella fujikuroi. *The dark ring is a deposit of the secondary metabolite bikaverin pigment, restricted to the L1 layer.*

idase or α-glucosidase). In other cases, the free mycelial morphology is preferred for optimal metabolite formation, in which case dispersion techniques should be employed[7,8].

Differentiation of mycelia during pellet formation results in striking effects on enzyme production. Thus, polygalacturonidase synthesis correlates well with the mycelial morphology of *A. niger*. The more compact the pellet, the greater the polygalacturonidase synthesis. Regardless of the medium used, an increase of almost two orders of magnitude in the polygalacturonidase concentration and production rates between the diffuse mycelium and the pellet mycelium was observed[27]. Similar increases were observed in α-galactosidase synthesis by mycelial pellets of *Mortierella vinaceae*[28]. Such phenomena may be related to diffusional limitations in pellets, which either reduce the extent of catabolic repression in pellets[29] or limit the oxygen supply[30] (thus, for example, preventing oxidative inactivation of a specific set of enzymes). The magnitude of the difference between enzyme production in pellets and in the free mycelia at different concentrations of catabolites[27] seems to indicate the existence of additional factors, such as gradients of metabolic products in pellets serving as biological signals (modulators).

Metabolic activators in mature pellets?

Metabolites such as organic acids directly regulate the polygalacturonidase production by *A. niger*[31] and cellulolytic enzymes from *Rhizoctonia solani*[32]. In a non-filamentous aggregated system (e.g. *Clostridium butyricum* flocs), the organic acid gradient within aggregates also induced metabolic changes[33]. Whereas dispersed growth of *Clostridium butyricum* resulted in the production of acids, aggregates of this microorganism produced alcohols, probably due to the accumulation of acids within the aggregate.

Certain secreted metabolites can accumulate in pellets (or in otherwise-immobilized mycelia), which are involved in overproduction of antibiotics in actinomycetes. Several low-molecular-weight, secreted antibiotic-biosynthesis regulators[34] have been detected in numerous species of *Streptomyces*: factor 1 in *S. aureofaciens*, factor IM (inducing material) in staphylomycin biosynthesis of *S. virginiae*, and A-factor of *S. griseus*. The ability of a specific *S. griseus* strain to produce streptomycin correlated well with A-factor formation[34].

In filamentous cultures of *S. tendae*, production of nucleoside–peptide antibiotics, nikkomycins (Nk), is limited by dissolved oxygen[35]. Small pellets would therefore be expected to be less effective than large ones in Nk production. We have, however, observed (Vecht-Lifshitz and Braun, unpublished) an increase in the apparent effectiveness (productivity versus size), for the production of Nk in pellets larger than 1 mm. Although Nk precursors are produced in small pellets and free mycelia, more mature secondary metabolites are characteristic of larger pellets. The cell lysis which occurs in the central layer of *S. tendae* pellets larger than 0.9 mm may release activators of secondary metabolism. If such a maturation phenomenon is general, it could be used to direct secondary-metabolite biosynthesis towards a specific metabolite by regulating the size of mycelial pellets.

A similar effect of the aggregate size upon productivity was observed in plant cell cultures of *Tagetes patula* (marigold)[36]. The thiophene production in these cell aggregates increased with increasing aggregate diameter over 3 mm. It reached a maximum at 11–13 mm and decreased at larger sizes. Calculation of oxygen profiles has shown the critical diameter for oxygen uptake to be

about 3 mm. The aggregates with a diameter exceeding 5.4 mm showed cavitation at the centre. Hulst *et al.*[36] have put forward two alternative explanations of this phenomenon, either (1) lack of oxygen directly stimulates the inner cell to produce thiophene, or (2) cell lysis in the aggregate centre impels the remaining viable cells to produce thiophene.

Gradients of nutrients and metabolites within the compact tissues, formed either by natural aggregation processes or by artificial immobilization of microorganisms, induce secondary metabolism within the pellet. The proliferating outer layer does not undergo this shift. Low oxygen tension in the innermost layers (L2–L4) makes them less productive. The synthesis of secondary metabolites would be expected to be restricted to a well-defined layer coinciding probably with the inner part of L1 layer. We have demonstrated this in pellets of *Gibberella fujikuroi* which produce a red water-insoluble pigment, bikaverin (Vecht-Lifshitz and Braun, unpublished). Deposits of bikaverin indeed form a well-defined layer at the depth of 0.24–0.39 mm (Fig. 3).

Prospects for productivity control

Effects of pellet morphology on productivity and composition of secondary metabolites remain unexplored and relatively obscure. Further research may reveal general trends in metabolite regulation patterns in aggregated microbial tissues of various origins. It may give the industrial microbiologist a tool for directing metabolism towards specific metabolites, unavailable under the conditions of free mycelial growth.

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