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REVIEW

# Heterogeneity in the mycelium: implications for the use of fungi as cell factories

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**Abstract** Fungi are widely used as cell factories for the production of pharmaceutical compounds, enzymes and metabolites. Fungi form colonies that consist of a network of hyphae. During the last two decades it has become clear that fungal colonies within a liquid culture are heterogeneous in size and gene expression. Heterogeneity in growth, secretion, and RNA composition can even be found between and within zones of colonies. These findings imply that productivity in a bioreactor may be increased by reducing the heterogeneity within the culture. The results also imply that molecular mechanisms underlying productivity of fungi in bioreactors should not be studied at the culture level but at the level of micro-colony populations or even at zonal or hyphal level.

**Keywords** Fungus · *Aspergillus* · Cell factory · Protein production · Heterogeneity · FlbA

## Introduction

Filamentous fungi efficiently degrade organic substrates. This ability is exploited in various biotechnological processes. For instance, filamentous fungi are used for biodegradation of waste products, solid state fermentation of raw materials, and as enzyme producers (Grimm et al. 2005). Moreover, they are used for the production of compounds from primary metabolism (e.g. organic acids) and secondary metabolism (e.g. antibiotics). Representatives of the *Aspergillus* genus are often used as a fungal cell factory. They have a high secretion capacity, which is illustrated by some strains of *Aspergillus niger* that secrete up to 30 grams per liter of glucoamylase (Finkelstein et al. 1989; Table 1). Moreover, aspergilli can be grown in large scale bioreactors, their physiology is relatively well understood, and their products are Generally Regarded as Safe. The fact that *Aspergillus* species are not very selective with respect to environmental conditions also contributes to their employability as a cell factory. Aspergilli tolerate a wide range of temperatures (10–50 °C), pH values (2–11), salt concentrations (0–34 %), and water activities (0.77–1) (Meyer et al. 2011; Krijgsheld et al. 2013a). Therefore, these fungi can be used for solid-state as well as submerged fermentations.

The fungal biomass within a bioreactor consists of different cell types. Conidia, that are used as inocula of the (pre)culture, aggregate within the liquid medium, a process known as primary aggregation (Lin et al. 2008). Initial pH, agitation, and medium composition determine the degree of coagulation (Metz and Kossen 1977). The aggregated spores germinate. The resulting clusters of germlings can also associate, which is known as secondary aggregation. Germlings of

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**Table 1** Examples of industrial products produced with *A. niger*

| | Product yield (g/l) | Reference |
|---|---|---|
| Citric acid | 130–140 | Papagianni et al. (1998) |
| Glucoamylase | 30 | Finkelstein et al. (1989) |
| Human interleukin | 0.15 | Punt et al. (2002) |
| Immunoglobulin IgG | 0.9 | Ward et al. (2004) |
| Itaconic acid | 2.5 | Li et al. (2013) |

primary and secondary aggregates grow out forming hyphae that extend at their apices and that branch subapically. Ultimately, the culture can have different macroscopic appearances, ranging from single hyphal elements, known as disperse mycelium, networks of hyphae, up to distinct micro-colonies known as pellets (Grimm et al. 2005). Bioreactor cultivations have been optimized for decades in terms of productivity. So far, there is not a simple relation between morphology and productivity. Some bioreactor cultivations are based on disperse growth, others on pelleted growth (Grimm et al. 2005). For instance, formation of large micro-colonies coincides with increased release of glucoamylase in *A. niger* (Papagianni and Moo-Young 2002). The appearance of the mycelium depends on the inoculum, fluid dynamics, medium composition, gradients of nutrients around and within the mycelium, and the physiological state of the fungus. Recent findings indicate that not only morphology but also heterogeneity within the culture determines productivity. This is the subject of this review, with emphasis on heterogeneity in *A. niger*.

## Heterogeneity between micro-colonies within a liquid culture

The complex object parametric analyzer and sorter (COPAS) device has been used to analyze and sort micro-colonies of liquid cultures of *A. niger* (de Bekker et al. 2011a; van Veluw et al. 2013). COPAS analysis and light microscopy showed that the micro-colonies differ in size (Fig. 1). In fact, statistical analyses demonstrates the existence of two populations of micro-colonies that differ in diameter. For instance, the average diameter of small and large micro-colonies is 505 and 595 μm, respectively, for one strain and 605 and 755 μm for another strain. Gene expression is also heterogeneous between micro-colonies within a liquid culture (de Bekker et al. 2011a). The population of micro-colonies highly expressing the glucoamylase gene *glaA* or the ferulic acid esterase gene *faeA* is smaller than the population of micro-colonies with a large diameter. This indicates that heterogeneity in *glaA* and *faeA* expression in a liquid culture is not only caused by the heterogeneity in the size of the micro-colonies. Indeed, heterogeneity in gene expression is observed between micro-colonies with one particular diameter (B Recter and HAB Wösten, unpublished results). Notably, the fraction of micro-colonies highly expressing *glaA* and *faeA* is under-represented in the liquid cultures.

Pigments in the cell wall of conidia contribute to heterogeneity in liquid cultures. This is exemplified by the deletion strains of *fwnA*, *olvA* and *brnA* that are affected in the biosynthesis of melanin (Priegnitz et al. 2012; Jørgensen et al. 2011). Gene *fwnA* encodes a polyketide synthase. Absence of this gene results in fawn-colored conidia. The Δ*olvA* and Δ*brnA* strains produce olive and brown conidia, respectively. The function of BrnA is not yet known, while OlvA is expected to convert the heptaketide, naphthopyrone YWA1, into 1,3,6,8-tetrahydroxynaphthalene, which is further modified into melanin (Fujii et al. 2004). The Δ*fwnA,* Δ*olvA* and Δ*brnA* strains form on average larger micro-colonies than a control strain (*i.e.* 790–858 versus 628 μm) (van Veluw et al. 2013), indicating that melanin impacts on colony size. This may be caused by the presence of a higher number of conidia per spore cluster, which is based on the finding that clusters of conidia leading to large micro-colonies contain more spores than those resulting in small micro-colonies (GJ van Veluw and HAB Wösten, unpublished results). The Δ*brnA* and Δ*olvA* strains form heterogeneous cultures like the control strain (van Veluw et al. 2013). However, the differences in average diameter between the populations of large and small micro-colonies is relatively small (i.e. 780 and 842 μm and 825 and 868 μm, respectively). The size of the micro-colonies of the Δ*fwnA* strain is even normally distributed with an average diameter of 818 μm. Taken together, the pigmentation mutants form larger and more homogenous micro-colonies than strains producing melanin. This may be of importance for the use of *A. niger* as a cell factory. For instance, pigmentation mutants may produce more glucoamylase (see above).

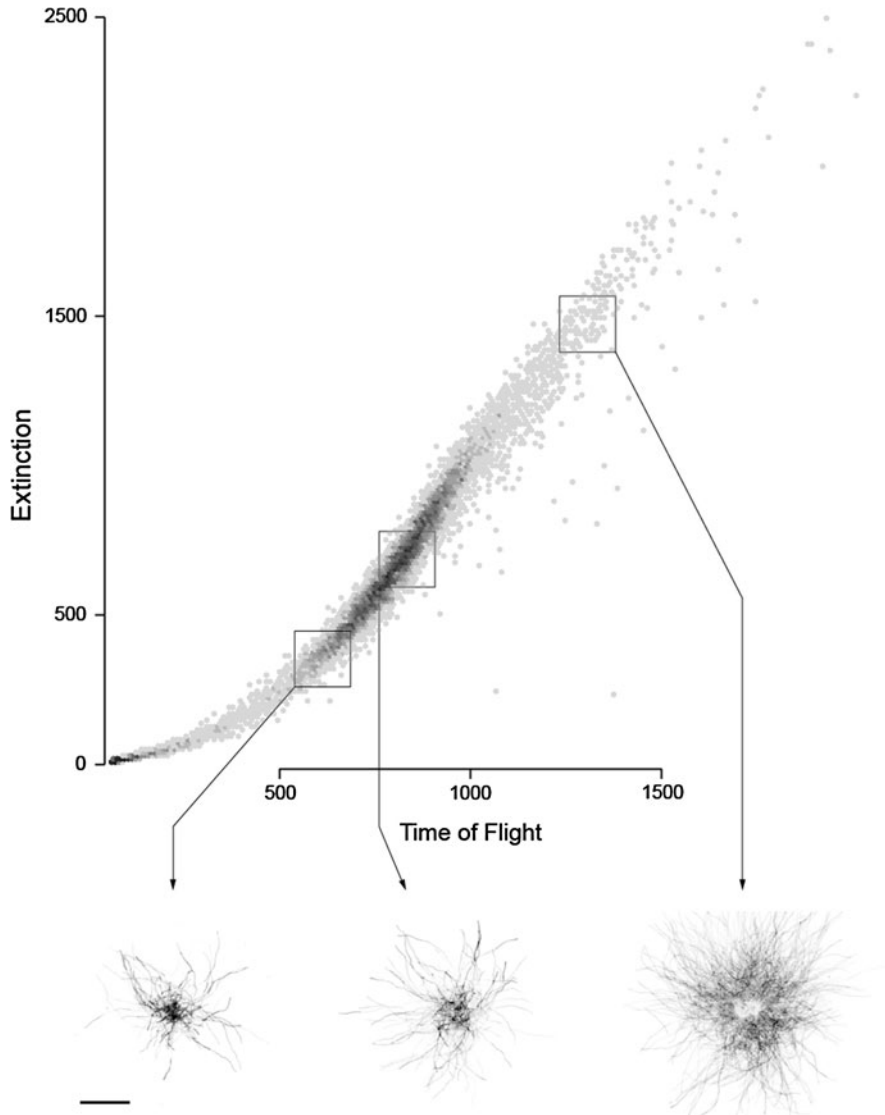


**Fig. 1** Micro-colonies of *A. niger* can be sorted based on size (X-axis) and density (Y-axis) using COPAS. Bar = 200 μm

Surface properties of the conidia of the pigmentation mutants were characterized to assess whether this could explain why these mutants form less heterogeneous cultures with respect to size. Conidia of the Δ*brnA* (van Veluw et al. 2013) and the Δ*fwnA* strains (Priegnitz et al. 2012; van Veluw et al. 2013) are similar in size and display a similar hydrophobicity as the control strain. The latter can be explained by the fact that rodlets are still present at the spore surface of these pigmentation mutants (van Veluw et al. 2013). The surface charge of the Δ*brnA* and the Δ*fwnA* conidia is also similar to that of the wild-type at pH 5–6. In contrast, the conidia of the Δ*olvA* strain have distinct properties when compared to the control strain (van Veluw et al. 2013). Conidia of the Δ*olvA* strain are larger, more negatively charged, highly hydrophilic, and rodlets are almost completely absent. The latter suggests that the pigment in the cell wall of conidia affects assembly of hydrophobins into rodlets, as was shown for glucan polymers (Scholtmeijer et al. 2009).

As mentioned above, the differences in biophysical and structural properties of the Δ*olvA* strain do not affect the size distribution of micro-colonies in liquid shaken cultures when compared to the other pigmentation mutants. This contradicts the work of Dynesen and Nielsen (2003). They showed that hydrophilic conidia of *A. nidulans* lacking the rodlet layer formed

smaller micro-colonies. From these and other data it was concluded that the reduced size of the micro-colonies of the hydrophobin deletion strain is not due to differences in electrostatic interactions but is caused by the surface hydrophobicity of *A. nidulans* conidia. This is in line with the finding that hydrophobic interactions are an important parameter for pellet formation in *A. niger* (Ryoo and Choi, 1999). The results of van Veluw et al. (2013) imply that mechanisms other than surface hydrophobicity can underlie spore aggregation and thus promote the formation of micro-colonies in liquid shaken cultures. It was shown that α-(1-3)-glucan mediates aggregation of swollen conidia of *Aspergillus fumigatus* (Fontaine et al. 2010). This polysaccharide becomes exposed during swelling of the spores. Possibly, α-(1-3)-glucan is already surface exposed in the case of conidia of the Δ*olvA* strain of *A. niger* but not in the hydrophobin deletion strain of *A. nidulans*. This would explain why the hydrophilic conidia of these strains result in different sized micro-colonies.

## Heterogeneity between zones of macro- and micro-colonies

In the previous section micro-colonies of *A. niger* have been discussed. Centimetre-scale macro-colonies that are formed on agar medium serve as a model system to understand heterogeneity within a mycelium such as a micro-colony. Growth, secretion and gene expression can be monitored easily by growing macro-colonies between porous polycarbonate membranes (Wösten et al. 1991). These so called sandwiched colonies of *A. niger* are heterogeneous with respect to growth and secretion. These processes mainly take place at the colony periphery of 5 cm wide colonies. Secretion is also observed in a more central zone, which overlaps with the secondary growth zone. From this and the fact that proteins are released at tips of growing hyphae only (Wösten et al. 1991), it was concluded that growth and secretion are strictly coupled. This is in line with the steady-state growth theory and the bulk flow theory (Wessels 1988; 1993). These theories describe that proteins are not able to diffuse through the highly cross-linked cell walls of non-growing hyphae. Protein release would be accommodated by co-migration with the plastic cell wall polymers that are extruded at the apex of growing hyphae. Interestingly, protein secretion can occur in non-growing zones of a colony (Levin et al. 2007a; Krijgsheld et al. 2013b). Growth was still localized at the periphery when sandwiched colonies had been transferred to fresh medium. In contrast, newly synthesized proteins, as assessed by labeling with radioactive amino acids, were now also secreted in the center. Only a sub-peripheral zone still did not release newly synthesized proteins in the medium. This is explained by the phenomenon of sporulation inhibited secretion (see below). How proteins are released into the culture medium by non-growing hyphae is not yet known. It may be that pores are created in the highly cross-linked cell walls of non-growing hyphae by the action of cell wall-modifying enzymes.

The composition of the secretome of five concentric zones of xylose-grown macro-colonies has been determined by quantitative mass spectrometry (Krijgsheld et al. 2012). To this end, sandwiched colonies had been transferred to a ring plate that consists of five concentric wells filled with liquid medium (Levin et al. 2007b). A total of 59 proteins were detected in the culture medium, of which 26 have a role in plant polysaccharide degradation. Notably, the quantitative composition of the secretome of the five different zones differs. For instance, 6 and 10 proteins were at least 4-fold more and less abundant, respectively in the outer zone when compared to an intermediate zone. Heterogeneity in protein secretion is accompanied by heterogeneous gene expression in the colony (Levin et al. 2007a). 9 % of the active genes in macro-colonies are expressed in only one of the five zones, while 25 % of these genes show at least a two-fold difference in expression between the outer and innermost zone of the colony (Levin et al. 2007a). Moreover, at least 50 % of the zonal heterogeneity in the secretomes can be explained by differences in RNA abundance (Krijgsheld et al. 2012; Krijgsheld 2013). For instance, *faeA* is 5-fold higher expressed at the periphery of xylose-grown colonies, while mRNA of *glaA* is more than 3-fold more abundant at the outer part of maltose-grown colonies (Levin et al. 2007a). The zonal differences in expression in *A. niger* can be explained by both medium dependent and medium independent mechanisms (Levin et al. 2007a). The medium composition (i.e. the concentration and nature of the carbon source) determines about half of the variation in gene expression, whereas the other half is attributed to differentiation processes in the vegetative mycelium.

The 50 most periphery-specific and center-specific genes within macro-colonies of *A. niger* consist of members of many different gene classes (Levin et al. 2007a). This indicates that the periphery and center of the colony are not specialized in for instance secretion (periphery) and uptake of nutrients (center). These data are in contrast with a gene expression study in *Aspergillus oryzae* (Masai et al. 2006). Genes encoding extracellular functions were expressed at the periphery only, whereas transporters were most active in the colony center. This difference in results may be caused by the experimental set-up that was used in *A. oryzae*. In this case, RNA was hybridized with microarrays containing cDNA that had been selected on the basis of an expressed-sequence-tag-sequencing project. This may have created a bias in and between specific gene sets. Still, the data of Masai et al. (2006) show that colonies of *A. oryzae* are heterogeneous in gene expression.

As mentioned above, the sub-peripheral zone of the macro-colony does not secrete newly synthesized proteins when the mycelium has been transferred to fresh medium. This zone starts to sporulate when the porous polycarbonate membrane overlaying the sandwiched colony is removed (Krijgsheld et al. 2013b), indicating that sporulation represses secretion in the vegetative mycelium. To test this hypothesis, zonal secretion in the sporulation deficient Δ*flbA* and Δ*brlA* strains of *A. niger* was monitored. BrlA is the central activator of asexual development in *Aspergillus* (Adams et al. 1988; Wieser et al. 1994). The stalks of the Δ*brlA* strain of *A. niger* and *A. nidulans* do not develop into mature conidiophores (Adams et al. 1988; Wieser et al. 1994; Krijgsheld et al. 2013b). Zonal growth and secretion are not affected in the *Δbrl*A strain of *A. niger* (Krijgsheld et al. 2013b). Apparently, sporulation-inhibited secretion acts upstream of BrlA. Indeed, growth and secretion patterns are changed in the case of the Δ*flbA* colony that does not initiate stalk formation at all (Krijgsheld et al. 2013b). Gene *flbA* encodes a Regulator of G-protein signaling that acts upstream of BrlA (Lee and Adams 1994; Yu et al. 1996). It hydrolyses the intrinsic activity of the active GTP-bound Gα-subunit FadA into its inactive GDP-bound conformation. As such, it represses growth and stimulates sporulation. Growth occurs throughout Δ*flbA* colonies of *A. niger*, irrespective whether they have been transferred to fresh medium or not (Krijgsheld et al. 2013b). This coincides with reduced zonal secretion heterogeneity; secretion takes place at the periphery and in central zones when colonies have been grown continuously on an agar medium. Thus, the non-transferred Δ*flbA* colonies show a secretion pattern similar to that of wild-type colonies that have been transferred to fresh medium. Such a transfer makes the Δ*flbA* colonies to secrete throughout the mycelium. Taken together, it is concluded that FlbA represses vegetative growth and secretion in intermediate and central zones of the colony.

Xylose-grown colonies of the Δ*flbA* strain release 138 proteins with a signal sequence for secretion into the culture medium, as well as 15 proteins with a motif for non-classical secretion (Krijgsheld et al. 2013b). Of these proteins, 30 had not been reported in any secretome study (Tsang et al. 2009; Braaksma et al. 2010; Lu et al. 2010; Ferreira de Oliveira et al. 2010; Ferreira de Oliveira et al. 2011; Krijgsheld et al. 2012). Only a few percent of the 138 proteins are differentially released in the colony (Krijgsheld et al. 2013b), which is about 5-fold less when compared to the wild-type (Krijgsheld et al. 2012). This again shows that FlbA impacts zonal secretion heterogeneity.

The build up of the cell wall explains at least partly why the Δ*flbA* colonies have a more complex secretome. The Δ*flbA* cell wall is thinner compared to that of wild-type hyphae. A thinner cell wall is expected to promote more efficient release of proteins into the culture medium because a large part of secreted proteins is associated with or trapped within the *A. niger* cell wall (Levin et al. 2007b). Such a mechanism was verified by using cycloheximide (Krijgsheld et al. 2012). This protein synthesis inhibitor was used to distinguish between proteins that had been synthesized and secreted into the culture medium immediately after transfer to fresh medium and proteins that had been formed before transfer and that were slowly released in the medium after transfer. Thus, newly secreted proteins in the secretome were expected to be identified by subtracting the secretome of cyclohemixide-treated colonies from non-treated cultures. As expected, less proteins are released at the periphery of cycloheximide-treated colonies (Krijgsheld et al. 2012). However, these colonies release more proteins in intermediate and central zones. A total of 124 and 59 proteins are present in the culture medium of wild-type colonies that have or have not been treated with cycloheximide (Krijgsheld et al.

2012). About 70 proteins are at least 4-fold more abundant in the medium of treated colonies when compared to untreated colonies, while 19 proteins had not been identified in the secretome of *A. niger* before (Tsang et al. 2009; Braaksma et al. 2010; Lu et al. 2010; Ferreira de Oliveira et al. 2010; Ferreira de Oliveira et al. 2011).

From these data, it was concluded that cycloheximide can be used to obtain a near complete qualitative and quantitative secretome of *A. niger*. Cycloheximide-increased protein release is explained by the fact that this protein synthesis inhibitor causes cell walls to become thinner in the inner zones of the colony. Cycloheximide induces partial cell wall degradation as was concluded from transmission electron microscopy studies. The degradation of the cell wall would release proteins that have been trapped in the cell wall or that are associated with cell wall polymers. Notably, cycloheximide treatment does not affect the width of the cell wall of Δ*flbA* colonies and no additional protein is released into the culture medium (Krijgsheld et al. 2013b). Apparently, the cell wall of the Δ*flbA* strain or its synthesizing machinery has a different composition compared to that of the wild-type.

The finding that zones of macro-colonies of *A. niger* are heterogeneous with respect to growth, secretion, and gene expression raises the question whether this is also the case in micro-colonies of this fungus. QPCR could not show zonal differences in the relative levels of 18S *rRNA*, and RNA of *actin*, *glaA*, and *faeA* (de Bekker et al. 2011a). However, the amount of RNA per individual hypha is 45 times higher at the periphery when compared to the center of micro-colonies. This demonstrates that also micro-colonies show zonal differences.

## Heterogeneity within a zone of macro- and micro-colonies

Glucoamylase is secreted at the periphery of macro-colonies of *A. niger* (Wösten et al. 1991). Only part of the hyphae in this zone actually secrete the protein. GFP was used to study the expression of the glucoamylase gene *glaA* in the outer zone of macro-colonies (Vinck et al. 2005). Fluorescence intensity distributions have demonstrated the existence of two populations of hyphae; one that highly and one that lowly expresses GFP. These experiments show that secretion of glucoamylase within the outer zone of the macro-colony is regulated at the transcriptional level. Similar results are obtained when a single spore, a single hypha, or a piece of mycelium is used as inoculum (Vinck et al. 2011). Apparently, variability in *glaA* expression arises during vegetative growth and is not the result of pre-programming in spores or hyphae from which the mycelium originates. A population of highly-expressing and a population of lowly-expressing hyphae also exists in the case of the acid amylase gene *aamA*, the α-glucuronidase gene *aguA*, and the feruloyl esterase gene *faeA* (Vinck et al. 2011). In all cases, the population of lowly-expressing hyphae contributes a high percentage of the total number of hyphae. This indicates that the number of hyphae that actually secrete a particular protein is low, probably far less than 1 % of the total macro-colony. Co-localization studies have shown that hyphae that highly express a gene encoding a secreted protein also highly express other such genes, even when they are induced by different transcriptional regulators (Vinck et al. 2011). These results suggest a higher level of regulation than a mere causative role for the transcriptional regulators of the genes encoding secreted proteins. Indeed, expression of these genes also correlates with expression of the glyceraldehyde-3-phosphate dehydrogenase gene, *gpdA,* and the abundance of 18S rRNA. These results indicate that there are at least two populations of hyphae at the periphery of a colony that can be discriminated by their transcriptional and translational activity (Vinck et al. 2011). Notably, these populations have a similar growth rate. Apparently, a "low" transcriptional and translational activity is sufficient to support growth, while a "high" transcriptional and translational activity would be needed for high secretion of proteins.

Single cell transcriptome analysis was performed to further explore differences in gene expression between neighboring hyphae at the outer zone of macro-colonies of *A. niger* (de Bekker et al. 2011b). RNA from the first compartment of five neighboring hyphae was amplified as cDNA and hybridized to micro-arrays. Up to 7 % of the genes yielded a present call in each of the hyphae. This is low when one considers that about 50 % of the genes are expressed in a sandwiched colony (Levin et al. 2007a). This is explained by the fact that the apical compartment of hyphae only contains 1 pg RNA and that only the most highly expressed genes had been sufficiently amplified to

yield a hybridization signal (de Bekker et al. 2011b). Still, a total of 2,608 genes were expressed in at least one of the five individual hyphae, of which 300 were differentially expressed. Both gene sets comprise all functional gene categories and include *glaA* (de Bekker et al. 2011b). Taken together, neighboring hyphae are highly heterogeneous with respect to gene expression. This heterogeneity may be caused by stochastic gene expression and/or by epigenetic processes.

Preliminary experiments indicate that epigenetic processes are indeed involved in heterogeneous expression of *glaA* (de Bekker 2011). Expression of *glaA* is no longer bimodal when macro-colonies are exposed to sodium butyrate or 5′-azacytidine. These compounds alter the chromatin structure by causing hyperacetylation of histones (Boffa et al. 1978; Sealy and Chalkley, 1978) and by inhibiting histone and DNA methylation (Jones 1985), respectively. Chromatin remodelling (DNA methylation, histone methylation, histone deacetylation and histone acetylation) has been studied to some extent in *Aspergillus*, but not yet in *A. niger*. So far, there is no evidence for DNA methylation in *Aspergillus* (Montiel et al. 2006; Lee et al. 2008). Yet, a predicted DNA methylase was shown to function in sexual development of *A. nidulans* (Lee et al. 2008). It was therefore proposed that the DNA methylases of *A. nidulans* have a low, so far undetectable, methylation activity (Lee et al. 2008). Aspergilli contain various genes encoding histone acetyl transferases (HATs) and histone deacetylases (HDACs). HDACs affect diverse processes in *Aspergillus* including germination, primary and secondary metabolism and growth under oxidative stress (Lee et al. 2009; Tribus et al. 2005; Shwab et al. 2007). The catabolite repressor CreA (which in turn regulates, among others, amylolytic gene expression) depends on the acetylation state of the chromatin. It could thus be that heterogeneous *glaA* expression in *A. niger* is indirectly regulated by the effect of the chromatin structure on CreA.

Distinct populations of hyphae can also be distinguished at the periphery of micro-colonies of liquid cultures (Fig. 2; van Veluw et al. 2013). However, statistical analysis indicates that the heterogeneity in gene expression is less robust. Possibly, signaling between hyphae is involved in promoting heterogeneity. Gradients of signaling molecules may be formed less easily between hyphae that are grown in liquid shaken cultures when compared to a solid medium.

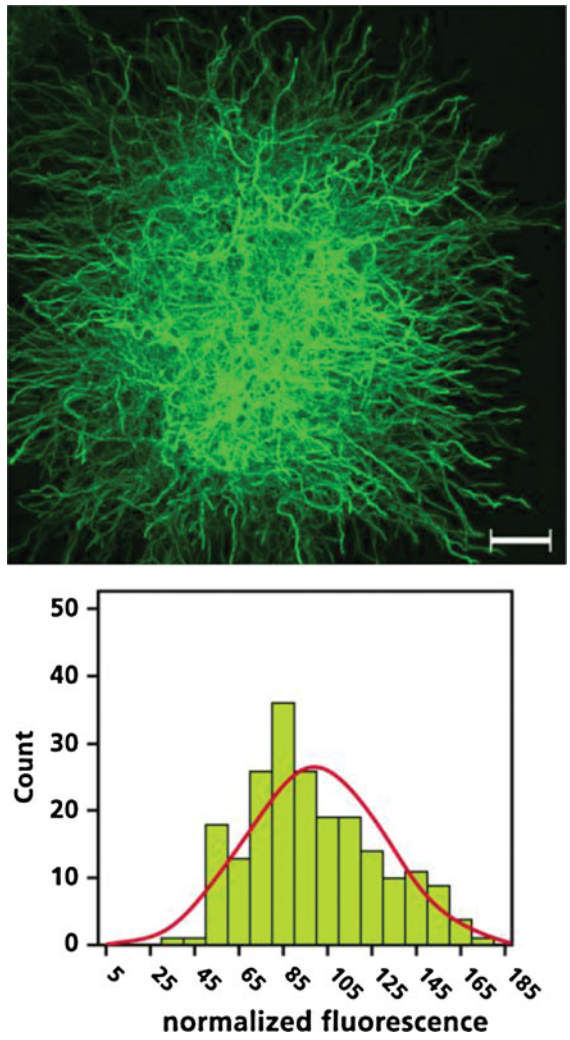


**Fig. 2** The distribution of fluorescence intensity (*bottom*) of hyphae at the periphery of a micro-colony of an *A. niger* strain expressing *GFP* under control of the *faeA* promoter (*top*) cannot be explained by a normal distribution (*red line* in *bottom* panel). There are more hyphae with a low fluorescence intensity as would be expected from such a distribution. Bar = 40 μm

Heterogeneity in gene expression between hyphae at the outer zone of micro- and macro-colonies is a surprising finding considering that it is generally believed that a fungal mycelium shares a common cytoplasm due to the presence of porous septa that allow cytoplasmic streaming. However, in *A. oryzae* about 40 % of the first three septa of hyphae at the periphery of a colony are closed due to plugging by peroxisome-like organelles called Woronin bodies (Bleichrodt et al. 2012). The plugging state of these apical septa neither depends on the plugging state of

neighboring septa nor on the environmental conditions. Woronin bodies also close about half of the apical septa of hyphae at the periphery of colonies of *A. niger*. In this case, temperature and osmolarity of the medium do affect the plugging state (Bleichrodt et al. 2012). Live cell imaging has shown that the presence of Woronin bodies near septa is dynamic, implying that septal closure is reversible. The dynamics of septal closure seems to be a stochastic process in *A. oryzae* but it may be controlled in *A. niger*.

Septal plugging in intact hyphae explains why hyphae can be heterogeneous with respect to RNA and protein composition. The fact that each septum of *A. oryzae* hyphae has a chance of 60 % to be open implies that only in about 1 % of the cases the cytoplasm of two hyphae is in physical contact when they are separated by 9 septa. Absence of Woronin bodies would result in a continuous cytoplasm and, consequently, hyphal heterogeneity would be abolished. Indeed, fluorescence of *glaA*- and *aguA*- driven expression is normally distributed in neighboring hyphae of the Δ*Aohex1* strain of *A. oryzae* that does not produce Woronin bodies. In contrast, distribution of GFP is heterogeneous in the wild-type background (Bleichrodt et al. 2012). We propose that heterogeneous gene expression still occurs in the Δ*Aohex1* mutant. However, since all septa are open, cytoplasmic streaming evenly distributes gene products within the mycelium. Indeed, GFP streams from the vegetative mycelium to aerial structures (Bleichrodt et al. 2013). In contrast, RNA of the reporter does not stream, at least not over these long distances. Future studies should systematically address which molecules in fact stream within and between hyphae.

## Future perspectives

The *A. niger* macro- or micro-colony is highly heterogeneous with respect to growth, secretion and gene expression. Heterogeneity is not only observed between micro-colonies but also between and within zones of colonies. There is quite some evidence that the phenomenon of heterogeneity within the fungal mycelium is widespread in the fungal kingdom (Moukha et al. 1993a,b; Teertstra et al. 2004; Masai et al. 2006; Kasuga and Glass 2008; Etxebeste et al. 2009; Bleichrodt et al. 2012).

Preliminary data indicate that heterogeneity between hyphae increases viability when the colony is exposed to stress conditions like the presence of antibiotics or high temperature (Bleichrodt et al. 2012). By varying the composition of the hyphae, there is a higher chance that some of the hyphae will survive such a particular stress condition. It is tempting to speculate that heterogeneity is not functional in a bioreactor. If so, strains of fungal cell factories can be developed that have reduced heterogeneity and thus would have increased productivity. The fact that the Δ*flbA* strain has a reduced secretion heterogeneity, a more complex secretome, and grows throughout the mycelium illustrates the potential of this strategy.

Heterogeneity in secretion and secretome complexity is partly regulated at the transcriptional level. For instance, part of the genes encoding the complex secretome of the Δ*flbA* strain are up-regulated in this deletion strain when compared to the wild-type (Krijgsheld 2013). Release of proteins in the culture medium is also affected by the cell wall. Cycloheximide treatment reduces cell wall thickness and increases secretome complexity. The complex secretomes of the Δ*flbA* strain and the cycloheximide-treated wild-type strain contain enzymes that had previously not been identified in the secretome of *A. niger*. They may have enzymatic activities that promote plant cell wall degradation, which could be useful in all kinds of industrial applications including bioethanol production. It would be of interest to unravel the transcriptional pathways leading to reduced cell wall thickness and up-regulation of genes encoding secreted proteins.

The fact that microbial micro-colonies are heterogeneous with respect to size and gene expression has implications for how analysis of RNA, proteins and metabolites from whole cultures should be interpreted. By using the whole culture, an average composition or activity of the micro-colonies is determined. This average may by far not reflect the composition or activity of each of the populations within the liquid culture or an individual colony. As a consequence, biological mechanisms may be easily overlooked. Therefore, individual populations or even individual hyphae should be studied to understand mechanisms underlying biological processes in a liquid culture. We have recently shown that FlbA represses directly or indirectly *brlA* in part of the colony of *A. niger*

(Krijgsheld 2013). This would not have been observed when the whole mycelium of the culture had been used for gene expression analysis simply because expression levels would have been leveled out. The fact that FlbA seems to repress *brlA* is interesting because so far FlbA has been described as an activator of this gene.

Future research should not only unravel the mechanisms underlying zonal and hyphal heterogeneity. It should also reveal to which extent compartments of hyphae are heterogeneous with respect to RNA and protein composition and which compartments actually contribute to secretion of proteins into the culture medium. These studies may identify new approaches to improve *Aspergillus* and other fungi as cell factories.

## References


Adams TH, Boylan MT, Timberlake WE (1988) *brlA* is necessary and sufficient to direct conidiophore development in *Aspergillus nidulans*. Cell 54:353–362

Bleichrodt R, van Veluw GJ, Recter B, Maruyama J, Kitamoto K, Wösten HAB (2012) Hyphal heterogeneity in *Aspergillus oryzae* is the result of dynamic closure of septa by Woronin bodies. Mol Microbiol 86:1334–1344

Bleichrodt R, Vinck A, Krijgsheld P, van Leeuwen MR, Dijksterhuis J, Wösten HAB (2013) Cytosolic streaming in vegetative mycelium and aerial structures of *Aspergillus niger*. Stud Mycol 74:31–46

Boffa IC, Vidali G, Mann RS, Allfrey VG (1978) Suppression of histone deacetylation in vivo and in vitro by sodium butyrate. J Biol Chem 253:3364–3366

Braaksma M, Martens-Uzunova ES, Punt PJ, Schaap PJ (2010) An inventory of the *Aspergillus niger* secretome by combining in silico predictions with shotgun proteomics data. BMC Genomics 11:584

de Bekker C (2011) Hyphal heterogeneity in *Aspergillus niger*. PhD Thesis, University of Utrecht, The Netherlands

de Bekker C, van Veluw GJ, Vinck A, Wiebenga LA, Wösten HAB (2011a) Heterogeneity of *Aspergillus niger* microcolonies in liquid shaken cultures. Appl Environ Microbiol 77:1263–1267

de Bekker C, Bruning O, Jonker MJ, Breit TM, Wösten HAB (2011b) Single cell transcriptomics of neighboring hyphae of *Aspergillus niger*. Genome Biol 12:R71

Dynesen J, Nielsen J (2003) Surface hydrophobicity of *Aspergillus nidulans* conidiospores and its role in pellet formation. Biotechnol Progress 19:1049–1052

Etxebeste O, Herrero-Garcia E, Araújo-Bazán L, Rodriguez-Urra AB, Garzia A, Ugalde U, Espeso EA (2009) The bZIP-type transcription factor FlbB regulates distinct morphogenetic stages of colony formation in *Aspergillus nidulans*. Mol Microbiol 73:775–789

Ferreira de Oliveira JM, van Passel MWJ, Schaap PJ, de Graaff LH (2010) Shotgun proteomics of *Aspergillus niger* microsomes upon D-xylose induction. Appl Environ Microbiol 76:4421–4429

Ferreira de Oliveira JM, van Passel MW, Schaap PJ, de Graaff LH (2011) Proteomic analysis of the secretory response of *Aspergillus niger* to D-maltose and D-xylose. PLoS ONE 6:e20865

Finkelstein DB, Rambosek J, Crawford MS, Soliday CL, McAda PC, Leach J (1989) Protein secretion in *Aspergillus niger*. In: Hershberger CL, Queener SW, Hegeman G (eds) Genetics and molecular biology of industrial microorganisms. American Society of Microbiology, Washington DC, pp 295–300

Fontaine T, Beauvais A, Loussert C, Thevenard B, Fulgsang CC, Ohno N, Clavaud C, Prevost MC, Latgé JP (2010) Cell wall alpha1-3glucans induce the aggregation of germinating conidia of *Aspergillus fumigatus*. Fungal Genet Biol 47:707–712

Fujii I, Yasuoka Y, Tsai HF, Chang YC, Kwon-Chung KJ, Ebizuka Y (2004) Hydrolytic polyketide shortening by Ayg1p, a novel enzyme involved in fungal melanin biosynthesis. J Biol Chem 279:44613–44620

Grimm LH, Kelly S, Krull R, Hempel DC (2005) Morphology and productivity of filamentous fungi. Appl Microbiol Biotechnol 69:375–384

Jones PA (1985) Effects of 5-azacytidine and its 2′-deoxy derivative on cell differentiation and DNA methylation. Pharmacol Ther 28:17–27

Jørgensen TR, Park J, Arentshorst M, van Welzen AM, Lamers G, Vankuyk PA, Damveld RA, van den Hondel CAMJJ, Nielsen KF, Frisvad JC, Ram AFJ (2011) The molecular and genetic basis of conidial pigmentation in *Aspergillus niger*. Fungal Genet Biol 48:544–553

Kasuga T, Glass NL (2008) Dissecting colony development of *Neurospora crassa* using mRNA profiling and comparative genomics approach. Eukaryot Cell 7:1549–1564

Krijgsheld P (2013). Sporulation inhibited secretion in *Aspergillus niger*. PhD Thesis, University of Utrecht, The Netherlands

Krijgsheld P, Altelaar AFM, Post H, Ringrose JF, Müller WH, Heck AJR, Wosten HAB (2012) Spatially resolving the secretome within the mycelium of the cell factory *Aspergillus niger*. J Proteome Res 11:2807–2818

Krijgsheld P, Bleichrodt R, van Veluw GJ, Wang F, Müller WH, Dijksterhuis J, Wösten HAB (2013a) Development in *Aspergillus*. Stud Mycol 74:1–29

Krijgsheld P, Nitsche BM, Post H, Levin AM, Müller WH, Heck AJ, Ram AF, Altelaar M, Wösten HAB (2013b) Deletion of flbA results in increased secretome complexity and reduced secretion heterogeneity in colonies of *Aspergillus niger*. J Proteome Res. doi:10.1021/pr301154w

Lee BN, Adams TH (1994) Overexpression of *flbA*, an early regulator of *Aspergillus* asexual sporulation, leads to activation of *brlA* and premature initiation of development. Mol Microbiol 14:323–334

Lee DW, Freitag M, Selker EU, Aramayo R (2008) A cytosine methyltransferase homologue is essential for sexual development in *Aspergillus nidulans*. PLoS ONE 3:e2531

Lee I, Oh JH, Shwab EK, Dagenais TR, Andes D, Keller NP (2009) HdaA, a class 2 histone deacetylase of *Aspergillus*

*fumigatus*, affects germination and secondary metabolite production. Fungal Genet Biol 46:782–790

Levin AM, de Vries RP, Conesa A, de Bekker C, Talon M, Menke HH, van Peij NNME, Wösten HAB (2007a) Spatial differentiation in the vegetative mycelium of *Aspergillus niger*. Eukaryot Cell 6:2311–2322

Levin AM, de Vries RP, Wösten HAB (2007b) Localization of protein secretion in fungal colonies using a novel culturing technique; the ring-plate system. J Microbiol Meth 69:399–401

Li A, Pfelzer N, Zuijderwijk R, Brickwedde A, van Zeijl C, Punt P (2013) Reduced by-product formation and modified oxygen availability improve itaconic acid production in *Aspergillus niger*. Appl Microbiol Biotechnol. doi: 10.1007/s00253-012-4684-x

Lin PJ, Grimm LH, Wulkow M, Hempel DC, Krull R (2008) Population balance modeling of the conidial aggregation of *Aspergillus niger*. Biotechnol Bioeng 99:341–350

Lu X, Sun J, Nimtz M, Wissing J, Zeng AP, Rinas U (2010) The intra- and extracellular proteome of *Aspergillus niger* growing on defined medium with xylose or maltose as carbon substrate. Microb Cell Fact 9:23

Masai K, Maruyama J, Sakamoto K, Nakajima H, Akita O, Kitamoto K (2006) Square-plate culture method allows detection of differential gene expression and screening of novel, region-specific genes in *Aspergillus oryzae*. Appl Microbiol Biotechnol 71:881–891

Metz B, Kossen NWF (1977) The growth of molds in the form of pellets—a literature review. Biotechnol Bioeng 19:781–799

Meyer V, Wu B, Ram AFJ (2011) *Aspergillus* as a multi-purpose cell factory: current status and perspectives. Biotechnol Lett 33:469–476

Montiel MD, Lee HA, Archer DB (2006) Evidence of RIP (repeat-induced point mutation) in transposase sequences of *Aspergillus oryzae*. Fungal Genet Biol 43:439–445

Moukha SM, Wösten HAB, Asther M, Wessels JGH (1993a) In situ localization of the secretion of lignin peroxidases in colonies of *Phanerochaete chrysosporium* using a sandwiched mode of culture. J Gen Microbiol 139:969–978

Moukha SM, Wösten HAB, Mylius E, Asther M, Wessels JGH (1993b) Spatial and temporal accumulation of mRNAs encoding two common lignin peroxidases in *Phanerochaete chrysosporium*. J Bacteriol 175:3672–3678

Papagianni M, Moo-Young M (2002) Protease secretion in glucoamylase producer *Aspergillus niger* cultures: fungal morphology and inoculum effects. Process Biochem 37: 1271–1278

Papagianni M, Mattey M, Kristiansen B (1998) Citric acid production and morphology of *Aspergillus niger* as functions of the mixing intensity in a stirred tank and a tubular loop bioreactor. Biochem Eng J 2:197–205

Priegnitz BE, Wargenau A, Brandt U, Rohde M, Dietrich S, Kwade A, Krull R, Fleissner A (2012) The role of initial spore adhesion in pellet and biofilm formation in *Aspergillus niger*. Fungal Genet Biol 49:30–38

Punt PJ, van Biezen N, Conesa A, Albers A, Mangnus J, van den Hondel CAMJJ (2002) Filamentous fungi as cell factories for heterologous protein production. Trends Biotechnol 20:200–206

Ryoo D, Choi C-S (1999) Surface thermodynamics of pellet formation in *Aspergillus niger*. Biotechnol Lett 21:97–100

Scholtmeijer K, de Vocht ML, Rink R, Robillard GT, Wösten HAB (2009) Assembly of the fungal SC3 hydrophobin into functional amyloid fibrils depends on its concentration and is promoted by cell wall polysaccharides. J Biol Chem 284:26309–26314

Sealy L, Chalkley R (1978) The effect of sodium butyrate on histone modification. Cell 14:115–121

Shwab EK, Jin WB, Tribus M, Galehr J, Graessle S, Keller NP (2007) Histone deacetylase activity regulates chemical diversity in *Aspergillus*. Eukaryot Cell 6:1656–1664

Teertstra WR, Lugones LG, Wösten HAB (2004) In situ hybridisation in filamentous fungi using peptide nucleic acid probes. Fungal Genet Biol 41:1099–1103

Tribus M, Galehr J, Trojer P, Brosch G, Loidl P, Marx F, Haas H, Graessle S (2005) HdaA, a major class 2 histone deacetylase of *Aspergillus nidulans*, affects growth under conditions of oxidative stress. Eukaryot Cell 4:1736–1745

Tsang A, Butler G, Powlowski J, Panisko EA, Baker SE (2009) Analytical and computational approaches to define the *Aspergillus niger* secretome. Fungal Genet Biol 46:S153–S160

van Veluw GJ, Teertstra WR, de Bekker C, Vinck A, van Beek N, Muller WH, Arentshorst M, van der Mei HC, Ram AFJ, Dijksterhuis J, Wösten HAB (2013) Heterogeneity in liquid shaken cultures of *Aspergillus niger* inoculated with melanised conidia or conidia of pigmentation mutants. Stud Mycol 74:47–57

Vinck A, Terlou M, Pestman WR, Martens EP, Ram AFJ, van den Hondel CAMJJ, Wösten HAB (2005) Hyphal differentiation in the exploring mycelium of *Aspergillus niger*. Mol Microbiol 58:693–699

Vinck A, de Bekker C, Ossin A, Ohm RA, de Vries RP, Wösten HAB (2011) Heterogenic expression of genes encoding secreted proteins at the periphery of *Aspergillus niger* colonies. Environ Microbiol 13:216–225

Ward M, Lin C, Victoria DC, Fox BP, Fox JA, Wong DL, Meerman HJ, Pucci JP, Fong RB, Heng MH, Tsurushita N, Gieswein C, Park M, Wang H (2004) Characterization of humanized antibodies secreted by *Aspergillus niger*. Appl Environ Microbiol 70:2567–2576

Wessels JGH (1988) A steady-state model for apical wall growth in fungi. Acta Bot Neerl 37:3–16

Wessels JGH (1993) Wall growth, protein excretion and morphogenesis in fungi. New Phytol 123:397–413

Wieser J, Lee BN, Fondon JW, Adams TH (1994) Genetic requirements for initiating asexual development in *Aspergillus nidulans*. Curr Genet 27:62–69

Wösten HAB, Moukha SM, Sietsma JH, Wessels JGH (1991) Localization of growth and secretion of proteins in *Aspergillus niger*. J Gen Microbiol 137:2017–2023

Yu JH, Wieser J, Adams TH (1996) The *Aspergillus* FlbA RGS domain protein antagonizes G protein signaling to block proliferation and allow development. EMBO J 15: 5184–5190