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Growth, branching and enzyme production by filamentous fungi in submerged culture

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Introduction

This chapter considers (a) the generation of highly branched (colonial) mutants during prolonged fermentation of the Quorn® myco-protein fungus, *Fusarium graminearum* A3/5, (b) the influence of hyphal branch frequency on the production of extracellular enzymes by *Aspergillus oryzae*, and (c) the stability of *Aspergillus niger gla* A transformants in prolonged continuous flow cultures. We believe that the results obtained with these three species can be extended to most, if not all, filamentous fungi.

Highly branched mutants do not have a selective advantage because of their morphology

When filamentous fungi or streptomycetes are grown in prolonged, continuous culture, it is common for the relatively sparsely branched parental strain to be supplanted by a relatively highly branched mutant (Fig. 5.1). Such mutants are called colonial because in Petri dish culture they form dense colonies that expand in radius more slowly than parental colonies. Selection of colonial mutants has been observed in continuous cultures of *Byssochlamys nivea*, *Paecilomyces variotii*, *Paecilomyces puntonii*, *Gliocladium virens*, *Trichoderma viride* (Forss *et al.*, 1974), *Penicillium chrysogenum* (Righelato, 1976), *F. graminearum* (Solomons & Scammell, 1976) and *Acremonium chrysogenum* (A. Trilli, personal communication).

When the Quorn® myco-protein fungus, *F. graminearum* A3/5, was grown in glucose-limited chemostat culture at a dilution rate of 0.19 h^{-1} (doubling time of 3.65 h), colonial mutants were first detected about 107 generations (*c*. 389 h) after the onset of continuous flow (Fig.
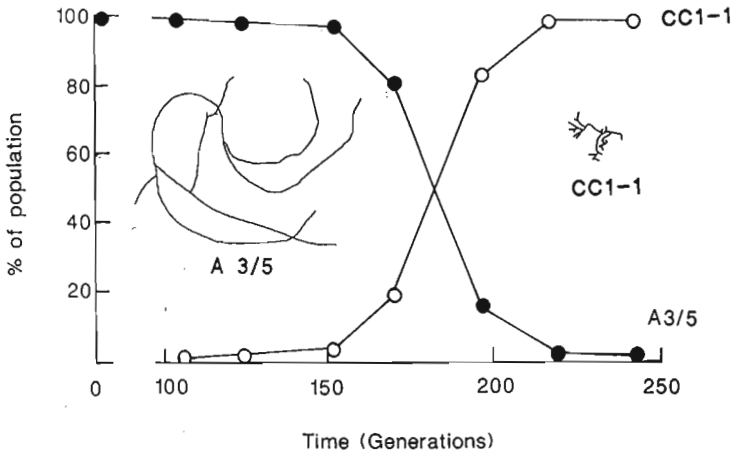
Fig. 5.1. Population of a highly branched (colonial) mutant CC1-1 (expressed as a percentage of the total population) generated during a glucose-limited, continuous flow culture of *F. graminearum* A3/5 grown at 25°C on modified Vogel's medium (Vogel, 1956) containing 3 g glucose l^{-1} at a dilution rate of 0.19 h^{-1} (pH of 5.8 and a stirrer speed of 1400 rpm). The morphology of representative mycelia of A3/5 and CC1-1 is also shown.

5.1; Trinci, 1994). Wiebe *et al.* (1991) isolated 20 morphological (colonial) mutants from two chemostat cultures; all were more highly branched (hyphal growth unit values from 14 to 174 µm) than A3/5 (hyphal growth unit of 232 µm) and produced colonies that expanded in radius more slowly (colony radial growth rate, K_r, values ranging from 18 to 105 µm h^{-1}) than A3/5 (K_r 135 µm h^{-1}). Given the recessive nature of the mutations (Wiebe *et al.*, 1991), the colonial phenotype of mutants can only be expressed once sufficient mutant nuclei have become separated from parental nuclei. This kind of separation may follow sporulation (macroconidia are formed from uninucleate phialides) or mycelial frag-mentation (Wiebe *et al.*, 1996).

The above results led to speculation about the reasons why highly branched mycelia have a selection advantage over sparsely branched mycelia in continuous flow cultures. The outcome of competition between the sparsely branched parental strain (A3/5) and a highly branched colonial mutant (CC1-1) under glucose- and sulphate-limita-tion in chemostat culture provided a crucial insight into this question (Fig. 5.2). For both nutrient limitations, CC1-1 retained its highly branched phenotype but behaved as a selectively advantageous mutant
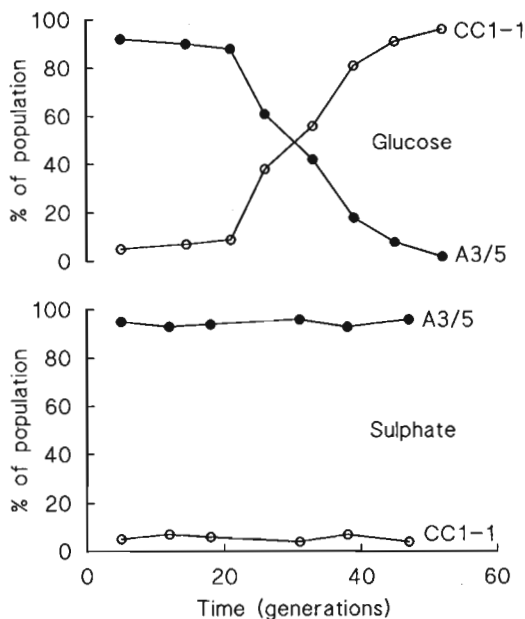
Fig. 5.2. Competition between *Fusarium graminearum* A3/5 and a highly branched (colonial) mutant CC1-1 in glucose-limited and sulphate-limited chemostat cultures grown at 25°C, pH 5.8 and a stirrer speed of 1400 rpm at a dilution rate of 0.19 h^{-1}.

under glucose-limitation but as a neutral mutant under sulphate-limitation. Thus, the highly branched phenotype does not confer a selection advantage on the mutant. Instead, the mutation alters glucose metabolism and it is this change which confers a selective advantage on CC1-1 when it is grown in mixed culture with A3/5 in glucose-limited chemostat culture (Wiebe *et al.*, 1992); thus, the highly branched phenotype is a pleiotropic effect of the CC1-1 mutation. Significantly, mutants of *Neurospora crassa* with altered activities of glucose-6-phosphate dehydrogenase (Brody & Tatum, 1966) and phosphoglucomutase (Brody & Tatum, 1967) also have highly branched phenotypes, as does *N. crassa* mycelium treated with compounds (paramorphogens like L-sorbose) which affect membrane or wall biosynthesis (Trinci, Wiebe and Robson, 1994). Finally, a putative protein kinase mutant of *N. crassa* forms a highly branched mycelium (Yarden *et al.*, 1992). These observations suggest that highly branched (colonial) phenotypes are the pleiotropic effects of mutations (or paramorphogens) whose primary effects are on the activities of enzymes involved in carbon metabolism, mem-

brane biosynthesis, wall biosynthesis, etc. Indeed we estimate that probably up to about 5% of all random mutations have colonial phenotypes. The knowledge gained from the results of the experiment shown in Fig. 5.2 facilitated the development of strategies to prevent or delay the appearance of colonial mutants during industrial production of biomass of *F. graminearum* A3/5 for Quorn® myco-protein production. It is hoped that the successful exploitation of these strategies will increase the cost-efficiency of the process (Trinci, 1994).

Increasing hyphal tip number per unit biomass does not result in an increase in the yield of extracellular enzymes

Following the observation of Wösten *et al.* (1991) that glucomylase is secreted (by a process known as 'bulk-flow') at the hyphal tips of mycelia of *A. niger* (Wessels, 1993), there has been speculation about whether or not the productivity of industrial enzyme fermentations can be increased by using highly branched fungal strains, i.e. by increasing the number of hyphal tips per unit fungal biomass. Figure 5.3 shows batch growth of *Aspergillus oryzae* and a more highly branched (colonial) mutant derived from this strain: the two strains had the same specific growth rate and approximately the same rate of α-amylase accumulation. A similar result was obtained with *A. oryzae* which, following treatment with echinocandin, was four times more highly branched than untreated control mycelia. However, some highly branched mutants of *A. oryzae* produced enzymes more rapidly than the sparsely branched parental strain during exponential growth in batch culture.

Of course, enzyme secretion may not be the factor limiting enzyme productivity in the strains shown in Fig. 5.3 and if this is so increasing branch frequency (and hence the number of points for enzyme secretion) will not increase enzyme productivity. Further, the percentage of the wall of a mycelium which is 'extensible' may not be changed by altering branch frequency since the hyphae of highly branched mycelia will have shorter extension zones than those of sparsely branched mycelia. Thus, the spatial distribution of 'extensible' wall in a mycelium may be changed in colonial mutants, but not its relative amount; if this is the case there is no reason to believe that enzyme secretion would occur at a faster rate in a highly branched mycelium than in a sparsely branched mycelium.
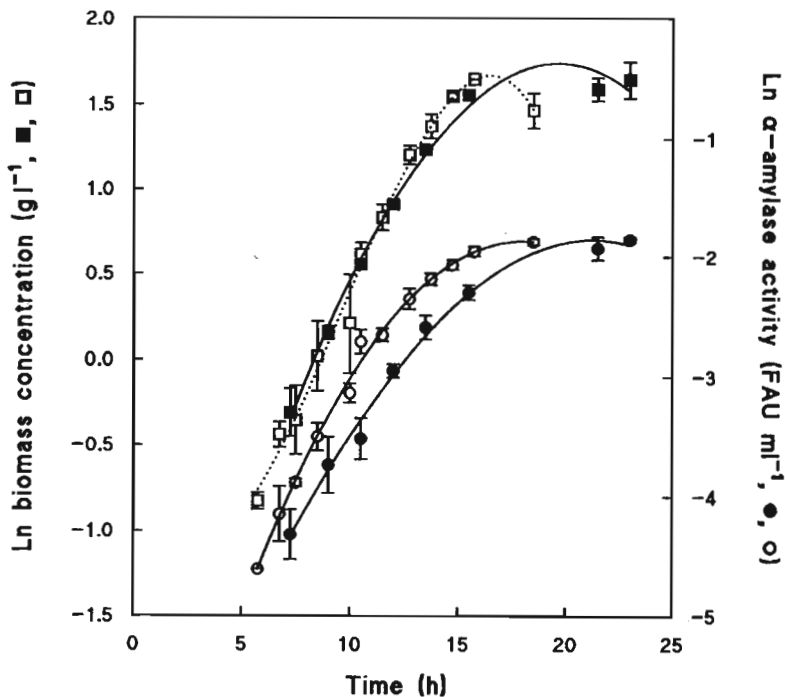
Fig. 5.3. Growth (■, □) and α-amylase production (●, ○) of a parental strain (IFO4177) of *A. oryzae* (■, ●) and of a more highly branched morphological mutant (HNP12) (□, ○) grown at 30°C in batch culture on Vogel's medium containing 10 g glucose l⁻¹. The parental strain and the more highly branched mutant had hyphal growth unit lengths of 101 ± 4 and 53 ± 2 μm respectively (mean of 5 replicates ± SEM).

Increasing hyphal tip number per unit biomass causes a decrease in culture viscosity

Although increasing hyphal tip number per unit biomass does not increase enzyme yield, it does have an appreciable effect on culture rheology. Figure 5.4 shows that for morphological mutants of *A. oryzae* culture viscosity (torque) increases with increase in hyphal growth unit length. Culture viscosity is important in industrial fermentations because oxygen solubility decreases with an increase in culture viscosity, and consequently steps have to be taken when culturing filamentous fungi to ensure that oxygen supply does not become a growth-limiting factor. Thus, it may be possible to increase the cost-efficiency of some industrial fungal fermentations by using highly branched strains to reduce culture viscosity and hence increase oxygen supply.
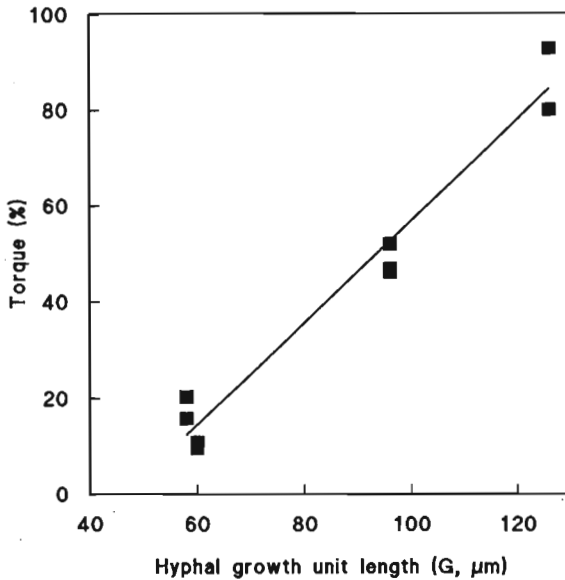
Fig. 5.4. Effect of hyphal branch frequency on the torque of biomass (4 g dry wt l^{-1}) of cultures of various strains of *A. oryzae* ($r^2 = 0.97$).

Production of growth-associated enzymes in continuous flow culture

A continuous flow culture operated at a high dilution rate is the most cost-effective way of producing a growth-associated product (Pirt, 1975); these culture systems include chemostats in which an organism can be grown at a value just below its maximum rate of growth (μ_{max}) and pH auxostats (Simpson *et al.*, 1995) in which an organism can be grown at μ_{max}. Therefore, to maximize productivity, growth-associated products (including at least some recombinant proteins) should be produced in continuous flow culture systems. However, although *F. graminearum* A3/5 is grown by Marlow Foods Ltd in a 155 m^3 continuous flow culture (a glucostat), and although the industrial potential of growing fungi in continuous flow systems to produce recombinant proteins is enormous, there is currently little information about the production of recombinant proteins by filamentous fungi in such cultures. Recombinant protein productivity of such systems is most meaningfully described in terms of specific production rate (q_p, g product [g biomass]$^{-1}$ h^{-1}). Continuous culture at a constant dilution rate is the simplest experimental system for determining q_p; for such systems, q_p is equal to $D \cdot C_p \cdot C_x^{-1}$ in which D is

dilution rate, C_p is product concentration and C_x is biomass concentration.

The *A. niger* B1 transformant used here originated from strain N402, a *cspA1* (conferring short conidiophores) derivative of ATCC 9029 and has been described by Verdoes *et al.* (1993, 1994). Transformation was based on a cosmid vector (42.8 kb) carrying four copies of the *A. niger glaA* (glucoamylase) gene and a single copy of the *amdS* (acetamidase) gene of *A. nidulans* as a selectable marker. Transformant N402 [pAB6-10]B1 (subsequently referred to as B1) carries an additional 20 copies of *glaA*.

Glucoamylase (GAM) production by A. niger B1 in batch and chemostat culture

A batch culture of *A. niger* B1 grown in an Infors IFS 100 fermenter (1.5 l volume of culture; pH 5.5 ± 0.2; aeration 0.7 l air [l culture]$^{-1}$ min^{-1}) on 5 g maltodextrin l^{-1} produced 320 ± 8 mg GAM l^{-1} (mean $\pm$ SEM), giving a specific production rate of 5.6 ± 0.6 mg GAM [g dry weight]$^{-1}$ h^{-1} (Table 5.1). Specific growth rate (dilution rate) had no significant ($P > 0.05$) effect on the concentration of GAM in maltodextrin-limited chemostat cultures (Fig. 5.5a) but did have a significant ($P < 0.05$) effect on the specific production rate (Fig. 5.5b). Table 5.1 compares the productivity of the batch and chemostat culture systems; it shows that since GAM is a growth-related product (i.e. productivity increases with specific growth rate), a continuous flow culture operated at a high dilution rate will be a more efficient system for GAM production than batch or fed-batch cultures. GAM production in glucose-limited chemostat cultures of *A. niger* B1 was only slightly less than in maltodextrin-limited cultures (Table 5.1).

Stability of transformant A. niger B1 in prolonged cultures

Table 5.1 shows the potential economical benefit of producing a growth-associated product (GAM, a recombinant protein) in a continuous flow culture operated at a high dilution rate. However, the extent of this benefit will be critically dependent on the stability of the transformed strain used in the production process (Trilli, 1977). Although industrial fermentations involve a substantial number of generations, even for high-value pharmaceutical proteins produced in reactors with a volume of 0.1–5 m^3, few studies have been made of the long-term stability of transformants of filamentous fungi (Dunn-Coleman *et al.*, 1994; Withers *et al.*,

Table 5.1. *Concentrations of GAM and specific GAM productivities for A. niger B1 grown on 5 g maltodextrin l^{-1} Vogel's medium in batch and in chemostat culture at a dilution rate of 0.13 h^{-1} (pH 5.4; 30°C; 0.7 l air [l culture]$^{-1}$ min^{-1}; 1000 rpm)*

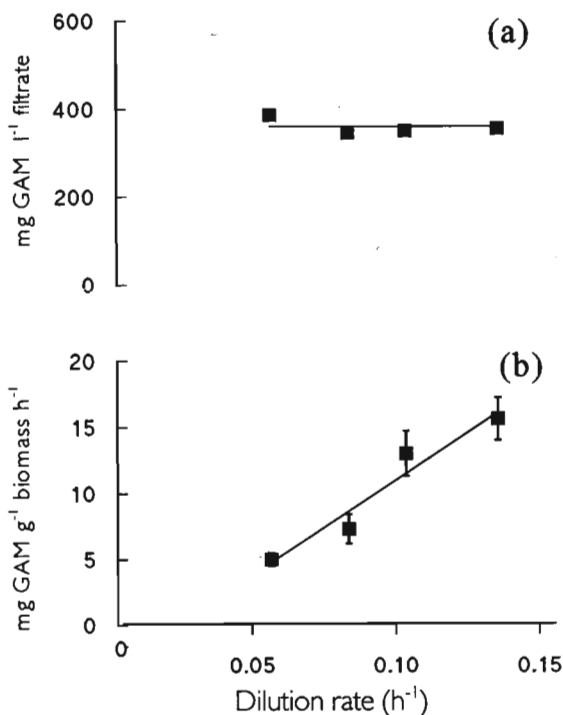| Type of culture	GAM concentration (C_p, mg l^{-1})	Specific production rate of GAM (q_p, mg GAM [g biomass]$^{-1}$ h^{-1})
Batch culture grown on maltodextrin	320 ± 8	5.6
Maltodextrin-limited chemostat culture	373 ± 9	16.0
Glucose-limited chemostat culture	303 ± 12	12.0

Mean ± S.E.M. (5 replicates)

Fig. 5.5. Effect of dilution rate on GAM production by *A. niger* B1 in a maltodextrin-limited chemostat culture (pH 5.5 ± 0.1; 30.0 ± 0.2°C; 0.7 l air [l culture]$^{-1}$ min^{-1}; 1000 rpm). (a) Concentration of GAM in the culture filtrate; (b) specific production rate of GAM. Error bars represent mean of 5 replicates ± SEM.

1995). Fed-batch fermentations are generally characterized by an initial phase of rapid growth followed by a period of deceleration during which production of the recombinant protein may be induced (Hensing *et al.*, 1995). However, instability in product formation will be enhanced if recombinant product formation is delayed until the final stages of the process. A continuous flow fermentation (chemostat or turbidostat) is more efficient than a fed-batch culture for the production of microbial biomass or a biomass-associated product (Pirt, 1975; Trinci, 1992) but such cultures demand even greater strain stability for product formation.

Importantly, numerous studies have shown that, in the absence of selective agents, plasmid carriage reduces the competitive fitness of microorganisms (Goodwin & Slater, 1979; Helling, Kinney & Adams, 1981; Lenski, Simpson & Nguyen, 1994) and plasmid-free segregants tend to overgrow plasmid-bearing strains (Cooper, Brown & Caulcott, 1987; Lenski & Nguyen, 1988). The selective advantage of one strain compared to another can be determined by calculation of the selection coefficient, which is based on the proportion of each colony type present in viable counts (Dykhuizen & Hartl, 1981). Selection coefficient (s) is defined as:

$$s = \frac{\ln\left[\dfrac{p_{(t)}}{q_{(t)}}\right] - \ln\left[\dfrac{p_{(o)}}{q_{(o)}}\right]}{t}$$

where $p_{(t)}$ is the concentration of the mutant at time t, $q_{(t)}$ *is the concentration of the parental strain at time t*, and $p_{(o)}$ and $q_{(o)}$ are the initial concentrations of each strain.

In bacteria and *S. cerevisiae*, two main factors have been found to influence plasmid stability in non-selective culture conditions: the segregation frequency of the plasmid and the effect of the plasmid's presence on growth rate (Goodwin & Slater, 1979). Segregation frequency has been shown to be dependent on various factors including dilution rate, nature of the growth-limiting substrate, and temperature (Roth, Noack & Geuther, 1985; Roth *et al.*, 1994; O'Kennedy, Houghton & Patching, 1995) as well as the host strain and the plasmid itself (Kumar *et al.*, 1991; Leonhardt & Alonso, 1991; D'Angio *et al.*, 1994; Roth *et al.*, 1994). In contrast to the self-replicating plasmids commonly used in the transformation of bacteria and *S. cerevisiae*, however, integrative plasmids are used to introduce extrachromasomal DNA into filamentous fungi. Although loss of the plasmid DNA as a result of unequal segregation during mitosis in filamentous fungi is therefore unlikely, deletions

and substitutions in both native and foreign DNA sequences may occur (Adams *et al.*, 1992; Gilbert *et al.*, 1994; Hensing *et al.*, 1995).

Figure 5.6 shows that the concentration of GAM (303 ± 3 mg l^{-1}) in *A. niger* B1 grown in a glucose-limited chemostat culture at a dilution rate of 0.14 h^{-1} (doubling time, 4.95 h) remained constant for 948 h (191 generations), having a mean specific production rate of 18.2 mg GAM [g biomass]$^{-1}$ h^{-1}. After 213 h (43 generations) the original B1 strain, which produced densely sporing black colonies, was completely displaced by a morphological mutant which produced less densely sporing, brown colonies. However, no change in GAM production was associated with the appearance of this mutant. Although other morphological mutants (forming fawn and white colonies, with few conidia on agar-solidified Vogel's medium) were occasionally observed in samples from the culture, the brown mutant continued to comprise over 90% of the population throughout the final 735 h of the fermentation. An isolate of the brown mutant (B1-E) was obtained at the end of the fermentation.
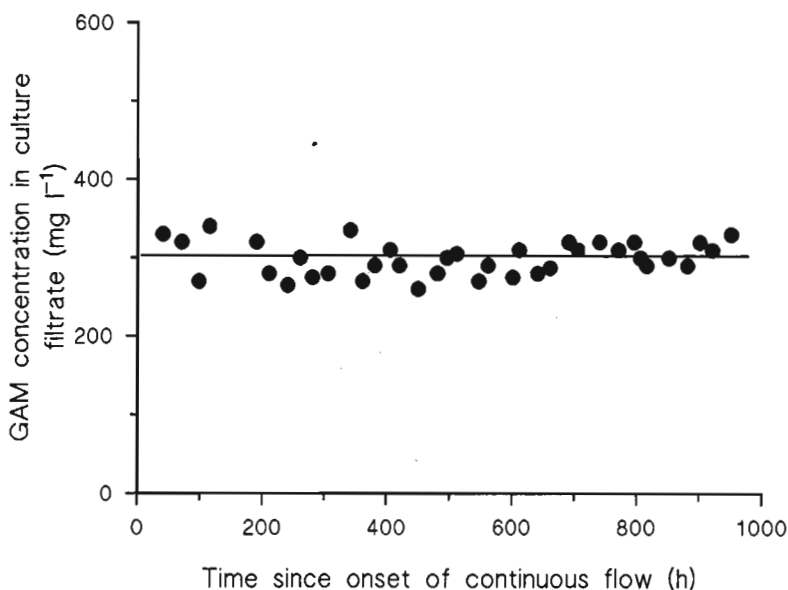

Fig. 5.6. GAM concentration in glucose-limited (5 g l^{-1}) chemostat cultures of *A. niger* B1 cultures grown on Vogel's medium at a dilution rate of 0.13 h^{-1}, $30.0 \pm 0.2°C$, pH 5.5 ± 0.1, aeration 0.6 l air [l culture]$^{-1}$ min^{-1}, agitation 1000 rpm). GAM assays were performed in quadruplicate for each sample. Error bars represent mean $\pm$ SEM.

Figure 5.7 shows the lack of stability of GAM production in cultures of *A. niger* B1 grown at a dilution rate of 0.14 h^{-1} for 354 h (71 generations) in a glucose-limited chemostat culture grown on Vogel's modified medium enriched with 5 g mycopeptone l^{-1}; all nutrients other than glucose were present in excess in these cultures and an increase in biomass concentration was observed when glucose was added to batch cultures grown on the same medium. Table 5.2 compares the production of biomass and GAM in mycopeptone-enriched and non-enriched medium during the first 130 h of chemostat culture and shows that GAM concentration and the specific production rate of GAM were increased by 64% and 37% respectively by the mycopeptone enrichment. However, after 136 h, GAM concentration in the mycopeptone-enriched culture decreased to 178 ± 14 mg l^{-1} following which no further significant change in the GAM concentration was observed (Fig. 5.6); this corresponded to a specific production rate of 9.1 mg GAM [g biomass]$^{-1}$ h^{-1}. The decline in GAM concentration was associated with the accumulation in the
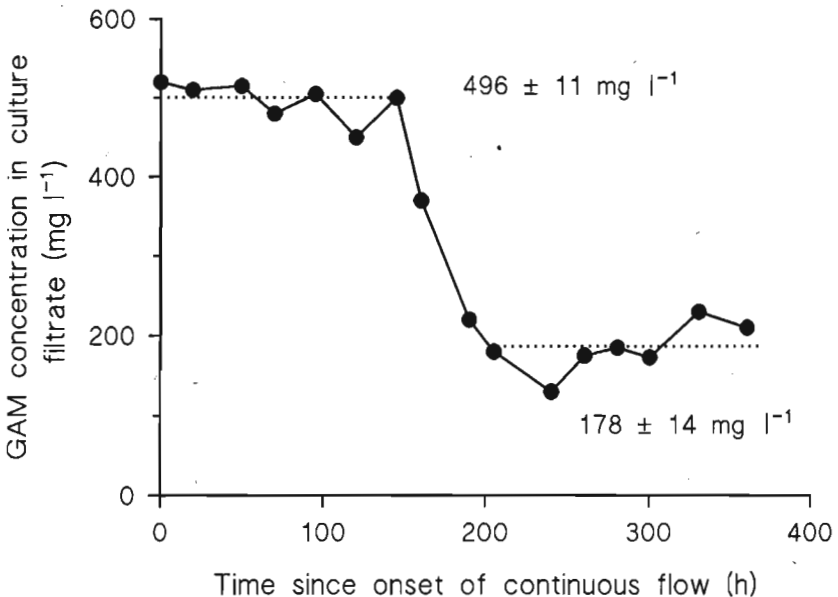

Fig. 5.7. GAM concentration in the culture filtrate of a glucose-limited (5 g l^{-1}) chemostat culture of *A. niger* B1 grown on modified Vogel's medium containing 5 g mycopeptone l^{-1} (cultures were grown at a dilution rate of 0.13 h^{-1}, 30.0 ± 0.2°C, pH 5.5 ± 0.1, aeration 0.6 l air [l culture]$^{-1}$ min^{-1}, agitation 1000 rpm).

Table 5.2. *GAM production for the first 130 h of the growth of A. niger B1 in glucose-limited and mycopeptone-supplemented glucose-limited chemostat culture at a dilution rate of 0.13 h^{-1} (pH 5.5; 30°C; 0.7 l air [1 culture]$^{-1}$ min^{-1}; 1000 rpm)*

Parameter of GAM production	Units of measurement	Modified Vogel's medium containing		% Increase
		5 g glucose l^{-1}	5 g glucose and 5 g mycopeptone l^{-1}	
Biomass	g l^{-1}	2.95 ± 0.2*	3.95 ± 0.2	34
GAM concentration (C_p)	mg l^{-1}	303 ± 12	496 ± 10	64
Specific production rate of GAM (q_p)	mg GAM [g biomass]$^{-1}$ h^{-1}	12.0 ± 0.4	16.4 ± 1.6	37

*Mean ± S.E.M. (5 replicates)

population of a brown mutant (B1-M) that was less densely sporulating than B1.

Selective advantage of strains B1-E and B1-M relative to N402 and B1

There was no selective advantage for either B1 or N402 when these strains were grown together in chemostat culture (Fig. 5.8). However, as shown in Table 5.3, each evolved strain (B1-E and B1-M) isolated from prolonged chemostat cultures had a selective advantage over the parental strain (B1); this selective advantage was largest under the cultural conditions in which the mutant had been originally isolated, i.e. glucose limitation for B1-E, and mycopeptone-enriched, glucose-limitation for B1-M. However, all evolved strains retained their selective advantage in the other conditions tested, including fructose limitation, which does not support GAM production. Further competition studies between B1-M and N402 (which produced similar levels of GAM to B1) demonstrated that B1-M also had a selective advantage over N402, although the selection coefficients were lower than for B1-M over B1.

glaA *gene copy number in fermenter-selected strains*

To analyse B1-E and B1-M for the presence and expression of the *glaA* gene copies the isolates were plated on acetamide/acrylamide and hygro-
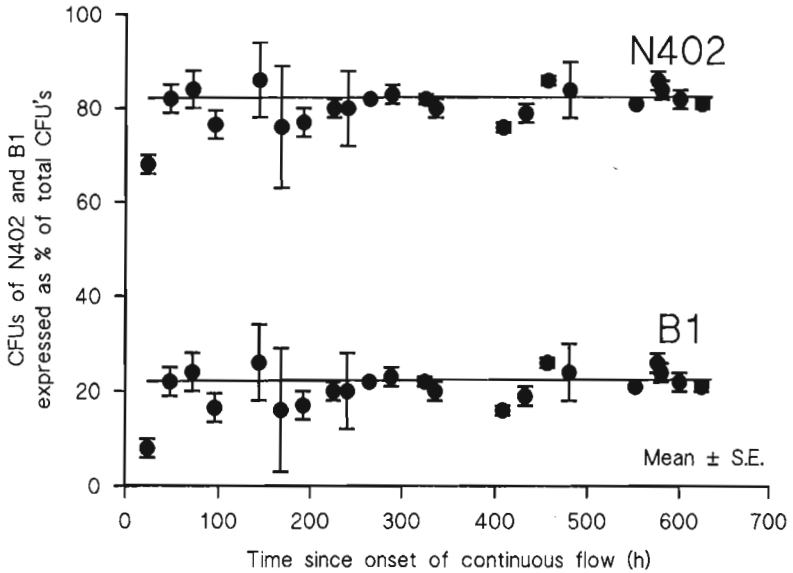
Fig. 5.8. Mixed culture of *A. niger* N402 and B1 grown in glucose-limited (5 g l^{-1}) chemostat cultures at a dilution rate of 0.13 h^{-1}, 30.0 ± 0.2°C, pH 5.5 ± 0.1, aeration 0.6 l air [l culture]$^{-1}$ min^{-1}, agitation 1000 rpm.

mycin B plates (Verdoes *et al.*, 1993, 1994) to verify the transformed phenotype. From this analysis it was clear that the B1-E had retained the transformed phenotype, whereas B1-M showed reduced growth on acrylamide plates, indicating reduced *amdS* expression and thus loss of introduced gene copies. This was further confirmed by Southern analysis using a *glaA*-specific probe (and the single-copy *gpdA* control probe). It was clear from these analyses that B1-M had lost most of the introduced *glaA* gene copies, while B1-E retained all or most of its *glaA* gene copies. Thus, although the specific production rate of GAM was significantly increased by the addition of mycopeptone to the medium, GAM production was unstable and decreased to less than half its previous level after 136 h (27 generations) of chemostat cultivation. B1-M, isolated from the end of this fermentation, produced significantly less GAM in mycopeptone-enriched, glucose-limited culture than B1. Rapid loss of productivity has been observed in cultures of *Streptomyces* sp. grown in a complex medium (Fazeli, Core & Baumberg, 1995) and more rapid plasmid loss has been observed from *S. cerevisiae* in complex than in defined medium

Table 5.3. *Selection coefficients (s, a measure of differences in specific growth rates during mixed culture in chemostat cultures; Dykhuizen & Hartl, 1981) for strains B1, B1-E, and B1-M relative to N402 and B1 in carbon-limited chemostat culture at a dilution rate of 0.13 h^{-1} (pH 5.5; 0.6 to 1.0 l air [l culture]$^{-1}$ min^{-1}; 30°C; 1000 rpm).*

Competition between		Carbon source limiting growth rate[a]	GAM produced?	Selection coefficient s for strain A (h^{-1})
Strain A	Strain B			
B1	N402	Glucose	Yes	0.000[b]
B1-E	B1	Glucose	Yes	0.058
B1-E	B1	Fructose	No	0.038
B1-M	N402	Glucose	Yes	0.055
B1-M	N402	Glucose + mycopeptone	Yes	0.077
B1-M	B1	Glucose	Yes	0.070
B1-M	B1	Fructose	No	0.069
B1-M	B1	Glucose + mycopeptone	Yes	0.144

[a]Glucose or fructose (5 g l^{-1}) were used as the carbon source. In addition, some cultures were enriched with mycopeptone (5 g l^{-1}). All cultures were inoculated with mycelial suspensions of the two strains used in the competition, to give initial concentrations of 5% to 50% of one of the strains and 50% to 95% of the other.
[b]i.e. B1 behaves as a neutral mutation.

(O'Kennedy *et al.*, 1995) but only when the culture was grown at a high dilution rate.

For stable integrated gene inserts, it is generally assumed that instability will primarily be the result of growth rate differences between the insert-bearing and insert-free cell (Devchand & Gwynne, 1991). Segregation instability (the unequal distribution of plasmids between daughter cells during cell division), a problem for non-integrated plasmids in bacteria, yeast and fungi (Roth *et al.*, 1994; Moreno *et al.*, 1994), is unlikely to occur, although structural instability (loss of heterologous DNA by deletions, insertions or rearrangements) may occur (Hensing *et al.*, 1995; Gilbert *et al.*, 1994; Numan, Venables & Wimpenny, 1991). As indicated by the measurement of selection coefficients for each of the evolved strains (B1-E and B1-M) relative to the parental strain (B1), growth rate differences probably accounted for the displacement of B1 in the long-term fermentations (Table 5.3). However, the growth rate differences between the original strains and the evolved strains cannot be explained on the basis of loss of gene copies and thus lower gene

expression, since no copy loss was observed in B1-E. Furthermore, the selective advantages observed were similar whether or not glucoamylase expression occurred (Table 5.3). Thus, the selective advantages of these strains result from other alterations which affect their physiology.

B1-M had a selective advantage of 0.055 h^{-1} relative to N402 in glucose-limited chemostat culture and a selective advantage of 0.069 h^{-1} relative to B1 in fructose-limited culture (Table 5.3). Thus, in this case, part of the growth rate increase may be GAM-expression independent. However, B1-M had also lost copies of the *gla*A gene, and this may have contributed to its growth rate advantage. Nevertheless, it is more likely that in B1-M, as in B1-E, a physiological alteration resulted in the selective advantage and that by chance the strain also suffered gene copy loss.

Concluding remarks

When an organism is cultured under constant conditions (Novick & Szilard, 1950*a*; Monod, 1950) in a continuous flow culture, the population evolves and progressively becomes better adapted to its environment (Novick & Szilard, 1950*b*). This evolution involves mutations which confer selective advantages (such as an increase in μ_{max} or decrease in K_s) to the mutants, and consequently, the mutants eventually replace the wild type. Since the chemostat cultures of *F. graminearum* A3/5 and *A. niger* described above were grown at relatively high dilution rates, the advantageous mutants selected probably had increased μ_{max} values.

Provided that the production strain is stable in prolonged culture, a continuous flow culture (chemostat or turbidostat) operated at high dilution rate (Fig. 5.5) is a cost-efficient system for the production of a growth related product. As far as the latter is concerned, it is noteworthy that 155 m^3 glucostat cultures are used to produce biomass of *F. graminearum* for Quorn® myco-protein production (Trinci, 1994). However, engineering problems related to scaling-up and strain stability and loss of productivity during long-term cultivation would need to be addressed before continuous cultures could be used on an industrial scale for recombinant protein production.

For *A. oryzae*, the use of highly branched mutants failed to improve the enzyme production (Fig. 5.3) although they did decrease culture viscosity (Fig. 5.4). Finally, recognition of the pleiotropic nature of the highly branched (colonial) phenotype of mutants arising in *F. graminearum*

A3/5 fermentations was an important step in developing strategies to prevent/delay the appearance of these mutants (Trinci, 1994).

The studies clearly show the importance of mycelial morphology and strain stability in the productivity of industrial fermentations of filamentous fungi.

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The fungal colony

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