Presidential address 1991

Myco-protein: A twenty-year overnight success story

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Fusarium graminearum for production of myco-protein is currently grown on a glucose–ammonia–biotin–mineral salts medium in a 40 m^3 air-lift fermenter. The resulting biomass is RNA reduced, harvested, texturized and sold for human consumption, either directly as a food or as meat or poultry alternatives in pre-prepared meals. Originally intended in the 1960s to combat the world's flagging supply of protein foods, Quorn® myco-protein was marketed in the middle 1980s as a low-calorie, high-fibre, food containing no cholesterol or animal fats. The continuous flow culture system currently used for myco-protein production is described, together with details of the 'evolution' of the fungus in prolonged culture.

Once described as a 'twenty-year overnight success story', Quorn® myco-protein now festoons the shelves of British supermarkets. The story began in the late 1950s when people were concerned that, in the coming years, traditional sources of protein foods, such as cattle, sheep, pigs and poultry, would no longer be able to meet demand, even in the western world, and that consequently there was an impending global shortage of protein foods. In addition, various childhood diseases associated with protein malnutrition, including kwashiorkor and marasmus, had been identified in underdeveloped countries. In 1955, the Protein Advisory Group (PAG) was created to help the World Health Organisation (WHO) advise the UN Food and Agriculture Organisation (FAO) and United Nations International Children's Emergency Fund (UNICEF) on the establishment of guidelines for safety, nutrition and palatability of new protein foods for human consumption. The United Nations (U.N. report, 1968: *International Action to Avert Impending Protein Crisis*) considered how the predicted 'protein deficit' might be met and assessed the possibility of using microbial protein to help meet future protein requirements. At that time, most microbial protein projects were concerned with producing single-cell protein (a term coined by workers at the Massachusetts Institute of Technology to avoid any unpleasant connotation which the public might associate with the terms bacterial or microbial protein) for animal, rather than human consumption, with the aim of using (the then) cheap by-products of the petroleum and agricultural industries as carbon and energy sources for microbial growth. By contrast, Ranks Hovis McDougall (RHM) decided in 1964 to develop a protein-rich food primarily for human consumption, but with the additional possibility of using it as an animal feed or pet food (Spicer, 1971); RHM decided that production of single-cell protein (SCP) for use as animal feed was not an economic proposition because of competition from feeds based on arable protein crops. The intention was to cultivate a fungus in a fermenter on a medium containing wheat starch as the carbon and energy source for growth, and to modify the fungal biomass to meet nutritional guidelines. Originally, the idea was to dry and powder the myco-protein and market it as a high-protein

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food (soya flour and dried milk are used in this way) but RHM decided that the texture of myco-protein was appealing enough to make it a new food in its own right. At the time, RHM estimated that an economic plant would consist of a large continous flow fermenter able to produce 20–30 000 tonnes of fungal protein per annum (Edelman, Fewell & Solomons, 1983).

In 1984, a joint venture (Marlow Foods) between RHM and ICI was initiated with the purpose of further developing the myco-protein process using ICI's expertise in scaling up fermentation technology. Today a fermenter with a capacity of 10 000 tonnes of myco-protein per annum is considered technically feasible. Quorn® is the registered trade name of the product and myco-protein, a term coined by the Foods Standards Committee, is the generic name of the food.

DEVELOPMENT OF MYCO-PROTEIN UP TO 1980

Reasons for choosing a filamentous fungus

The Egyptians and Romans prized mushrooms and there are records of their being eaten in China between 26 BC and AD 220 (Wang, 1985). In particular, mushrooms have traditionally been associated with meats, and indeed in Malawi they are regarded as a meat analogue (Morris, 1984). The latter view was also held by Francis Bacon who, in his *Sylva Sylvarum* of 1627 described mushrooms as yielding 'so delicious a meat'. In addition, long-standing, fermented foods such as tempeh and miso have a meat-like flavour. Thus, one reason why RHM chose a fungus as the basis of its new food was because of the long history (Hesseltine, 1965, 1983) of humans eating mushrooms and foods fermented with fungi. RHM felt that this widespread tradition would result in much less consumer resistance to a fungal based food, than to one derived from bacteria. Even the idea of edible fungi grown in fermenters is not new, as in World War II biomass from a filamentous fugus grown on milk whey was used in Germany to supplement human diets (Robinson, 1952).

Compared with many bacteria and yeasts, the relatively slow growth rate of most filamentous fungi is a disadvantage for biomass production (Solomons, 1985); however, because of this slow growth rate, fungi generally have a lower nucleic acid content than bacteria, and this is an advantage (see below). In general, Quorn® fungal protein has a Net Protein Utilization (NPU; defined as retained nitrogen/intake nitrogen × 100) value of 75 and, after supplementation with 0·2% (w/w) methionine, this is increased to 100, making it equal to egg protein (Table 1). Further, although yeast protein can only replace *ca* 10% of the protein required in our diet, myco-protein can provide a total replacement (Steinkraus, 1986). However, the most important reason why RHM chose to base its new protein food on a filamentous fungus rather than on a yeast or a bacterium, was because of the organoleptic (smell/taste/feel) properties of the biomass. When harvested, *F. graminearum* biomass looks like uncooked pastry and has a very mild, almost bland wheaty-cum-mushroom flavour. The texture of food products made from myco-protein is imparted by the fungal mycelium which is composed of filaments similar to the fibres of meat (Fig. 1). In the final product, even the longitudinal alignment of the structural fibres of meat can be uncannily mimicked by the fungal biomass (Fig. 1). Consequently, the filamentous nature of the fungus gives myco-protein products a chewiness and succulence akin to meat and facilitates the fabrication of myco-protein products of different textures and forms. Remarkably, the textures of myco-protein and meat are so similar that myco-protein has been used as a reference standard for comparative tests on meat (Edelman *et al.*, 1983). Importantly, myco-protein retains colourings and flavourings even when cooked. In this respect

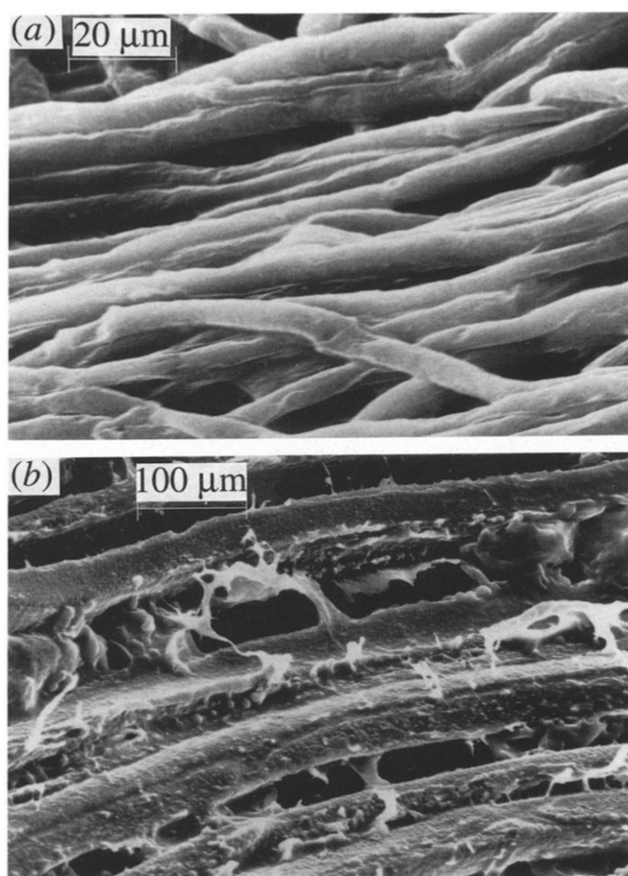

Fig. 1. SEM image of (*a*) myco-protein prepared from *F. graminearum* A 3/5, and (*b*) beefsteak. Photographs kindly provided by Dr R. Angold (The Lord Rank Research Centre, High Wycombe).

Table 1. Comparison of Net Protein Utilization (NPU) of Quorn® myco-protein and some common food proteins

Food	Biological assessment (NPU)
Egg	100
Skimmed milk protein	85
Fish	83
Beef	80
Quorn® myco-protein	75*
Cow's milk	75
Wheat flour	52
Beans	47

* When supplemented with 0·2% (w/w) methionine, the NPU value of myco-protein is increased to 100.

myco-protein differs from meat which exudes juices when cooked.

Strain selection

The strain selected by RHM for the production of myco-protein had to satisfy a number of criteria, including safety, nutritional value and organoleptic properties. These included protein content [originally set at 30% (w/w) of biomass, but subsequently increased to 45%], amino acid composition, NPU value (a minimum NPU of 75 was set which is equivalent to milk protein), growth rate and yield coefficient (= unit biomass produced: unit carbohydrate consumed for growth), and of course it had to be completely non-toxic. Initially, RHM used a strain of *Penicillium notatum-chrysogenum* (a strain of uncertain identity) isolated from a farmer's field which had been occasionally sprayed with surplus starch slurry (a potential substrate for the fermentation) from the nearby RHM starch plant in Ashford, Kent (Solomons & Spicer, 1973). However, although *P. notatum-chrysogenum* had a specific growth rate greater than $0{\cdot}175\ h^{-1}$ (doubling time $< 4{\cdot}0$ h) and contained sufficient protein (25–28% w/w) of excellent nutritional quality (NPU of 70) to compete successfully with conventional sources of protein, in continuous flow culture the fungus overgrew all the internal surfaces of the fermenter and this resulted in a decrease in the supply of oxygen to the biomass, and consequently a decrease in the yield coefficient from glucose.

In 1968, the same year as ICI screened 10000 micro-organisms to find its Pruteen bacterium (*Methylophilus methylotrophus*), RHM initiated a £1M development programme which included a three-year screening project to select an alternative fungus for myco-protein production. The preliminary screening of isolates was designed to eliminate toxic strains, and this was then followed by characterization of the isolates in shake flask culture. During the screening programme, 3000 isolates obtained worldwide (mainly from soil samples) were examined, and of these, 20 were considered sufficiently promising to be tested further in small-scale animal feeding trials. Remarkably, of these 20 isolates, 8 were species of *Fusarium*, and one of these (*Fusarium graminearum* Schwabe) was finally chosen for further development. Ironically, this isolate (coded A 3/5) was obtained from the third soil sample collected from a field in Marlow (hence the name of the company currently producing Quorn® myco-protein) only three miles from the RHM Laboratories at High Wycombe. When grown on a glucose–ammonia–mineral salts medium containing biotin (a vitamin essential for the growth of this strain), *F. graminearum* A 3/5 has a high protein content (minimum of 42% w/w) and grows optimally at *ca* 30 °C with a maximum specific growth rate of $0{\cdot}28\ h^{-1}$ (a doubling time of about 2·5 h). Extensive toxicology testing using both animals and human subjects (see below) showed the strain to be non-toxic to animals, non-pathogenic to wheat and maize seedlings and of excellent nutritional value.

Choice of the carbon and energy source for growth of Fusarium graminearum

RHM's decision to use food-grade glucose derived from starch as the carbon and energy source for myco-protein production was timely and prudent, since the Protein Advisory Group Committee was reluctant to sanction SCP for human consumption when the micro-organism used was grown on petroleum fractions. Wheat-starch, the substrate chosen by RHM as the carbon and energy source for myco-protein production is a by-product of the production of wheat gluten (protein) and wheat flour is used as an ingredient in a variety of foods; before use in myco-protein production, the wheat starch is first hydrolysed to sugars. Whereas in many countries the glucose feed-stock for the fermentation can be derived from hydrolysed wheat or corn starch, myco-protein fermentations in Ireland might utilize potatoes, whilst tropical countries might use cassava, rice or cane sugar (Anderson *et al.*, 1975; Steinkraus, 1986). *F. graminearum* converts each kg of carbohydrate into *ca* 136 g of protein; whilst chickens, pigs and cattle fed the same amount of carbohydrate would produce about 49, 41 and 14 g of protein respectively. Thus, fungal fermentations are a very efficient way of converting carbohydrate into protein.

Reason for choosing a continuous flow culture system for myco-protein production

Common to all SCP fermentations is the need for low capital and running costs, high-yield coefficient on the limiting substance, near complete utilization of the substrate and ease of recovery of the biomass (Solomons, 1985). Continuous flow culture systems (Fig. 2) avoid the fluctuating conditions inherent in batch cultures (Pirt, 1975) and enable perpetual exponential growth of the organism to be maintained at a specific growth rate (μ) approaching its maximum rate of growth (μ_{max}) for the prevailing conditions. Consequently, a higher productivity of biomass can be achieved in a continuous flow culture system than in a sequential series of batch cultures (Pirt, 1975). Trilli (1977) developed a model to predict the cost of production of biomass and secondary metabolites in continuous flow cultures. He found that at dilution rates higher than a critical value, the unit cost of biomass production decreases with time towards a finite value, unique for the system (Fig. 3). The economic disadvantage associated with the first part of each continuous culture run (Fig. 3) is repeated each time the fermenter is started afresh and a further economic disadvantage accrues from the down time between consecutive fermentation runs. Thus, for maximum myco-protein productivity, the continuous flow culture should be maintained for as long as possible (Fig. 3). In practice, myco-protein fermentations are usually run for about six weeks and yield a productivity which is some five-fold greater than that which could be achieved using a series of separate batch fermentations (Sadler, 1988).

Growth of Fusarium graminearum *in continuous flow culture in a stirred tank fermenter*

F. graminearum for myco-protein production is grown under strictly defined conditions (with temperature, pH, nutrient concentration, dissolved oxygen tension and growth rate all being maintained constant) in a continuous flow culture system. RHM used two $1{\cdot}3\ m^3$ stirred tank reactors as fermentation

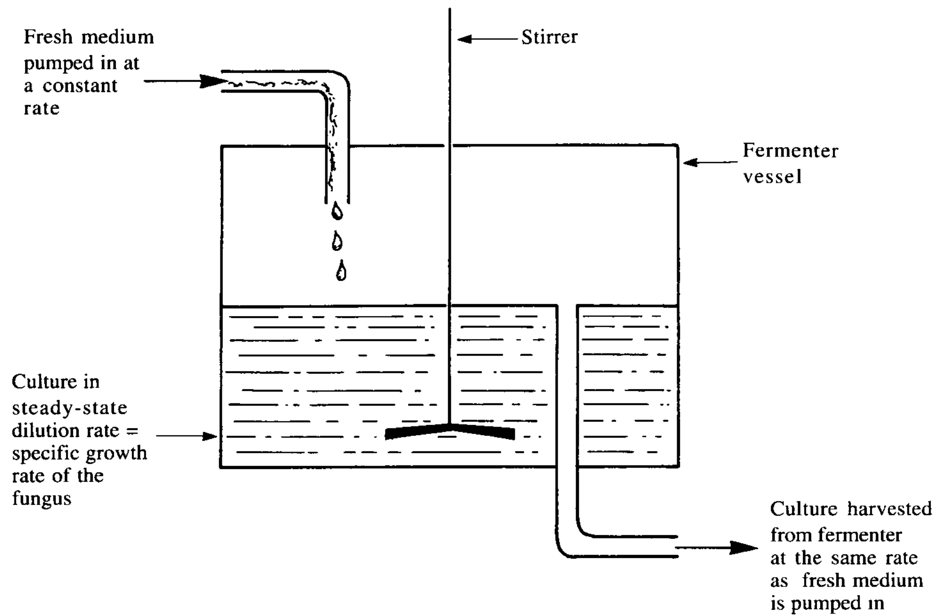

Fig. 2. Diagrammatic representation of a continuous flow (chemostat) culture system.

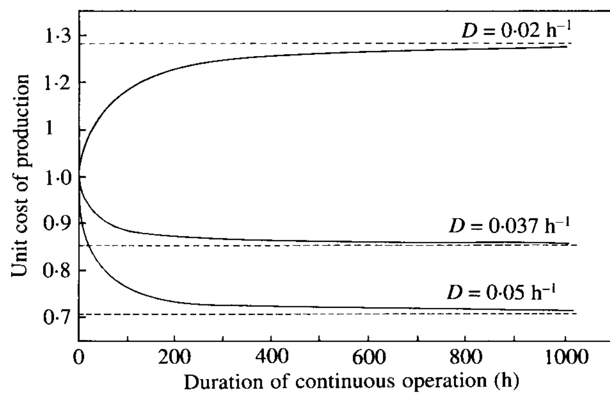

Fig. 3. Predicted effects of dilution rate (*D*) and the duration of continuous operation on the unit cost of biomass production in a continuous flow culture system. Solid line = unit cost of the product, expressed as the ratio, cost at time *t*:cost at time 0; broken line = asymptotic value of unit cost (redrawn from Trilli, 1977).

vessels and these provided a production capacity of *ca* 50–100 tonnes dry weight of myco-protein per annum (Solomons, 1983).

In a continuous flow culture, growth of the fungus can be restricted by the supply of any nutrient (the growth-limiting nutrient), but is usually limited by the concentration of the carbon and energy soruce (e.g. glucose), with all other nutrients being present in excess. Medium containing the growth-limiting nutrient is fed into the fermenter vessel at a constant rate and culture is removed from the vessel at the same rate, so that the volume of culture remains constant (Fig. 2). At steady state, the concentrations of biomass and growth-limiting nutrient in the vessel remain constant, and the specific growth rate of the organism equals the rate (D) at which the culture is diluted with fresh medium (Pirt, 1975). The relationship between an organism's specific growth rate and the concentration of the growth-limiting nutrient in the medium is given by the Monod (1942) equation,

$$\mu = \mu_{max}\, s/(s+K_s), \quad (1)$$

where μ = the specific growth rate of the organism, μ_{max} = the maximum specific growth rate of the organism in the absence of nutrient limitation, s = the concentration of the growth-limiting nutrient, K_s = the saturation constant of the organism, and is the concentration of the growth limiting nutrient at which $\mu = \mu_{max}/2$. *F. graminearum* has two uptake systems for glucose (Anderson & Solomons, 1983), and in common with other filamentous fungi (Steensland, 1973; Fiddy & Trinci, 1975; Robinson & Smith, 1976) its high-affinity uptake system (the one which is operational in glucose-limited continuous flow cultures) results in a very low K_s (2·5 μM) value for glucose (M. G. Wiebe, G. D. Robson and A. P. J. Trinci, unpublished), i.e. *F. graminearum* has a high affinity for glucose and can therefore maintain high growth rates even at very low glucose concentrations.

RNA reduction

Nucleic acids tend to be present in high concentrations in microbial cells having a high growth rate and if human food contains too much nucleic acid blood uric acid values rise and the excess accumulates as crystalline deposits in joints and tissues, leading to gout-like manifestations and calculi in the urinary tract (Rivière, 1975; Sinskey & Tannenbaum, 1975). In man, uric acid is produced as a result of breakdown of nucleic acids whereas in other vertebrates the sparingly soluble uric acid is converted to the highly soluble acid allantoin. Because of this, PAG laid down a recommendation for human ingestion of RNA from SCP sources which, for adults, was defined as 2 g RNA d^{-1}, with a total nucleic acid ingestion from all sources not exceeding 4 g d^{-1}.

F. graminearum biomass cultured at a specific growth rate of 0·19 h^{-1} has a RNA content of 8–9% (w/w) which would

limit the ingestion of myco-protein to not more than 20 g per day. Consquently, a heat shock process was developed to reduce the RNA content of myco-protein whilst minimizing loss of protein and fibrous structure. This method is carried out in the culture broth with no other adjustments, since a pH of 5 to 6 is optimal for RNAase activity (Solomons, 1983). In this process, the temperature of the biomass is raised rapidly (< 10 s) to 64° (for 20–30 min) to stop growth, disrupt ribosomes, and activate endogenous RNAases which break down cellular RNA to 5′ nucleotides which diffuse through the hyphal wall into the culture broth; importantly, RNAases are more heat resistant than proteases, reducing protein loss. However, RNA reduction is bound to have substantial economic penalties attached to it, since as well as removing RNA, other cell constituents are inevitably lost during this process, including perhaps up to one third of the biomass dry weight (Solomons, 1983). After this treatment, the myco-protein contains only *ca* 1% (w/w) RNA, similar to that present in animal liver and well within the 2% upper limit recommended by WHO, allowing consumption of more than 100 g of myco-protein d^{-1}.

Harvesting, texturization and storage

One of the advantages of using a filamentous fungus rather than a bacterium or yeast for SCP is the comparative ease with which the fungal biomass can be harvested. After RNA reduction, the filter cake of fungal biomass is harvested by vacuum filtration on a horizontal belt filter where liquid is removed to give a product which contains 30% (w/w) total solids (Anderson & Solomons, 1983). RHM developed a mechanical process in which the filaments of myco-protein are aligned so that the required fibrous structure is attained. Other natural ingredients are added which impart colour and flavour together with small amounts of egg white (a protein binder) which is then 'heat set' to stabilize the alignment of the filaments in myco-protein. Finally, the product is size reduced and frozen for short- or long-term storage. Quality control checks are carried out at every stage of the process to ensure that the end product is of a consistently high quality.

Safety evaluation of myco-protein

A ten-year (1970–80) myco-protein safety evaluation programme resulted in the submission to the U.K. regulatory authority, the Ministry of Agriculture, Fisheries and Food (MAFF) of a 26-volume, two-million-word report requesting permission for RHM to sell myco-protein for human consumption. The trials showed that myco-protein caused no adverse changes when fed as the sole source of protein to eleven species of animal, including pigs, calves and baboons, with none of the studies showing animal responses that would be considered as toxicological in origin (Duthie, 1975; Solomons, 1983, 1986; Sadler, 1988). Subsequently, RHM and the Massachusetts Institute of Technology conducted human feeding trials which, when continued in the U.K., involved the product being consumed by about 2500 people on more than one occasion. A particularly important finding in this study was the lack of immunological response of the volunteers to being fed myco-protein. Tests on the bacteriological safety of myco-protein were also made and it was found that the product had slightly less propensity to support the growth of bacteria than did chicken or fish (Solomons, 1986). RHM rightly claims that no other food had been subjected to such rigorous testing. Indeed, the protocol developed to test myco-protein was completely novel and will probably serve as the basis for future testing of new foods and food ingredients.

PRESENT-DAY PRODUCTION OF MYCO-PROTEIN

Growth of Fusarium graminearum *in an air-lift fermenter*

After MAFF had approved myco-protein for marketing in the U.K., there was a need to increase production. This was achieved in 1984 by the use of a 40 m^3 air-lift fermenter at Billingham which had been built previously by ICI as a pilot scale fermenter during their development of the animal feed, Pruteen (Gow *et al.*, 1975). Use of this fermenter enabled Marlow Foods, the joint venture formed in 1984 between ICI and RHM, to develop the market for Quorn® myco-protein products.

The Billingham fermenter differs from the stirred tank reactors used by RHM for myco-protein production. Because of their filamentous morphology, cultures of moulds are much more viscous than bacterial cultures (Righelato, 1979) and therefore more difficult to mix. RHM used a novel impeller system to mix the culture and to achieve heat and mass transfer (important for efficient supply of O_2 and nutrients to the growing organism and for removal of CO_2). By contrast, the air-lift or pressure-cycle fermenter developed by ICI does not have an impeller but instead uses rising air bubbles (Fig. 4) to mix the culture and to provide heat and mass transfer (Gow *et al.*, 1975). Because of the different ways used to mix the culture, less heat is generated (and therefore less cooling is required) in an air-lift fermenter than in a stirred tank reactor.

The Billingham air-lift fermenter currently used for myco-protein production consists of an elongated loop *ca* 30 m tall in which the culture is continuously circulated (Fig. 4). Because of their high viscosity, it is difficult to supply cultures of filamentous fungi with sufficient oxygen to maintain exponential growth (Righelato, 1979); oxygen has a very low solubility in water (7 mg l^{-1} at 30°) but production of one g dry weight of *F. graminearum* requires 0·78 g of O_2! It is therefore essential that myco-protein cultures should be provided with a good supply of O_2 and without such a supply, growth would become oxygen-limited, yield coefficient would decrease, sporulation would increase, and other products such as ethanol would be formed (Anderson & Solomons, 1983). In an air-lift fermenter most of the oxygen transfer takes place near the base of the relatively wide 'riser' where sterile air is introduced (Fig. 4) and where the height of the fermenter creates a high hydrostatic pressure. This, together with turbulence (and consequently small bubble size) provides excellent conditions for O_2 transfer from the gaseous to the liquid phase. In the design and operation of the pressure cycle fermenter, control of initial bubble size is vital since this

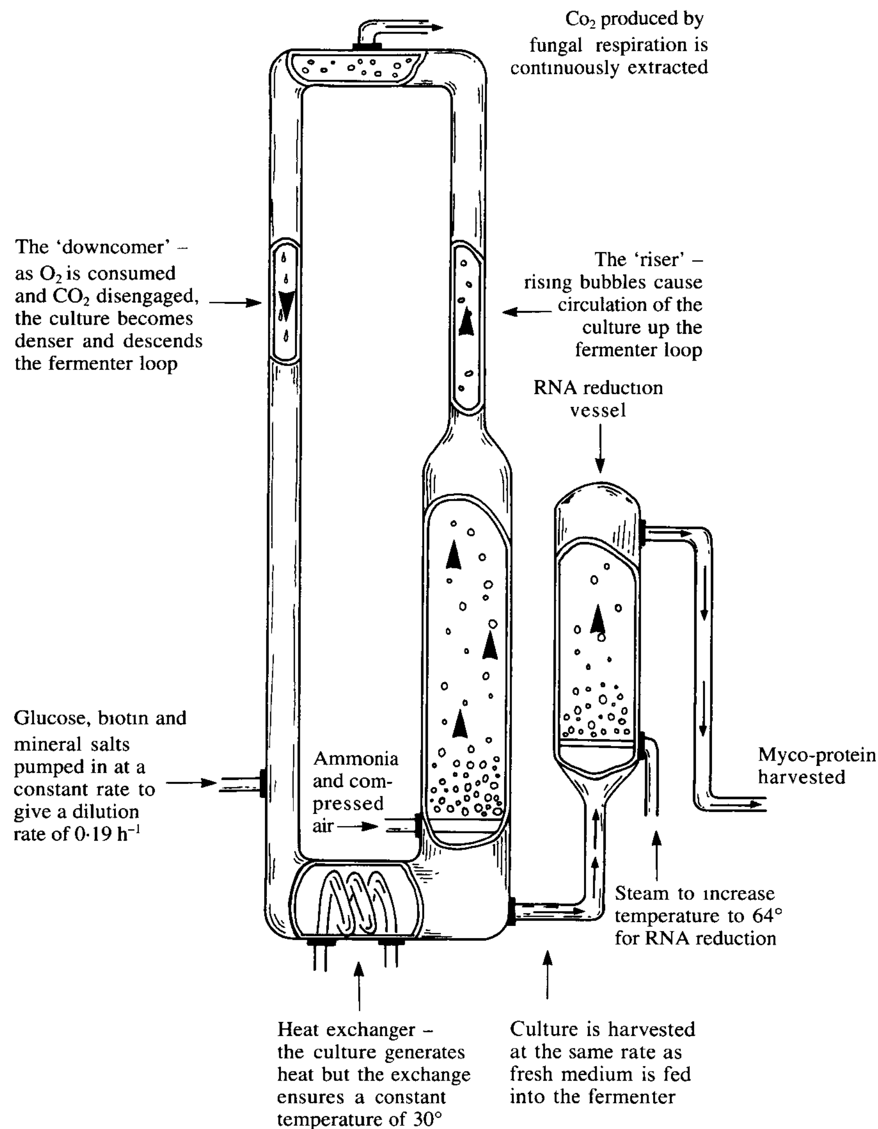

Fig. 4. Diagrammatic representation of the air-lift fermenter used by Marlow Foods at Billingham for the production of myco-protein in continuous flow culture.

has a considerable effect on the rate of O_2 transfer (Gow *et al.*, 1975). Thus, the riser contains a two-phase mixture of air and culture flowing concurrently at voidages up to 50% (Gow *et al.*, 1975). The rate of transfer of O_2 from the gaseous to the liquid phase decreases as the culture flows to the top of the riser where the gas contains *ca* 10% O_2. The low-pressure region at the top of the riser causes release of CO_2 and the culture then enters the 'downcomer' where, at the bottom, it is directed into the riser and is again charged with air, glucose and other nutrients, thereby completing the pressure cycle. The difference in specific gravity (hydrostatic pressure differential) of the aerated culture in the 'riser' and the air-depleted culture in the 'downcomer' ensures that the culture circulates continuously around the fermenter loop. The nutrient solution is fed to the culture to give a dilution rate in the range 0·17 to 0·20 h^{-1}, well below the organism's μ_{max}(0·28 h^{-1}). The nitrogen supply (ammonia) for growth is fed into the fermenter with the sterile compressed air, at the base of the 'riser'; the rate of supply of ammonia to the culture is regulated by a pH monitor set to give a culture pH of 6·0. The culture is maintained at *ca* 30° by a heat exchanger set into the 'downcomer' (Fig. 4) and is harvested continuously and RNA reduced as described above.

Although the shear forces experienced by mycelia in stirred tank (the RHM method) and air-lift (the Marlow Foods method) fermenters may differ, the morphology and growth of mycelia of *F. graminearum* in the two types of fermenter are similar, and morphological (colonial) mutants arise (see later) in both systems at approximately the same time after inoculation.

Effect of dilution rate and nutrient-limitation on the morphology of **Fusarium graminearum**

Fungal morphology can influence the texturization of the final products and therefore morphological control during the myco-protein fermentation is an important feature of the overall process. Because of this, a study has been made of the effect of dilution rate and nutrient-limitation on mycelial morphology (Wiebe, 1989; Wiebe & Trinci, 1991). For any

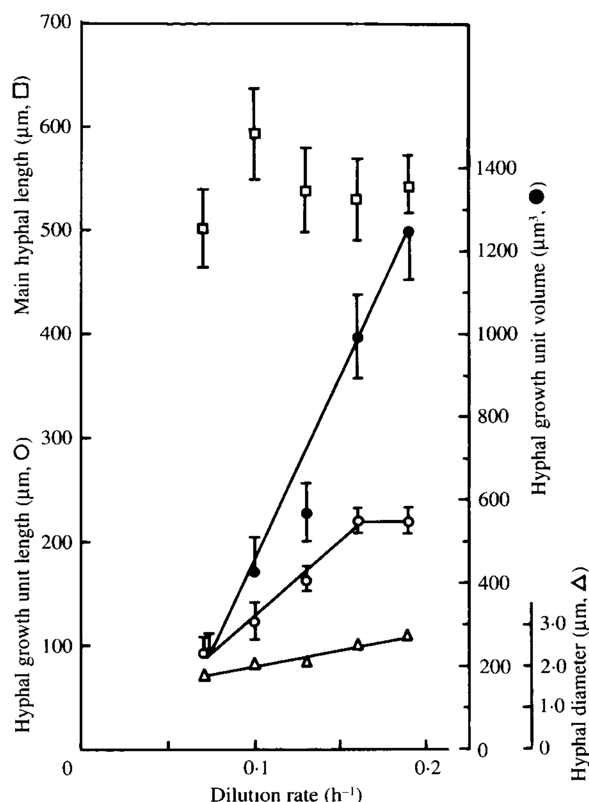

Fig. 5. Effect of dilution rate on main hyphal length (□), hyphal growth unit length (○), hyphal diameter (△) and hyphal growth unit volume (●) of *F. graminearum* A 3/5 grown at 25° in a glucose-limited continuous flow culture on modified Vogel's medium containing 3 g glucose l^{-1} at pH 5·8 and 1400 rpm (from Wiebe & Trinci, 1991).

given steady state, the concentration of *F. graminearum* fragments in glucose-limited continuous flow cultures remained constant with time, but, in general, fragment concentration decreased with increase in dilution rate (Wiebe & Trinci, 1991). The observation that at given dilution rate fragment concentration remains approximately constant suggests that mycelia grown in stirred tank reactors fragment in a regular manner, either spontaneously or because of shear forces. Figure 5 shows the effects of dilution rate on main hyphal length (the longest hypha in a mycelium), hyphal growth unit length (G, the ratio between the length of a mycelium and the number of growing tips and is a measure of mycelial branching; Trinci, 1974), hyphal diameter, and hyphal growth unit volume (Trinci, 1984) of a glucose-limited chemostat culture of *F. graminearum*. Importantly, main hyphal length was not affected by dilution rate, but both hyphal diameter and hyphal growth unit length increased with increase in dilution rate. Robinson & Smith (1979) also found that, in glucose-limited continuous flow cultures, the hyphal diameter of *Geotrichum candidum* increased with increase in dilution rate, but for this fungus, hyphal growth unit volume remained constant and hyphal growth unit length decreased with the increase in dilution rate.

When *F. graminearum* was grown in shake flask (batch) culture on a glucose-'limited' medium (i.e. glucose was the first nutrient to become exhausted), it had a hyphal growth unit length of 276 ± 11 μm. However, when batch cultures of *F. graminearum* were grown in a fermenter at a stirred speed of 1400 rpm, the length of the hyphal growth unit was reduced to 227 ± 6 (Table 2). In batch cultures grown in fermenters, the nature of the nutrient (glucose, NH_4 or Mg^{2+}) first exhausted affected both hyphal growth unit length and main hyphal length (Table 2). Similarly, in continuous flow culture, mycelial morphology was affected by the nature of the limiting nutrient (Table 2).

F. graminearum produces macroconidia in batch and continuous flow culture (Table 2), and in glucose-limited continuous flow cultures, spore concentration ($4{\cdot}8 \times 10^5$ ml^{-1}) was at its highest at a dilution rate of 0·13 h^{-1}, decreasing at higher dilution rates (Wiebe & Trinci, 1991). The nature of the

Table 2. Effect of nutrient limitation on the morphology of *F. graminearum* A 3/5 grown at 1400 rpm, pH 5·8 and 25° on modified Vogel's medium in a 2 l Braun Biostat M fermenter (from Wiebe, 1989 and Wiebe & Trinci, 1991)

(a) Glucose-, ammonium- and magnesium-'limited' batch (1 l culture volume) cultures

	Nutrient first exhausted		
	Glucose (10·0 g l^{-1})	NH_4‡ (0·2 g l^{-1})	Mg§ (1·5 mg l^{-1})
Length of main hypha* (μm); maximum value observed	725 ± 33†	627 ± 36	861 ± 37
Hyphal growth unit length (G, μm)	227 ± 6	205 ± 5	327 ± 3
Hyphal diameter (μm)	3·9 ± 0·1	3·8 ± 0·1	4·2 ± 0·1

(b) Glucose-, ammonium- and magnesium-'limited' continuous flow cultures (1·85 l culture volume) grown at a dilution rate of 0·16 h^{-1}

	Limiting nutrient		
	Glucose (4·5 g l^{-1})	NH_4‡ (0·2 g l^{-1})	Mg§ (1·5 mg l^{-1})
Length of main hypha* (μm)	744 ± 23	504 ± 16	655 ± 12
Hyphal growth unit length (G, μm)	223 ± 6	146 ± 4	195 ± 7
Hyphal diameter (μm)	3·18 ± 0·04	3·16 ± 0·003	3·42 ± 0·03
Sporulation (macroconidia ml^{-1})	$1{\cdot}0 \pm 0{\cdot}06 \times 10^5$	$2{\cdot}9 \pm 0{\cdot}12 \times 10^5$	$< 0{\cdot}01 \times 10^5$

* The longest hypha in a mycelium.
† Standard error of the mean.
‡ Provided as $(NH_4)_2SO_4$.
§ Provided as $MgSO_4$

nutrient limitation had a profound effect on sporulation of *F. graminearum*, with very few macroconidia being produced under conditions of magnesium-limitation (Table 2).

Effect of choline on mycelial morphology

Although choline has no effect on the specific growth rate of *F. graminearum*, Wiebe, Robson & Trinci (1989) showed that its inclusion in the medium at a concentration of 1 to 10 µM increased both hyphal growth unit length (from 323 to 515 µM) and colony radial growth rate (from 144 to 331 µm h^{-1}). Addition of betaine, ethanolamine, monomethylethanolamine or dimethylethanolamine (but not serine, glycine, dimethylglycine, methylamine, hydroxylamine or β-hydroxethylhydrazine) to the medium also resulted in appreciable increases in the colony radial growth rate of *F. graminearum*. However, addition of 100 µM choline to the medium had no significant effect on phospholipid composition of the fungus. At present no explanation can be offered to account for the dramatic effect of small concentrations of choline on the morphology of mycelia of *F. graminearum*.

EVOLUTION OF *FUSARIUM GRAMINEARUM* IN CONTINUOUS FLOW CULTURE

Appearance of selectively favoured mutants

When an organism is cultured under constant conditions (Novick & Szilard, 1950*a*; Monod, 1950) in a continuous flow culture, the population evolves and 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 a decrease in K_s) to the mutants compared to the wild type, and, consequently, the mutants eventually replace the wild type. According to Solomons (1983), Harrison (1976) described an improvement in yield factor from 0·40 to 0·47 for a strain of *Pseudomonas extorquens* grown in methanol-limited continuous culture, and it is possible that a selective advantage may also be conferred by mutations which provide a more efficient conversion of the growth-limiting substrate to cell biomass, i.e. mutations which increase yield coefficient.

When moulds or streptomycetes are grown in prolonged, continuous flow culture, it is common for the relatively sparsely branched parental strain to be supplanted by a relatively highly branched mutant (Fig. 6); such mutants are called 'colonial' because in Petri dish culture they form dense colonies which expand in radius more slowly that parental colonies (Fig. 7). Selection of colonial mutants has been observed in continuous flow 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, pers. comm.). In *P. chrysogenum*, but not in *A. chrysogenum*, appearance of the colonial mutant was associated with a reduction in antibiotic productivity. According to Edelman *et al.* (1983) one reason why *F. graminearum* A 3/5 was chosen for myco-protein production was because its sparsely-branched mycelia enabled an appropriately textured product to be obtained. However, after *ca* 500–1000 h in steady state, A 3/5 starts to become supplanted by highly branched, (colonial) mutants (Fig. 8), and because of this phenomenon, industrial myco-protein fermentations have to be terminated prematurely.

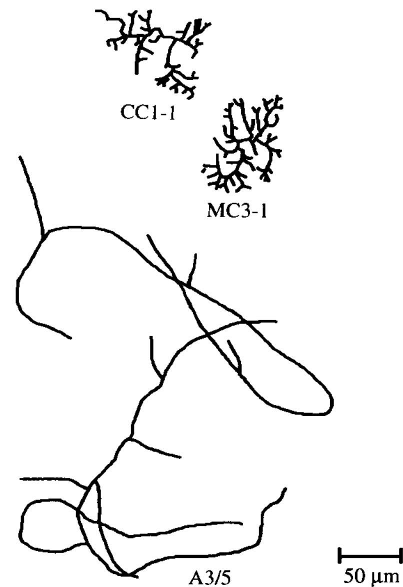

Fig. 6. Comparison of the morphology of mycelia of *F. graminearum* A 3/5 and of two morphological (colonial) mutants (CC1-1 and MC3-1) which arose spontaneously in two separate glucose-limited continuous flow cultures grown at 25° on modified Vogel's medium containing 3 g glucose l^{-1} at a dilution rate of 0·19 h^{-1}, a stirrer speed of 1400 rpm, and a pH of 5·8.

Wiebe *et al.* (1991) isolated twenty morphological (colonial) mutants from prolonged (220 to 600 h in steady state), glucose-limited, continuous flow cultures of *F. graminearum*. All the mutants were more highly branched (hyphal growth unit values ranged from 14 to 174 µm) than the wild type (hyphal growth unit of 232 µm) and produced colonies which expanded in radius more slowly [colony radial growth values (K_r) ranging from 18 to 105 µm h^{-1}] than wild-type colonies (K_r, 135 µm h^{-1}). However, with the exception of two isolates, the μ_{max} values of the mutants were not significantly different from the wild type; the apparent reduced μ_{max} of these two highly branched mutants in batch culture may have been an artifact caused by their forming pellets in submerged, batch culture. The 20 colonial mutants, which were all recessive to the wild type, were assigned to three complementation groups (M. G. Wiebe, G. D. Robson and S. G. Oliver, unpublished). Since the mutants are recessive, the colonial phenotype will only be expressed in continuous flow cultures of *F. graminearum* once the mutant nuclei have become separated from the parental nuclei, as may occur during sporulation (macroconidia are formed from uninucleate phialides) or mycelial fragmentation.

Figure 8 shows the spontaneous appearance of colonial mutants in glucose-limited continuous flow cultures of *F. graminearum*. In these four experiments, colonial mutants were first detected 360 to 672 h after the onset of continuous flow, and, after initial detection, the proportion of colonial mutants

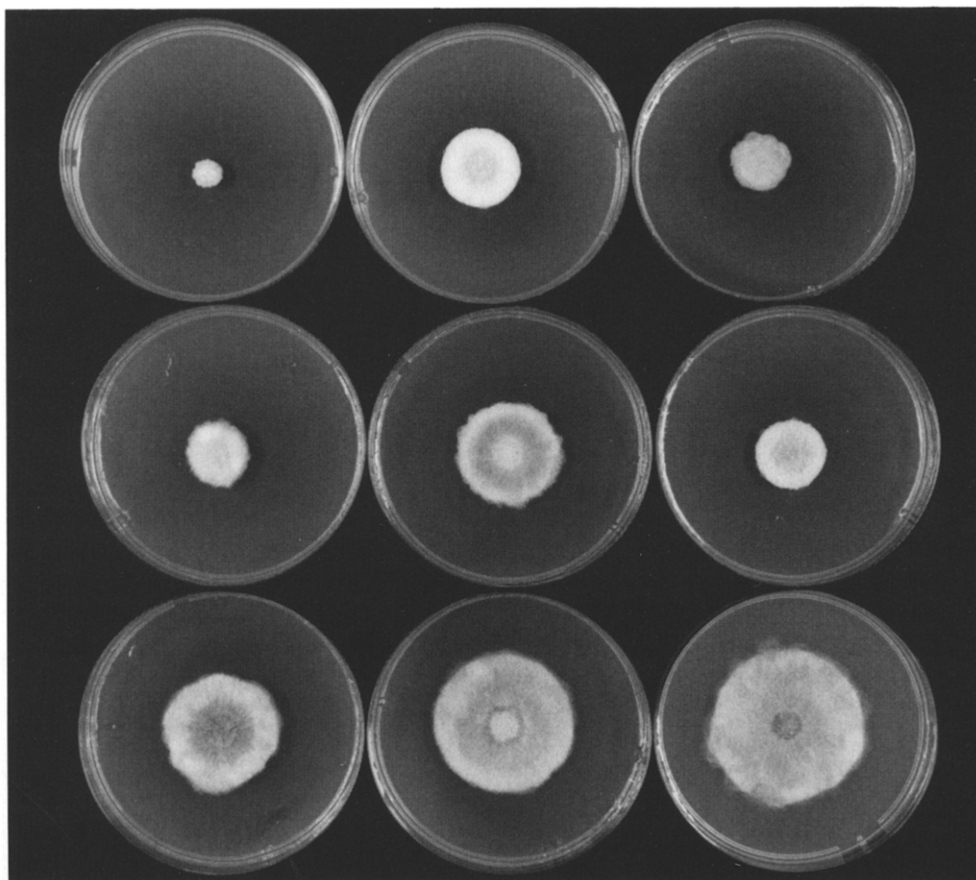

Fig. 7. Colonial mutants isolated from a glucose-limited continuous flow culture (CC1) of *F. graminearum* A 3/5 grown at 25° on modified Vogel's medium containing 3 g glucose l^{-1} at a dilution rate of 0·19 h^{-1}, a pH of 5·8 and a stirrer speed of 1400 rpm, and then cultured for 6 days in 9 cm diam. Petri dishes at 25° on modified Vogel's medium containing 10 g glucose l^{-1}. Strains from left to right and from top to bottom row: CC1-1, CC1-2, CC1-3, CC1-4, CC1-5, CC1-7, CC1-8, CC1-9, and A 3/5 (wild-type strain). (From Wiebe *et al.*, 1991).

in the population increased rapidly (Fig. 8). Figures 6 and 7 show the phenotypes of some of the colonial mutants isolated from two of these cultures. In each experiment (CC1 and MC3) the predominant colonial mutant observed (CC1-1 and MC3-1, was very highly branched (Fig. 6) and had a much slower colony radial growth rate than the parental strain or even the other colonial mutants isolated (Fig. 7). When the MC3 and CC1 experiments were terminated, the predominant colonial mutants (MC3-1 and CC1-1 respectively) represented 80% (experiment MC3) and 97% (experiment CC1) of the total colonial mutant population (Wiebe *et al.*, 1991). The method of Dykhuizen & Hartl (1981) was used to determine the selection coefficients of colonial mutants compared with the parental strain. Plots of the ratio of colony forming units (c.f.u.) of colonial mutants to c.f.u. of the parental strain for these experiments are shown in Fig. 9 and selection coefficients (based on time measured in hours) of 0·017 to 0·034 were obtained.

As indicated above, in steady-state continuous flow cultures, the growth-limiting substrate exerts a selection pressure, so that any mutation which confers upon the organism the ability to reduce the concentration of the limiting substrate will possess a competitive advantage. Reduction of the limiting substrate (*s*) can be brought about by changes which increase

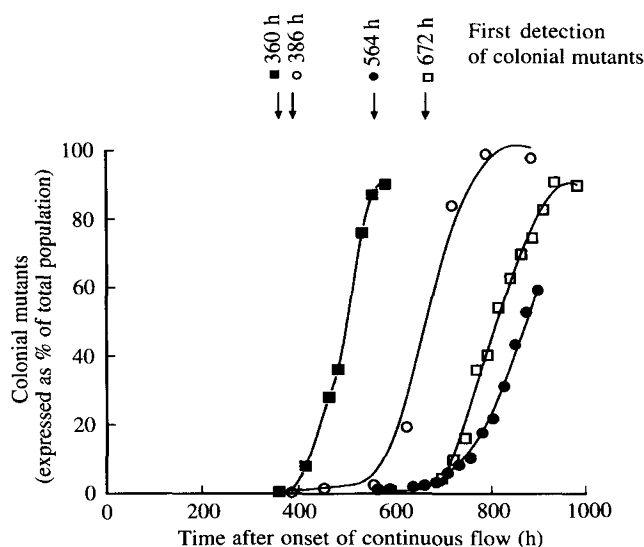

Fig. 8. Population of morphological (colonial) mutants (expressed as a percentage of the total population) generated during four glucose-limited, continuous flow cultures of *F. graminearum* A 3/5 grown at 25° on modified Vogel's medium containing 3 g glucose l^{-1} at a dilution rate of 0·19 h^{-1}, a pH of 5·8 and a stirrer speed of 1400 rpm. Data from four separate experiments (■, CC2; ○, CC1. □, AC20; ●, MC3).

Table 3. Chlorate-resistant mutants of *F. graminearum* which arose spontaneously in macroconidia harvested from colonies grown at 25° for 10 days in plate cultures (G. D. Robson & A. P. J. Trinci, previously unpublished results)

Growth on nitrogen source						
NO_3	NO_2	Hypoxanthine	Glutamine	Locus	Number of isolates	Number of isolates as % of total
−	+	+	+	*nia*D	44	19·0
−	+	−	+	*cnx*	4	1·7
−	−	+	+	*nir*A	66	28·6
+	+	+	+	*CRUN*	109	47·2
+	−	+	+	?	8	3·5

Growth (+), and no-growth (−). Macroconidia harvested from 10-day-old colonies were plated onto modified Vogel's medium containing 300 mM potassium chlorate (each plate was inoculated with about 2×10^5 macroconidia). The chlorate-resistant colonies which appeared were subcultured onto malt extract agar and then tested on modified Vogel's medium containing $NaNO_3$, glutamine, hypoxanthine (all at 2 g l^{-1}) or $NaNO_2$ (0·4 g l^{-1}) as the sole nitrogen source.

μ_{max} and/or decrease K_s (equation 1). Although the selective advantage of some colonial mutants has been attributed to increased μ_{max} values (Forss *et al.*, 1974; Righelato, 1976), the reason why highly branched (colonial) mutants of *F. graminearum* displace the parental strain is not known. Using formulae given by Powell (1958), it can be shown that the percentage increase in the μ_{max} of mutant strains (compared with the parental strain) required to yield selection coefficients identical to those observed experimentally (Figs 8 and 9), is so small (5 and 7% for MC3 and CC1, respectively) that, using present methods of measuring biomass, they would be experimentally indistinguishable from the μ_{max} of the parental strain. Similar experimental difficulties occur when trying to demonstrate that the K_s of the parental strain and colonial mutants are significantly different. Consequently, it has not proved possible to use direct methods to identify the selective advantage of the highly branched, colonial mutants. Nevertheless, indirect evidence suggests that one colonial mutant (MC1-1) isolated from a glucose-limited continuous flow culture of *F. graminearum* has a higher μ_{max} than the wild type, whilst a second (CC1-1) apparently has an altered glucose metabolism (M. G. Wiebe, G. D. Robson, A. P. J. Trinci and S. G. Oliver, unpublished results). However, it is not known

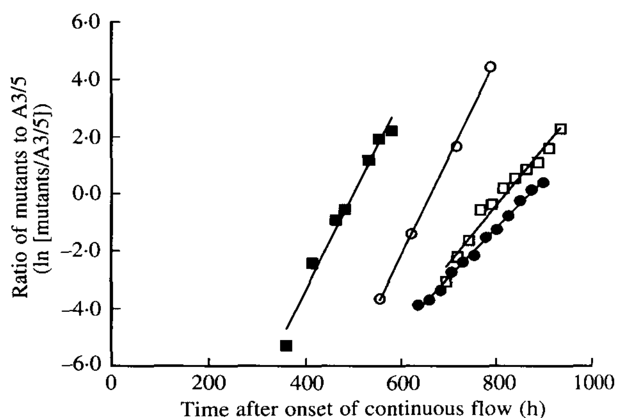

Fig. 9. Ratio of ln of total colonial mutant population to A 3/5 population during four (same as for Fig. 8 above) glucose-limited continuous flow cultures of *F. graminearum* A 3/5 grown at 25° on modified Vogel's medium containing 3 g glucose l^{-1} at a dilution rate of 0·19 h^{-1}, a pH of 5·8 and a stirrer speed of 1400 rpm. Data from four separate experiments (■, CC2; ○, CC1; □, AC20; ●, MC3). Selection coefficients: ■, 0·034; ○, 0·034; □, 0·020; ●, 0·017.

if or how these physiological/biochemical changes are correlated with the altered morphology of the mutants. It is possible that mutations causing a change in mycelial morphology (such as increased branching) are independent from those causing changes in μ_{max} or K_s. Further, the morphological mutation itself may confer some unknown selective advantage to the mutant, although there is no direct evidence supporting this suggestion.

Appearance of selectively neutral (chlorate-resistant) mutants

Mutation in seven genes in *Aspergillus nidulans* results in chlorate resistance and an inability to use nitrate as a nitrogen source (Cove, 1976*a*). Of these seven genes, *nia*D and five *cnx* genes together contribute to the activity of nitrate reductase, the *nia*D gene being the structural gene for nitrate reductase, with the *cnx* genes directing the synthesis of a molybdenum co-factor necessary for the activity of nitrate reductase and xanthine dehydrogenase I and II (Cove, 1979). Mutation in the *nir*A gene in *A. nidulans* results in chlorate resistance and in an inability to use nitrate or nitrite as a nitrogen source. However, some chlorate-resistant (*CRUN*) mutants of *A. nidulans* are still able to utilize nitrate as a nitrogen source, and the existence of these mutants suggests that, contrary to the hypothesis of Åberg (1947), chlorate is not rendered toxic by conversion to chlorite by the activity of nitrate reductase. Instead, Cove (1976*b*) suggests that toxicity may result from chlorate mimicking nitrate in mediating a shut-down of nitrogen catabolism.

Table 3 shows the classification of five chlorate-resistant mutants which had arisen spontaneously in macroconidia harvested from 10-day-old cultures of *F. graminearum* grown in Petri dishes.

In addition to the appearance of selectively advantageous mutants, the occurrence of random, spontaneous mutations in continuous flow cultures leads to a gradual accumulation of selectively neutral mutants, i.e. mutants which have neither a selective advantage nor disadvantage compared with the parental strain. Three chlorate-resistant, neutral mutants (*nir*A, *nia*D and *CRUN*) accumulated at linear rates in a glucose-limited continuous flow culture of *F. graminearum* grown at a dilution rate of 0·19 h^{-1} on modified Vogel's medium containing ammonia as the sole nitrogen source (Trinci, 1990).

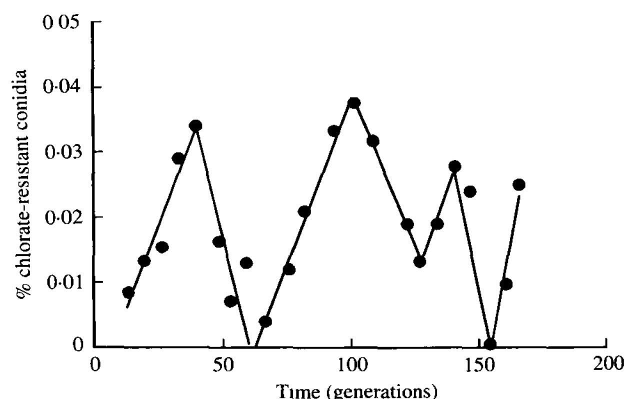

Fig. 10. Frequency of chlorate-resistant macroconidia of *F. graminearum* A 3/5 produced in a glucose-limited continuous flow culture grown at 25° on modified Vogel's medium containing 3 g glucose l^{-1} and 1 g $(NH_4)_2SO_4$ l^{-1} at a dilution rate of 0·19 h^{-1}, a pH of 5·8 and a stirrer speed of 1400 rpm. Macroconidia and mycelial biomass produced in the fermenter were separated and the spores were plated onto modified Vogel's medium containing 10 g glucose l^{-1} and 300 mM potassium chlorate; the wild-type strain (A 3/5) is inhibited by potassium chlorate.

Under these conditions mutations which result in chlorate resistance would be expected to be selectively neutral. These results are consistent with the predictions of Novick & Szilard (1950*b*) and Kubitschek (1970) that, in continuous flow culture, neutral mutants will increase in the vessel at linear rates determined by the relative rates of forward (for example, from chlorate sensitive to chlorate resistant) and back (from chlorate resistant to chlorate sensitive) mutations. For *F. graminearum*, the results suggests that the *nir*A, *nia*D and *CRUN* genes mutate spontaneously at frequencies between 4 to 7×10^{-7} [calculations made assuming no back mutations; some chlorate-resistant mutants of *A. nidulans* arise from deletions (Tomsett & Cove, 1979)]. Figure 10 shows the number of chlorate-resistant macroconidia harvested from a prolonged, glucose-limited, continuous flow culture of *F. graminearum* grown on modified Vogel's medium containing, 1 g $(NH_4)_2SO_4$ l^{-1} instead of 2 g NH_4NO_3 l^{-1}. The observed oscillations in the frequency of chlorate-resistant macroconidia in the culture shown in Fig. 10 may be caused by the appearance at intervals of selectively favoured mutations that arise in chlorate sensitive strains (the vast bulk of the population) and displace the neutral, chlorate-resistant strains (Paquin & Adams, 1983).

PRESENT-DAY MARKETING OF QUORN® MYCO-PROTEIN AS A 'HEALTH' FOOD

Although myco-protein was originally developed to supplement the world's flagging supply of conventional protein foods, by the late 1970s and early 1980s, when the production techniques had been fully established, and when it had been approved for sale to the public, the predicted global protein shortage had not materialized. However, in the western world, Quorn® myco-protein now satisfies the needs of the 1990s in that it provides a low-calorie (80 kcal 100 g^{-1}) food which lacks animal fats and cholesterol and is low in saturated fats and high in dietary fibre. The composition of Quorn® myco-protein and braised beef are compared in Table 4. The protein content of myco-protein is comparable to that of milk and cottage cheese and it contains the correct proportions of the eight essential amino acids required by man (Duthie, 1975; Solomons, 1985; Sadler, 1988); indeed, its amino acid composition is close to the ideal recommended by the FAO, with, however, methionine and cysteine being the limiting amino acids (Sadler, 1988). Even though many of the vitamins etc. are lost in the RNA reduction process, myco-protein is biotin-rich (16 μg 100 g^{-1}), and contains most B vitamins although it is lacking in B_{12}, a vitamin provided by meats and other animal products; its B_6 content (0·13 mg 100 g^{-1}) is equivalent to that of lamb, its riboflavin content (0·23 mg 100 g^{-1}) is similar to meats, and it contains about one tenth of the nicotinic acid present in meats. The mineral content of myco-protein also compares favourably with meat, and the iron content (1·3 mg 100 g^{-1}) of myco-protein is equivalent to pork, and is higher than chicken. However, iron is present in an inorganic form, in contrast to the well adsorbed haem-iron present in meats. Myco-protein is very rich in zinc, which would be useful to vegetarians who generally have a low zinc intake (Sadler, 1988). Finally, Quorn® myco-protein has approximately the same dietary fibre content (5·1% w/w) as fresh green vegetables such as peas, and has more dietary fibre than wholemeal bread. In the development of myco-protein, meat analogues have been favoured since meat is a high-value protein food.

Table 4. Comparison of the composition of braised beef and Quorn® myco-protein

	Component as % of total (by weight)	
Component	Braised beef	Quorn® myco-protein
Protein	30·9	12·2
Dietary fibre	0	5·1*
Fat, total	11·0	2·9
Fat, saturated	4·6	0·4
Polyunsaturated fatty acids: saturated fatty acids ratio	0·1	2·5
Carbohydrate	0	1·3
Cholesterol	0·08	0

* Mainly chitin and β-glucans in hyphal walls.

The first retail product to contain Quorn® myco-protein was a savoury pie (Fig. 11) which was sold by Sainsbury's in January 1985. The decision to make the first myco-protein product in a traditional rather than a completely novel form was to generate consumer interest and because pies are a popular form of food. Fish analogues made from myco-protein have been investigated but to date lack of a suitable flavouring additive has hampered their development (Edelman *et al.*, 1983; Sadler, 1988). According to Quorn® Information Services, in 1989, there were 35 myco-protein products on offer to the consumer, including entrées, casseroles, desserts and salads but with a predominance of highly spiced foods because of Quorn® myco-protein's ability to absorb and enhance the flavours of herbs, spices and sauces used in

Fig. 11. A chicken style and a potato topped savoury pie containing Quorn® myco-protein. Reproduced with permission of the copyright holder, J. Sainsbury PLC.

Fig. 12. Uncooked, unflavoured Quorn® myco-protein (with top covering on and off) marketed by Tesco's. Reproduced with permission of the copyright holder, Marlow Foods.

cooking. Currently, Quorn® myco-protein is increasingly available in ready meals and pies and as an ingredient for home cooking. The estimated retail sales value of Quorn® myco-protein is currently in excess of £12M p.a. Advertising of Quorn® myco-protein is currently being targeted at A, B, C1 and C2 females aged 25–45 who are reducing the meat content in their diets (this group covers 50% of all women). Sales of the newly marketed Quorn® myco-protein for home cooking (Fig. 12) have soared and have encouraged Marlow Foods to plan for increased production. Due to this success in the U.K. approvals are now being sought to enable Quorn® myco-protein to be sold in other countries. Thus, although it has taken a long time (1964–85) to bring Quorn® myco-protein to the marketplace, it has the distinction of being the sole survivor of the many SCP programmes initiated in the 1960s, and can be regarded as a significant 'success story'.

I am grateful to Mr T. W. Naylor (Marlow Foods), Professor S. G. Oliver (UMIST), Dr G. D. Robson (University of Manchester), Dr G. L. Solomons (formerly of RHM) and Dr M. G. Wiebe (University of Manchester) for valuable discussions, and to the SERC Biotechnology Directorate and Marlow Foods for financial support.

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