CONTROL OF FUNGAL MORPHOLOGY IN CONTINUOUS CULTURE.

by

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The control of Fungal Morphology in Continuous Culture.

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The aim of this project was the development of a system for the continuous production of fungal spores by the control of mycelial morphology and the optimisation of environmental factors stimulating sporulation.

Spores, considered a dormant stage in the life cycle of fungi, have been shown to contain enzymes unrelated to those required for germination, but which perform a variety of commercially important metabolic activities. The production of spores in large numbers as stable enzyme packages therefore has considerable commercial potential.

Sporulation of Aspergillus niger and Aspergillus ochraceus was induced by growth restriction using nutrient and environmental limitations. Citrate supported poor sporulation while ammonium nitrogen inhibited sporulation at all concentrations. Examination of the pH, temperature, carbon dioxide and ferrocyanide ion tolerance of both organisms indicated that no physical factor alone induced sporulation. However, a transient sporulation phase occurred in response to gradual carbohydrate and to shock nitrate limitations.

To increase spore productivity, the transient sporulation phases were induced semi-continuously by cycling nutrient supplies. Continuous production was then achieved in a two-stage fermentation system.

For these systems to operate successfully, continuous growth of the fungus during sporulation was essential, and there was no upper limit of growth rate which prevented sporulation. Spore production occurred at specific carbohydrate supply rates above the calculated maintenance requirements for fungal survival, but values could not be calculated for nitrogen maintenance because growth continued without an external nitrogen supply.

Both organisms displayed variable and much-simplified sporulation apparatus, indicating that complex structures were not required in a submerged environment. Sporulation was controlled by a multivariate and delicate interaction between growth rate and the nutritional and physiochemical environment.

The continuous tower fermenter proved ideal for controlling fungal morphology and enabled a system for the controlled continuous induction of sporulation to be developed.

Sporulation Filamentous Fungi Continuous Fermentation

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1.0 INTRODUCTION.

1.1.0. The development of biotechnology.

The origins of the industrial uses of fungi are lost in the mists of time. Fermentation was practised as an art by the Egyptians and Babylonians long before any scientific implications of the process were understood. Alcohol was known to be produced by the fermentation of sugars, although the role of microorganisms was unknown, and the methods of production of cheese, yoghurt, soy sauce, vinegar, beer and bread had likewise been perfected by many races of the world with no scientific knowledge.

The ability of living organisms to produce fermentation reactions was unveiled by Pasteur in the latter half of the 19th Century, and concommitant biochemical studies, particularly those of Wehmer (1893) who first described the production of citric acid by Aspergillus niger, helped explain the scientific basis of fermentation. This somewhat theoretical subject was transformed into 'industrial fermentation' at the outset of the first world war, primarily due to the research of Chaum Weizmann at Manchester University. The war demands for acetone were met by the developmen of an industrial scale acetone production process based upon microbiological degradation of maize starch, which was subsequently adopted by the Admiralty. This is one example of the way in which the urgent needs of raw materials for the war effort during the two wars stimulated research into the cheap and rapid production of protein, fats, organic acids, vitamins and antibiotics by new biological means. Although the principle of fungal citric acid production was known, it was not until the 1920's, some 40 years after the discovery by Wehmer, that methods for commercial production were developed. The discovery of Penicillin by Fleming in 1928 lead to intensive research into antibiotic

production by fungi, and to subsequent development of fermentative production systems which are now well - established techniques. The mould fermentation industry expanded rapidly in post - war years, aided by new biochemical information. For example, Krebs (1937) had published his fundamental work on the tricarboxylic acid cycle which clarified the relationships of many of the simpler fungal metabolic products. In the last 50 years, while many chemicals have been produced more economically by the oil industry, microbial technology has enabled the development of commercially successful industrial systems for the production of valuable products, including antibiotics, vitamins, organic acids, enzymes, amino acids and, particularly relevant to this work, modified steroids. Most steroids of chemical importance in therapy are physiologically active only if hydroxylated, but most natural steroids readily available are not so hydroxylated. The clinical transformation of these compounds is difficult, long and expensive, while in the 1950's steroid - transformation was found to be within the metabolic potential of many fungi, and many medically important steroids are now activated by fungi, and particularly by fungal spores. Many of these aspects of industrial mycology are comprehensively described by various authors in 'The Filamentous Fungi, Vol. 1' (Smith and Berry, 1975). Within the last decade the recognition of the critical food, energy and raw material shortages, and the way in which these must be overcome for future survival, have lead to the development of the relatively new discipline of biotechnology. This study encompasses all forms of commercial exploitation of the interaction between living organisms, (particularly microorganisms) and their environment for industrial

processes. The production of single - cell protein and alcohol as an alternative fuel source are two examples of intensive research within this field, likewise fungal transformations will undoubtedly receive increased attention. Kristiansen and Bu'lock (1980) have reviewed developments in industrial fungal biotechnology and conclude that as fermentation products find new application, so fungal technology has to move into new areas. While this represents the greatest challenge to biotechnologists, the advanced level of technology now available means the future is met from a position of strength.

1.2.0. Fermentation systems

Hatch (1975) defined a fermenter as 'a vessel which provides the nutritional and physiological environment required for single cell growth.' The choice of fermenter design for a particular process will depend on many factors which include :

1) The final product.
2) The nature and availability of substrate.
3) The services available to the fermentation plant once established.
4) The degree of technical supervision that the process will require and the availability of such suitable technical personnel.

Many designs have now been developed, but before any basic design can be chosen, the commercial performance criteria of the different kinds must be known, and there is at present little comparative data available (Emery 1976). There is a need for more performance data for systems based upon economic objectives, so that meaningful comparisons can be made. Katinger (1977) discusses new fermenter configurations, and lists the desired

properties of fermenters :

Table 1.1.

A. Functional requirements.

1. High gas/liquid mass transfer.
2. Creation of gas/liquid interfaces without causing foaming problems.
3. Sufficient gas, liquid or solid hold - up.
4. Reasonable heat transfer.
5. The avoidance of 'dead' zones to maintain efficient mixing.
6. Aggregation prevention without damage to the organism.

B. Economic requirements.

1. Cheap, robust and simple mechanical design.
2. Ease of operation and automation.
3. Well understood scale up characteristics.
4. Flexibility with respect to process requirements.
5. Stable operation ability.
6. Lower specific power consumption.

Thus the commercial viability will be based not only upon a balance between income from the process and expenditure on running costs and, principly, capital investment, but also on reliability, stability, flexibility and ease of control. Many traditional examples of fermentation processes are successfully operated by unskilled personnel, and do not require expensive plant. These could be of use to many countries who would benefit from fermentation technology, but do not wish to invest in expensive and complex fermentation equipment. One should

therefore aim to develop systems which at first need sophisticated biochemical engineering knowledge in order to eventually attain stability and simplicity of operation.

In the early years of the fermentation industry the mechanically agitated fermenter became standard (Hatch 1975) primarily because the stirred tank reactor was adopted during the war for penicillin production, and subsequent manufacturers adopted what was the established technology. The stirred tank reactor is mechanically agitated, fully baffled fermenter with an open - blade turbine mixer. Many modifications of the design exist and have been reviewed by Solomons (1968), Rowley and Bull (1973) and the industrial applications of the system are outlined by Hospodka (1966). Stirred tanks, however, have several drawbacks. Under viscous conditions (mycelial fermentations) it is difficult to achieve adequate mixing, and the efficiency of aeration decreases. Donovick (1960) states that aeration is not the only parameter governed by agitation, that solute concentration gradients are largely governed by agitation and that physical agitation and shearing of mycelium must also play an important role in their own right. Laboratory fermentations often have to be terminated due to growth of mould on the impellers etc., (Evans 1965) and much of the energy supplied by the impeller is dissipated as heat, again decreasing dissolved oxygen levels, but also creating the need for cooling apparatus which imposes severe economic penalties upon the system, particularly in the tropics (Greenshields 1980). These vessels also have scale - up problems in that parts become very expensive as size increases (Solomons, 1972 and Cocker and Greenshields, 1977). An alternative to these vessels is the air - lift fermenter, which works on the principle

of air introduction at or near the base, which provides both aeration and mixing as it rises up the vessel. The varieties of air - lift fermenters are described by Ewen (1980). The simplest form is the bubble column reactor, which is versatile, cheap to construct and has low energy requirements although these have been only slowly adopted by the fermentation industry despite their valid biotechnological advantages. The tubular bubble column used in this work was the continuous tower fermenter described by Greenshields et al. (1971); Greenshields and Smith (1971 & 1974); Smith and Greenshields (1974) and Cocker and Greenshields (1975). For a detailed description of the system see section 2.1.0. In his review of bubble - column reactors, Oestergaard (1968) classes this type of fermenter as a gas/liquid fluidised reactor where the solid phase is suspended by the upward movement of gas and liquid. Greenshields and Smith (1971 & 1974) review the applications of the tower fermenter. Some of the earliest attempts to use the towers for metabolite production were for the production of citric acid from the inhibited fermentation of molasses by A. niger. This is a non - growth associated metabolite, and so only semi - continuous operation was possible (Horitsu 1971), but the pellet form of growth was found to be particularly advantageous for the fermentation. The system was also found to be suitable for the production of bacterial and fungal biomass (the mass production of yeast is described by Rosen, 1968) and to represent a remarkable increase in the efficiency of the production of alcoholic liquids when used without aeration/agitation. This was due to the heterogenous nature of the population in non - aerated towers (dense at the bottom, sparse at the top) fermenting the

substrate at the optimum rate as it passed up the tower. In this way the alcoholic charging wort for acetification in malt vinegar manufacture could be made, and the tower fermenter has also proved ideal for continuous acetic production from this wort (Greensh ields 1972, Nerantzis 1978), having a lower aeration requirement than conventional systems. Morris (1972) undertook a detailed investigation into the growth of A. niger in batch tower fermenters and this work was continued by Cocker (1975) in continuous culture, where filamentous organisms are shown to form a variety of floc formations depending upon the cultural conditions (Cocker 1980). At present, the towers are considered particularly suitable for the fermentation of large columes of dilute substrates (Pannell and Greenshields 1976) and recent workers have concentrated upon this application. The towers are easily scaled - up, have the advantage of low capital investment, are adaptable and easily automated and the strongest motive for their use in fermentation is to establish them as a means for the rapid and cheap evaluation and exploitation of new applications. The towers impose very little shear force upon the mould, while aerating the culture adequately, and are therefore ideally suited for the controlled development of mould morphologies (Morris et al. 1973). The problems of scale - up are discussed by Emery (1955, 1976), Gaden (1960) and Solomons (1972) who concludes that specific proposals for scale - up methods with supporting data are scarce and that more attention should be applied to the scale - up of the fermentation process rather than just the reactor vessel or its aeration capabilities.

1.3.0. The advantages and disadvantages of continuous culture.

The advantages of continuous culture have been reviewed by

Maxon (1955), Beran (1966), Malek (1966) and Langlykke (1970) and are summarised here. Tempest (1970) discusses the place of continuous culture in microbial research, and questions why it has found so little favour among microbiologists. After all, it offers a rigidly controlled environment, which is the only way to study the physiology of growing functioning organisms which are able to quickly change themselves chemically and physically in response to environmental change.

1.3.1. Advantages.

1) A marked decrease in processing time, using the same capacity or, conversely a smaller fermenter size for equivalent product formation. This is a primary consideration in the economic plans of a fermentation system. Unproductive phases of batch cultivation, namely the lag periods after inoculation, harvesting, cleaning, refilling, sterilization and reinoculation, are avoided. There are several types of continuous fermenter that have been used with fungi. The best known is the chemostat, in which the flow rate of medium is controlled such that the dilution rate has any value below the maximum specific growth rate, and one or more substrates of the medium is growth - limiting. The turbidostat operates at the maximum growth rate by adjusting the flow rate to maintain the organism concentration at a level at which growth is not substrate limited. The principle of tower fermenter operation falls between these two, as fermenter design and mould morphology combine to retain biomass in the vessel, enabling operation at dilution rates far in excess of growth rate, with all nutrients of the medium in excess.

2) Steady state operation gives a more homogeneous population and greater organism uniformity and synchrony of development and

biochemistry of the organism.

3) Equilibrium conditions can be established in which the culture population density, environment and growth rate do not change with time. The growth rate can therefore be varied by alteration of the environment, which, since it is rigidly controlled, allows the development of a whole range of cultures containing phenotypically different organisms, and hence the selective enhancement of each morphological stage of the life cycle of the organism. This allows study of the biochemistry and physiology of the growth stages which are normally transient in batch culture, but which can be maintained in continuous culture. Beran (1966) says that for any stage of a batch culture, there is a point at which continuous cultivation can proceed at steady state (this theoretically includes sporulation). If a steady state is changed, a new one will develop (Hough & Wase, 1966) at new physiological conditions, and this enables greater product control especially when a product is only obtained during a narrow physiological state, which can be maintained in continuous culture.

4) The effect of each factor of the environment upon the organism may be studied alone or in combination and the causal relationship between environment and morphology/physiology, and the functional significance of precise changes in morphology and physiology in response to the environment may be assessed.

5) The great variety of unusual physical and chemical conditions which would greatly handicap other biological systems may be applied to favour unusual products, e.g. antibiotics, physiologically active drugs, chemicals, and enzymes. The novelty and variety of such products particularly recommends

the extension of fermentation into this area (Langlykke 1970).

6) The culture is physiologically 'permanently young', as any time factor is eliminated.

7) The kinetics are simpler than those in batch culture.

8) Being self - regulating and self - perpetuating it can fit into a production line of continuous medium make - up, sterilization, production, extraction and processing of products. As a result, it will be more economic, effective and efficient.

9) Continuous culture is ideally suited to automation and is therefore easier to control.

10) Any population can be obtained by careful manipulation of the environment and so the influence of population density upon growth and production can be studied.

11) There is generally easier separation of the product. This has greater importance at industrial level, but will be particularly important in spore production at all scales.

12) The number of stage multiples is theoretically unlimited. For the production of non-growth associated metabolites, and those associated with the death phase of an organism, a two stage system is generally required. In such cases, the dilution rate may exceed the growth rate, enabling the study of new developmental cycles (eg sporulation). This application is considered further in section 4.9.

1.3.2. Disadvantages.

Tempest (1970) and Pirt (1972) reviewed the prospects and problems of continuous culture, which will obviously vary from system to system.

1) Mutation. - During continuous fermentation, whether at industrial or laboratory scale, stability of the organism strain

is essential. One of the problems with continuous culture is the random mutation of a strain that will change, often detrimentally, the growth characteristics. The kinetics of mutation formation and wash - out are discussed by Gerhardt and Bartlett (1959). This has not proved a great problem in this system, due to the low mutation rates found and the continuous wash - out effect of the process. Only a very high mutation rate would cause problems.

2) Infection. - The environment is by nature selective, and any infecting organism which is better suited to the conditions or has a higher growth rate, will gradually replace the original culture. Again, this effect is rare, due to the wash - out effect of continuous culture and the generally low culture pH. Contamination will of course interefere technically with the fermentation and impair the final product quality, but fermentations at dilution rates at or above 0.2 h^{-1} were run aseptically without infection. At lower dilution rates, sterile techniques were used, but slight infection was unavoidable, particularly when unfavourable conditions were being applied to the culture.

3) Fouling of the system. - Uncontrolled growth of organisms on fermenter walls, baffles and impellors has obvious disadvantages, but this was not a problem in the tower fermenter used, except for mycelial aggregation around sensory probes.

4) If the contents of a continuous fermenter die, and the culture is lost, then this is a greater setback than if one batch fermentation is lost.

5) Steady - state instability. - Theoretically, an aerobic continuous culture producing biomass is a stable system, but a steady state may be practically difficult to attain.

Fuld et al. (1961) attributed their unsteady - states of Aspergillus ochraceus culture to the slow growth rate of the organism. Likewise, synchrony of cell division, and undamped oscillations interfere with the assumption of a true steady - state. Technical problems with the fermenter itself contribute to erra tic results, and the morphology of the mould will change with time, which will cause oscillations of the steady - state. (Pannell 1976).

1.4.0. Fungal nutrition.

Fungi need a carbon (energy) source, a nitrogen source and trace elements to maintain their viability, and to grow. Requirements differ between species, even between strains, both qualitatively and quantitatively, but the growth and morphology of a mould is partially governed by the availability of the essential nutrients and the ability to synthesise other requirements from the environment. This project will primarily examine the relationship between type and availability of carbon and nitrogen sources, and a brief consideration of the ways in which these nutrients are metabolised and of how this metabolism is regulated, is therefore pertinent.

1.4.1. Nitrogen supply and metabolism.

There have been few in - depth studies of nitrogen metabolism in fungi, and thus the few well - investigated species can only provide an indication of the metabolic activities of others. Major reviews of nitrogen metabolism in the fungi are by Nicholas (1965), Pateman and Kinghorn (1976, 1977) and Kinghorn and Pateman, (1977). Most fungi can use nitrate as a sole nitrogen source, and the equivalent inorganic source ammonia (Steinberg

1937), although the form in which the ammonia is supplied can be important. This is due to the low pH of the growth medium when ammonia ions are utilized, which can be inhibitory to some fungi. Nitrite is not preferentially used by most fungi, although the ability to do so is present in those resistant to the toxicity. In one sense, nitrite is utilized by all fungi that can use nitrate as it is an intermediary in the metabolism of nitrate. Hawker (1950) reports that A. niger is able to utilize nitrite if necessary. Urea, while neither inorganic nor an amino acid, is another commonly utilized source of nitrogen, and Steinberg (1937) considers it equal in nutritional value to nitrate and ammonia ions, when the trace elements contents of the medium are adjusted accordingly. Theoretically, ammonia is the optimum inorganic source, because nitrate and nitrite are both reduced to ammonia prior to use in amino acid synthesis (Kostychev and Tsvetkova 1920). Ammonia is a weak electrolyte, and can enter the cell by passive diffusion; but an active transport system has been characterized in Aspergillus nidulans (Pateman et al. 1974).

The reduction of nitrate to ammonia. Nitrate is reduced via nitrite and hydroxylamine to ammonia in a series of steps. The enzymes involved contain a number of cofactors and metals, and utilize NADPH as a hydrogen donor.

The sequence :

$$NO_3 \xrightarrow{\text{Nitrate reductase}} NO_2 \xrightarrow{\text{Nitrite reductase}} (NOH?) \xrightarrow{\text{Hyponitrite reductase?}} NH_2OH \xrightarrow{\text{Hydroxylamine reductase}} NH_3$$

The nature of the intermediate between nitrite and hydroxylamine is uncertain. The assimilatory nitrate reductase enzyme appears to be activated by molybdenum (Mo). Steinberg (1937) examined

the role of Mo. in the utilization of ammonium and nitrate nitrogen, by A. niger and concluded that Mo. is essential for the activation of nitrate reductase, and that, although there is generally sufficient Mo. present as impurities in most media, a Mo. deficiency in a nitrate medium will have the same effect as nitrogen deficiency.

Nitrite reductase activity has been reported to require copper (Nicholas et al. 1960) and a ferric ion for the haem prosthetic group in the enzyme (Garret and Amy 1978). Pateman and Kinghorn (1976) suggest that manganese and magnesium may be necessary for the formation of the hydroxylamine reductase molecule in vivo. There has been no unequivocal demonstration that a nitrate transport system other than diffusion exists in any fungus, but a nitrate assimilation system is claimed for P. chrysogenum (Goldsmith et al. 1973) and N. crassa (Schloemer and Garrett 1974a). Schloemer and Garrett (1974b) also report a nitrite uptake system for N. crassa.

Regulation: Nitrate accumulates in the cell in a medium also containing ammonia, due to the repression of nitrate reductase activity with a functioning uptake system (Goldsmith et al. 1973, Garrett and Amy 1978) induced by the presence of nitrate. This effect led Schloemer and Garrett (1974a) to suggest that the uptake and reductase activity are separate processes in the assimilation of nitrate. Ammonia assimilation is unaffected by the presence of nitrate. Nitrite uptake is unaffected by the presence of either NH_3 or NO_3, because it is in the own interests of any organism to continually remove toxic nitrite, (Morton and Macmillan 1954). Cove and Pateman (1969) propose a model for the regulation of nitrate and nitrite production in A. nidulans

Amino Acids and proteins are available to fungi as a nitrogen source. Amino acids may be assimilated and incorporated into proteins, although some deamination usually occurs. Proteins are broken down to amino acids prior to utilization by extracellular proteases. An exogenous carbon supply must always be present to permit any nitrogen utilization. The synthesis of nucleic acid and protein by filamentous fungi from their nitrogen supply is reviewed by Berry & Berry (1976) and Anderson et al. (1973) analyse the distribution of nitrogen in the cells of microfungi, splitting the content into non - protein nitrogen, amino nitrogen and total nitrogen values.

1.4.2. Carbon metabolism.

Carbohydrates are usually the major source of carbon for fungi, although fatty acids and organic acids may also be utilized. Most fungi can utilize a range of monosaccharides, oligosaccharides and polysaccharides although uptake is usually restricted to monosaccharides. Disaccharides and polysaccharides are normally hydrolysed by extracellular amylases, cellulases or invertase. Hasija and Wolf (1969) studied the growth of A. niger on a variety of carbon sources, and obtained good growth with most hexoses and pentoses, although growth with sorbose and lactose was poor. Brannon (1923) reported that fructose was metabolised by Aspergilli and Penic-illia in favour to glucose. Anderson et al. (1973) reviewed the growth of microfungi on carbohydrates and stated that growth rates on pentoses were generally lower than on hexoses. Carbohydrate catabolism is traditionally divided into three phases; glycolysis, pyruvate metabolism and the tricarboxylic acid (TCA) cycle, and oxidative phosphorylation. The detailed processes of glycolysis are described by Blumenthal (1965) and Cochrane (1976), the TCA cycle by Niederpruem, (1965) and oxidative phosphorylation by Lindenmeyer (1965). Available

evidence indicates that these pathways are the same in fungi as in other organisms. A review of carbon metabolism in A. nidulans is given by McCullough et al. (1977). Glycolysis denotes the metabolic sequences by which sugars are converted to pyruvate or lactate for subsequent entry into the pathways of terminal oxidation. In fungi, there are three pathways; the Embden - Meyerhof-Parnas (EMP) pathway, the Pentose Phosphate (PP) pathway and the Entner - Doudoroff (ED) pathway, which is rarely encountered in fungi. Glucose is metabolised to pyruvate via the E.M.P. pathway, and this is the major pathway in most species of fungi. The pentose phosphate (or hexose monophosphate pathway, HMP) has no one end - product. Instead, a pool of triose, pentose, hexose and heptose phosphates is formed, and the principal role of the pathway is the provision of reduced nicotinamide adenine dinucleotide phosphate (NADPH) for synthetic reactions.

The second stage in the oxidation of carbohydrates in aerobic cells is the oxidation of pyruvate to CO_2 via the TCA cycle. Acetate (from oxidative carboxylation of pyruvate) is the main substrate of the TCA cycle, although other intermediates supplied exogeously can be metabolised. The TCA cycle is inactive in aerobic conditions. In the presence of glucose the TCA cycle is maintained by carboxylation reactions from phosphoenol pyruvate, and the glyoxylate cycle is non - fuctional. (This synthesises oxaloacetate from isocitrate and acetate and operates when acetate is the sole carbon source). (Berry 1975)

1.4.3. The carbon/nitrogen ratio.

Morris (1973) examined the effect of the C : N ratio upon the growth rate of A. niger in batch culture and concluded that the ratio had a significant effect. Optimal mycelial growth rate

lay between C : N ratios of 7:1 and 13:1 and this ratio was maintained in the basal medium used for the cultivation of this mould for this project.

1.4.4. Trace elements.

There is no evidence for qualitative differences between mineral needs for sporulation and vegetative growth. Sporulation at no time requires something not essential to growth, but there are quantitative differences between the two processes, as sporulation often needs higher concentrations than vegetative growth (Valenzuela - Perez 1968).

Most trace metals are present in sufficient quantities for growth as impurities in other compounds. The effect of trace metals on the growth of fungi has been reviewed by Foster (1939) and specifically for A. niger by Steinberg (1939). Magnesium, Manganese, Potassium, Molybdenum, Zinc, Copper and Iron are all essential in trace amounts, but are easily toxic. (Foster 1939, states that Gallium is also essential for the growth of A. niger.)

The change in trace metal content between vegetative mycelium and spores of A. niger was shown by Dalby and Gray (1974) ;

	Cu.	Zn.	Mg.	K.
Mycelium:	8	468	1239	18,638
Spores:	51	651	450	38,144

(concentrations of element in μg element g dry weight^{-1} of fungus)

Zinc is a well - known enzyme activator and is essential for the correct functioning of the EMP and TCA cycles of A. niger (Valenzuela - Perez 1968) and cannot be replaced in the metabolism. Zinc is also essential for sporulation (Steinberg 1935) and the first sign of lack of zinc in the medium of a growing culture is the decreased growth rate and increased sporulation, and only when

the deficiency is complete is sporulation inhibited (Foster 1939). Tomlinson et al. (1950) found zinc to be essential for citric acid production by A. niger, although its' presence may have an adverse effect upon some strains (Foster 1939). The most striking effect of copper, appears to be upon sporulation and pigmentation where an increased level is required by A. niger. If copper is absent from the medium, sporulation is reduced (Foster 1939). Although calcium does not appear to be essential for growth of A. niger, it is required for phialide and conidium production during sporulation of A. niger in submerged culture (Valenzuela - Perez 1968) and this requirement has also been shown for the sporulation of Penicillium notatum in submerged culture (Foster et al. 1945, Hadley and Harrold 1958). Foster (1939) gives a detailed account of the role of iron in the growth and sporulation of A. niger. The black spore pigment of A. niger (aspergillin) is a haemoglobin - like molecule and contains iron. The role of molybdenum in the activation of nitrate reductase has already been described, and this element is essential to growth when the nitrogen source for the organism is nitrate. Phosphorus plays a well - known role in ATP production and is incorporated into the nucleic acids (RNA, DNA) and into polyphosphates which are used as energy reserves, particularly during spore formation. Bajaj et al. (1953) report that spores of A. niger have a higher phosphate content than the mycelium, with 25 - 34% of the mycelium total phosphate being transferred to the spores as phosphoric esters as easily hydrolysed energy reserves. There is an increased uptake of phosphate from the medium at the onset of sporulation, even though the mycelial content is decreasing.

Sulphur is used in the synthesis of sulphur - containing amino acids, and Steinberg (1939) suggests that sulphurous compounds should contain oxygen to support A. niger growth, ie; be supplied as sulphate.

Manganese, while essential, is required in very small amounts, and this makes biochemical studies of its' effect upon growth and sporulation very difficult. Steinberg (1939) reports that absence of manganese from a medium causes a marked decrease in spore formation. The effect of aluminium upon the growth of A. niger was described by Bertrand (1963). In pure medium, when aluminium is lacking, growth rate is poor, but increases as aluminium is added as a function of aluminium concentration up to saturation level. The reason for this is unknown.

Potassium and sodium: Tresner and Hayes (1971) report that Aspergillus and Penicillium species are the most tolerant fungi to sodium chloride, particularly A. ochraceus. NaCl has been shown to be essential for sporulation of A. ochraceus (Vezina et al. 1965). Potassium has a growth promoting effect which cannot be replaced by the related elements, sodium and lithium. All three of these elements promote sporulation in the order Li > Na > K.

The role of lipids, amino acids, alcohols, growth promoters and other aspects of the chemical environment in the growth of A. niger are reviewed by Cocker and Greenshields (1977). The requirement by a fungus for vitamins and growth promoters depends upon the ability of the fungus to synthesise its' requirements from the medium constituents and yeast extract was supplied in the medium during this work as a source of these factors and of many trace elements.

1.5.0. Secondary metabolism and differentiation.

Growth and secondary metabolism are frequently complementary alternatives, but that is not to say that the two are incompatible activities. Growth is dependent upon a number of biosynthetic activities, and the absence of any one of several nutrients may restrict it, and the remaining non-limiting nutrients may then be diverted into biosyntheses which are not related to growth. The biosynthesis of secondary metabolites or specific compounds required during differentiation may be stimulated in this manner, indeed Bu'lock (1975) states that secondary metabolism is an aspect of the differentiation which limited growth usually implies. The similarity between physiological and biochemical control of secondary metabolism and differentiation, suggests that they are closely related processes each of which can benefit from being studied in the context of the other. The onset of differentiation, and production of non growth - associated secondary metabolites is characterized by new metabolite production and by qualitative or quantitative enzymatic changes. The pattern of secondary metabolism, (and thus differentiation), is determined not merely by the existence of a limitation upon growth, but more particularly by the intensity of that limitation, and different secondary metabolic pathways will have different metabolic requirements (Bu'lock 1975). The capacity to control the metabolic state of the cell by environmental manipulation is therefore important whether for production of metabolites or induction of differentiation. In several fungi, the production of secondary metabolites is associated with the development of specific reproductive sturctures, and there are also examples of the requirement of the reproductive unit itself. Mushroom cultivation (frequently of Agaricus bisporus) relies upon the induction of a fruiting body from vegetative mycelium, and

there are several reports of hallucinogens produced from the fruit bodies of Basidiomycetes (legally and otherwise) and cephalosporin is produced only in the arthrospores of Cephalosporium acremonium (Smith and Berry 1974). Continuous culture has removed the requirement for some other reproductive phases for metabolite production. For example, penicillin used to be produced in batch culture in the decreasing growth phase of Penicillium spp. but is now produced in continuous culture and penicillin production is directly proportional to growth rate, because catabolite repression which operated in the batch system, is removed (Pirt & Righelato 1967). Citric acid production, while not a secondary metabolite, is associated with the restriction of growth of A. niger by the addition of various ferrocyanide ion concentrations. The production of fungal spores for mutation studies, for the storage of inoculum, for seed stages and for use as a stable form of biomass represents a more obvious application of fungal differentiation to industrial mycology, but the cultivation of mushrooms must remain as the most conspicious example of exploitation of fungal differentiation for economic gain (Smith & Berry, 1974).

1.6.0. The biochemistry of sporulation

The results of any biochemical investigations into the sporulation process must be treated with caution (Valenzuela - Perez 1968), due to the transient nature of the process and the inherent errors of the analysis of the crude homogenates of hyphae along which there is spatial distribution of differing biochemical activities, particularly during conidiation (Smith and Berry 1974). Qualitative and/or quantitative changes in the rate of enzyme

synthesis are believed to play a critical role in determining morphogenetic change. A key variable in the control of this enzyme activity will be the availability of precursors or substrates, the changing levels of which will regulate developmental patterns. The biochemistry of conidiation is reviewed by Smith and Galbraith (1971), Smith and Anderson (1973), Smith and Berry (1974) and Smith et al. (1977a). There has been extensive examination of carbon catabolism during conidiation of A. niger (Valenzuela - Perez, 1968; Valenzuela - Perez and Smith, 1971; Smith et al., 1971; Smith and Valenzuela - Perez, 1971; Ng et al., 1972). During growth and sporulation, both EMP and PP pathways operate at all times. The highest activity of the EMP pathway occurs during vegetative growth while the PP pathway predominates and has maximum activity during conidiophore development. The PP pathway has been reported to be stimulated by nitrate reduction in A. niger (Smith and Galbraith, 1971). These results are in agreement with other general conclusions that the PP pathway is the major glycolytic route during sporulation (Carter and Bull, 1969, for A. nidulans). The direct oxidation of glucose through the pentose phosphate pathway may be so important during conidiophore development because the high biosynthetic demands of the sporulation process cannot be met by the normal vegetative metabolism. Oxidative metabolism appears to be essential to the sporulation of most fungi. Conditions which block the TCA cycle and favour glycolysis tend to inhibit conidiation, a situation described by some authors as a morphological expression of the Pasteur effect. There have been several examinations of the TCA and glyoxylate cycles during fungal sporulation; Galbraith and Smith (1969b) and Ng et al. (1973a). Some TCA cycle intermediates are reported to enhance sporulation, while if the TCA cycle is

inhibited, vegetative growth will still continue, but sporulation will not occur. Vegetative growth therefore appears to be adequately supported by energy from glycolysis, and the TCA cycle appears to be only 'ticking over' during this phase. (Valenzuela - Perez, 1968). The major difference between sporing and non - sporing tissue is the synthesis during sporulation of glyoxylate by isocitrate dehydrogenase and isocitrate lyase. These enzymes have been shown to have much higher specific activities at the period preceeding conidiophore development of A. niger than in vegetative mycelium in flask culture (Galbraith and Smith, 1969b) replacement culture (Ng et al. 1973a) and in continuous culture (Ng et al. 1974).

1.7.0. The environmental control of sporulation.

The effect of physiochemical environment on fungi has been well - reveiwed; Cochrane (1958), Ainsworth and Sussman (1965), Hawker (1966) and Bull and Bushell (1976), particularly with respect to sporulation : Smith and Galbraith (1971), Turian and Bianchi (1972) Smith and Anderson (1973), Smith and Berry (1974) and Smith et al. (1977a). Almost all environmental factors can be shown to influence fungal development, and include temperature, pH, gaseous and nutritional status and, in continuous culture, dilution rate.

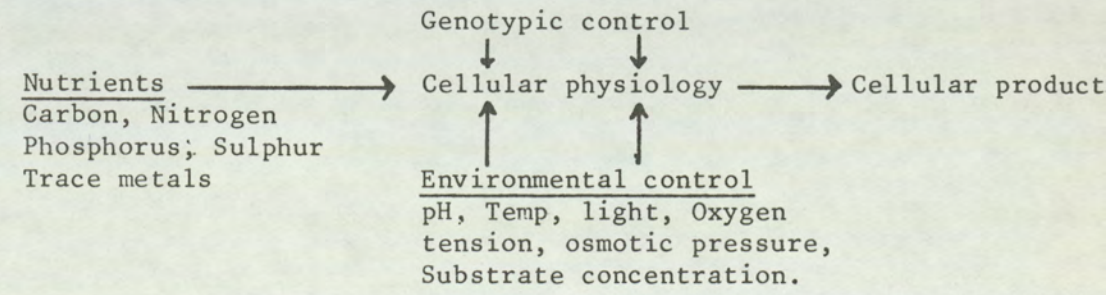


Fig 1.1 Diagram illustrating the relationship between the genetic and environmental control of microbial metabolism (from Berry 1975).

1.7.1. Surface culture.

Surface culture techniques have yielded much information on the influence of the environment on sporulation, and these techniques have commonly been used for industrial and laboratory scale mass production of conidia. Raper and Fennell (1965) report the use of grain as a sporulation substratum for moulds dating back to the early Koji manufacture in the Orient. Singh et al. (1968) obtained spores of A. ochraceus and Septomyxa affinis for transformation processes from barley and bran. High yields are obtained (7×10^{12} conidia kg bran^{-1}) and it affords homogeneity and purity of spores (Singh and Rakhit 1971). Vezina and Singh (1975) describe the elaborate precautions needed for the industrial scale surface culture production of A. ochraceus spores, many of which, (unit under negative pressure, exhaust hoods, air incineration, the wearing of bacteriological masks) are directly due to the airborne nature of the production process. Most of these precautions would be unnecessary with the alternative submerged cultivation system proposed in this project. Grain has also been used as a substrate for the mass propogation of Verticillium lecanii spores, which are subsequently used as biological pest control agents. (Hall and Burges 1979).

1.7.2 Submerged culture

Most studies on sporulation of filamentous fungi have been carried out in submerged shake - flask culture, which allows the development of a more homogeneous mycelium and easier biochemical analysis, although this method is rarely used for industrial spore production. It will therefore be difficult to compare the production levels of the proposed continuous submerged system with any previously reported systems. It is characteristic of

submerged culture that fungi remain entirely vegetative, but sporulation can be achieved by manipulation of the medium components and other environmental parameters. The details of these processes will be discussed later, but the general conclusion is that sporulation is a response to conditions which restrict vegetative growth. These may be :

a) Most commonly, the exhaustion of a nitrogen supply in the presence of an assimilable carbohydrate supply. (Morton et al. 1960, Morton 1961).

2) Exhaustion of the carbon (energy) source while other nutrients remain in excess.

3) Addition of growth - inhibiting factors to the growth medium.

4) Manipulation of the environmental conditions.

The level of sporulation appears to depend upon the type and concentration of medium components and upon the physiochemical environment. Vezina et al. (1965) reviewed the submerged sporulation of filamentous fungi and described several sporulation media for A. ochraceus, and concluded that submerged sporulation required very specific conditions and was dependent upon a delicate equilibrium between medium composition and physiochemical conditions. Studies that rely upon exhaustion of nutrients, while controlling the induction of conidiation at the end of the growth phase, have no influence over further maturation. More precise control over both conidiophore induction and subsequent maturation has been developed by the replacement - fermenter technique (Anderson and Smith 1971b) in which each stage of conidiation is selectively induced by sequential medium replacement. Four major stages were characterised for the conidiophore maturation of A. niger. The initial stage, foot cell formation, is induced in a nitrogen - limited medium with available carbon source to prevent autolysis.

Elongation of the conidiophore is then induced in the absence of exogenous nitrogen although a carbon supply is still required, when translocation of nutrients from the supporting mycelium to the conidiophore is occurring. No further development will occur in this medium, and replacement of the culture to a new medium containing a nitrogen source and a TCA cycle intermediate as a carbon source, induces vesicle and phialide formation. Although these are clearly two distinct morphological stages they appear to have no environmental separation. No spores develop in this medium, and they are best induced by replacement to a medium containing glucose and nitrate as the nitrogen source. This control of structural changes, achieves synchronous maturation of the conidiophores and the method has subsequently facilitated detailed examination of the sporulation process by Ng et al. (1972), Lloyd et al. (1972) and Ng et al. (1973a, 1974). Synchronous sporulation of Neurospora crassa has been achieved by Stine and Clark (1967) by transferring mycelium from liquid culture to a petri dish and allowing spores to develop in the aerial environment and by Zeidler and Margalith (1972 & 1973).

1.7.3. Continuous culture.

Batch cultivation has a number of drawbacks in the study of sporulation, including the heterogeneous nature of the mycelium, poor aeration and the confusion of whether sporulation is the result of nutrient limitation or of decreased growth rate imposed by the conditions. If meaningful interpretations are to be made about sporulation development, then experimental conditions should impose rigorous control over developmental patterns (Smith and Berry, 1974). Chemostat culture will permit study of microbial populations at various growth rates and under various metabolic steady - states, and the development of techniques is needed to

study a single developmental growth phase at steady - state rather than as a transient phase. Smith (1978) however, observes that, regrettably, with the exception of industrial antibiotic systems, the use of fermenter techniques in basic filamentous studies, let alone complex developmental studies such as sporulation, is still the exception rather than the rule. There have been a few studies using chemostat culture that have shown the conidiation of fungi to be due to an interaction between growth rate and the carbon/ nitrogen status of the medium.

1.7.4. Microcycle conidiation.

One final aspect of the study of the sporulation process is the development of microcycle conidiation. This is basically immediate recapitulation of conidiation following spore germination, without an intervening phase of mycelial growth, and has been demonstrated with A. niger (Anderson and Smith 1971a& 1972), N. crassa (Cortat and Turian 1974), Penicillium urticae (Sekiguchi et al. 1975 a, b & c), Claviceps purpurea (Pazoutova et al. 1978) and others. This technique avoids much of the vegetative growth which creates an heterogeneous population, and has been examined in most detail for A. niger. When incubated at elevated temperature (44^{o}C) in basal medium plus glutamate, the spores of A. niger lose their ability to germinate, but swell to produce large spherical cells. When incubated at this temperature for 48 hours, and transferred to germination medium at 30^{o}C, direct outgrowth of conidiophores from the spores occurs, and maturation follows. The expression of conidiation requires glutamate and is stimulated by other factors which inhibit apical growth. As a culture technique for studies on conidiation this was seen as a method that could simplify the studies of regulatory

mechanisms of conidiation, and several detailed studies of the process have followed (Davis et al. 1977, Deans and Smith 1979, Deans et al. 1980). The loss of ability of spores to produce vegetative growth in a complete growth medium while retaining the ability to sporulate is still not fully clear (Smith et al., 1977a) but the phenomenon of microcycle conidiation is consistent with the theory that conidiation is associated with the state of the physiochemical environment and with restricted growth rate. Kuboye et. al. (1976) achieved microcycle conidiation at fermenter scale, and Smith et al. (1977a) describe this process of a sequence of batch cultivations in a 5 litre fermenter producing up to 10^8 spores ml^{-1} from 10^6 giant cells. The synchronous control of microcycle development in this system will allow much more accurate appraisal of the biochemical fluctuations causally related to differentiation (Smith et al. 1977a), and also provides an attractive alternative to conventional bulkspore production.

1.8.0. The uses of fungal spores.

The mass propogation and purification of fungal spores is necessary for many areas of microbiological and biochemical research (Sansing and Ciegler 1973).

1.8.1. Microbial transformation.

A review of the transformation of organic compounds by fungal spores is given by Vezina et al. (1968) and Vezina and Singh (1975). In microbial transformations of chemical compounds, the substrates are added to microbial cultures and usually undergo a simple, specific modification. The earliest reports of transformations by microrganisms were by Fried et al. (1952), Peterson and Murray (1952) and Perlman et al. (1952), but the

first observation of transformation by fungal spores was made by Gehrig and Knight (1958) with the conversion of fatty acids to 2 - heptanone by spores of Penicillium roquefortii. 2 - heptanone is important in the aroma and flavour of Roquefort cheese. Gehrig and Knight (1961) later found that spores of many Aspergilli and Penicillia could also carry out this conversion. Schleg and Knight (1962) reported the 11α - hydroxylation of progesterone by conidia of A. ochraceus, which is an important step in the synthesis of corticoids, which is often difficult, lengthy and expensive to do chemically, and Knight (1966) concluded that far from spores merely being a dormant stage of a life cycle, they possessed enzymes for the transformation of substrates apparently unrelated to their metabolism. Several monosaccharides may be oxidised by the spores during the transformation, but glucose is most effective and considered essential for conversions by A. ochraceus spores by Singh et al. (1968). A list of steroid conversions carried out by A. ochraceus is given by Knight (1966) and Jones (1973). The 11α - hydroxylation of progesterone by spores of A. ochraceus has been reported by many authors; Haines and Collingworth (1953), Dulaney (1959), Vezina et al. (1963) and Sehgal et al. (1968) to name but a few.

Although most authors report that spore transformations are more efficient than those by vegetative mycelium or cell - free extracts (Vezina and Singh, 1975, state that spores are three to ten times more active than mycelium on a dry weight basis), Shibahara et al. (1970) did report the 11α - hydroxylation of progesterone by cell - free extracts of A. ochraceus. The transformation of steroids by spores of Mucor griseo - cyanus is described by Singh et al. (1967) and by spores of Septomyxa affinis by Singh et al. (1965) and Singh and Rakhit (1967). Other developments of the

transformation process are the continuous transformation of steroids (Matales and Fuld 1959, and Reusser et al. 1961), transformation of organic compounds by immobilised spores (Johnson and Ciegler 1969, Chibata and Tosa 1977), the transformation of sterols (Martin 1977, and Schoemer and Martin 1980) and the hydroxylation of steroids by spores of A. niger (Fried et al. 1952). Perlman (1977) lists the companies using fermentation techniques for steroid conversion. Generally, the germination of spores during the transformation process is inhibited by the lack of nitrogenous nutrients, and they can be re - used up to five times before losing any of their transforming ability and may be stored at $4^{o}C$ for more than three months with no loss of hydroxylating activity (Schleg and Knight 1962). The process is therefore commercially feasible on a large scale but the process is presently limited to species that sporulate abundantly, in order to obtain an adequate supply of spores. This project is directly aimed at overcoming this problem.

1.8.2. Mass inocula

A. niger spores are needed for the various industrial fermentations in which A. niger is used (e.g. citric acid and gluconic acid production) and other spores are required for the uses of other filamentous fungi in industrial biotechnology, which are reviewed by Kristiansen and Bu'lock (1980). Asexual spores are more stable for storage than vegetative mycelium, and thus the production of large quantities of spores is important in the preparation and storage of inocula of commercially important strains.

1.8.3. Biological control agents.

In 1979, Hall and Burges described the control of aphids in glasshouses on chrysanthemum crops with the fungus Verticillium

lecanii. The treatment involves the spraying of a spore suspension of 5×10^6 - 1×10^8 spore ml^{-1} over the crop, which requires a regular and plentiful supply of conidia (the same effect can be obtained by fragmented mycelium, but spores have a much better shelf - life). At present, conidia are cultured on Sabouraud dextrose agar, and the blastospores in batches of aerated agitated Sabouraud liquid medium. A continuous spore producing system, if possible to develop, would be an advantage for this application.

1.8.4. Secondary metabolites.

The study of sporulation may well indicate the way in which the production of secondary metabolites may be induced (section 1.5.0.) A. ochraceus produces toxins particularly effective against insects (Kodaira 1961), the antibacterial penicillic acid (Brian 1951) which is also slightly antifungal, mellein (Brian 1951, Blair 1955) which is weakly inhibitory to Staphylococcus aureus, and Turner (1971) described several other secondary metabolites of both A. ochraceus and A. niger. A. ochraceus also produces ochratoxins, which have the same origin as penicillic acid. Nesheim (1967) gives the structure of ochratoxin A, which has the same toxicity as aflatoxin B, and may be carcenogenic (Ciegler and Lillehoj 1968) but ochratoxins B and C are comparatively weak. Bacillus infections of guinea pigs were cured by injection of an unnamed antibiotic from A. ochraceus (Couzi and Werner 1948) and there were subsequent reports of the successful treatment of typhoid, paratyphoid, dysentery, enterocolitus and gastroenteritus in new-born children (Couzi 1949).

1.9.0. Evidence for the possibility of continuous sporulation.

This project was conceived as a result of observations by co - workers Spensley (1977) and Stockbridge (1979) who reported

the sporulation of A. niger in a tower fermenter over a period of several hours under conditions of carbohydrate limitation (starch supply reduced to below the maintenance level) and nitrogen limitation, respectively. The aim of the project was to identify and optimise the factors causing this sporulation, and so to develop a commercially viable system for the continuous production of fungal spores.

2.0. MATERIALS AND METHODS

2.1.0. Fermenter Systems

Two fermenters were used for these studies either individually or in combination, one of 5.0 litres (l.) total volume, the other of 11.2 l. total volume.

2.1.1. The 5 litre fermenter.

This was constructed by the University glassblowers and had a working volume of 4.2 l. at an aeration rate of one volume per volume per minute (v.v.m.). The fermenter (Fig.2.1) consisted of three primary sections. The base contained ports for air admission and drainage and this was connected by a gasket to the main fermenter body which was a one - piece glass tower, 95 c m. long x 8 cm. internal diameter providing ports for sample collection, dissolved oxygen, pH, pH reference and thermistor probes and inlets for thermometer, acid/alkali addition and medium. At the top the tower narrowed to a 25 cm. diameter outlet. To this was connected an 8 mm. diameter glass swan - neck for overflow of spent medium, gases and biomass.

2.1.2. The 11.2 litre fermenter.

This fermenter (Fig.2.2.) had a working volume of 10 l. at an aeration rate of one v.v.m. and was constructed from modified 10.0 cm. internal diameter pyrex glass pipework (Corning Process Systems, Corning Ltd. Stone. Staffs.) The main body of the fermenter consisted of three 40 cm. length (QVF PS4/400) sections connected with polytetra - fluoroethylene (P.T.F.E.) gaskets. The bottom section contained ports (Quickfit screw - cap fittings SW13 and SW28) for medium inlet, thermometer and sampling.

Fig. 2.1. THE 5 LITRE FERMENTER.

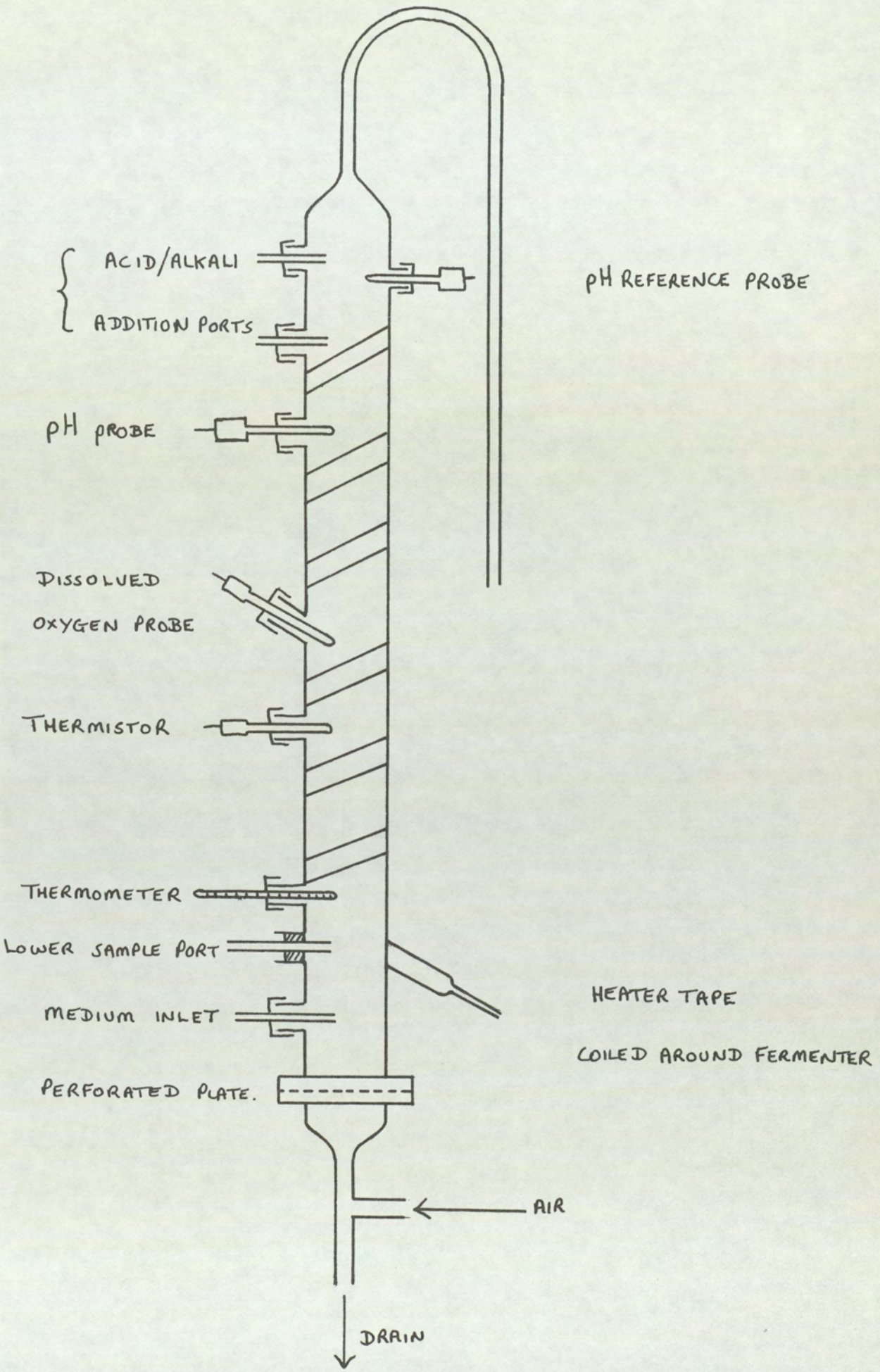
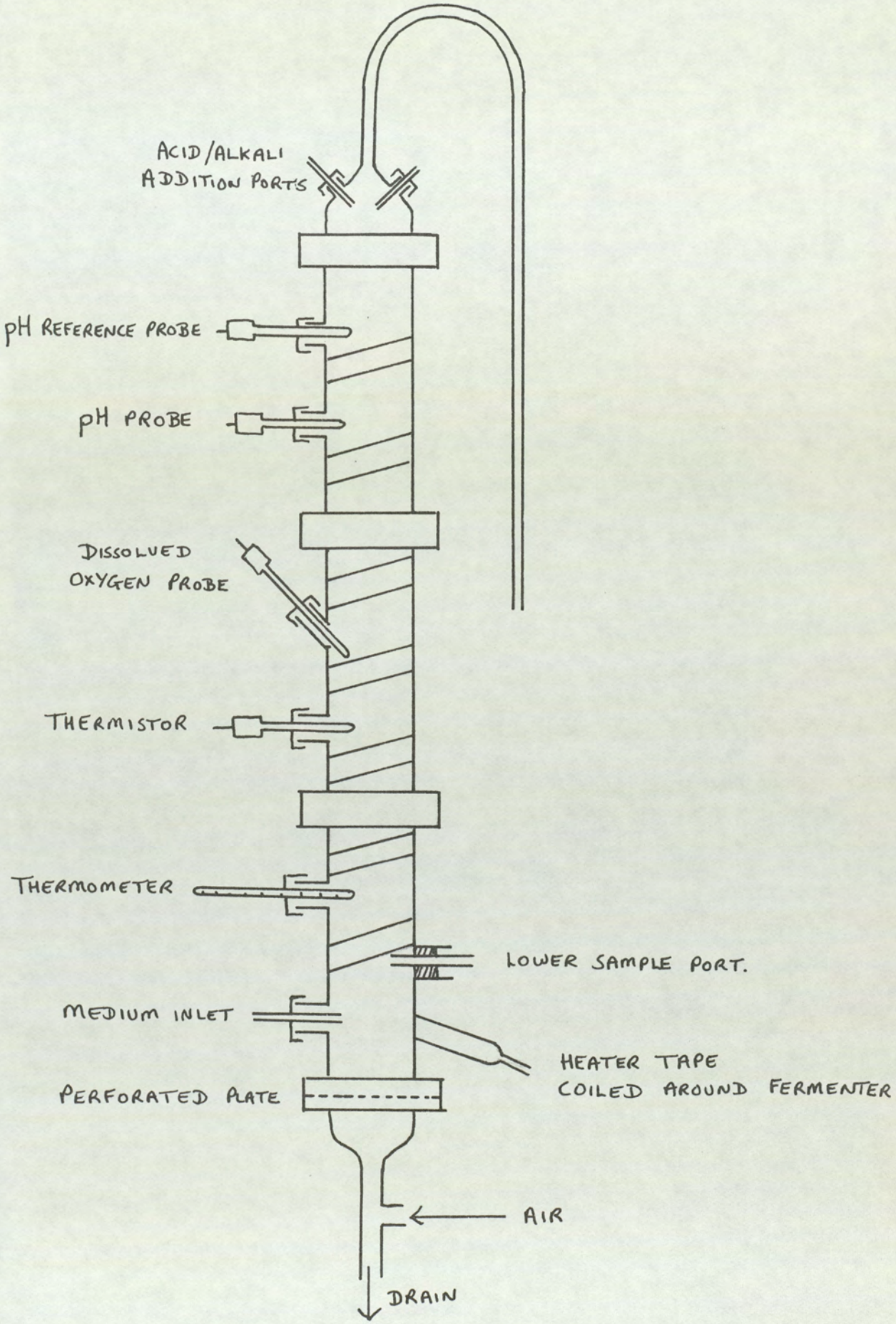


Fig. 2.2. THE 11.2 LITRE FERMENTER



The mid - section had ports for the thermistor probe and dissolved oxygen probe, while the upper section provided ports for pH and pH reference probes and an upper sample port. The effluent/gas outlet was constructed from a 10.0 cm. to 2.5 cm. pipe reducer section (QVF PR4/1) which contained ports for acid/alkali addition for pH control. This was connected to a 2.5 cm. end fitting (PHC1/0.5) modified to lead to an 8.00 mm. internal diameter swan - neck. The base section of the tower was also constructed from a pipe reducer section (QVF PR4/1) and as with the 5.0 1. fermenter, this section contained ports for air inlet and drainage.

2.1.3. Air supply and dispersal.

Compressed air was supplied from a piston compressor (Groom and Wade Ltd. High Wycombe.) via a pressure reducer/oil filter (Air Power Minnett Ltd. Birmingham.) to a second pressure reducer and oil filter on the fermenter framework (Spirax - Monnier, Cheltenham.). Air flow to the fermenter was controlled and monitored by a 0.25 1. per minute flow meter (Gapmeter Ltd.) which fed air to a Whatman Gamma - 12 filter steriliser unit, fitted with a 0.3 μ grade filter (ex. Gallenkamp Ltd.). From here air flowed through vinyl tubing (Portex Ltd. Hythe, Kent.) to a level above the highest point of the fermenter, and back down to the air inlet in the base, thus protecting the air supply system from fermenter contents in the event of air pressure failure. The air passed through the base section of the fermenter, and into the tower via a perforated plate air distributor. This was constructed in our laboratories from a 2.0 mm. thick P.T.F.E. plate, perforated on a 2.0 cm. triangular grid by 1.5 mm. holes, and was positioned between the gasket connecting base

and fermenter base. These were cheap and easy to construct, easy to remove and clean, and experience has shown them to be less susceptible to mycelial blockage than glass sinters. Air was always supplied to both fermenters at a rate of one v.v.m.

2.1.4. Medium supply.

Medium was supplied to each fermenter through 2.0 mm. internal diameter silicone rubber tubing (Jencons Scientific Ltd. Hemel Hempstead, Herts.), by a Baron Yemm BYO 800 peristaltic pump (Norris Industries, Rushton Ltd. Rushton. Northants.).

All media were supplied from 10.0 l or 20.0 l. pyrex aspirators in which medium could be sterilized at 103.5 kilopascals for 15 minutes. At dilution rates above 0.1 h^{-1} (section 2.6.1.), sterilization of the medium was unneccesary after initial "start - up" due to the rapid wash - out effect upon organisms (as discussed previously) and infection of the medium was then suppressed for 24 hours by the addition of ice. Media constituents are summarized in Appendix 1, and reasons for selection are discussed in section 3. Effluent from the tower was collected in 40.0 l. polypropylene containers.

2.1.5. Heating systems.

The fermenter contents were heated by a silicone - rubber sheathed 1.1 kilowatt flat heating tape (type G.W. 50 - 70. Hotfoil Ltd. Wolverhampton), which was coiled around the body of the fermenter. This was regulated by a thermistor - controlled zero voltage switch designed by Radiospares Ltd. (1975) and constructed by the University workshop in conjunction with P.J. Stockbridge (1979). This system maintained the temperature to within 0.1^{o}C of the set temperature under all conditions.

2.1.6. pH measurement and control

pH was measured by a toughened, steam - sterilizable probe

in conjunction with a remotely positioned reference electrode (E.I.L., Richmond. Surrey.). The pH of the broth was continuously plotted by a pH meter - recorder (Analytical Measurements Ltd., Feltham. Middx.) model R4/69, and pH was monitored and controlled by a meter - controller (E.I.L. model 918). This recorded and adjusted pH to set values within $\pm$ 0.1 pH unit between 1.0 and 9.0 by controlling two Delta peristaltic pumps (Watson - Marlow Ltd., Falmouth. Cornwall.) which fed control solutions of $400gl^{-1}$ sodium hydroxide solution or concentrated orthophosphoric acid into the fermenter.

2.1.7. Dissolved oxygen measurement

The dissolved oxygen (D.O.) content of the fermentation broth was measured by a steam - sterilizable probe with a replaceable polypropylene membrane (Uniprobe Instruments Ltd., Cardiff.) connected to a D.O. meter - recorder (New Brunswick Scientific Co. Ltd., New Jersey, U.S.A. - Model D.O. 50). The meter was calibrated by immersing the probe in 0.01 M borax solution (pH 9.2) containing 100 mg. sodium sulphite per 5.0 ml. (This gives the point zero D.O.). After transferring the probe to the tower, maximum aeration was taken as the point at which the indicator stabilized when sterile medium was fully aerated. Aeration could then be expressed as a percentage along this arbitrary scale. It was not, however, found possible to make accurate measurements of D.O. with this equipment, because of gradual mould growth on the probe, but the system did give a useful indication of the state of the fermentation.

A diagrammatic representation of the system, and a key are presented over the page.

2.2.0. The organisms.

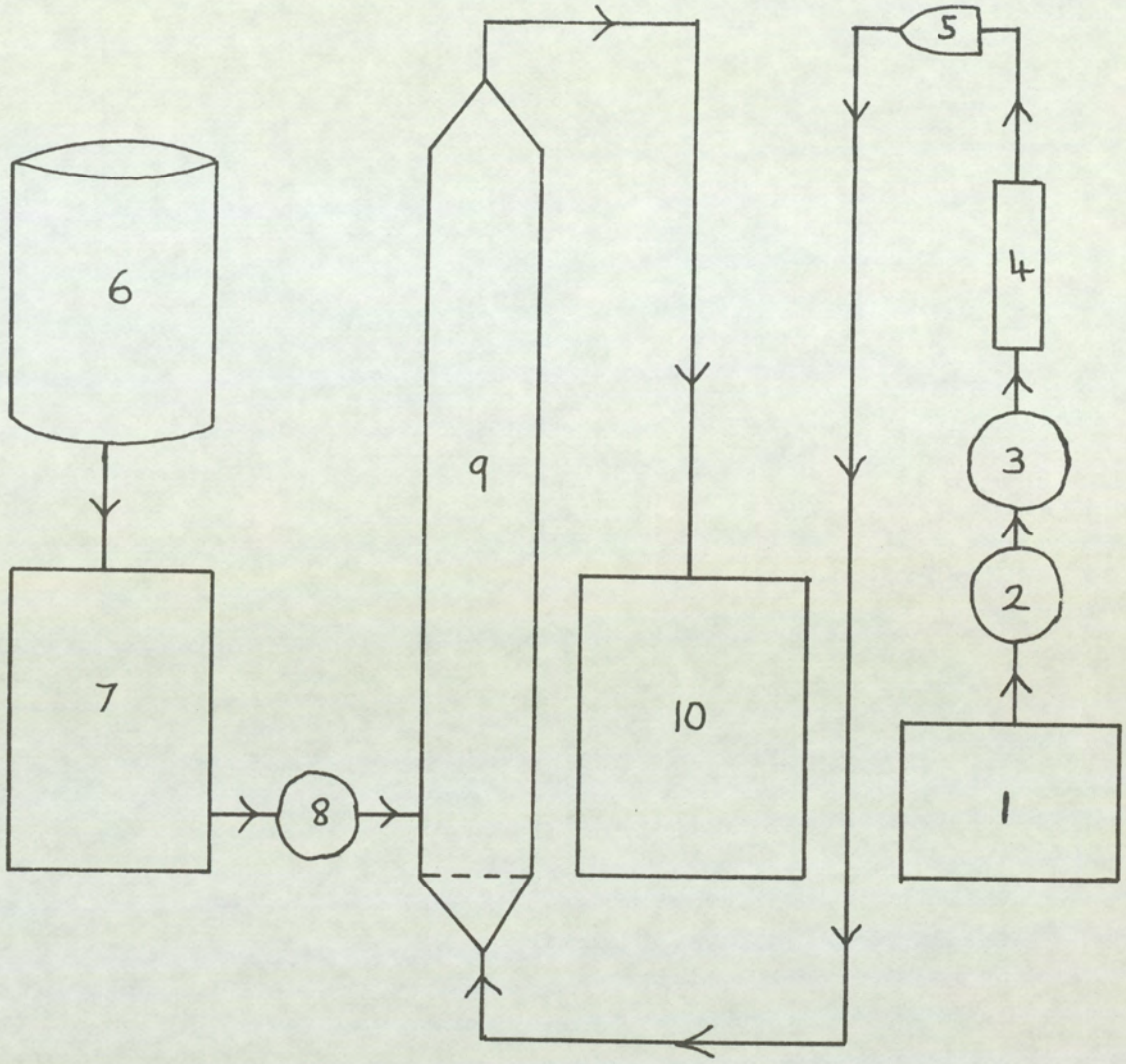


Fig 2.3. Diagrammatic representation of single-stage fermentation system.

1: Air compressor.
2: Pressure reducer/oil filter.
3: Pressure reducer/oil filter of fermenter framework.
4: Flow-meter control.
5: Gamma-12 filter sterilizer unit.
6: Autoclave.
7: Medium reservoir.
8: Peristaltic pump.
9: Tower fermenter.
10: Effluent receiver.

2.2.1. Selection.

One primary organism used was Aspergillus niger Van Tiegham 38 from the Fermentation Laboratory Culture Collection. (ex Dr. Drysdale, Department of Genetics, Birmingham University.). This organism had been the subject of a number of previous studies by members of the fermentation research group (Davies 1971, Pannell 1976, Spensley 1977, Stockbridge 1979.) and its suitability for use in the tower fermentation system was already confirmed. The nutritional requirements of A. niger (Van Teigham) M1 (ex Tate and Lyle Ltd.) have also been studied (Morris 1973, Cocker 1975). The second organism studied in depth was Aspergillus ochraceus Wilhelm (Commonwealth Mycological Institute No. 16247 iv). This was chosen because of its' physical similarity to A. niger and the well reported importance of its' spores in pharmaceutical reactions. (Dulaney et al., (1955), Schleg and Knight (1962), Sehgal et al., (1968), Singh et al., (1965), (1967) and (1968), Haines and Collingworth (1953). Other organisms used during the project were Penicillium javanicum Van Beijma isolated from soil by Ewen (1980) and selected for use in the fermentation of milk wastes, and Verticillium lecanii, Zimmerman, (C.M.I. No. 179172) Macrosiphoniella sanborni - R.A. Hall 1973). P. Javanicum was studied briefly after the reported observation of sporulation in a tower fermenter by Ewen (personal communication) during an experiment to study the effect of temperature upon growth of the organism. V.lecanii was also used briefly after study of a report by Hall and Burges (1979) on the use of spores of V.lecanii as biological control agents against aphids on glasshouse crops.

2.2.2. Culture maintenance.

Master cultures of all organisms were maintained on malt extract

agar (Oxoid Ltd.) slopes at 4°C, and were regularly sub - cultured. When required, a sample was transferred from the slopes to malt extract agar plates, and incubated at 30°C (25°C for V.lecanii) for several days to allow spores to develop. These spores were then removed and used in the preparation of inoculum mats. Malt extract agar mats were prepared in 250.0 ml. conical flasks and aseptically inoculated with the required organism by removing the spores from the plates with a sterile needle and streaking them across the agar in the flask. These were then incubated at 30°C (25°C for V. lecanii) for between two weeks and two months, until good spore mats had formed. Malt extract agar plates were used for the solid - culture pH experiment and for spore and mycelium viability tests.

2.2.3. Viability tests.

Pannell (1976) and Dawes and Thornley (1970) report the gradual loss of sporulative capacity of a mould continuously cultured in the C.T.F. for long periods. This effect was monitored by the occasional plating out of mycelial samples from the tower, incubating at optimum temperature and allowing spores to form. If the culture showed any sign of not producing an even spore - mat, then the tower culture was renewed. The viability of spores produced during the experiments, was assessed by suspending spores in an optimum liquid nutrient medium (Appendix 1) in a 250.0 ml. conical flask, and incubating on an orbital shaker at 100 revs/minute and optimum temperature. Samples were observed regularly and the percentage of germinating spores was recorded by counting on the haemacytometer. Germination was deemed to have occurred when a germ tube was visible.

2.3.0. Microscopy and Photography.

The morphology of the mould was assessed by direct observation

of fresh mycelial samples, using a Leitz - Wetzler binocular microscope and a Patholux microscope with an automatically operated camera (Vickers Instruments Ltd. York and Croydon.). All photographs were taken on Ilford FP4 or HP500 black and white film. Other non-microscopic photographs were taken with a Yashica T.L. electra camera, and floc morphology photographs were taken by this camera fitted with a 2.0 cm. extension tube. The samples were poured into a 10.0 cm. x 6.0 cm. x 1.5 cm. perspex viewing cell, and diluted with either distilled water or formalin to suspend the flocs. Illumination was by two 250 watt photoflood lamps.

2.3.1. Spore counts.

Spore numbers were assessed by counting free conidia in an effluent sample after vigorous shaking of the sample (to dislodge spores) followed by filtration through glass wool to remove mycelial fragments. Spores in the filtered samples were then counted using an Improved Neubauer haemacytometer, and numbers expressed either as number per ml. or as number per g. dry weight fermenter mycelium concentration or as number per g. carbon source utilized. The calculation of these values is described in section 2.6.5.

2.4.0. Fermenter 'start - up' procedures.

2.4.1. Fermenter preparation.

Before use, the fermenter was steam sterilized by steaming for 24 hours at atmospheric pressure. A small amount of steam was allowed to escape from all ports to ensure complete sterilization. The medium tubing and air filters were autoclaved at 103.5 kilopascals for 15 minutes and then connected to the fermenter. Sterile air was then passed into the fermenter as the steam was

slowly turned off. The vessel was allowed to cool before filling with sterile medium which was brought to the required temperature for inoculation.

2.4.2. Spore inoculation.

Onto the spore mats prepared as in 2.2.2., was poured 100 ml. solution of sterile distilled water plus one drop of 'Tween 80' wetting agent. (polyoxyethylenesorbitan mono - oleate. Hopkins and Williams Ltd.). The flask was shaken to dislodge the spores, which were then aseptically injected into the fermenter from a polypropylene syringe and needle through a 'subaseal' self - sealing rubber bung. The mould was then allowed to grow batch - wise for up to 12 hours (usually overnight) with slight aeration, after which time, the medium and air were supplied at the desired rate.

2.4.3. Mycelial inoculum.

As a time - saving alternative to the above, up to one litre of growing mycelium could be pumped directly from one fermenter to another (previously cleaned but not necessarily sterilized) and the medium supply to the second fermenter immediately started at the maximum rate. This proved an effective and relatively infection - free procedure for quick start - ups.

2.4.4. Antifoam.

Foaming of the tower contents was frequently observed, particularly when acid was being added to maintain a low culture pH value. This foaming was prevented by manual injection of a silicone - based antifoam agent (I.C.I. silicones, Ambersil Ltd., Basingstoke. Hants.). Pannell (1976) comments on the adverse effects of antifoam upon the performance of the C.T.F. (i.e. decreased gas holdup, air bubble coalescence, decreased X_F) and so addition was drop - wise until the foaming was prevented in order to minimise the amount used.

2.4.5. Fermenter sampling.

500.0 ml. of fermenter effluent were collected and filtered through fine muslin and the resultant solids dried at 105°C to constant weight. This gave the effluent biomass concentration (X_E) when converted to gl^{-1}. The sample collecting time was used as a guide to the dilution rate. (Section 2.6.1.)
After discarding the first 20.0 ml., 100.0 ml. samples were taken from the fermenter sampling port. Stockbridge (1979) reported that the faster such a sample is withdrawn, the deeper within the tower the effective sampling area extends. Samples were therefore drawn as quickly as possible. When filtered, dried and weighed as above, these samples gave the fermenter biomass concentration (X_F gl^{-1}). Several previous workers (Pannell 1976, Spensley 1977, Stockbridge 1979) reported that the liquid portions of the fermenter contents and effluent were nutritionally identical. Only effluent samples were therefore analysed. These were compared with the analysis of medium samples.

2.5.0. Analytical techniques.

2.5.1. Total sugars.

Glucose and sucrose were determined by the phenol - sulphuric acid method of Dubois et al. (1956). Liquid samples were volumetrically diluted to give sugar concentrations of between 20.0 and 100.0 mgl^{-1}. 1.0 ml. of the diluted sample was added to 1.0 ml. of 5% Analar phenol solution in a pyrex text - tube, followed by the addition of 5.0 ml. concentrated Analar sulphuric acid. The mixture was allowed to stand for a minimum for 20 minutes to allow the colour to develop. The colour was then measured in an E.E.L. Spectra (Evans Electro - selenium Ltd.

Halstead, Essex.) spectrophotometer at 488 nm. against the appropriate sugar standard. To analyse starch, 10.0 ml. sample plus 1.0 ml. concentrated hydrochloric acid were boiled together for 10 minutes. Spensley (1977) has shown that hydrolysis of the starch is complete after 6 minutes. 10 minutes therefore allows sufficient time for hydrolysis while avoiding caramelisation. The sample was then cooled and neutralised with 10M sodium hydroxide before analysis as for glucose/sucrose. Results were expressed as gl^{-1} glucose equivalent.

2.5.2. Ammonia nitrogen.

Ammonia nitrogen was determined by Kjeldahl distillation. 5.0 ml. of sample were run into a Markham still followed by 5.0 ml. of 400.0 gl^{-1} sodium hydroxide solution. The first 20.0 ml. of distillate were collected into 10.0 ml. saturated boric acid which was back - titrated against 0.01M HCl using Tashiro's indicator (a mixture of methyl red, 2.0 gl^{-1} and methyl blue 1.0 gl^{-1} in absolute ethanol). The nitrogen concentration was calculated by the following equation :

$$N\ gl^{-1} = 0.028 \times \text{Titration volume (ml.)}.$$

2.5.3. Total nitrogen/crude protein.

Total nitrogen content of the mould was determined by the micro - kjeldahl method (Markham 1942). 30.0 - 40.0 mg. of sample and approximately 20.0 mg. kjeldahl catalyst (potassium sulphate/copper sulphate/selenium 32:5:1) were digested with 1.0 ml. of concentrated analar sulphuric acid for three hours. The sample was then distilled as above and the total N. content of the mould and crude protein content were calculated from the following equations :

$$\text{Total N} = \frac{0.014 \times \text{titration value (v)}}{\text{(w) sample weight (g)}}$$

$$\% \text{ crude protein} = \frac{0.0875 \times v}{w}$$

Anderson et al. (1973) consider this expression of crude protein content to be misleading, in that organisms can have a high total N. content without necessarily being rich in protein, a better measure of which is amino nitrogen.

2.5.4. Nitrate nitrogen.

The nitrate level of the medium and effluent was measured in a special instruments laboratory using a Technicon Autoanalyser (Technicon Corporation, New York.) after the method of Chapman et al. (1967). Nitrate present is first reduced to nitrite with hydrazine sulphate under alkaline conditions, using copper ions as a catalyst. The nitrite ion then reacts with sulphanilamide under acidic conditions, to form a diazo compound. This compound then couples with N - 1 - Naphthyl - ethylene - diamine dihydrochloride to form a reddish purple azo dye which is measured at 520 nm. The determination of nitrite alone in a solution containing both nitrate and nitrite ions is performed by replacing the hydrazine - copper sulphate reagent with distilled water. Nitrate may then be calculated by subtraction.

2.5.5. Citric acid.

De - proteinized samples (from total nitrogen analysis) were analysed by the method of Saffran and Denstedt (1948) using the modification of the technique reported by Davies (1971). This involves dehydration of the sample with acetic anhydride at 60°C for 10 minutes followed by the addition of 1.0 ml. pyridine to form a chromophore at either 60°C or at 0°C, followed by recording the absorbance of the sample at 400 nm.

and comparing the results with standard curves. The pyridine added at 60°C reacts with both citric and aconitic acids if present, while at 0°C the pyridine reacts only with aconitic acid. Citric acid can then be calculated by subtraction.

2.5.6. Ferrocyanide estimation.

The modified method of Marier and Clarke (1960) developed by Davies (1971) was used to estimate ferrocyanide concentration. This involved the formation of the blue chromophore ferric ferrocyanide. A sample containing 5 - 100 mg. of ferrocyanide ion was made up to 5.0 ml. with distilled water. To this, 1.0 ml. of ferric chloride solution ($FeCl_3 6H_2O$ 6.0 gl^{-1} in 0.1M HCl) and 2.0 ml. of citric acid solution (500.0 gl^{-1}) were added. After shaking, the absorbance of the mixture after 60 minutes was recorded at 690 nm. The level was then compared with a standard calibration curve.

2.5.7. Carbon dioxide.

During experiments to test the effect of increased CO_2 in the air supply, the levels of carbonate/bicarbonate ions in the fermentation broth were measured using Official method number 33.076 (Horowitz 1975). This involved adding phenolphthalein indicator to detect carbonate ions, and titrating against 0.05M HCl until clear, followed by addition of methyl orange and continued titration against 0.05M HCl to the end point. The HCO_3/CO_3 ion content was calculated by the following equations:

1st titration x 3 = mg. CO_3 ions per 100.0 ml.

If CO_3 present :

Total titration - 2(initial titration) x 3.05

= mg. HCO_3 ions per 100.0 ml.

If no CO_3 present :

Total titration x 3.05 = mg. HCO_3 per 100.0 ml.

Mean nutrient levels and utilization rates were calculated from at least three analytical replicates.

2.6.0. Calculation of Fermentation parameters.

2.6.1. Growth rate and productivity.

The rate of growth of an organism in batch culture = a constant multiplied by organism concentration

$$\text{ie: } \frac{dx}{dt} = \mu x \qquad (1)$$

where μ = growth rate, h^{-1}. x = organism concentration gl^{-1}.

Monod (1950) developed the basic growth rate equation:

$$\mu = \frac{1}{x} \cdot \frac{dx}{dt} \qquad (2)$$

Taking into account the limiting nutrient:

$$\mu = \mu \text{ max.} \left(\frac{S}{Ks + x}\right) \qquad (3)$$

where μ max. = maximum specific growth rate, h^{-1}, Ks = saturation constant of substrate, ie: that concentration allowing half the maximum growth rate $\left(\frac{\mu \text{ max.}}{2}\right)$ gl^{-1}, S = limiting substrate concentration in the vessel gl^{-1}. The Ks value, although small, is always greater than zero, therefore $\mu \neq \mu$ max. in practice. In continuous culture, the same situation applies, but loss of cells in the effluent must be considered. The average time a cell will remain in the vessel is governed by the volume of the vessel and the flow rate. This mean residence time is inversely proportional to the dilution rate (D, units h^{-1}) which = $\frac{f}{v}$ where f - flow rate, lh^{-1}, v = fermenter volume, l.

The kinetics of growth in homogeneous continuous culture have been described by Monod (1950), Herbert et al. (1956), Pirt (1965), Malek and Fencl (1966) and Righelato (1975) and are applicable to the growth of filamentous moulds (Pirt and Callow 1960, Righelato et al. 1968).

If the cells in the vessel were not growing, overflow would be a function of flow rate :

$$-\frac{dx}{dt} = D.x. \qquad (4)$$

But, when growing there will be exponential multiplication of cells to replace the lost cells ($\frac{dx}{dt} = \mu.x.$).

Therefore, the net change in cell mass is given by :

$\frac{dx}{dt} = x.(\mu - D)$. (Hough and Wase 1966).

If, therefore, $\mu > D$ the organism mass will increase until substrate becomes limiting. If $D > \mu$, growth rate will increase until there is a maximum substrate utilization (μ max.), beyond which the organism will wash - out with any further increase in D. Within this range are the theoretical steady states, where $\frac{dx}{dt} = 0$.

At steady state, input = output (law of conservation).

The steady - state biomass balance is given by:

Input (by growth) = $V.\mu.X_F$. Output (by flow) = $f.X_E$.

where X_F = fermenter biomass concentration, gl^{-1} and

X_E = effluent biomass concentration, gl^{-1}.

$$\therefore V.\mu.X_F = f.X_E \qquad \text{at steady state.}$$

Since dilution rate (D) has already been defined as $\frac{f}{v}$, by rearrangement:

$$\mu.X_F = D.X_E. \qquad (4)$$

The organism specific growth rate in this system at steady state is therefore given by:

$$\mu = \frac{D.X_E}{X_F} \; h^{-1} \qquad (5)$$

Although this equation works for steady state results, it cannot be applied to transient state conditions. In this work, a steady state is frequently deliberately disturbed by

alteration of environmental parameters, and the response of parameters, including growth rate, to this change, monitored. In a transient phase, the parameters vary progressively with time before either, a) a new steady state develops or b) the organism dies or is washed out of the system. (Hough and Wase 1966). A transient state should not be confused with an unsteady state, where parameter values oscillate about a mean value. Sterkin et al. (1973) examined transitional changes in Escherichia coli fermentations after rapid changes of temperature, dilution rate and substrate concentration, and state that the formation and duration of transient states in continuous culture are likely to be affected by the age of the population, and prevailing conditions at the time of a sudden change. Growth rate during a transient phase is given by the equation :

$$\mu\ (h^{-1}) = \frac{1}{X_{F\ TOTAL}} \times \frac{\text{New biomass produced}}{\Delta t} \qquad (6)$$

where New biomass produced $= f.X_E.\ t + V(X_{F2} - X_{F1})$.

where X_E = mean effluent [biomass] between 0 and t (gl^{-1})

Δt = time elapsed between 0 and t (h)

X_{F1} = fermenter [biomass] at time 0. (gl^{-1})

X_{F2} = fermenter [biomass] at time t. (gl^{-1}), and

$$X_{F\ TOTAL} = V.\ \frac{X_{F1} + X_{F2}}{2} \quad (g)$$

The factor $D.X_E$ represents the biomass productivity, Y $gl^{-1}h^{-1}$. Mean growth rate and productivity values were calculated for each sampling time from triplicate X_E and X_F samples.

The biomass concentration of the C.T.F. is a function of the floc sedimentation rate due to gravity, the effect of the upward flow of medium and gases and the substrate concentration.

As in batch culture, equation (2) applies and μ will always be less than the μ max. because the factor $\frac{S}{Ks + S}$ must be less than unity.

In practice, therefore, $\mu \neq D$. at steady state.

2.6.2. Yield coefficient.

The biomass yield coefficient (Ysub) represents the amount of biomass resulting from the utilization of unit mass of nutrient, ie: g. cells g. substrate^{-1}, and is used primarily in this work to describe the conversion of carbon source into biomass. The yield (or economic coefficient as it is sometimes called) is calculated by:

$$Ysub = \frac{-\Delta x}{\Delta s} = \frac{dx}{ds} \quad g.g^{-1} \qquad (7)$$

This is represented by $\frac{X_E}{dS}$ in this system where dS = substrate utilization, gl^{-1}.

2.6.3. Substrate supply and utilization.

The sugar (Sm) and nitrogen (Nm) concentrations of the medium and effluent (Se/Ne) were expressed as gl^{-1} glucose equivalent and gl^{-1} atomic nitrogen respectively, as were the sugar (dS) and nitrogen (dN) utilization levels. eg:

$$dS = Sm - Se, \qquad dN = Nm - Ne.$$

The substrate balance equation is:

Input = Output + Consumption by microorganisms.

$$D.Sm = D.Se. + \mu \text{ max.} \frac{x}{Ysub} \cdot \left(\frac{S}{Ks + S} \right)$$

Utilization therefore = D.Sm - D.Se.

This is a rate expression of substrate utilization, and was expressed in two ways in this work. Where the dilution rate varies during experiments, substrate supply and utilization are

conveniently represented by :

Supply rate, kS (sugar), kN (nitrogen) gh^{-1}

= Sm or Nm. f.

where Sm/Nm = Sugar/nitrogen medium content gl^{-1} and f = flow rate lh^{-1}.

Utilization rate; ΔS (sugar), ΔN (nitrogen) gh^{-1} = dS or dN. f

where dS/dN = Sugar/nitrogen utilization, gl^{-1}.

These values alone, however, may at times be misleading because equal values may be obtained by a high flow rate and low nutrient concentration, or vice versa; two sets of conditions which will have a markedly different effect upon fermentation characteristics. The specific substrate supply and utilization rates, relating nutrient supply and utilization rates to the biomass content of the fermenter remove this ambiguity, and are calculated by the equations:

$$\text{Specific sugar supply rate } (Q_S) = \frac{Sm.D}{X_F} \; gg^{-1}h^{-1} \qquad (8)$$

where Sm = Sugar medium concentration, gl^{-1}.

D = dilution rate, h^{-1}.

X_F = fermenter biomass concentration, gl^{-1}.

$$\text{Specific nitrogen supply rate } (Q_N) = \frac{Nm.D}{X_F} \; gg^{-1}h^{-1} \qquad (9)$$

where Nm = Nitrogen medium concentration, gl^{-1}.

$$\text{Specific sugar utilization rate } (q_S) = \frac{dS.D}{X_F} \; gg^{-1}h^{-1} \qquad (10)$$

where dS = sugar utilization, gl^{-1}.

$$\text{Specific nitrogen utilization rate } (q_N) = \frac{dN.D}{X_F} \; gg^{-1}h^{-1} \qquad (1$$

where dN = nitrogen utilization, gl^{-1}.

2.6.4. Maintenance energy.

The concept of maintenance energy to represent that quantity of a particular substrate consumed by cells for functions other than growth, is reviewed by Pirt (1965). Metabolic activities may be divided into growth - rate dependant and growth - rate independant components, and an organism consumes a constant level of energy source purely to maintain the integrity and viability of the cell, ie sufficient energy for essential metabolic activities; macromolecule turnover, osmotic regulation and organelle maintenance (Righelato et al. 1968). This energy requirement should not be confused with endogenous metabolism where an organism utilizes its' own resources for maintaining viability or growth. As the growth rate of an organism decreases, the maintenance level assumes an ever - increasing proportion of the total energy requiremnt. Pirt (1965) introduces a term of 'true growth yield' (Y_G) to represent the amount of substrate consumed purely for growth:

$$Ysub = \frac{\Delta x}{(\Delta s)_G + (\Delta s)_m} \qquad Y_G = \frac{\Delta x}{(\Delta s)_G}$$

where $(\Delta s)_G$ = substrate utilized for growth

$(\Delta s)_m$ = substrate utilized for maintenance

Δx = [organism]

The overall rate of substrate utilization = the rate of utilization for growth + the rate of utilization for maintenance, expressed by:

$$\frac{ds}{dt} = \left(\frac{ds}{dt}\right)_G + \left(\frac{ds}{dt}\right)_m$$

But, $\frac{ds}{dt} = \frac{-\mu x}{Ysub}$, $\left(\frac{ds}{dt}\right)_G = \frac{-\mu x}{Y_G}$ and $\left(\frac{ds}{dt}\right)_m = -mx$

$\therefore$ the equation rearranges to :

$$\frac{1}{Ysub} = \frac{m}{\mu} + \frac{1}{Y_G} \qquad (12)$$

where Ysub = Total biomass yield coefficient (gg^{-1})

m = maintenance coefficient ($gg^{-1}h^{-1}$)

μ = specific growth rate (h^{-1})

Y_G - True growth yield (gg^{-1})

Equation (12) forms the basis of a method used by Pirt (1965) and Rokem et al. (1978) for maintenance coefficient calculation. If m and Y_G are considered constant, the plot of $\frac{1}{Ysub}$ against $\frac{1}{\mu}$ will be a straight line, with the slope = m and the intercept on the ordinate = $\frac{1}{Y_G}$. In a reciprocal plot, the least reliable data, - that at low growth rates - has the greatest influence upon the regression equation, (14) and therefore upon the slope of the line which = m. For a valid result using this method the very high and very low values must consequently be omitted (Veldkamp 1976).

Equation (12) may be reduced to :

$$q = \frac{\mu}{Y_G} + m \qquad (13)$$

where q = specific substrate utilization rate.

This equation is the basis of the method of maintenance energy calculation used by Schulze and Lipe (1964) and subsequently by Tempest and Herbert (1965), Righelato et al. (1968), Von Meyenherg (1969) and Carter et al. (1971). A plot of specific substrate utilization (q, $gg^{-1}h^{-1}$) against growth rate (μ, h^{-1}) gives a straight line with an intercept on the ordinate, when μ = zero, which represents the substrate used for maintenance, m ($gg^{-1}h^{-1}$).

For the methods of both Pirt and Schulze and Lipe an accurate value of m is calculable from the regression analysis equation :

$$b = \frac{n\Sigma xy - \Sigma x \Sigma y}{n\Sigma x^2 - (\Sigma x)^2} \qquad (14)$$

In the method of Pirt, substitute $\frac{1}{Ysub}$ for y, $\frac{1}{\mu}$ for x, b = slope, m. In the method of Schulze and Lipe, substitute q for y and μ for x, then b = slope of the line. Then from equation $a = \bar{y} - b\bar{x}$, the intercept on the ordinate, a, may be calculated. This = m.

In the above derivations, the whole of the culture is assumed to contribute to the production of new cellular material, but a proporation of the population consists of dead cells, particularly at low growth rates. The estimation of this proportion is impossible, and therefore the values of growth rate and maintenance coefficient will be be underestimates.

2.6.5. Expression of spore numbers.

The number of spores ml^{-1} was calculated from the counts in the haemacytometer by simple multiplication. These values were converted to the sporulation index (β) by :

$$\frac{\text{spores } ml^{-1}}{X_F\ (gl^{-1})} \times 1000$$

The number of spores per g. carbon source utilized were calculated by :

$$\frac{\text{spores } ml^{-1}}{dS\ (gl^{-1})} \times 1000$$

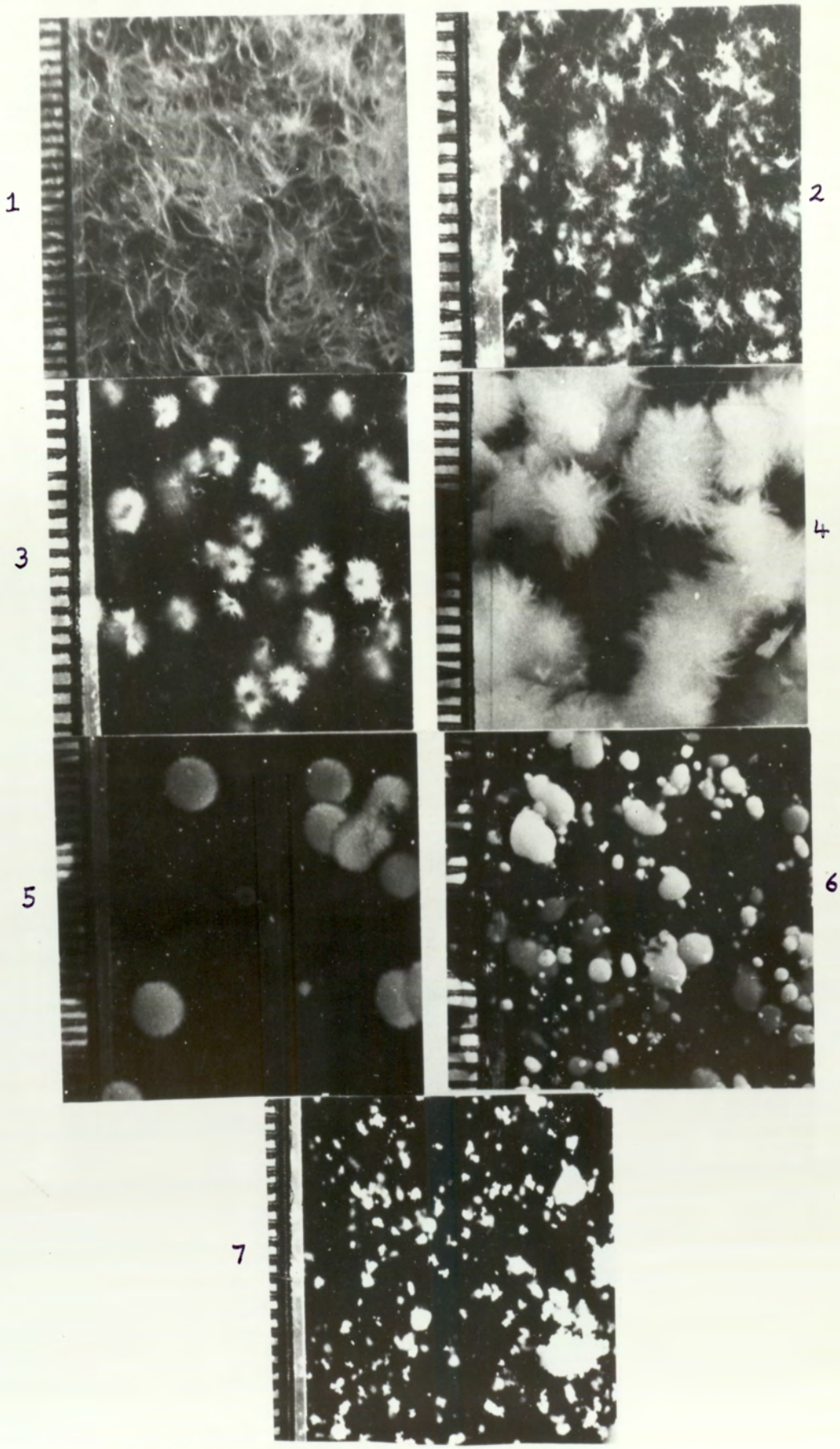
2.7.0. Replication.

Where no time scale appears on a graph, a result represents the mean of steady - state values recorded over at least three days although true steady - states were not maintainable at parameter extremes due to the adverse effects upon mould growth. When an experiment monitored the transient change of parameters

with time, experiments were generally repeated (at least twice) and the standard deviation and mean value of the means of these replicates calculated. In the case of a single experiment, results are the means of triplicate samples. During all fermentations, while at ' steady - state', measured parameters did not remain constant relative to time, but oscillated about a mean value, the period of oscillation being approximately two days. As a result, measurements were taken for three days to give a better sample, but an unavoidable result was that standard deviations were consequently higher than would be expected from a constant steady - state.

2.8.0. Floc morphology.

Through the text, the floc morphology type is referred to the scale 1 - 7 established by Cocker (1975) during his PhD research and discussed by Atkinson and Daoud (1976). See Plates 1 - 7.



PLATES 1 - 7 Morphological development of A.niger flocs in batch tower fermenter. Plates represent sequence of types 1-7. Scale ruled in 1mm. (From Cocker, 1975.)

SECTION 3 - PROJECT PLANNING

3.0 Project Planning

Work of this nature, where the effect of each environmental factor was examined in turn, needed an arbitrary starting-point. The initial fermentation had to be carried out at a known dilution rate, with a medium supply of known composition and at defined temperature and pH values. While the starting-point was arbitrary, the choice of initial parameter values was based upon the experience of previous workers, and on practical and theoretical considerations of project requirements.

3.1 Dilution rate

This was the first parameter value selected from the original work carried out in the C.T.F. by Pannell (1976). Under conditions of nutrient excess (27.5 gl^{-1} sucrose) he demonstrated the linear relationship between dilution rate and growth rate in the dilution rate range 0 to 0.24h^{-1}. (Fig 3.1.) Growth rate, while approximately equal to dilution rate, was consistently lower for the reasons discussed in section 2.6.1.

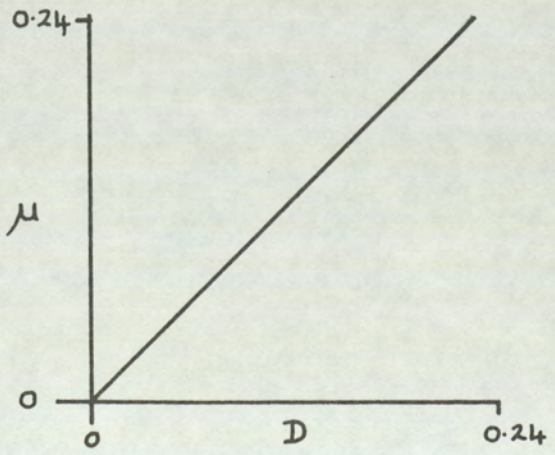


Fig 3.1. Specific growth rate v dilution rate ($D<0.24h^{-1}$) From Pannell (1976).

From these results, the growth rate of A. niger at a dilution rate of $0.09h^{-1}$ was $0.065h^{-1}$, and although the theoretical maximum growth rate of A. niger is thought to be $0.2h^{-1}$, this can clearly be exceeded in this system at high dilution rates. A dilution rate of $0.1h^{-1}$ was selected for use in the majority of experiments in this project for a combination of several reasons :

a) Sporulation is associated with zero or low growth rate, which in turn is associated with a low dilution rate. Dawes and Thornley (1970) and Ng et al. (1973b) confirmed this link by stating that conidiation is inversely proportional to dilution rate. These are reasons for employing a low dilution rate.

2) A low dilution rate assists prevention of organism wash-out after the imposition of a shock growth limitation.

3) At high dilution rates, filamentous fungi assume a pellet - type morphology in the C.T.F. and these are primarily vegetative and hence unfavourable for sporulation. For maximum spore production, all the mycelium of the tower needs to contribute to sporulation, however, only the peripheral zone of a pellet would be able to do so. At dilution rates up to $0.2h^{-1}$ the mould remains filamentous in this system (depending upon other environmental factors), a feature considered preferable for biochemical work (Galbraith & Smith, 1969c).

4) The dilution rate must be sufficiently high to prevent infection. At dilution rates below $0.2h^{-1}$ there is a much greater chance of an infecting organism becoming established in the tower due to its' higher natural growth rate. Consequently at a dilution rate of $0.1h^{-1}$, sterilized medium was used at all

times to minimise infection, while at a dilution rate of $0.2h^{-1}$ (used when autoclave facilities were unavailable) the wash-out effect was sufficient to prevent infection without sterile medium.

5) From a commercial aspect, if conidiation is inversely proportional to dilution rate, it is desirable to reach a compromise between the concentration of spore production (spores ml^{-1}) and the flow rate (mlh^{-1}) at which spore production (spores h^{-1}) is maximum.

6) A practical consideration. At a dilution rate of $0.1h^{-1}$, a 10 litre fermenter used 24 litres of sterilized medium per day, which, with no technical assistance and limited autoclave facilities, represented the maximum possible production in the available preparation time.

3.2. Selection of carbon concentration.

This was again based upon the results of Pannell (1976). In a conventional stirred tank reactor the carbon source is often the growth limiting nutrient and it directly controls biomass production and therefore at dilution rates below the critical value, effluent carbon concentration tends to zero. In the C.T.F., when substrate is supplied in excess, the utilization is governed by dilution rate and substrate concentration only determines the organism growth rate when supplied in limiting quantities. The data of Pannell (1976), Fig. 3.2., shows that carbohydrate utilization is independant of influent concentration. At each dilution rate there is a maximum carbohydrate concentration which can be utilized, and this is determined by fermenter biomass concentration, maintenance

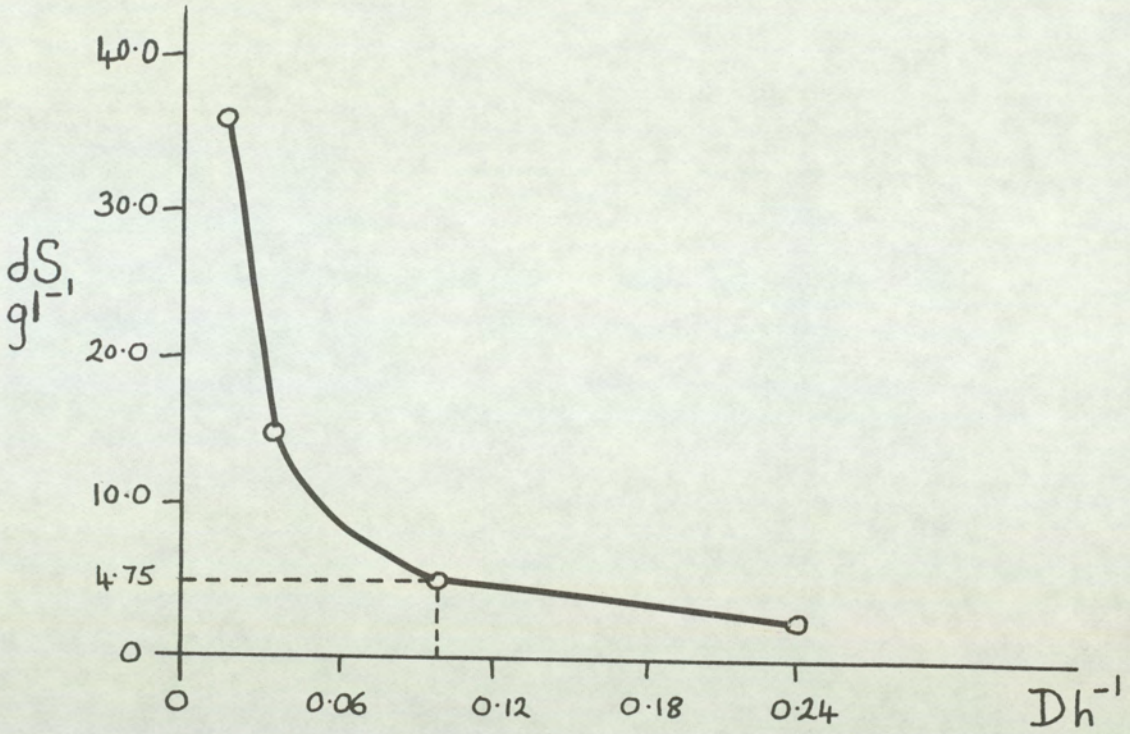


Fig 3.2. Sucrose utilization (dS) v. Dilution rate (D) for A. niger. From data of Pannell (1976).

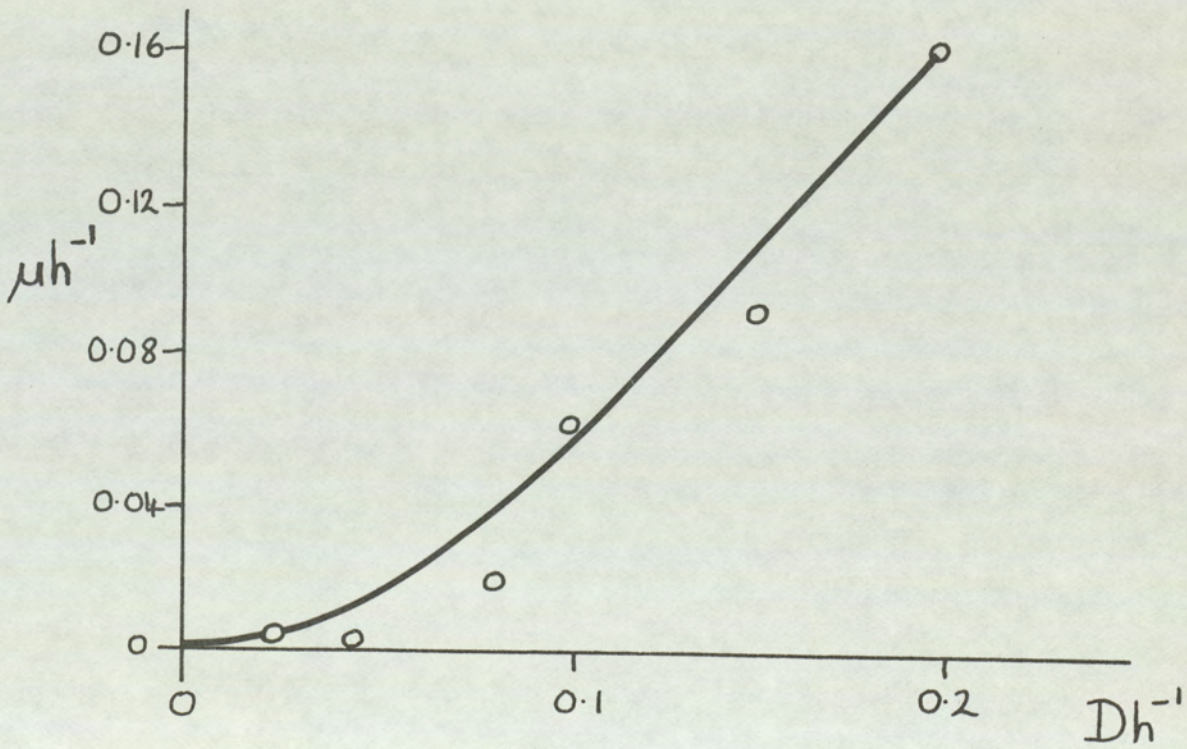


Fig 3.3. Growth rate of A. niger (μ) v. Dilution rate (D) on 5.0gl^{-1} sucrose.

requirements and metabolite production. From Fig 3.2. at a dilution rate of $0.1h^{-1}$, maximum sucrose utilization was $4.75gl^{-1}$. A medium concentration of $5.0gl^{-1}$ was therefore chosen as excess at a dilution rate of $0.1h^{-1}$ and above, and this would not have a limiting effect upon growth, while at the same time minimising substrate wastage.

Using the basic sucrose - salts medium (Appendix 1), the growth rate of A. niger at various dilution rates was recorded (Fig. 3.3., Table 1). This demonstrated that when using this medium, growth rate was proportional to dilution rate at or above $D = 0.1h^{-1}$ when the carbon source was in excess, but growth was inhibited at dilution rates below $0.1h^{-1}$ as the energy source became limiting.

3.3. Selection of type and concentration of nitrogen source.

The nitrogen concentration of the medium was again based upon that used by Pannell (1976) in the $5.0gl^{-1}$ sucrose - salts medium; ie $1.0gl^{-1}(NH_4)_2SO_4 \equiv 0.213g$ atomic Nl^{-1}. There was no reason to alter this concentration as results from experiment 3.3. showed that average ammonium utilization was 0.157 g atomic Nl^{-1}. The substrate was therefore in excess at a dilution rate of $0.1h^{-1}$ and above, with little wastage. As mentioned in section 1.7.2., extensive studies by Galbraith & Smith (1969a and 1969c) have provided much information on the growth of A. niger in submerged culture and conidiation has been shown to occur at the end of a rapid growth phase due to the exhaustion of a limiting nutrient, usually nitrogen, while assimilable carbohydrate is still present. Sporulation of A. niger

was induced in shake - flasks by varying the nitrogen source and concentration of a synthetic liquid medium. When glucose was the limiting nutrient, conidia induction did not occur in the presence of ammonium ions, although a wide range of nitrate levels permitted sporulation. Acetate, pyruvate, TCA cycle intermediates, glyoxylate and some amino acids overcame this ammonium inhibition. Weiss and Turian (1966) observed that conidiation of Neurospora crassa was inhibited by ammonium ions but that conidiation was abundant when nitrate was the sole nitrogen source, although Martinelli (1973) reported that NH_4 Cl enhanced sporulation of Aspergillus nidulans more than $NaNO_3$. Ng et al. (1973b) in studies on the sporulation of A. niger in continuous culture, reported that no conidiation was induced under ammonium limitation, while nitrate limitation stimulated conidiophore formation, but not maturation. In consideration of these findings, the ammonium sulphate of the medium was replaced with equivalent sodium nitrate (1.3 gl^{-1} ≡ 0.21 g atomic Nl^{-1}). To confirm that this did not adversely affect the organisms, continuous cultures of A. niger and A. ochraceus were grown under optimum ammonium and nitrate conditions using the medium described in Appendix 1. (Table 3). The mean growth rates of A. niger and A. ochraceus in $(NH_4)_2SO_4$ medium were $0.062h^{-1}$ and $0.051h^{-1}$ respectively, while in $NaNO_3$ medium the respective growth rates were $0.06h^{-1}$ and $0.05h^{-1}$. These results show that while the growth rate of A. ochraceus is lower than that of A. niger in this system, nitrate nitrogen has no adverse effect upon either organism. The greatest difference between the two nitrogen sources was the effect that their utilization had upon the pH of the fermentation broth. If the pH of the growth medium

was not controlled, much lower pH levels were obtained with ammonium sulphate as the nitrogen source than with nitrate. Ammonium sulphate utilization involves the production of sulphuric acid (H_2SO_4) which lowered the pH to approximately 2.5 - 3.0 while sodium nitrate utilization involves no acid formation. Organism growth rate is higher at low pH (Section 4.4.), low pH is a major factor preventing infection of the fermentation by bacteria and yeasts, and a pellet - type morphology is induced at high pH values. These factors obviously alter the characteristics of the fermentation, and therefore the culture broth was always continually maintained at a low pH value during experiments with sodium nitrate as the nitrogen source.

3.4. pH, temperature and aeration.

The levels of these three parameters were initially set at the values found to be optimal for the growth A. niger in the C.T.F. by previous workers. These values were; pH = 3.0, temperature = $30^{o}C$ and aeration = 1 v.v.m.

3.5. Nutrient limitation.

The final critical factor remaining to be defined was the available nutrient concentration that constituted a limiting concentration, and the way in which that limitation was to be imposed.

3.5.1. Carbon

It can be seen from section 3.2. that either a decrease in dilution rate or in medium sugar concentration would cause the carbon level to become limiting. These factors were used singly or in combination to inflict limitation upon the culture, with

the intention of lowering the carbon supply until just above, equal to or below the maintenance requirements of the organism. It was anticipated that sporulation would occur at or just above the maintenance value (Righelato et al., 1968, Ng et al. 1973b

3.5.2. Nitrogen.

The criteria for nitrogen limitation were determined in two ways. Firstly, by lowering the nitrogen content of the medium to below the level of utilization at maximum growth rate under optimum conditions at steady state, while maintaining all other parameters optimal. These values were $0.141gl^{-1}$ for A.niger and $0.13gl^{-1}$ for A. ochraceus. Secondly, limitation was imposed by decreasing the nitrogen level to that used by other workers to induce sporulation in batch culture. Galbraith and Smith (1969a) state that conidium formation is inhibited by nitrate ion concentrations at or above $60mgl^{-1}$ (atomic N) and conidiophores are inhibited by levels at or above $120mgl^{-1}$. In these experiments the nitrate content ofthe medium was decreased to $20mgl^{-1}$ (atomic N) and at times to zero (Anderson and Smith, 1971a, used a nitrogen - free medium to induce conidiophore elongation). Ng et al. (1973b) used a continuous supply of approximately $60mgl^{-1}$ atomic N ($0.386gl^{-1}$ $NaNO_3$) in their study of conidiation of A. niger in continuous culture.

To provide gradual nitrogen and carbon limitation, step - wise reductions in the nutrient content of the medium were carried out between the establishment of each steady state. Shock treatment was introduced by removing the medium supplying the optimum nutrient supply and replacing this immediately with one containing the extreme limiting concentration without altering any other environmental parameter.

Details of each limitation are given with the results.

3.6. Summary of selected parameter values for initial fermentations.

Dilution rate = $0.1h^{-1}$ or $0.2h^{-1}$. Temperature = $30^{o}C$

pH = 3.0 Aeration = 1 v.v.m.

Carbon source = $5.0gl^{-1}$ sucrose

Nitrogen source = $1.3gl^{-1}$ sodium nitrate ($\equiv$ $0.21gl^{-1}$ atomic N).

SECTION 4 - EXPERIMENTAL RESULTS

4.1. Carbon limitation.

The exhaustion of the carbohydrate supply in batch culture was used by Carter & Bull (1969), Martinelli (1972, 1976) and Saxena and Sinha (1973) to study the submerged sporulation of A. nidulans. Glucose limited batch cultures have been used by many workers to study various biochemical aspects of sporulation, but there have been relatively few carbon - limited continuous culture studies. Righelato et al. (1968) achieved conidiation of P. chrysogenum in a glucose - limited chemostat when the glucose feed rate decreased to, or just above the maintenance requirement (0.022 g glucose g mycelial dry $wt^{-1}h^{-1}$) at an optimal growth rate of $0.009h^{-1}$. The optimum glucose supply rate for sporulation was 0.038 $gg^{-1}h^{-1}$ which is approximately 1.7 x the maintenance requirement. If the glucose supply was removed completely, the mould autolysed but with a limitation that prevented autolysis and facilitated sporulation, the rate of sporulation was inversely proportional to the growth rate prior to the onset of nutrient limitation. The sporulation of Clostridium thermosaccharolyticum was induced under glucose - limited conditions by Hsu & Ordal (1969). After allowing the culture to grow batch - wise until the glucose was exhausted, a limiting 0.1% per hour glucose supply was fed continuously to the culture, and this induced sporulation after only four hours. Sporulation did not occur in the starved culture, nor if the glucose feed was too great. Ng et al. (1973b) reported that conidiation of A. niger did not occur above a dilution rate

of $0.013h^{-1}$ under glucose limitation, and was greatest at $0.008h^{-1}$, but at the latter dilution rate the culture was difficult to maintain due to autolysis. Productivity in the C.T.F. is lower than the continuous stirred tank reactor as a result of the larger overall maintenance requirement of biomass in the C.T.F. due to the lower growth rate and internal biomass recycle. There is therefore a lower efficiency of conversion of energy substrate to biomass during a carbon - limited fermentation, which is a disadvantage if biomass production is required, but may act in favour of sporulation induction.

4.1.1. Shock sucrose limitation.

These experiments were originally conducted at a dilution rate of $0.2h^{-1}$ due to the lack of medium sterilization facilities. Using $5.0gl^{-1}$ sucrose as the carbon source, an A. niger fermentation was allowed to reach steady state at $30^{o}C$, pH 3.0, aeration 1 v.v.m. and a nitrate supply of 0.21 g atomic N. l^{-1}. The medium was then abruptly changed to one supplying only $0.25gl^{-1}$ sucrose (supply rate (kS) down from $4.2gh^{-1}$ to $0.21gh^{-1}$) and the fermentation monitored (Table 4). The responses of growth rate and productivity are shown in Fig. 4.1. At this dilution rate, the limitation was too severe, and wash - out occurred within three days with no sign of sporulation. The peaks of growth rate and productivity after limitation was imposed are false values due to the dramatic decrease in fermenter biomass concentration during wash - out.

The experiments were repeated with A. niger, imposing a similar carbon supply rate limitation (kS $4.2gh^{-1}$ to $0.19gh^{-1}$) but by a

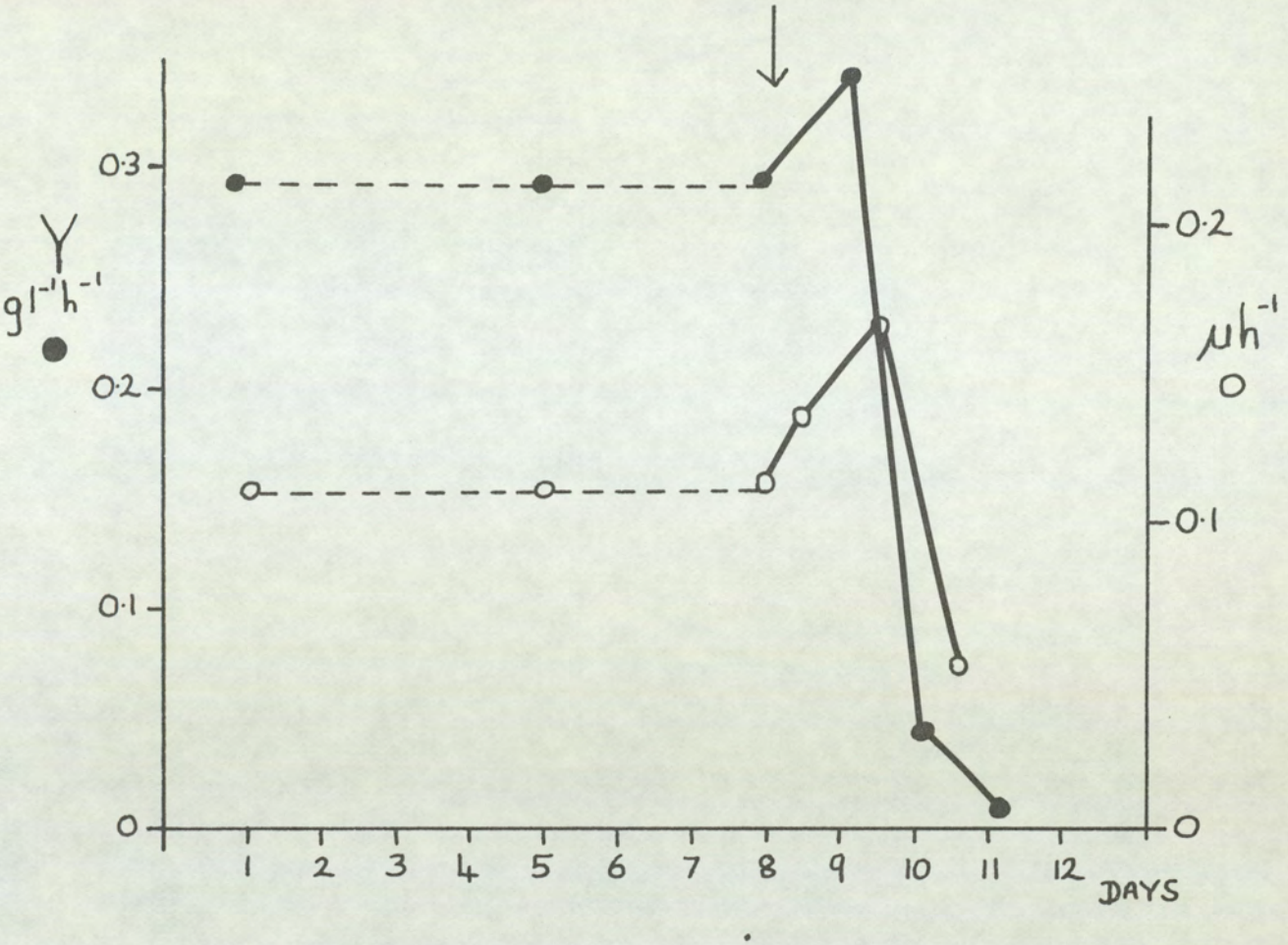


Fig 4.1. Growth rate (μ) and productivity (Y) of A. niger after shock sucrose limitation. (Sm 5.0 - 0.25gl^{-1}) D = 0.2h^{-1}

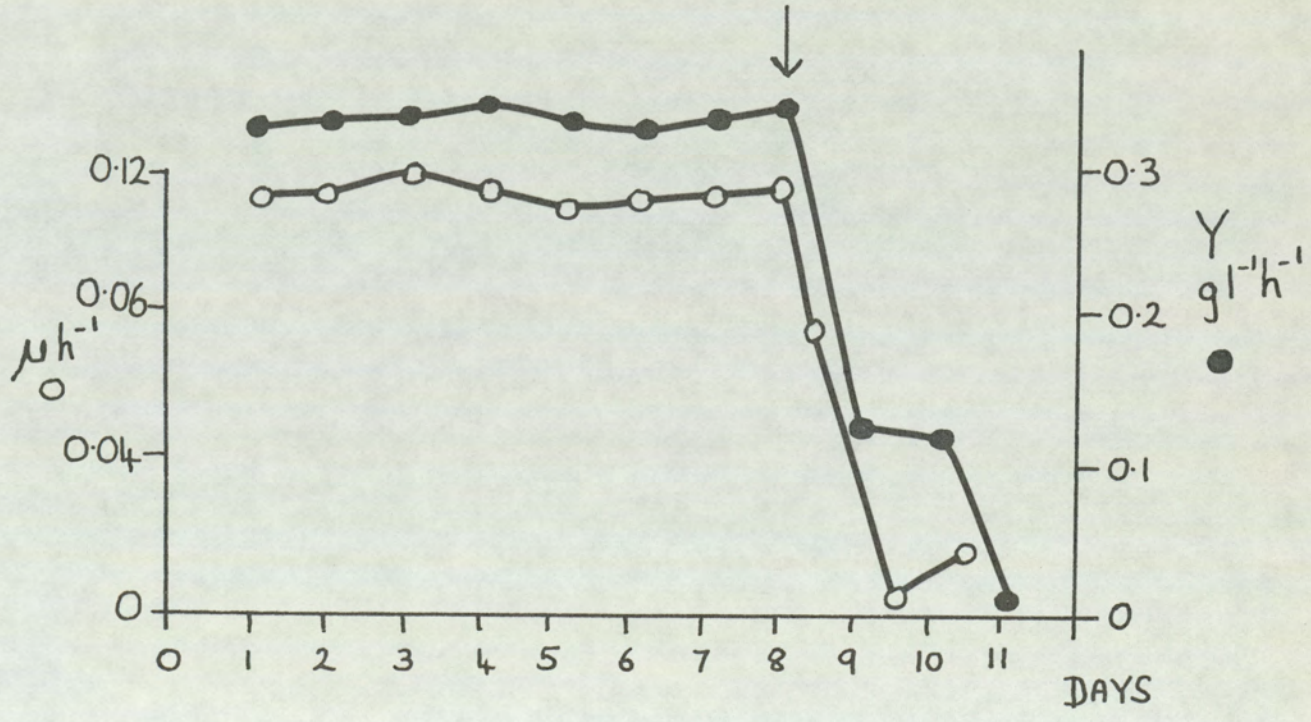


Fig 4.2. Growth rate (μ) and productivity (Y) of A. niger after shock sucrose limitation. (Sm 5.0 - 0.47gl^{-1}, D 0.2 - 0.1h^{-1}).

combination of lowered dilution rate ($0.2h^{-1}$ to $0.1h^{-1}$) and lowered medium sucrose concentration ($5.0gl^{-1}$ to $0.47gl^{-1}$). The effect of this limitation upon the growth rate and productivity are shown in Fig. 4.2. (Table 5). This was again unsuccessful, and no sporulation resulted before the organism was washed out, although the decrease in fermenter biomass concentration was less dramatic. This meant that there were no false increases in growth rate and productivity after limitation was imposed.

A sudden large decrease in the availability of the major energy source at a dilution rate of $0.2h^{-1}$ appeared too great. The growth rate of the organism was dramatically reduced by the limitation and the organism was washed out of the fermenter by the relatively high dilution rate before it was able to recover. Although the decrease of the carbon source concentration was the reason for wash - out (the fermenter can only support a certain population density at a particular medium sucrose concentration at a particular dilution rate), the decrease did not actually physically cause mould removal. The physical wash - out was due to the change in floc morphology caused by carbon limitation, which was transformed from loose filaments (Type 1 - 2, Cocker 1976) to small, tight fragments via a series of forms shown in plates 8 - 13. The final morphology corresponds with a type 7 floc, characterised by extremely high branching frequency, and these flocs were more easily removed from the fermenter.

PLATE 8

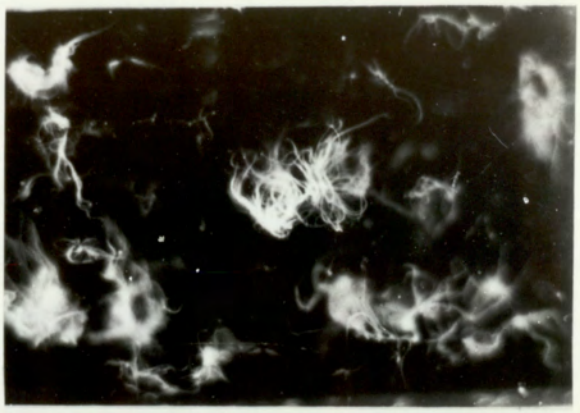


PLATE 9

PLATE 10

PLATE 11

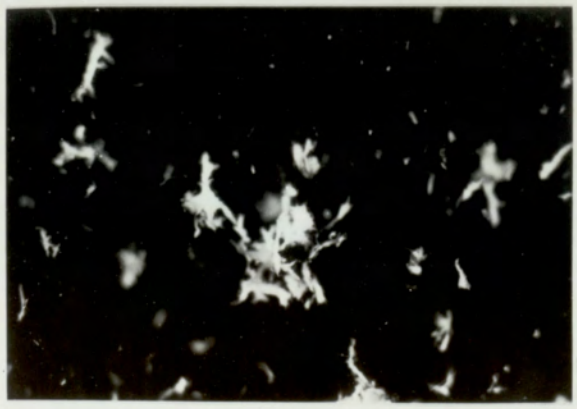


PLATE 12

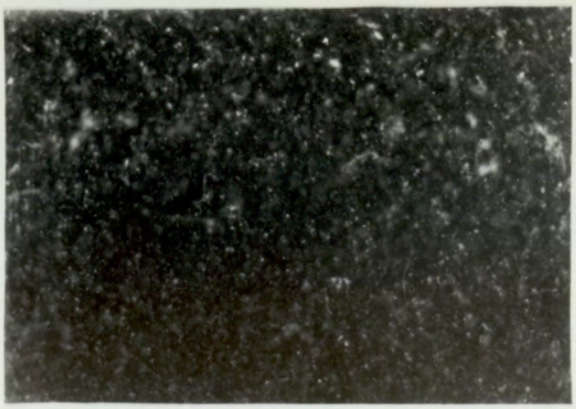


PLATE 13

PLATES 8 - 13. Sequence of floc morphologies exhibited by A.niger in continuous culture in the tower fermenter after a shock decrease in sucrose supply ($5.0gl^{-1}$ to $0.25gl^{-1}$ at Dilution rate $0.2h^{-1}$), leading to wash-out. Each plate shows an area 40mm. x 25mm.

4.1.2. Gradual sucrose limitation.

Sporulation occurred during the gradual decrease of sucrose supply to A. niger by a combination of decreased dilution rate and medium sucrose concentration (Table 2). The effects of this limitation upon growth rate and productivity (Fig. 4.3) and nitrogen and carbon utilization (Fig 4.4) are shown. Initially, the carbon supply rate (kS) was sufficient for maximum growth rate which remained constant as carbon supply decreased until a critical point was reached, beyond which growth rate decreased rapidly, while the relationship between productivity and carbon supply rate is approximately linear. Nitrogen utilization closely followed the trend of growth rate. Sporulation was observed only upon the introduction of a carbon supply rate of $0.4gh^{-1}$, when developing structures were seen after 8 hours, and spores after 12. All structures (Plate 14) were similar to the sub - aerial form and while many spores were attached to the phialides, their numbers in the medium were too few to count accurately. Spore production continued in this system for up to five days before mould death and infection. However, only one of two subsequent replicates was successful.

Data during sporulation:

Mean specific substrate supply rate (Q_S)	= $0.071\ gg^{-1}h^{-1}$
Maintenance requirement (m) Fig. 4.5.	= $0.0185\ gg^{-1}h^{-1}$
Mean specific substrate utilization rate (q_s)	= $0.026\ gg^{-1}h^{-1}$
Mean growth rate (μ)	= $0.025h^{-1}$
By subtraction, $(\Delta s)_G$ (qs - m)	= $0.0485\ gg^{-1}h^{-1}$

It is significant that sporulation occurred at a substrate supply rate above the maintenance value, which agrees with

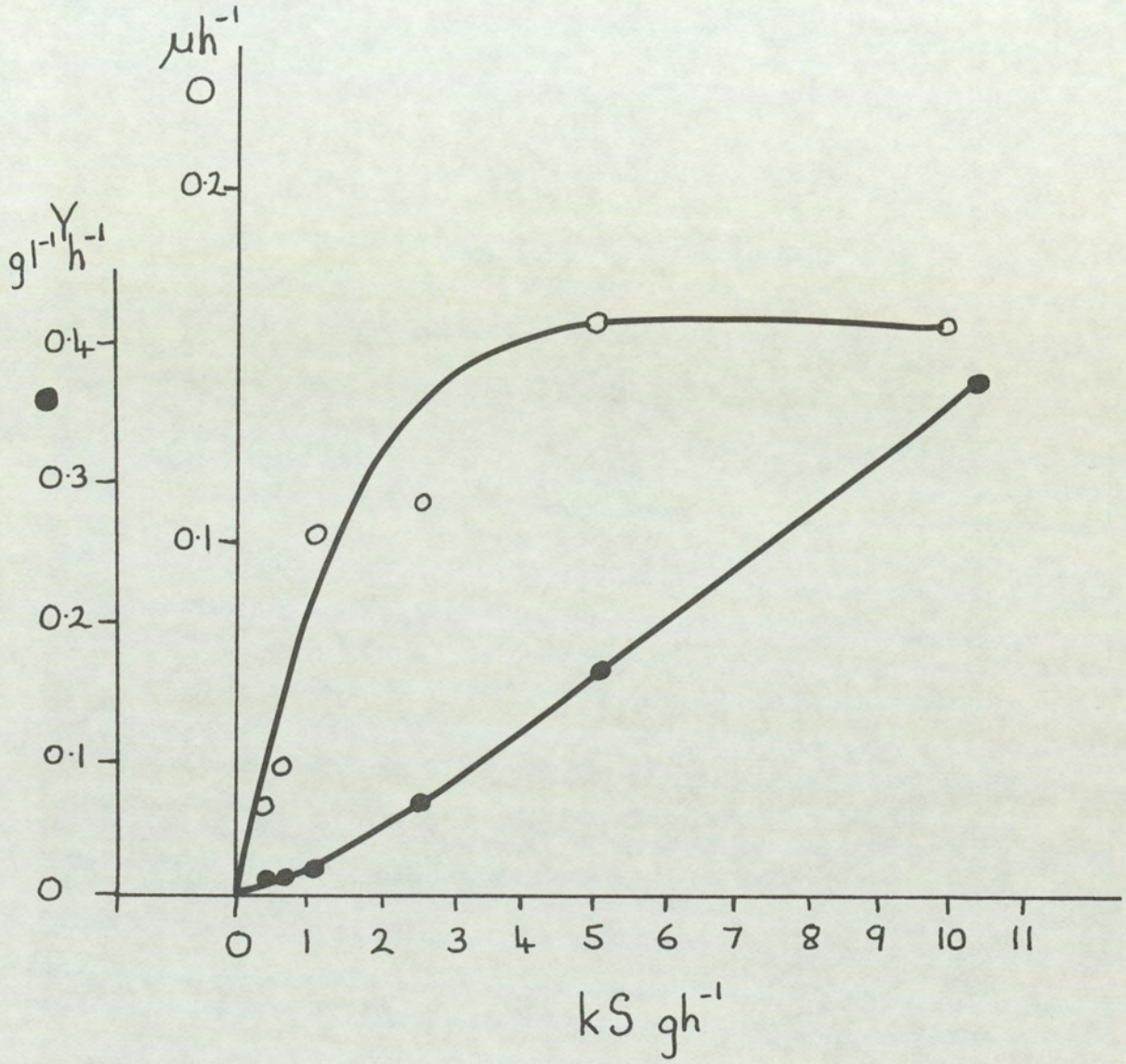


Fig 4.3. The effect of gradual sucrose limitation, Sm (by combination of decreased dilution rate and decreased sucrose medium concentration) upon the growth rate (μ) and productivity (Y) of A. niger.

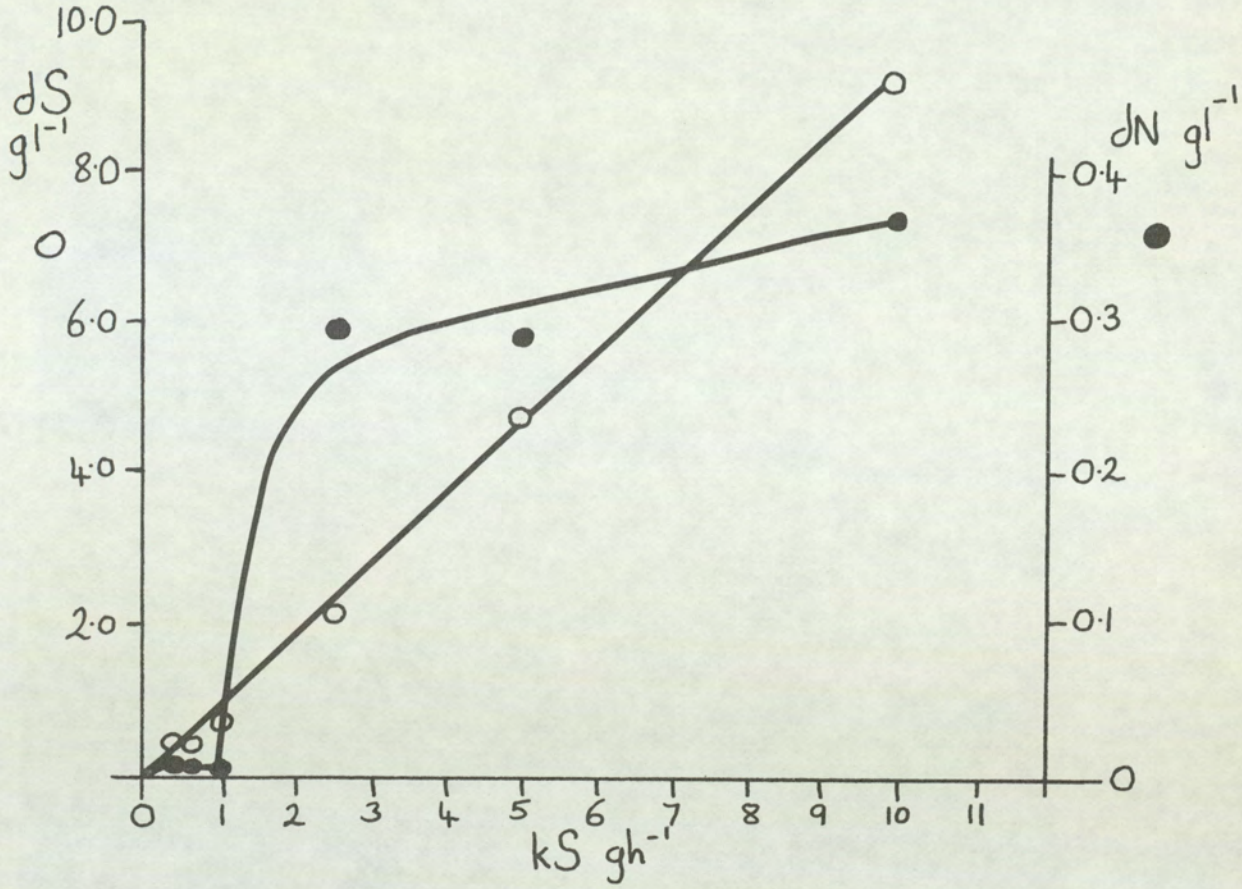


Fig 4.4. The effect of gradual sucrose limitation upon sucrose (dS) and nitrogen (dN) utilization by A. niger.

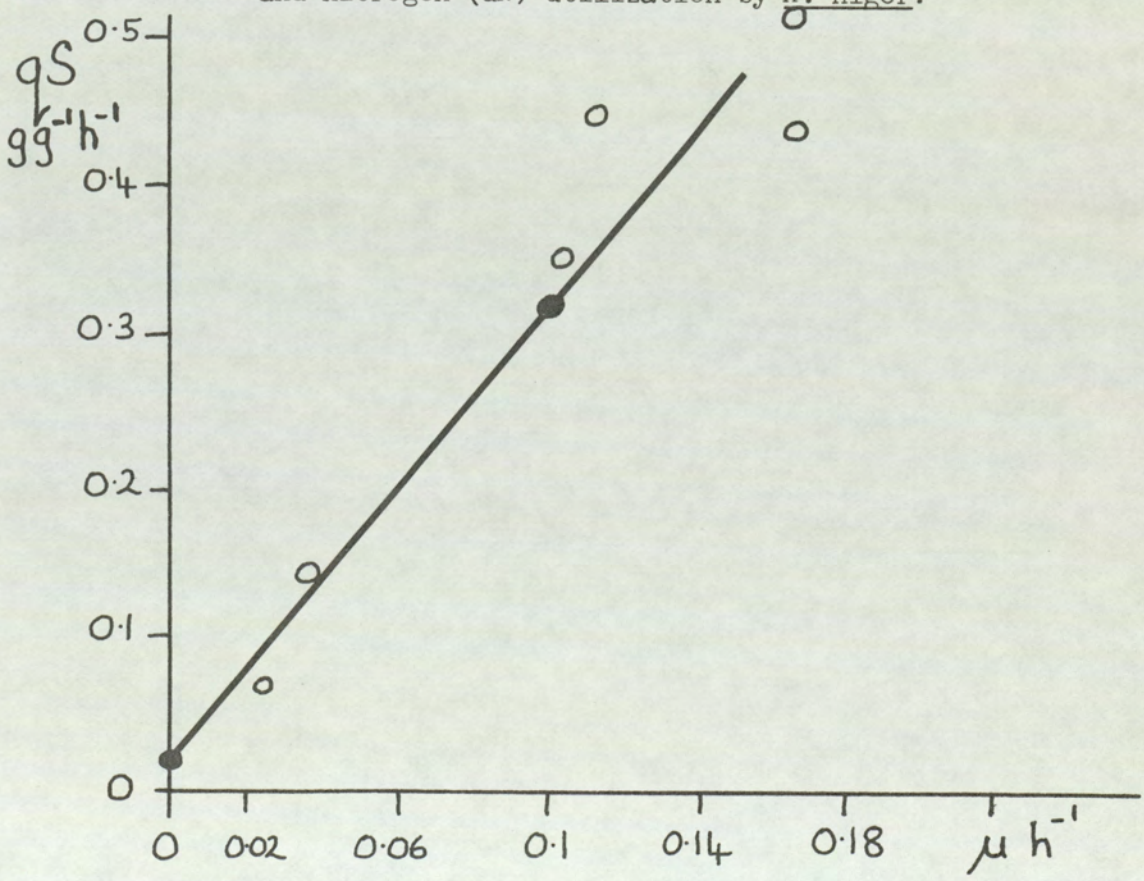


Fig 4.5. Calculation of maintenance coefficient of sucrose ($0.0185 gg^{-1}h^{-1}$ for A. niger by plot of specific substrate utilization rate (q_s) v. growth rate (μ).

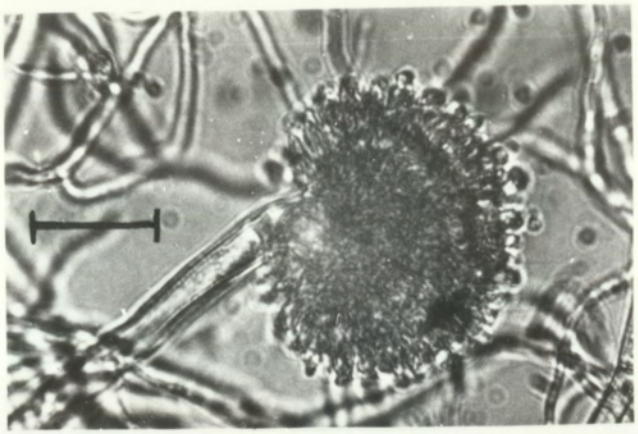


PLATE 14

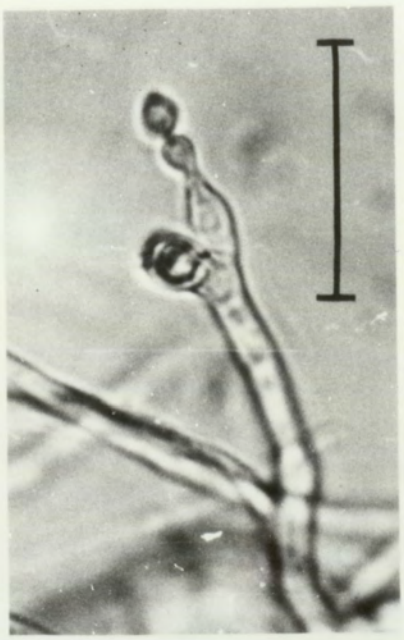


PLATE 15

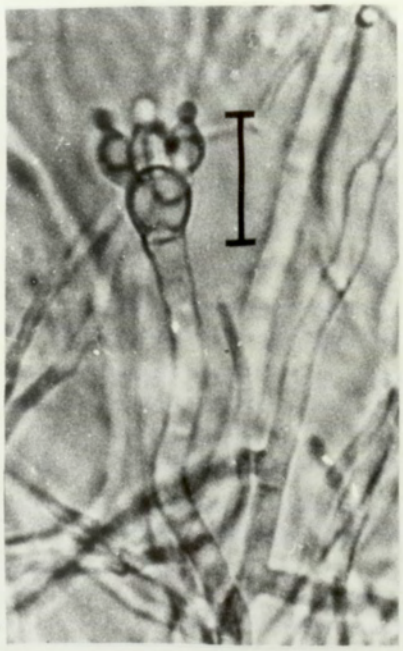


PLATE 16

PLATES 14, 15 & 16 Sporulative structures of A.niger formed in continuous submerged culture.

14 - Sub - aerial type formed during gradual sucrose limitation. (4.1.2.)

15 - Simplified structure formed during gradual glucose limitation. (4.1.3.)

16 - Sub - aerial type with reduced phialide numbers formed during gradual starch limitation. (4.1.4.)

BAR = 30 µm.

the observations of Righelato et al. (1968) and Ng et al. (1973b). The loose filamentous nature of the flocs (Type 1-2) did not alter throughout the experiment, which accounts for the persistently low fermenter biomass concentration. The fact that sporulation occurred at or below a growth rate of $0.025h^{-1}$, above which the mycelium was vegetative, supports the theory that the two processes are not mutually exclusive, but need to occur simultaneously for this type of system to operate (Larmour and Marchant 1977).

When this experiment was repeated with A. ochraceus, the organism was washed out of the fermenter when the sucrose supply rate was decreased to $1.0gh^{-1}$ or lower, before any sign of sporulation was observed.

4.1.3. Comparison of gradual sucrose and glucose limitation.

To compare the effect of gradually reducing the supply of sucrose or glucose to A. niger, experiments were performed (Table 6) in which each carbon source concentration was lowered step - wise while maintaining a single dilution rate, $0.1h^{-1}$, and all other parameters optimal. As a result, any change in the culture was due to the effects of reduced carbon supply alone.

The effect of medium sucrose concentration upon growth rate and productivity for both sucrose and glucose are shown in Fig. 4.6. and Fig. 4.7. respectively. Again, biomass productivity was proportional to sucrose concentration of the medium, while the restriction upon growth progessively increased as sucrose concentration decreased. Growth rate of A. niger in this system was greater with sucrose than with glucose under

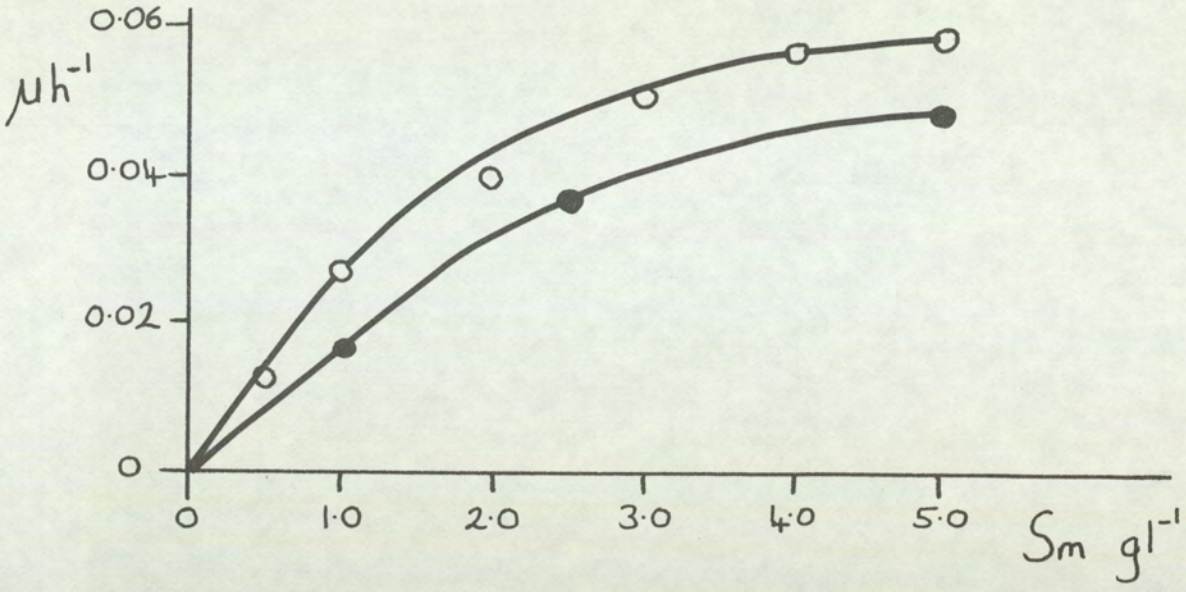


Fig 4.6. The effect of sucrose (o) or glucose (●) supply concentration (Sm) upon growth rate (μ) of A. niger at D = 0.1h^{-1}.

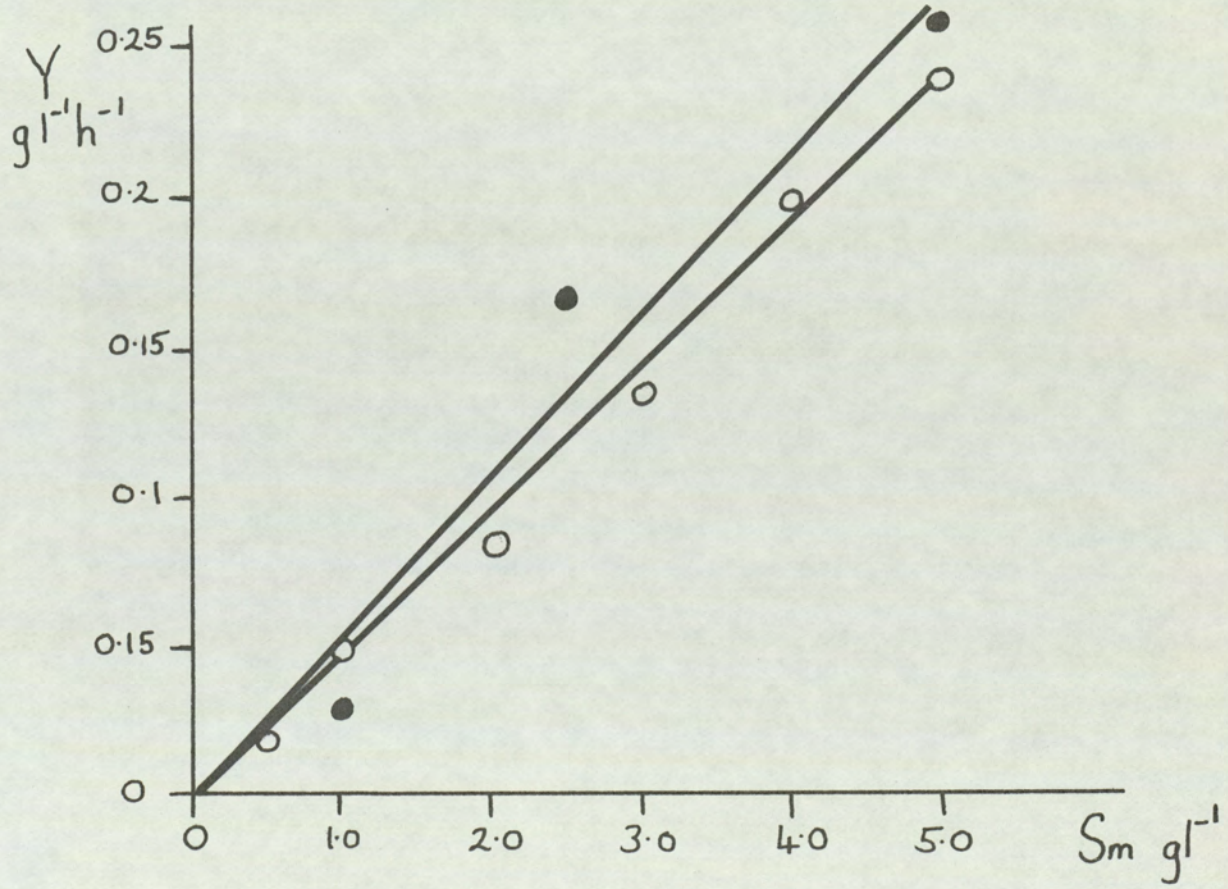


Fig 4.7. The effect of sucrose (o) or glucose (●) supply concentration (Sm) upon productivity (Y) of A. niger at D = 0.1h^{-1}.

all conditions of carbohydrate supply. Sporulation occurred with both substrates.

Sucrose: Spores were first observed 18 hours after the introduction of $1.0 gl^{-1}$ medium sucrose concentration, (supply rate $0.42 gh^{-1}$) and production continued for 3 days during the period of $0.5 gl^{-1}$ sucrose concentration supply. Spores were again formed on sub-aerial type structure, but numbers were too few for accurate counts.

Glucose: Sporulation was much enhanced under glucose limitation, and began initially 48 hours after the introduction of $2.5 gl^{-1}$ medium glucose concentration. Intensity increased immediately upon the supply of $1.0 gl^{-1}$ glucose, and reached a peak of 4×10^6 spores ml^{-1} (= 9.5×10^8 spores g mycelium $^{-1}$) which represented a production of approximately 4×10^9 spores g glucose^{-1} utilized. Sporulation continued for several days in each of the replicated experiments, with decreasing intensity until sporulation ceased and the fermentation was terminated. Sporulation morphology was of a type previously unobserved (Plate 15). Single phialides were borne on hyphal tips with no evidence of normal sporing structure development. The formation of this structure is discussed in section 5. Mycelial flocs remained filamentous throughout the study.

Data during sporulation :

Q_S	(sucrose)	=	$0.039\ gg^{-1}h^{-1}$	Q_S (glucose)	$0.04\ gg^{-1}h^{-1}$
q_s	"	=	$0.039\ gg^{-1}h^{-1}$	q_s "	$0.044\ gg^{-1}h^{-1}$
m	"	=	$0.016\ gg^{-1}h^{-1}$	m "	$0.0123\ gg^{-1}h^{-1}$
$(\Delta s)_G$	"	=	$0.023\ gg^{-1}h^{-1}$	$(\Delta s)_G$ "	$0.0317\ gg^{-1}h^{-1}$

These results again support the views that sporulation occurs at a substrate supply rate above the maintenance requirement

and occurs simultaneously with growth at rates between $0.1h^{-1}$ and $0.028h^{-1}$. (sucrose) and between $0.008h^{-1}$ and $0.05h^{-1}$ (glucose).

This experiment was copied twice using A. ochraceus, but when the concentration of either sucrose or glucose reached $1.0gl^{-1}$, the organism washed out of the fermenter with no sign of sporulation.

4.1.4. Gradual starch limitation to A. niger.

Starch is a carbohydrate source which is biochemically less available to the Aspergilli due to the requirement for hydrolysis before utilization. It was therefore anticipated that starch limitation would prove more severe at higher substrate concentrations than sucrose or glucose which would facilitate the maintenance of the state between autolysis and vegetative growth.

The starch supply was reduced step - wise to A. niger growing in otherwise optimum conditions ($D = 0.1, h^{-1}$, $T = 30^{o}C$, pH = 4.0, Air = 1 v.v.m. and $N_m = 0.21gl^{-1}$). At each stage the fermentation was allowed to reach steady - state over 3 days except at the lowest medium starch concentration of $0.6\ gl^{-1}$, when growth rate showed a progressive decline with time (Fig. 4.8.). Growth rate values were calculated for a transient phase after the onset of $0.6\ gl^{-1}$ concentration. The results (Table 7) show that growth rate altered little with each starch concentration until $0.6\ gl^{-1}$ was introduced, when conidiophore initials were first observed 48 hours after the onset of the lowest feed rate (Day 12). Spores were produced 18 - 24 hours later, indicating an overall production

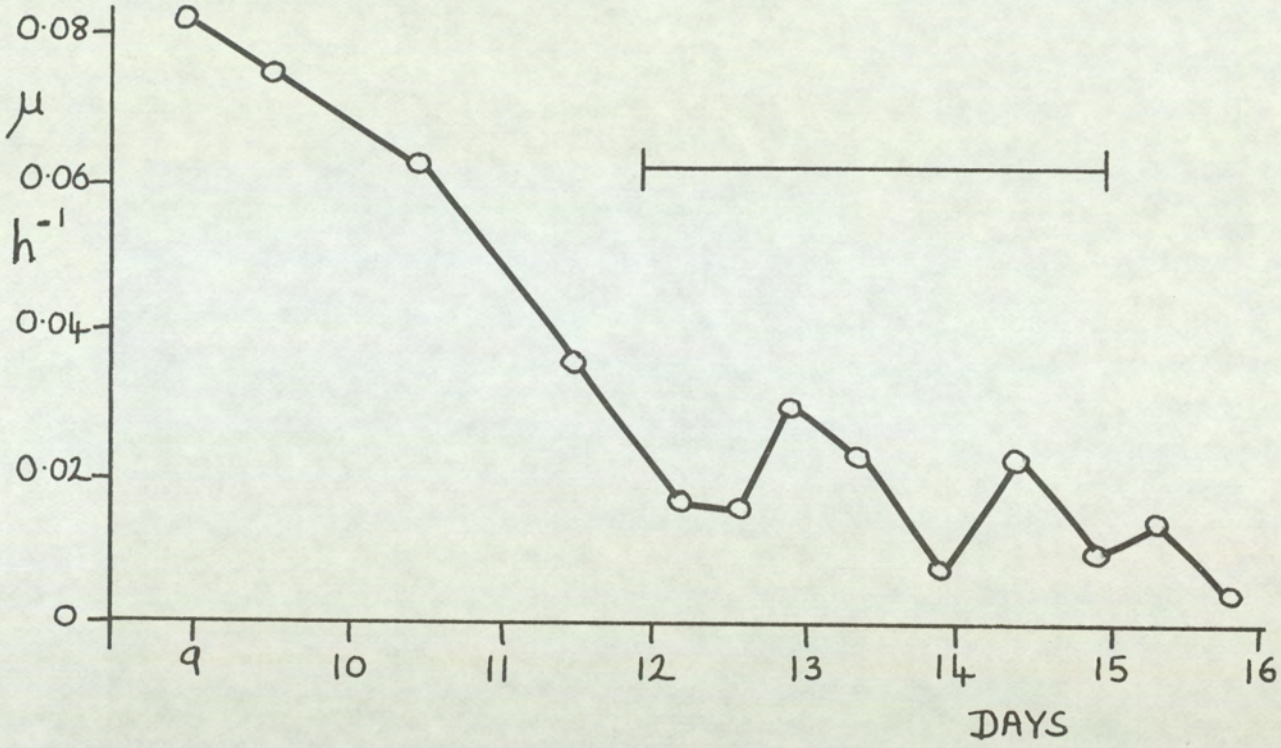


Fig 4.8. The gradual decrease of growth rate (μ) of A. niger after the onset of a starch supply of $0.6 gl^{-1}$ glucose equivalent.
$D = 0.1 h^{-1}$ ⟷ = period of sporulation.

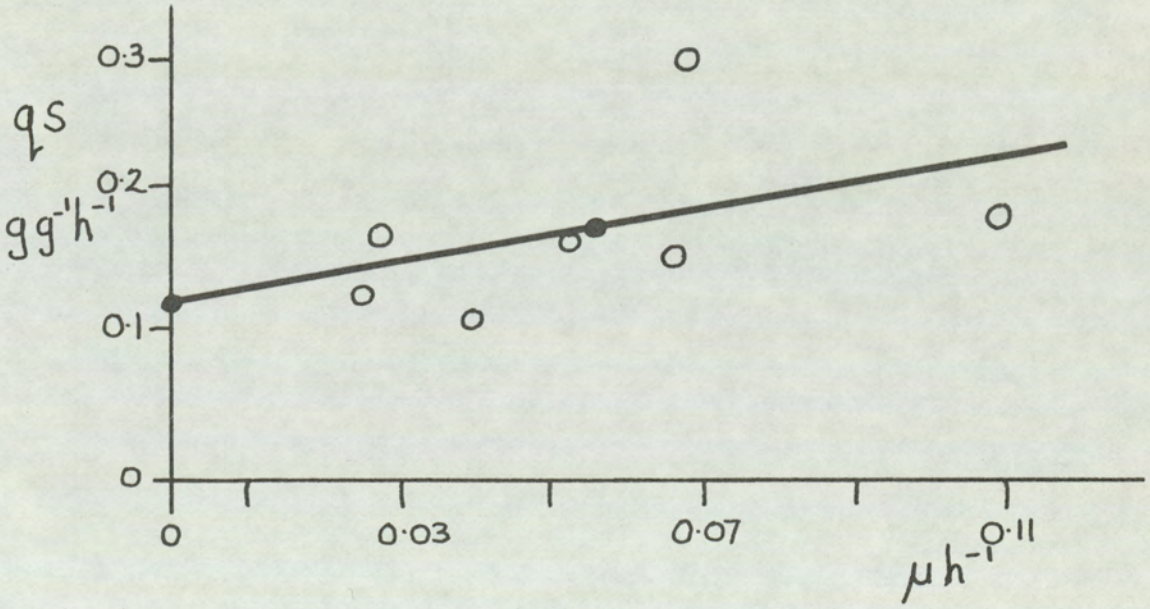


Fig 4.9. Calculation of maintenance coefficient ($0.123 gg^{-1}h^{-1}$) of starch for A. ochraceus. Plot of specific starch utilization rate (q_s) v. growth rate (μ).

time of 60 - 72 hours, but a development time from vegetative hyphae to spores of some 18 - 24 hours. Spores were produced asynchronously on sub - aerial type structures, although a degree of simplification was evident in that only 3 or 4 phialides were produced on each vesicle (Plate 16). Sporulation was evident for up to 72 hours but insufficient free spores were produced for accurate counts. Throughout the experiments, the floc morphology remained filamentous (Type 1-2).

Data during sporulation :

Mean substrate supply rate (Q_S) - starch = $0.044\ gg^{-1}h^{-1}$

Mean substrate utilization rate (q_s) = $0.0422\ gg^{-1}h^{-1}$

m (by method of Schulze & Lipe, 1964. Fig 4.9)= $0.0383\ gg^{-1}h^{-1}$

m (by method of Pirt, 1965) = $0.0145\ gg^{-1}h^{-1}$

By subtraction, $(\Delta s)_G$ = $0.0039\ gg^{-1}h^{-1}$ or $0.0217\ gg^{-1}h^{-1}$

These figures again demonstrate that sporulation occurred when the substrate supply rate was decreased to just above the maintenance requirement of the organism. Spore production occurred over a growth rate range of $0.0079\ h^{-1}$ to $0.0287\ h^{-1}$ (wash - out occurred at growth rate $0.002\ h^{-1}$) and conidiophores were formed at a growth rate $0.036\ h^{-1}$ above which growth was vegetative. It is interesting to note that the fermenter biomass concentration was unaltered after the $0.6\ gl^{-1}$ concentration of starch was fed to the culture even though productivity (Y) and yield coefficient (Ysub) (Fig. 4.10.), and nitrogen utilization (Fig 4.11) gradually decreased similarly to growth rate with nitrogen utilization eventually reaching zero.

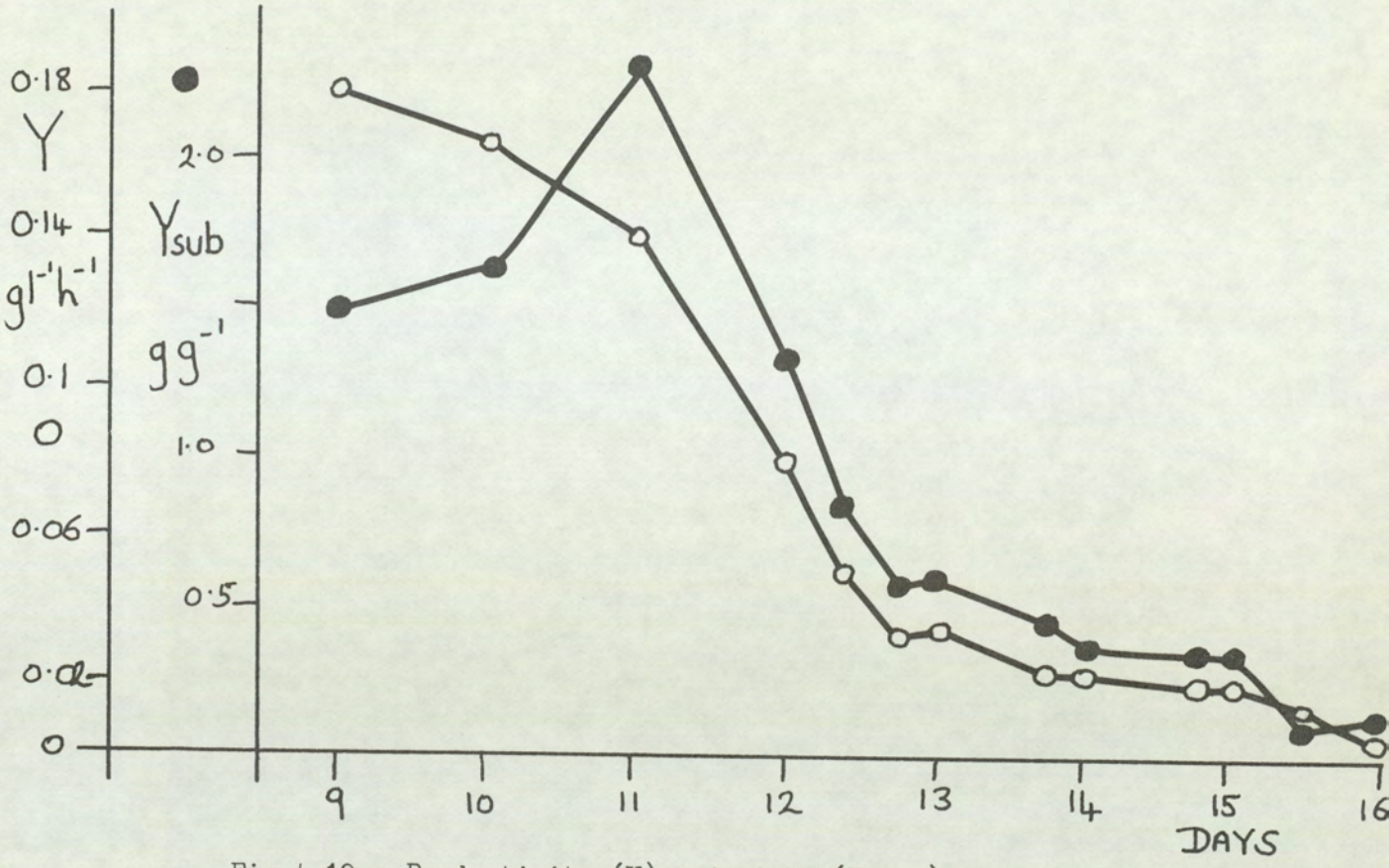


Fig 4.10. Productivity (Y) and yield (Y_{subs}) after the onset of $0.6gl^{-1}$ starch supply to A. niger v. time.

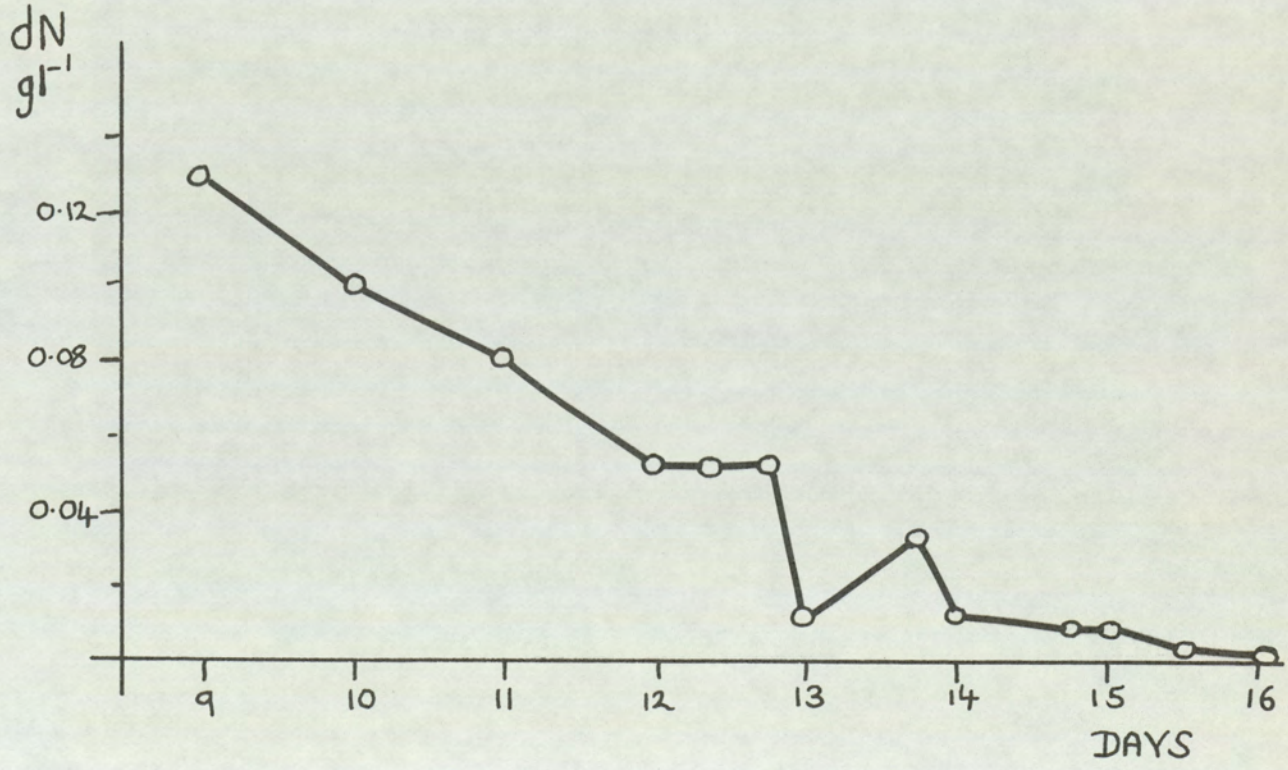


Fig 4.11. Starch reduction to A. niger. The decay of N utilization (dN) with time after onset of $0.6gl^{-1}$ supply.

4.1.5. Gradual starch limitation to A. ochraceus

Starch supply to A. ochraceus was reduced step - wise by a combination of decreased dilution rate ($0.1h^{-1}$ to $0.02h^{-1}$) and decreased medium starch concentration (4.0 gl^{-1} to $2.0gl^{-1}$) while all other parameters were optimal. No sporulation was achieved as the organism washed out when the substrate supply rate was reduced to 0.4 gl^{-1} (Table 8.) This was initially surprising as this was at a relatively high starch concentration (2.0 gl^{-1} glucose equivalent) and low dilution rate ($0.02h^{-1}$) and the specific substrate supply rate (Q_S) was 0.121 $gg^{-1}h^{-1}$ at the time of wash - out. However, the calculation of the maintenance coefficient (Fig. 4.9 .) revealed that the value for A. ochraceus was 0.123 $gg^{-1}h^{-1}$ which is considerably higher than the requirement of A. niger in this system. Thus at the time of wash - out, the specific supply rate had dropped below the calculated maintenance requirement.

The calculation of the maintenance energy requirement of 0.117 $gg^{-1}h^{-1}$ by the method of Pirt (1965) shows close agreement with that of 0.123 $gg^{-1}h^{-1}$ calculated by the Schulze and Lipe (1964) method, and both these values are considerably higher than carbohydrate maintenance requirements of other fungi calculated by other workers.

4.2. Nitrogen limitation.

As previously stated, the most common stimulus to sporulation in submerged culture is the exhaustion of the nitrogen source in the presence of assimilable carbohydrate. Morton et al. (1960) and Morton (1961) concluded that sporulation of

Penicillium griseofulvum was inhibited by high concentrations of nitrogen, which promote vegetative growth, while conidiation was induced by nitrogen exhaustion and the shake flask results of Galbraith and Smith (1969a) have already been discussed (3.3). In the continuous culture studies of nitrate limitation upon A. niger by Ng et al. (1973b), conidiophore induction, but not maturation was stimulated at dilution rates of 0.016 and $0.022h^{-1}$ but wash - out occurred at $D = 0.034h^{-1}$ using a $NaNO_3$ concentration of $0.386\ gl^{-1}$. Conidiation was shown to occur at a growth rate between zero and a critical value above which vegetative growth predominated, and to depend upon the interaction between growth rate and the carbon and nitrogen sources of the medium.

4.2.1. Gradual nitrogen limitation to A. niger and A. ochraceus

These experiments involved reducing the nitrate concentration of the media in a step - wise manner, while all other parameters remained optimal for growth ($T = 30^{o}C$, pH = 3 - 4, Air = 1 v.v.m. $Sm = 5.0\ gl^{-1}$ sucrose) and $D = 0.2h^{-1}$ (A.niger) or $0.1h^{-1}$ (A. ochraceus), and allowing the establishment of a steady state at each nitrogen level. From the experimental results (Tables 9 & 10) :

Mean nitrogen utilization by A. niger @ D = 0.2 = $0.136\ gl^{-1}$

Mean nitrogen utilization by A. ochraceus & D = 0.1 = $0.125\ gl^{-1}$

The figures relate closely to those obtained in section 3.

Mean nitrogen utilization by A. niger @ D = 0.1 = $0.141\ gl^{-1}$

Mean nitrogen utilization by A. ochraceus @ D = 0.1 = $0.131\ gl^{-1}$

From these values it was anticipated that growth rate would be

constant at medium nitrogen concentrations above this average utilization in optimum conditions, and that a limitation upon growth rate would be imposed once levels below this were introduced. However, Figures 4.12 and 4.13 show that this was not the case. Despite the different dilution rates, the graphs have a similar pattern, in that the plateau of maximum growth rate is reached at approximately 0.04 gl^{-1} medium nitrogen concentration beyond which it is constant, and below which it decreases rapidly. With A. niger, therefore, the growth rate is similar when available nitrogen (0.042gl^{-1} - 0.21gl^{-1}) permits a utilization of 0.04gl^{-1} or 0.136gl^{-1}, and for A. ochraceus growth rate is similar at utilization rates of 0.037 gl^{-1} or 0.125 gl^{-1} (supply of 0.038 - 0.2 gl^{-1}). The concentration of nitrogen supply at which growth rate becomes inhibited probably represents the point at which replication becomes restricted due to an inability to synthesise sufficient proteins, RNA and DNA. Above this level, excess nitrogen may be used in increasing the mould protein content. To check this hypothesis, the total nitrogen content of A. ochraceus mycelium at various stages of nitrogen limitation was determined and converted to crude protein content ;

Medium nitrogen concentration (gl^{-1})	0.21	0.18	0.042	0.03	0.005
Mould crude protein (percent)	18.1	17.5	17.0	13.8	13.3
S.D.	1.0	0.85	1.1	0.42	0.77

These figures show that there is a significant increase in protein content of the mould when nitrogen limitation is removed but only a slight increase with increasing nitrogen availability. Estimation of a 'maintenance ration' of nitrate for A. niger and A. ochraceus was not possible from these experiments,

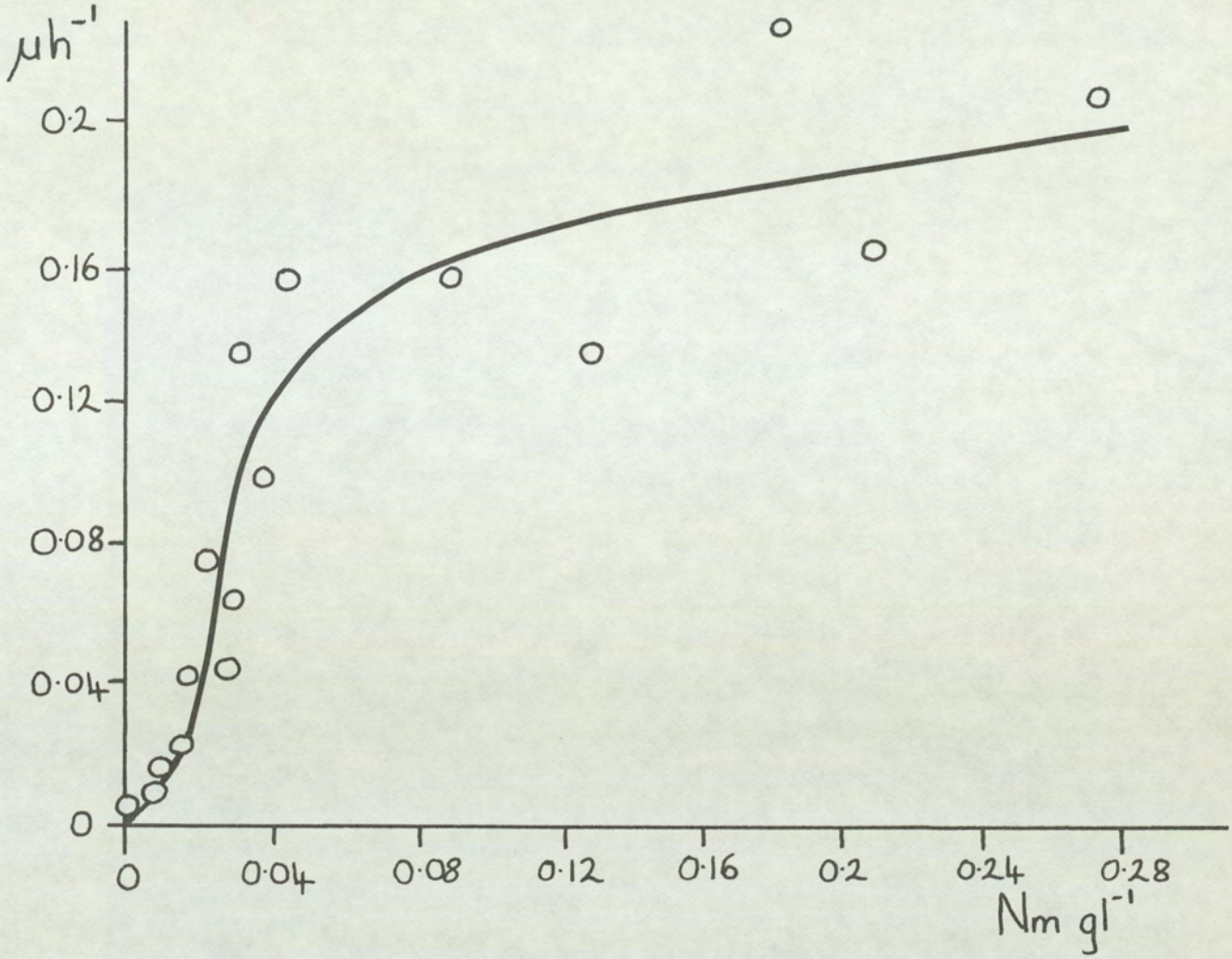


Fig 4.12. The effect of gradual reduction of medium nitrogen concentration (Nm) upon the growth rate (μ) of A. niger. $D = 0.2h^{-1}$.

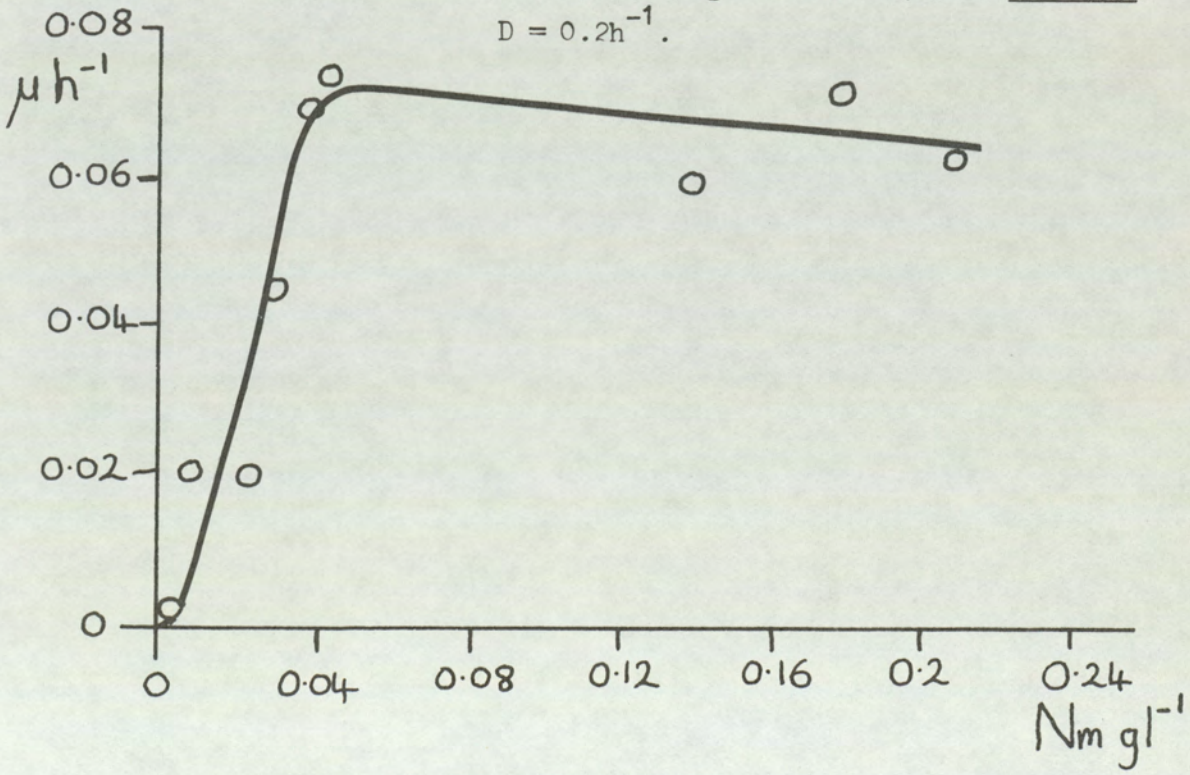


Fig 4.13. Gradual nitrate reduction (Nm). The effect upon growth rate (μ) of A. ochraceus. $D = 0.1h^{-1}$.

because growth continued when nitrogen supply and utilization were apparently zero.

One particular fermentation was run for 1536 hours on a nitrogen supply of 0.02 gl^{-1} or less, with no sign of sporulation. It is suggested that the organism can obtain sufficient nitrogen supply for maintenance and growth from the autolysis of dead cells at this low dilution rate. Pannell (1976) also observed this effect. This condition cannot last indefinitely, because only sufficient nitrogen is available for the maintenance of existing cells, and not for production of new cells, ie. there is no increase in mycelial mass. The existing cells therefore gradually die without replacement, although this is a slow process. This observation is discussed further in section 5. The floc morphology remained unchanged throughout these experiments, exhibiting loose filamentous structures (Type 1-2) of a light beige colour.

4.2.2. Shock nitrate limitation to A. niger

The stimulation of sporulation of A. niger was induced by the sudden removal of the nitrogen supply from a healthy culture growing under optimum conditions. After allowing the fermentation to reach steady state conditions at D = 0.1h^{-1}, T = 30°C, pH = 3, Air = 1 v.v.m., Sm = 5.0 gl^{-1} sucrose and Nm = 0.21 gl^{-1} atomic N for several days, the favourable medium supply was suddenly replaced by one containing only 0.025 gl^{-1} atomic N while maintaining all other parameters constant. Once nutrient limitation was imposed, growth rate values were calculated by the transient state equation, and the change in this and other

parameters was then monitored for up to 10 days (Table 11). Sporulation was induced in this system after 48 hours and Fig 4.14 shows the effect of the reduction of nitrogen availability upon growth rate and the intensity of sporulation. For 48 hours after limitation was imposed, the growth rate was unaffected. A lag time of approximately 12 hours was expected while residual nitrate was washed out of the tower, but in 48 hours there were almost 5 replacements of the fermenter volume. A. niger mycelium would therefore appear to contain nitrogen which is still available to contribute to growth of the organism when conditions are severely limiting. This nitrogen was eventually utilized and could not be replaced and growth rate then began to decrease, at which point intense sporulation was stimulated, reaching a maximum intensity (5×10^6 spores ml^{-1}) within 12 hours. (The spore numbers are values given from one experiment as a typical example of the progress of the fermentation). After the initial acute decrease, growth rate continued to decline gradually while spore production continued at approximately 3.5×10^6 spores ml^{-1} (a production of 8.62×10^8 spores g. $sucrose^{-1}$ utilized) for up to 5 days, at which point sporulation ceased abruptly. When the fermentation was continued, the mould existed at a low growth rate for many days as in section 4.2.1., but eventually died with no further sporulation. Subsequent replicates that achieved transient sporulation were therefore terminated once sporulation ceased.

The effect of shock limitation upon other parameters is shown in Fig. 4.15. Sucrose utilization remains constant, exhibiting normal oscillations about a mean value, supporting the fact that a carbohydrate may still be used by fungi in the absence

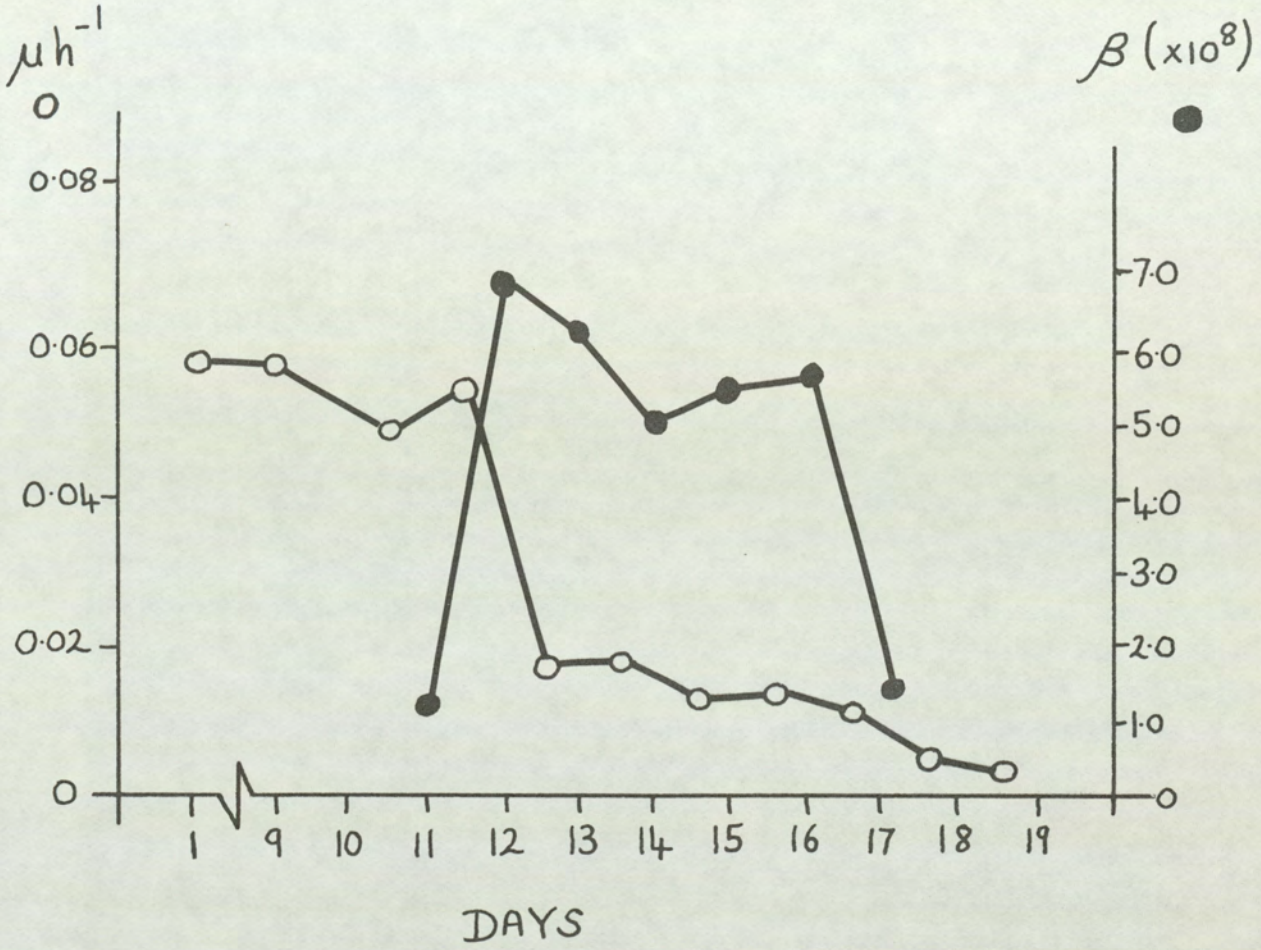


Fig 4.14. The effect of shock nitrate limitation (0.21 - 0.025gl^{-1}) upon the growth rate (μ) and sporulation (β, spores g^{-1} dry weight mycelium) of A. niger.

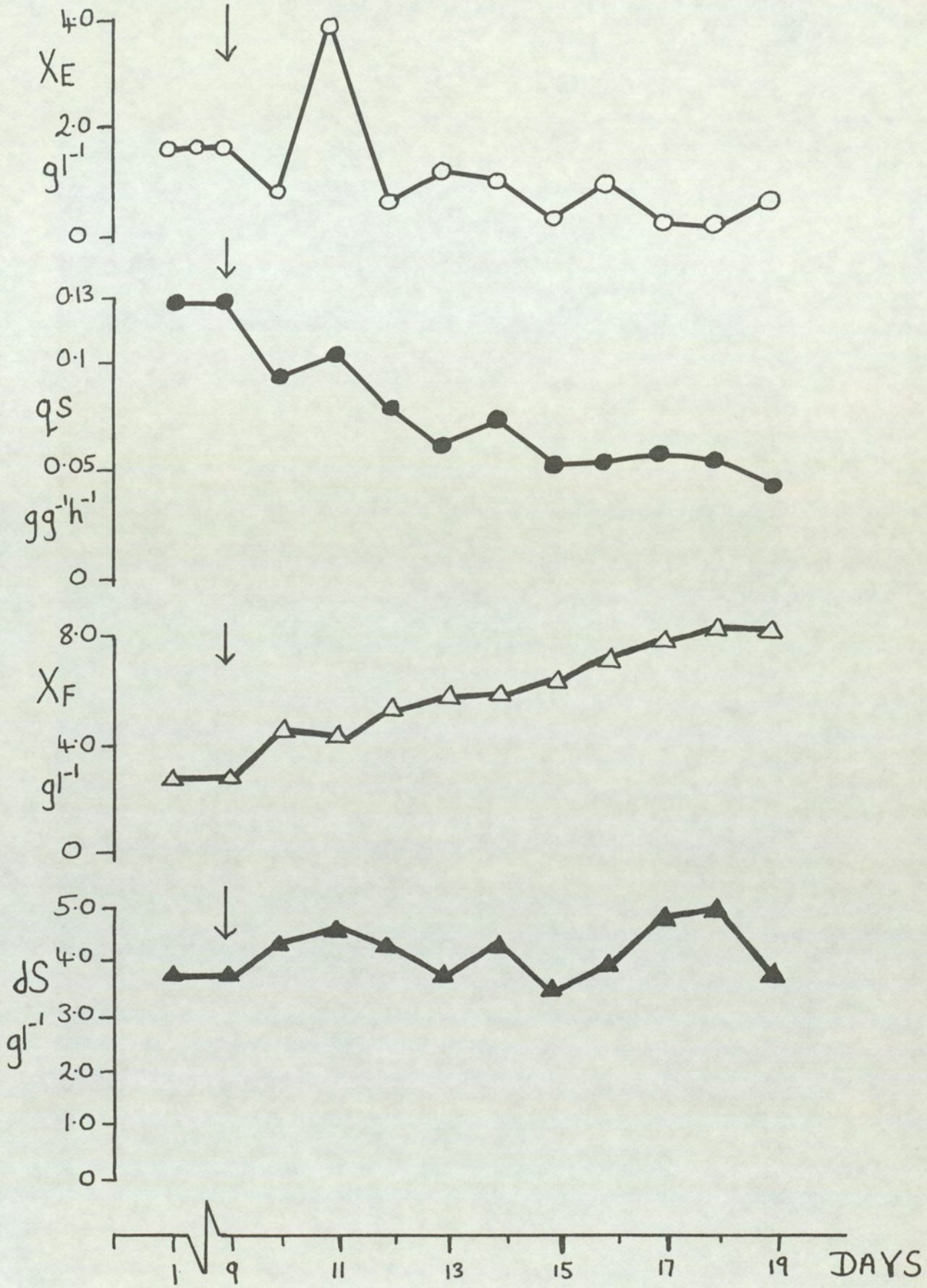


Fig 4.15. Response of fermentation parameters of A. niger to shock nitrate limitation (0.21 - 0.025gl^{-1}). D = 0.1h^{-1}.

of a nitrogen source. The fermenter biomass concentration (X_F) gradually increased throughout the experiment, and with sucrose utilization constant, the specific utilization rate (qs) decreased accordingly. There was no visible change in floc morphology, which remained filamentous (Type 1-2), to account for this increased fermenter biomass concentration, but the colour of the mould changed from cream to dark yellow.

The mould was being retained in the tower, as shown in the decrease in effluent biomass concentration. The increase on day 3 of the limitation was associated with a fall in the natural pH of the culture which then required less acid addition to maintain the fixed value. The acid caused foaming of the culture, which had a mould - retaining effect and the sudden removal of the acid addition caused excess mould to be washed from the tower. All available nitrogen supplied in limiting quantities was utilized, and the specific nitrogen utilization rate consequently dropped by a factor of ten from the steady state value.

This experience was found to be easily repeatable, with sporulation occurring between 12 and 36 hours after imposition of limitation. A variety of sporing structures were produced ranging from complex sub-aerial type spores heads, through the type seen under starch limitation, to single phialides on hyphal tips (Plate 17, Fig. 5.1.). These appeared identical in structure to those of A. niger under glucose limitation, and were the most abundant and rapidly formed structures (12 hours). No spore chains were observed, sporulation was not synchronous, and a range of these structures were frequently produced together. (Plate 18).

One should note that growth rate did not reach zero during

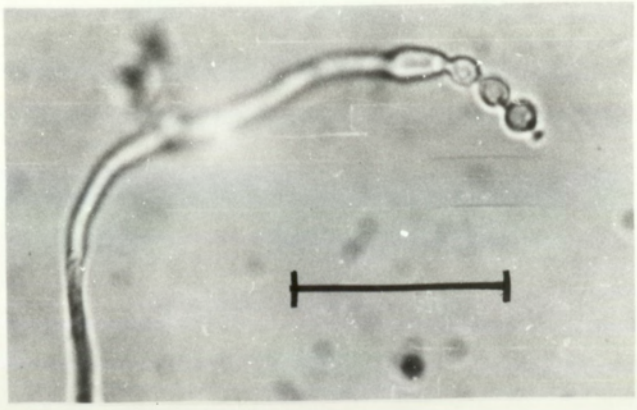


PLATE 17

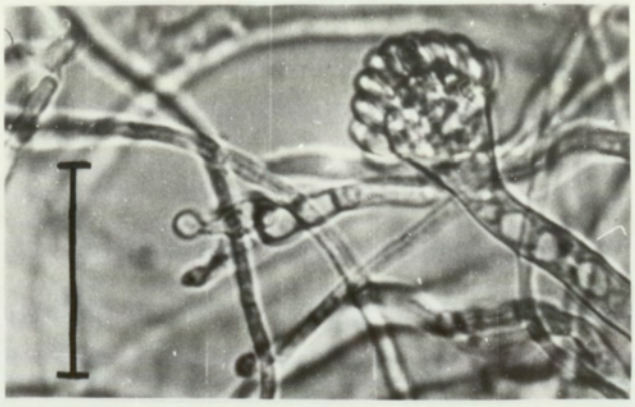


PLATE 18

PLATES 17 & 18. Sporulative structures of A.niger formed in continuous culture.

17 - Reduced complexity of structures after shock nitrate limitation. (4.2.2.)

18 - Simplified and complex structures produced simultaneously after shock nitrate limitation. (4.2.2.)

BAR = 30μm.

sporulation, which again supports the view that these two processes are required simultaneously for this system to operate. Two other shock reductions in the nitrate concentration of the medium were also examined using A. niger cultures, to discover the most effective shock concentration for induction and prolongation of sporulation. Identical experiments to that described above were performed, except that the nitrate concentration was decreased in one instance to 0.08 gl^{-1} and in the other to 0.06 gl^{-1}. In both cases there was no visible effect upon the culture and sample analysis indicated that fermentation parameters remained constant, although after the shock of 0.06 gl^{-1} nitrate the growth rate of the A. niger culture decreased from 0.063h^{-1} to 0.059h^{-1} before adopting new steady - state conditions. A shock decrease in nitrate concentration to approximately 10% of the original (0.21 gl^{-1} to 0.021 gl^{-1}) was therefore concluded to be the most effective, and was used in subsequent experiments.

4.2.3. Shock nitrate limitation to A. ochraceus

A fermentation of A. ochraceus was established over several days in the 4.2 litre fermenter and was allowed to reach a steady state at D = 0.1h^{-1}, T = 30°C, pH = 4, Air = 1 v.v.m., Sm = 5.0gl^{-1} sucrose and Nm = 0.21gl^{-1} nitrate nitrogen. This optimum medium supply was then suddenly replaced by one containing only 0.021 gl^{-1} atomic N while maintaining all other parameters constant. The progressive changes of the transient state after the limitation were then monitored (Table 12). Sporulation was again repeatedly induced in this system, at a greater intensity than that of A.niger. The effect of the shock upon growth rate and sporulation is shown in Fig. 4.16.

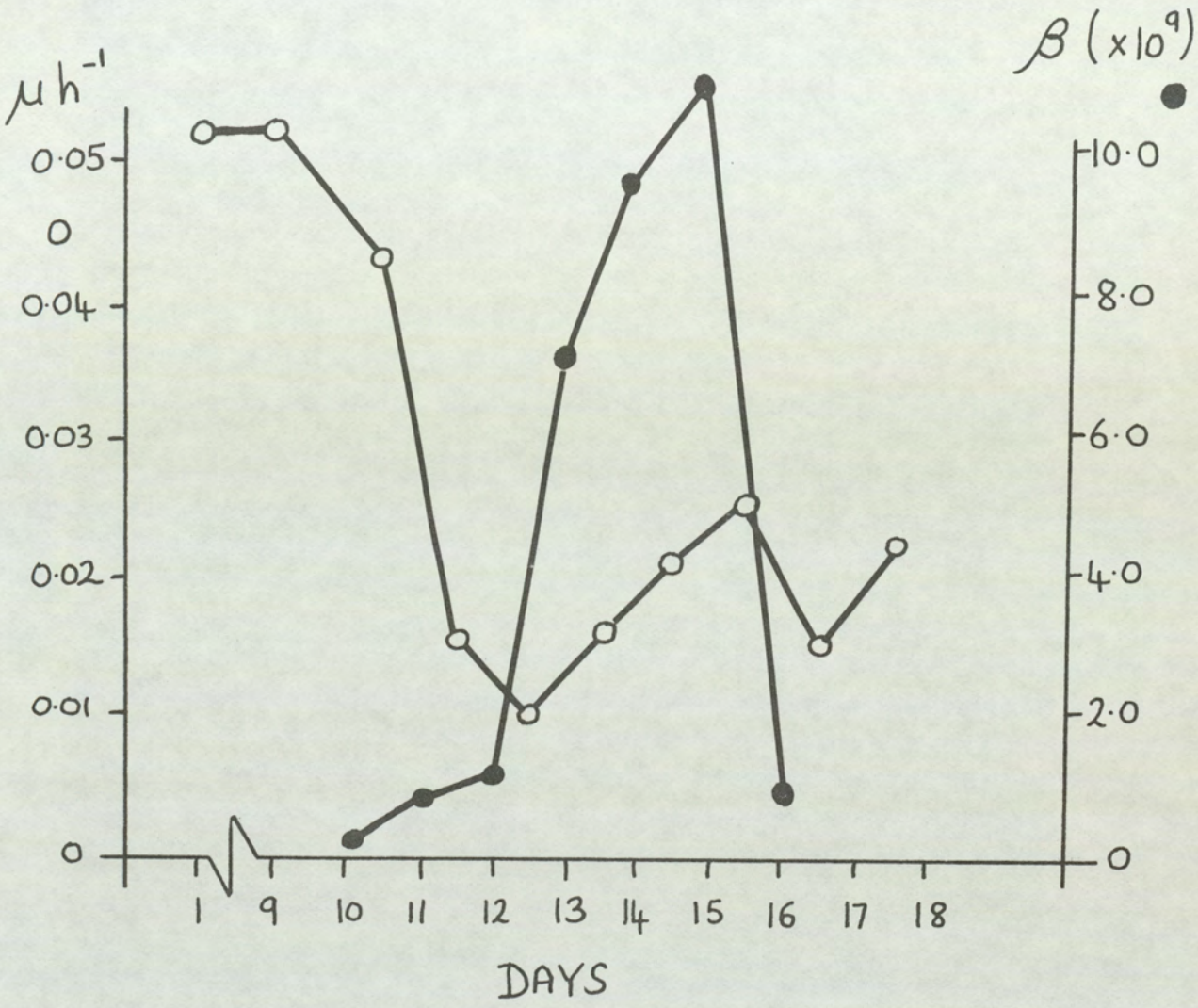


Fig 4.16. The effect of shock nitrate limitation (0.214 - 0.0214gl^{-1}) upon the growth rate (μ) and sporulation (β spores g^{-1} dry weight mycelium) of A. ochraceus. D = 0.1h^{-1}.

A decrease in growth rate was immediately apparent. Conidiophore initials were observed after 10 hours, vesicles after 16 hours and spores approximately 18 hours after onset of limitation. At a flow rate of 0.42 $1h^{-1}$ this time lapse represents only one through - put before intials are formed, and thus A. ochraceus does not appear to have the 'reserve' capacity of nitrogen shown by A. niger.

After the initial rapid decrease, growth rate oscillated around a value of $0.015h^{-1}$, but it was not until it dropped below $0.01h^{-1}$ that intense sporulation occurred. Spore production was not synchronous, reached a peak of 3.6×10^7 spores ml^{-1} (representing 9.7×10^9 spores g. sucrose^{-1} utilized) and continued for 7 - 8 days, although high productivity was limited to 4 or 5 days. At the end of this time, sporulation ceased abruptly, and the organism became vegetative and remained so for long periods without further sporulation. Spore production in one experiment was monitored frequently;

Table 4.1.

hours :	0	6	12	18	24	30	36	42	48
Sporulation coefficient (β) x 10^9: spores g^{-1}	0.49	0.8	1.1	0.3	2.8	3.4	1.1	1.1	1.1

This outlines the cyclic nature of the spore production caused by cyclic nutrient levels, ie sporulation consumes available limiting nutrients, which are then exhausted and sporulation stops. Limiting nutrients then build up until sufficient to permit sporulation, and the cycle is repeated. The same process has been reported in carbon - limited cultures (Grisman 1978). The period of the cycle above is approximately 18 hours, much shorter than that of 30 hours reported by Larmour & Marchant (1977)

for Fusarium culmorum. These cycles of nutrient level will vary with dilution rate : the lower the dilution rate, the longer the cycle.

The effect of shock nitrate limitation on other parameters; (Fig. 4.17) Fermenter biomass concentration and productivity dropped initially and then oscillated around a new value during sporulation. Specific nitrogen utilization rate (qN) again dropped approximately by a factor of 10, and then remained constant.

The floc morphology remained unchanged (filamentous type 1-2) although an olive - green pigment was produced both intra and extracellularly during sporulation, but it did not appear to be localised in spores or spore structures. After several days limitation, a few sub-aerial type reproduction structures were observed (Plate 19) but the vast majority of the structures were unusual, in that they had a variably shortened conidiophore (in some cases not apparent at all) bearing a normal - sized vesicle which produced only approximately 2 - 20 phialides (Fig. 5.1. Plate 20). The spore size was normal, and again no spore chains were seen.

An important observation during these fermentations was that of apparent microcycle conidiation (Plate 21). The incidence of these structures was relatively low, but their presence indicates that the environemnt of the tower was capable of supporting the germination of spores produced in the system and also such that immediate resporulation of the germ tube was induced. The nutrient interactions here are complex and an immediate explanation was not apparent.

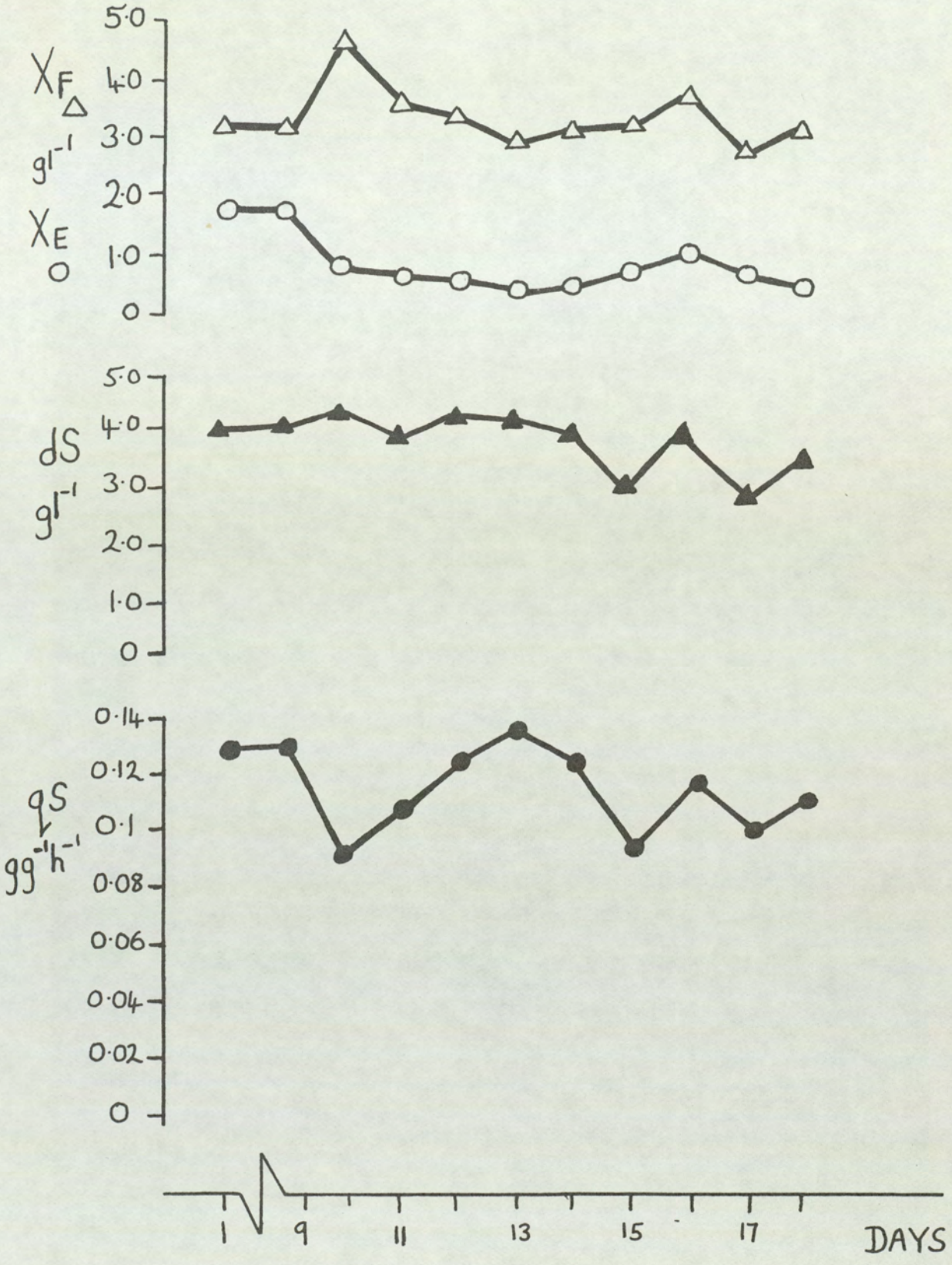


Fig 4.17. Response of fermentation parameters of A. ochraceus to shock nitrate limitation (Nm 0.21 - 0.021gl^{-1}). D = 0.1h^{-1}.

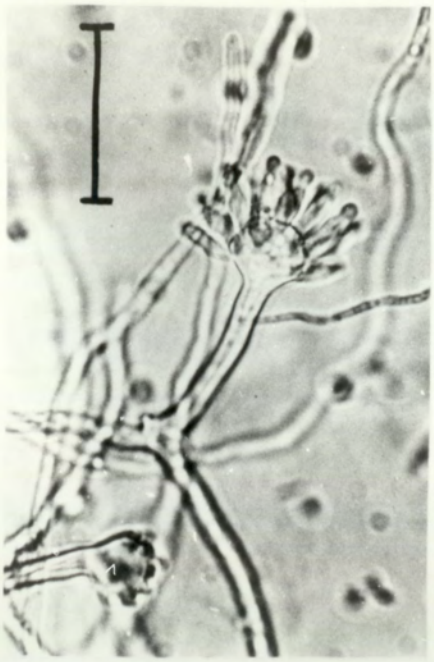


PLATE 19

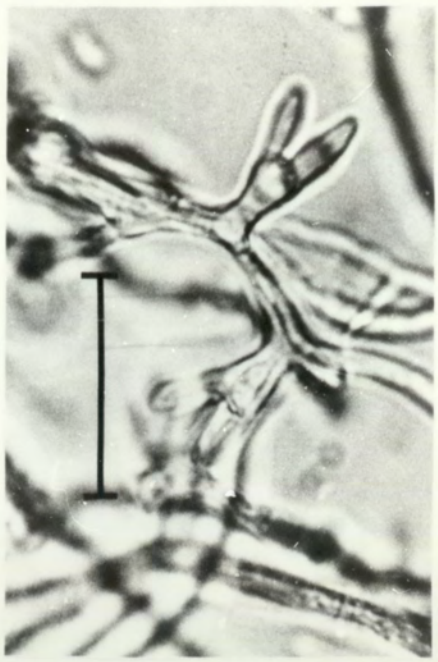


PLATE 20

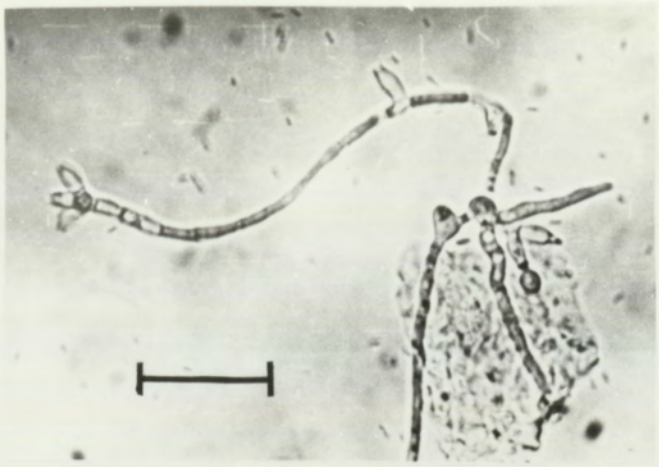


PLATE 21

PLATES 19, 20 & 21. Sporulative structures of A.ochraceus formed in continuous culture.

19 - Sub - aerial type with decreased phialide number formed after shock nitrate limitation. (4.2.3.)

20 - Reduced complexity also formed after shock nitrate limitation. (4.2.3.)

21 - Microcycle conidiation after shock nitrate limitation (4.2.3.) Group of spores germinate ; hypha / conidiophore produced directly, which in turn produces phialides and spores in simplified manner.

BAR = 30 µm.

4.2.4. Shock nitrate limitation of A. ochraceus with glucose.

Identical shock nitrate experiments to those in section 4.2.3. were conducted on A. ochraceus (Table 14) but using glucose as the carbon source ($5.0 g l^{-1}$). The effect of the shock upon growth and sporulation is shown in Fig. 4.18. The decrease in growth rate was less pronounced than with sucrose, but again occurred immediately after the limitation was imposed.

The initiation of intense sporulation occurred approximately 36 hours after the limitation and reached a peak of 4×10^6 spores ml^{-1} (representing 8.9×10^8 spores g glucose^{-1} utilized) over 3 - 4 days, after which there was an abrupt end and no further sporulation, as the culture reached a new steady state. The culture turned dark brown during sporulation with the pigment in both mould and broth. All sporulation structures were sub - aerial forms (Plate 22) but there were very few free spores and spore counts were obtained from vigorously agitated samples. The floc morphology changed from loose filamentous to type 4 flocs, which were denser, tighter pellet - like flocs.

Effect of shock on other paramenters :

Fermentation biomass concentration, glucose utilization and specific glucose utilization rate remained constant as in previous experiments. Effluent biomass concentration and productivity followed the declining trend of growth rate, in that their decline was gradual, which suggests that the nitrate limitation had less effect with glucose as the carbon source. Nitrogen utilization decreased dramatically immediately after limitation but then recovered to utilize most of the available nitrogen during sporulation. During very low nitrogen utilization, the nitrogen for conidiation probably comes from

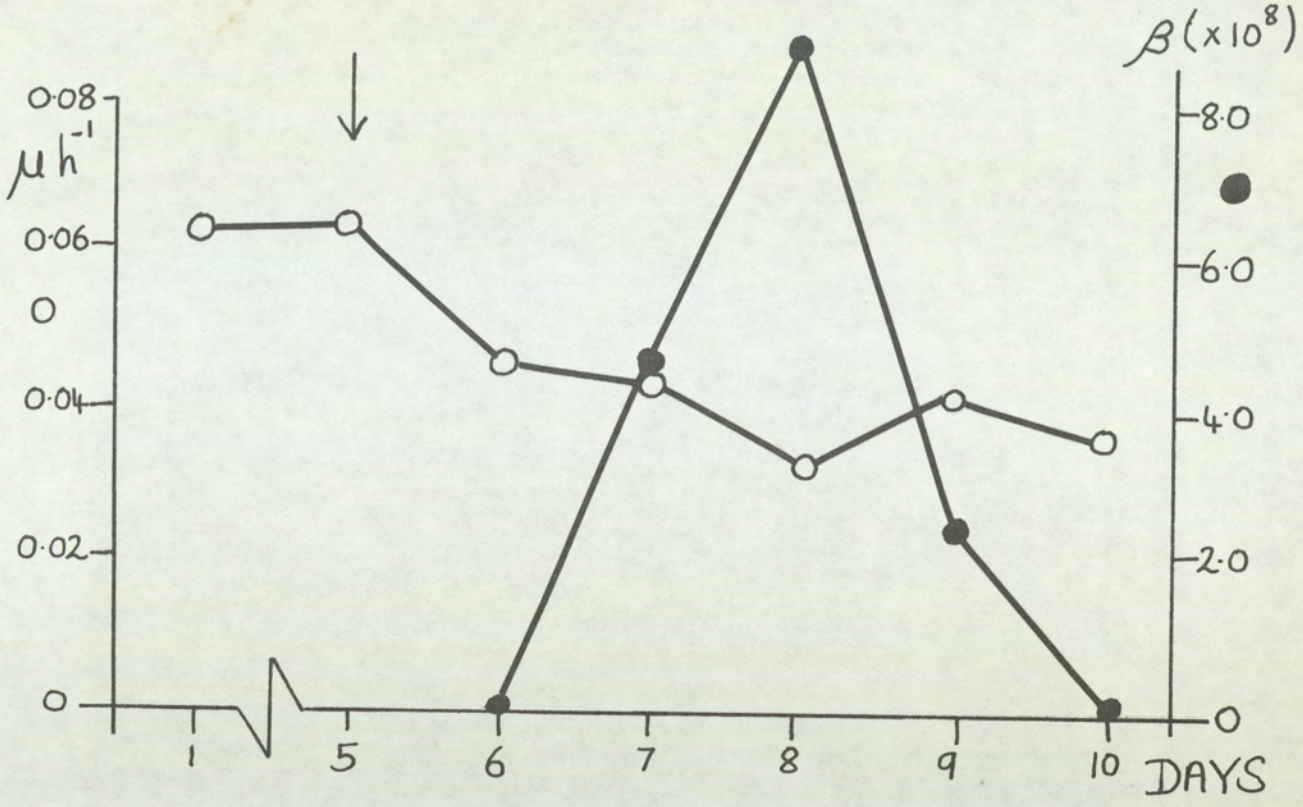


Fig 4.18. The effect of shock nitrate limitation (0.21 - 0.021gl^{-1}) upon growth rate (μ) and sporulation (β) of A. ochraceus. D = 0.1h^{-1}. Sm = 5.0gl^{-1} glucose.

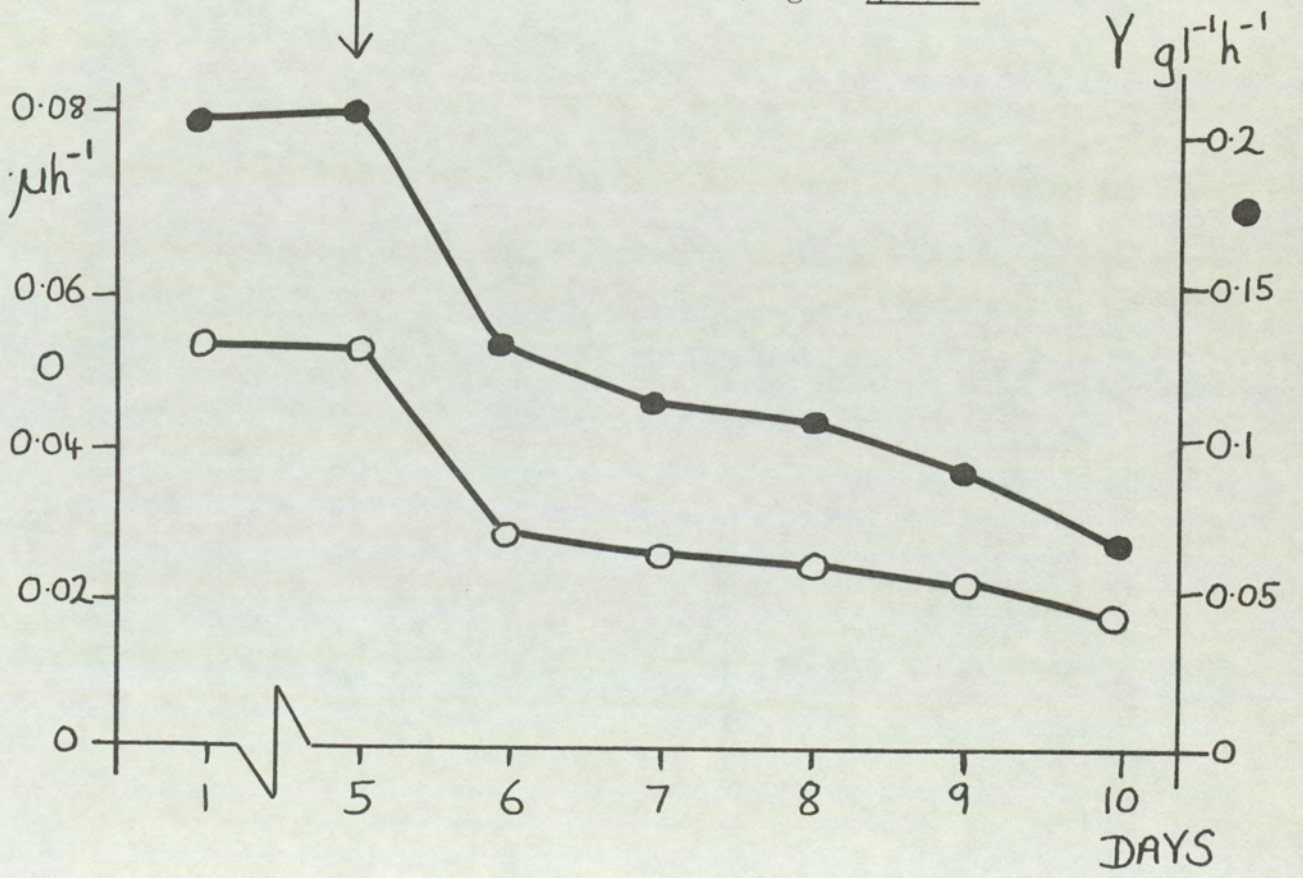


Fig 4.19. Effect of shock ammonium limitation (0.213 - 0.021gl^{-1}) upon growth rate (μ) and productivity (Y) of A. ochraceus. D = 0.1h^{-1}.

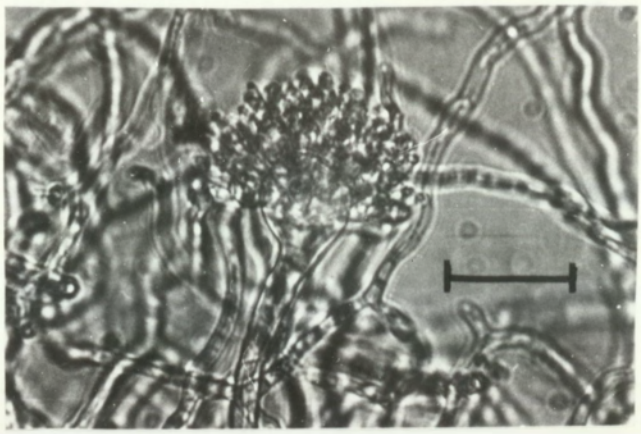


PLATE 22

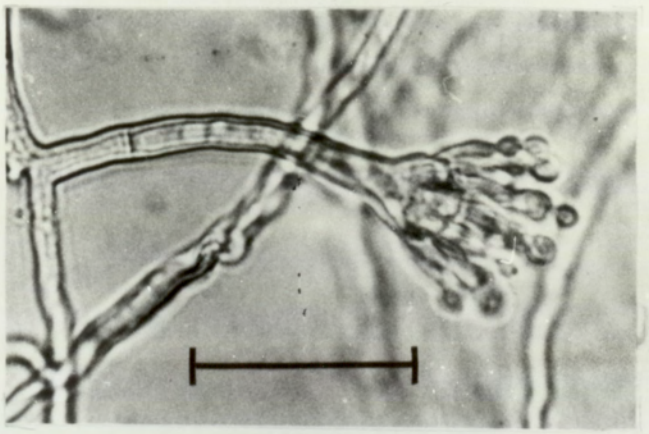


PLATE 23

PLATES 22 & 23. Sporulative structures of A.ochraceus formed in continuous submerged culture.

22 - Sub - aerial type formed after shock nitrate limitation with glucose as the carbon source. (4.2.4.)

23 - Sub - aerial type with decreased phialide number after shock nitrate limitation in final combination limitation experiment. (4.7.2.)

BAR = 30 μm.

intracellular proteins, as proteinases are stimulated during conidiation (Morton et al. 1960).

4.2.5. Shock ammonium limitation to A. ochraceus

This was to compare ammonium and nitrate limitation to confirm or dispute the reported ammonium ion inhibition of submerged sporulation. An identical experiment to the shock nitrate limitation to A. ochraceus was performed (Table 13) except that the steady state on $0.213 g l^{-1}$ ammonium nitrogen was disturbed by introduction of an otherwise optimum medium containing $0.021\ g l^{-1} N$. The effect upon growth rate and productivity is shown in Fig 4.19. There was an immediate drop in both values, which continued for several days until establishment of a new steady state on day 10.

No sporulation resulted, although conidiophore initials were once observed immediately after the shock, but on two occasions the culture remained entirely vegetative. This confirms the ammonium inhibition of sporulation.

Again, X_F, dS and qS were almost constant, while the yield coefficeint (Y_{sub_s}) showed an initial decrease before levelling out at the new steady state value. Nitrogen utilization followed an identical pattern to that of nitrate limitation with glucose, i.e. an initial dramatic drop followed by a recovery to utilize what nitrogen there was available. Mycelial flocs remain loosely filamentous at all times.

4.2.6. Nitrate limitation to A. niger at D = $0.4h^{-1}$

These are triplicate results of one fermentation, which was conducted as a possible improvement on the system developed at D = $0.1h^{-1}$. The A. niger culture, growing under optimum conditions (Sm = $5.0gl^{-1}$ sucrose, T = $30^{o}C$, pH = 3.0 Air = 1 v.v.m. Nm = $0.21gl^{-1}$ atomic N.) at D = $0.4h^{-1}$, was suddenly supplied with a medium containing only 0.021 gl^{-1}N. The results (Table 15) show that growth rate was virtually unaffected and no sporulation was observed. The steady state was interrupted because X_F, X_E(hence Y), dS and Y_{sub_s} all decreased and assumed new levels. The lack of effect at this dilution rate was not surprising, as nitrogen utilization was already low (a shorter residence time and lower fermenter biomass concentration) and so any reduction will be less severe. It was surprising, however, to find such a high growth rate ($0.13h^{-1}$) with such low nitrogen utilization after the shock. Autolysis would not provide many nutrients at this dilution rate, but the state appeared to be steady when the fermentation was terminated when no sporulation had been induced.

4.3. The effect of temperature upon A. niger and A. ochraceus

There are strict temperature limits within which the metabolic processes of any fungal organism can take place. (Hawker 1950) Metabolism has a basic dependence upon temperature, which is an important inducer of specific developmental change in filamentious fungi, and Giona et al. (1976) point out that as

maintenance energy requirements depend upon cellular processes and osmotic regulation, then this requirement is really termperature dependent. Each micro organism activity has cardinal temperature points; optimum, maximum and minimum. The minimum level is determined by inactivation of solute transport systems, while the maximum level depends upon the sensitivity of the protoplasmic membrane, and particularly upon enzyme denaturation. The optimum level depends upon substrate supply and environmental conditions. Hawker (1950) states that the cardinal points of the Aspergilli are higher than those of Penicillia, and that the range of temperatures permitting sporulation is smaller than that permitting vegetative growth (Hawker 1966). The effects of elevated temperatures on spore germination have been reported by workers examining microcycle conidiation, and a review of temperature effects on filamentous fungi is given by Anderson & Smith (1976). The effect of temperature on fungal growth is complex, because other factors, particularly oxygen solubility, are affected by a variation in temperature. Chemostat culture studies will enable temperature effects to be studied in isolation, although in this work, no attempt was made to maintain dissolved oxygen levels constant, as oxygen was always supplied in excess. Hunter & Rose (1972) report that when temperature decreases, RNA production increases to produce the same amount of protein at a lower growth rate, and polysaccharide and unsaturated fatty acid levels also increase. Rowley & Pirt (1972) increased the temperature to A. nidulans at steady state in continuous culture from 23°C to 37°C, and found the oxygen uptake

rate of the organism to increase, due to an increased maintenance requirement due to higher protein and nucleic acid turnover at an increased temperature. Trinci (1969) found the growth rate of the organism to increase from $0.09h^{-1}$ to $0.37h^{-1}$ over the same temperature range in submerged culture.

To examine the effect of temperature upon A. niger and A. ochraceus each organism was allowed to establish steady - state growth at a range of temperatures which were altered in sequence from low to high levels. The values in Table 16 are means of readings taken over a period of 3 days at steady state, except at 40°C and 42.5°C where no steady state was established before wash - out.

4.3.1. Effect of temperature on A. ochraceus

Fermentations were run at a dilution rate of $0.083h^{-1}$ in the 10 litre fermenter with a nutrient supply of $5.0gl^{-1}$ sucrose and $0.21gl^{-1}$ nitrate atomic nitrogen. Growth rate was maximum at 32.5°C and productivity at 30°C. (Fig. 4.20). Growth increases as temperature rises, as enzyme catalysed reactions are directly proportional to temperature, but as temperature rises beyond the optimum cardinal point, thermal denaturation of cellular proteins restricts growth. The object of the experiment was to select a temperature unfavourable for growth, while maintaining good fermentation conditions. For example, growth rate decreased as temperature rose above 32.5°C, but yeast infection was high at 35°C and 37.5°C, while at 22.5°C and 25°C the culture was 'clean' while still reducing growth rate.

The culture also appeared 'clean' at 40°C but this temperature was considered too high to permit the enzymatic reactions

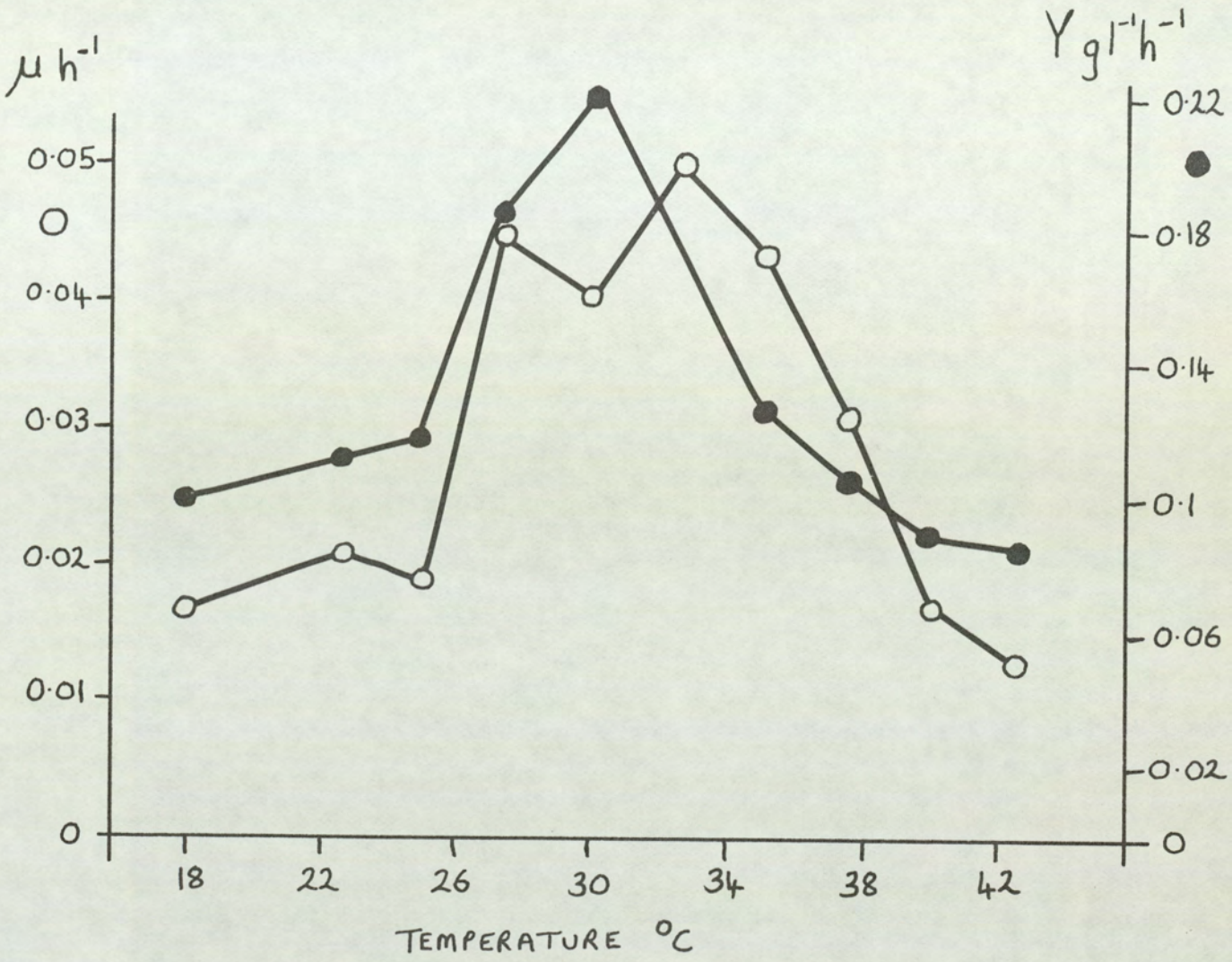


Fig 4.20. Effect of temperature on growth rate (o) (μ) and productivity (●) (Y) of A. ochraceus. D = 0.083 (Sm = 5.0gl^{-1} Nm = 0.21gl^{-1}).

required for sporulation. The temperatures selected for use in the final combination limitation section were therefore 22.5°C and 39°C. The lower temperature is preferable from the aspect of system efficiency (low heat requirement) but this may be unfavourable for sporulative metabolism. Fig. 4.21 shows that carbon utilization is largely unaffected by temperature, but nitrogen utilization is maximum in the range 25 - 35°C, and thus limitation imposed at the temperatures selected may have less effect that at 30°C, although growth rate will be lower. (It was shown in experiment 4.2.6. that nitrogen limitation has less effect at high growth rates). The floc morphology changed from loose filaments (Type 1 - 2) to a pellety restricted growth form (Type 4) either side of the optimum temperature. This accounts for the increase in fermenter biomass concentration (X_F - Fig 4.21) either side of the optimum temperature.

4.3.2. Effect of temperature on A. niger

The effect of temperature upon growth rate and productivity is shown in Fig. 4.22., and this experiment was similar to that conducted by Stockbridge (1979) although he used a dilution rate of $0.4h^{-1}$. In this case, a dilution rate of $0.1h^{-1}$ was used, with a nutrient supply of $5.0gl^{-1}$ sucrose and $0.21gl^{-1}$ nitrate nitrogen (Table 16). Growth rate was maximum at 30°C and productivity at 35°C, and the shape of the curves agrees closely with those of Stockbridge. Two temperatures were selected as unfavourable for growth ; 18-20°C and 38°C.

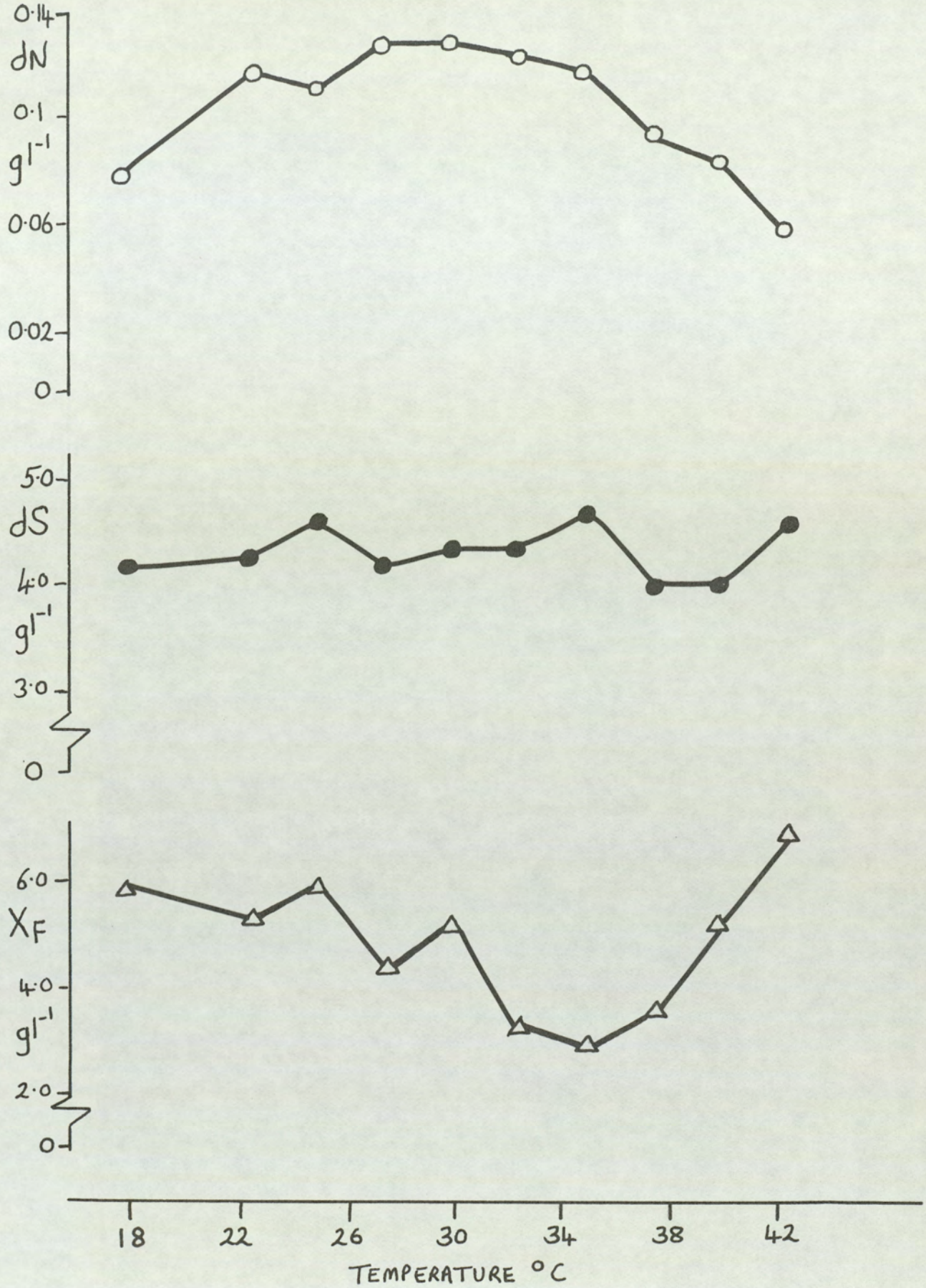


Fig 4.21. The effect of temperature upon the sucrose (dS) and nitrate (dN) utilization and fermenter biomass concentration (X_F) of A. ochraceus. $D = 0.083h^{-1}$

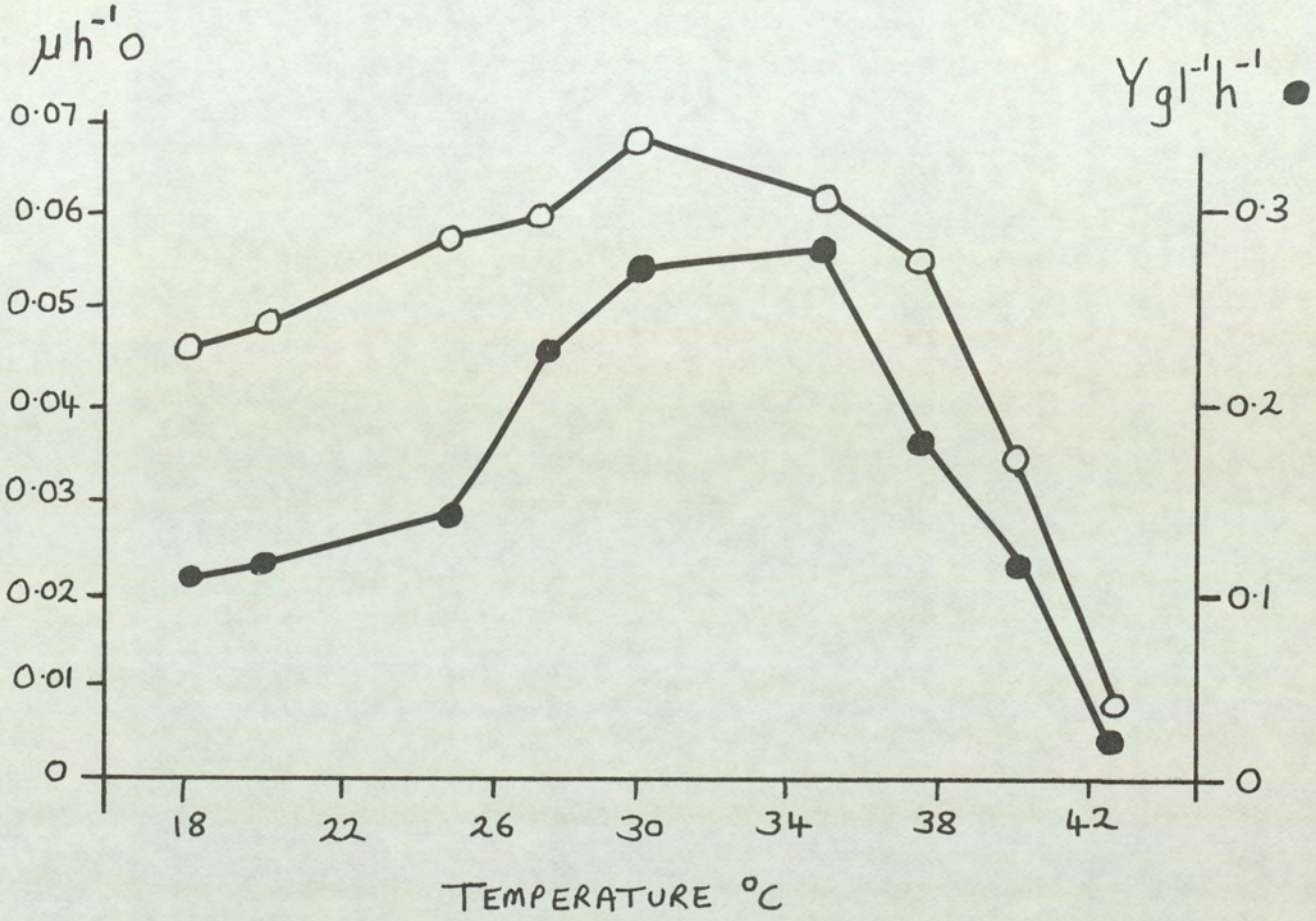
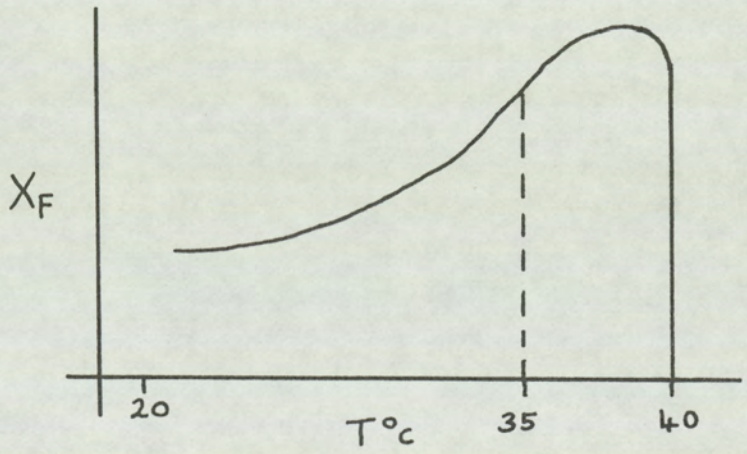


Fig 4.22. The effect of temperature upon growth rate (μ) (o) and productivity (Y) (●) of A. niger at $D = 0.1h^{-1}$.
($Sm = 5.0gl^{-1}$ sucrose, $Nm = 0.21gl^{-1}$).

At these points, enzymes are able to function, growth rate is low and the culture remained clean. Again, as Fig. 4.23 shows, nitrogen utilization was low at these two temperatures, the maximum of $0.12gl^{-1}$ being at $27.5^{o}C$, and carbon utilization gradually increased as temperature increased to $37.5^{o}C$, beyond which there was a rapid decline. Both these trends are similar to those observed by Stockbridge (1979) although the values differ due to the different dilution rate. The floc morphology did not alter throughout the experiment and remained loosely filamentous, which accounts for the decrease in fermenter biomass concentration each side of the optimum value ($4.5gl^{-1}$) at $35^{o}C$. Stockbridge observed the following effect of temperature upon fermenter biomass concentration of A. niger :



The graph in Fig 4.23 follows this trend up to $35^{o}C$ (shown by the dotted line) but at $D = 0.1h^{-1}$, myceliumis washed from the fermenter beyond $35^{o}C$, but retained at $D = 0.4h^{-1}$ causing increased X_F. This is due to the increased sedimentary nature of floc pellets at $D = 0.4h^{-1}$.

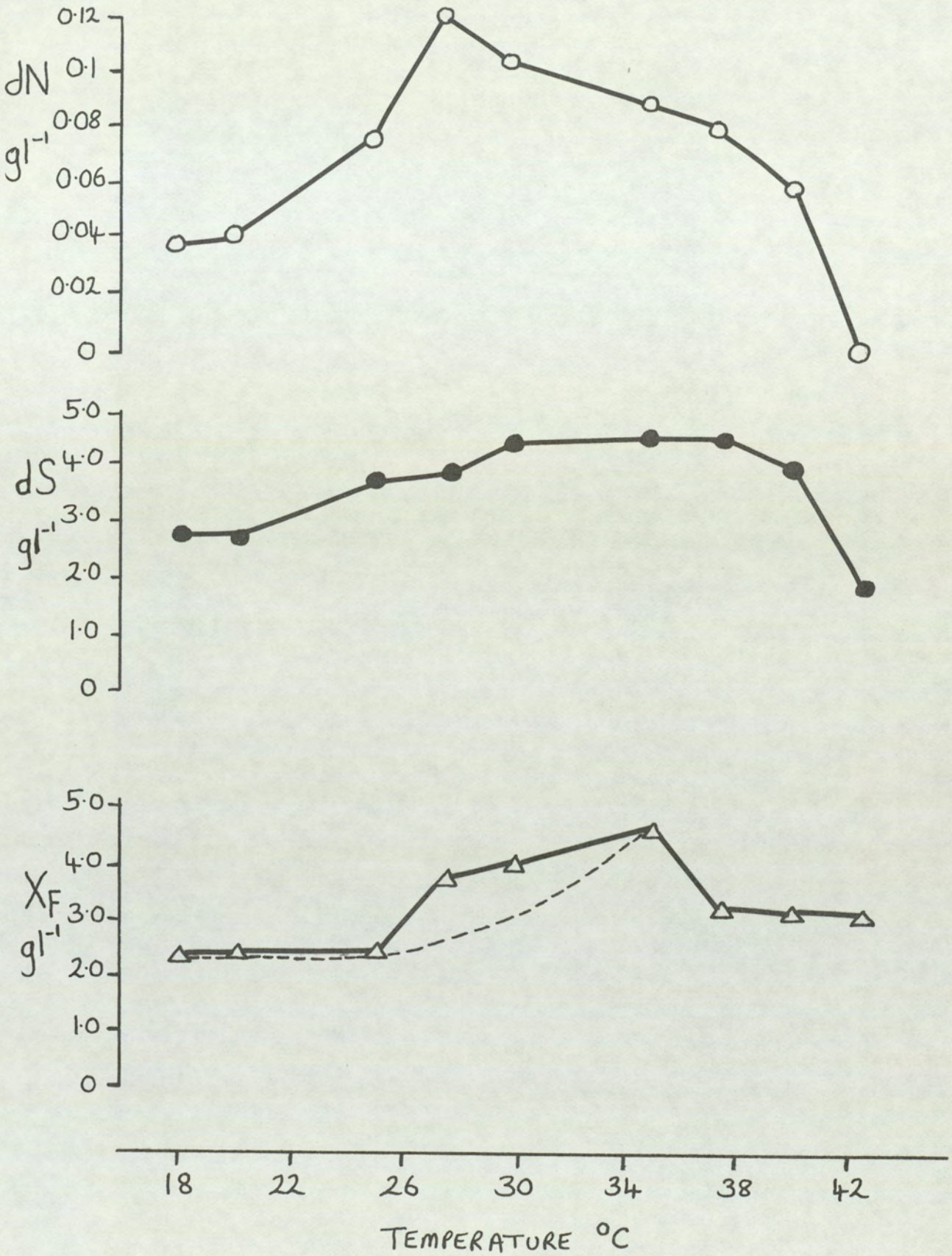


Fig 4.23. Effect of temperature on sucrose (dS) and nitrate (dN) utilization and fermenter biomass concentration (X_F) of A. niger. $D = 0.1h^{-1}$.

No sporulation of either organism occurred at any temperature, but a bright yellow pigment was produced by A. ochraceus at low temperatures and by A. niger below and above the optimum temperature. (This was also observed by Stockbridge, 1979). A yeast infection invariably developed at temperatures of 38°C and above, despite sterile precautions.

4.3.3. Temperature shock to P. javanicum

This organism was used after sporulation was observed during an investigation of the effect of temperature upon its' growth by Ewen (1980) when under all experimental conditions the mould grew in spherical flocs (pelleted form) of approximately 0.1cm. diameter. As temperature decreased, pellet size increased, and as temperature was increased to 37°C the pellets became smooth - surfaced and intitiation of phialides occurred for several days along with the production of an olive green/grey pigment. The mould was killed at 38°C. As gradual temperature reduction had failed to induce sporulation, shock increases from 30°C to 37°C and from 30°C to 17°C were attempted in these experiments.
A culture was allowed to reach a steady state at a dilution rate of $0.2h^{-1}$ with a nutrient feed of $5.0gl^{-1}$ sucrose and $0.21gl^{-1}$ nitrate atomic nitrogen, and the temprature was then suddenly altered. The effects of both up and down shocks at time = 0 on growth rate and sugar and nitrogen utilization are shown in Fig. 4.24 (Table 18). After the temperature increase, growth rate decreased rapidly and exhibited a typical oscillatory response to a change in steady state, which continued for less than 24 hours in every case, before the mould washed out. After only $2\frac{1}{2}$ hours,

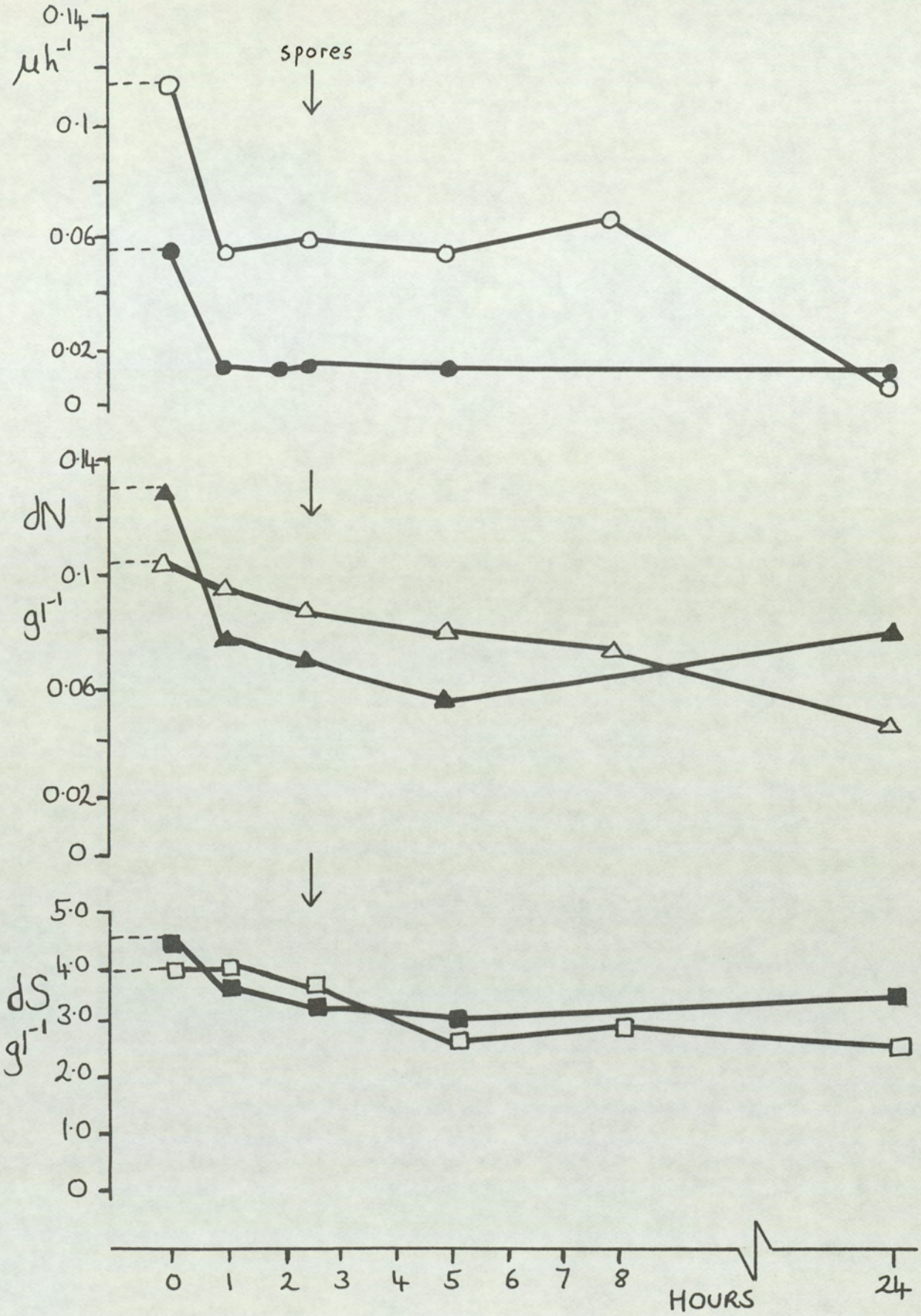


Fig 4.24. Effect of temperature shock at time 0 on P. javanicum.

○ △ □ 37° shock

● ▲ ■ 17° shock

single or paired phialides were visible on hyphal tips and after 5 hours spores were formed from these phialides, although few were seen free in the medium. 1.25×10^6 spores ml^{-1} ($\beta = 3.8 \times 10^8$ spores g^{-1} dry wt. of mycelium) were recorded after vigorous agitation of the samples after 6 hours, but thereafter spore number decreased, the hyphae became shortened and thickened and flocs formed pellets (Type 4-5) and heavy yeast infection developed.

When the low temperature shock was applied to a steady - state optimum P. javanicum culture, no sporulation was induced and there was no change in hyphal or floc morphology. Growth rate and sugar and nitrogen utilization decreased after the shock, but quickly assumed new steady - state values. (Fig. 4.24). The mould was capable of continued growth at 17°C without sporulation, albeit at a low growth rate, while the 37°C shock caused the mould to be washed from the tower, although Ewen (1980) states that P. javanicum can survive at this temperature if it is introduced gradually. A disadvantage of the low - temperature system was that the sudden temperature drop caused shrinkage of the glass fermenter body so that leaks developed at the joins around the air distributor plate, which is obviously undesirable in as aseptic fermentation.

4.4. The effect of pH upon A. niger and A. ochraceus

Galbraith and Smith (1969c) discussed the critical influence of pH upon filamentous growth of A. niger in submerged culture. Growth was filamentous at low pH (less than 2.3) and became increasingly pelleted as pH increased, with maximum growth rate in the pH range 2.1 to 4.5.

Filamentous fungi have a wide pH tolerance range with sharply defined limits, but the range permitting sporulation is usually narrower than that for vegetative growth (Hawker 1950). The effect of pH may be critical for sporulation in some strains, yet other strains can support sporulation throughout the range that will support growth. Many workers have reported that pH exerts an effect upon mould morphology, and that organism growth rate is a function of pH (Brown et al. 1975) and it was therefore anticipated that pH would have a controlling influence over sporulation in this system. The pH of the growth medium affects solubilities of nutrients (Steinberg, 1937 states that increased acidity increases trace element availability which increases growth rate) and certain extracellular enzymes. However, the internal pH of the fungal cell is maintained at around 5 - 6 independant of the medium pH, and thus effects of changes in external pH are probably restricted to membrane permeability and other surface phenomena rather than on intracellular enzymatic systems. pH influences the dissolved bicarbonate ion level, which has an influence upon fungal growth (Maccauley and Griffin, 1969) and high pH values tend to induce pellet formation in submerged culture, which is to be avoided if possible in this system. A low pH value is a key factor in enabling the tower fermenter to be run septically without infection at high dilution rates, and Stockbridge (1979) concluded that the best fermentations for biomass production were those with no pH control. Maximum spore production by Giberella zeae was reported at pH 8.0 by Capellini and Peterson (1969) within the range 5 - 9; few spores were produced at pH9

and none at pH4. Carels and Shepherd (1978) found a low pH inhibited conidiation of Monascus spp. and concluded that the nitrogen source dictates which pH has the greatest effect upon conidiation. pH exerts an external effect upon organic phosphate and potassium uptake which are both optimal at high pH. Potassium and phosphate activate the EMP enzyme phosphofructokinase while phosphate inhibits the HMP enzyme glucose-6-phosphate-dehydrogenase. Thus at a high pH, the EMP pathway is stimulated and the HMP inhibited, which results in less production of NADPH, required for nitrate reduction. Nitrogen limitation is therefore more severe at high pH when nitrate is the source, and nitrate uptake has a pH optimum of 6.0.

As with temperature, there is a need to study the isolated effects of pH upon fungal growth at steady state in continuous culture, as opposed to the ill-defined unsteady state conditions of batch culture. Larmour & Marchant (1977) studied the induction of conidiation of Fusarium culmorum in continuous culture, and found that in a glucose - limited culture with no pH control, low pH existed and the organism remained vegetative. When pH was increased to above 5.0 for 6 hours or more, maximum conidia production was induced. This was either a transient response to a short pH increase (6 hours) or a continuous response if the pH was maintained at 6.5.

4.4.1. pH effects upon A. niger.

These experiments were similar to those conducted by Stockbridge (1979), except that in this case, nitrate was the nitrogen source

and dilution rate was $0.1h^{-1}$ (compared with ammonium at $D=0.4h^{-1}$) which gave an entirely different original organism growth rate. Using the 4.2 litre fermenter, a culture was allowed to reach a steady state under optimum conditions ($T = 30^{o}C$ pH = 3.5, $D = 0.1h^{-1}$, Air = 1 v.v.m. Sn = $5.0gl^{-1}$ sucrose and Nm = $0.21gl^{-1}$ nitrate N.) and pH was then altered step - wise throughout the range 2 - 9, allowing the culture to reach steady state at each pH value, and parameters were recorded over three days (Table 17). Fig 4.25. shows the response of growth rate and productivity to pH, and the trends agree closely with those obtained by Stockbridge, with growth rate maximum at pH = 3 and productivity maximum at pH = 2.0. The object of the experiment was to select a pH unfavourable for growth while maintaining good fermentation conditions. Fig 4.26 shows that nitrate utilization was optimum in the pH range 5 - 8 (which agrees with the theory) and thus nitrogen limitation was expected to have maximum effect in this range, where utilization is high but growth rate low. At this pH, however, the culture invariably suffered bacterial infection, and so pH 4.0 was selected for use in the final limit combination experiment, which prevented infection at sub-optimal growth rates. At pH 4.0, filamentous growth occurred which was preferable for a sporing system, while as pH increased, the floc morphology showed a gradual transition to pellets (Type 5-6 at pH9) which were undesirable due to their primarily vegetative nature. Effluent biomass concentration showed the same trend as productivity (Fig. 4.25) but fermenter biomass concentration progressively decreased from $5.3gl^{-1}$ to $1.2gl^{-1}$ as pH increased from 2 to 9. Carbon utilization was unaltered across the whole

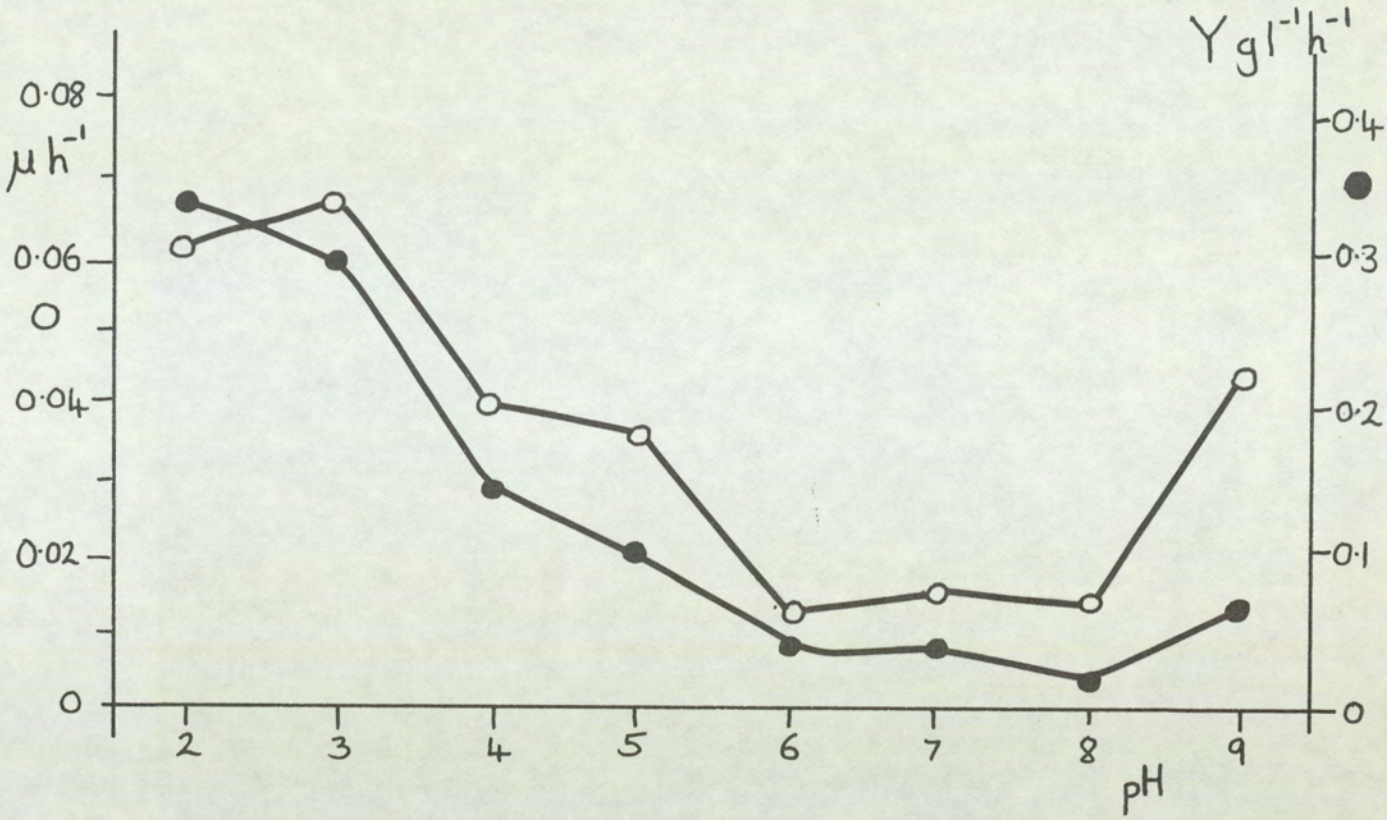


Fig 4.25. The effect of pH upon the growth rate (μ) and productivity (Y) of A. niger. D = 0.1h^{-1}

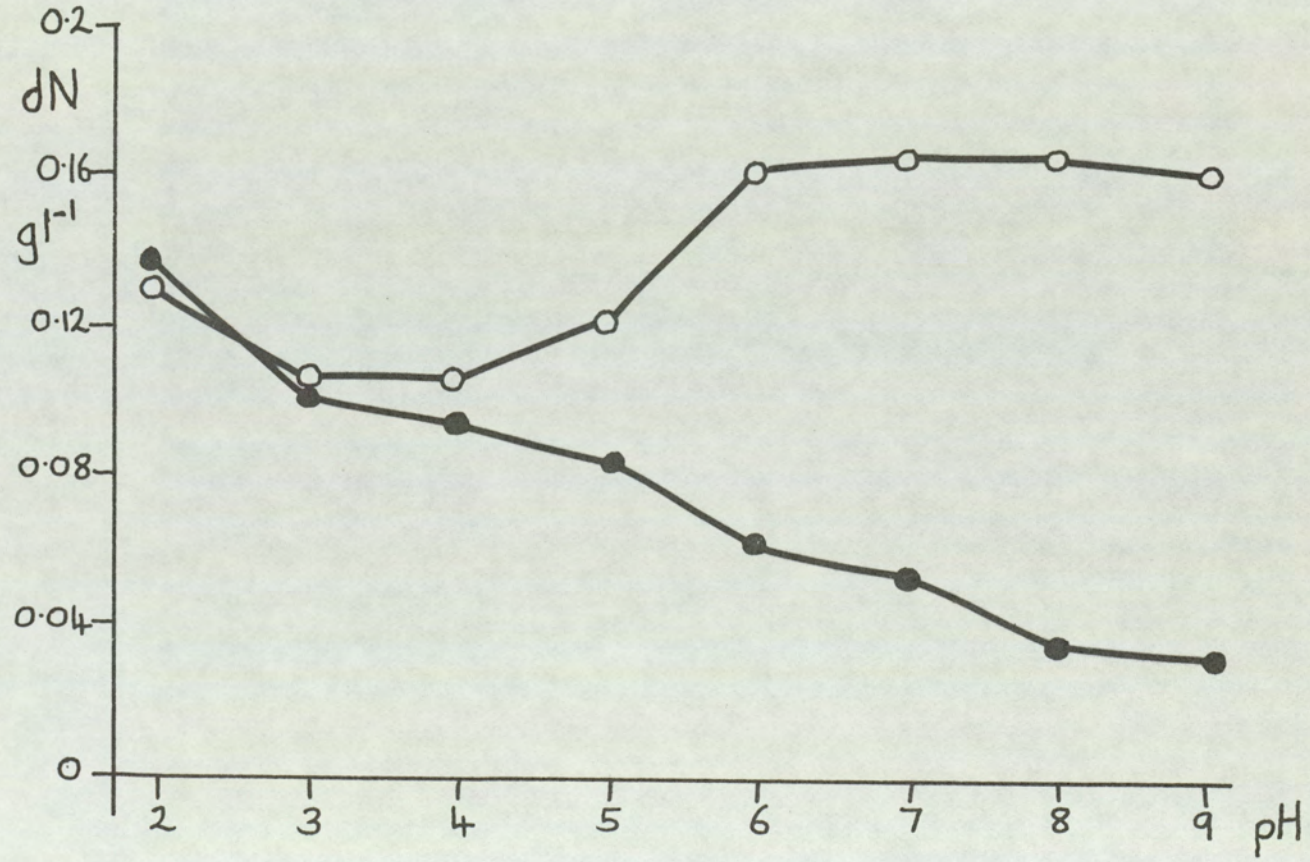


Fig 4.26. The effect of pH upon nitrogen utilization (dN) by A. niger (o) and A. ochraceus (•). D = 0.1h^{-1}.

pH range (mean = $4.12 g l^{-1}$) and consequently specific carbon utilization rate (qs) gradually increased with pH (Fig. 4.27). No sporulation was induced by any pH, but a deep yellow pigment was produced at all sub-optimal pH levels. The growth of A. niger in continuous culture versus pH was compared with growth on solid culture. Malt extract agar plates of various pH were inoculated with mycelial plugs taken from a healthy colony, and the radial spread of the colony across the plate was then monitored for 6 days. The results of 5 replicates were plotted in Fig. 4.28, which shows that the results correspond closely with those from continuous submerged culture in that growth rate is maximum at pH 3 and then progressively decreases as pH increases. The growth rate of A. niger on agar plates would have been expected to be maximum at around pH 5, but the culture grew well on all low - pH plates (below 7) and retained its ability to sporulate on all plates although at pH 9 sporulation was poor.

4.4.2. pH effects upon A. ochraceus

An identical experiment was performed using A. ochraceus (Table 17) and the responses of growth rate and productivity to pH are shown in Fig. 4.29. These are similar to those of A. niger with growth and productivity maximum at pH 2.0 and gradually decreasing as pH increased. Fig. 4.26. shows that nitrate utilization by A. ochraceus also decreased as pH increased which suggests that the effect of pH upon potassium and phosphate uptake described by Carels and Shepherd (1978) was not the limiting effect upon nitrate uptake by this organism in this system. Nitrate

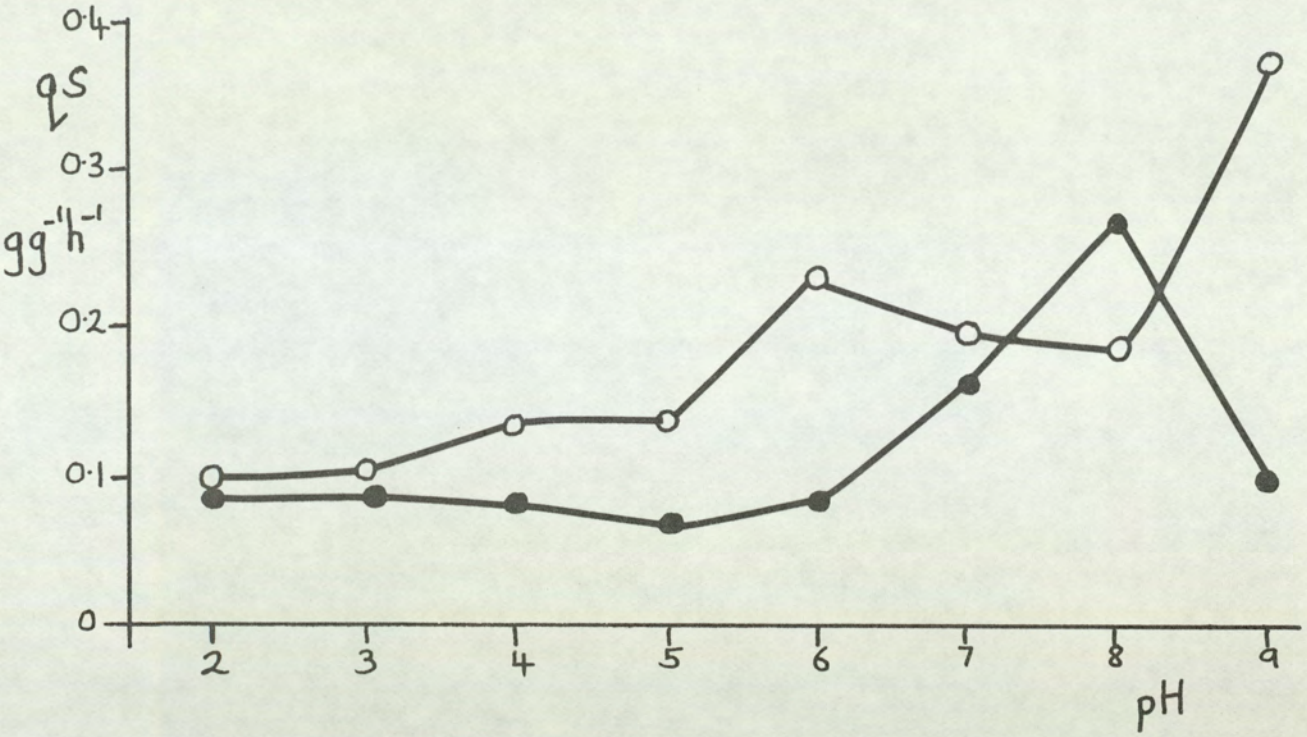


Fig 4.27. The effect of pH upon specific sucrose utilization rate (q_s) of A. niger (o) and A. ochraceus (•). $D = 0.1h^{-1}$.

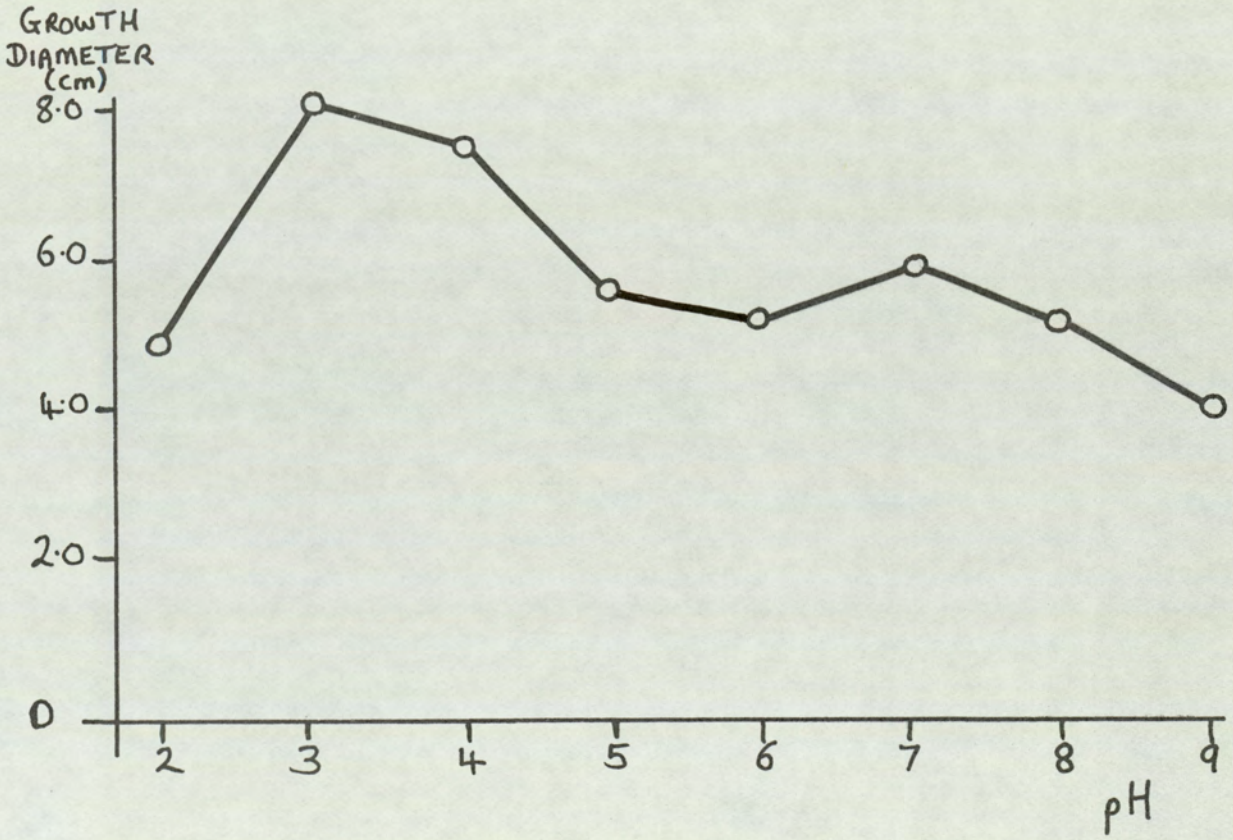


Fig 4.28. Surface pH experiment. Growth of A. niger on malt extract agar at various pH.

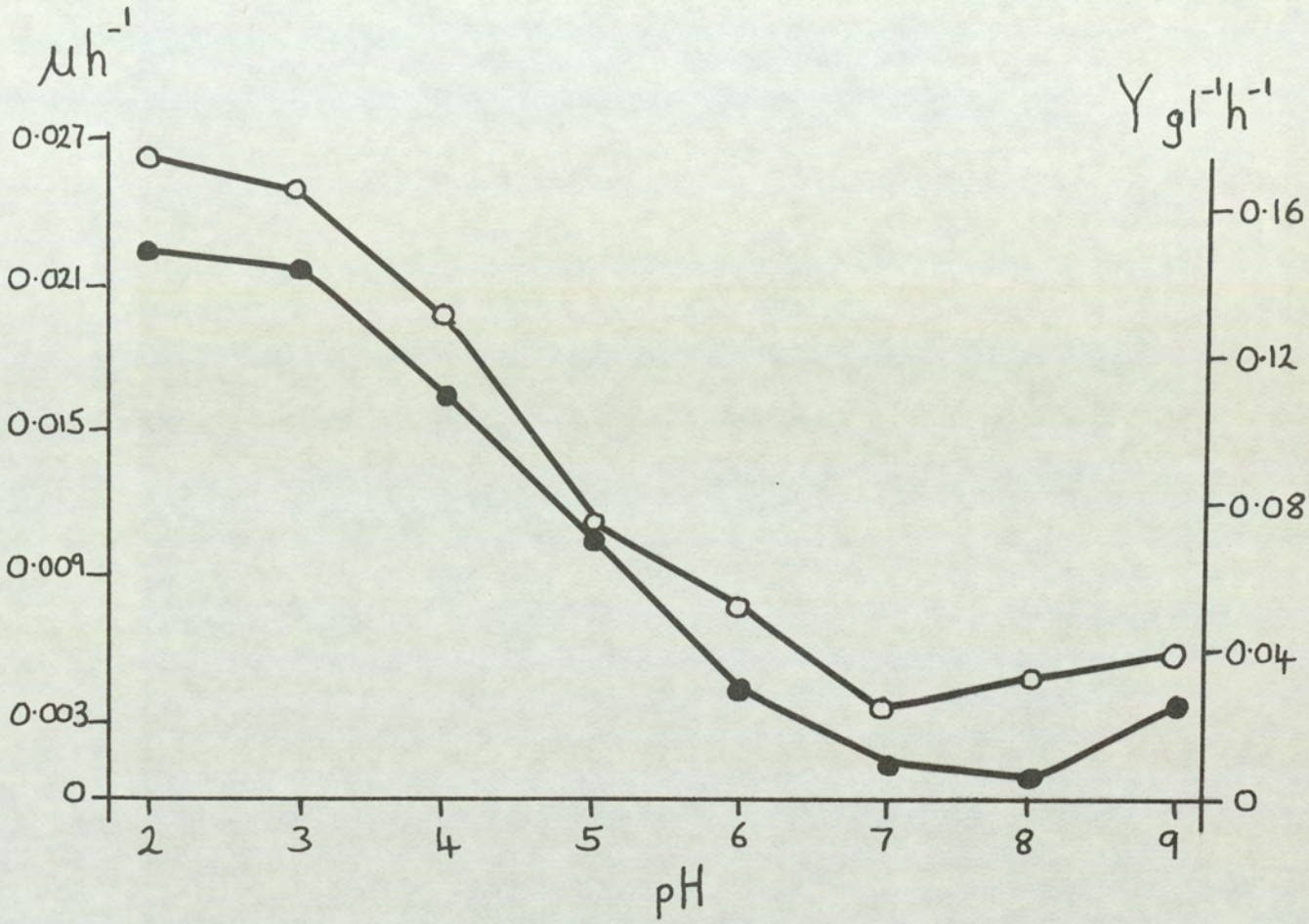


Fig 4.29. The effect of pH upon growth rate (μ) (O) and productivity (Y) (●) of A. ochraceus.
D = 0.1h^{-1}. T = 30°C Sm = 5.0gl^{-1} sucrose Nm = 0.21gl^{-1} nitrate N.

utilization was probably affected by decreased permeability of the cellular membrane as pH increased. Once again, the object of the experiment was to select a pH value that restricted growth but maintained good fermentation conditions. Growth rate was lowest at pH 7 and 8, but bacterial infection occurred at these levels and consequently pH 6 was selected for use in the final limit combination experiment, because this maintained the culture infection - free, restricted growth rate and allowed the mould to grow in Type 3 flocs which were preferable for sporulation over the pellets induced at pH 7 and 8. Effluent biomass decreased from $1.5gl^{-1}$ to $0.07gl^{-1}$ as pH increased and fermenter biomass concentration decreased from a mean of $6.0gl^{-1}$ at pH 2 - 5 to 1.76 at pH 8. The increase in effluent fermenter biomass concentration at pH 9.0 is unexplained. Carbon utilization again remained constant throughout the experiment and specific carbon utilization rate consequently increased as pH increased (Fig 4.27) except at pH9 when fermenter biomass concentration was high. No sporulation was induced at any pH, but a yellow/green pigment was produced at high pH levels.

4.4.3. pH shock to A. niger

As the gradual pH change had induced no sporulation, a shock change in pH was tested by allowing a culture to reach steady - state growth under optimum conditions at pH = 4, and then suddenly altering the pH of the culture to either pH = 2.0,

or to pH = 8.0. The results (Table 19) show that after the increase in pH, growth rate dropped dramatically and then exhibited the damped oscillatory response to shock seen in previous experiments. A new steady - state was not reached before the culture washed out, and no sporulation was induced. Hyphal morphology remained apparently normal, although increased branching undoubtedly contributed to the formation of a 'fluffy' pellet morphology from the original filamentous culture. The dark yellow pigment was again produced although this disappeared after 48 hours, shortly before wash - out occurred. It is interesting that nitrogen utilization dropped when the pH was increased suddenly (to a mean of $0.02gl^{-1}$) while utilization was much higher at pH 8 than at pH 4 when the change was made step - wise in experiment 4.4.1. This suggests that the most immediate effect of pH change is upon membrane permeability rather than upon trace element uptake and their subsequent activity upon enzyme activity.

The decrease in pH only served to improve the growth of the culture as was expected, and no sporulation response was observed. Sudden acid addition to the tower during this experiment caused excessive foaming of the growth medium, which tended to retain mycelial flocs in the tower for a period, until acid addition slowed when pH 2 was reached. As a result, fermenter biomass concentration was originally found to increase and effluent biomass concentration to decrease, giving falsely low growth rate values. The mould morphology remained filamentous, nitrogen and carbon utilization were unaffected, and a new steady state quickly developed.

4.5. The effect of the Carbon dioxide/oxygen ratio.

This experiment was designed to examine the effects of alteration in the relative proportions of oxygen and carbon dioxide in the influent air upon the growth of A. niger and A. ochraceus. Fungi obtain oxygen in the dissolved state, but a discussion of oxygen supply to fungal cultures is beyond the scope of this project, due to the complex interactions of oxygen solubility, temperature and broth viscosity/floc morphology which determine oxygen supply. The non-Newtonian, high viscosity filamentous cultures encountered during this work are considered difficult to aerate, but in all experiments the air supply was such that dissolved oxygen concentration was maximum. The interaction of aeration/agitation in the tower fermenter has been examined by Stockbridge (1979) who found no oxygen limitation at various air supply rates, because above a critical level the fermenter liquid was saturated with dissolved oxygen (this level depends upon environmental conditions and is not a constant). The centre of tight flocs may be oxygen limited, but these were rarely seen during this work, and the deficiency cannot be overcome by increasing the air flow rate. Hawker (1950) states that fungi are markedly insensitive to oxygen tensions due to their low but essential requirements, although sporulation is associated with higher oxygen levels than vegetative growth as the greatest stimulus to sporulation is exposure of mycelium to the aerial environment. The mechanism of oxygen regulation in induction and repression roles in filamentous fungi is still poorly understood. Carter & Bull (1971) studied the effect of oxygen tension on a glucose - limited continuous culture of

A. nidulans at a dilution rate of $0.05h^{-1}$ and found that oxygen had negligable effect upon growth kinetics in the range 10 - 156 mm Hg., but at tensions below 3.5 mmHg, free conidia (but no conidiophores) appeared in the medium.

Carbon dioxide plays an important role in controlling the differentiation of many fungal species, and also has a major role in carboxylation reactions in the formation of TCA cycle amino acids. Carbon dioxide influences the activity of the TCA cycle and its' associated pathways, and as such is important in the commercial production of organic acids by fungi. Carbon dioxide is reported to increase the activity of the glyoxylate cycle during conidiation by Smith & Galbraith (1971) who review the effect of CO_2 on fungal morphogenesis. Hawker (1950) states that 10% carbon dioxide checks most fungal growth, (although Tabak and Cooke (1968) state 95% inhibits growth), and that sporulation is more readily suppressed by increased carbon dioxide than is vegetative growth. Carbon dioxide dissolves in aqueous solution to form carbonic acid, which dissociates to form bicarbonate and carbonate ions. Maccauley and Griffin (1969) suggest that the bicarbonate ion, rather than carbon dioxide directly, affects the growth of fungi in submerged culture, but the bicarbonate ion concentration is extremely sensitive to pH, which has made it difficult to interpret the effect of the ion when pH is not rigidly controlled. Generally, high concentrations of carbon dioxide inhibit growth, and Hodgekiss and Harvey (1972) found that an increase in carbon dioxide level advanced the sporulation of coprophilous pyrenomycetes, although sporulation and secondary

metabolite production is usually inhibited by concentrations of carbon dioxide lower than those needed to inhibit vegetative growth.

To increase the percentage of carbon dioxide supplied to the culture, the gas was metered through a series of valves and a flow meter as for compressed air (section 2.1.3.,) and the air flow was decreased by the equivalent amount to that at which carbon dioxide was supplied, in order to maintain the overall gas supply, and hence agitation, constant. For example, under normal conditions, air was supplied to the 4.2 litre tower at 1 v.v.m. i.e. 4.2 l minute $^{-1}$, but when 40% carbon dioxide was supplied, the air flow rate was decreased to 2.52 l minute $^{-1}$ and pure carbon dioxide was supplied at 1.68 l minute^{-1}. The content of the influent air was altered step - wise to each organism, and the culture was allowed to establish a steady - state at each value. The results in table 20 are the means of values taken over three days at these steady states.

4.5.1. Effect of carbon dioxide upon A. niger.

Figure 4.30 shows that growth rate immediately decreased as the carbon dioxide level increased, while productivity decreased beyond 40% carbon dioxide. At 20% carbon dioxide, very sedimentary type 5 flocs had formed within 24 hours of introduction of the gas and the characteristic yellow pigment was produced. 40 hours later simplified sporulation structures were observed, consisting of one to four phialides formed at a hyphal tip, each bearing a spore, although no free spores were seen in the medium.

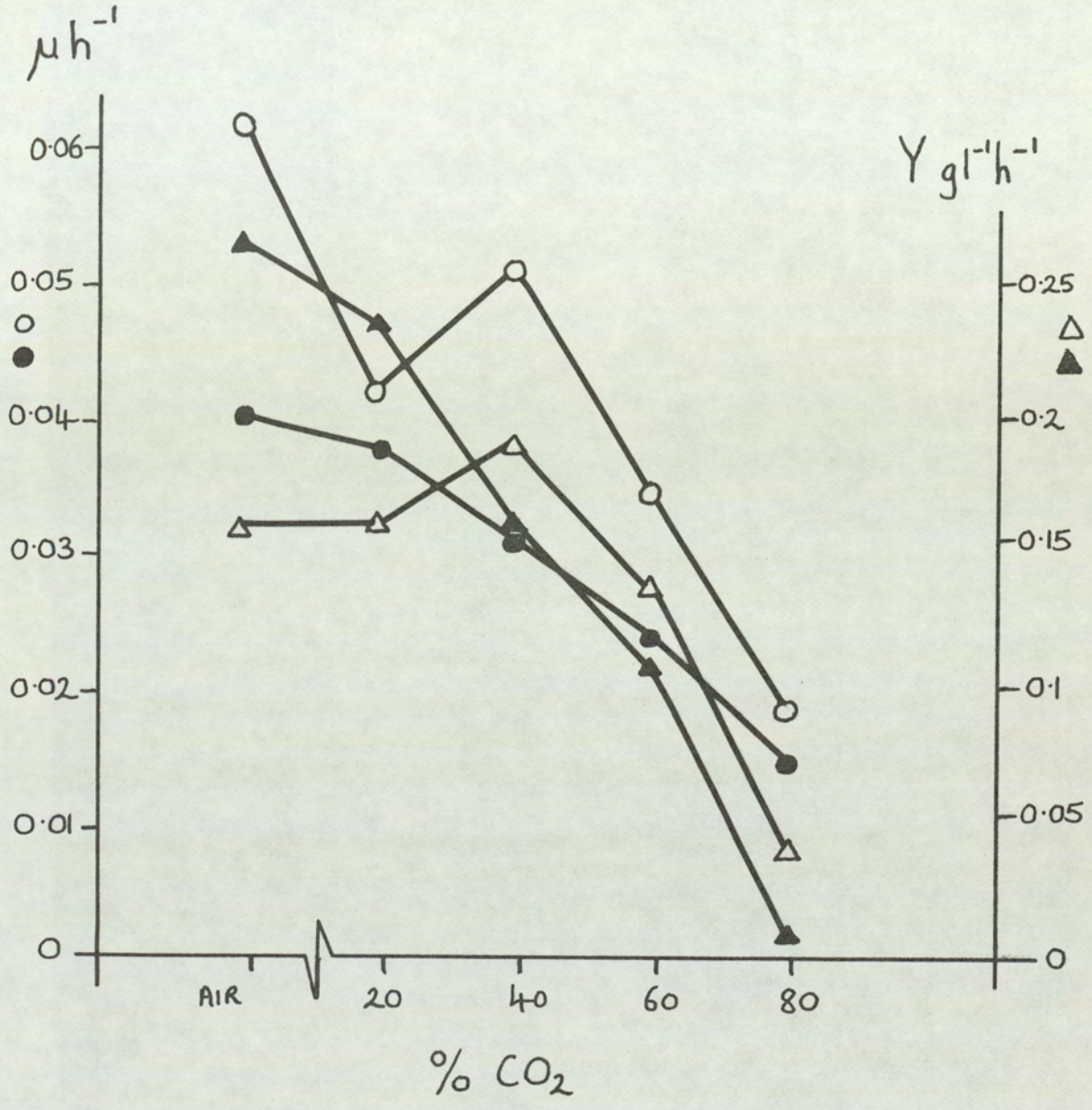


Fig 4.30. The effect of carbon dioxide concentration in the influent air supply upon the growth rate (μ) and productivity (Y) of A. niger (o) and A. ochraceus (●). $D = 0.1h^{-1}$ $T = 30^{\circ}C$ $Sm = 5.0gl^{-1}$ sucrose $Nm = 0.21gl^{-1}$ nitrate N.

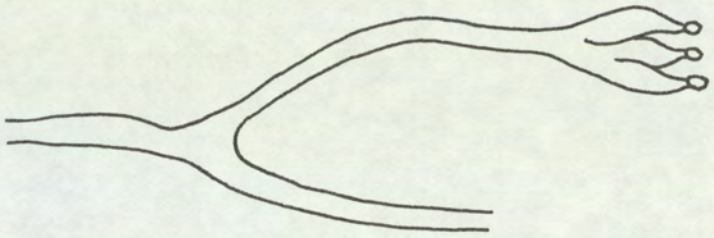


Fig. 4.31. Sporulation morphology of A. niger at 20% carbon dioxide.

These structures persisted for 36 hours, but then no more were formed. At 40% and 60% carbon dioxide there was no apparent visual change in floc morphology from that seen normally, i.e. loose filamentous strands, and there was no sporulation, although the yellow pigment was produced. At 80% carbon dioxide the growth rate of the organism decreased greatly, inhibited pellet type flocs forms (type 6) which were retained in the tower, and productivity almost ceased. This condition appeared stable over a period of several days, but although the growth rate was low ($0.018h^{-1}$), no sporulation was observed.

4.5.2. Effect of carbon dioxide upon A. ochraceus

Fig 4.30 shows that growth rate and productivity of A. ochraceus progressively declined as the carbon dioxide level of the influent air was increased. Although growth rate decreased to $0.014h^{-1}$ at 80% carbon dioxide, no sporulation was observed at any stage and the only apparent effect upon floc morphology was a progressive decrease in the floc size. This resulted in small, fine flocs at 80% carbon dioxide but they were loosely filamentous and showed no signs of inhibition or pellet formation. This morphology was easily removed from the fermenter, and accounts

for the low fermenter biomass concentration observed ($1.0gl^{-1}$). Fig 4.32 illustrates the carbon and nitrogen utilization levels of both organisms as the carbon dioxide concentration increased. Nitrogen utilization of A. ochraceus decreased from $0.03gl^{-1}$ to $0.015gl^{-1}$ as carbon dioxide percentage increased, while the utilization by A. niger only decreased at 80% carbon dioxide, when it dropped from 0.128 to 0.09 gl^{-1}. The same trend occurred for carbon utilization. That by A. ochraceus decreased from 4.2 to $0.4gl^{-1}$ as carbon dioxide percentage increased while that by A. niger was constant until 80% carbon dioxide was introduced when utilization decreased from 4.53 to 2.1 gl^{-1}. The bicarbonate ion concentration of the fermentation broth was almost constant throughout the experiments ($36.6mgl^{-1}$) but the free carbon dioxide level increased with increasing CO_2 supply. This was probably due to the rigidly controlled pH of the broth preventing further bicarbonate formation, because bicarbonate ion is a function of pH and carbon dioxide partial pressure in the gas bubble within the liquid (Maccauley & Griffin 1969) and this partial pressure may have had negligable effect in this system because of the rate of air passage through the liquid. The gas bubbles also coalesced or 'slugged' so that mixing of gas with liquid was minimised, and Nyiri and Lengyel (1968) state that the concentration of the bicarbonate ion in culture broths is influenced by the viscosity of the system, along with the buffer capacity of the medium.

4.6. The effects of potassium ferrocyanide and citrate.

The use of the ferrocyanide ion to inhibit A. niger growth in the citric acid fermentation is well known, and there are many

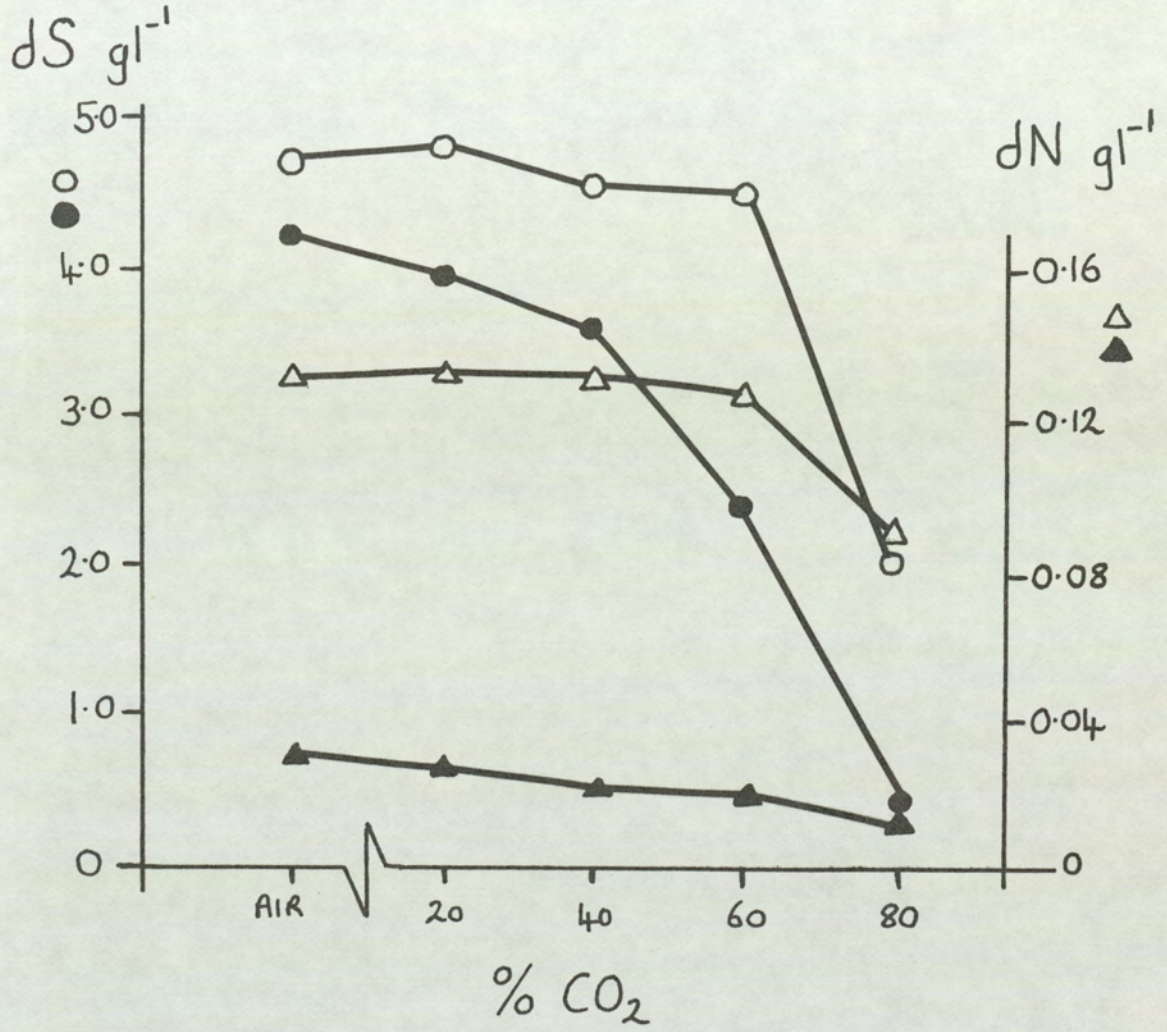


Fig 4.32. The effect of carbon dioxide concentration in the influent air supply upon sucrose (dS) and nitrogen (dN) utilization by A. niger (o) and A. ochraceus (●).
$D = 0.1h^{-1}$ $T = 30^{\circ}C$ $Sm = 5.0gl^{-1}$ sucrose $Nm = 0.21gl^{-1}$ nitrate N.

references in the literature to methods used (Martin, 1955, Clark 1962a + b, + c and Horitsu and Clark 1966). As citric acid is a non-growth associated metabolite, it was anticipated that ferrocyanide might inhibit growth and promote sporulation of the fungi under examination. The action of the cation is 3-fold; Firstly, its' principal effect is the 'medium modifying' action, by precipitation of the insoluble heavy metal - ferrocyanide complex formed in solution, which removes zinc, copper, iron and manganese ions from the solution, leaving a medium that is only capable of supporting a deficient metabolism. Secondly, excess ferrocyanide ions directly inhibit the activity of aconitase. Thirdly, hydrocyanic acid may be formed in solution, which is itself inhibitory. Martin (1955) stated that growth of A. niger in simple synthetic medium may be inhibited by less than 1 p.p.m. ferrocyanide, although more than 100 p.p.m. may be needed in a molasses medium, due to the greater amounts of metals present. Clark (1962 a + b) states that 20 p.p.m. ferrocyanide are required at the start of a citric acid fermentation, later increased to 80 - 150 p.p.m. for highest yields. 60 p.p.m. severely retards inoculum development, but up to 400 p.p.m. does not inhibit a healthy fermentation. An important feature of increased ferrocyanide concentration is the development of mycelial pellets (Clarke 1962c) essential for citric acid production, but undesirable for sporulation. The mould is reported to remain filamentous below 10 p.p.m. ferrocyanide. In these experiments, a medium solution containing 1×10^{-5}M, 1×10^{-4}M or 1×10^{-3}M $K_4Fe(CN)_6 3H_2O$ was supplied continuously to the culture. These values correspond approximately to 4.0, 40.0, and 400.0 p.p.m. ferrocyanide.

Citric acid was supplied as a carbon source to some fermentations, in an effort to increase the intensity of sporulation as reported by other workers. Galbraith and Smith (1969a) reported that citrate was one of the most effective inducers of conidiation when added to a basal medium supplemented with ammonium nitrate. Ng et al. (1973b) achieved conidiation of A. niger in continuous citrate - limited culture at dilution rates above $0.013h^{-1}$ when $10gl^{-1}$ citrate was supplied to the culture. In the same system, conidiation was difficult to achieve under glucose limitation, and the success of citrate was attributed to the fact that it is metabolised slowly by A. niger, and thus the concentration used provided sufficient energy for sporulation but insufficient for growth. Conversely, Martinelli (1973, 1976) reported that with the exception of ethanol, all carbon sources proved to be poorer sources for conidiation than glucose, and that citrate did not support conidiation. Dawes and Mandelstam (1970) also report that sporulation of B. subtilis occurs at high frequency under glucose limited conditions, but that sporulation is low with citrate as the limiting carbon source.

4.6.1. Potassium ferrocyanide addition to optimum medium for A. niger.

Having allowed an A. niger culture to grow at a steady - state for several days at a dilution rate of $0.2h^{-1}$ the optimum medium ($5.0gl^{-1}$ sucrose, $0.21gl^{-1}$ nitrate N. plus salts at T= $30^{o}C$, pH = 3, Air = 1 v.v.m.) was supplemented with potassium ferrocyanide solutions of $10^{-5}M$, $10^{-4}M$ and $10^{-3}M$ in a step - wise

manner. The culture was allowed to establish a new steady - state growth pattern with each ferrocyanide supply, and results (Table 23) were recorded over 3 days minimum. Although increasing levels of ferrocyanide ion had increasingly restrictive effects upon growth rate, productivity, sucrose and nitrate utilization, there was no sporulation induced at any concentration. The mould turned a deep blue shortly after ferrocyanide introduction, but there was no other apparent effect upon the morphology. Higher concentrations of potassium ferrocyanide were not used because the quantities involved became impractical for a commercial system at higher concentrations at a dilution rate of $0.2h^{-1}$.

4.6.2. Ferrocyanide and nitrogen limitation upon A. niger

The failure of ferrocyanide ions to induce sporulation of a culture growing in a optimum medium, led to the development of a system to examine the effect on sporulation intensity of reducing the nitrate supply as before (4.2.2.) to a culture with an already inhibited growth rate due to ferrocyanide presence. (Table 24). Thus, A. niger was allowed to reach steady - state growth in a optimal medium (D = 0.2, T = $30^{o}C$, pH = 3.0, Air = 1 v.v.m. Sm = $5.0gl^{-1}$ sucrose, Nm = $0.2gl^{-1}$ nitrate N and standard salts) plus 10^{-3}M potassium ferrocyanide. Growth rate was sub-optimal but steady under these conditions ($0.076h^{-1}$). After several days, the nitrogen content of the medium was suddenly reduced to $0.022gl^{-1}$ nitrate atomic N, and the subsequent transient growth phase was monitored. The results are shown in Fig. 4.33. Growth rate initially rose after the shock, but then

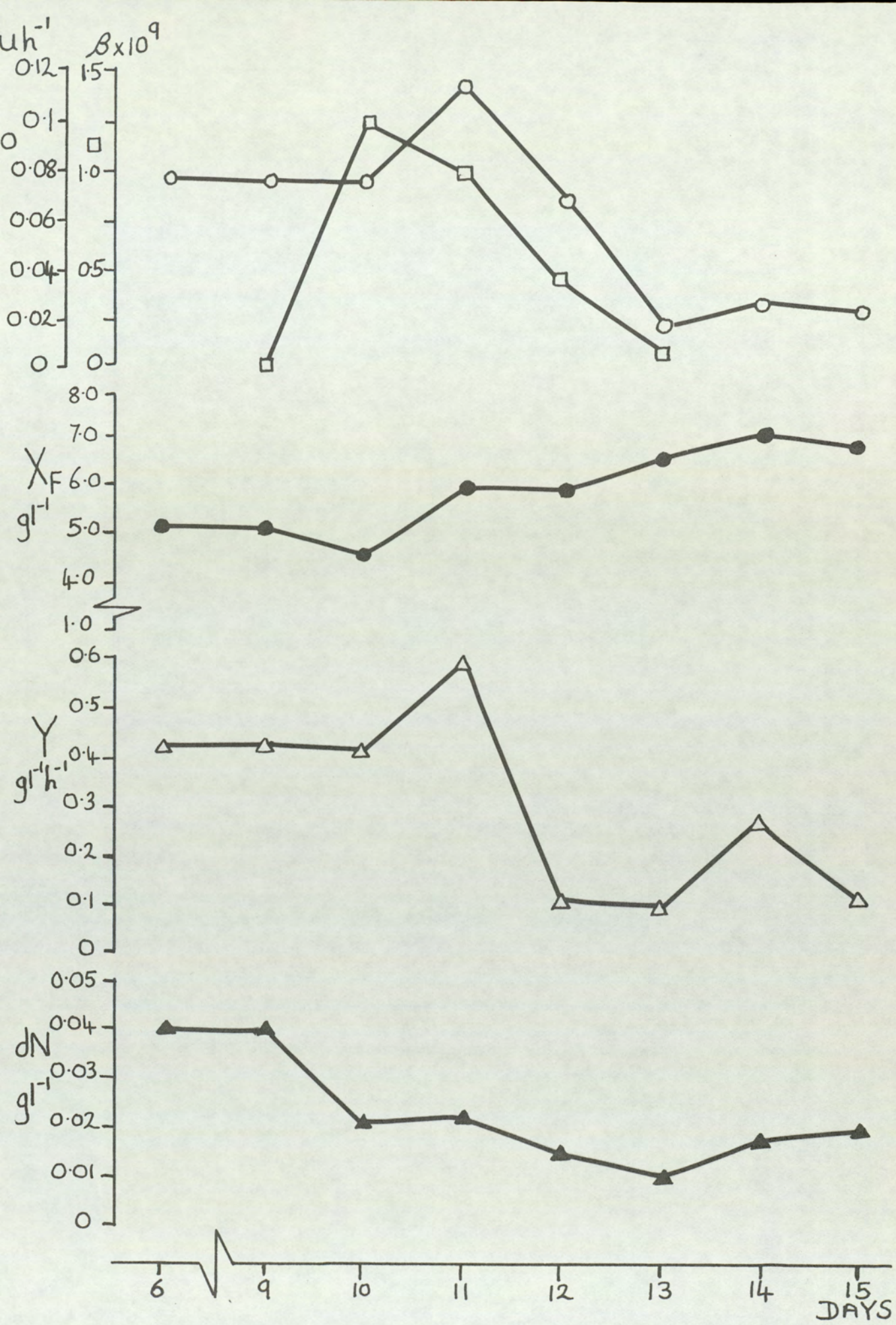


Fig 4.33. Response of ferrocyanide-limited fermentation parameters of A. niger to shock nitrate limitation. (0.2 - 0.022gl^{-1})

decreased to a new steady level ($0.027h^{-1}$), as did productivity, although the after - shock oscillations were greater. Nitrogen utilization dropped immediately to a new steady level ($0.015gl^{-1}$), but carbon utilization was unaffected by the change. (This was observed in normal nitrogen limitation experiments). Fermenter biomass concentration (X_F) gradually increased after the shock, although there was no apparent floc morphology change to account for this. The flocs remained filamentous throughout the experiment. These trends agree closely with those of experiment 4.2.4. (Fig 4.15) although values differ due to the different dilution rates used.

Sporulation was induced in this system, and occurred within 24 hours of the imposition of nitrogen limitation, when production of spores was maximum (6×10^6 spores ml^{-1}), and continued for up to four days with progressively decreasing spore numbers. The culture appeared stable in this condition, and growth continued for up to two weeks with no further sporulation, and thus subsequent replicates were terminated when sporulation ceased. If these results are compared with those of experiment 4.2.2. (Maximum spores $ml^{-1} = 4 \times 10^6$, max $\beta = 6.9 \times 10^8$, mean spores g sucrose^{-1} utilized $= 9.9 \times 10^8$) it can be seen that the pre-addition of ferrocyanide led to increased spore production (maximum $= 1.25 \times 10^9$) at a more efficient mean substate utilization rate (mean spores g sucrose^{-1} utilized $= 1.14 \times 10^9$). The most significant difference, however, was that the ferrocyanide presence removed the requirement of the 48 hour lag period before sporulation observed in experiment 4.2.4. As in 4.2.2., there were a variety of sporing structures produced, although the

majority were simplified with single phialides on hyphal tips.

4.6.3. Nitrogen limitation and ferrocyanide upon A. niger.

The combination of ferrocyanide addition and nitrogen limitation was tested in the reverse sequence. A culture of A. niger was allowed to establish steady - state growth at a dilution rate of $0.2h^{-1}$ (Sm = $5.0gl^{-1}$ sucrose, Nm = $0.21gl^{-1}$ nitrate N) and then the medium nitrogen supply was suddenly reduced to $0.021\ gl^{-1}$ nitrate N. The effects of this shock were then recorded. (Table 25). As expected, growth rate was immediately inhibited and dropped to a new mean value of $0.021h^{-1}$ from $0.069h^{-1}$, while fermenter biomass concentration gradually increased and sugar utilization was unaffected as previously observed. (Fig 4.34). Sporulation was induced, although only after a lag period of 24 - 36 hours after limitation was imposed, which supported the evidence of the effect of ferrocyanide in reducing this lag time in experiment 4.6.2.. Spore production was relatively low (max spores $ml^{-1} = 6 \times 10^5$, max $= 8.7 \times 10^7$) but continued at these levels for 6 - 7 days. 6 days after the introduction of limitation, with growth rate at the new level and sporulation occurring, 10^{-3} M potassium ferrocyanide was added to the growth medium (Day 11 on graphs). The inhibitory effect upon growth rate, productivity, fermenter biomass concentration and sucrose utilization was immediate. Although sporulation continued for two days after ferrocyanide addition, the mould was rapidly killed, and remaining mycelium was washed from the fermenter after 5 - 6 days. The cessation of sporulation

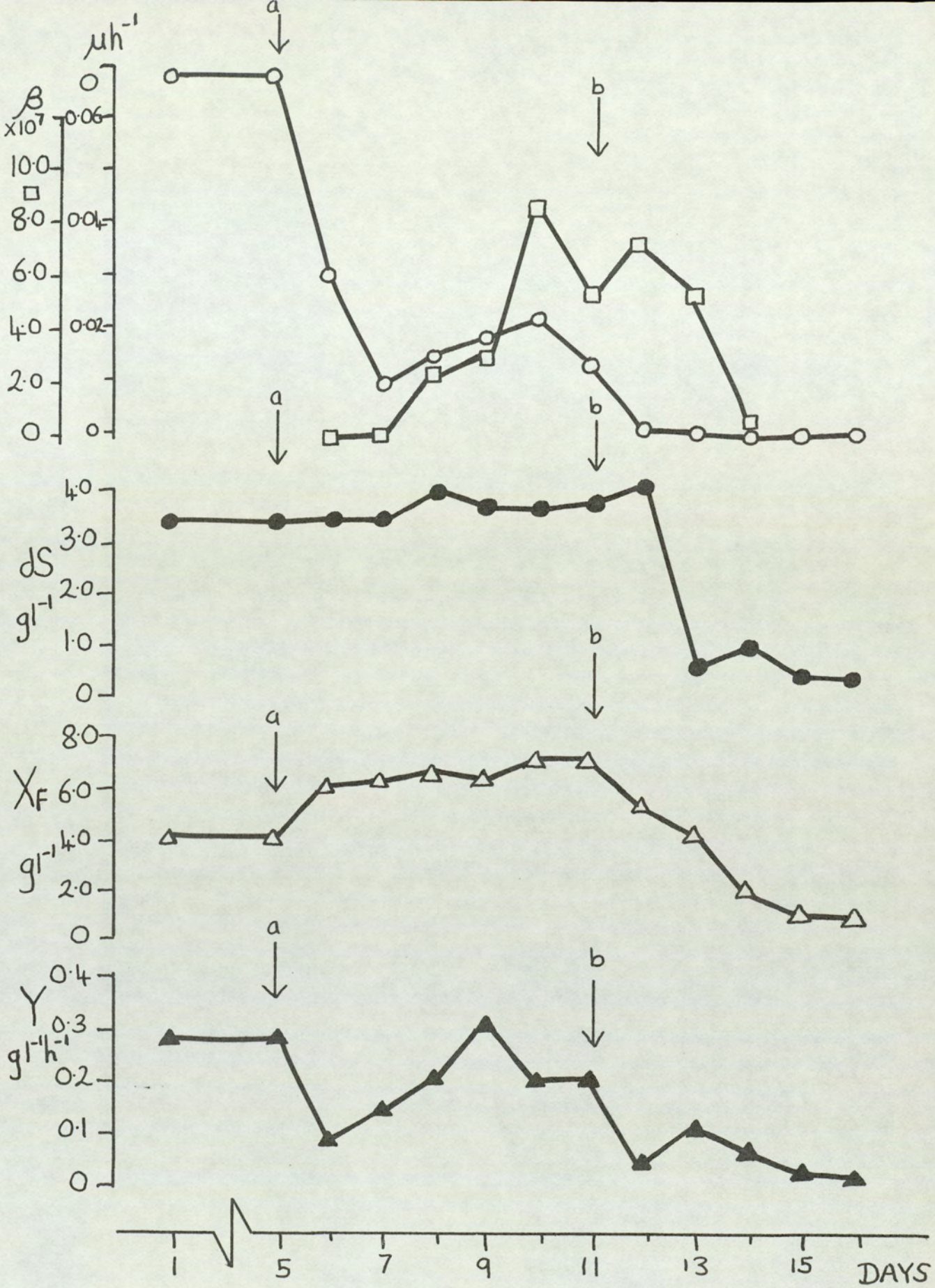


Fig 4.34. The effect of nitrogen limitation (0.21 - 0.021gl^{-1}) (a) followed by ferrocyanide addition (b) upon growth rate (μ) sporulation (β) sucrose utilization (dS) fermenter biomass concentration (X_F) and productivity (Y) of A. niger.

coincided with zero nitrogen utilization and followed 12 hours after growth rate had reached zero. This observation supports the theory that growth and sporulation are required simultaneously for this system to operate, and suggests that the two processes are closely connected and far from mutually exclusive.

The combination of nitrate limitation and ferrocyanide addition in this sequence was obviously undesirable for the operation of a continuous sporing system.

4.6.4. Nitrogen limitation to A. niger on citrate.

A culture of A.niger was allowed to reach steady - state growth in an optimum medium where sucrose had been replaced by $5.0gl^{-1}$ citrate as the carbon supply. ($D = 0.1h^{-1}$, $T = 30^{o}C$, pH = 3.5, Air = 1 v.v.m. Nm = $0.21gl^{-1}$ nitrate N and standard salts). Growth was poor on citrate and a culture had to be grown up from a mycelial inoculum for 10 days before steady growth was achieved. This was probably due to genetic alteration of the organism which had been grown on sucrose for long periods prior to this experiment, and needed time to code for and produce the enzyme systems required for citrate utilization. A lower growth rate would be expected also, because citrate is less available biochemically to A. niger than is sucrose or glucose. Table 26 shows that the mean optimum growth rate was $0.048h^{-1}$, although the majority of the citrate ($4.75gl^{-1}$) had been utilized. When the nitrate supply was suddenly reduced to $0.021gl^{-1}$ atomic N,

the growth rate was drastically reduced ($0.0044h^{-1}$) and growth stopped 24 hours after limitation was imposed. All parameters (productivity, X_E, X_F, dS, $Ysub_s$ and dN) were decreased by the limitation, with nitrogen utilization ending immediately after the limitation. Spores were seen briefly on the first day after limitation (3×10^3 spores ml^{-1}) and were produced from sub-aerial type structures, but production was low and lasted only a few hours. A. niger grew in larger flocs with well - defined centres (type 3) under citrate, in contrast to the loose filamentous strands observed in sucrose, and these flocs did not visually change during the experiment. In two repeats of the experiment, no sporulation at all was observed, before mould death and it was concluded that this nitrogen limitation was too severe for the mould when its' only energy source was citrate. However, when a fourth fermentation was attempted using a shock nitrate supply of 0.08 gl^{-1} nitrate, no sporulation was observed, probably due to nitrogen inhibition. It is interesting to note that the sporing structures of A. niger induced in the nitrogen - limited citrate culture were all complex sub - aerial types, whereas in nitrogen - limited sucrose culture sporulation structures were predominantly simplified with phialides on hyphal tips.

4.6.5. Citrate supply to A. niger after nitrogen limitation.

This experiment was to establish whether sporulation of the normal nitrogen limitation system was enhanced by replacing the sucrose with the less avialable carbon source, citrate. A culture of A. niger was allowed to reach steady - state growth under

optimum conditions ($D = 0.1h^{-1}$, $T = 30^{o}C$, pH = 3.5, Air = 1 v.v.m. $Sm = 5.0gl^{-1}$ sucrose, $Nm = 0.21gl^{-1}$ nitrate N, plus standard salts) before the nitrate supply of the medium was suddenly reduced from 0.21 to $0.021gl^{-1}$ atomic N, as in previous experiments. Table 27 shows that the expected decrease in growth rate and subsequent sporulation by simplified structures (Fig 5.1) was achieved. Sporulation reached a peak of 3.4×10^{6} spores ml^{-1} ($\beta = 8.1 \times 10^{8}$) approximately 48 hours after limitation was imposed, and occurred for several days. When sporulation was proceeding normally, and growth rate had settled at a new steady state condition ($0.036h^{-1}$), the sucrose medium was suddenly replaced by a $5.0gl^{-1}$ citrate medium, and the effect upon sporulation monitored. Day 9 in table 27, approximately 24 hours after introduction of the citrate medium, shows that parameters remained constant for that time, although sporulation intensity decreased. This, however, would have been expected in an undisturbed sucrose system because this was the fourth day of sporulation, and this phase had seldom lasted for longer, and so the decrease was considered not directly attributable to citrate addition. Subsequent days, showed a considerable fall in growth rate ($0.04 - 0.014h^{-1}$) and productivity ($0.14 - 0.052\ gl^{-1}h^{-1}$) and the cessation of spore production, and the culture was washed from the fermenter within 5 days of citrate addition without further sporulation.
The experiments therefore demonstrated that changing carbon supply from sucrose to citrate in a nitrogen - limited sporing system did not enhance sporulation, but rather caused a sufficient decrease in growth rate to disturb the limited

steady state, resulting in organism wash - out. The system was therefore considered unsuitable for further development.

4.7. Combination of growth rate limitations.

In an attempt to increase the intensity of sporulation in the single - stage fermentation system, it was proposed to restrict the growth rate, and hence induce sporulation, by a combination of the environmental parameters examined previously. It has been shown that sporulation does not result simply from the restriction of growth rate to the greatest extent, but depends upon a complex combination of nutritional and environmental factors. The temperature, pH, aeration rate, dilution rate, sugar and nitrogen concentrations used in these experiments were not therefore simply those that had the maximum inhibitory effect upon growth rate, but those that favoured good fermentation conditions, ease of running, favourable floc morphologies, and economy, while also inducing sporulation. The decrease in growth rate during these experiments was more a consequence of these particular combinations rather than a value specifically aimed at to induce sporulation. It was appreciated that in each type of system there was a critical growth rate above which vegetative growth occurred and below which was necessary for sporulation, but this point was not clearly defined in the nitrogen - limited system used, as sporulation had already been achieved at various growth rates between 0.09 and $0.0003h^{-1}$. Two basic shock limitations were used for each organism, one at high temperature and one at low temperature. The limiting

temperatures selected from section 4.3. were :

A. niger : $20^{o}C$ and $38^{o}C$ A. ochraceus : $22^{o}C$ and $38^{o}C$

The limiting pH levels selected in section 4.4. were :

A. niger : 4.0 A. ochraceus : 6.0

No excess CO_2 was supplied, as section 4.5 showed it to be expensive and ineffective. Compressed air was therefore supplied at 1.v.v.m. to maintain excess oxygen availability and adequate mixing. No mycelial blockage of any fermenter inlet or outlet had occurred during previous experiments, but there was a gradual build - up of mould on the P.T.F.E. air distributor plate which progressively blocked the holes, but which was removed by occasional short bursts of high aeration. (approximately each 2 - 3 weeks). No ferrocyanide ion was added as part of the shock as a result of experiment 4.6.3. but ferrocyanide was supplied to the steady - state phase of the A.niger culture before and during the shock as an initial growth rate inhibitor, as shown by experiment 4.6.2. The established system of nitrate limitation with constant sucrose supply was used to induce sporulation as glucose and citrate had been shown to be less effective in promoting sporulation. A dilution rate of $0.1h^{-1}$ was used throughout the experiments.

4.7.1. Combined limitation to A. niger

The culture was allowed to reach steady - state at $D = 0.1h^{-1}$, $T = 30^{o}C$, pH = 3.0, Air = 1 v.v.m., $5.0gl^{-1}$ sucrose, $0.21gl^{-1}$ nitrate N. standard salts and $10^{-3}M$ $K_4 Fe(CN)_6 3H_2O$. The conditions were then suddenly altered to : a) $T = 20^{o}C$, pH = 4.0, Nm - $0.021gl^{-1}$ nitrate or;b) $T = 38^{o}C$, pH = 4.0, Nm = $0.021\ gl^{-1}$ nitrate, while maintaining all other parameters constant.

The effect of these changes was then monitored (Table 21). Fig. 4.35. shows the effect of limitation imposed on day 5 upon growth and sporulation at both temperatures. The rise in growth rate immediately after the 38°C shock was due to sudden removal of acid addition maintaining pH at 3.0, which was causing foaming. When the acid was stopped while pH increased to 4.0, foaming ceased and mycelium that had been held in the tower was washed out, giving the effect of increased growth rate. The trends of growth rate and sporulation at high temperature were typical in that growth decreased to a new level ($0.022h^{-1}$) around which it oscillated, and sporulation intensity increased rapidly in response to the rapid growth rate decrease, and then gradually declined. The response of other parameters to both shocks is shown in Fig 4.37. At high temperature, X_F, dS and qS were largely unaffected, while productivity (Y) and specific nitrogen utilization rate (qN) decreased rapidly to a new steady level. Spores were produced for up to 4 days by simplified structures consisting of single phialides on hyphal tips, and peak production was 5×10^7 spores ml^{-1}. This level corresponded to a production of 1.1×10^{10} spores g $sucrose^{-1}$ utilized, and represented the highest productivity observed in any single stage fermentation with A. niger. Fig. 4.35. also indicates the change in growth rate after limitation was imposed at 20°C, and while there was an immediate and progressive decrease, no sporulation was induced. In the high temperature experiment, flocs were filamentous with well - defined centres (type 3) throughout the experiment, probably due to the presence of ferrocyanide. In the low - temperature experiment, mycelial flocs remained

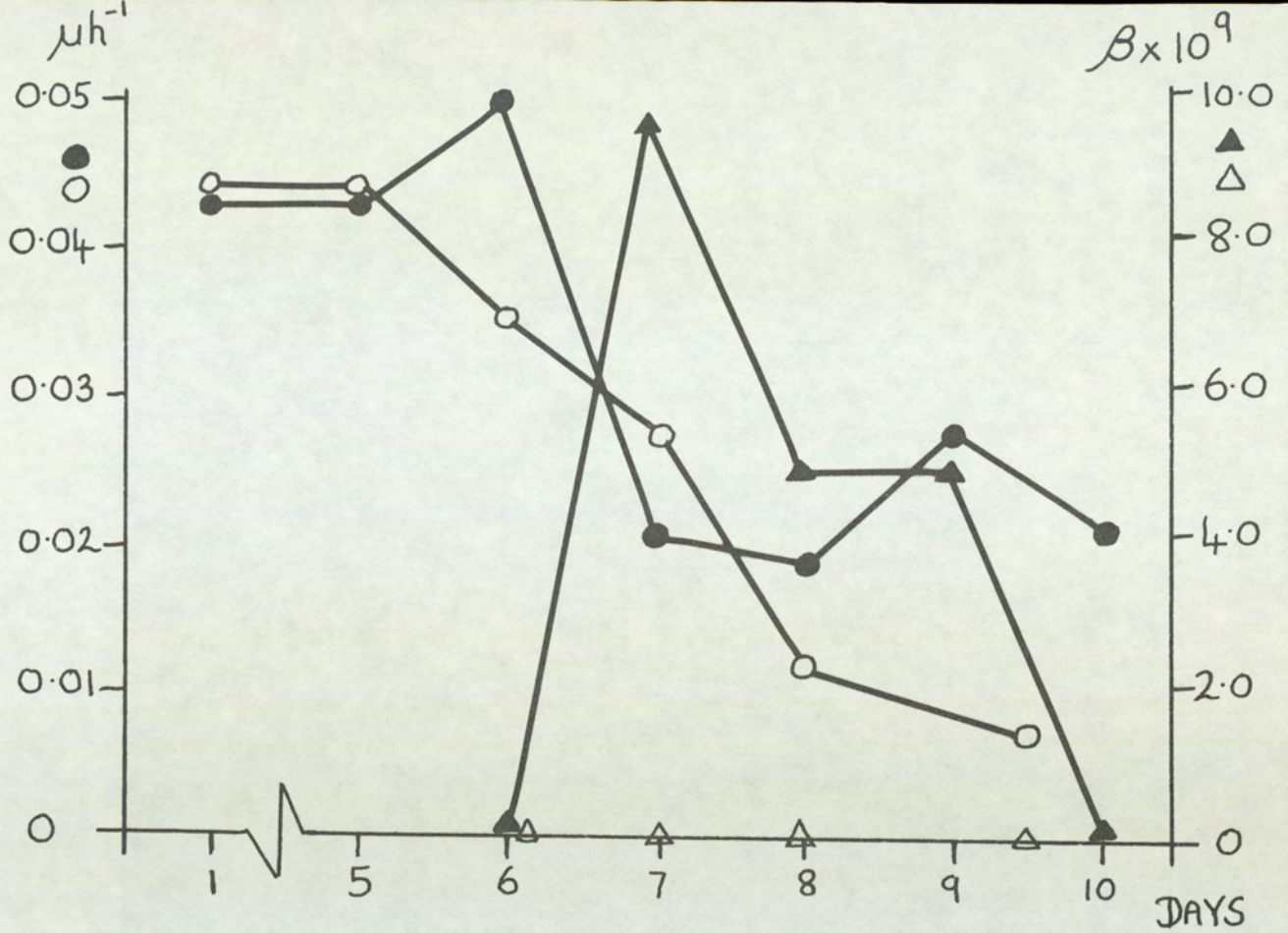


Fig 4.35. Growth rate (μ) and sporulation (β) of A. niger in response to shock combination limitation. $D = 0.1h^{-1}$.

△ ○ = low temperature ▲ ● = high temperature

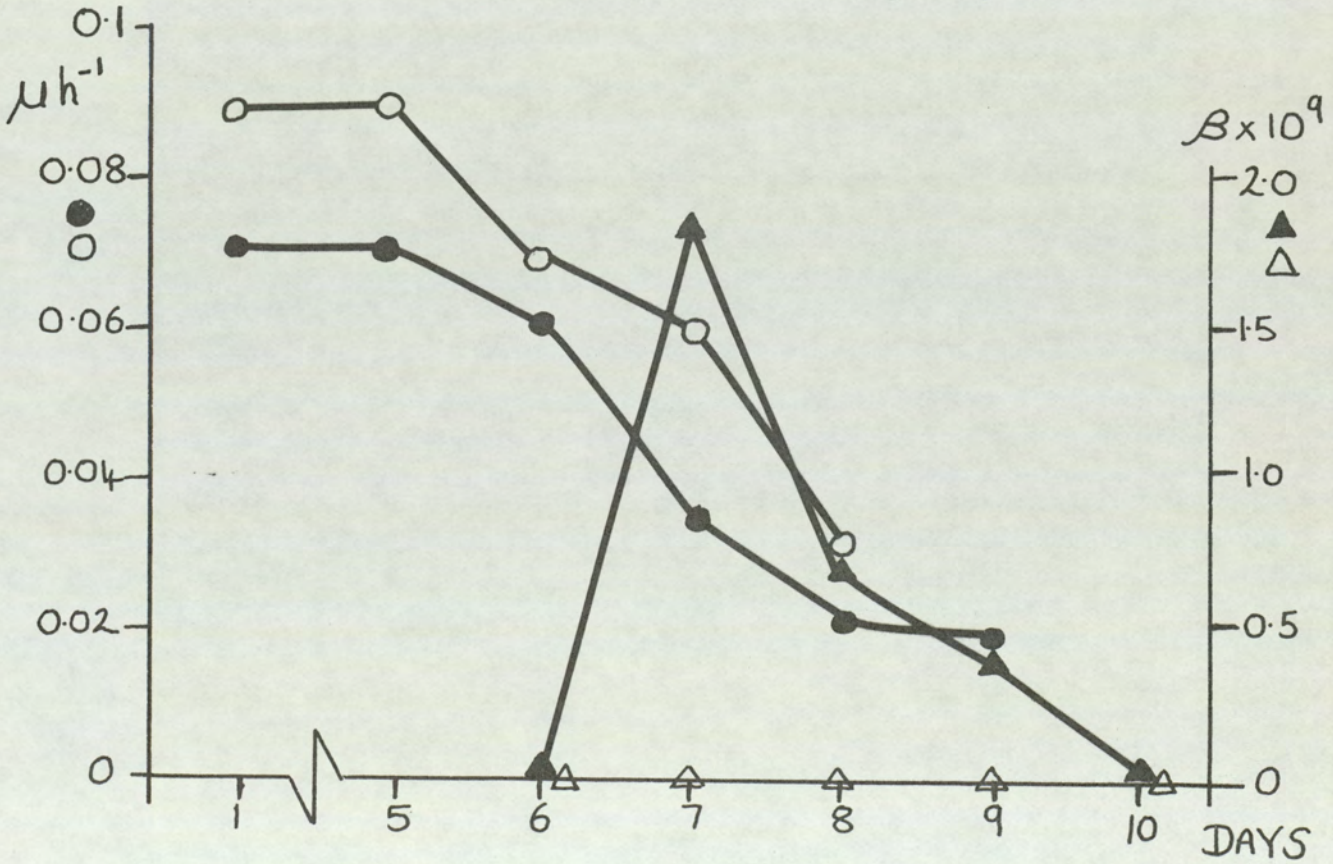


Fig 4.36. Growth rate (μ) and sporulation (β) of A. ochraceus in response to shock combination limitation. $D = 0.1h^{-1}$.

△ ○ = low temperature ▲ ● = high temperature.

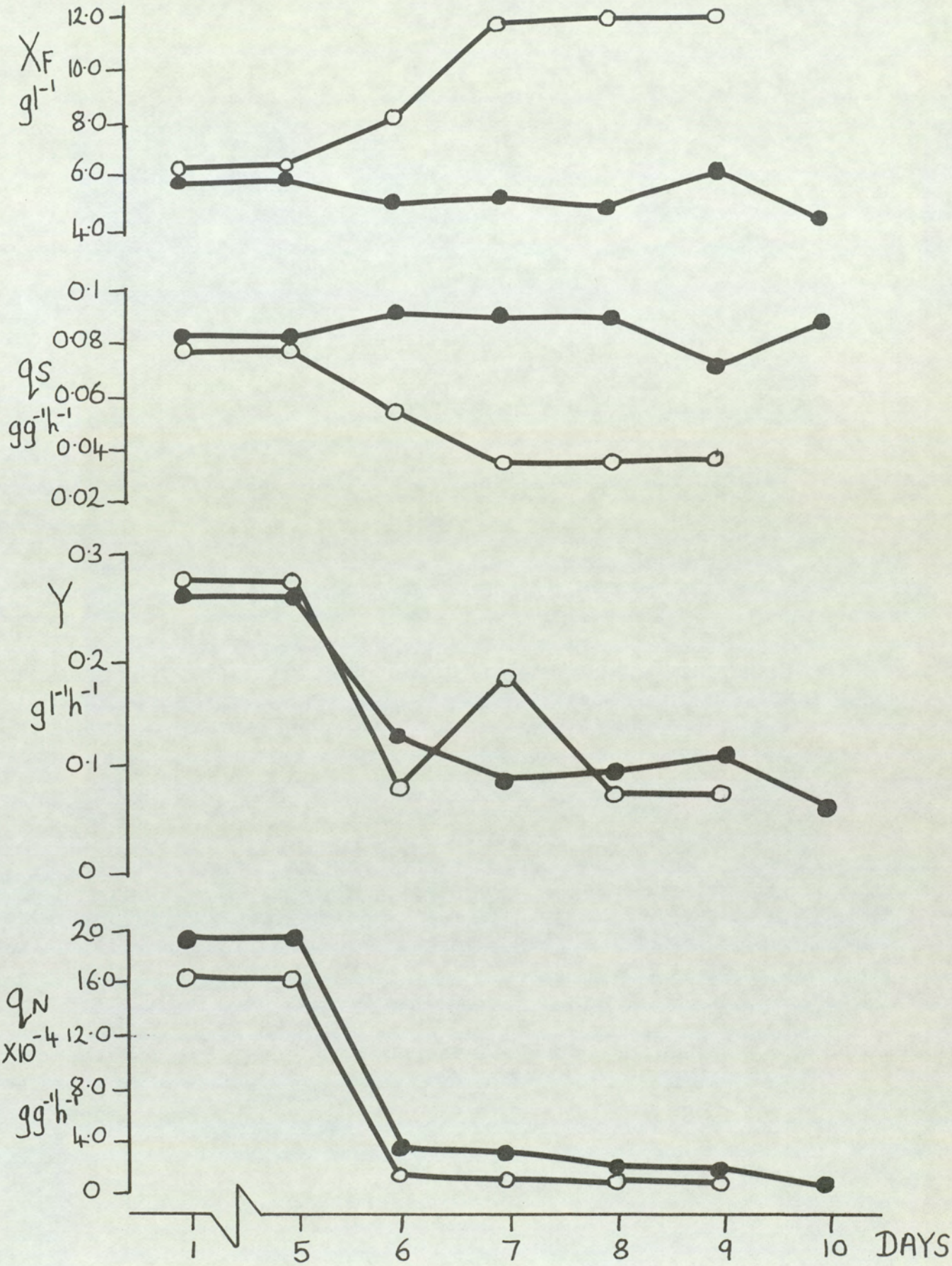


Fig 4.37. Response of A. niger fermentation parameters to shock combination limitation. $D = 0.1h^{-1}$. ○ = low ● = high

loose filaments throughout the experiment.

Fig 4.37 illustrates the contrasting reactions of other fermentation parameters to the nitrogen limitation at high and low temperatures. Sucrose utilization remained approximately constant at all times, but fermenter biomass concentration (X_F) at low temperatures increased causing a corresponding decrease in specific sucrose utilization rate (q_S). This was unexpected with the fine floc morphology induced, which would not be retained in the fermenter due to high sedimentation rates. Productivity (Y) and specific nitrogen utilization rate (qN) at 20^oC followed similar patterns to those at 38^oC with a sudden rapid decrease to a new approximate steady state.

4.7.2. Combined limitation to A. ochraceus

A culture of A. ochraceus was allowed to reach steady state at $D = 0.1h^{-1}$, $T = 30^oC$, pH = 3.5, Air = 1 v.v.m. $5.0gl^{-1}$ sucrose standard salts and $0.21gl^{-1}$ nitrate atomic N. These conditions were suddenly altered to : a) $T = 22^oC$, pH = 5.0, $Nm = 0.021gl^{-1}$ nitrate or; b) $T = 38^oC$, pH = 6.0, $Nm = 0.021gl^{-1}$ nitrate, while maintaining all other parameters constant. The effect of these changes was monitored (Table 22). Fig 4.36 shows the response of growth rate and sporulation to the high temperature shock limitation. There was an immediate decrease in growth rate which stabilized at approximately $0.02h^{-1}$ and sporulation began after a time lag of approximately 24 hours

corresponding with the most rapid decline phase of growth rate. As with A. niger, sporulation reached a peak after 24 hours, beyond which there was a gradual decline, and lasted for up to 4 days. Maximum productivity was 1×10^7 spores ml^{-1}, which corresponded with a production of 2.2×10^9 spores g sucrose^{-1} utilized. These levels were both exceeded consistently in experiment 4.2.4., (3.6×10^7 spores ml^{-1}, 1.15×10^{10} spores g sucrose $^{-1}$) and thus the high temperature combination shock did not improve sporulation of this organism. Spores were produced from an equal mixture of sub - aerial type structures, and the inhibited form described in 4.2.4., with reduced conidiophore. It was a characteristic of all these structures, that a reduced number of phialides were produced from each vesicle (Plate 23). There was no change in the loosely filamentous nature of the mycelium during the experiment, but a typical dark olive green pigment was produced after the limitation, and for the duration of the experiment (not only during sporulation). Again, no sporulation occurred in the low - temperature system, although the limitation caused an immediate and progressive decrease in growth rate, which eventually (after 6 days) stabilised at $0.015h^{-1}$.

The effects of both high and low temperature nutrient limitations upon other fermentation parameters are shown in Fig. 4.38. As with A. niger, at 38°C, fermenter biomass concentration sucrose utilization and hence specific sucrose utilization rate were relatively constant while productivity and specific nitrogen utilization rate both initially decreased after the shock before settling at a new steady - state ($Y = 0.1 gl^{-1} h^{-1}$, $qN = 4 \times 10^{-4}$ $gg^{-1} h^{-1}$). At 22°C, sucrose utilization was

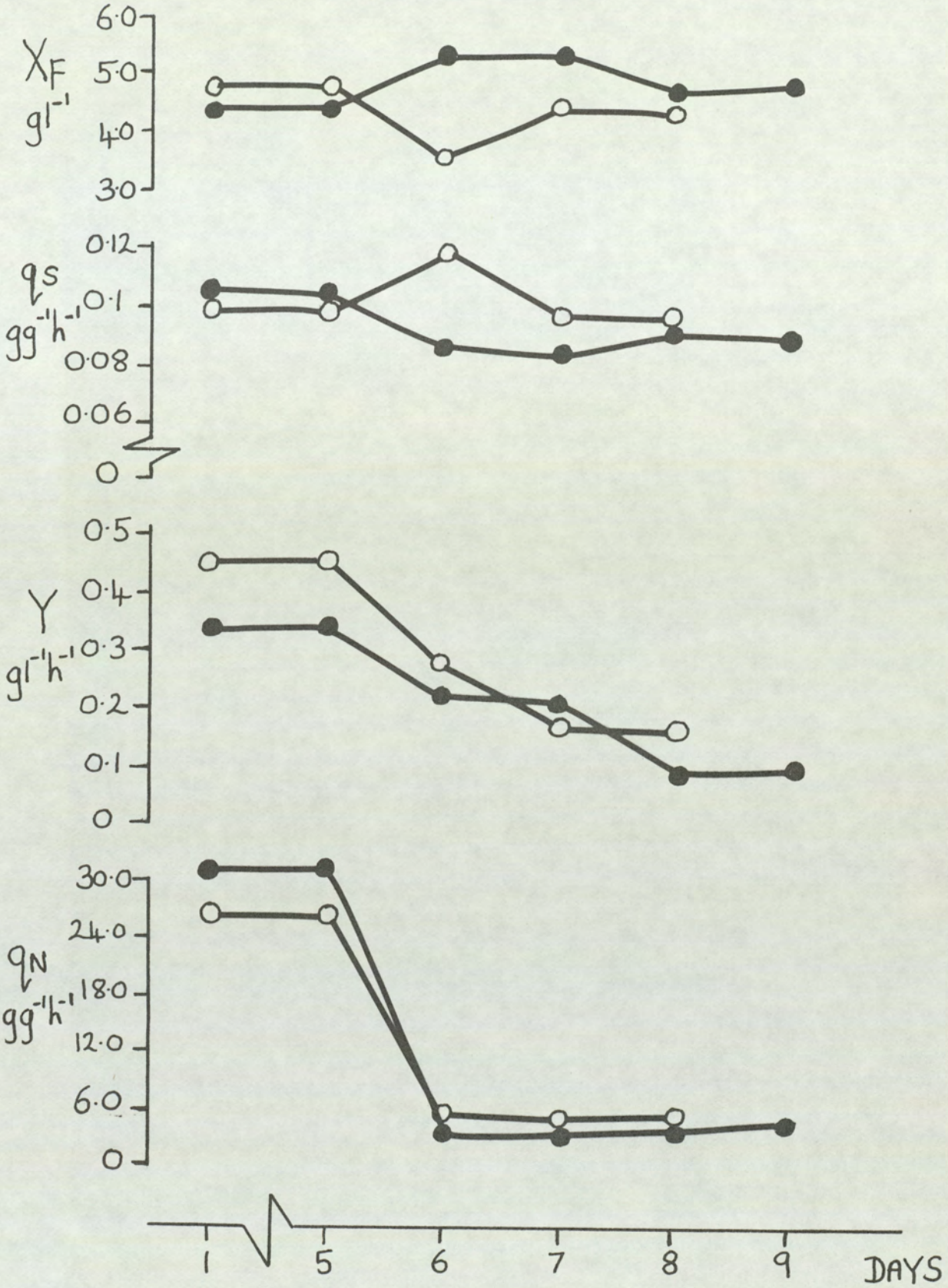


Fig 4.38. Response of A. ochraceus fermentation parameters (Fermenter biomass concentration, X_F, productivity, Y, specific carbon and nitrogen utilization rate, q_s, q_N) to shock combination limitation. $D = 0.1h^{-1}$.

○ low temp. ● high temp.

constant as in most other cases, but fermenter biomass concentration decreased and specific sucrose utilization rate consequently increased initially, before returning to their original values. This may be accounted for by the washing out of the loose floc morphology of A. ochraceus as opposed to the more sedimentary nature of the A. niger flocs under ferrocyanide limitation. Productivity and specific nitrogen utilization rate dropped to new levels ($0.16 g l^{-1} h^{-1}$ and $4.5 \times 10^{-4} g g^{-1} h^{-1}$ respectively) as previously observed. These experiments had therefore shown that no sporulation could be induced at low temperatures, even with shock nitrate limitation and sub-optimal pH levels. This is probably due to the low enzyme activity at these temperatures, and may also be due to greater tolerance by filamentous fungi of low temperatures. All experiments continued for many days at steady state after the points shown on the graphs and tables before deliberate termination of the fermentation, but no further sporulation occurred, and these periods were not therefore carefully recorded. While the combination of limitations succeeded in increasing sporulation intensity of A. niger, the spore production by A. ochraceus was lower than in other experiments. This may have been because nitrogen limitation at 38°C had less effect than at 28 - 32°C where utilization was maximum, and the restrictive effect of high temperature upon growth rate could not compensate for this.

4.8. Cyclic limitations

Sporulation had only occurred as a transient response to a

shock nitrate and gradual carbon limitation, and no culture was induced to spore for more than 5 - 6 days. In an attempt to overcome this problem and to at least achieve semi - continuous sporulation, optimum and limiting nutrient media were supplied cyclically to cultures of A. niger growing at a dilution rate of either $0.1h^{-1}$ or $0.2h^{-1}$. Once again, the basic nitrate shock limitation developed in section 4.2.2. was employed to induce sporulation. A culture of A. niger was allowed to reach steady state growth at : T = $30^{o}C$, pH = 3.0, Air = 1 v.v.m. $5.0gl^{-1}$ sucrose, standard salts, $0.21\ gl^{-1}$ nitrate atomic N. at either D = $0.1h^{-1}$ or D = $0.2h^{-1}$. The nitrate supply was then suddenly reduced to $0.021gl^{-1}$, while all other parameters remained constant and the growth rate and sporulation response monitored. This sporulation induction method proved successful and easily repeatable. When spore numbers declined, full medium was restored until an optimum (or near optimum) steady state was re-established, and then limitation was re-introduced, and so on. Originally, the limitation also involved a temperature increase to $38^{o}C$, and an increase of pH to 4.0, but these changes, when applied and removed frequently proved too disruptive to the growth of the organism and infection frequently resulted.

4.8.1. Cyclic conditions at $0.1h^{-1}$ dilution rate

The effects of these cycles at D = $0.1h^{-1}$ upon fermentation parameters are given in Table 28, and the responses of growth rate and sporulation are illustrated in Fig. 4.39. Table 28 gives details of the first two cycles in the sequence, while

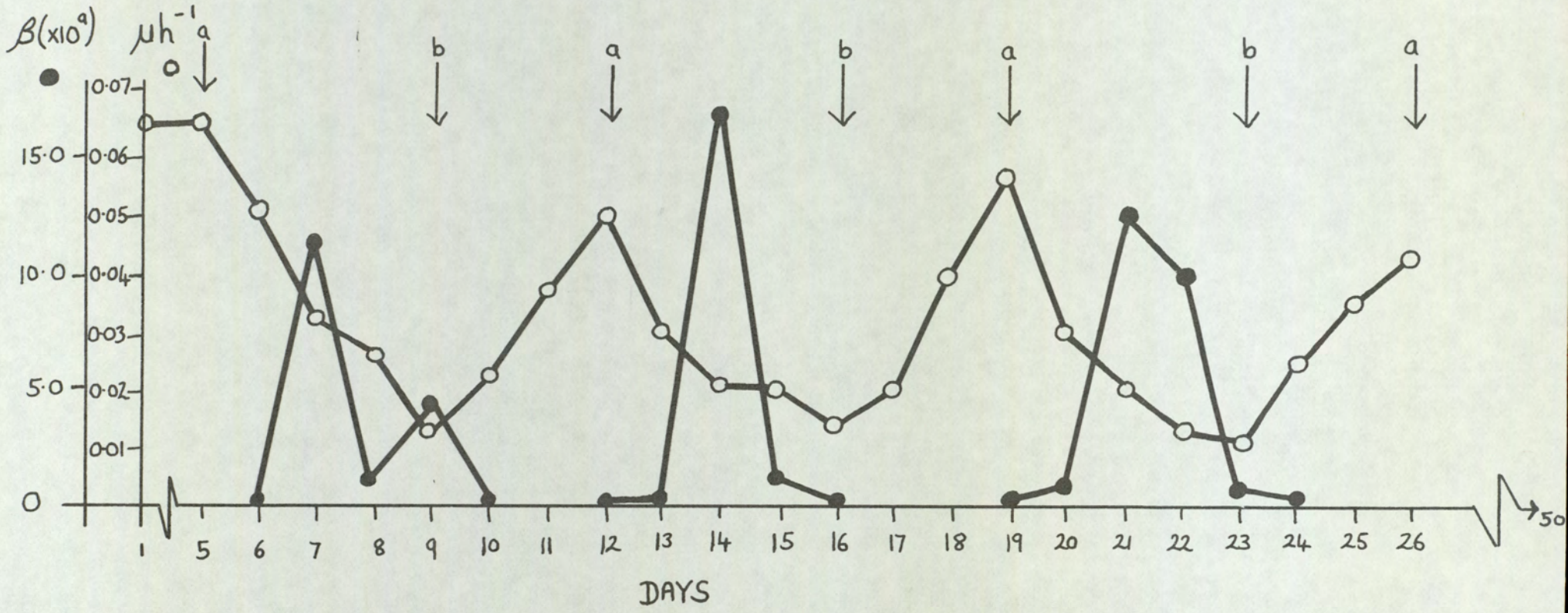


Fig 4.39. Growth rate (μ) and sporulation (β) of A. niger in response to repeated cycles of nitrate limitation (Nm 0.21 - 0.021gl^{-1}) followed by full-medium recovery.

Arrow (a) indicates point of nitrate limitation

" (b) " " " restoration of optimal medium

Fig. 4.39. illustrates 3½ cycles for convenience of space but it should be stressed that the sequence was twice run for 6 successful cycles and appeared to be able to run indefinitely. Characteristically of the nitrogen - limited system, growth rate immediately decreased upon the shock introduction of the limiting medium (there was no lag period) and in the first cycle the first spores were observed approximately 18 hours after the limitation was imposed, although in subsequent cycles spores were evident as soon as growth rate began to decline. As in other experiments, sporulation intensity rapidly reached a peak and then gradually declined, and when spore production ceased (or just before) the optimum medium was restored and growth rate recovered to optimum levels in approximately 3 days. As can be seen from Fig 4.39., the time for one complete cycle was 7 days. The floc morphology was loose filamentous strands (Type 1 - 2) and did not visibly alter at any point during the experiment. The mean maximum sporulation intensity was 8.3×10^{7} spores ml^{-1} (1.93×10^{10} spores g sucrose^{-1} utilized) which represented the highest and most efficient production observed in any experiment. A crude estimation of the production capacity of this system may be calculated :

On average, 2.5 days per cycle at mean production level.

$\therefore$ 60h. per week at 3.6×10^{7} spores ml^{-1}

Flow rate = 420.0 mlh^{-1} (at D = $0.1h^{-1}$ in 4.21 fermenter)

$= 1.5 \times 10^{10}$ spores h^{-1}.

= approx. 9×10^{11} spores $cycle^{-1}$ = spores $week^{-1}$.

All spores were produced from simplified structures of a single phialide on a hyphal tip, and a deep yellow pigment was

produced in medium and mould during each limitation phase, but at each recovery stage the mould resumed the normal beige colour.

The effect of the shock on other parameters :

Fermenter biomass concentration, (X_F) increased during nitrogen limitation and decreased during the return to optimum conditions before increasing again during the next limitation. This can only be explained by an increased density of the flocs, as no physical changes were apparent. Sucrose utilization was unaffected, as observed before, and the response of specific substrate utilization rate (qS) was therefore a mirror image of that of X_F. Effluent biomass concentration and productivity decreased during each limitation, and returned to the normal level during each recovery phase. The same trend was exhibited by the yield (Ysub, gg^{-1}), but these trends would be expected, because although substrate utilization remained constant, the amount used for biomass productivity (increase in mycelial mass) during a nutrient limited period, decreases. The energy is required for the energy - consuming sporing process, storage compounds in spores and other secondary metabolic functions which are associated with a decreased growth phase. This change in utilization of the energy source is discussed further in section 5. This system was therefore successful for semi - continuous spore production, and produced a high spore yield and removed the requirement for the lag period between decrease in growth rate and spore production. In an attempt to increase spore production in terms of spores per week, the same system was run at $D = o.2h^{-1}$.

4.8.2. Cyclic conditions at $D = 0.2h^{-1}$

The effect of identical oscillations of nutrient supply at a dilution rate of $0.2h^{-1}$ is shown in Table 29. Growth rate followed similar fluctuating responses to those at $D = 0.1h^{-1}$, but at higher values; $0.165h^{-1} \longrightarrow 0.068h^{-1} \longrightarrow 0.13h^{-1} \longrightarrow 0.063h^{-1}$ etc. Consequently, no sporulation was induced in this system as a result of the interaction of several factors preventing sporulation for which the high dilution rate was ultimately responsible. The high growth rates support the theory that in a certain system there is a critical growth rate above which growth is vegetative, but this fact alone was not responsible for repression of sporulation. At this dilution rate, nitrogen limitation caused a significant decrease in sucrose utilization, dS, ($4.58gl^{-1}$ to $2.7\ gl^{-1}$) which probably limited the availability of energy required for sporulation as discussed in the previous section. The floc morphology at this dilution rate was filamentous flocs with well defined centres (Type 4) which did not alter through the experiment, and which were less desirable than loose filaments for promoting submerged sporulation.

Table 29 lists the results from two complete cycles at $D = 0.2h^{-1}$, although the experiment was repeated for 4 cycles which did not induce sporulation. Effluent biomass concentration, productivity and fermenter biomass concentration responded to the shocks

in a similar manner to those at D = $0.1h^{-1}$, and as sucrose utilization decreased while fermenter biomass concentration increased, specific sucrose utilization rate showed a significant decrease during limitation. The yield coefficient (Y_{sub_s}) increased during limitation, which was opposite to that at D = $0.1h^{-1}$, but this may be explained by the fact that all available energy was still being used for biomass production at this growth rate, (none for sporulation) and if nitrogen limitation was imposing a restriction upon carbon utilization, the efficiency of conversion was probably increased to compensate for this. This could be done by temporarily ' shutting off' some pathways of oxidiation and diverting the carbon source towards pathways for incorporation into structural units. This would result in an increased yield of biomass per g. sucrose utilized.

An increased dilution rate was not therefore a solution to increasing the rate of spore production and improving the yield from the sucrose supplied. If required, the spore production could be increased by increasing the number of fermenters or, preferably, by increasing the size of the fermenter, although spores would be produced at the efficiency of sucrose conversion observed.

4.9 Two - stage fermentations

Maxon (1960) stated that the solution to a continuous process where conditions optimum for product formation do not equal those for vegetative growth is the development of a multi - stage fermentation system. He added that one can only expect

better control of such a system using a continuous fermenter, and not increased productivity of the non growth - associated products. As discussed in section 1.5., spore production may be considered equivalent to production of a non - growth associated metabolite and, as for any product involving the inhibition of growth, spore production must be examined in a continuous two - stage system. In a single stage continuous system, the equilibrium between environments inducing vegetative growth and sporulation had proved difficult to maintain and only transitory sporulation had been achieved. This was not surprising considering the differing optimum requirements of vegetative growth and sporulation which could not both be supplied at a steady state in a single stage fermenter. As the physiological state of sporulation represents a product resulting from extensive metabolic transformation, the application of a two - stage system for spore production was examined, based upon the principle of mycelial production in the first stage and sporulation induction in the second.

A multistage system may be developed in two ways :

a) Partitioning of one vessel into different sections.

b) Increasing the number of vessels.

The multistage tower fermenters described by Prokop et al. (1969), Falch and Gaden (1969) Kitai et al. (1969) and Paca and Gregr (1976) belong to the first category. These are one - piece towers divided into compartments by perforated plates through which air and medium flow concurrently from bottom to top at sufficiently high rates to prevent backflow.

Each section or 'stage' has its' own stirrer and environmental conditions (dilution rate governed by the volume of the section) and the overall system represents a series of stirred tank reactors stacked on top of one another. The individual control of conditions allows selection of different phases of the batch growth curve in different sections, and the system may therefore be used for both growth and non - growth associated antibiotic production, and for single - cell biomass production. However, the requirement for passage of organisms through the perforated plates makes this system impractical for filamentous fungi, which are restricted to the second category of multistage systems. Pirt and Callow (1959) suggested the use of a two - stage fermentation system for the continuous production of penicillin, due to the pH separation of the phases of growth and antibiotic production. Pencillium chrysogenum had an optimum pH for growth of 6.7, while maximum penicillin production occurred at pH 7.4, and therefore the two stages proposed were for growth at pH 6.7 providing a continuous supply of mould for penicillin production at pH 7.4. Ezhov & Luzina (1978) suggested the use of a two stage system for production of extracellular ribonuclease by Penicillium brevi - compactum, as production was found to occur just prior to and during the stationery growth phase in batch culture. Callow and Pirt (1961) described the design of a laboratory two - stage continuous flow apparatus, and stated that most two - stage systems require a longer residence time in the second stage, as product formation usually takes longer than growth, and thus

the second stage is usually larger than the first. If the dilution rate is fixed for the first stage, then dilution rate in the second stage may only be altered by changing the volume of the vessel or by the addition of another inflow. Fencl et al. (1972) warned that the greater the difference between cultural conditions and residence time of the two vessels, then the more difficult were predictions of the course of fermentation. The two stages used in this system were the 4.2 litre (Stage 1) and 10.0 litre (Stage 2) fermenters described in Section 2.1. The outflow from tower 1 was fed directly to the base of tower 2 (Fig.4.40) and it was proposed to use the first fermenter for biomass production under optimum steady state conditions, and the second fermenter for the restriction of growth - rate and sporulation induction by provision of steady state unfavourable environments. In some experiments, the second stage received an additional nutrient supply, but in others, the flow rate through both vessels was the same.

P.T.O.

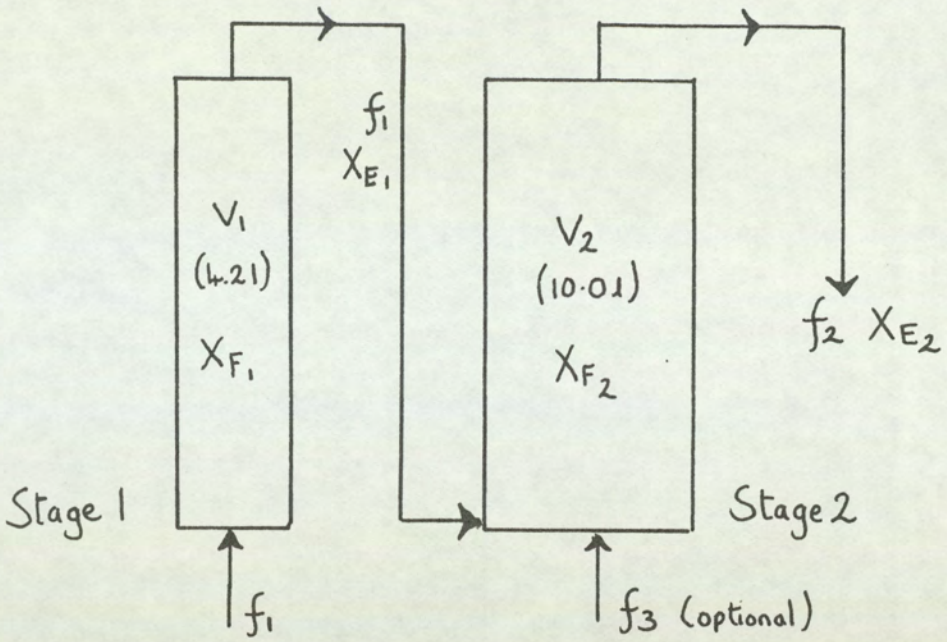


Fig. 4.40. The two stage fermentation system, where :

V_1 = volume of stage 1, (litres). V_2 = volume of stage 2, (litres)

X_{F_1} = fermenter biomass concentration of Stage 1 (gl^{-1})

X_{F_2} = fermenter biomass concentration of Stage 2 (gl^{-1})

X_{E_1} = effluent biomass concentration of Stage 1 (gl^{-1})

X_{E_2} = effluent biomass concentration of Stage 2 (gl^{-1})

$f1$ = Flow rate through stage 1 ($1h^{-1}$)

$f2$ = Flow rate through stage 2 ($1h^{-1}$)

$f3$ = Optional flow rate into stage 2 ($1h^{-1}$)

The growth rate in stage 1 was calculated as described in section 2.6.1. (equation 5). For growth rate in the second stage (μ_2) the law of conservation still applies ;

(with reference to notations in Fig. 4.40)

Input by flow + Input by growth = output by flow.

$$f1 \cdot X_{E_1} + f3 \cdot 0 + \mu_2 \cdot V_2 \cdot X_{F_2} = f2 \cdot X_{E_2}$$

$$\therefore \mu_2 = \frac{f2 \cdot X_{E_2} - f1 \cdot X_{E_1}}{V_2 \cdot X_{F_2}}$$

$$\text{as } D_2 = \frac{f2}{V_2}, \qquad \mu_2 = \frac{D_2 X_{E_2} - f1 \cdot X_{E_1} / V_2}{X_{F_2}} \quad (15)$$

This equation was also used by Ricica et al. (1967) for calculation of growth rate in a second stage when $f1 \neq f2$ (i.e. $f3$ positive). Where $f1 = f2$ (i.e. $f3$ zero) the same equation applies, but may be simplified :

$$\mu_2 = \frac{D_2 X_{E_2} - \frac{f2 X_{E_1}}{V_2}}{X_{F_2}} = \frac{D_2 (X_{E_2} - X_{E_1})}{X_{F_2}} \quad (16)$$

Productivity of the second stage (Y) = $D_2 (X_{E_2} - X_{E_1})$

Yield coefficient of the second stage ($Ysub_s$ or $Ysub_N$) = $\frac{X_{E_2} - X_{E_1}}{dS}$ or $\frac{X_{E_2} - X_{E_1}}{dN}$

4.9.1. Temperature alteration to the second stage.

A. niger was used in all two - stage experiments and sufficient time was allowed for the parameters of the second stage to reach steady - state before limitations were imposed. In this series of experiments, mycelium was supplied from vessel 1, where it was cultured at $D = 0.1h^{-1}$, $T = 30^{o}C$, pH = 3.0, Air = 1 v.v.m., $Sm = 5.0gl^{-1}$ sucrose, $Nm = 0.21gl^{-1}$ nitrate atomic N. and standard salts. The mould passed directly into the second vessel which had no nutrient supply, $T = 30^{o}C$, pH = 4.0, Air = 1 v.v.m. ($4.21h^{-1}$ from vessel 1, + $5.81h^{-1}$ supplied separately to stage 2) and $D = 0.042h^{-1}$ $\frac{f2}{V_2} = \frac{0.42\ 1h^{-1}}{10.0\ 1.}$

When a steady - state in both towers was established at 30°C, samples were taken over a period of 4 days, before the temperature of the second stage was altered to 20°C, 40°C, and 43°C allowing establishment of a new steady - state at each temperature. The results are presented in Table 30. Second stage growth rate was low ($0.007h^{-1}$ at 20°C, $0.01h^{-1}$ at 40°C) or zero (at 30°C and 43°C) which was expected with no nutrient supply, but sporulation was not stimulated. The mean sucrose and nitrogen supplies to the second stage were equal to the effluent concentrations from vessel 1 :

20°C	$\bar{S}m$	=	$0.43\ gl^{-1}$	$\bar{N}m$	= $0.092\ gl^{-1}$
30°C	$\bar{S}m$	=	$0.22\ gl^{-1}$	$\bar{N}m$	= $0.114\ gl^{-1}$
40°C	$\bar{S}m$	=	$0.37\ gl^{-1}$	$\bar{N}m$	= $0.085\ gl^{-1}$
43°C	$\bar{S}m$	=	$0.48\ gl^{-1}$	$\bar{N}m$	- $0.08\ gl^{-1}$

These were not utilized in the second stage, which was probably the reason for the lack of sporulation, due to the lack of an adequate energy source. These experiments demonstrated both the importance of an adequate nutrient supply and a sufficiently high growth rate for sporulation to occur in continuous culture. In all cases when growth rate reached zero in the second stage, mould autolysis occurred.

4.9.2. Variable sucrose supply to the second stage

The conclusion from experiment 4.9.1. was that the lack of an energy source prevented sporulation. A series of experiments were therefore conducted in which various sucrose concentrations

were continuously supplied to stage 2 to supplement the nutrients in the effluent from stage 1. Again, vegetative mycelium was grown under optimum conditions in stage 1 ($T = 30^{o}C$, $D = 0.1h^{-1}$, pH = 3.0, Air = 1 v.v.m., $Sm = 5.0\ gl^{-1}$ sucrose, $Nm = 0.21\ gl^{-1}$ nitrate and standard salts) and continuously supplied to stage 2. ($T = 30^{o}C$, pH = 4, Air = 1 v.v.m.). The supply (ƒ3) to stage 2 contained only sucrose (no nitrogen or salts) at a flow rate of $0.58\ lh^{-1}$. When combined with the flow from stage 1 ($0.42\ lh^{-1}$) this gave an overall flow rate of $1.0\ lh^{-1}$, giving a dilution rate of $0.1h^{-1}$. The steady-state results of both stages when 5.0, 4.0, 3.0 and 2.0 gl^{-1} sucrose were supplied to stage 2 are presented in Table 31.

4.9.2.1 5.0 gl^{-1} sucrose concentration.

Originally, once the two stages achieved steady state, the organism grew adequately in both stages without any sign of sporulation. When the media were analysed, the effluent of stage 1 was found to contain an average of 0.35 gl^{-1} sucrose and 0.08 gl^{-1} nitrate. Thus the second stage was continuously supplied with 5.35 gl^{-1} sucrose (5 + 0.35) and 0.08 gl^{-1} nitrate N, which would normally be expected to support adequate growth at $0.1h^{-1}$ dilution rate. (A reduction in nitrate concentration from 0.21 gl^{-1} to 0.08 gl^{-1} was shown to be ineffective for sporulation induction in experiments 4.2.2. and 4.6.4.) To overcome this problem the nitrate supply to stage 1 was reduced to $0.1525gl^{-1}$, a level just above that of average utilization under optimum conditions at a dilution rate of

$0.1h^{-1}$. When the 2 - stage fermentation reached steady state, stage 1 was unaffected and growth was still optimal, but analysis of the effluent showed that $0.3gl^{-1}$ sucrose, and only $0.025\ gl^{-1}$ nitrate were present. Hence, the second stage received $5.3gl^{-1}$ sucrose (5 + 0.3) and $0.025gl^{-1}$ nitrate at a dilution rate $0.1h^{-1}$, which was similar to nutrient levels used for sporulation induction in single - stage cultures. As a result, sporulation continually developed in the second stage with a mean spore production of $3.22 \times 10^{7} ml^{-1}$. ($\beta = 7.16 \times 10^{9}$ spores g mycelium^{-1}) although the numbers varied by a factor of 10. The development time for spores was difficult to estimate in a culture with a complete range of sporulation development, but the period taken for spore formation had to be less than the residence time in the second stage, which was 10 hours.

Spore production was continuous, and the culture appeared to have settled at a steady state at a growth rate of $0.042h^{-1}$ and sucrose and nitrogen utilization of 4.73 and $0.016gl^{-1}$ respectively. The efficiency of production, 3.4×10^{9} spores g sucrose^{-1} utilized, was lower than that in the cycled limitation of experiment of section 4.8.1. because both carbon supplies to both stages had to be taken into account.

The floc morphology of A. niger in stage 2 was large loose yellow/brown flocs with dense centres (Type 3 - 4) and remained constant throughout the experiment. Spores were produced primarily from simplified structures of single phialides on hyphal tips, but there were some undeveloped sub - aerial structures present at all times. Sporulation

continued for 3 weeks, after which time the fermentation was terminated although the situation appeared tobe stable and sporulation would have continued if time had allowed. There was a gradual build - up of bacterial and yeast infection in stage 2 and it was anticipated that this would be the factor to cause the eventual end of sporulation after several weeks.

4.9.2.2 $4.0gl^{-1}$ sucrose concentration

This experiment was conducted identically to the previous one, except that stage 2 was continuously supplied with 4.0 gl^{-1} sucrose. At steady state the mean sucrose and nitrogen concentrations of the stage 1 effluent were 0.25 and 0.036 gl^{-1} respectively, and thus the mean principal nutrient supply to stage 2 was $4.25gl^{-1}$ sucrose (4 + 0.25) and 0.036 gl^{-1} nitrate N. The mean growth rate in stage 2 was $0.039h^{-1}$, and steady state sporulation was again induced at an average intensity of 8.0×10^{6} spores ml^{-1} ($\beta = 1.8 \times 10^{9}$ spores g^{-1} mycelium). This represented an efficiency of 1.07×10^{9} spores g $sucrose^{-1}$ utilized, which indicated that spore production was lower, at a lower efficiency than when $5.0gl^{-1}$ sucrose was supplied to stage 2. This may have been due to either the lower energy substrate supply, or the higher nitrate availability due to the lower mean utilization by the mould in stage 1.

Floc morphology and reproductive structures were as in experiment 4.9.2.1. and production appeared to be indefinite, although there was a gradual build - up of infection once again.

4.9.2.3 $3.0gl^{-1}$ sucrose concentration

The experiments were repeated with a sucrose supply of $3.0gl^{-1}$ to the second stage which permitted a mean growth rate of A. niger of $0.044h^{-1}$ when at steady state. An average of $2.52gl^{-1}$ sucrose was utilized in stage 2 from the $3.5gl^{-1}$ (3.0 + 0.5) supplied, and an average $0.029gl^{-1}$ nitrate of the $0.0325gl^{-1}$ supplied was utilized. Some spores were induced on simplified structures only, but numbers were too few to count, which appeared to be due to the lack of an energy source rather than high nitrate source, as only $0.0325gl^{-1}$ nitrate atomic N was supplied from stage 1.

4.9.2.4 $2.0\ gl^{-1}$ sucrose concentration.

At steady state, with a supplementary supply of $2.0gl^{-1}$ sucrose, the mean growth rate in stage 2 was $0.04h^{-1}$. $1.42gl^{-1}$ of the $2.25\ gl^{-1}$ sucrose supplied were utilized, and $0.018\ gl^{-1}$ nitrate were utilized from the $0.0345gl^{-1}$ supplied in the effluent from stage 1. No sporulation was induced over a per iod of 10 days, and the floc morphology differed from previous experiments in that it was loosely filamentous (Type 1) and hence more easily removed from the fermenter, which accounted for the low fermenter biomass concentration during these experiments.

These results indicate a relationship between medium carbon source concentration and the ability of A. niger to sporulate during nitrogen limitation at a constant growth rate.

This is discussed further in section 5.

4.9.3. Comparison of cyclic and two - stage sporulation efficiency.

Although sporulation was continuous in the two - stage system, it was stated that production efficiency was lower than that of the nitrogen limited cycle system in one stage. However, if the number of spores per week from the continuous system are compared with the number per cycle (= 1 week) of the one - stage system, it can be seen that this is not the case;

Mean production in $5.0gl^{-1}$ 2-stage system = 3.22×10^{7} spores ml^{-1}

$f = 1.01h^{-1}$ $\therefore$ 3.22×10^{10} spores h^{-1} produced.

In 1 week, $\therefore$, $7.24.3{\cdot}22 \times 10^{10}$ spores produced.

$= 5.41 \times 10^{12}$ spores $week^{-1}$

From section 4.8.1., at $0.1h^{-1}$ dilution rate, 9.0×10^{11} spores $week^{-1}$ produced from nitrate limitation cycle.

During the cycle, an average $4.5gl^{-1}$ sucrose is utilized, at a flow rate of $0.42lh^{-1}$

$\therefore$ 4.5 . 0.42 . 24.7 g sucrose utilized in 1 week

= 317.5 g

During the 2-stage fermentation;

Stage 1 utilized an average $4.7gl^{-1}$ sucrose at a flow rate of $0.42lh^{-1}$

Stage 2 utilized an average $4.73gl^{-1}$ sucrose at a flow rate of $0.58lh^{-1}$

$\therefore$ total utilization = $4{\cdot}7.0{\cdot}42.24.7 + 4{\cdot}73.0{\cdot}58.24.7$

= 792.5 g sucrose utilized in 1 week.

Therefore, by cyclic methods, in 1 week 9.0×10^{11} spores were produced from 317.5 g sucrose utilized. Overall efficiency = 2.83×10^{9} spores g sucrose^{-1}.

by 2 - stage methods, in 1 week 5.41×10^{12} spores were produced from 792.5 g sucrose utilized. Overall efficiency = 6.83×10^{9} spores g sucrose^{-1}.

Thus the 2 - stage system is shown to be more than twice as efficient as the cyclic system over a period of one week when run under the conditions stated.

4.9.4. Potassium ferrocyanide supply to the second stage.

Table 32 shows the results of supplying different concentrations of potassium ferrocyanide continuously to the second stage of the 2 stage system. The principles of the experiment were identical to those of the previous sections. Vegetative mycelium was supplied from the 4.2 litre first stage under optimum conditions ($T = 30^{o}C$, pH = 3.0, Air = 1 v.v.m. $D = 0.1h^{-1}$, $Sm = 5.0gl^{-1}$ sucrose, $Nm = 0.21gl^{-1}$ nitrate N, and standard salts) to the second stage at $T = 30^{o}C$, pH = 4.0, Air = 1 v.v.m. and a feed of ferrocyanide solution only at a rate of $0.1lh^{-1}$.

The dilution rate of the second stage was therefore

$$\frac{f1 + f2}{V_2} = \frac{0.42 + 0.1}{10} = 0.052h^{-1}$$

The culture was initially allowed to establish a steady state growth pattern with distilled water added to stage 2 in place of ferrocyanide. (This maintained a constant dilution rate in

stage 2 throughout the experiment). Growth rate in the second stage was low ($0.0072h^{-1}$) as expected with a principal nutrient supply of only $0.4gl^{-1}$ sucrose and $0.0425gl^{-1}$ N from the effluent of stage 1.

4.9.4.1. Addition of 10^{-5}M potassium ferrocyanide.

The results for this part of the experiment were not included in Table 32, as there was no visible effect upon the mould when the distilled water supply was replaced by one of 10^{-5}M potassium ferrocyanide, and there was virtaully no change in fermentation parameters over a period of 10 days.

4.9.4.2. Addition of 10^{-4}M potassium ferrocyanide.

When the 10^{-5}M ferrocyanide was replaced by the 10^{-4} concentration, spores were observed in stage 2 after approximately 36 hours. The addition of ferrocyanide at this concentration appeared to stimulate the growth rate of A. niger in two replicates of this experiment, which was unexplained. The trace metal complexing activity of the ferrocyanide ion may have been negligible due to the low levels of traces entering the second stage, and the potassium may have stimulated growth sufficiently for sporulation to occur, but this would be an unusual effect of what is normally considered a growth inhibitor. $0.105gl^{-1}$ of the $0.51gl^{-1}$ sucrose supplied from stage 1 were utilized as were $0.013gl^{-1}$ of the $0.0455gl^{-1}$ nitrate supplied. This low nutrient utilization and low growth rate caused the low spore production of 7.1×10^5 spores ml^{-1} ($\beta = 4.6 \times 10^7$ spores g $mycelium^{-1}$). The mould turned an olive green/blue colour in

the second stage, and the culture appeared to stabilise at a steady state and sporulation was continuous from simplified single phialide on hyphal tip structures. Infection was absent from the culture after 17 days, when the fermentation was deliberately terminated due to lack of time, but spore production was too low for this system to be considered competitive with the cyclic and sucrose supplemented 2 - stage system discussed previously.

4.9.4.3. Addition of 10^{-3}M potassium ferrocyanide.

This experiment was identical to those previously described, except that 10^{-3}M potassium ferrocyanide was supplied. Approximately 12 hours after the addition of this highest ferrocyanide concentration and the removal of the distilled water supply, spores were observed in the second stage. However, sporulation intensity was low (4.1×10^{5} spores ml^{-1}, $\beta = 1.1 \times 10^{8}$ spores g mycelium^{-1}), and only occurred for two days, after which time no further sporulation was seen. The culture then stabilised at a steady state (growth rate = $0.0044h^{-1}$) utilizing $0.45gl^{-1}$ of the $0.5gl^{-1}$ sucrose and $0.016gl^{-1}$ of the $0.0465gl^{-1}$ nitrate supplied in the effluent from stage 1. This effect was observed in two further repeats, and appears to represent a shock response to the ferrocyanide supply, as there was no subsequent steady state sporulation. The inhibition of sporulation may again have been due to the relatively high nitrogen availability ($0.0465gl^{-1}$) and the low growth rate induced, although why the initial shock should not apply to all new mycelium entering stage 2 was unclear.

4.10. Additional fermentations

The applicability of the basic single - stage nitrate - limited system to the induction of sporulation in other fungal genera was examined. The experiments were merely guides as to whether fungi other than the Aspergilli could be induced to spore by shock removal of nitrate from a culture growing under optimum conditions, and as such, the experiments were not analysed, and no data produced.

As Penicillium javanicum was being used in the study of whey and lactose permeate effluent treatment by Ewen (1980) in the same laboratories, this organism was tested first. Mycelial samples taken from a working fermenter were used as an inoculum, and the organism was grown under optimum conditions (pH = 3.0, T = $30^{o}C$, Air = 1 v.v.m, D = $0.1h^{-1}$, Sm = $5.0gl^{-1}$ sucrose, Nm = $0.21gl^{-1}$ nitrate atomic N + standard salts). It took 7 days for the organism to adapt from growth on lactose to growth on sucrose and for it to establish approximately steady state growth, and after this time lag, the nitrate concentration of the medium was suddenly decreased. The development of sporulation structures occurred after approximately 18 hours and spores were present after 24 hours. All structures closely resembled those produced sub - aerially, consisting of a conidiophore, phialides and spores, although no spore chains were observed due to medium agitation. Sporulation intensity was low (5×10^{5} spores ml^{-1} after vigorous agitation) and continued for four days before all spores and sporing structures were removed from the vessel. The mould stabilised at a steady state after this time and no further sporulation was observed.

The second organism tested was the insect pathogen, Verticillium lecanii. Hall and Burges (1977) reported the use of conidia and blastospores of this organism as biological agents in the control of aphids on glasshouse chrysanthemum crops. When the spores were sprayed over the crop (at 5×10^6 - 1×10^8 spores ml^{-1}) they were picked up by the aphids, germinated, penetrated the insects' body wall, and caused death by growing throughout the body cavity. Blastospores and conidia were equally effective in the control of the aphids (Hall, pers. comm.) but blastospores had a short shelf - life and were therefore less suitable for the development of commercial spore solutions. Although submerged batch fermentations had been used for the production of spores, only blastospores were produced in submerged culture, and the more practical conidia could only be produced on inoculated grain plates which was a time - consuming method. The organism was therefore grown in the tower fermenter to study the type of spore induced by nitrate limitation, and to evaluate the potential application of the system to meet the requirements of mass spore production for crop treatment. The organism grew poorly in continuous culture, and took more than two weeks before an adequate vegetative stage developed. The mould grew in thin, loose, filamentous flocs (Type 1) which resulted in a low fermenter biomass concentration both before and after nitrate limitation. The shock removal of the nitrogen source caused the segmentation of the hyphae in the characteristic method of blastospore formation and after 24 hours almost all the hyphae had divided into blastospores of various size. Production of blastospores resulted

in the termination of mycelial growth and consequently no further biomass was produced and the spores were gradually washed from the fermenter and the fermentation ended. This experiment was repeated, with the same result, which indicated that the system operated in a similar way to a batch fermentation in that most of the mycelial content of the vessel, formed under optimum conditions, divided to form blastospores when a particular nutrient became limiting (in this case nitrogen). This meant that continuous spore production was impossible in one stage cyclic systems as no mycelial phase was left for recovery and growth after nutrient limitation, but a two stage system of growth followed by spore formation appeared feasible. However, only blastospores were produced, and there was no sign of the characteristic conidial formation structures of verticillium, although these would have been difficult to identify in submerged culture. The spores produced in this system were not therefore those most suited to use as commercial biological control agents.

5.0. DISCUSSION AND CONCLUSIONS

The successful production of fungal spores requires a detailed knowledge of the growth characteristics and physiology of the fungus concerned, and one aspect of the differentiation process of A. niger and A. ochraceus that was revealed by this research was the complex interaction between growth and sporulation. It has been generally accepted that fungal differentiation is stimulated by factors which restrict vegetative growth (Smith et al. 1977a). The two phases of growth and sporulation, while antagonistic, are not necessarily incompatible. Vegetative growth and sporulation should perhaps be considered as cellular processes competing for limiting metabolic intermediates rather than as mutually exclusive phenomena (Smith et al. 1977a). As growth and sporulation do not normally occur simultaneously, they can be considered as separated by metabolic shifts (i.e. a change from vegetative metabolism to sporulative metabolism) caused by a change in an environmental factor, although sporulation must be considered as an integration of several biological factors. In contrast to the theory that growth and sporulation are mutually exclusive, their simultaneous occurrence was essential for the successful operation of the continuous system. The organism in question had to maintain a certain growth rate in order to prevent death and subsequent onset of autolysis, and to prevent wash - out from the fermenter.

The need for an adequate growth rate of the organism, (as calculated by an increase in mycelial dry weight) for sporulation to occur was demonstrated by several experiments. After the

addition of ferrocyanide to a nitrogen - limited culture (4.6.3.) the growth rate decreased to zero, sporulation ceased and autolysis began. During fermentations in which the growth rate fell below $0.002h^{-1}$ (4.1.4, 4.9.1.) mould death and wash - out occurred before sporulation was induced.

The results summarised in table 5.1. show that sporulation occurred over a wide range of organism specific growth rates and it was not possible to define a specific growth rate below which sporulation was induced in every system. This supports the view of Dawes and Mandelstam (1970) that there is no overall threshold effect of growth rate upon sporulation. Rather, for each particular system there appeared to be a critical growth rate above which vegetative growth occurred and below which sporulation could be induced. In the system used, dilution rate governed the incidence of sporulation, and several authors have reported clear correlation between increased sporulation and decreased growth rate, and hence dilution rate (Dawes and Thornley, 1970, Ng et al. 1973b). However, Larmour & Marchant (1977) reported that increased growth rate led to increased conidium production by Fusarium culmorum. While sporulation intensity at various dilution rates was not examined, the results throughout this project suggested that sporulation intensity increased as dilution rate decreased.

Sporulation appeared to be a shock response to a sudden decrease in the growth rate of the organism (due to nitrogen limitation) and reached peak intensity during the phase of decreasing growth rate, after which spore production gradually ceased as the growth

TABLE 5.1. Comparative data from experiments that induced sporulation.

	Specific carbon supply rate Q_s $gg^{-1}h^{-1}$	Specific carbon utilization rate q_s $gg^{-1}h^{-1}$	Specific nitrogen supply rate Q_N $gg^{-1}h^{-1}$	Specific nitrogen utilization rate q_N $gg^{-1}h^{-1}$	Maintenance coefficient m $gg^{-1}h^{-1}$
			A. NIGER		
4.1.2. Gradual sucrose	0.071	0.026	0.015	0.01	0.0185
4.1.3. Gradual sucrose	0.039	0.039	0.016	0.0023	0.016 (Schulze) 0.016 (Pirt)
4.1.3. Gradual glucose	0.04	0.044	0.0085	0.0004	0.0123 (Schulze) 0.043 (Pirt)
4.1.4. Gradual starch	0.044	0.042	0.016	0.0017	0.0383 (Schulze) 0.014 (Pirt)
4.2.2. Shock nitrate	0.078	0.07	3.9×10^{-4}	3.5×10^{-4}	-
4.6.2. Ferrocyanide + nitrate shock	0.166	0.119	7×10^{-4}	5.66×10^{-4}	-
4.6.3. Nitrate shock + ferrocyanide	0.151	0.11	6.34×10^{-4}	3.14×10^{-4}	-
4.7.1. Combination limitation	0.045	0.04	1.9×10^{-4}	1.82×10^{-4}	-
4.8.1. Cycles at D = 0.1	0.07	0.07	3.2×10^{-4}	3.1×10^{-4}	-
4.9.2.1. 2-stage + 5.0g sucrose	0.115	0.103	5.43×10^{-4}	3.6×10^{-4}	-
4.9.2.2. 2-stage + 4.0g sucrose	0.095	0.061	8×10^{-4}	7.5×10^{-4}	-
4.9.4.2. 2-stage + ferrocyanide	0.01	0.002	8.7×10^{-4}	2.4×10^{-4}	-
			A. OCHRACEUS		
4.2.3. Shock nitrate	0.143	0.11	6×10^{-4}	5.6×10^{-4}	-
4.2.4. Shock nitrate + glucose	0.13	0.119	5.47×10^{-4}	3.3×10^{-4}	-
4.7.2. Combination limitation	0.12	0.104	5×10^{-4}	4.95×10^{-4}	-

TABLE 5.1. (continued)

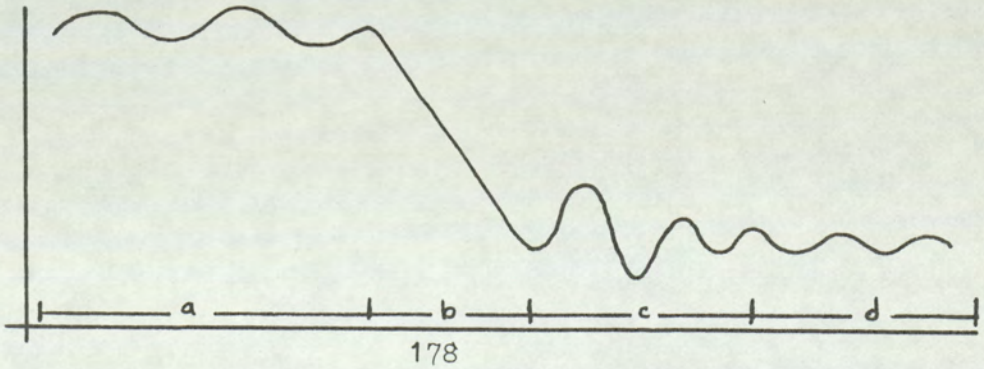
	Growth rate at sporulation $\mu\ h^{-1}$	Growth rate prior to sporulation $\mu\ h^{-1}$	Time of spore development h	Mean number spores produced spores ml^{-1}	Efficiency of spore production spores g substrate^{-1}
			A. NIGER		
4.1.2 Gradual sucrose	0.025	0.036	12	-	-
4.1.3. Gradual sucrose	0.01 - 0.028	0.04	12	-	-
4.1.3. Gradual glucose	0.008 - 0.05	0.037	12	4×10^6	4×10^9
4.1.4. Gradual starch	0.008 - 0.0287	0.074	60 - 72	-	-
4.2.2. Shock nitrate	0.029	0.058	12 - 48	4×10^6	9.9×10^8
4.6.2. Ferrocyanide + nitrate shock	0.02	0.076	<24	6×10^6	1.14×10^9
4.6.3. Nitrate shock + ferrocyanide	0.01 - 0.0003	0.069	24 - 36	6×10^5	1.65×10^8
4.7.1. Combination limitation	0.022	0.043	30	5×10^7	1.1×10^{10}
4.8.1. Cycles at D = 0.1	various	0.066	18h	max 8.3×10^7 3.6×10^7	max 1.93×10^{10} 8×10^9
4.9.2.1. 2-stage + 5.0g sucrose	0.042	0.056	<10	3.22×10^7	3.4×10^9
4.9.2.2. 2-stage + 4.0g sucrose	0.039	0.042	<10	8×10^6	1.07×10^8
4.9.4.2. 2-stage + ferrocyanide	0.01	0.041	<19	7.1×10^5	1.44×10^8
			A. OCHRACEUS		
4.2.3. Shock nitrate	0.015	0.052	18	3.6×10^7	1.15×10^{10}
4.2.4. Shock nitrate + glucose	0.04	0.064	36	4×10^6	8.3×10^8
4.7.2. Combination limitation	0.02	0.07	24	1×10^7	2.2×10^9

rate oscillated and eventually settled to a new steady state. Sporulation was not induced by the gradual restriction of growth rate by nitrogen limitation, while only gradual restriction by carbon source limitation induced sporulation.

Righelato et al. (1968) stated that the growth rate of P. chrysogenum prior to the onset of sporulation affected the subsequent rate of spore formation. The data in table 5.1. show that there was no correlation between original growth rate and spore production time in nitrogen - limited cultures, but the results from carbon - limitation experiments suggest that a high original growth rate prolongs spore development time.

Another feature of growth in continuous culture observed throughout the project was that, in common with previous workers in the fermentation laboratories, when at 'steady state', organism growth rate and other fermentation parameters oscillated about a mean value and were never truly constant. The period of this oscillation was approximately 18 hours and the amplitude increased as dilution rate decreased. All steady states were therefore recorded over a period of three days so that fluctuations due to the oscillation would cancel out, but it also meant that inevitably high standard deviation values were often obtained. The oscillation may be due in part to sampling error, but the frequency was too regular for this to be the only factor involved. Larmour & Marchant (1977) also reported the cyclic fluctuation of conidial production, hyphal biomass concentration and residual glucose concentration during continuous sporulation of F. culmorum in continuous culture.

Many parameters exhibited a damped oscillatory response after the imposition of a shock growth - limiting factor. The transient state after introduction of the lowest starch supply to A. niger in experiment 4.1.4. (Fig 4.8) best demonstrates the typical oscillatory response of growth rate after a severe limitation. A similar effect was observed for growth rate, productivity, effluent biomass concentration, yield coefficient and nitrogen utilization after shock nitrate limitation, but carbohydrate utilization and fermenter biomass concentration did not fluctuate. In experiment 4.2.3. the culture growth rate of A.ochraceus oscillated after the sudden decrease following the onset of nitrogen limitation, and sporulation occurred during the initial growth rate decrease and subsequent oscillatory phases. Spore production ceased as the amplitude of the oscillations decreased and the culture assumed a new steady state. Sterkin et al. (1973) studied the transient stages of micro organism growth in continuous culture after shock parameter changes (temperature or substrate concentration) and concluded that the form and duration of such a transient phase were dependant upon the age of the population and environmental conditions at the time of the change. They also presented the concept of 'biological inertia' to explain the damped oscillatory responses observed. The typical steady - state and transient oscillations seen in this work are illustrated below.



a = 'steady state' oscillations.
b = progressive decline in response to limitation.
c = damped oscillations at end of decline phase
d = new 'steady state' oscillations.

While growth rate may be a central factor in the determination of sporulation, several experiments illustrated that sporulation is by no means due to decreased growth rate alone. The rate at which growth rate declines is genetically specific and environmentally dependent, and should reflect the degree to which the normal vegetative growth metabolism of the organism has been disrupted. Morphological expression is the result of these changes induced in the metabolic pathways by the growth - restricting stimulus. Smith (1978) stated that movement away from balanced growth is a pre - requisite for sporulation, but that unbalanced growth does not always cause sporulation. The nature of the unbalanced growth is the important factor, as demonstrated by the different effects that nitrogen and carbon nutrient limitations had upon growth and sporulation during this work. The equilibrium between mould death and vegetative growth was difficult to maintain under carbon limitation and therefore the majority of experiments were based upon the nitrate - limited system. However, the initial carbon - limited experiments demonstrated the necessity of an energy source to survival of the organism. Under nitrogen limitation, sporulation was triggered by a combination of decreased growth rate and decreased nitrogen utilization, because carbon utilization remained virtually constant throughout the experiment. However, little is known of the true sequence of events between perception

of an external stimulus and its' translation into a reproductive phase.

The low growth rate of A. niger in the second stage in the temperature variation experiment (4.9.1.) was caused by the lack of adequate nutrients, and the lack of sporulation may have been equally due to insufficient nutrients as to inadequate growth rate. Sporulation has already been described as an energy - consuming process, and the importance of an adequate nutrient supply was well demonstrated by experiment 4.9.2. in which different carbon concentrations were supplied to the second stage of a two - stage fermentation. Growth rate had no effect upon sporulation in this case, as it remained approximately constant ($0.04h^{-1}$) but sporulation intensity was proportional to the concentration of carbon source (sucrose) supplied to the second stage. This relationship applied under nitrate limited conditions, but the effect of glucose limitation upon sporulation was shown in section 4.1.3. While sporulation of A. niger occurred at growth rates as high as $0.05h^{-1}$ when glucose supply concentration was reduced to $0.5gl^{-1}$, no sporulation occurred at growth rates of $0.049h^{-1}$ and $0.037h^{-1}$ with a glucose supply concentration of $1.0gl^{-1}$. However, when the glucose supply was removed completely, the organism was washed out of the fermenter before sporulation occurred, showing that sporulation was not a response to glucose starvation. Sporulation may have been the result of the removal of catabolite repression (Hsu & Ordal, 1969). The combination limitation experiments did not improve the spore production of A. ochraceus by simply combining environmental conditions to restrict growth, and production by A.niger was not greatly improved.

The importance of the type and concentration of the nitrogen source was demonstrated in many cases. Ammonium ions inhibited sporulation (4.2.5.) at the same concentration at which nitrate ions permitted sporulation, and the requirement for a sufficiently low nitrate ion concentration was shown in experiment 4.9.2., when the concentration of nitrate in the effluent stream from stage one originally prevented the onset of sporulation in stage two. The importance of the carbon/nitrogen ratio was also particularly well shown by experiment 4.9.2.

Sporulation was therefore shown to depend upon an interaction between growth rate and the nutrient status of the environment, but sporulation is also dependant upon the nature of the physiochemical environment. Individual factors of the physiochemical environment were shown to be ineffective in the induction of sporulation, yet sporulation is known to occur within particular limits of temperature, pH, aeration etc., outside which spore production is inhibited.

At an aeration rate of one v.v.m. the dissolved oxygen concentration of the fermentation broth was maximum and on an industrial scale, high operating costs restrict the use of high aeration levels and oxygen - transfer is more efficient. For these reasons the aeration rate of the laboratory scale system was not increased above one v.v.m. so that scale - up problems would be minimised. The replacement of compressed air by carbon dioxide to induce sporulation was found to be both expensive and inefficient and was not therefore considered further. High carbon dioxide levels suppressed growth of both A. niger and A. ochraceus, but sporulation response was poor.

Section 4.4. confirmed that growth rate was a function of pH, and the experimental results agreed with those of Steinberg (1937) that a low pH stimulates growth of A. niger. A pellet - type floc morphology was induced by a pH level above 7.0, but the decrease in fermenter biomass concentration of A. niger as pH increased was unexplained. Although pellets are thought to be retained in the tower more easily than small filaments the A. niger flocs formed at high pH were obviously more easily removed from the fermenter. This may have been due to autolysis of the pellet centres, creating a lower - density floc. In agreement with the work by Ewen (1980) on P. javanicum, both A. niger and A. ochraceus exhibited a wide pH range of carbon substrate utilization.

Section 4.3. demonstrated that temperature alone was not a sufficiently strong stimulus to promote sporulation at sub - optimal temperatures. This was not altogether surprising, as the enzyme systems required for sporulation are similar to those for vegetative growth and thus these operate best at the optimal temperature point for a particular mould. High temperatures also reduce the dissolved oxygen concentration of the growth medium which is detrimental to sporulation. This may explain why the final combination fermentations did not greatly improve spore production, as output may have been improved at optimum temperatures.

Sporulation therefore depends upon a delicate equilibrium between growth rate and the physiochemical and nutritional nature of the environment. However, while the continuous tower fermenter was

ideal for the study of the effect of individual aspects of the environment upon growth and sporulation, one should be wary of drawing too many conclusions from such investigations. Future work could well involve the examination of a continuous range of paired parameters (i.e. nitrogen limitation and temperature, nitrogen limitation and pH).

Growth rate observations may be distorted because fermenter productivity and hence growth rate is influenced not only by the physical and nutritional parameters, but also by the organism morphology induced by these restraints. As a consequence of nutrient limitation, the gross mycelial floc morphology may alter to a type 1 - 2 form (Cocker 1980) which is more easily removed from the fermenter, which results in gradual wash - out and decrease in the fermenter biomass concentration and falsely increased fermenter output. Growth rate is then artificially high. Conversely, if the floc morphology alters to the pelleted form and is retained in the tower, although growth may occur, fermenter biomass concentration increases and output decreases causing a decrease in the calculated growth rate. Using the calculation for growth rate under transient states, this loss or accumulation was accounted for. The formation of a mycelial floc in the tower fermenter during the 'start - up' batch phase before a medium feed is supplied, is illustrated by plates 24 - 28. The process is identical to that described by Burkholder and Sinnot (1945) and Galbraith and Smith (1969c) in that germinating spores agglutinate and aggregates then intertwine to form a larger floc. Initially

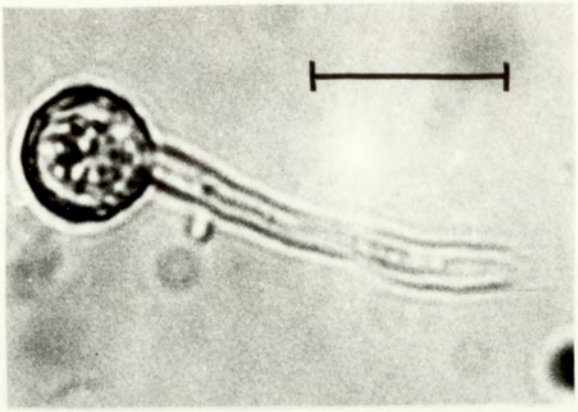


PLATE 24

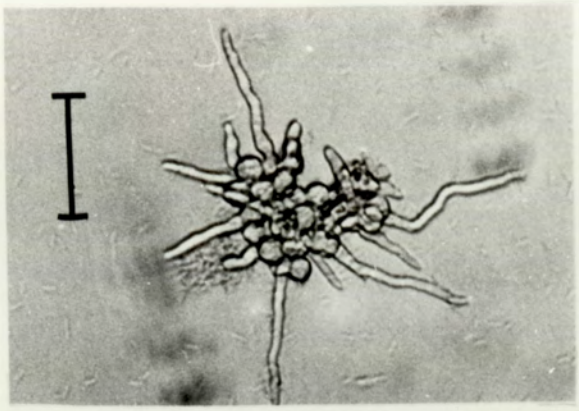


PLATE 25

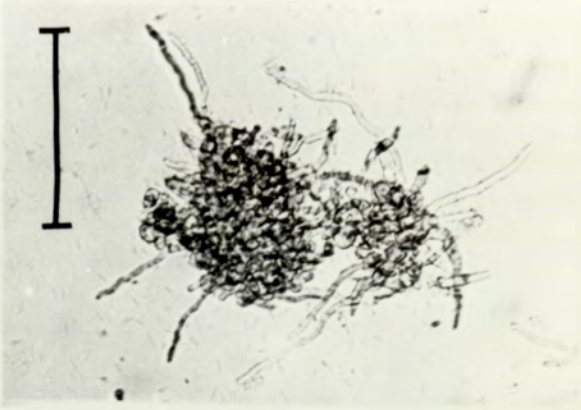


PLATE 26

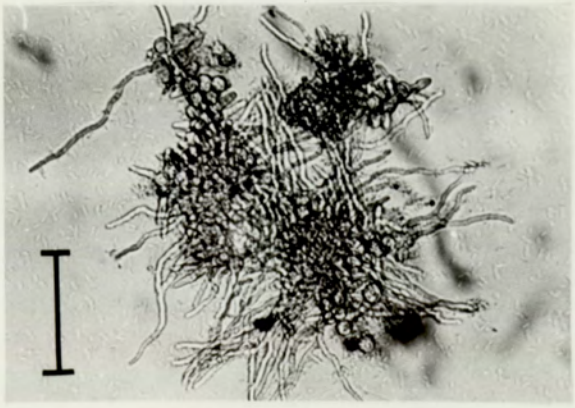


PLATE 27

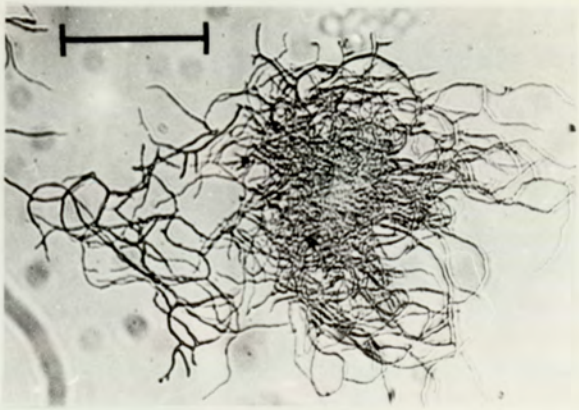


PLATE 28

PLATES 24 - 28 Series of photographs illustrating floc formation during the batch phase at the start of a fermentation.

24 - Spore germination (Bar = 20 µm.) 25 - Aggregation of germinating spores (Bar = 50 µm.) 26 - Germ tubes of aggregates intertwine. (Bar = 100 µm.) 27 - Floc enlarges. (Bar = 100 µm.) 28 - Complete floc. Future morphology depends upon environmental and nutritional factors. (Bar = 2.0 mm.)

these flocs formed distinct pellets due to even exposure of the floc to all factors of the environment (gravity, nutrient concentration gradients, abrasion etc.) and remained as pellets if the dilution rate used was above $0.4h^{-1}$. The theories of pellet formation in submerged culture are reviewed by Pirt (1966), Whitaker & Long (1973) and Metz & Kossen (1977). However, at low dilution rates, the pellets soon broke up due to excessive abrasion during the long residence time and filamentous growth was established by continuously broken mycelium (Pannell 1976). This form was preferable for submerged sporulation investigations due to the primarily vegetative nature of pellets.

A problem encountered when working at low growth rates, was that a fermentation often had to be terminated due to excessive bacterial infection, and this problem was frequently encountered, despite medium sterilization. This could perhaps have been overcome by more sophisticated sterilization apparatus and techniques. Improved fermentation conditions would have been obtained by a continuous medium sterilization unit, as the major source of infection was at the point of changing - over of medium reservoirs. This was not adopted because of the expense to our own laboratories and the cost that such a system would add to the final process when developed.

Nitrogen limitation had a less dramatic effect upon the morphology of the mould than did carbon limitation, and as a result the artificially high fermenter biomass concentration remained constant, allowing a more rapid response of the culture to the environmental stimuli. The value of mould retention in

the tower was demonstrated repeatedly after shock nitrate limitation when, after an initial decrease in growth rate, the organism was able to establish a new steady - state growth pattern. An even more striking effect was the prolonged survival of an A. niger fermentation for more than two months on a standard sucrose - salts medium, but without any nitrogen supply. Although there were normal hyphae present at all times during the two months, the concentration of biomass in the fermenter slowly decreased. It was assumed that maintenance, and maybe growth, of some hyphae was occurring at the expense of other hyphae and that A. niger was thus able to survive for long periods on its own nitrogen lysis products. This form of 'cryptic' growth was observed by Trinci & Righelato (1970) with P. chrysogenum and by Bainbridge et al. (1971) with A. nidulans. This condition could not last indefinitely because the lysis nitrogen products (principally amino acids) could only maintain the existing cells without production of new cells. Thus existing cells gradually died and were not replaced and mycelial mass consequently decreased. During the period of cryptic growth, extensive intra - hyphal hyphae formation occurred, and this was also observed during starch limitation of A. niger (4.1.4.) and temperature shock to P. javanicum (4.3.3.) Plate 29 illustrates the formation of intra - hyphal hyphae during conidiophore and vesicle formation by A. niger under starch limitation. Intra - hyphal hyphae were observed in glucose - starved cultures of P. chrysogenum (Trinci & Righelato, 1970) and A. nidulans (Bainbridge et al. 1971) accompanied by extensive vacuolation of the hyphae.

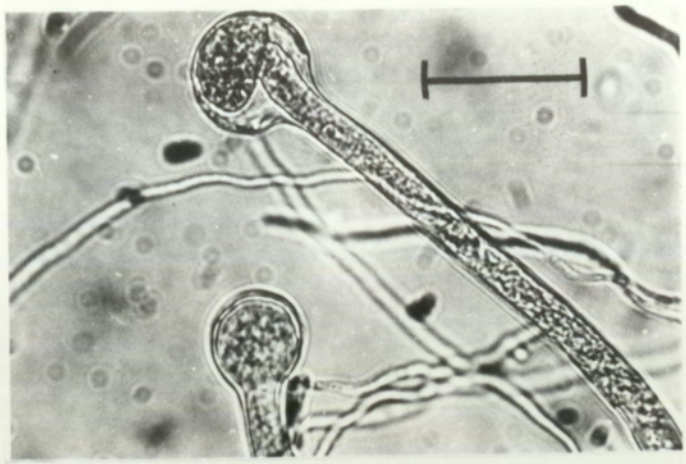


PLATE 29

PLATE 29 . Intra - hyphal hyphae produced by A.niger during conidiophore and vesicle formation after the onset of gradually - induced starch supply (0.044 $gg^{-1}h^{-1}$) just above the maintenance requirement (0.0383 $gg^{-1}h^{-1}$). (4.1.4.)

BAR = 30 μm.

The values in table 5.1 indicate that the specific carbohydrate utilization rate was always greater than the maintenance requirement of the organism during sporulation, and the carbohydrate utilization beyond the maintenance level was used for production of the sporulation apparatus, which has a high energy requirement. Some energy was also used for the essential maintenance of the cell, and some was diverted into secondary metabolic pathways to form various secondary metabolites, organic acids and pigments. The carbohydrate yield coefficient decreased during nitrogen - limited growth, indicating that while the same amount of substrate was utilized, less was directed towards biomass production. Here again one may draw a close analogy between differentiation and secondary metabolism. Bu'lock (1975) discusses pigment formation by fungi and the relation to secondary metabolism. Frequently the role of a secondary metabolite in metabolism is unknown, but the production of a metabolite may provide a mechanism by which excess intermediates or carbohydrates in the medium may be metabolised during adverse conditions. Such a mechanism would serve to maintain the cell in a functional state, and it is this function that the bright yellow pigment production by A. niger and A. ochraceus in adverse conditions is thought to serve. Anderson & Smith (1971b) identified the bright yellow pigment produced by A. niger after nitrogen exhaustion as a carotenoid with wavelengths of maximum absorbance at 376, 396 and 410 mμ. No analysis of the pigment was undertaken during this project, but it was produced under all types of adverse conditions, particularly sub - optimal temperatures, although production

was quickly reversed by restoration of favourable growth conditions. The yellow pigment was also observed by Stockbridge (1979) with A. niger at a temperature of 40°C and by Ewen (1980) at high and low thermal death points of P. javanicum.

The colour of the culture changed during sporulation from creamy white to grey/brown (A. niger) or olive green (A. ochraceus), but the pigment was not localised in any reproductive structure and was also released into the medium. The black spore pigment of A. niger spores was absent in submerged culture, and this lack of pigment was also observed by Anderson and Smith (1971a).

It was possible with the continuous tower fermenter system to estimate the maintenance requirements for various carbohydrate supplies of A. niger and A. ochraceus. This was calculated by two methods; firstly by the method of Schulze & Lipe (1964) which involved the plot of growth rate (μ) against specific substrate utilization rate (qs) and extrapolation to the point of zero growth, and secondly by the method of Pirt (1965) which involved the reciprocal plot of yield coefficient (Ysub) against growth rate (μ) when the slope of the line was equivalent to the maintenance coefficient. As table 5.1 shows, where both methods were used on the same set of data, different maintenance values were obtained. For the reasons outlined in section 2.6.4. it was felt that the values calculated by the method of Schulze & Lipe (1964) were the most reliable. The maintenance values of 0.0185 $gg^{-1}h^{-1}$ (by the method of Schulze) and 0.016 $gg^{-1}h^{-1}$ (by the methods of Schulze and Pirt) of sucrose; 0.0123 $gg^{-1}h^{-1}$ (by the method of Schulze) and 0.043 $gg^{-1}h^{-1}$ (by the method of Pirt) of glucose; and 0.0383 $gg^{-1}h^{-1}$ and 0.014 $gg^{-1}h^{-1}$ (the latter

by the method of Pirt), of starch for A. niger are similar to previously reported values for the carbodydrate maintenance requirements of other filamentous fungi (Table 5.2.) The values of 0.123 $gg^{-1}h^{-1}$ (by the method of Schulze) and 0.117 $gg^{-1}h^{-1}$ (by the method of Pirt,) of starch for A. ochraceus are considerably higher than any previously calculated value, however, this may be due to the slow growth rate of this organism. It was not possible to calculate a maintenance coefficient value for nitrogen as growth rate and productivity were still positive when utilization had apparently reached zero with no nitrogen supply. Righelato et al.(1968) reported a specific substrate supply rate (Q_s) of 0.038 g glucose $g^{-1}h^{-1}$ during sporulation of P. chrysogenum in continuous culture. This compares with 0.04 $gg^{-1}h^{-1}$ calculated from experiment 4.1.3. for A. niger. Spensley reported a Q_s of 0.025 g starch $g^{-1}h^{-1}$ during sporulation of A. niger in a tower fermenter, and this compares with 0.044 $gg^{-1}h^{-1}$ calculated from experiment 4.1.4. All these results confirm the theory that sporulation occurs at a substrate supply rate slightly above the maintenance requirement of the organism. The calculated values for sucrose supply rate to to A. niger (specific substrate supply rate = 0.071 $gg^{-1}h^{-1}$ or 0.039 $gg^{-1}h^{-1}$, maintenance = 0.0185 $gg^{-1}h^{-1}$ or 0.016 $gg^{-1}h^{-1}$) also support this theory.

Spore production by shock nitrate limitation was increased in two ways. Firstly, by the cycling of the limitation so that between each short period of sporulation in response to the shock nitrate removal, the mould could recover to its former

TABLE 5.2.

Calculated maintenance coefficients of nutrient supplies for filementous fungi.

Organism	Substrate	Maintenance coefficient $gg^{-1}h^{-1}$ (m)	Reference
P. chrysogenum	glucose	0.022	Righelato et al. (1968)
A. nidulans	glucose	0.018	Carter et al. (1971)
A. nidulans	glucose	0.029	Bainbridge et al.(1971)
A. niger	sucrose	0.015	Pannell (1976)
A. niger	citrate	0.045	Ng et al. (1973)
P. chrysogenum	hexose equivalent	0.024	Ryu & Hospodka (1980)
P. chrysogenum	Ammonia - N	0.002	Rye & Hospodka (1980)
A. aerogenes	glucose	0.054	Solomon & Erickson (1981)

steady state. This produced a semi - continuous system oscillating between peak spore production and optimal vegetative growth on a cycle length of approximately one week. The cycle lengths used were the minimum possible to enable full recovery of the vegetative culture between each sporulation phase, and any shorter time period would have had a progressively restrictive effect upon starting growth rate, leading to eventual termination of the sequence, and the fermentation would not then have been truly semi - continuous. There was no correlation between the peak height of recovery growth rate and subsequent sporulation intensity. Output from the system could have been increased by decreasing the length of the sporulation phase, and thus reducing the cycle time. The maximum spore output occurred within the first 48 hours, and the subsequent 48 hours produced considerably fewer spores and this time could have been removed from the cycle with only a small effect upon the number of spores produced. In practical terms, production could also have been increased by increasing the number of fermenters which would be expensive, or, more preferably, by increasing the size of the existing fermenter.

In their studies on sporulation of B. subtilis , Dawes & Thornley (1970) found that cells were committed to sporulation immediately that glucose limitation was initiated, and an instant restoration of the glucose supply, while halting further initiation, could not prevent the development of those cells already committed to sporulation. Under nitrogen limitation,

initiation and development were both arrested by restoration of the full nitrogen supply. This committment may be explained by an environmental change triggering an internal response which effectively insulates the system so that further differentiation processes are governed by internal factors. Ng et al. (1973b) found that conidiation of A. niger was a process with marked committment although Bu'lock (1975) stated that any transient feature of a batch fermentation maintained indefinitely in a chemostat does not involve committment. In the cycled nitrogen limitation experiment, there was a positive expression of committment in that spores continued to be produced for up to 18 hours after full nutrient medium was restored to the culture, in which time all residual free spores would have been washed from the fermenter if production had abruptly ceased. One can conclude that that portion of the mycelium committed to sporulation before restoration of the full nutrient medium, produced spores regardless of the nutrient environment, before switching again to vegetative growth.

The second method used to increase spore production by the basic nitrate limitation technique was the development of a two - stage fermentation system. Once established, spore production in this system was truly continuous and thus led to an overall increased efficiency of production in terms of substrate utilized and to increased production of spores per day (although this may not have applied if the cycled limitation experiment had been modified in the way discussed). To compare the production by this system with that of other methods used for mass propogation of spores is difficult due to the nature of the solid substrate used in the other methods. The spores

ml^{-1} production, and the efficiency of each method used during this project are listed in table 5.1. On an industrial scale, Singh, et al. (1968) produced 7×10^{12} spores kg^{-1} bran, which compares with 8×10^{12} spores kg^{-1} sucrose in the cyclic system and 3.4×10^{12} spores kg^{-1} sucrose in the two - stage system, but the commercial advantages of one system over the other involve not only the relative costs of the substrates, but also the capital investment needed to establish the apparatus required for each mode of production, and the subsequent running costs and ease of operation. It was mentioned in the introduction that a submerged production system should provide great financial savings over solid substrate production systems, particularly in the area of safety precautions which are so extensive with aerially - borne spores. (Vezina & Singh 1975). An advantage of the continuous system is that spore production is constant, 24 hours a day after the establishment of a steady - state (approximately 7 - 10 days). Thus in the example in section 4.9., 5×10^{12} spores were produced in 168 litres of medium per week, from only 0.84kg sucrose supplied. To obtain a production of 5×10^{12} spores from the production by bran (7×10^{12} spores kg^{-1}), a total of 0·77 kg of bran would be required. A production of 4 to 9×10^{7} spores ml^{-1} was reported by Vezina & Singh (1975) for A. ochraceus in batch submerged culture, and this is similar to the intensities obtained during the transient production phases of this work. The efficiency of the two - stage system developed in this project may be improved by the study of a combination of limitations to the second stage,

in particular the addition of low ferrocyanide ion concentrations to maintain an infection - free second stage and thus improve both duration and quality of the fermentation. It should be noted that for the maximum production, all spores were produced on the simplified sporing structures of A. niger, although Smith & Berry (1974) stated that the simplified form of production cannot compare quantitatively with the normal sub - aerial morphology.

When studying the submerged conidiation structures of fungi, one must bear in mind that these structures are not the natural developmental form of the fungus and so information derived from fermenter studies must be treated with caution, particularly in direct comparisons with the traditional sub - aerial development. A detailed account of the normal asexual development of Aspergillus is given by Smith et al. (1977b) and diagnostic descriptions of A. niger and A. ochraceus are recorded by Thom & Church (1926) and Raper & Fennell (1965). Normally the first morphological indication of sporulation is the formation of a foot - cell, from which the conidiophore is produced perpendicularly to the axis of the hypha. The conidiophore is usually unbranched and aseptate and sized 1.5 - 3.00 mm x 15 - 20μm (A. niger) or 1 - 1.5mm x 10μm (A. ochraceus). A multinucleate vesicle forms at the apex of this conidiophore, (30 - 75μm A. niger, 45 - 75μm A. ochraceus), and produces two rows of sterigmata, primary (the metulae) and secondary (the phialides). The bottle - shaped phialides develop over the surface of the vesicle, and conidia are produced sequentially from the 'neck' of the phialides as phialospores.

Conidia are approximately 4 - 5μm diameter.

There are conflicting reports on the submerged reproductive structures of filamentous fungi. Foster et al. (1945) reported that the submerged sporulation structure of P. notatum was similar to that produced on solid culture, and similar observations were made by Hadley & Harrold (1958) with P. notatum, Morton et al. (1958) with P. griseofuluum, Vezina et al. (1965) with A. ochraceus, Carter & Bull (1969) with A. nidulans, Galbraith & Smith (1969a) with A. niger, Anderson & Smith (1971b) with A. niger, Saxena & Sinha (1973) with A. nidulans, Smith & Berry (1974) with A. niger and Larmour & Marchant (1977) with F. culmorum.

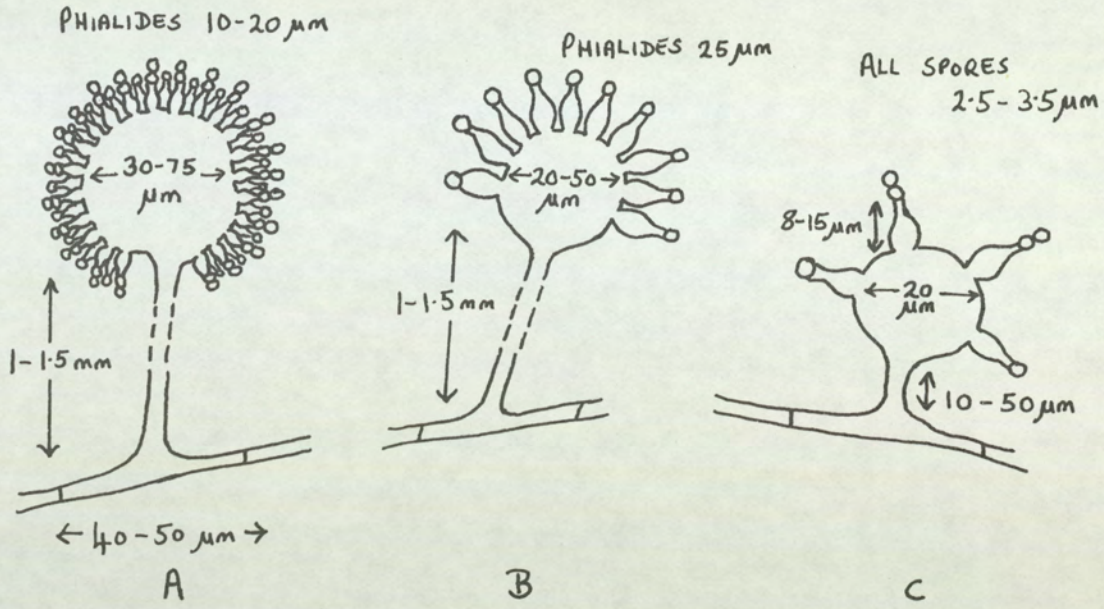
In all cases, submerged structures were reported as similar in shape to sub - aerial structures although frequently smaller and always lacking conidial chains due to medium agitation. Evidence for the reduction of complexity of submerged sporing structures is equally well documented. Cappellini & Peterson (1965) observed macroconidia of Giberella zeae borne singly upon simple conidiophores and at numerous points along the hyphae, while Morton (1961) and Carter & Bull (1971) observed free conidia in the medium before conidiophore production by P. griseofulvum and A. nidulans respectively. Righelato et al. (1968) reported the reduction in complexity of P. chrysogenum sporulation in submerged culture, by the production of spores from single or paired phialides on hyphal tips, a feature also reported by El Kotry (1970) for A. oryz and by Martinelli (1972, 1976) for A. nidulans. Anderson & Smith (1971a) observed direct production of phialides on the tips

of germ tubes of A. niger and Ng et al. (1973b) reported considerable reduction of complexity of the conidial apparatus of A. niger in chemostat culture, characterized by a small vesicle with few phialides, and occasionally conidia produced directly from hyphal tips. Reduced complexity is also represented by the various microcycle sporulation structures described for A. niger (Anderson & Smith, 1971a) and P. urticae (Sekiguchi et al. 1975). Anderson & Smith (1971a) compare the conidiophores of microcycle conidiation with those of normal surface - grown cultures of A. niger . Overall, the microcycle conidiophore and whole fruiting body was smaller, the metulae were absent and no pigment was produced in the spores.

During this project simplified reproductive structures of both A. niger and A. ochraceus were frequently observed. Structures of A. niger produced by sucrose or starch limitation were similar in shape to the sub - aerial type, and any reduction in complexity was reflected in a decreased number of phialides on the vesicle (e.g. starch limitation).. However, under gradual glucose limitation, A. niger produced spores from single phialides borne directly upon hyphal tips. Under nitrate limitation, A. niger produced predominantly simplified single phialides on hyphal tip structures, although sub - aerial complex reproductive structures were frequently observed in low numbers in a sporing culture. The two types of structure also occurred side by side on many occasions. In response to shock nitrate limitation A. ochraceus produced a mixture of complex structures with reduced phialide numbers, and previously

unobserved structures in which the vesicle and phialides were retained, but were borne almost directly on the hypha with no conidiophore production. These reproductive structures are summarized in Fig. 5.1. The results suggest that the formation of a conidiophore is not essential for sporulation, but that its' formation is the method by which the organism lifts the spores above the surrounding mycelium in a sub - aerial environment, to promote spore production and dispersal. Consequently, the production of a conidiophore is not required in submerged culture. Ng et al. (1973b) suggested that the simplification of the morphology was due to a partial switch - on of the conidial mechanism with the morphological and biochemical events of development precluding spore formation being by - passed. They also suggested that the residence time of the mycelium in the fermenter may determine the morphology of sporulation, but this seems unlikely on the evidence of this work. Dilution rate, and hence residence time, determined incidence of sporulation (under nitrogen - limited conditions, a dilution rate of $0.4h^{-1}$ inhibited sporulation while spores were formed under identical conditions at $D = 0.2h^{-1}$ and $0.1h^{-1}$) but exerted no control over the morphology of sporulation. Sporing structures were produced primarily at the edge of a mycelial floc with the centre of the floc remaining vegetative, which indicated that those hyphae at the edge of the floc had access to the limited nutrients available, and used them to produce spores. It was significant that the simplified structures were the most rapidly - formed structures in a sporing culture that exhibited both complex and simplified forms. This suggests that simplified structures are produced

ASPERGILLUS OCHRACEUS



ASPERGILLUS NIGER

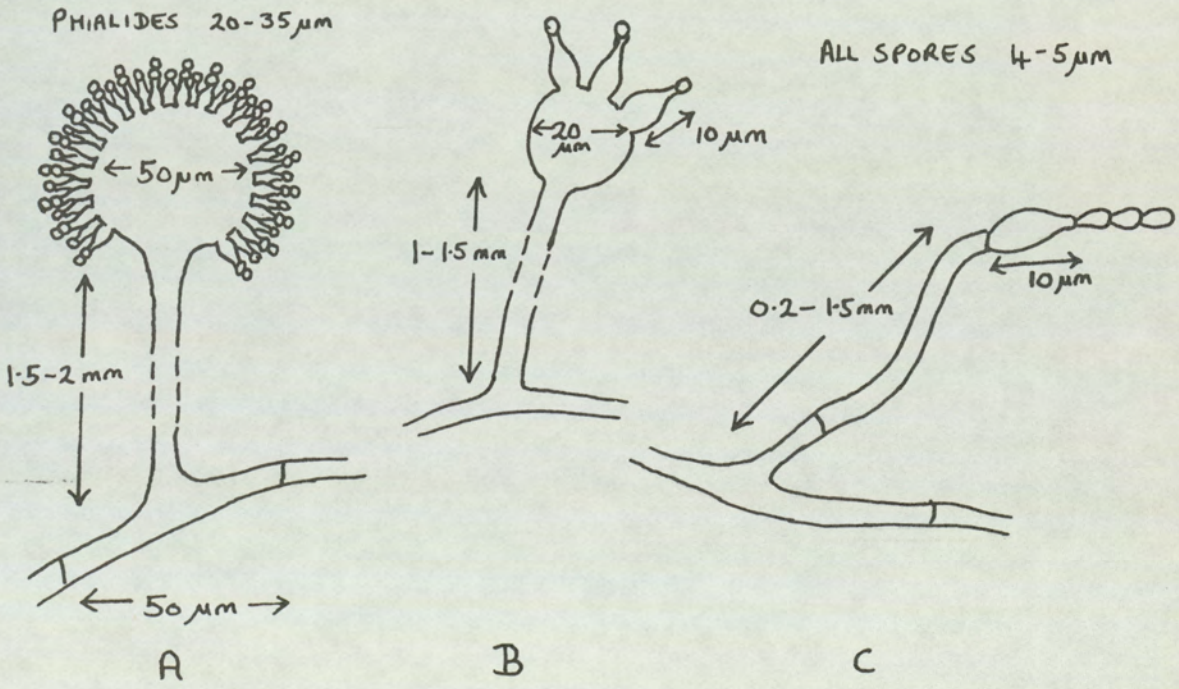


Fig. 5.1. Sporulative structures of A.ochraceus and A.niger.

A = Sub aerial type structures. B & C = Simplified structures.

in order to form spores quickly when the growth rate of the organism is suddenly severely restricted and available nutrient resources are low. If the mould is later able to adapt to grow under the new conditions (as frequently seen after a shock nitrogen limitation) then spores are produced by the more energy - consuming method and eventually sporulation ceases altogether. It is as if the rapid,simplified spore production process in response to restricted growth is a 'panic' measure undertaken by the organism to ensure survival should nutrient limitation prove too severe for vegetative growth.

The observation of adjacent complex and simplified structures suggests that the sporulation morphology produced depends upon nutrient and oxygen availability along the hypha within the floc. Hence those structures near the hyphal tip, which have greater access to the nutrients, would be complex, while further back along the hypha, structures would become increasingly simplified as nutrient availability decreased. However, no firm evidence in support of this theory resulted from observations during this project. The different formations may have been influenced by hyphal age. Bu'lock (1975) stated that hyphal tips are permanently young while more distal portions get progressively older. Thus there are permanent structural and biochemical differences along the length of a hypha, and this may well influence the type of structure that the hypha is capable of forming.

The observation of micro cycle conidiation of A. ochraceus

during nitrogen limitation was interesting. The structures were observed on two occasions in two separate fermentations, but their induction was not controlled. The incidence of the structures was relatively low but their presence indicated that the environment of the tower was capable of supporting the germination of spores produced in the system (which normally washed out of the fermenter without germinating), and also such that almost immediate re - sporulation of the germ tube was induced. The photograph (Plate 21) shows that the germinating spores were not swollen to the giant cells induced by high temperature treatment in normal microcycle conidiation induction, although they were slightly larger than ungerminated spores. The germ tube produced phialides directly, which again differed from the sub - aerial type of morphology produced by the germ tube of A. niger during microcycle conidiation (Anderson & Smith 1971a). The nutrient interactions causing this response were complex and an immediate explanation of this phenomenon was not apparent, although nutrient fluctuation in the micro environment within the floc must have played a part. Periodically high nutrient concentration gradients within the floc could stimulate germination of a spore produced in the normal system which then grows vegetatively for a short period of time before the nutrient fluctuations impose a limiting environment and simplified spore production from the hypha is induced. This situation would represent an uncontrolled version, on a small scale, of the cyclic nutrient supplies examined in section 4.8.

The induction of the reproductive phase of the organism was considered analagous to the induction of a non - growth associated secondary metabolite and as such the production of the desired product (i.e. spores) continued only for a finite period of time. Dawes & Thornley (1970) reported that the steady state conditions of spore formation by B. subtilis were not maintained indefinitely due to the spontaneous appearance of asporagenous mutants which replaced the sporing organism. This was not observed with either fungus tested, because samples taken regularly from the tower and plated out on malt extract agar, produced perfect spore mats in 10 - 12 days. 98 percent of all spores produced in the system were viable when plated out on malt extract agar. It is a feature of submerged continuous culture of filamentous fungi that the capacity for sporulation is gradually lost, but Pannell (1976) considers this to be physiological rather than genetic although the precise cause is unknown. The loss of sporulation capacity was only noticed in this work with any fermentation conducted for six months or longer. Pannell (1976) found that sporulative ability was restored in sub - cultures of the low - sporing strains, as was the case in these experiments, but new fermentations were always started from a new master culture. The loss of sporulative capacity is considered to be a response to the physiochemical stress and continual growth restraint imposed upon the fungus in the C.T.F. system.

In conclusion, there are several statements that may be made concerning the induction and continuous production of asexual spores of A. niger and A. ochraceus in continuous culture;

1) Growth and sporulation must occur simultaneously for the continuous spore production system to operate.

2) Adequate growth rate is needed for sporulation to occur. Sporulation will not occur at zero growth rate.

3) Sporulation intensity increases as dilution rate (hence growth rate) decreases.

4) There is no overall threshold effect of growth rate upon sporulation.

5) For a particular continuous culture system, there is a critical growth rate above which growth is vegetative and below which sporulation is induced if other conditions are favourable.

6) An energy source is required for sporulation and can be utilized in the absence of nitrogen, although the reverse is not possible.

7) Ammonium ions inhibit sporulation of A. ochraceus at the same concentration at which nitrate ions permit sporulation.

8) Sporulation occurs at specific substrate supply rates above the maintenance requirement.

9) Sporulation is a transient shock response to shock growth rate restriction due to nitrate limitation, and may be made semi continuous by cyclic nutrient levels, or continuous using two - stage fermentation.

10) Sporulation is the response to gradual carbon limitation. The shock removal of the vital energy source is too severe upon growth rate and wash - out occurs before sporulation can develop.

11) Sporulation is the result of complex interaction between organism growth rate, the physiochemical environment and the nutritional status of the growth medium.

13) Simplification of sporulative morphology occurs in submerged culture, partly as a more rapid response, partly as a response requiring lower nutrient concentrations, and partly due to the lack of requirement in liquid culture for the complex structures designed for improved spore dispersal when the fungus grows on its more natural solid culture.

14) The continuous tower fermenter was ideal for controlling fungal morphology in continuous culture and for examining the effects of isolated and combined aspects of the environment upon growth and sporulation. As a result, a system for the controlled continuous induction of sporulation was developed.

There are several future directions in which this work could be expanded . With specific reference to the experiments carried out during the project, one needs to examine new methods for the increased production of spores to improve the system developed. These methods would include investigating a range of paired parameters (e.g. nitrate limitation plus temperature effects, nitrate limitation plus pH effects) in single - stage culture, and the combination of growth - limiting and sporulation promoting parameters in the second stage of a two - stage system to increase spore production. These investigations may be combined with biochemical tests designed to locate the receptor sites for chemical and physical stimuli within the organism.

The variation of cycle lengths in the cycled nutrient method

for semi - continuous sporulation could well lead to an increased efficiency of production, and a more commercially attractive system, (although the cycled system requires greater operator attention).

Finally, the optimisation of dilution rates in single or two - stage systems may improve spore production, although the dilution rate of $0.1h^{-1}$ used during this project was close to the optimum.

In general terms, the system developed could be applied to further calculation of the maintenance requirements of commercially important strains (particularly fungi). Solomon & Erickson (1981) present the most recent review of biomass yields and maintenance requirements for a variety of organisms on carbohydrates and there is clearly scope for more information about the maintenance value of particular substrates for particular organisms as this is a key point in the control of physiology and morphology of the organism.

The system for continuous spore production should be optimised for other commercially important organisms. V.lecanii was briefly tested in the system with poor results due to lack of time available, but one cannot simply apply a set collection of limiting parameters to every organism. Each fungus is unique in anatomical, morphological and physiological development, and therefore for each fermentation, the precise physiological conditions must be established for induction of the correct stage of development.

Finally, the system for continuous spore production must be tested on an industrial scale. The size of the system will depend essentially upon the approach to the marketing of the system and the product. Due to the stable nature of the spores, one could design a large system for production and subsequent storage and distribution of vast quantities of spores of fungal strains in demand in industry. Alternatively one could market the system itself for construction and use on a smaller scale *in situ* by those having a use for spores of a particular strain or strains.

APPENDIX 1.

Medium constituents and analytical chemicals.

Chemical	Grade	Source
Sucrose	Standard	F & W Berisford (Sugar) Lt
		Kings Heath, Birminghm.
Ammonium sulphate $(NH_4)_2SO_4$	Technical	Various.
Potassium chloride KCl	S.L.R.	Fisons Scientific Appara
		Loughborough, Leics.
Sodium nitrate $NaNO_3$	S.L.R.	"
Ammonium nitrate NH_4NO_3	S.L.R.	"
Magnesium sulphate $MgSO_4 7H_2O$	S.L.R.	"
Sodium Di-hydrogen orthophosphate $(NaH_2PO_4 2H_2O)$	S.L.R.	"
Phenol (detached crystals) C_5H_5OH	A.R.	"
Pyridine C_5H_5N	A.R.	"
Acetic anhydride $(CH_3CO)_2O$	A.R.	"
Orthophosphoric acid H_3PO_4	S.L.R.	"
Citric acid $C(OH)(COOH)(H_2COOH)_2H_2O$	S.L.R.	"
Calcium chloride $Ca\ Cl_2$	L.R.	B.D.H. Chemicals Ltd.
		Poole, Dorset.
Potato Starch	L.R.	"
Sodium hydroxide NaOH	L.R.	"
Sulphuric acid H_2SO_4	Analar	"
Hydrochloric acid HCl	"	"
Dilute HCl, in 0.1M 500ml. C.V.S.	"	"
Potassium ferrocyanide $K_4Fe(CN)_6\ 3H_2O$	"	Hopkin & Williams.
		Romford, Essex.
Ferric chloride $FeCl_3\ 6H_2O$	L.R.	"
Glucose	Technical	A. Gallenkamp & Co. Ltd.
		Birmingham.
Yeast extract	Food Grade	Bovril, Burton-on-Trent.

This semi - defined medium has been found suitable for cultivation of A. niger in C.T.F.

Basal medium composition : (gl^{-1})

Sucrose	5.0
$(NH_4)_2SO_4$	1.0
NaH_2PO_4 $2H_2O$	0.5
KCl	0.25
$MgSO_4$ $5H_2O$	0.1
$CaCl_2$	0.05
Yeast extract	0.5

The mi n or element composition of Difco yeast extract reported by Grant & Pramer (1962):

Element in μg. g. dry wt^{-1}

Al.	Ba.	Cd.	Co.	Cr.	Cu.	Fe.	Ga.	Mg.
3.1	1.3	1.5	3.5	12.0	71.3	150.0	0.09	1270.0
Mn.	Mo.	Ni.	Pb.	Sn.	Sr.	Ti.	V.	Zn.
2.3	5.9	18.2	6.8	0.09	1.1	3.0	43.7	74.0

This is therefore an ideal substrate for supplying all trace element requirements of the mould, outlined in the nutrition section (1.4.4.), but this could not be used if accurate measurements were to be made upon the effect of trace elements on the growth of the mould.

Alterations:

$(NH_4)_2SO_4$ normally replaced by 1.3 gl^{-1} $NaNO_3$.

Sucrose replaced by starch (5.0 gl^{-1} glucose equivalent) or glucose (5.0 gl^{-1}) or citrate (5.0 gl^{-1} glucose equivalent) for particular carbon limitation experiments.

Levels of carbon and nitrogen varied during many experiments.

Medium was sterilised or had ice added to suppress infection in the medium reservoir.

APPENDIX 2

TABLES OF EXPERIMENTAL RESULTS.

Symbols and Units used in tables 1 - 32:

Parameters.	Abbreviation.	Units.
Dilution rate	D	h^{-1}
Sugar medium concentration	Sm	gl^{-1}
Nitrogen medium concentration	Nm	gl^{-1}
Effluent biomass concentration	X_E	gl^{-1}
Fermenter " "	X_F	gl^{-1}
Productivity	Y	$gl^{-1}h^{-1}$
Growth rate	μ	h^{-1}
Sugar utilization	dS	gl^{-1}
Nitrogen utilization	dN	gl^{-1}
Sugar supply rate	kS	gh^{-1}
Sugar utilization rate	S	gh^{-1}
Nitrogen utilization rate	N	gh^{-1}
Specific sugar utilization rate	q_s	$gg^{-1}h^{-1}$
Specific nitrogen utilization rate	q_N	$gg^{-1}h^{-1}$
Yield coefficient (sugar)	Y_{sub_s}	gg^{-1}
" " (nitrogen)	Y_{sub_N}	gg^{-1}
Sporulation coefficient	β	spores g^{-1}

Table 1. Growth of A. niger v Dilution rate. T = 30°C, pH = 2.5, Air = 1 vvm. S_m = 5.0 gl^{-1} sucrose, N_m = 0.21 gl^{-1} ammonium N. V = 4.21.

$\bar{D}$	$\overline{X_E}$	$\overline{X_F}$	$\bar{Y}$	$\bar{\mu}$	$\overline{dS}$	$\overline{q_s}$	$\overline{Ysub}$	$\overline{dN}$	$\overline{qN}$ x 10^{-3}
0.02	0.063	0.6	0.0013	0.0046	3.95	0.337	0.016	0.014	1.1
S.D.	0.032	0.5	0.0006	0.0048	0.156	0.41	0.008	0.0015	1.3
0.04	0.06	1.15	0.0011	0.0022	3.7	0.13	0.017	0.063	2.4
S.D.	0.014	0.35	0.0012	0.0011	0.6	0.337	0.0071	0.014	1.1
0.08	0.92	3.3	0.073	0.021	4.09	0.098	0.251	0.117	2.7
S.D.	0.6	0.46	0.048	0.012	0.52	0.0026	0.132	0.021	0.1
0.1	2.23	3.6	0.223	0.062	4.7	0.131	0.47	0.157	4.3
S.D.	0.24	0.14	0.024	0.0042	0.035	0.0042	0.042	0.0035	0.1
0.15	1.2	2.0	0.18	0.09	4.1	0.307	0.293	0.12	9.0
S.D.	0.11	0.08	0.016	0.006	0.05	0.003	0.14	0.021	1.0
0.2	2.9	3.6	0.56	0.160	3.4	0.19	0.85	0.12	6.7
S.D.	0.155	0.085	0.031	0.011	0.011	0.018	0.106	0.005	1.0

Table 2. Gradual sucrose limitation to A. niger by a combination of decreased D and decreased S_m. T = 30°C, pH = 3.0, Air = 1 vvm. V = 10l. Nm = 0.21 gl^{-1}.

D	$\overline{Sm}$	$\overline{Ks}$	$\bar{Y}$	$\bar{\mu}$	$\overline{\Delta s}$	$\overline{q_s}$	$\overline{Ysub_s}$	$\overline{\Delta N}$	$\overline{qN}$
0.2	5.0	10.0	0.365	0.165	9.21	0.438	0.396	0.371	0.017
S.D.	--	-	0.095	0.0086	0.203	0.112	0.11	0.011	0.0048
0.2	2.5	5.0	0.162	0.166	4.76	0.512	0.34	0.29	0.029
S.D.	-	-	0.04	0.005	0.136	0.129	0.08	0.106	0.004
0.2	1.25	2.5	0.065	0.113	2.13	0.448	0.3	0.3	0.065
S.D.	-	-	0.038	0.026	0.187	0.211	0.16	0.035	0.037
0.1	1.0	1.0	0.021	0.104	0.71	0.35	0.298	-	-
S.D.	-	-	0.0042	0.023	0.003	0.002	0.06	-	-
0.05	1.0	0.5	0.012	0.036	0.355	0.104	0.365	0.001	0.0016
S.D.	-	-	0.004	0.014	0.087	0.023	0.17	-	0.0028
0.04	1.0	0.4	0.013	0.025	0.35	0.067	0.374	0.05	0.01
S.D.	-	-	0.0048	0.01	0.03	0.019	0.154	0.025	0.0064

Table 3. The growth of A. niger and A. ochraceus with ammonium and nitrate nitrogen supply. $D = 0.1h^{-1}$, pH = 3.2, Air = 1v.v.m. T = 30°C, Sm = $5.0gl^{-1}$ sucrose, V = 4.21.

	$\overline{X_E}$	$\overline{X_F}$	$\overline{Y}$	$\overline{\mu}$	$\overline{dS}$	$\overline{qS}$	$\overline{Ysub_s}$	$\overline{dN}$	$\overline{qN} \times 10^{-3}$	$\overline{Ysub_N}$
A. niger	2.23	3.6	0.223	0.062	4.72	0.131	0.47	0.157	4.3	14.2
$(NH_4)_2SO_4$	0.24	0.14	0.024	0.0042	0.035	0.004	0.042	0.0035	0.1	1.2
A. niger	2.1	3.47	0.21	0.06	4.63	0.14	0.466	0.141	4.3	14.9
$NaNO_3$	0.51	0.9	0.051	0.0038	0.061	0.035	0.088	0.0014	1.1	3.72
A. och.	1.97	3.87	0.197	0.051	4.8	0.125	0.41	0.13	3.4	15.1
$(NH_4)_2SO_4$	0.21	0.208	0.021	0.0026	0.18	0.006	0.038	0.0087	0.4	2.68
A.och.	1.85	3.7	0.185	0.05	4.7	0.127	0.39	0.131	3.5	14.2
$NaNO_3$	0.22	0.132	0.022	0.0078	0.026	0.005	0.043	0.0046	0.1	2.1

Table 4. Shock sucrose limitation to A. niger by decreased Sm. D = 0.2, pH = 3.0 T = 30°C, Air = 1 v.v.m. V = 4.2l. Nm = $0.21\ gl^{-1}$ nitrate N.

Day	$\overline{X_E}$	$\overline{X_F}$	$\overline{Y}$	$\overline{\mu}$	$\overline{Sm}$	$\overline{dS}$	$\overline{q_s}$	$\overline{Ysub_s}$	$\overline{dN}$	$\overline{qN}$
1-8	1.46	2.57	0.293	0.116	5.0	2.55	0.2	0.58	0.117	0.009
S.D.	0.163	0.316	0.033	0.024	-	0.2	0.025	0.1	0.003	0.0012
9	1.7	1.22	0.34	0.139	0.25	0.25	0.041	6.8	0.03	0.005
S.D.	0.28	0.32	0.056	0.026	-	-	0.011	1.13	0.021	0.0023
10	0.2	0.8	0.04	0.17	0.25	0.25	0.125	0.8	0.024	0.013
S.D.	0.21	0.21	0.04	0.06	-	-	0.067	0.8	0.008	0.01
11	0.01	0.4	0.002	0.0525	0.25	0.09	0.045	0.11	-	-
S.D.	0.0035	0.17	0.0014	0.0048	-	0.056	0.011	0.18	-	-

Table 5. Shock sucrose limitation to A. niger by combination of decreased D and decreased Sm. T = 30°C, pH = 3.0, Air = 1v.v.m. V = 4.2l, Nm = $0.213\ gl^{-1}$ nitrate N.

Day	$\overline{X_E}$	$\overline{X_F}$	$\overline{Y}$	$\overline{\mu}$	$\overline{\Delta S}$	$\overline{q_s}$	$\overline{Ysub_s}$	$\overline{\Delta N}$	$\overline{qN}$	$\overline{Ks}$
1-8	1.7	3.0	0.34	0.113	2.44	0.193	0.586	0.151	0.012	4.2
S.D.	0.18	0.34	0.044	0.017	0.181	0.03	0.1	0.0018	0.001	-
9	1.25	4.55	0.125	0.056	0.167	0.009	3.1	0.011	0.0006	0.196
S.D.	0.25	0.636	0.025	0.011	0.018	0.0003	0.97	0.0045	0.0001	-
10	1.2	1.7	0.12	0.0012	0.167	0.023	3.0	-	-	0.196
S.D.	0.056	0.37	0.0056	0.001	0.004	0.0057	0.08	-	-	-
11	0.1	0.7	0.01	0.019	0.042	0.014	1.0	-	-	0.196
S.D.	0.028	0.084	0.0028	0.0056	0.035	0.011	1.77	-	-	-

Table 6. Comparison of gradual sucrose/glucose limitation on A. niger.
$D = 0.1h^{-1}$, $T = 30^{\circ}C$, pH = 3.0, Air = 1v.v.m. $Nm = 0.21gl^{-1}$ nitrate N.
V = 4.2l.

Sm	$\overline{X_E}$	$\overline{X_F}$	$\overline{Y}$	$\overline{\mu}$	$\overline{dS}$	$\overline{q_s}$	$\overline{Ysub_s}$	$\overline{dN}$	$\overline{qN} \times 10^{-3}$	$\overline{Ysub_N}$
					SUCROSE					
5.0	2.4	4.1	0.24	0.059	4.88	0.12	0.49	0.13	3.17	18.5
S.D.	0.165	0.35	0.0165	0.0012	0.029	0.01	0.037	0.017	0.7	3.5
4.0	2.0	3.6	0.2	0.056	3.78	0.105	0.53	0.11	3.05	18.2
S.D.	0.26	0.07	0.026	0.0085	0.11	0.005	0.066	0.005	0.2	1.67
3.0	1.6	3.2	0.16	0.051	2.88	0.09	0.55	0.055	1.7	29.1
S.D.	0.2	0.436	0.02	0.012	0.13	0.015	0.046	0.009	0.2	7.3
2.0	0.86	2.15	0.086	0.04	1.95	0.091	0.44	0.04	1.86	21.5
S.D.	0.058	0.22	0.006	0.004	0.09	0.008	0.01	-	0.16	1.4
1.0	0.49	1.8	0.05	0.027	1.0	0.056	0.49	0.02	1.1	24.5
S.D.	0.017	0.087	0.002	0.001	-	0.003	0.017	0.004	0.25	5.5
0.5	0.17	1.3	0.017	0.013	0.5	0.039	0.34	0.03	2.3	7.1
S.D.	0.026	0.2	0.0026	0.002	-	0.007	0.053	0.016	1.0	4.1
					GLUCOSE					
5.0	2.58	5.0	0.26	0.05	4.75	0.095	0.544	0.124	2.45	20.94
S.D.	0.23	0.316	0.023	0.0084	0.115	0.004	0.052	0.013	0.4	2.6
2.5	1.675	5.1	0.167	0.037	2,5	0.05	0.67	0.045	0.88	35.4
S.D.	1.38	1.27	0.138	0.036	-	0.012	0.55	0.005	0.3	26.5
1.0	0.3	2.48	0.03	0.017	0.98	0.044	0.3	0.011	0.44	60.7
S.D.	0.28	0.9	0.028	0.023	0.04	0.018	0.28	0.011	0.4	115.4

Sporulation on Glucose $1.0gl^{-1}$

spores ml^{-1}	β
4.0×10^6	9.5×10^8
3.6×10^6	1.2×10^9
2.7×10^6	7.5×10^8
1.5×10^6	5.77×10^8
1.0×10^6	5.5×10^8

Table 7. Gradual starch limitation to A. niger.
$D = 0.1h^{-1}$, $T = 30^{\circ}C$, pH = 3.0, Air = 1v.v.m. V = 4.2l, $Nm = 0.21gl^{-1}$ nitrate N.

	$\overline{X_F}$	$\overline{Y}$	$\overline{\mu}$	$\overline{dS}$	$\overline{q_s}$	$\overline{Ysub_s}$	$\overline{dN}$	$\overline{qN} \times 10^{-3}$	$\frac{1}{\overline{Ysub_s}}$	$\frac{1}{\overline{\mu}}$
Sm	2.8	0.22	0.078	2.4	0.088	0.92	0.13	4.6	1.1	12.8
2.5	0.24	0.032	0.006	0.08	0.011	0.12	0.016	0.5	-	S.D.
Sm	2.7	0.2	0.074	1.75	0.065	1.14	0.12	4.4	0.88	13.5
1.8	0.132	0.018	0.006	0.05	0.002	0.08	0.018	0.4	-	S.D.
Sm	2.2	0.18	0.082	1.2	0.054	1.5	0.13	5.95	0.67	12.2
1.2	0.49	0.014	0.013	-	0.013	0.113	-	1.4	-	S.D.
Sm	2.25	0.166	0.074	1.0	0.044	1.66	0.1	4.4	0.6	13.5
0.6	0.25	0.01	0.008	0.05	0.001	0.11	-	0.8	-	S.D.
Day	2.4	0.14	0.0625	0.6	0.025	2.33	0.08	3.3	0.43	16.0
11	0.17	0.007	0.007	-	0.0014	0.12	0.007	0.07	-	S.D.
Day	1.8	0.08	0.036	0.6	0.033	1.33	0.05	2.8	0.75	27.85
12	1.0	0.071	0.018	-	0.021	1.17	0.035	0.5	-	S.D.
+ 8	1.5	0.05	0.0167	0.59	0.039	0.85	0.05	3.5	1.17	59.9
hrs	0.31	0.015	0.003	0.011	0.009	0.28	0.017	1.8	-	S.D.
+ 18	1.29	0.034	0.015	0.58	0.045	0.586	0.05	3.9	1.71	66.7
hrs	0.014	0.022	0.017	0.008	-	0.38	0.0085	0.7	-	S.D.
+ 24	1.31	0.034	0.0287	0.56	0.043	0.607	0.01	0.76	1.65	34.8
hrs	0.014	0.015	0.013	0.014	0.007	0.29	0.014	1.0	-	S.D.
+ 42	1.3	0.024	0.0218	0.54	0.041	0.44	0.03	2.3	2.27	45.87
hrs	0.17	0.0014	0.001	0.034	0.0085	0.054	0.02	1.3	-	S.D.
+ 48	1.21	0.022	0.0064	0.58	0.048	0.38	0.01	0.82	2.63	156.2
hrs	0.55	0.007	0.0014	0.003	0.024	0.12	-	0.4	-	S.D.
+ 66	1.3	0.021	0.0211	0.58	0.044	0.36	0.006	0.46	2.78	47.4
hrs	-	0.002	0.0014	-	-	0.036	0.0056	0.4	-	S.D.
+ 72	1.24	0.019	0.0079	0.56	0.045	0.34	0.006	0.48	2.94	126.6
hrs	0.155	0.0014	0.003	0.01	0.005	0.031	0.0085	0.6	-	S.D.
+ 84	1.36	0.013	0.011	0.51	0.0375	0.11	0.00	-	9.1	90.9
hrs	0.11	0.009	0.002	0.01	0.005	0.02	-	-	-	S.D.
+ 96	1.25	0.0025	0.002	0.2	0.016	0.125	0.00	-	8.0	500.0
hrs	0.11	0.003	0.001	-	-	0.025	-	-	-	S.D.

Table 8. Gradual starch limitation to A. ochraceus. $D = 0.1h^{-1}$, $T = 30^{\circ}C$ pH = 3.0, Air = 1v.v.m. V = 10l. Nm = 0.21 gl^{-1} nitrate N.

Ks	$\overline{X_E}$	$\overline{X_F}$	$\overline{Y}$	$\overline{\mu}$	$\overline{\Delta S}$	$\overline{q_s}$	$\overline{Ysub_s}$	$\overline{\Delta N}$	$\frac{1}{Ysub_s}$	$\frac{1}{\mu}$
4.0	1.97	2.28	0.197	0.086	3.2	0.172	0.6	0.087	1.66	11.57
S.D.	0.86	1.23	0.086	0.077	0.336	0.085	0.23	0.013	-	-
3.0	0.95	2.4	0.095	0.04	2.56	0.106	0.37	0.074	2.7	25.0
S.D.	-	0.8	-	-	-	-	-	-	-	-
2.0	0.2	1.13	0.02	0.018	1.54	0.163	0.128	0.04	7.8	56.5
S.D.	0.17	0.61	0.017	0.034	0.226	0.086	0.106	0.013	-	-
1.25	0.8	0.75	0.05	0.066	0.865	0.115	0.64	0.034	1.35	15.15
S.D.	-	0.07	-	0.005	0.39	0.04	0.28	0.003	-	-
0.8	0.4	0.25	0.016	0.068	0.675	0.275	0.24	0.018	4.16	14.8
S.D.	-	0.07	-	0.018	0.021	0.078	-	0.013	-	-
0.6	0.5	0.35	0.015	0.053	0.49	0.16	0.315	0.0025	3.17	19,05
S.D.	-	0.21	-	0.032	0.099	0.068	0.064	0.0015	-	-
0.4	0.33	0.33	0.007	0.025	0.325	0.123	0.22	0.002	4.5	40.0
S.D.	0.2	0.15	0.004	0.025	0.057	0.047	0.17	0.0026	-	-

Table 9. Gradual nitrate limitation to A. ochraceus. T = 30°C, pH = 3.2, $D = 0.1h^{-1}$, Air = 1v.v.m. Sm = 5.0gl^{-1}, sucrose, V = 4.2l.

Nm	$\overline{X_E}$	$\overline{X_F}$	$\overline{Y}$	$\overline{\mu}$	$\overline{dS}$	$\overline{q_s}$	$\overline{Ysub_s}$	$\overline{dN}$	$\overline{qN} \times 10^{-4}$	$\overline{Ysub_N}$
0.21	2.1	4.1	0.21	0.051	4.75	0.116	0.442	0.125	30.5	16.8
S.D.	0.1	0.2	0.01	0.005	0.07	0.004	0.027	0.005	2.7	0.1
0.18	3.1	4.4.	0.31	0.071	4.7	0.107	0.66	0.106	24.0	29.2
S.D.	0.4	0.2	0.04	0.006	-	0.005	0.085	0.006	0.25	2.1
0.14	2.6	4.37	0.26	0.06	4.57	0.105	0.57	0.11	25.0	23.6
S.D.	0.4	0.25	0.021	0.008	0.18	0.01	0.025	0.014	1.8	5.0
0.042	3.5	4.8	0.35	0.074	4.7	0.098	0.74	0.04	8.3	87.4
S.D.	0.28	0.07	0.028	0.016	0.155	0.004	0.14	0.002	0.56	14.8
0.038	3.4	4.95	0.34	0.07	4.35	0.09	0.78	0.037	7.57	90.7
S.D.	0.14	0.21	0.014	0.004	0.071	0.0056	0.02	-	0.32	3.7
0.03	2.1	4.75	0.21	0.045	4.15	0.087	0.51	0.03	6.2	71.2
S.D.	0.49	0.21	0.05	0.008	0.21	0.008	0.143	-	0.28	16.7
0.021	1.02	5.1	0.102	0.02	4.25	0.083	0.24	0.02	4.0	49.7
S.D.	0.03	0.14	0.003	-	0.21	0.002	0.006	0.0003	0.06	0.63
0.005	1.0	5.0	0.1	0.02	3.3	0.066	0.3	0.0045	0.9	222.2
S.D.	0.14	0.42	0.014	0.001	0.56	0.0056	0.007	-	0.07	31.4
0	0.05	5.0	0.005	0.001	2.45	0.049	0.02	-	-	-
S.D.	0.07	0.13	0.007	0.001	0.28	0.004	0.03	-	-	-

Table 10. Gradual nitrate limitation to A. niger. V = 4.2l. D = $0.2h^{-1}$, T = 30°C, pH = 3.2, Air = 1v.v.m. Sm = $5.0gl^{-1}$ sucrose.

Nm	$\overline{X_E}$	$\overline{X_F}$	$\overline{Y}$	$\overline{\mu}$	$\overline{dS}$	$\overline{q_s}$	$\overline{Ysub_s}$	$\overline{dN}$	$\overline{qN} \times 10^{-3}$	$\overline{Ysub_N}$
0.21	3.11	3.75	0.622	0.166	4.85	0.26	0.64	0.13	6.93	23.9
S.D.	0.155	0.14	0.03	0.0113	-	0.05	0.028	0.01	0.39	3.0
0.182	4.03	3.5	0.81	0.23	4.8	0.274	0.84	0.143	8.15	28.2
S.D.	0.247	0.28	0.05	0.0007	0.03	0.018	0.056	0.0007	0.45	1.6
0.13	2.36	3.45	0.472	0.137	4.75	0.275	0.5	0.12	6.96	19.7
S.D.	0.042	0.07	0.008	0.0002	0.21	0.018	0.032	-	0.14	0.28
0.09	2.92	3.7	0.58	0.158	4.68	0.253	0.62	0.08	4.32	36.5
S.D.	0.113	0.14	0.023	-	0.04	0.07	0.018	-	0.17	1.4
0.042	3.34	4.25	0.667	0.157	4.7	0.22	0.71	0.04	1.88	83.5
S.D.	0.51	0.35	0.1	0.01	0.01	0.018	0.106	0.0014	0.23	15.7
0.034	2.2	4.4	0.44	0.1	4.65	0.211	0.47	0.033	1.5	66.7
S.D.	-	0.35	-	0.008	0.04	0.015	0.004	-	-	-
0.032	2.71	3.95	0.54	0.137	4.6	0.233	0.59	0.03	1.57	87.4
S.D.	0.16	0.071	0.032	0.01	-	0.004	0.034	-	0.038	4.3
0.028	1.44	4.5	0.29	0.064	4.25	0.19	0.34	0.027	1.2	52.7
S.D.	0.127	-	0.025	0.0056	0.21	0.01	0.047	0.0004	-	3.8
0.025	1.01	4.5	0.2	0.045	3.85	0.17	0.2	0.0245	1.09	41.2
S.D.	0.014	0.14	0.028	0.002	0.14	0.008	0.002	-	0.035	0.59
0.021	1.64	4.25	0.328	0.077	4.15	0.195	0.395	0.0205	0.96	80.0
S.D.	0.27	0.07	0.054	0.011	0.28	0.016	0.09	-	0.018	13.1
0.016	1.0	4.8	0.201	0.042	3.8	0.16	0.27	0.015	0.64	64.5
S.D.	0.071	0.5	0.014	0.0014	0.28	0.028	0.036	-	0.028	4.5
0.014	0.48	4.8	0.096	0.02	3.5	0.063	0.14	0.0135	0.56	35.5
S.D.	0.17	0.07	0.34	0.007	-	0.002	0.05	-	0.085	12.5
0.0084	0.29	4.75	0.058	0.0123	3.6	0.152	0.08	0.0074	0.31	39.2
S.D.	0.02	0.07	0.004	0.0005	0.14	0.003	0.003	0.0003	0.014	4.35
0.0056	0.2	5.0	0.04	0.008	2.75	0.11	0.07	0.005	0.20	39.2
S.D.	0.07	2.87	0.014	0.003	0.21	0.015	0.02	-	0.001	13.8
0	0.058	4.6	0.012	0.0025	2.75	0.12	0.02	-	-	-
S.D.	0.03	-	0.006	0.0014	0.14	0.006	0.014	-	-	-

Table 11. Shock nitrate limitation to A. niger. V = 4.2l. D = $0.1h^{-1}$, T = 30°C, pH = 3.0, Air = 1v.v.m. Sm = $5.0gl^{-1}$ sucrose.

Day	$\overline{X_E}$	$\overline{X_F}$	$\overline{Y}$	$\overline{\mu}$	$\overline{dS}$	$\overline{q_s}$	$\overline{Ysub_s}$	$\overline{dN}$	$\overline{qN} \times 10^{-4}$	$\beta \times 10^8$
1-9	1.67	2.87	0.167	0.058	3.85	0.134	0.43	0.13	45.0	-
S.D.	0.14	0.25	0.014	0.002	0.82	0.11	0.25	0.032	40.0	
10	0.95	4.7	0.095	0.055	4.43	0.094	0.214	0.012	2.5	
S.D.	0.07	0.28	0.007	-	0.33	0.013	0.032	0.003	0.76	low
11	3.9	4.4	0.39	0.05	4.65	0.106	0.84	0.025	5.2	
S.D.	0.28	0.64	0.028	0.005	0.07	0.02	0.11	0.0007	0.24	1.14
12	0.6	5.6	0.06	0.055	4.43	0.08	0.135	0.025	4.0	
S.D.	0.21	0.07	0.021	0.004	0.33	0.004	0.007	0.005	0.45	6.9
13	1.2	6.0	0.12	0.018	3.86	0.064	0.55	0.0247	4.0	
S.D.	0.28	0.28	0.028	0.004	0.13	0.005	0.21	0.0006	0.1	6.3
14	1.0	6.0	0.1	0.018	4.43	0.074	1.06	0.0246	3.4	
S.D.	0.35	0.14	0.035	0.0045	0.18	0.005	0.042	0.0014	0.14	5.0
15	0.3	6.6	0.03	0.014	3.56	0.054	0.08	0.025	3.7	
S.D.	0.07	0.14	0.007	0.0007	0.2	0.004	0.03	-	0.08	5.4
16	1.0	7.3	0.1	0.013	4.04	0.055	0.25	0.024	3.3	
S.D.	0.7	0.85	0.07	0.008	0.08	0.005	0.011	0.003	0.75	5.5
17	0.2	8.1	0.02	0.012	4.91	0.06	0.04	0.0246	3.0	
S.D.	-	0.42	-	0.0001	0.01	0.003	0.008	-	0.16	1.23
18	0.15	8.7	0.015	0.005	5.0	0.057	0.03	0.0247	2.8	
S.D.	0.07	1.84	0.007	0.0003	-	0.012	0.008	-	0.61	low
19	0.6	8.4	0.06	0.003	3.86	0.046	0.155	0.024	3.0	
S.D.	0.42	1.27	0.042	0.0028	0.18	0.009	0.085	-	0.45	-

Table 12. Shock nitrate limitation to A. ochraceus. V = 4.2l. D = $0.1h^{-1}$ T = 30°C, pH = 4.0. Air = 1v.v.m. Sm = $5.0gl^{-1}$ sucrose.

Day	$\bar{X}_E$	$\bar{X}_F$	$\bar{Y}$	$\bar{\mu}$	$\bar{dS}$	$\bar{q}_s$	$\bar{Y}sub_s$	$\bar{dN}$	$\bar{qN}$ x 10^{-4}	β x 10^9
1-9	1.75	3.2	0.175	0.053	4.03	0.128	0.41	0.168	53.0	
S.D.	1.52	0.56	0.152	0.004	0.53	0.024	0.305	0.023	8.5	-
10	0.9	4.8	0.09	0.049	4.3	0.089	0.91	0.009	2.0	0.21
S.D.	0.35	0.2	0.035	0.013	0.16	0.0005	0.09	0.003	0.67	
11	0.7	3.7	0.07	0.043	3.95	0.107	0.18	0.02	5.6	
S.D.	0.5	0.17	0.05	0.01	0.14	0.0007	0.13	-	0.25	0.89
12	0.6	3.45	0.06	0.015	4.24	0.123	0.14	0.02	5.5	
S.D.	0.07	0.41	0.007	0.0025	0.34	0.024	0.0054	0.0003	0.61	1.1
13	0.4	3.05	0.04	0.011	4.1	0.134	0.097	0.02	6.7	
S.D.	0.22	0.21	0.022	0.005	0.28	0.02	0.062	0.0003	0.55	7.2
14	0.5	3.15	0.05	0.016	3.92	0.124	0.127	0.021	7.0	
S.D.	0.014	0.042	0.001	-	0.3	0.008	0.006	0.0006	0.27	9.5
15	0.75	3.3	0.075	0.021	3.12	0.094	0.24	0.021	6.0	
S.D.	0.35	0.42	0.035	0.009	0.17	0.007	0.126	0.0003	0.25	1.1
16	1.05	3.8	0.105	0.025	4.0	0.117	0.26	0.021	5.0	
S.D.	0.5	0.42	0.05	0.007	-	0.014	0.123	0.0003	0.19	1.92
17	0.7	2.9	0.07	0.015	2.86	0.1	0.25	0.021	7.0	
S.D.	0.3	0.35	0.03	0.005	1.61	0.07	0.3	-	0.89	low
18	0.4	3.2	0.04	0.022	3.56	0.11	0.112	0.021	5.0	
S.D.	0.028	0.28	0.003	0.001	0.08	0.012	0.005	-	0.57	-

Table 13. Shock ammonium limitation to A. ochraceus. V = 4.2l. D = $0.1h^{-1}$ T = 30°C, pH = 3.5, Air = 1v.v.m. Sm = $5.0gl^{-1}$ sucrose.

Day	$\bar{X}_E$	$\bar{X}_F$	$\bar{Y}$	$\bar{\mu}$	$\bar{dS}$	$\bar{q}_s$	$\bar{Y}sub_s$	$\bar{dN}$	$\bar{qN}$ x 10^{-4}
1-5	2.1	4.0	0.21	0.053	4.64	0.116	0.45	0.11	27.5
S.D.	0.26	0.35	0.026	0.035	0.22	0.007	0.04	0.026	5.2
6	1.32	4.6	0.132	0.028	4.5	0.1	0.29	0.0005	0.1
S.D.	0.113	0.56	0.011	0.0007	0.106	0.015	0.03	0.0007	0.14
7	1.15	4.6	0.115	0.025	4.33	0.09	0.265	0.013	3.08
S.D.	0.07	0.7	0.007	0.003	0.042	0.015	0.02	0.008	0.21
8	1.05	4.4	0.105	0.024	4.12	0.09	0.25	0.016	3.95
S.D.	0.35	0.85	0.035	0.0035	0.035	0.018	0.08	0.005	2.0
9	0.9	4.3	0.09	0.021	3.82	0.09	0.243	0.011	2.57
S.D.	0.11	0.42	0.011	0.0007	0.32	0.001	0.005	-	0.25
10	0.65	3.7	0.065	0.017	3.83	0.103	0.17	0.008	2.24
S.D.	0.07	0.39	0.007	0.0003	0.106	0.007	0.014	0.006	1.75

Table 14. Shock nitrate limitation to A. ochraceus with Sm = 5.0gl^{-1} glucose. V = 4.2l. D = 0.1h^{-1}, T = 30°C. pH = 4.0, Air = 1v.v.m.

Day	$\overline{X_E}$	$\overline{X_F}$	$\overline{Y}$	$\overline{\mu}$	$\overline{dS}$	$\overline{q_s}$	$\overline{Ysub_s}$	$\overline{dN}$	$\overline{qN}$ x 10^{-4}	β x 10^8
1-5	2.05	3.2	0.205	0.064	4.72	0.15	0.43	0.14	44.0	-
S.D.	0.2	0.25	0.02	0.011	0.115	0.009	0.04	0.012	6.1	
6	1.55	3.65	0.155	0.046	4.28	0.12	0.36	0.005	1.5	0.01
S.D.	0.78	0.9	0.078	0.03	0.18	0.036	0.17	0.0007	2.0	
7	1.7	3.9	0.17	0.043	4.57	0.12	0.37	0.018	4.6	4.3
S.D.	0.42	1.0	0.042	0.0007	0.32	0.023	0.07	0.004	0.14	
8	1.35	4.1	0.135	0.032	4.5	0.11	0.3	0.016	3.8	8.9
S.D.	0.49	0.6	0.05	0.008	0.27	0.01	0.11	0.007	1.1	
9	1.5	3.7	0.15	0.04	4.61	0.128	0.32	0.013	3.4	2.3
S.D.	0.49	0.85	0.05	0.004	0.13	0.025	0.15	0.0056	0.71	
10	1.25	3.4	0.125	0.036	4.5	0.14	0.28	0.012	3.35	-
S.D.	0.21	0.56	0.021	0.0007	-	0.013	0.008	0.005	0.78	

Table 15. Shock nitrate limitation to A. niger at D = 0.4h^{-1}. V = 4.2l. T = 30°C. pH = 3.0, Air = 1v.v.m. Sm = 5.0gl^{-1} sucrose.

Day	$\overline{X_E}$	$\overline{X_F}$	$\overline{Y}$	$\overline{\mu}$	$\overline{dS}$	$\overline{q_s}$	$\overline{Ysub_s}$	$\overline{dN}$	$\overline{qN}$ x 10^{-5}
1-4	1.37	3.7	0.56	0.156	3.34	0.31	0.44	0.078	1020.0
S.D.	0.18	0.9	0.07	0.053	0.41	0.15	0.075	0.017	415.0
5	0.9	3.7	0.36	0.11	2.8	0.35	0.32	0.0003	6.35
S.D.	0.14	1.9	0.06	0.043	0.14	0.2	0.067	0.0001	3.3
6	1.1	3.5	0.45	0.12	3.06	0.38	0.37	0.0003	2.95
S.D.	0.034	1.5	0.01	0.058	0.08	0.16	-	0.0001	0.49
7	0.92	2.42	0.37	0.15	3.0	0.5	0.315	0.0003	6.3
S.D.	0.035	0.6	0.01	0.046	0.07	0.14	0.007	0.0002	2.4
8	0.8	2.5	0.32	0.128	2.7	0.43	0.29	0.0004	6.4
S.D.	0.075	0.08	0.03	0.017	0.09	0.12	0.05	0.0002	1.0
9	1.0	2.1	0.4	0.149	2.8	0.53	0.36	0.0004	7.6
S.D.	0.05	0.11	0.02	0.04	0.09	0.17	0.007	0.0006	2.8

Table 16. The growth of A. niger ($D = 0.1h^{-1}$, V = 4.2l. pH = 3.5) and A. ochraceus ($D = 0.083h^{-1}$, V = 10l. pH = 4.0) v. temperature. Air = 1v.v.m. Sm = $5.0gl^{-1}$ sucrose. Nm = $0.21gl^{-1}$ nitrate N.

$T^{o}C$	$\overline{X_E}$	$\overline{X_F}$	$\overline{Y}$	$\overline{\mu}$	$\overline{dS}$	$\overline{q_s}$	$\overline{Ysub_s}$	$\overline{dN}$	$\overline{qN} \times 10^{-3}$
				A. N I G E R					
18.0	1.1	2.4	0.11	0.046	2.78	0.116	0.4	0.038	1.5
S.D.	0.07	-	0.007	0.003	0.035	0.001	0.07	0.017	0.71
20.0	1.17	2.44	0.117	0.048	2.65	0.108	0.45	0.041	1.7
S.D.	0.18	0.75	0.018	0.011	0.64	0.017	0.18	0.0056	0.35
25.0	1.4	2.45	0.14	0.057	3.76	0.153	0.372	0.077	3.15
S.D.	-	0.07	-	0.001	0.51	0.025	0.05	0.004	0.05
27.5	2.25	3.8	0.225	0.059	3.86	0.102	0.58	0.12	2.4
S.D.	0.2	0.28	0.02	0.0014	0.226	0.012	0.09	-	0.14
30.0	2.7	4.03	0.27	0.067	4.38	0.108	0.616	0.105	2.6
S.D.	0.21	0.1	0.021	0.007	0.22	0.011	0.004	0.015	0.44
35.0	2.8	4.6	0.28	0.061	4.5	0.098	0.622	0.089	1.9
S.D.	-	0.21	-	0.003	0.254	0.01	0.035	0.027	0.49
37.5	1.74	3.18	0.174	0.055	4.48	0.14	0.39	0.08	2.5
S.D.	0.3	0.25	0.037	0.007	0.02	0.012	0.084	0.0085	0.07
40.0	1.08	3.14	0.108	0.034	3.97	0.126	0.27	0.059	1.9
S.D.	0.03	0.4	0.003	0.003	0.1	0.019	0.014	0.0085	0.07
				A. O C H R A C E U S					
18.0	1.2	5.8	0.1	0.017	4.2	0.06	0.286	0.08	1.14
S.D.	-	-	-	-	-	-	-	-	-
22.5	1.3	5.2	0.11	0.021	$4\frac{1}{2}25$	0.068	0.31	0.119	1.9
S.D.	0.18	0.28	0.014	0.0014	0.3	0.0014	0.014	0.001	0.07
25.0	1.36	5.8	0.113	0.019	4.54	0.065	0.3	0.112	1.6
S.D.	0.78	0.35	0.064	0.012	0.07	0.004	0.168	0.039	2.2
27.5	2.2	4.36	0.182	0.045	4.18	0.079	0.53	0.13	2.5
S.D.	0.7	1.23	0.059	0.023	0.104	0.03	0.167	0.055	1.6
30.0	2.8	5.1	0.232	0.045	4.35	0.07	0.643	0.13	2.1
S.D.	0.11	0.21	0.008	0.0035	0.035	0.003	0.016	0.014	0.28
32.5	2.0	3.2	0.162	0.05	4.34	0.113	0.46	0.125	3.2
S.D.	0.49	0.28	0.04	0.0085	0.05	0.011	0.113	0.01	-
35.0	1.5	2.9	0.126	0.043	4.65	0.133	0.32	0.12	3.3
S.D.	0.74	0.14	0.06	0.019	-	0.008	0.16	-	-
37.5	1.28	3.45	0.106	0.031	3.98	0.096	0.33	0.097	2.3
S.D.	0.95	0.07	0.08	0.024	0.323	0.007	0.27	0.0014	0.07
40.0	1.08	5.1	0.09	0.017	3.97	0.065	0.27	0.086	1.4
S.D.	0.71	1.0	0.06	0.009	0.216	0.015	0.185	0.0083	0.4

Table 17. The growth of A. niger and A. ochraceus v pH. V = 4.2l, T = 30°C, Air = 1v.v.m. D = $0.1h^{-1}$ Sm = $5.0gl^{-1}$ sucrose. Nm = $0.21gl^{-1}$ nitrate N.

pH	$\overline{X_E}$	$\overline{X_F}$	$\overline{Y}$	$\overline{\mu}$	$\overline{dS}$	$\overline{q_s}$	$\overline{Ysub_s}$	$\overline{dN}$	$\overline{qN} \times 10^{-3}$
				A. N I G E R					
2	3.3	5.3	0.33	0.061	4.66	0.094	0.71	0.13	2.4
S.D.	1.25	1.47	0.125	0.01	0.17	0.034	0.29	0.03	0.3
3	2.93	4.4	0.293	0.067	4.38	0.1	0.67	0.105	2.4
S.D.	0.21	0.53	0.02	0.007	0.12	0.013	0.062	0.004	0.35
4	1.38	3.26	0.138	0.04	4.48	0.14	0.32	0.107	3.3
S.D.	1.12	0.23	0.112	0.033	0.232	0.018	0.26	0.019	0.5
5	1.02	2.9	0.102	0.036	3.81	0.135	0.27	0.12	4.2
S.D.	0.3	0.56	0.03	0.012	0.235	0.03	0.09	0.015	0.94
6	0.23	1.93	0.037	0.012	4.16	0.23	0.056	0.16	8.2
S.D.	0.15	0.6	0.015	0.007	0.206	0.07	0.038	0.195	0.3
7	0.37	1.66	0.037	0.015	3.6	0.19	0.1	0.166	13.2
S.D.	0.39	1.27	0.039	0.015	0.83	0.104	0.086	0.01	10.0
8	0.24	2.0	0.024	0.014	3.54	0.182	0.068	0.166	8.5
S.D.	0.24	0.44	0.024	0.013	0.26	0.04	0.08	0.021	2.3
9	0.63	1.2	0.063	0.044	4.3	0.372	0.16	0.16	13.5
S.D.	0.77	0.3	0.077	0.05	0.27	0.08	0.163	0.008	4.1
				A. O C H R A C E U S					
2	1.5	5.7	0.15	0.027	4.18	0.073	0.37	0.136	2.4
S.D.	1.2	0.14	0.12	0.022	0.4	0.006	0.32	0.023	0.49
3	1.4	5.7	0.143	0.025	4.4	0.079	0.32	0.112	2.0
S.D.	0.84	1.13	0.084	0.012	0.12	0.018	0.185	0.013	0.3
4	1.13	5.4	0.11	0.02	4.21	0.078	0.27	0.094	1.7
S.D.	0.58	0.2	0.58	0.011	0.26	0.004	0.123	0.016	0.26
5	0.72	6.6	0.072	0.011	4.43	0.067	0.16	0.082	1.25
S.D.	0.03	0.3	0.003	0.001	0.285	0.003	0.007	0.001	0.58
6	0.3	4.83	0.03	0.008	3.7	0.08	0.09	0.06	3.0
S.D.	0.51	0.96	0.051	0.014	0.39	0.008	0.15	0.034	2.8
7	0.1	2.6	0.01	0.004	4.02	0.155	0.025	0.052	2.0
S.D.	-	-	-	-	0.1	0.003	0.001	0.004	0.14
8	0.07	1.76	0.007	0.005	4.15	0.258	0.017	0.035	2.4
S.D.	0.06	0.76	0.006	0.005	0.43	0.086	0.015	0.02	1.5
9	0.26	4.3	0.026	0.006	4.0	0.093	0.065	0.03	6.9
S.D.	0.04	0.35	0.004	0.001	0.2	0.011	0.006	0.004	0.23

Table 18. Temperature shock to P. javanicum. V = 4.2l. D = $0.2h^{-1}$ pH = 3.5, T = 30°C. Air = 1v.v.m. Sm = $5.0gl^{-1}$ sucrose Nm = $0.2gl^{-1}$ nitrate N.

Time	$\bar{X}_E$	$\bar{X}_F$	$\bar{Y}$	$\bar{\mu}$	$\bar{dS}$	$\bar{q}_s$	$\bar{dN}$	$\bar{qN} \times 10^{-3}$
				OPTIMUM				
S.S.	2.13	3.63	0.43	0.115	3.95	0.226	0.105	6.1
S.D.	0.78	0.85	0.155	0.071	0.05	0.05	0.018	2.2
				38°C SHOCK				
1h	0.9	3.3	0.18	0.054	3.88	0.235	0.095	5.76
S.D.	0.065	0.11	0.013	0.005	0.5	0.035	0.011	0.35
2.5h	0.9	3.0	0.18	0.06	3.6	0.24	0.087	5.8
S.D.	0.055	0.08	0.011	0.005	0.45	0.008	0.011	1.2
5h	0.9	3.3	0.18	0.054	2.8	0.17	0.08	4.8
S.D.	0.4	0.07	0.08	0.007	0.08	0.04	0.007	0.14
8h	1.15	3.35	0.23	0.068	2.9	0.173	0.072	4.3
S.D.	0.021	0.11	0.004	0.007	1.1	0.007	0.007	0.71
24h	0.165	3.9	0.033	0.008	2.46	0.126	0.047	2.4
S.D.	0.042	0.08	0.008	0.0004	0.08	0.035	0.011	0.042

Table 19. pH shock to A. niger. V = 4.2l. D = $0.1h^{-1}$ pH = 3.0 T = 30°C. Air = 1v.v.m. Sm = $5.0gl^{-1}$ sucrose. Nm = $0.21\ gl^{-1}$ nitrate N.

Time	$\bar{X}_E$	$\bar{X}_F$	$\bar{Y}$	$\bar{\mu}$	$\bar{dS}$	$\bar{q}_s$	$\bar{dN}$	$\bar{qN} \times 10^{-4}$
				OPTIMUM				
S.S.	5.0	6.2	0.5	0.08	4.85	0.078	0.15	24.0
S.D.	0.23	0.35	0.023	0.01	0.07	0.003	0.04	1.9
				pH 8.0				
30min	1.1	5.2	0.11	0.021	3.95	0.76	0.025	4.8
90min	1.3	4.8	0.13	0.027	3.4	0.071	0.033	6.9
150min	2.0	3.8	0.2	0.053	2.67	0.07	0.015	3.9
5 h	1.8	3.7	0.18	0.048	2.6	0.07	0.015	4.0
24h	0.93	3.1	0.093	0.03	2.45	0.079	0.02	6.45
48h	1.06	3.1	0.106	0.034	3.7	0.12	0.11	35.0

Table 20. The effect of CO_2 upon the growth of A. niger and A. ochraceus. $D = 0.1h^{-1}$, V = 4.2l. T = 30°C. pH = 3.0, Air = 1v.v.m. $Sm = 5.0gl^{-1}$ sucrose. $Nm = 0.21gl^{-1}$ nitrate N.

$\%CO_2$	$\bar{X}_E$	$\bar{X}_F$	$\bar{Y}$	$\bar{\mu}$	$\bar{dS}$	$\bar{q}_s$	$\bar{Y}sub_s$	$\bar{dN}$	$\bar{qN} \times 10^{-3}$
				A. NIGER					
Air	1.6	2.67	0.16	0.062	4.69	0.2	0.34	0.13	5.3
S.D.	0.3	0.97	0.03	0.017	0.144	0.09	0.07	0.007	2.3
20	1.6	3.98	0.16	0.042	4.81	0.124	0.328	0.132	3.4
S.D.	0.75	0.69	0.075	0.022	0.233	0.027	0.146	0.007	0.7
40	1.9	3.7	0.19	0.051	4.55	0.123	0.417	0.133	3.57
S.D.	0.17	0.1	0.017	0.0055	0.087	0.0046	0.032	0.006	0.09
60	1.37	3.97	0.137	0.034	4.53	0.114	0.302	0.128	3.24
S.D.	0.15	0.15	0.015	0.005	0.046	0.004	0.033	0.009	0.29
80	0.39	2.1	0.039	0.0185	2.1	0.1	0.189	0.09	4.3
S.D.	0.01	0.1	0.001	0.0001	0.3	0.02	0.034	0.015	0.55
				A. OCHRACEUS					
Air	2.64	6.6	0.264	0.04	4.2	0.064	0.63	0.03	0.45
S.D.	0.05	0.132	0.005	-	0.05	0.002	0.019	0.0087	0.11
20	2.35	6.1	0.235	0.038	3.95	0.065	0.61	0.025	0.41
S.D.	0.65	0.36	0.065	0.01	0.435	0.008	0.248	0.005	0.05
40	1.6	5.1	0.16	0.031	3.6	0.071	0.44	0.02	0.39
S.D.	0.5	0.95	0.05	0.006	0.167	0.01	0.13	-	0.07
60	1.1	4.4	0.11	0.024	2.4	0.054	0.47	0.02	0.45
S.D.	-	0.2	-	0.0015	0.36	0.0078	0.074	-	0.021
80	0.15	1.0	0.015	0.0147	0.4	0.041	0.38	0.015	1.6
S.D.	0.07	0.28	0.007	0.0032	0.007	0.011	0.17	0.01	0.32

Table 21. Combined limitation (Nm = 0.021gl^{-1} nitrate N. pH = 4.0, T = 20°C/38°C to A. niger. V = 4.2l. D = 0.1h^{-1}. Sm = 5.0gl^{-1} sucrose.

Day	$\overline{X_E}$	$\overline{X_F}$	$\overline{Y}$	$\overline{\mu}$	$\overline{dS}$	$\overline{q_s}$	$\overline{Ysub_s}$	$\overline{dN}$	$\overline{qN}$ x 10^{-4}	$\beta \times 10^9$
1-5	2.7	6.25	0.27	0.044	4.89	0.078	0.55	0.104	16.6	-
S.D.	0.05	0.05	0.005	0.0001	0.02	0.0001	0.013	0.008	1.2	
				Maximum limitation Low Temperature						
6	0.7	8.2	0.07	0.035	4.47	0.054	0.16	0.019	2.3	-
S.D.	0.14	0.71	0.014	0.0014	0.056	0.005	0.03	0.001	0.035	
7	1.7	11.9	0.17	0.027	4.16	0.035	0.41	0.019	1.6	-
S.D.	0.18	0.14	0.018	0.006	0.1	0.0014	0.05	0.0004	0.035	
8	0.72	12.0	0.072	0.011	4.23	0.035	0.17	0.0203	1.7	-
S.D.	0.08	0.32	0.008	0.001	0.07	0.0004	0.02	0.0003	0.97	
9	0.72	12.0	0.072	0.0062	4.23	0.035	0.17	0.0203	1.7	-
S.D.	0.03	0.14	0.003	0.0014	0.042	0.0008	0.005	0.0003	0.042	
				O P T I M U M						
1-5	2.6	6.15	0.26	0.043	4.93	0.08	0.53	0.12	19.0	-
S.D.	0.21	0.17	0.021	0.009	-	0.026	0.045	0.008	3.0	
				Maximum limitation High Temperature						
6	1.2	5.0	0.12	0.049	4.6	0.092	0.26	0.08	3.6	0.1
S.D.	0.2	2.1	0.02	0.0015	0.035	0.005	0.041	0.0013	0.37	
7	0.8	5.2	0.08	0.02	4.67	0.09	0.17	0.013	2.5	9.61
S.D.	0.43	0.95	0.043	0.014	0.1	0.018	0.09	0.002	0.1	
8	0.9	4.9	0.09	0.018	4.38	0.089	0.2	0.011	2.25	4.9
S.D.	0.21	0.18	0.02	0.002	0.21	0.008	0.035	-	0.084	
9	1.1	6.1	0.11	0.027	4.29	0.07	0.26	0.012	1.97	4.9
S.D.	-	0.24	-	0.005	0.035	0.001	0.014	0.0014	0.31	
10	0.6	4.3	0.06	0.02	3.8	0.088	0.16	0.003	0.7	0.07
S.D.	0.24	0.28	0.024	0.0025	0.18	0.01	0.07	0.0028	0.69	

Table 22. Combined limitation (Nm = 0.02gl^{-1} nitrate N. pH = 5.0 T = 21°C/37.5° to A. ochraceus. V = 4.2l. D= 0.1h^{-1} Sm = 5.0gl^{-1} sucrose.

Day	$\bar{X}_E$	$\bar{X}_F$	$\bar{Y}$	$\bar{\mu}$	$\bar{dS}$	$\bar{q}_s$	$\bar{Y}sub_s$	$\bar{dN}$	$\bar{qN} \times 10^{-4}$	$\beta \times 10^9$
					OPTIMUM					
1-5	4.3	4.8	0.43	0.09	4.75	0.1	0.9	0.125	26.0	-
S.D.	0.28	0.21	0.028	0.01	0.07	0.006	0.042	-	1.9	
					Maximum limitation Low Temperature					
6	2.7	3.6	0.27	0.07	4.3	0.12	0.63	0.0205	5.7	-
S.D.	0.15	0.1	0.015	0.003	0.06	0.002	0.06	-	0.25	
7	1.4	4.5	0.14	0.059	4.37	0.097	0.32	0.0205	4.5	-
S.D.	0.28	0.31	0.028	0.002	0.042	0.005	0.07	0.0003	0.42	
8	1.3	4.4	0.13	0.029	4.25	0.096	0.31	0.0205	4.66	-
S.D.	0.04	-	0.004	0.008	0.07	0.003	0.014	-	-	
					OPTIMUM					
1-5	3.1	4.4	0.31	0.07	4.6	0.104	0.67	0.14	31.8	-
S.D.	0.35	0.21	0.035	0.005	0.1	0.008	0.092	0.01	3.2	
					Maximum limitation High Temperature					
6	2.0	5.4	0.2	0.06	4.65	0.086	0.43	0.0207	3.83	0.07
S.D.	0.07	0.42	0.007	-	0.03	0.008	0.014	0.0003	0.35	
7	1.8	5.4	0.18	0.035	4.51	0.083	0.4	0.0205	3.8	1.85
S.D.	0.06	0.13	0.006	0.006	0.015	0.002	0.014	0.0001	0.4	
8	0.9	4.8	0.09	0.021	4.4	0.092	0.204	0.0207	4.3	0.71
S.D.	0.27	0.18	0.027	0.006	-	0.002	0.062	-	0.14	
9	0.87	4.9	0.087	0.019	4.44	0.09	0.196	0.0202	4.1	0.4
S.D.	0.1	0.12	0.01	0.001	0.012	0.003	0.02	0.0003	0.15	

Table 23. Supply of $K_4Fe(CN)_6$ to A. niger with otherwise optimum medium. $D = 0.2h^{-1}$, $T = 30^{\circ}C$. pH = 3.0, Air = 1v.v.m. $Sm = 5.0gl^{-1}$ sucrose. $Nm = 0.21gl^{-1}$ nitrate N.

$\bar{X}_E$	$\bar{X}_F$	$\bar{Y}$	$\bar{\mu}$	$\bar{dS}$	$\bar{q}_s$	$\bar{Y}sub_s$	$\bar{dN}$	$\bar{qN} \times 10^{-3}$	$\bar{Y}sub_N$
				OPTIMUM					
2.6	4.0	0.52	0.13	4.55	0.227	0.57	0.135	6.7	19.2
0.21	0.28	0.043	0.01	0.18	0.02	0.06	0.03	0.1	7.5
				10^{-5} M FERROCYANIDE					
2.0	4.8	0.4	0.083	4.34	0.18	0.46	0.109	4.5	18.3
0.28	0.37	0.06	0.012	0.2	0.02	0.06	0.023	1.2	7.3
				10^{-4} M FERROCYANIDE					
1.87	4.59	0.373	0.082	3.2	0.148	0.58	0.1	4.3	19.0
0.14	0.6	0.028	0.012	0.24	0.02	0.06	0.01	0.6	2.85
				10^{-3} M FERROCYANIDE					
1.07	4.85	0.215	0.045	3.19	0.135	0.34	0.08	3.4	13.3
0.38	0.96	0.075	0.017	0.23	0.03	0.114	0.007	0.73	3.9

Table 24. Growth of A. niger: Addition of $K_4Fe(CN)_6$ to full medium then nitrate limitation. $D = 0.2h^{-1}$, $T = 30^{\circ}C$. pH = 3.5, Air = 1 v.v.m. V = 4.2l. $Sm = 5.0gl^{-1}$ sucrose.

Day	$\bar{X}_E$	$\bar{X}_F$	$\bar{Y}$	$\bar{\mu}$	$\bar{dS}$	$\bar{q}_s$	$\bar{Y}sub_s$	$\bar{dN}$	$\bar{qN} \times 10^{-4}$	$\beta \times 10^9$
					OPTIMUM					
1-5	1.62	3.72	0.324	0.085	3.66	0.206	0.19	0.075	37.0	-
S.D.	0.26	1.17	0.05	0.008	0.67	0.042	0.05	-	8.2	
					+ FERROCYANIDE (10^{-3} M)					
6-9	2.09	5.41	0.42	0.076	3.76	0.142	0.49	0.04	15.0	-
S.D.	0.87	0.75	0.17	0.025	0.27	0.029	0.34	0.01	4.2	
					+ FERROCYANIDE + NITROGEN LIMITATION					
10	2.07	4.8	0.41	0.076	3.9	0.162	0.53	0.021	8.3	1.25
S.D.	0.035	0.07	0.007	0.0007	0.035	0.035	0.014	0.0001	0.42	
11	2.47	6.2	0.59	0.095	3.53	0.114	0.84	0.022	6.85	1.0
S.D.	0.106	0.28	0.021	0.01	0.1	0.008	-	0.0001	0.21	
12	0.56	6.2	0.113	0.07	3.1	0.1	0.185	0.015	4.7	0.48
S.D.	0.09	0.14	0.018	0.0035	0.14	0.0014	0.035	0.0042	0.28	
13	0.49	6.83	0.098	0.02	3.46	0.102	0.142	0.01	2.8	0.14
S.D.	0.015	0.25	0.003	-	0.19	0.002	0.003	0.0007	0.35	
14	1.37	7.33	0.275	0.029	3.76	0.103	0.36	0.018	3.1	-
S.D.	0.41	0.4	0.093	0.011	0.14	0.0014	0.11	0.0056	3.8	
15	0.58	7.15	0.116	0.026	3.6	0.1	0.16	0.02	5.6	-
S.D.	0.113	0.071	0.023	0.003	0.13	0.004	0.027	0.0027	0.64	

Table 25. Growth of A. niger: Shock nitrate limitation, then addition of $K_4Fe(CN)_6$ to medium. $D = 0.2h^{-1}$, $T = 30^{\circ}C$, pH = 3.5, Air = 1v.v.m. V = 4.21. $Sm = 5.0gl^{-1}$ sucrose.

Day	$\overline{X_E}$	$\overline{X_F}$	$\overline{Y}$	$\overline{\mu}$	$\overline{dS}$	$\overline{q_s}$	$\overline{dN}$	$\overline{qN} \times 10^{-4}$	$\beta \times 10^7$
				OPTIMUM					
1-5	1.4	4.1	0.28	0.069	3.38	0.165	0.14	68.0	-
-	0.28	0.14	0.056	0.016	0.44	0.027	0.047	2.0	
				NITROGEN LIMITATION					
6	0.4	6.1	0.08	0.033	3.4	0.11	0.023	7.7	-
S.D.	0.107	0.15	0.021	0.008	0.125	0.008	0.002	0.28	
7	0.7	6.3	0.14	0.01	3.42	0.108	0.023	7.4	0.1
S.D.	0.073	0.506	0.015	0.001	0.083	0.007	-	0.21	
8	1.0	6.6	0.2	0.015	3.95	0.12	0.0007	0.2	2.3
S.D.	0.29	0.103	0.058	0.0007	0.153	0.014	0.0001	0.044	
9	1.55	6.4	0.31	0.018	3.66	0.114	0.0007	0.2	3.12
S.D.	0.123	0.42	0.025	0.004	0.105	0.011	0.0001	0.028	
10	1.0	6.9	0.2	0.022	3.66	0.106	0.0007	0.2	8.7
S.D.	0.077	0.1	0.015	0.0085	0.09	0.003	0.0001	0.071	
11	1.0	6.9	0.2	0.014	3.7	0.11	0.0007	0.2	5.43
S.D.	0.08	0.08	0.016	0.0003	0.11	0.007	0.0001	0.014	
				FERROCYANIDE ADDITION					
12	0.2	5.5	0.04	0.0003	4.11	0.149	0.0003	0.1	7.3
S.D.	0.014	0.06	0.003	-	0.115	0.007	0.0003	0.014	
13	0.5	4.2	0.1	zero	0.58	0.028	0.0004	0.2	5.24
S.D.	0.09	0.3	0.018	-	0.206	0.004	0.0004	0.014	
14	0.3	2.1	0.06	zero	0.975	0.093	-	-	-
S.D.	0.042	0.11	0.008	-	0.11	0.015	-	-	
15	0.05	1.3	0.01	zero	0.45	0.07	-	-	-
S.D.	0.009	-	0.002	-	0.058	0.011	-	-	
16	0.02	1.2	0.004	zero	0.38	0.1	-	-	-
S.D.	0.01	0.13	0.002	-	0.03	0.008	-	-	

Table 26. Shock nitrate limitation to A. niger grown on 5.0gl^{-1} citrate. D = 0.1h^{-1}, T = 30°C, pH = 3.0, Air = 1v.v.m. V = 4.2l.

Day	$\bar{X}_E$	$\bar{X}_F$	$\bar{Y}$	$\bar{\mu}$	$\bar{dS}$	$\bar{q}_s$	$\bar{Y}sub_s$	$\bar{dN}$	$\bar{qN} \times 10^{-3}$	$\beta \times 10^6$
					CITRATE OPTIMUM					
1-5	2.1	4.4	0.21	0.048	4.75	0.108	0.44	0.13	2.95	-
S.D.	0.087	0.15	0.009	0.012	0.12	0.009	0.08	0.025	1.1	
					NITROGEN LIMITATION					
6	1.35	3.8	0.135	0.0044	2.5	0.066	0.54	-	-	1.0
S.D.	0.216	0.04	0.021	-	-	-	0.085	-	-	-
7	0.5	1.5	0.05	zero	1.2	0.08	0.417	-	-	-
S.D.	0.09	0.1	0.009	-	0.16	0.004	0.138	-	-	
8	zero	0.9	-	zero	0.7	0.07	-	0.006	0.5	-
S.D.	-	0.52	-	-	0.177	0.021	-	0.005	0.28	

Table 27. Growth of A. niger: Shock nitrate limitation, then addition of citrate to medium. D = 0.1h^{-1}, T = 30°C, pH = 3.5, Air = 1v.v.m. V = 4.2l. Sm = 5.0gl^{-1} sucrose/citrate.

Day	$\bar{X}_E$	$\bar{X}_F$	$\bar{Y}$	$\bar{\mu}$	$\bar{dS}$	$\bar{q}_s$	$\bar{Y}sub_s$	$\bar{dN}$	$\bar{qN} \times 10^{-4}$	$\beta \times 10^8$
					OPTIMUM					
1-5	2.62	4.17	0.262	0.063	4.6	0.11	0.57	0.11	27.0	-
S.D.	0.106	0.21	0.011	0.004	0.045	0.005	0.024	0.016	4.9	
					NITROGEN LIMITATION					
6	1.58	5.4	0.158	0.055	4.3	0.08	0.37	0.0207	3.83	4.4
S.D.	0.028	0.1	0.003	0.0013	0.113	0.001	0.018	0.001	0.26	
7	1.85	4.2	0.185	0.035	4.4	0.116	0.415	0.019	4.8	8.1
S.D.	1.06	1.62	0.106	0.005	0.22	0.05	0.22	0.0014	1.5	
8	1.37	4.0	0.137	0.037	3.43	0.094	0.4	0.019	5.6	6.3
S.D.	0.6	2.5	0.06	0.005	1.2	0.027	0.035	0.002	3.0	
					CITRATE SUPPLY + NITROGEN LIMITATION					
9	1.4	5.05	0.14	0.04	0.9	0.024	1.57	0.015	4.13	1.0
S.D.	-	3.3	-	0.002	0.14	0.018	0.25	0.0034	0.33	
10	0.6	5.0	0.06	0.019	1.32	0.033	0.44	0.019	4.56	-
S.D.	0.49	3.1	0.05	0.002	0.106	0.023	0.35	0.0015	0.25	
11	0.525	5.35	0.052	0.014	1.22	0.03	0.41	0.019	4.5	-
S.D.	0.39	3.75	0.039	0.002	0.106	0.024	0.276	0.0015	0.28	

Table 28. Cycles of shock nitrate limitation at $D = 0.1h^{-1}$ to A. niger. $V = 4.2l$. $T = 30^{\circ}C$, $pH = 3.0$, Air = 1v.v.m. $Sm = 5.0gl^{-1}$ sucrose.

Day	$\overline{X_E}$	$\overline{X_F}$	$\overline{Y}$	$\overline{\mu}$	$\overline{dS}$	$\overline{q_s}$	$\overline{Y_{sub_s}}$	$\overline{dN}$	$\overline{qN} \times 10^{-5}$	$\beta \times 10^9$
1-5	2.5	3.85	0.25	0.066	4.63	0.117	0.545	0.137	350.0	-
S.D.	0.71	0.35	0.07	0.024	0.09	0.0042	0.163	0.011	71.0	
6	1.8	5.4	0.18	0.051	4.58	0.085	0.39	-	-	-
S.D.	0.17	0.2	0.017	0.0035	0.1	0.003	0.037	-	-	
7	1.35	6.0	0.135	0.033	4.6	0.077	0.29	0.0005	0.75	11.3
S.D.	0.06	0.42	0.006	0.001	0.04	0.005	0.02	0.0001	0.03	-
8	1.15	7.2	0.115	0.026	4.4	0.061	0.26	0.0005	0.347	1.18
S.D.	0.05	0.25	0.005	0.0064	-	0.002	0.01	0.0001	0.13	
9	0.9	7.0	0.09	0.013	4.4	0.063	0.2	0.0001	0.143	4.5
S.D.	0.13	0.27	0.013	0.0023	0.02	0.002	0.03	-	0.008	
10	2.4	6.8	0.24	0.022	4.69	0.069	0.51	0.14	210.0	-
S.D.	0.04	-	0.004	0.0035	-	-	0.01	0.01	20.0	
11	3.5	5.2	0.35	0.037	4.75	0.09	0.74	0.147	280.0	-
S.D.	0.07	0.47	0.007	0.0035	-	0.008	0.01	0.003	25.0	
12	1.95	5.8	0.195	0.05	4.55	0.078	0.428	-	-	0.02
S.D.	0.06	0.11	0.0056	0.003	0.2	0.0042	0.03	-	-	
13	1.4	6.0	0.14	0.03	4.55	0.076	0.31	0.0004	0.583	1.0
S.D.	0.42	-	0.042	0.0035	-	-	0.092	0.0001	0.073	
14	1.15	6.0	0.115	0.021	4.4	0.073	0.26	0.0001	0.17	17.0
S.D.	0.2	0.56	0.02	0.006	0.02	0.008	0.113	-	0.014	
15	1.25	6.1	0.125	0.02	4.25	0.07	0.29	0.0001	0.164	1.3
S.D.	0.11	0.71	0.011	0.0014	0.01	0.0085	0.028	-	0.02	
16	0.88	6.3	0.088	0.014	4.4	0.07	0.2	0.002	0.317	-
S.D.	0.1	0.66	0.01	0.003	-	0.004	0.008	-	0.008	

Table 29. Cycles of shock nitrate limitation at D = $0.2h^{-1}$ to A. niger.
V = 4.21. T = 30°C, pH = 4.0, Air = 1v.v.m. Sm = $5.0gl^{-1}$ sucrose.

Day	$\overline{X_E}$	$\overline{X_F}$	$\overline{Y}$	$\overline{\mu}$	$\overline{dS}$	$\overline{q_s}$	$\overline{Ysub_s}$	$\overline{dN}$	$\overline{qN} \times 10^{-5}$
1-5	2.55	3.1	0.51	0.165	4.58	0.306	0.56	0.173	1200.0
S.D.	0.578	0.78	0.115	0.01	0.04	0.065	0.126	0.012	320.0
6	2.3	5.8	0.46	0.127	2.62	0.09	0.88	-	-
S.D.	0.109	1.9	0.038	0.0064	0.62	0.023	0.03	-	-
7	1.7	5.7	0.34	0.069	2.72	0.095	0.625	0.0001	0.35
S.D.	0.04	0.32	0.008	0.0085	0.52	0.013	0.025	0.0001	0.46
8	1.9	4.8	0.38	0.061	2.72	0.113	0.7	0.0001	0.42
S.D.	0.24	0.1	0.048	0.0014	0.12	0.003	0.008	-	-
9	1.9	4.8	0.38	0.079	2.72	0.113	0.7	0.0001	0.42
S.D.	0.13	1.59	0.026	0.0085	0.17	0.008	0.011	-	-
10	1.4	4.5	0.28	0.068	2.7	0.12	0.5	0.0001	0.44
S.D.	0.15	0.19	0.03	0.0014	-	0.029	0.008	0.0001	0.6
11	1.9	4.5	0.38	0.073	3.2	0.14	0.59	0.11	490.0
S.D.	0.21	0.1	0.042	0.007	0.19	0.003	0.04	0.01	49.0
12	3.0	4.1	0.6	0.11	4.25	0.207	0.7	0.14	680.0
S.D.	-	0.4	-	0.0056	0.16	0.03	0.008	0.015	140.0
13	2.7	4.9	0.54	0.13	3.1	0.126	0.87	0.0003	1.22
S.D.	0.22	0.3	0.044	0.018	0.02	0.01	0.015	-	0.1
14	1.95	4.9	0.39	0.095	3.0	0.122	0.65	0.0002	0.81
S.D.	0.15	0.1	0.03	0.0056	-	0.002	0.007	0.0001	0.537
15	1.5	4.6	0.3	0.07	3.0	0.13	0.5	0.0002	0.87
S.D.	0.16	0.16	0.032	0.007	0.35	0.019	0.055	-	0.042
16	1.31	4.8	0.302	0.063	2.6	0.108	0.58	0.0001	0.42
S.D.	0.08	0.1	0.008	0.0056	0.16	0.003	0.01	-	0.056

Table 30. 2-stage fermentation of A. niger, with temperature alteration in 2nd stage. V_1 = 4.2l. V_2 = 10l. D_1 = 0.1h^{-1}, D_2 = 0.042h^{-1} pH_1 = 3.0, pH_2 = 4.0, Air_{1+2} = 1v.v.m. T_1 = 30°C, Sm_1 = 5.0gl^{-1} sucrose. Nm_1 = 0.21gl^{-1} nitrate N.

Stge	$\bar{X}_E$	$\bar{X}_F$	$\bar{Y}$	$\bar{\mu}$	$\bar{dS}$	$\bar{q}_s$	$\bar{Y}sub_s$	$\bar{dN}$	$\bar{qN} \times 10^{-3}$
				2ND STAGE = 20°C					
1	1.57	2.27	0.157	0.07	4.57	0.21	0.34	0.118	5.6
S.D.	0.29	0.6	0.029	0.01	0.103	0.055	0.054	0.016	2.2
2	1.9	1.95	0.014	0.007	-	-	-	-	-
S.D.	0.4	0.48	0.007	0.007	-	-	-	-	-
				2ND STAGE = 30°C					
1	1.65	1.85	0.165	0.089	4.78	0.26	0.343	0.096	5.2
S.D.	0.07	0.07	0.007	0.007	0.18	-	0.024	0.03	1.4
2	1.05	1.95	-	-	-	-	-	0.009	0.19
S.D.	0.07	0.35	-	-	-	-	-	-	-
				2ND STAGE = 40°C					
1	1.27	2.67	0.127	0.05	4.63	0.212	0.27	0.125	5.7
S.D.	0.32	1.18	0.032	0.015	0.03	0.128	0.07	-	3.4
2	1.67	1.6	0.017	0.01	0.36	0.007	1.11	-	-
S.D.	0.7	0.69	0.008	0.008	0.54	0.011	0.85	-	-
				2ND STAGE = 43°C					
1	1.67	2.43	0.167	0.071	4.52	0.2	0.366	0.12	5.3
S.D.	0.75	0.115	0.075	0.027	0.074	0.02	0.16	0.005	0.4
2	0.75	1.75	-	-	-	-	-	-	-
S.D.	0.13	0.41	-	-	-	-	-	-	-

Table 31. 2-stage fermentation of A. niger, with various carbon supplies to 2nd stage. V_1 = 4.2l. V_2 = 10l. $D_{1+2} = 0.1h^{-1}$, pH_{1+2} = 3.5, $T_{1+2} = 30^{\circ}C$ Air_{1+2} = 1v.v.m. $Sm_1 = 5.0gl^{-1}$ sucrose. $Nm_1 = 0.21gl^{-1}$ nitrate N.

C.to 2nd.	Stge	$\bar{X}_E$	$\bar{X}_F$	$\bar{Y}$	$\bar{\mu}$	$\bar{dS}$	$\bar{q}_s$	$\bar{dN}$	$\bar{qN} \times 10^{-3}$	$\beta \times 10^9$
5.0		2.0	3.6	0.2	0.056	4.7	0.13	0.13	3.58	-
	1 S.D.	0.25	0.11	0.025	0.008	0.095	0.002	0.004	0.15	-
		2.78	4.6	0.078	0.042	4.73	0.103	0.016	0.36	7.16
	2 S.D.	0.58	0.43	0.058	0.01	0.072	0.01	0.006	0.15	7.48
4.0		2.7	6.4	0.27	0.042	4.75	0.074	0.118	1.8	-
	1 S.D	0.25	0.5	0.025	0.006	0.03	0.007	0.005	0.11	-
		2.9	4.47	0.02	0.039	2.75	0.062	0.033	0.75	1.8
	2 S.D.	0.058	0.29	0.006	0.007	0.3	0.009	0.009	0.22	0.177
3.0		3.1	5.32	0.31	0.06	4.5	0.087	0.1	2.0	-
	1 S.D.	0.3	0.97	0.03	0.016	0.05	0.015	-	0.38	-
		3.17	4.25	0.007	0.044	2.52	0.06	0.049	1.15	+
	2 S.D	0.55	0.37	0.055	0.011	0.23	0.009	0.0001	0.1	-
2.0		2.55	6.15	0.255	0.042	4.75	0.077	0.118	2.0	-
	1 S.D.	0.21	0.35	0.021	0.0007	0.007	0.004	-	0.14	-
		2.4	3.25	-	0.04	1.42	-	0.018	0.56	-
	2 S.D.	0.14	0.35	-	0.0085	0.61	-	0.0068	0.27	-

Table 32. 2-stage fermentation of A. niger, with continuous $K_4Fe(CN)_6$ supply to 2nd stage. V_1 = 4.2l. V_2 = 10l. $D_1 = 0.1h^{-1}$, $D_2 = 0.052h^{-1}$ pH_{1+2} = 4.0 $T_{1+2} = 30^{\circ}C$ Air_{1+2} = 1v.v.m. $Sm_1 = 5.0gl^{-1}$ sucrose. $Nm_1 = 0.21gl^{-1}$ nitrate N.

Stge	$\bar{X}_E$	$\bar{X}_F$	$\bar{Y}$	$\bar{\mu}$	$\bar{dS}$	$\bar{q}_s$	$\bar{Y}sub_s$	$\bar{dN}$	$\bar{qN} \times 10^{-3}$	$\beta \times 10^9$
			No $K_4Fe(CN)_6$ TO 2ND STAGE							
	1.85	4.1	0.185	0.045	4.6	0.112	0.4	0.11	2.7	-
1 S.D.	0.2	0.5	0.02	0.005	0.063	0.023	0.063	0.01	1.4	-
	1.95	3.3	0.005	0.0072	0.25	0.004	0.4	0.06	0.94	-
2 S.D	0.25	0.11	0.005	0.001	0.05	0.001	0.007	0.04	0.1	-
			10^{-4} M $K_4Fe(CN)_6$ TO 2ND STAGE							
	1.38	3.38	0.138	0.041	4.49	0.14	0.307	0.107	3.36	-
1 S.D.	0.45	0.93	0.045	0.003	0.05	0.037	0.1	0.002	0.85	-
	1.64	2.7	0.013	0.01	0.105	0.002	2.48	0.013	0.24	0.46
2 S.D.	0.5	0.17	0.005	0.0085	0.12	0.002	1.1	0.017	0.33	0.63
			10^{-3} M $K_4Fe(CN)_6$ TO 2ND STAGE							
	1.89	4.6	0.189	0.041	4.5	0.098	0.42	0.106	2.33	-
1 S.D.	0.11	0.34	0.011	0.0032	0.094	0.008	0.025	0.006	0.23	-
	1.84	3.7	-	0.0044	0.45	0.006	-	0.016	0.32	1.1
2 S.D.	0.15	0.25	-	0.001	0.09	0.001	-	0.014	0.22	1.46

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"Sporulation of *Aspergillus niger* and *Aspergillus ochraceus* in continuous submerged liquid culture"

by A. J. Broderick and R. N. Greenshields.

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Sporulation of *Aspergillus niger* and *Aspergillus ochraceus* in continuous submerged liquid culture.

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RUNNING TITLE:

Continuous submerged fungal sporulation

SUBJECT CATEGORY:

Physiology and Growth

INDEX SUGGESTIONS

Sporulation of A. niger and A. ochraceus in continuous culture

Aspergillus niger. sporulation in continuous culture

Aspergillus ochraceus. sporulation in continuous culture

Continuous submerged fungal sporulation.

Sporulation of Aspergillus niger and Aspergillus ochraceus was induced in a continuous tower fermenter by restricting growth by nutrient limitation. Shock carbon limitation produced no sporulation, but the gradual decrease of sucrose or starch supply to A. niger produced slight sporulation. Gradual nitrate limitation produced no sporulation, while a shock decrease in nitrate concentration caused heavy sporulation of both organisms. The previously unobserved morphology of the sporulating structures produced was much simplified under nitrate limitation, but similar to sub-aerial morphology under carbon limitation. Maintenance energy values for sucrose and starch were calculated for A. niger and for starch for A. ochraceus. The continuous tower fermenter system was found to be ideal for controlling organism morphology and thus sporulation.

INTRODUCTION

Suppression of submerged conidiation of filamentous fungi has been described as a feature characteristic of shake cultures (Cochrane 1958). Several studies since have examined the induction of submerged sporulation by manipulation of cultural conditions (Smith et al. 1977). Conidiation is considered to be a response to environmental conditions which severely restrict vegetative growth. Vezina et al. (1965) achieved conidiation of several filamentous fungi, including Aspergillus ochraceus, in submerged culture, and emphasised the importance of equilibrium between medium composition and physiochemical conditions. Sporulation may be induced by exhaustion of the carbohydrate source while nitrogen is in excess (Galbraith & Smith, 1969a, for A. niger), or by exhaustion of available nitrogen in the presence of assimilable carbohydrate (Morton et al., 1960 for Penicillium griseofulvum; Morton, 1961, for P. griseofulvum; Hadley & Harold, 1958 for P. notatum; Carter & Bull, 1969, for Aspergillus nidulans; and Weiss & Turian, 1966 for Neurospora crassa). Galbraith and Smith (1969a) concluded that conidiation of A. niger depended upon the type and concentration of the nitrogen source. Batch cultivation consists of transient environments, while chemostat culture allows steady state selective enhancement of specific morphological life cycle stages. Ng et al. (1973) described the conidiation of A. niger in continuous culture under citrate and nitrogen limitation, which showed that growth and sporulation are not necessarily mutually exclusive. Righelato et al. (1968) and Hsu & Ordal (1969) have also studied sporulation of fungi in chemostat culture, and indicate that both nature and concentration balance of the medium are important. The aim of this study was to develop a commercial fermentation system for the continuous production of conidia of A. niger and A. ochraceus using the continuous tower fermenter, and to study the sporulation morphologies induced.

METHODS

Organisms Master cultures of Aspergillus niger, Van Tieghem, 38 of the fermentation laboratory culture collection (ex Dr. Drysdale, Department of Genetics, Birmingham University) and Aspergillus ochraceus, Wilhelm (C.M.I No.16247 iv) were maintained on malt extract agar slopes at 4°C and sub-cultured six-monthly. The suitability of A. niger for work in the C.T.F. system has previously been confirmed, and A. ochraceus was used because of its similarity to A. niger and the importance of the spores in pharmaceutical reactions has been reported (Dulaney et al., 1955; Schleg & Knight, 1962; Sehgal et al., 1968; Haines & Collingworth, 1953).

Fermentation equipment. Fermentations were conducted in one of two glass tubular fermenter vessels, one of 10 litres working volume, the other of 4.2 litres. The design, construction and applications of the tower fermenter are described by Greenshields & Smith (1971, 1974), Smith & Greenshields (1974) and Cocker & Greenshields (1975). The basic 10 litre design consists of three 40.0 cm lengths of standard 10.0 cm internal diameter pyrex glass pipework, joined by polytetrafluoroethylene (P.T.F.E) gaskets and clamped water tight. Spent medium and gases pass out of the top of the vessel via an 8.0 mm internal diameter glass swan-neck and sterile compressed air (supplied to each fermenter at one volume per volume per minute) enters at the base and is dispersed before passing upwards by a P.T.F.E. air distributor plate. The 5.0 litre fermenter has an identical aspect ratio to the 10 litre (90.0 cm x 7.5 cm) but the main body of the fermenter is in one piece. Both vessels have facilities for continuous monitoring and control of pH, temperature and dissolved oxygen.

Preparation of inocula. A malt extract agar mat was prepared in the base of a 250 ml conical flask, inoculated with the appropriate mould from the sub-master culture and incubated at 30 - 35°C for three weeks. After this time, a full spore mat had formed and the spores were removed by addition of

100 ml 0.1% v/v solution of 'Tween 80' wetting agent (sterile) and vigorous agitation, before the spore suspension was aseptically injected into the fermenter.

Cultural conditions. Basal medium constituents, gl^{-1}; Sucrose 5.0; $(NH_4)_2SO_4$ 1.0; $NaH_2PO_4H_2O$ 0.5; KCl 0.25; $MgSO_45H_2O$ 0.1; $CaCl_2$ 0.05; yeast extract 0.5. Variations: Sucrose replaced in some experiments by glucose or starch equivalent. $(NH_4)_2SO_4$ was sometimes replaced by 1.3 gl^{-1} $NaNO_3$. Media were sterilised by autoclaving in 20 litre batches at 103.5 Kilopascals for 20 minutes.

Methods of nutrient limitation. Limiting levels selected were based upon the principle of decreasing nutrient supply until equal to or below the maintenance requirements of the organism. The criteria for nitrogen limitation were determined in two ways. Firstly by lowering the nitrogen content of the medium to below the level of utilization at maximum growth rate under optimum conditions at a steady state while maintaining all other parameters optimal. Secondly by decreasing nitrogen level to that used by other workers to induce sporulation in batch culture. Galbraith and Smith (1969a) state that conidium formation is inhibited by the presence of NO_3^- ion concentrations at or above 60 mgl^{-1} (atomic N) and conidiophores are inhibited by concentrations at or above 120 mgl^{-1}. In these experiments the level of nitrogen content of the medium was therefore decreased to 20 mgl^{-1} (atomic N) and at times to zero (Anderson & Smith, 1971a, used a nitrogen-free medium to induce conidiophore elongation). To provide gradual nitrogen limitation, step-wise reductions in the nitrogen content of the medium were made between the establishment of each steady state. Shock treatment was introduced by removing the medium supplying the optimum nitrogen supply (0.21 gl^{-1}) and replacing this immediately with one containing approximately 20.0 mgl^{-1} without altering any other environmental parameter. The effect upon growth of the organism was then monitored. The principles of carbon limitation (gradual and shock) were identical to those of nitrogen,

except that a concomitant decrease in dilution rate with carbon level was examined for both shock and gradual limitation. Details of each limitation are given with the results.

Dilution Rate: The majority of experiments were conducted at dilution rates of 0.2 h^{-1} or 0.1 h^{-1}, although lower rates were used during carbon-limited fermentations.

Analytical Methods. Total sugar concentration of medium and effluent was determined by the method of Dubois et al. (1956) with reference to a standard curve constructed for each kind of carbohydrate supply (expressed as gl^{-1} sugar). Nitrate and nitrite content of medium and effluent were determined by the method of Chapman et al. (1967) and expressed as g. atomic nitrogen l^{-1}. Ammonium nitrogen (g. atomic N l^{-1}) was determined by the Kjeldahl distillation method of Markham (1942).

Fermenter sampling. 100 ml samples were collected from (a) the effluent stream and (b) the tower, filtered through fine muslin and the mycelial dry weight (gl^{-1}) determined by drying the residual biomass at 105°C for 24 hours. These gave (a) the effluent biomass concentration, (X_E), and (b) the fermenter biomass concentration, (X_F).

Microscopy. The production of sporulation structures was assessed by direct observation of fresh mycelial samples. Spore numbers were recorded by counting free conidia in an effluent sample after vigorous agitation, using an Improved Neubaur counting chamber. All observations were made using a Leitz-Wetzler binocular microscope.

Fermentation Parameters. Dilution rate (D),

was calculated by $\frac{f}{V}$ where f = flow rate of medium supply lh^{-1}
V = volume of fermenter, l.

The specific growth rate during a steady state was then calculated from the equation:

Growth rate $\mu = D\frac{X_E}{X_F}$ where X_E = effluent [biomass] gl^{-1}
and X_F = fermenter [biomass] gl^{-1}

The factor $X_E.D$ represents the productivity (Y) of the system, $gl^{-1}h^{-1}$.

Growth rate in transient states was calculated by the equation:

$$\mu = \frac{1}{X_F \text{ TOTAL}} \times \frac{\text{New biomass produced}}{\Delta t}$$

where 'New biomass produced' $= f. \bar{X}_E.\Delta t + V(X_{F2} - X_{F1})$, where $\bar{X}E$ = mean effluent biomass concentration between time O and t., Δt = time elapsed $O \rightarrow t$ (hours) and $X_F \text{ TOTAL} = V \frac{X_{F1} + X_{F2}}{2}$ g. where X_{F1} = fermenter biomass concentration at time = O, and X_{F2} = fermenter biomass concentration at time = t.

Where dilution rate varied during an experiment, the substrate supply rates (Ks for sugars, K_N for nitrogen) were expressed as gh^{-1}, calculated by:

Ks = Sm.f where Sm = sugar medium concentration, f = flow rate.

KN = Nm.f where Nm = nitrogen medium concentration.

The maintenance coefficient, m ($gg^{-1}h^{-1}$) representing the quantity of substrate consumed by cells for functions other than growth, was calculated by the method of Schulze and Lipe (1964) using regression analysis. The specific substrate utilization rate for sugar (qs) was calculated from the equation $qs = \frac{\Delta S.D}{X_F}$ $gg^{-1}h$ where ΔS = sugar utilization, gl^{-1}. To express the intensity of sporulation, a sporulation index (β) was used (Hadley & Harrold, 1958) which represents the number of spores per g. dry weight mycelium.

Replication. Where no time scale appears on the graphs, a result represents the mean of steady state values recorded over at least three days. Mean nutrient levels were calculated from at least three analytical replicates, and mean growth rate and productivity values were obtained from triplicate X_E and X_F sample analyses. Where a time scale monitors a transient state, a point represents the mean value of the means of two or three replicates of the experiment.

RESULTS

Several authors have expressed the opinion that ammonium ions are either inhibitory or detrimental to sporulation (Galbraith & Smith, 1969a). The $(NH_4)_2SO_4$ of the basal medium was therefore replaced with $NaNO_3$. without any adverse effect upon the optimum growth rates of either organism at a dilution rate of $0.1h^{-1}$ in continuous culture.

The mean growth rates of A. niger and A. ochraceus in $(NH_4)_2SO_4$ medium were 0.062 h^{-1} and 0.051 h^{-1} respectively while in $NaNO_3$ medium the respective growth rates were 0.06 h^{-1} and 0.05 h^{-1}. These results show that the growth rate of A. ochraceus is lower than that of A. niger in this system.

Carbon limitation

Shock reductions

Several fermentations were performed using sucrose as the carbon source at D = 0.2 h^{-1}, and after achieving steady state (on 5gl^{-1} sucrose plus normal basal medium) the sucrose concentration of the medium (Sm) was lowered to 0.25 gl^{-1}, i.e. Ks decreased from 4.2 gh^{-1} to 0.21 gh^{-1} (Fig.1). In this case limitation was imposed on day 8, but the limitation was too severe at this dilution rate, and washout occurred within 3 days with no sign of sporulation. The peaks of productivity (Y) and growth rate (μ) the day after limitation was imposed are false values due to the high X_E values resulting from wash-out.

These experiments were repeated imposing a similar shock carbon limitation (Ks 4.2 gh^{-1} to 0.19 gh^{-1}) but with higher Sm values and lower D values. This was again unsuccessful and no sporulation resulted. Wash out was more gradual but at longest the fermentation only lasted three days after initiation of sucrose limitation.

Gradual Carbon Limitation

Dilution rate and sucrose concentration (Sm) were gradually reduced to A. niger growing in otherwise optimal conditions (Fig.2). Sporulation was achieved during this type of fermentation. In one example, sporing structures became visible in the tower contents on day 16 (Ks 0.4 gh^{-1}) although spores were too few to count. All structures produced were complex sub-aerial forms with long conidiophores and large vesicles supporting many phialides, each producing spores. No spore chains were seen. Sporulation continued until the fermentation was terminated due to yeast infection. The gross morphology

of the mycelium during sporulation corresponded to the type I - II form as proposed by Cocker (1980) accounting for the low X_F values encountered. A maintenance coefficient of 0.018 $gg^{-1}h^{-1}$ was calculated. This experiment was repeated using A. ochraceus but the organisms washed out of the fermenter when Ks was lower than 1.0 gh^{-1}.

Starch is a carbohydrate source which is biochemically less available to the Aspergilli due to the need for hydrolysis before utilization, and it was anticipated that starch limitation would prove more severe than sucrose limitation and thus lead to increased sporulation. The effect of the lowest starch supply to A. niger at D = 0.1h^{-1} and T = 30°C is shown in Fig.3a. Sporulat was first noticed on day 12 (48 hours after the onset of the lowest carbon feed state) when conidiophore initials were produced from foot cells. Eight hours later vesicles were visible, and 24 hours later phialides and spores were present. Sporulation was not synchronous, all structures being present together, but a sporulation development time of approximately 18 - 24 hours from vegetative mycelium to spore production was indicated. The morphology of the sporing structures was simplified by only 3 - 4 phialides being produced on each vesicle.

Sporulation was evident for 72 hours, but insufficient free spores were produced for accurate counts. At the time of sporulation the floc morphology was type I - II (Cocker, 1980). After 72 hours, sporulation ceased and the culture died. This starch limitation experiment was repeatedly copied using A. ochraceus, but the organism died when Ks was decreased to 0.4 gh^{-1} and no sporulation resulted.

The calculation of maintenance values of 0.0383 $gg^{-1}h^{-1}$ (A. niger) and 0.123 $gg^{-1}h^{-1}$ (A. ochraceus) were carried out as in Fig.3b.

Nitrogen limitation

Gradual limitation

Maintaining a carbon supply of 5.0 gl^{-1} sucrose, at D = 0.2h^{-1} and T = 30°C, the nitrogen content of the medium was reduced stepwise, allowing

the fermentation to approximate to a steady state between each stage; during nutrient limitation, steady state operation is rarely encountered. The results for A. niger and A. ochraceus are presented in Figs.4a and 4b. In both cases there was no sporulation, although growth was very slow, the mould adapting to the change in environment. One particular A. niger fermentation was run for 1536 hours on Nm of 0.02 gl^{-1} or less with no sign of sporulation. It is suggested that the organism obtains sufficient nitrogen supply for maintenance and growth from the autolysis of dead cells at the low dilution rate. Pannell (1976) also observed this effect.

Shock nitrogen limitation

After several days at optimum conditions and at a steady state, the nitrogen supply for A. ochraceus was suddenly reduced from 0.214 gl^{-1} to 0.0214 gl^{-1} (Fig.5a). Sporulation began approximately 10 hours after the initiation of nitrogen limitation when conidiophore initials were seen. After 16 hours vesicles were present and sporulation was complete after 18 hours upon the production of phialides and spores. Again, sporulation was not synchronous and continued for 7 days after which time the fermentation was stopped due to infection. Although sub-aerial sporing structures were seen, the majority of structures were unusual, having a much reduced conidiophore bearing an apparently normal vesicle which held few (as low as two) phialides (Fig.5b). The spore size was normal (2 - 3 µm diameter) and no spore chains were seen. The sporulation index figures from one experiment (Fig.5a) show that spore production reached a peak after six days and then declined rapidly until, after 9 days, there were no spores visible, when the fermentation was stopped. An important observation during this fermentation was that of microcycle conidiation. The incidence of these structures was relatively low but their presence indicates that the environment of the tower was capable of supporting the germination of spores produced in that system, and also such that almost immediate re-sporulation of the germ tube was induced. The nutrient interactions here are complex and an immediate explanation of this

phenomenon was not apparent.

The experiment of shock nitrate limitation was repeated using A. niger, and an optimum steady state was achieved on a full medium supply at $D = 0.1h^{-1}$ and $T = 30^{o}C$. The nitrate supply was then decreased to one-tenth and results are shown in Figure 6a. As with the carbon limitation experiments, μ decreases very shortly after nutrient limitation due to wash-out of the organism (at a particular dilution rate and nutrient concentration, the fermenter is only capable of supporting a limited fermenter population). Sporulation occurred approximately 12 hours after the onset of limitation and continued for 8 days when sporulation ceased and the fermentation was terminated. Spore production was not as heavy as with A. ochraceus (sporulation index, Fig 6a) but was found to be equally easily repeated. There were a variety of sporing structures produced ranging from complex (sub-aerial type) to much simplified structures (those produced in the shortest time of 12 hours) of single large phialides borne on hyphal tips (Fig. 6b). Sporulation was not synchronous and a complete range of structures were produced together. One should note that μ never reaches zero during sporulation and carbon utilization does not drop appreciably at any time during nutrient limitation. The fermentation parameters exhibit damped oscillations after the shock stimulus, during which time sporulation occurs, and later gradually level out to an almost steady state, by which time sporulation has ceased.

DISCUSSION

It was possible with the continuous tower fermenter system to estimate the maintenance level of the carbohydrate supply to the fungi

by extrapolating graphs of growth rate (μ) against specific substrate utilization rate (q) to the point of zero growth. The values of 0.018g sucrose $g^{-1}h^{-1}$ and 0.0383g starch $g^{-1}h^{-1}$ for A. niger calculated from our results are similar to previously stated values for the carbohydrate maintenance requirement of other filamentous fungi (Table 1) but the value of 0.123g starch $g^{-1}h^{-1}$ for A. ochraceus is considerably higher than any previously calculated value. This may be due to the slow growth rate of this organism. It was not possible to calculate a maintenance co-efficient value for nitrogen as growth rate and productivity were still positive when utilization had apparently reached zero with no nitrogen supply to the culture. This agrees with the fact that the organism can utilize a carbohydrate source for respiration and growth in the absence of a nitrogen supply while the reverse is not possible. Thus growth ceases when sufficient carbohydrate is removed from the supply, but continues when all nitrogen is removed from the medium. At this point the organism is assumed to obtain sufficient nitrogen from autolysis of dead cells. This was most evident in the A. niger cultures which were able to survive for long periods on their own lysis nitrogen products with medium that contained no nitrogen source but had all other nutrients in excess. This condition could not last indefinitely, because only sufficient nitrogen was available for the maintenance of existing cells and not for the production of new cells i.e. there was no increase in mycelial mass. The existing cells therefore gradually die and are not replaced, although this is a very slow process.

The results support evidence that sporulation occurs at substrate levels slightly above the maintenance ration (sucrose 0.102 g $g^{-1}h$ to A. niger, starch 0.093 g $g^{-1}h^{-1}$ to A. ochraceus and 0.04 g $g^{-1}h^{-1}$ to A. niger)

which was also noted by Righelato et al. (1968) and Ng et al. (1973).

We agree with Smith et al. (1977) that is 'better to consider that vegetative growth and sporulation are cellular processes competing for limiting metabolic intermediates rather than as mutually exclusive phenomena', because growth continued throughout each sporulative phase. This observation may be distorted because fermenter productivity and hence growth rate is influenced not only by the physical and nutritional parameters but also by the organism morphology induced by these restraints. As a consequence of nutrient limitation, the gross mycelial floc morphology alters to a form type I to II (Cocker, 1980), more easily removed from the fermenter which results in a gradual wash out which lowers the fermenter biomass concentration and falsely increases fermenter output. Growth, as calculated by the increase in mycelial dry weight, is probably due to the formation of sporulation structures and not to hyphal biomass.

The most intriguing aspect of this work was the unusual morphology of the sporulating structures observed. There are conflicting reports on the submerged reproductive structures of filamentous fungi. Carter and Bull (1969) describe structures of Aspergillus nidulans as identical to sub-aerial structures. Galbraith and Smith (1969a) also state that the sporulation structures observed were identical to those of static cultures although lacking conidial chains. The similarity between submerged and sub-aerial structures has also been reported by Righelato et al. (1968), Vezina et al. (1965) and Hadley and Harrold (1958). Evidence for the reduction of complexity of submerged sporing structures is equally well documented. Anderson and Smith (1971b) observed direct production of phialides on tips of germ tubes of A. niger and Ng et al. (1973) report considerable reduction of complexity

of conidial apparatus of A. niger in chemostat culture. There are also various microcycle sporulation structures described for A. niger (Anderson & Smith, 1971b) and Penicillium urticae (Sekiguchi et al. 1975). These experiments produced much simplified reproductive structures of both species. Structures produced in continuous culture under carbon limitation were all similar to the sub-aerial form, and any simplification (e.g. A niger on starch) was reflected in the decreased number of phialides and spores. The structures produced by A. niger and A. ochraceus during nitrogen limitation were predominantly much simplified. However, in the nitrogen limited cultures, a range of reproductive structures from complex to very simple were frequently observed, along the same hypha. Consequently, explanation of the simplification of sporulation morphology is complicated and we consider that the exact switch-over point to a sporulative metabolism has not yet been defined. Ng et al. (1973) suggest the simplification of morphology is due to a partial switch-on of the conidial mechanism with the morphological and biochemical events of development precluding spore formation being by-passed. They also suggest that the residence time of the mycelium in the fermenter may determine the morphology of sporulation, but this seems unlikely on the evidence of this work. Dilution rate and hence residence time determined the incidence of sporulation (under nitrogen limited conditions, a dilution rate of 0.4 h^{-1} inhibited sporulation, while spores were formed under identical conditions at D = 0.2h^{-1} and 0.1h^{-1}) but exerted no control over the morphology of sporulation. Sporing structures were produced primarily at the edges of a mycelial floc with the centre of the floc remaining vegetative which indicated that those hyphae at the edge of the floc which have access to the limited nutrients

available, use these to produce spores. The observation of adjacent complex and simplified structures suggests that the morphology produced depends upon nutrients and oxygen availability along the hypha within the peripheral zone of the floc. Hence those structures near the hyphal tip, which has greater access to nutrients, would be complex, while further back along the hypha, structures would become increasingly simplified. However, there is as yet no evidence of this. The induction of the reproductive phase of the organisms is considered analagous to the induction of a non growth-associated secondary metabolite, and as such the production of the desired product (i.e. spores) continued only for a finite period of time. Dawes and Thornley (1970) report that the steady state conditions of spore formation by Bacillus subtilis were not maintained indefinitely due to the spontaneous appearance of asporagenous mutants, which replaced the sporing organism. This was not the case with either of the fungi tested, because samples taken after the sporulation phase, and plated out at 30^{o}C produced perfect spore mats in 10 - 12 days. It is a feature of submerged continuous culture of filamentous fungi that the capacity for sporulation is gradually lost, but Pannell (1976) considers this to be physiological rather than genetic although the precise cause is not known. This loss of sporulative capacity was only noted in this work with any fermentation conducted for six months or longer. Pannell (1976) found that sporulative ability was restored in sub-cultures of the low-sporing strains, as was the case in these experiments, but new fermentations were always started from a new master-culture. The loss of sporulative capacity is considered to be

a response to the physico-chemical stress and continual growth restraint imposed upon the fungus in this system.

During sporulation, overall growth rate decreases and therefore X_f decreases. As a result, the substrate supply per gram of mycelial dry weight increases, so slowly removing the stimulus of nutrient limitation and ending sporulation. A cycling of nutrient levels in this way may explain the micro-cycle conidiation observed.

It is proposed to establish a semi-continuous system by cyclic medium supply using one medium to induce conidiation, and a second to increase growth (and X_f) after sporulation has ceased, before imposing new growth restraint. The effects of physical parameters ,pH, temperature, dilution rate, O_2/CO_2 supply which are easily controlled in the C.T.F. will also be investigated. It is already known that pH exerts a major effect upon mould morphology (Galbraith & Smith 1969b, Stockbridge 1979).

The effect of nutrient limitation upon sporulation is a complex phenomenon and is closely linked with the influence of the physiochemical environment of the continuous tower fermenter.

We consider that continuous sporulation is the result of the summation of two or more elements of the physiochemical environment . However, this work indicated that control over sporulation in continuous submerged culture is possible using this system, and leads to previously unobserved morphological forms.

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Organism	Substrate	Maintenance coefficient $gg^{-1}l^{-1}$	Reference
P.chrysogenum	Glucose	0.022	Righelato et al. (1968)
A.nidulans	Glucose	0.018	Carter et al. (1971)
A.nidulans	Glucose	0.029	Bainbridge et al. (1971)
A.niger	Citrate	0.045	Ng et al. (1973)
A.niger	Sucrose	0.015	Pannell (1976)
A.niger	Starch	0.04	Spensley (1977)

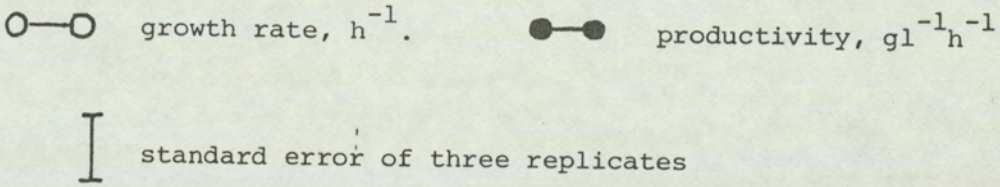
TABLE 1

Calculated maintenance levels (m) of carbohydrate supply for filamentous fungi in continuous culture.

Figure 1.

The effect of shock medium sucrose limitation, 5.0 gl^{-1} to 0.25 gl^{-1}, upon the growth rate (μ) and productivity (γ) of A. niger.

Dilution rate = 0.2 h^{-1}, Temperature = 30°C and medium [nitrogen] = 0.21 gl^{-1} atomic N.



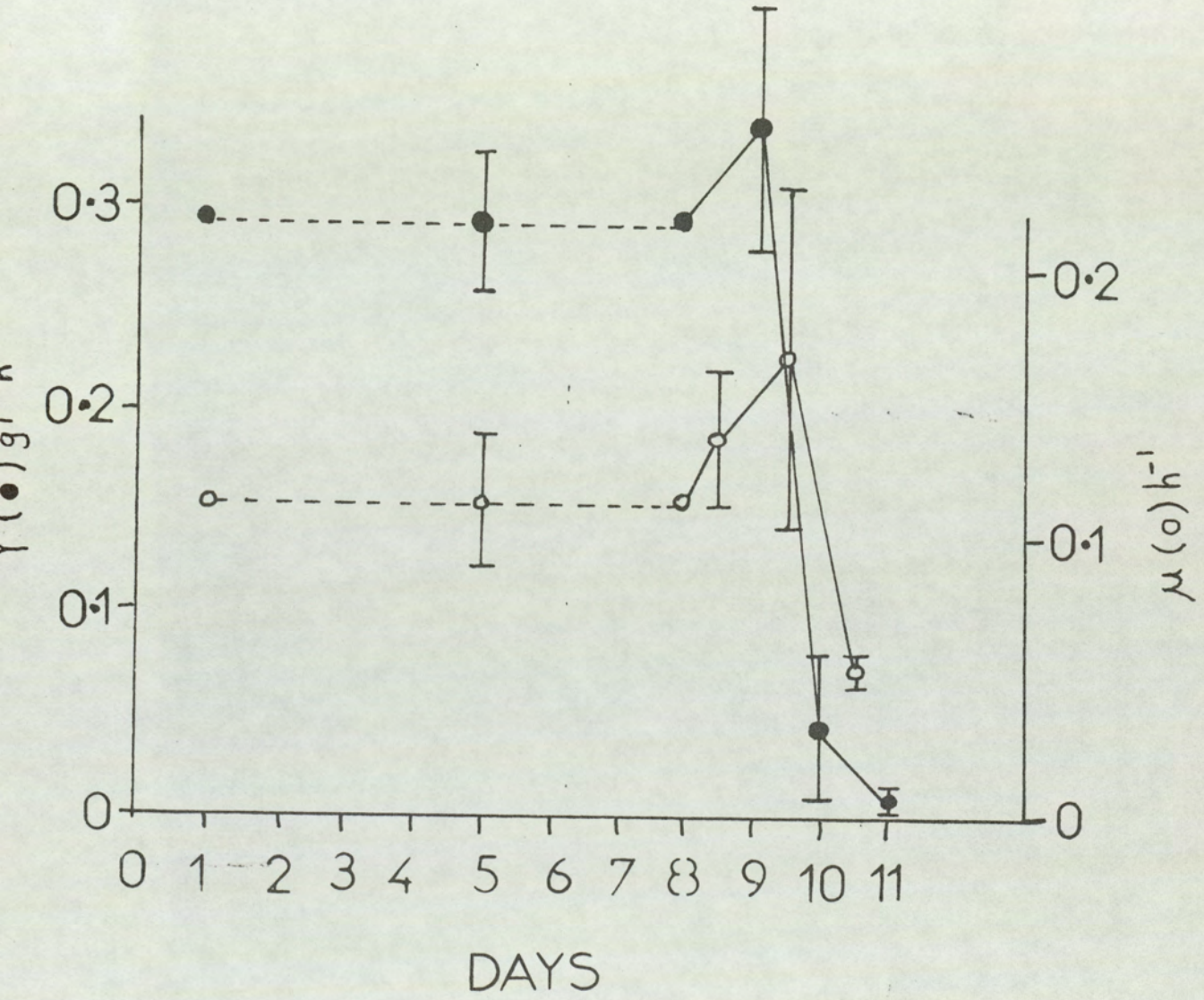
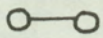
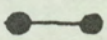

0·3
0·2
0·1
0
0·2
0·1
0
0 1 2 3 4 5 6 7 8 9 10 11
DAYS
μ (○) h⁻¹

Figure 2.

The combined effect of decreased medium [sucrose], 5.0 gl^{-1} to 1.0 gl^{-1} and decreased dilution rate, 0.2 h^{-1} to 0.04 h^{-1} upon the growth rate (μ) and productivity (γ) of A. niger. (Substrate supply rate, K_s decreased from 10.0 gh^{-1} to 0.4 gh^{-1}). Temperature = 30°C. Medium [nitrogen] = 0.21 gl^{-1}.



growth rate h^{-1}



productivity $gl^{-1}h^{-1}$

standard error of four replicates.

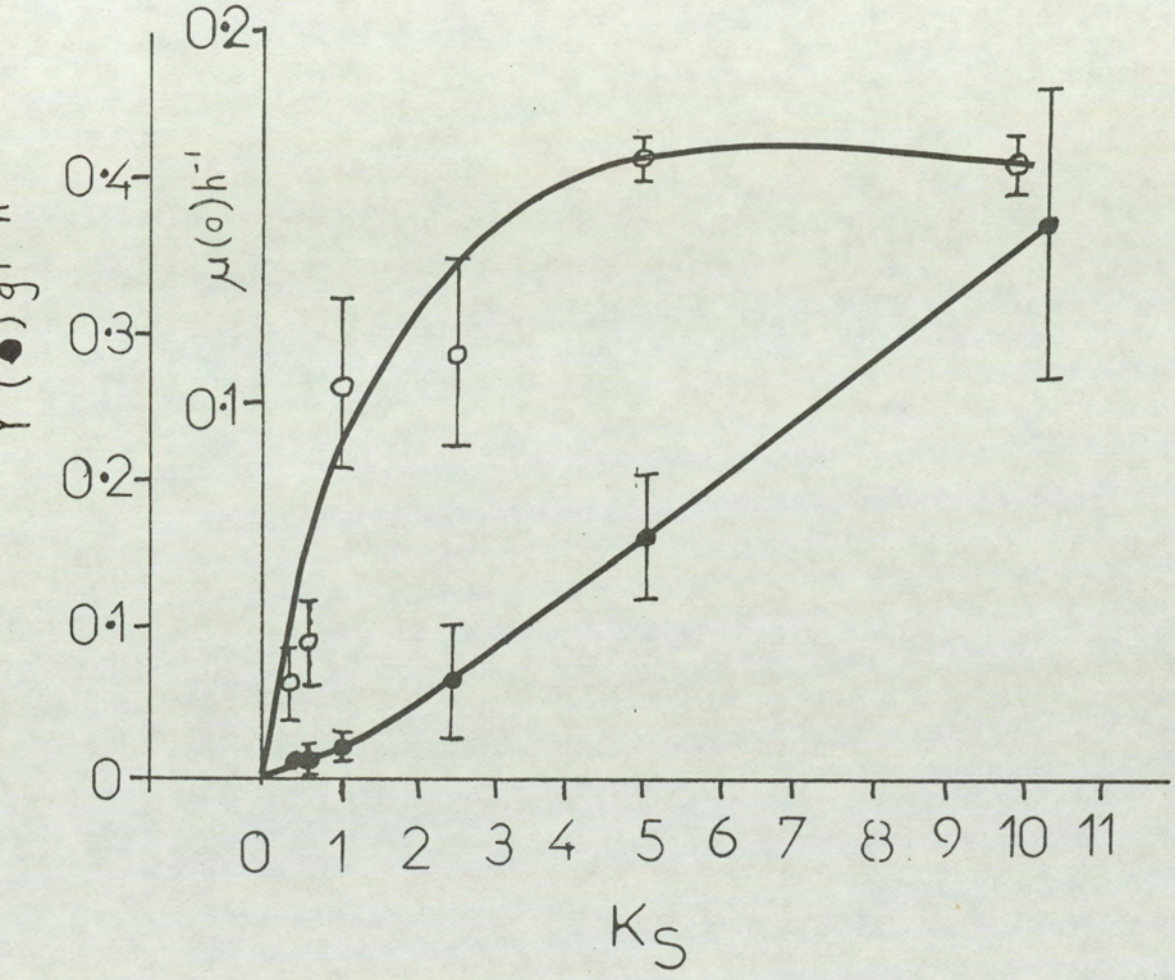
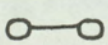
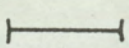

0·2
0·4
0·3
0·1
0·2
0·1
0
μ(o)h⁻¹
0 1 2 3 4 5 6 7 8 9 10 11
Ks

Figure 3a

The gradual decrease of growth rate (μ) h^{-1} of A. niger after the onset of a starch supply of 0.6 gl^{-1} glucose equivalent at a dilution rate of 0.1 h^{-1}.

Temperature = 30°C [nitrogen] = 0.2| gl^{-1}

 growth rate h^{-1}  period of sporulation

 Standard error of 3 replicates.

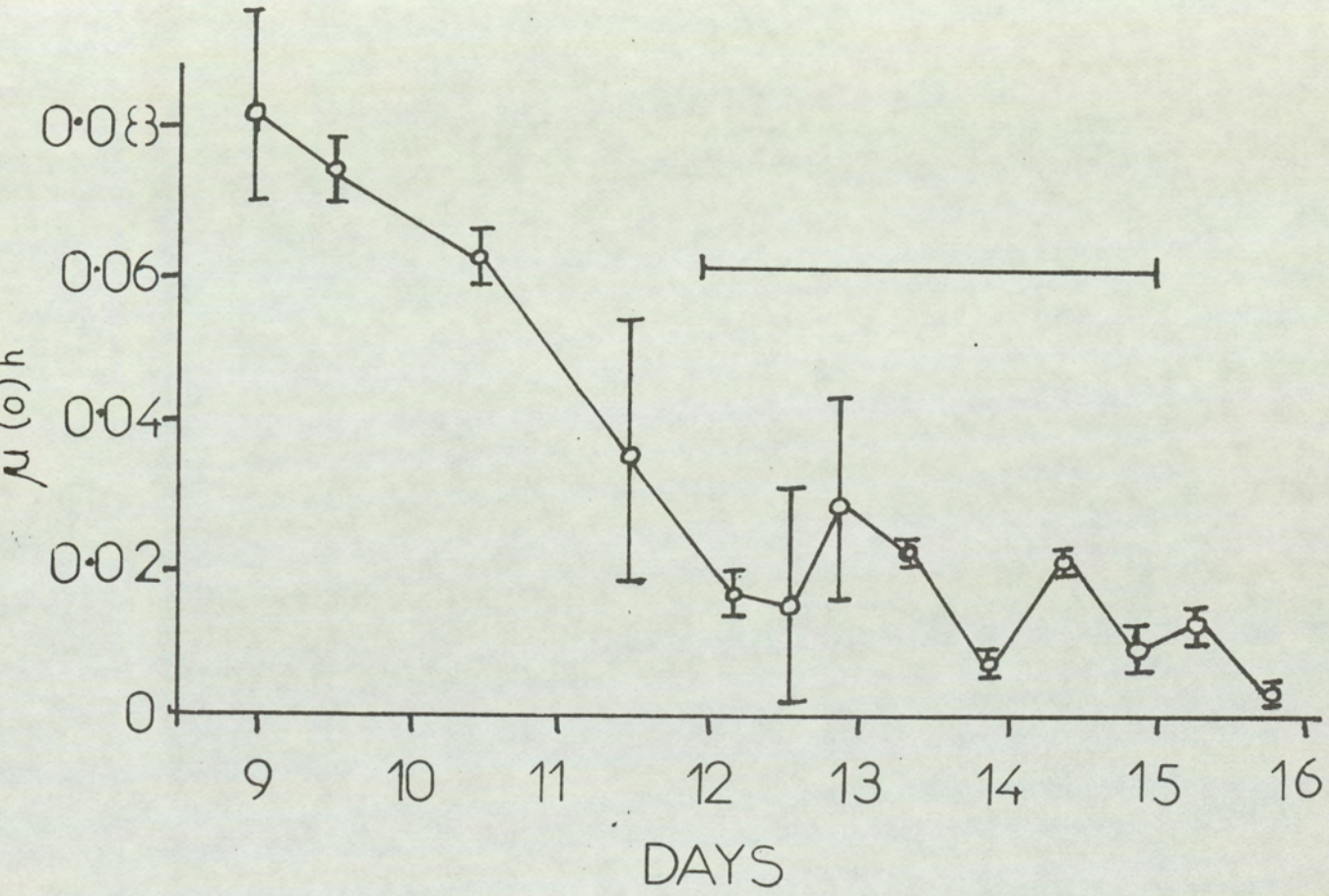
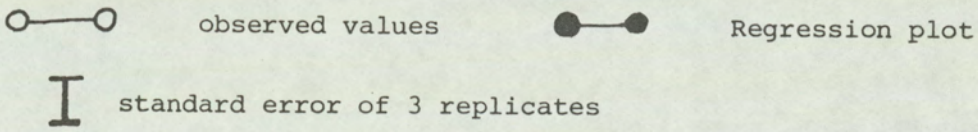

0·08
0·06
0·04
0·02
0
μ (o) h⁻¹
9
10
11
12
13
14
15
16
DAYS

Figure 3b

The estimation of the maintenance coefficient (m) of 0.0383 $gg^{-1}{}_{h}{}^{-1}$ of starch for A. niger from the data of experiments in Fig.3a. Specific substrate utilization rate (q) against growth rate (μ).



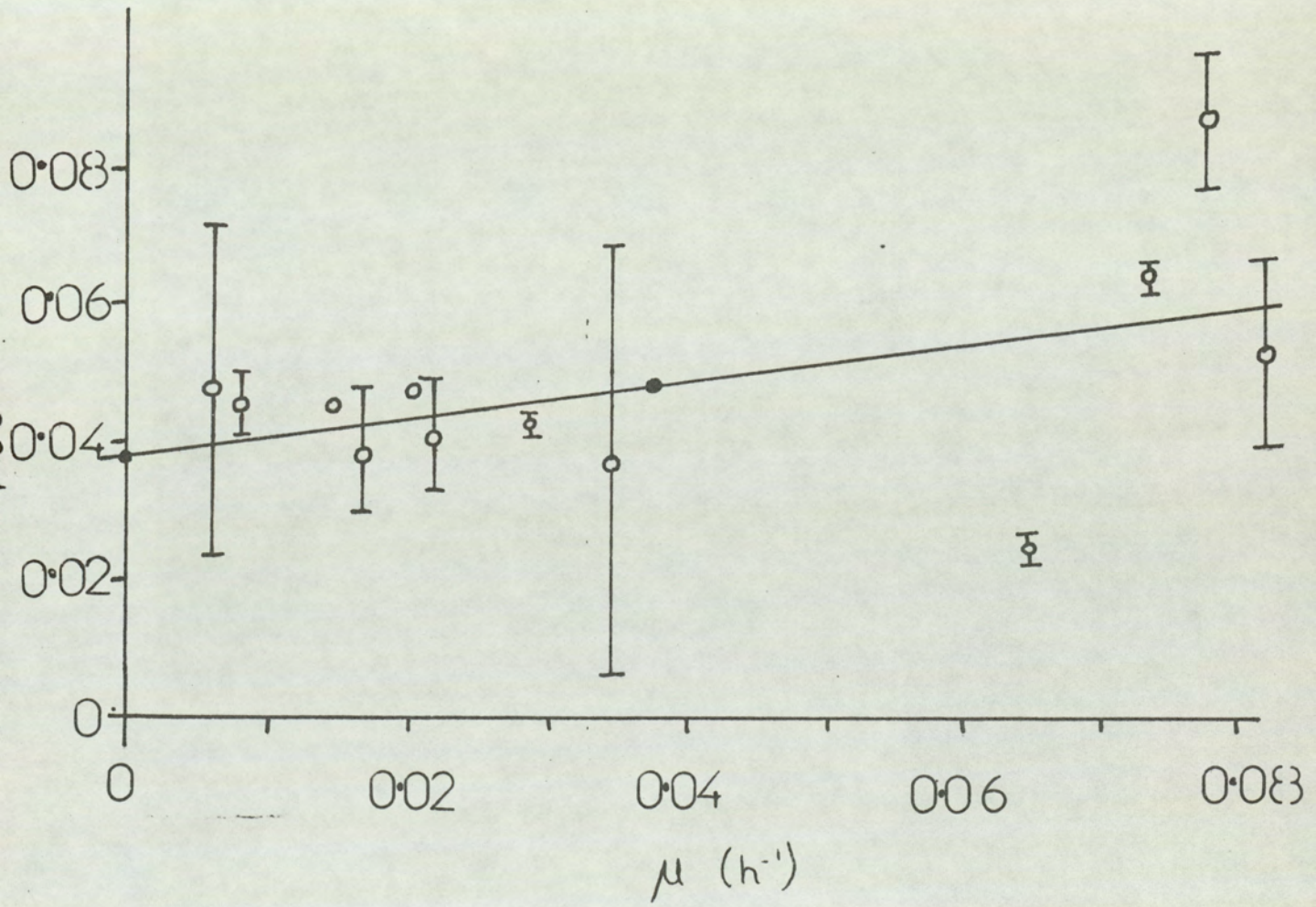

0·08
0·06
0·04
0·02
0
0
0·02
0·04
0·06
0·083
μ (h⁻¹)

Figure 4a

The effect of gradual reduction of medium nitrogen concentration upon the growth rate (μ, h^{-1}) of A. ochraceus.
Temperature = 30°C., Dilution rate = 0.1 h^{-1}, and medium sucrose concentration = 5.0 gl^{-1}.

Standard error of 3 replicates

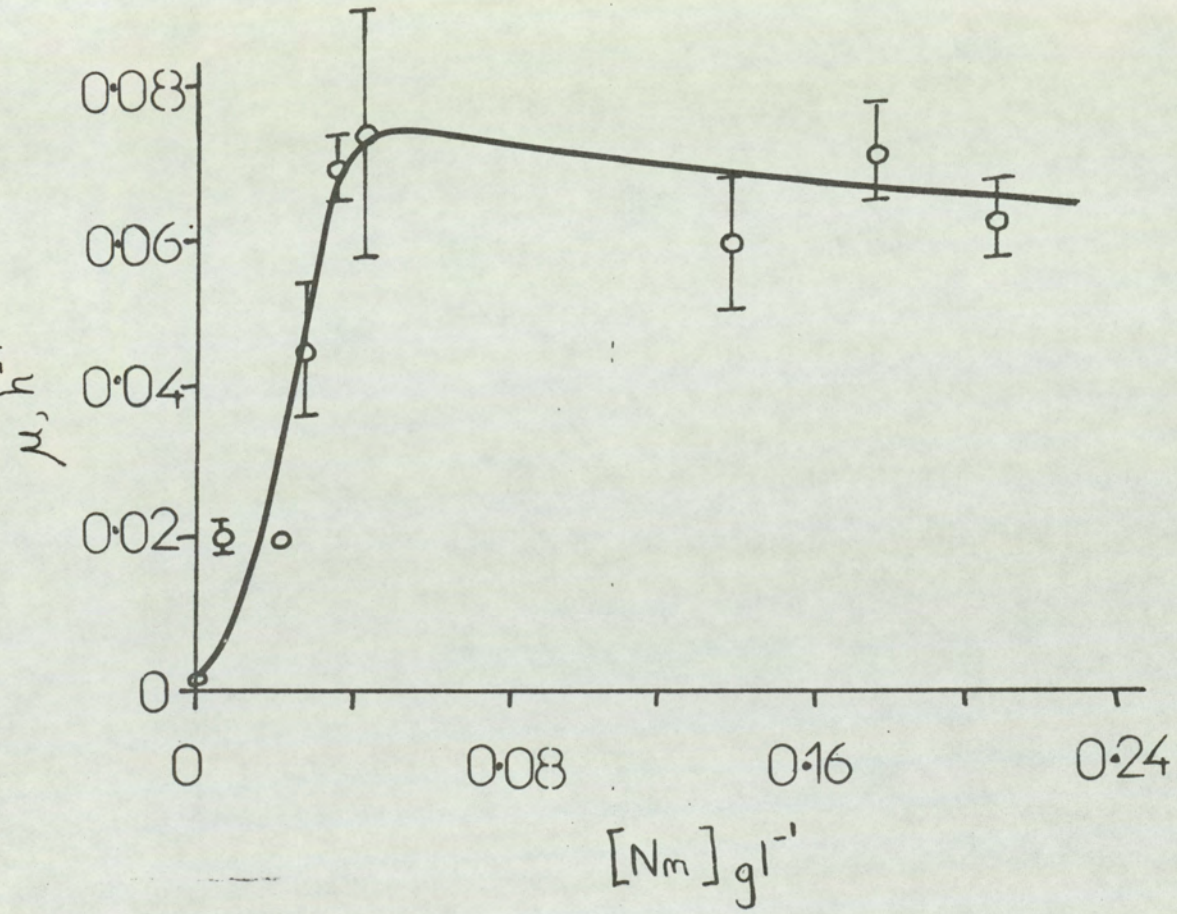

0·08
0·06
0·04
0·02
0
μ, h⁻¹
0
0·08
0·16
0·24
[Nm] gl⁻¹

Figure 4b

The effect of gradual reduction of medium nitrogen concentration upon the growth rate (μ, h^{-1}) of A. niger.

Temperature = 30°C, dilution rate = 0.2 h^{-1} and medium sucrose concentration = 5.0 gl^{-1}.

I Standard error of 3 replicates.

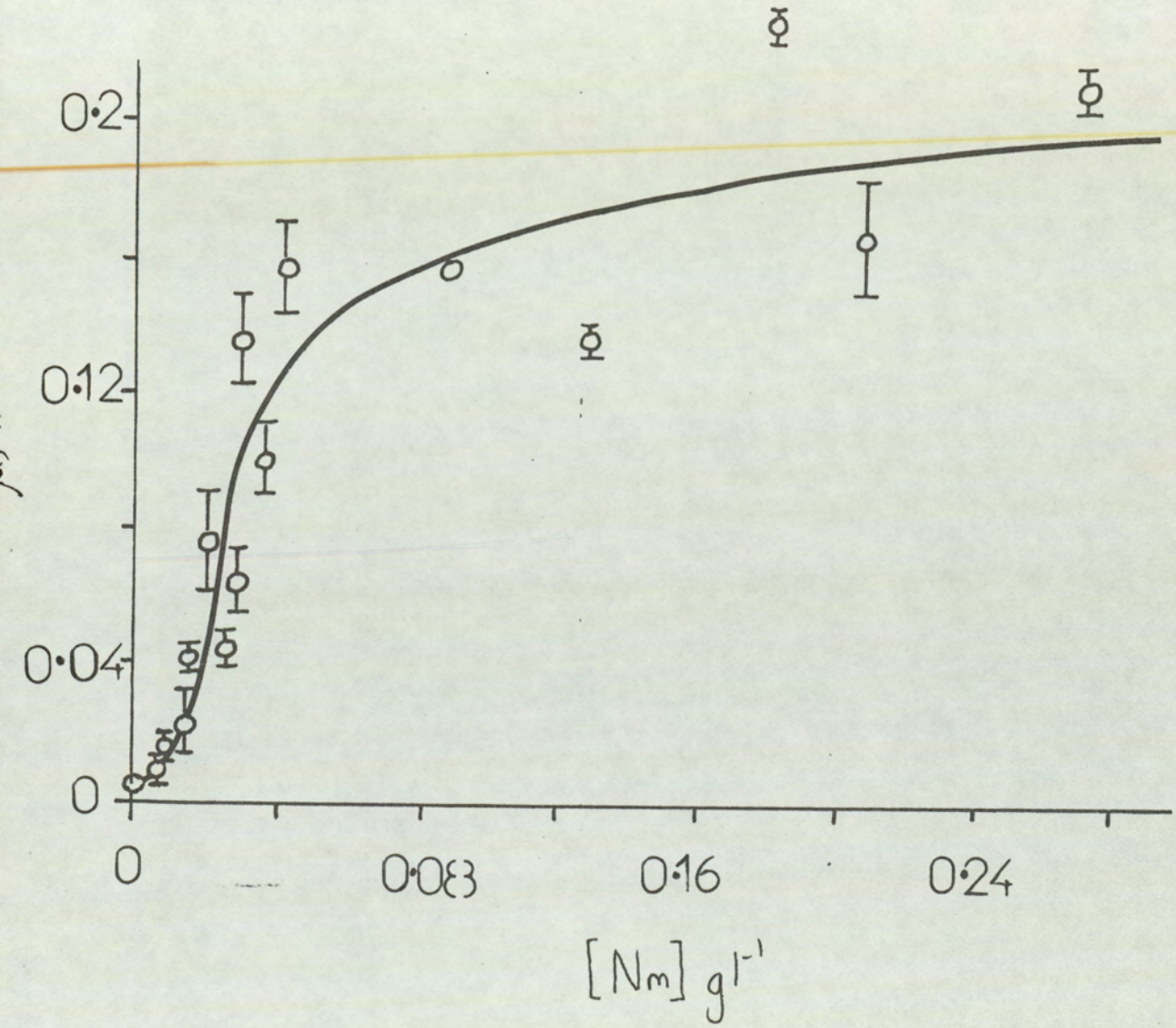

0·2
0·12
0·04
0
0
0·083
0·16
0·24
[Nm] g l⁻¹

Figure 5a

The effect of shock nitrate limitation (medium nitrogen concentration suddenly reduced from 0.24 gl^{-1} to 0.0214 gl^{-1} upon the growth rate (μ, h^{-1}) of A. ochraceus.

Dilution rate = 0.1 h^{-1}, temperature = 30°C and medium sucrose concentration = 5.0 gl^{-1}.

O—O growth rate, h^{-1}

●—● sporulation index (β)

spores g. dry wt. mycelium^{-1}

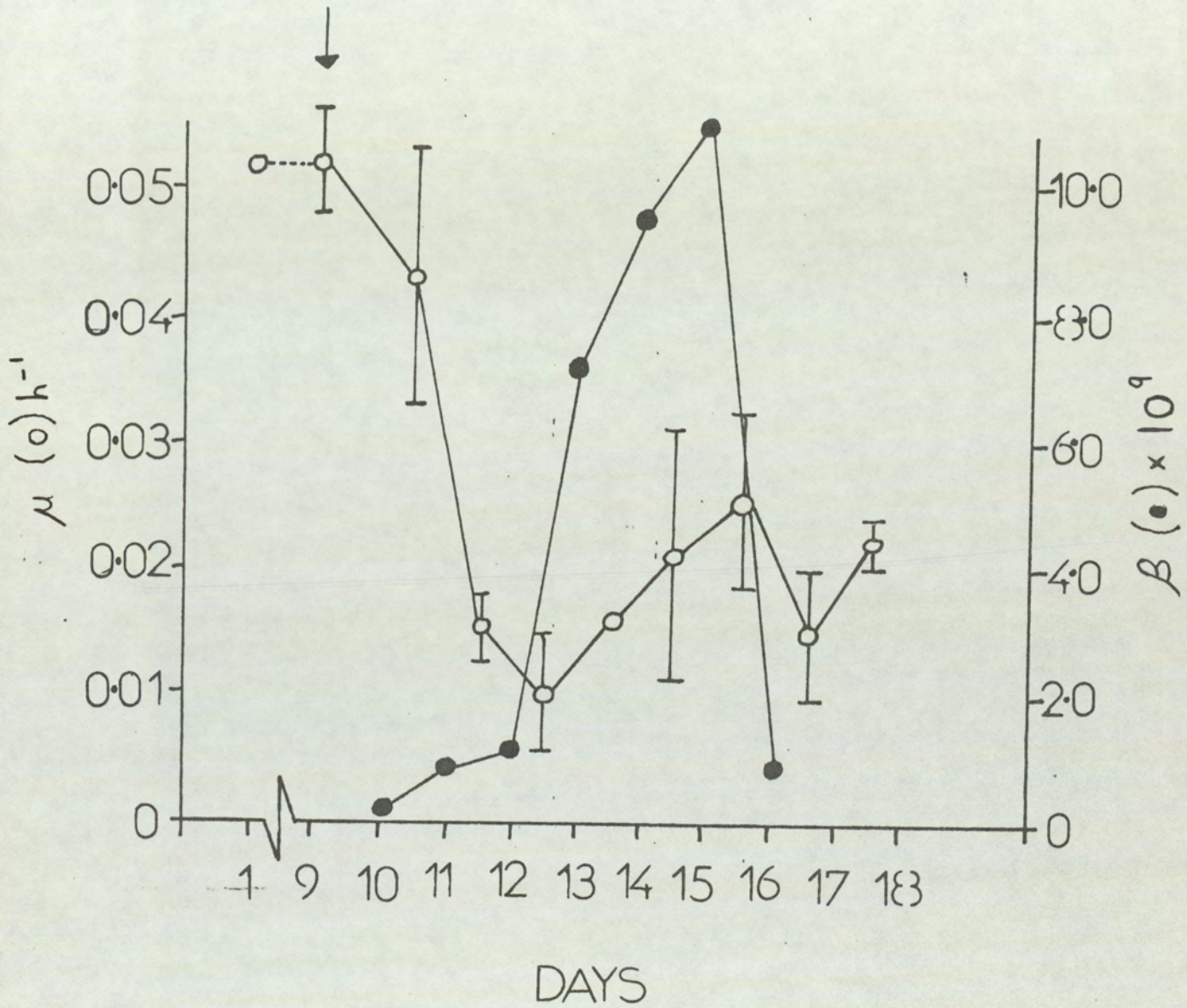
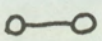
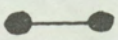

0·05
0·04
0·03
0·02
0·01
0
μ (○) h⁻¹
10·0
8·0
6·0
4·0
2·0
0
β (●) × 10⁹
1 9 10 11 12 13 14 15 16 17 18
DAYS

Figure 6a

The effect of shock nitrate limitation (medium nitrogen concentration suddenly reduced from 0.21 gl^{-1} to 0.021 gl^{-1} upon the growth rate (μ, h^{-1}) of A. niger. Dilution rate = 0.4 h^{-1}, temperature = 30°C and medium sucrose concentration = 5.0 gl^{-1}.

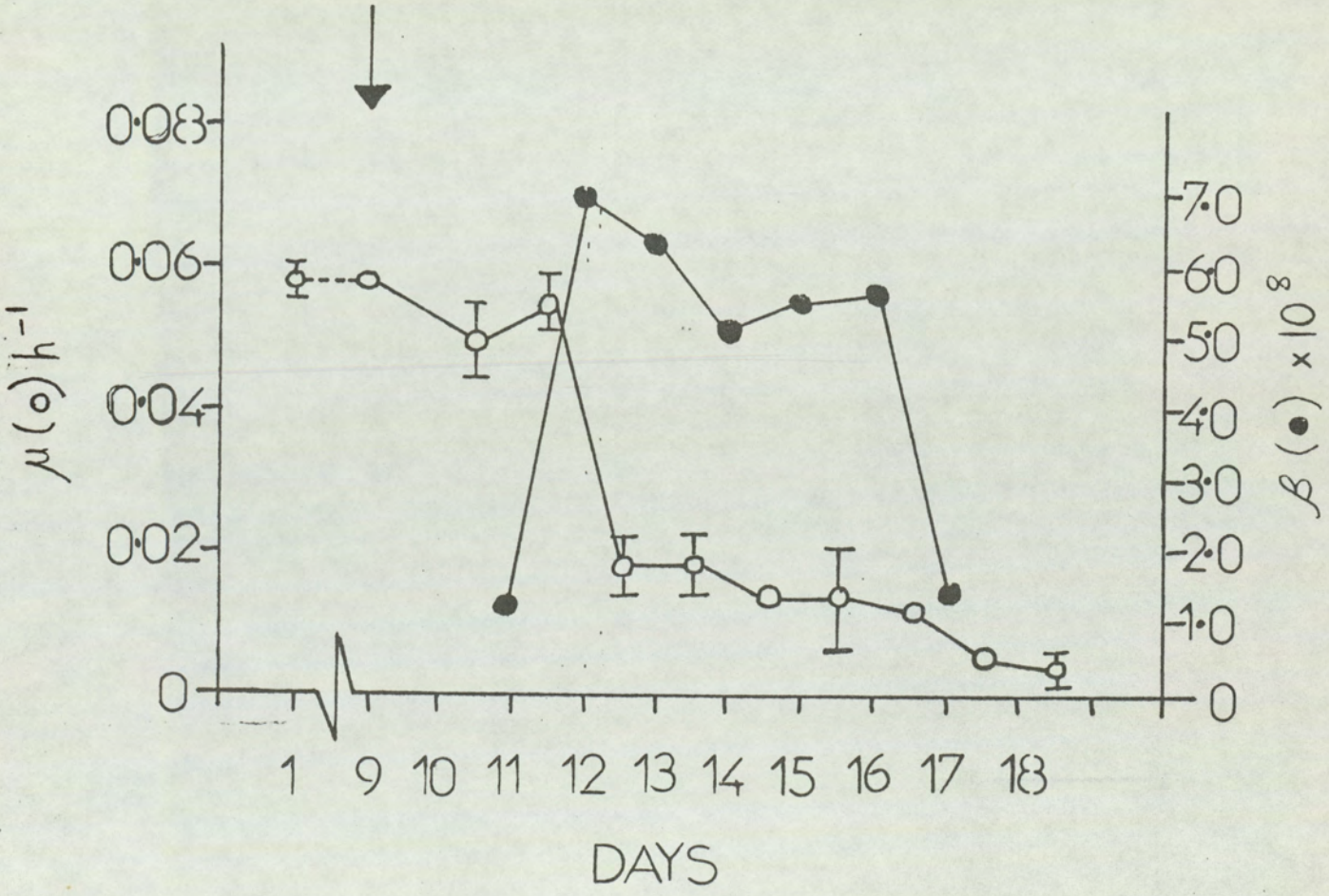


growth rate, h^{-1}



sporulation index (β)

spores g. dry wt. mycelium^{-1}


0·08
0·06
0·04
0·02
0
μ(○) h⁻¹
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0
β(●) ×10⁸
1 9 10 11 12 13 14 15 16 17 18
DAYS

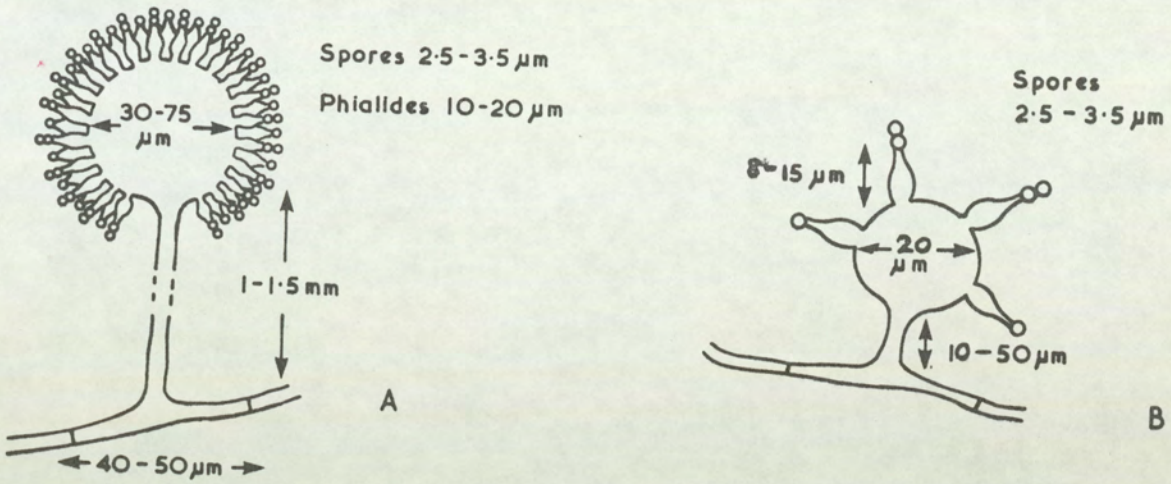


Figure 5b : Submerged sporulation structures of A. ochraceus

A. Sub-aerial form. B. Simplified form in continuous culture

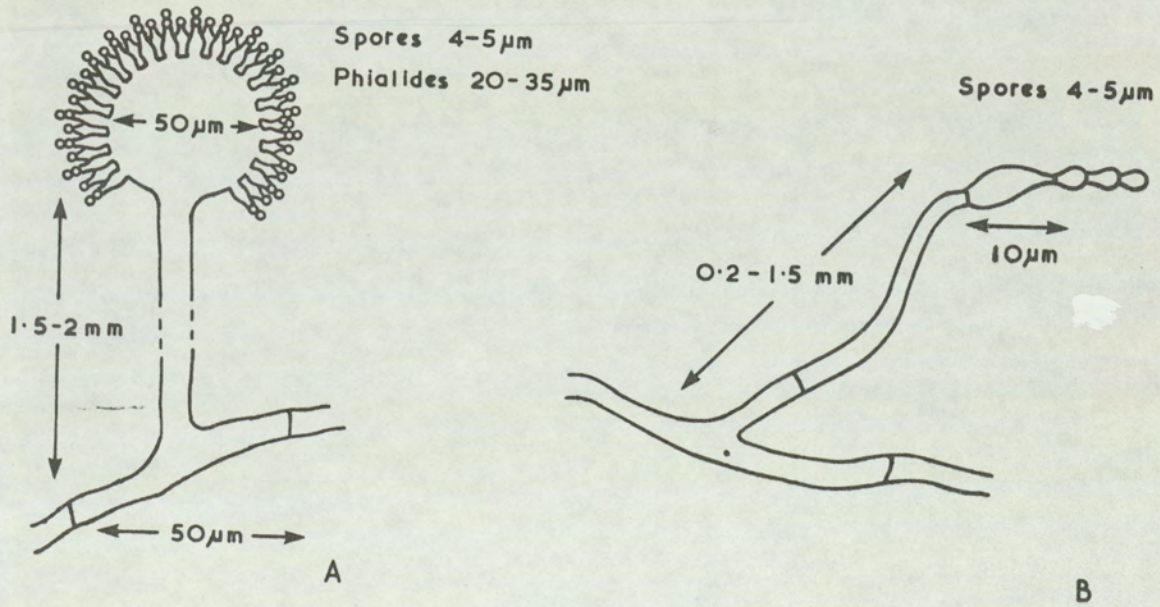


Figure 6b : Submerged sporulation structures of A. niger

A. Sub-aerial form B. Simplified form in continuous culture