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THE EFFECT OF CATABOLITE REPRESSION ON A KNOWN
SECONDARY METABOLITE STREPTOMYCETE

BY

P. MILNER

A Dissertation Submitted in Partial Fulfilment
of Requirements for
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ABSTRACT

The possible involvement of carbon catabolite repression in control of synthesis of the polyether antibiotic M139603 was examined by culturing the producing organism S. longisporoflavus in a variety of media containing a range of different carbon sources at different concentrations. From the results it has been tentatively concluded that carbon catabolite repression is not mediated by any of the carbon sources tested.



Introduction

The Actinomycetes are Gram-positive bacteria belonging to the Order Actinomycetales. Typically the Actinomycetes form branching hyphal filaments (usually 0.5 to 1.0 μ in diameter) resulting in colonies with a fungal appearance. Indeed, when first discovered, their morphological characteristics led to the belief that they were related to both fungi and bacteria (Williams and Wellington, 1982), though the issue has been decided in favour of the bacteria (Lechevalier and Lechevalier, 1967).

Actinomycetes reproduce either by almost total fragmentation of the hyphae or by the production of spores on specialised hypae. Most species are chemo-organotrophic, aerobic, mesophilic and grow optimally at pH's approaching neutral (Williams and Wellington, 1982).

The Actinomycetes may be separated into families and genera on the basis of their physiology (oxidative versus fermentative metabolism); morphology (type and number of spores, formation of specialised bodies such as sclerotia and sporangia); physical properties (e.g. heat resistance) and chemistry (cell wall composition and type of lipid), (Lechevalier and Lechevalier, 1981. See tables 1 to 4.

Actinomycetes can be found in a wide variety of habitats. Many are obligate or facultative parasites of plants and animals, including Man. Others may be found in aquatic and marine environments, but it is the soil which is their most important habitat (Williams and Wellington, 1982), where numbers may be anywhere between 7 and 10^6 to 46×10^6 per gram dry weight of soil (dependant on the proximity of plant roots), Gray and Williams, 1971. Numbers in anaerobic, water logged, and acidic soils are usually far reduced.

Within the Order Actinomycetales there are a number of families (divided on the criteria mentioned above), including Streptomycetaceae; Actinomycetaceae and Nocardiaceae. It is with a member of the

Table 1. Major Constituents of Cell Walls of Actinomycetes

Cell Wall Type	Major Constituents ^{a)}	Example	Total Number ^{b)}
I	L-DAP ^c , Glycine	<u>Streptomyces</u>	11
II	meso-DAP, Glycine	<u>Micromonospora</u>	5
III	meso-DAP	<u>Actinomadura</u>	13
IV	meso-DAP, arabinose, galactose.	<u>Nocardia</u>	8
V	Lysine, ornithine	<u>Actinomyces israelii</u>	1
VI	Lysine (aspartic acid; galactose) ^d	<u>Oerskovia</u>	4
VII	DAB ^e glycine (lysine)	<u>Agromyces</u>	1
VIII	Ornithine	<u>Bifidobacterium</u>	2
IX	meso-DAP, numerous amino acids	<u>Mycoplana</u>	1

a) All cell wall preparations contain major amounts of alanine, glutamic acid, glucosamine, and muramic acid.

b) Total number of actinomycete genera known to have this cell wall type.

c) DAP = 2,6-diaminopimelic acid.

d) Bracketed constituents are variable.

e) DAB = 2,4-diaminobutyric acid.

(After Lechevalier and Lechevalier, 1981)

Table 2. Genera of Actinomycetales with a Type 1 Cell Wall.

<u>Generic Name</u>	<u>Morphological Characteristics</u>
<u>Streptomyces</u>	Aerial mycelium with chains (usually long) of non motile conidia
<u>Streptoverticillium</u>	Same as Streptomyces, but the aerial mycelium bears verticils consisting of at least three side branches, which may be chains of conidia or hold sporulating terminal umbels.
<u>Nocardioides</u>	Both substrate and aerial mycelia fragment into rod and coccus-shaped elements.
<u>Chainia</u>	Same as Streptomyces, but sclerotia are also formed.
<u>Actinopyonidium</u>	Same as Streptomyces, but pyonidia-like structures are also formed.
<u>Actinosporangium</u>	Same as Streptomyces, but spores accumulate in drops.
<u>Elytrosporangium</u>	Same as Streptomyces, but merosporangia are also formed.
<u>Microellobosporia</u>	No chains of conidia; merosporangia with nonmotile spores are formed.
<u>Sporichthya</u>	No substrate mycelium is formed; aerial chains of motile, flagellated conidia are held to the surface of the substratum by holdfasts.
<u>Intrasporangium</u>	No aerial mycelium; substrate mycelium forms terminal and subterminal vesicles.
<u>Arachnia</u>	No aerial mycelium; substrate mycelium is branched and may fragment.

(After Lechevalier and Lechevalier, 1981)

Table 3. Morphological Criteria of Genera of Streptomycetaceae and Some Other Actinomycetales.

Genus	Substrate mycelium				Aerial mycelium				Sporangia like vesicles (few spores)	Motility of Spores
	Fragmenting into rod or coccoid Elements	Chains of Spores	Pod-shaped spore vesicles: mero-sporangia few spores	Sclerotia	Chains of arthrospores within a thin fibrous sheath		Fibrous sheath			
					Verticillate arrangement	Smooth	Hairy or Spiny			
No	Yes	No	Yes	No	Yes	No	Yes			
Streptomycetaceae										
<u>Streptomyces</u>	-	(+)	-	-	+	-	+	+	-	-
<u>Streptoverticillium</u>	-	-	-	-	-	+	+	-	-	-
<u>Chainia</u>	-	-	-	+	+	-	+	-	-	-
<u>Microellobosporia</u>	-	-	+	-	-	-	ND ^a	ND	+	-
<u>Kitasatoa</u>	-	-	+	(+)	+	-	+	-	+	+
Actinomycetaceae										
<u>Arachnia</u>	+	-	-	-	no aerial mycelium					
Nocardiaceae										
<u>Nocardia</u>	+	-	-	-	+	-	+	-	-	-
<u>Micropolyspora</u>	-/+	+	-	-	+	-	+	+	-	-
Thermomonosporaceae										
<u>Actinomadura</u>	-	-	-	-	+	-	+	+	-	-
<u>Nocardiopsis</u>	(+)	-	-	-	+	-	+	-	-	-
Genera without a family										
<u>Nocardioides</u>	+	-	-	-	Fragmenting aerial hyphae					
<u>Sporichthya</u>	No substrate mycelium				Aerial hyphae become chains of spores					
<u>Intrasporangium</u>	-	-	-	-	No aerial mycelium					

a) no data (After Kutzner, 1981)

Table 4. Biochemical markers of Streptomycetaceae and Some Other Actinomycete Genera

Family and Genus	G+C (mol%)	DAP	Sugars in Whole Cell Hydrolyate					Fatty Acid Spectrum				
			Glycine in IPD ^a	Ara+ Gal	Mad	Xyl	Fucose	Satur. Straight	Iso- and anteiso- branched	Unsatur- ated	10-Methyl branched	Mycolic acids
Streptomycetaceae												
<u>Streptomyces</u>	69-73	LL	+	-	-	-	-	+	-	-	-	-
<u>Streptoverticillium</u>	69-73	LL	+	-	-	-	-	+	+	-	-	-
<u>Chainia</u>	71	LL	+	-	-	-	+	+	+	-	-	-
<u>Microellobosporia</u>	67-70	LL	+	-	-	-	-	+	+	-	-	-
<u>Kitasatoa</u>		LL	+	-	-	-	-	+	+	-	-	-
Actinomycetaceae												
<u>Arachnia</u>	70-72	LL	+ ^b	-	-	-	-	+	+	-	-	-
Nocardiaceae												
<u>Nocardia</u>	67-69	DL	-	+	-	-	-	+	-	+	+	+
<u>Micropolyspora</u>												
<u>Brevicatena</u>	66-69	DL	-	+	-	-	-	+	-	+	+	+
Thermomonosporaceae												
<u>Actinomodura</u>	77	DL ^c	-	-	+	-	-	+	+	+	+	-
<u>Nocardiopsis</u>		DL	-	-	-	-	-	+	+	+	+	-
Genera without a family												
<u>Nocardioides</u>	67	LL	+	-	-	-	-	+	+	+	+	-
<u>Sporichthya</u>		LL	+	-	-	-	-	+	+	+	+	-
<u>Intrasporangium</u>		LL	+	-	-	-	-	+	-	+	-	-

a IPB Interpeptide bridge: b Together with aspartic acid: c Often additional small quantities of LL-DAP
(After Kutzner 1981)

Streptomycetaceae that this study is concerned.

Streptomycetes (members of the Streptomycetaceae) are common gram-positive sporoactinomycetes, with a highly oxidative metabolism, and which form non-fragmenting, extensively branched aerial and substrate hyphae. Characteristically their cell walls contain LL-Diaminopimelic acid (DAP) and diamino acid, possess glycine as the crosslinking amino acid and have no characteristic wall sugars. This is known as wall chemotype I, Goodfellow and Cross, 1983.

Streptomycetes are further characterised by a high G C content of their DNA (see table 4), the sugar pattern of whole cell hydrolysates and fatty acid spectrum of cell lipids, Kutzner, 1981. The streptomycetes are divided into a number of genera, for example Streptomyces (Waksman and Henricic, 1943); Streptoverticillium (Baldacci, 1958); Chainia (Thirumalachar, 1955); Microellobosporia (Cross, Lechevalier and Lechevalier, 1963) and Kitasatoa (Matsume et al 1968); of which Streptomyces, Streptoverticillium and Chainia are very closely related.

The genus Streptomyces is by far the most important genus of the family, in terms of economic value (due to the production of antibiotics and other secondary metabolites) and in terms of environmental abundance. Streptomyces species are so common in soil that many investigations into soil actinomycetes have been, in effect, studies on Streptomyces (Williams and Wellington 1982). This is well illustrated by the work Lechevalier and Lechevalier (1967) who found that of 5,000 isolates (from 16 soils) Streptomyces had a frequency of occurrence of 95.34%

Although Bergey's manual of Determinative Bacteriology (Buchanan and Gibbons, 1974) lists only 463 species of Streptomyces (which includes a number of species of Chainia, which the authors consider

'synonymous with Streptomyces, Kutzner, 1981) many more 'species' have been described.

During the period 1940-1957, over 1,000 species of Streptomyces had been described (Pridham et al., 1958), whilst by 1970 the number had risen to 3,000 (Trejo, 1970).

This large number of species had come about due to the use of such characters as aerial mycelium colour; spore chain morphology; spore ornamentation; soluble melanoid pigment production (on media containing tyrosine); carbohydrate utilisation spectra and proteolytic activities. Using these characters in their many permutations, hundreds of 'new' species were identified, many of them solely for the purpose of patenting (due to the secondary metabolite producing capabilities of 'species').

In the 1960's it became clear that the genus was over classified and so in 1964 the International Streptomyces Project was set up in order to resolve some of the problems encountered in correctly classifying the genus.

Over 450 species were rediscrbed and much of this information was used in the present volume of Bergey's Manual (Buchanan and Gibbons, 1974). However, Silvestri et al (1962), using numerical taxonomic techniques concluded that the genus was still overclassified and that there might be as few as 25 centres of variation. In the more recent phenetic study, by Williams et al (1983) it is suggested that there are relatively few cluster groups or species within the genus, and that the heavy weighting given to morphological characters, in the past, is no longer justified.

As stated above, the genus Streptomyces is of great economic value due to the wide range of commercially useful secondary metabolities (Metabolic products which appear to play no part in cell growth, and which are formed maximally under conditions of restricted or zero

growth, Singleton and Sainsbury, 1973), including antibiotics, it produces. The economic importance of Streptomyces can easily be demonstrated if one considers that of 5,000 antibiotics identified in 1977, 3,000 were produced by actinomycetes (the majority by Streptomyces). Also of the 5,000 only 100 have been marketed, and of these 69 were products of streptomycetes, (figures from Ahronowitz and Cohen, 1981).

The following report is concerned with the production of a polyether antibiotic (termed ML39603) by an industrial strain of Streptomyces longisporoflavus, NC1B11426 (see Materials and Methods).

at present the process involves growing S. longisporoflavus on a complex medium in fed batch culture. The protocol used in the current industrial process differs from that used in the following experiments (see Materials and Methods), in that, at the time of inoculation the production medium contains 10% w/v Starch (see Materials and Methods) and that after an appropriate time, as determined by the state of the culture, Morsweet, (a mixture of glucose, maltose and maltotriose, supplied by C.P.C. UK Ltd) is fed at an appropriate rate.

The maximum level of ML39603 production achieved so far by this method is in the region of 15 g l^{-1} . However, in order to achieve greater economic viability for the process, it has been decided to try to increase the yield to 20 g l^{-1} .

There are two methods by which the maximum yield of an antibiotic fermentation (or any fermentation process) may be increased. Firstly there is genetic manipulation of the producing organism, whether by 'classical' mutation and selection procedures; genetic recombination; or more recently by utilising the techniques of genetic engineering to circumvent many types of barrier to increased production. Secondly by altering the culture conditions of the fermentation process, and hence by-passing such physiological regulatory controls as catabolite repression (see below).

Industrial microbiology or biotechnology is not a new field of entrepreneurial activity; its development can be traced back to the production of alcohol and vinegar by the Sumerians before the year 5000 B.C. Today biotechnology is an established factor in the world economy with an annual value of hundreds of billions of dollars (Old and Primrose, 1985).

However, despite its long history the first steps towards controlling and improving microbiological processes were taken little more than 100 years ago, when the bacteria and fungi that made desirable commodities were isolated and grown in pure culture, and it became possible to select strains particularly suited to a given task (Hopwood, 1981).

The purposeful breeding of special industrial strains only became possible much later, as our basic knowledge of microbial genetics improved.

The first deliberate attempts at changing the genetic composition of microbial cultures came about as early as 1927 when it was discovered that x-rays could induce mutations. After 1945 a wide range of other potent mutagenic radiations and chemical mutagens were discovered and this gave microbiologists a powerful set of tools for changing the genetic composition of their cultures.

Probably the best known example of the efficacy of mutation and selection procedures is that of the Penicillin fermentation, where the productivity of the producing organism Penicillium chrysogenum has been increased over 55 times. Coupled with current fermentation practices the yield is now in excess of 20 g l^{-1} (Hopwood 1981; Aharonowitz and Cohen 1981). In the same way the yield of tetracycline from Streptomyces aureofaciens has been increased from a few milligrams to over 20 g l^{-1} .

It is probably true to say that, to date, most if not all, industrial strains of micro-organisms used in the production of economically useful microbial metabolites, have undergone strain development via a mutation

and selection programme.

The mid 1940's also saw advances in microbial genetics that made it possible to modify the genotype of the producing organism via genetic recombination.

Whilst mutation alters a micro-organisms genes, recombination (the other basic approach to genetic programming), rearranges genes or parts of genes and brings together in an individual organism genetic information from two or more organisms. The techniques involved in recombination have been reviewed by Hopwood, 1981.

Within the last 15 years the techniques of genetic engineering have assumed increasing importance in the development of fermentation processes.

There are many ways of classifying industrial fermentations, which can be subdivided as follows: production of cells; production of proteins; destruction of toxic compounds; and the production of small molecules including antibiotics. Genetic engineering techniques have been used in the development of each of the above categories (see Old and Primrose 1985), however, it is with the production of antibiotics that this report is concerned. The current state of gene cloning of antibiotic producing organisms has been most recently reviewed by Fayerman, 1986.

The second method used to increase the productivity of a Fermentation process is to manipulate the physio-chemical environment such that the producing organism is cultured under optimum physical conditions with all its nutritional requirements fulfilled without inhibiting biosynthesis of the desired product.

It is also important to develop inoculum and medium sterilization procedures, so as to introduce the producing organism into the culture broth, in the optimum (for product formation) physiological state, and to ensure medium sterilization without altering the nutritional quality of the medium.

The protocol used in medium sterilization and inoculum development for the following experiments will be discussed in the conclusion of this report. For general reviews see Wang et al, 1979; Crueger and Crueger, 1982 and Sikyta, 1983.

The factors which comprise the physical environment of a fermentation include temperature, pH, aeration and agitation. The optimum level for each of these parameters can be determined experimentally by culturing the producing organism under a range of different levels of each of the parameters. The optimum levels for these parameters have been determined previously by I.C.I workers and are those used in Materials and Methods.

As with the physical environment the chemical environment can also be determined experimentally. This is often done at an empirical level, with a wide range of carbon and nitrogen sources being tested at various concentrations.

As well as the source and concentration of carbon and nitrogen in the medium, the concentration of inorganic phosphate and minor elements are also of importance, especially phosphate.

Again previous work by I.C.I personnel have determined what would appear to be the optimum concentrations and source of nitrogen, phosphate and minor elements in the medium for ML39603 production (see Materials and Methods).

This report is concerned with the development of an optimum source and concentration of carbon for the production of ML39603.

It should be noted at this point that genetic manipulation of the producing strain and development of the culture environment occur simultaneously. This is because a higher producing strain obtained by mutation may only express its true potential under different optimum conditions to the previous strain.

Data published by large manufacturers of microbial products indicate that the increases in productivity of industrial microbial processes are due almost equally to genetic manipulation of the producing organism and to the development of the technological process itself. Hence data on the productivity of industrial strains and the methods used to increase productivity are closely guarded secrets (Sikyta, 1983).

The question must now be asked how do the source and concentration of nutrients affect the production of microbial metabolism and M139603 in particular. It is known that the source and concentration of carbon, nitrogen and phosphate have a definite effect on the biosynthesis of M139603 production (P. Willis, personal communication). These effects are due to one or more of the regulatory controls of metabolism coming into effect. These mechanisms have been reviewed by a number of workers: Demain, 1972; Drew and Demain, 1977; Demain et al., 1979; Aharonowitz, 1980; Martin and Demain, 1980.

As stated above this report is concerned with the development of an optimum carbon regime, by trying to investigate the possible role of one of the above regulatory mechanisms, carbon catabolite repression, in the biosynthesis of M139603.

Epps and Gale (1942) first discovered that the synthesis of enzymes involved in primary metabolism (metabolic processes needed and directly involved in cellular growth) is influenced by glucose and raised the question of the biological significance of this observation. It was Magasanik (1961) who saw a survival advantage in this phenomenon and termed it 'catabolite repression', i.e. the inhibition of synthesis of inducible enzymes by intermediates produced by the rapid catabolism of glucose (Demain et al., 1979).

Probably the best known example of catabolite repression involves an organism grown on a mixture of lactose and glucose. In such a case glucose is metabolized preferentially by the organism since the sugar is capable of maintaining a higher growth rate than lactose. Since there is no

advantage in synthesizing the enzymes necessary for lactose metabolism whilst the glucose is in a plentiful supply, glucose, through a catabolic intermediate represses (i.e. prevents transcription of) the genes responsible for lactose metabolism (Demain et al., 1979)

Readily metabolized carbon sources, such as glucose can suppress antibiotic production by preventing the synthesis of a key enzyme in the biosynthetic pathway (Chatterjee and Vining, 1981). Because of parallels with the well known suppression by glucose of catabolic enzymes for using less preferred substrates, Demain (1972) referred to the above as 'catabolite repression', (Chatterjee and Vining, 1982). Consequently although glucose is often an excellent carbon and energy source for microbial growth, it is infrequently used as the major carbon and energy source in secondary metabolite fermentations (Drew and Demain, 1972).

During studies on fermentation medium development, polysaccharides and oligosaccharides are often found to be better than glucose as a carbon source for antibiotic production. For example the current production process for ML39603 involves a fermentation medium containing starch as the primary carbon source, with moursweet being used as a carbon feed (P. Willis, personal communication).

Materials and Methods

1. Strain

The micro-organism used in all the following experiments was

Streptomyces longisporoflavus NC1B 11426 strain 83/E6. This was supplied as second generation slope cultures by I.C.I. Pharmaceutical Division.

2. Shake Flask Experiments

i) Inoculum development

Production of ML39603 was based on initial growth in an inoculum medium consisting of;

4%	(w/v)	Milou Pro Bio	(Supplied by: Milouot M.P. Ashwat Israel)
0.4%	(w/v)	Chalk	(" " Brito Mayo, Violet label).
0.4%	(w/v)	Minor Elements	
0.1%	(v/v)	Polypropylene glycol, antifoam	(Diamond Shamrock, EEA 142)

The minor elements present were:

1.00	gl ⁻¹	FeSO ₄	7 H ₂ O
1.00	gl ⁻¹	ZnSO ₄	7 H ₂ O
0.15	gl ⁻¹	CuSO ₄	5 H ₂ O
0.10	gl ⁻¹	MnSO ₄	7 H ₂ O
0.10	gl ⁻¹	K ₂ MoSO ₄	

The inoculum flasks were 1 litre non-baffled conical containing 200 mls of medium, which were sterilized for 30 minutes at 121°C. The sterile flasks were inoculated from slope cultures. Using aseptic technique 10 mls of the sterile medium was transferred from the flask and added to the slope culture, using a wide bore 10 mls plastic pipette (Sterilin). The scraping of the slope, with the pipette, caused a release of spores. The spore suspension was then withdrawn, by means of the pipette, and used to inoculate the flask.

In all the experiments, duplicate inoculum flasks were prepared in case of contamination. The inoculum flasks were incubated at 28°C on an orbital shaker at 220 rpm (L.H. orbital shaker) for 48 hours. After 24 hours of the 48 hour incubation period, purity tests were

performed on the inoculum. Firstly, a Gram stain was performed on the culture broth to ensure no contaminants were present. Secondly, check the broth using the streak plate method on nutrient agar (Oxoid). Duplicate plates were used, with one being incubated at 28°C and the other at 37°C.

After 48 hours, the flasks are used to inoculate production flasks by transferring 10 mls of inoculum to each production flask.

ii) M139603 Production Flasks

The production flasks were 500 mls non-baffled conical flasks containing 100 mls of the production medium. This medium was based on the inoculum medium (mentioned above) except that Milou Pro was present as a 2% (w/v) concentration. Also a carbon source of choice was present. These flasks were sterilized at 121°C for 30 minutes. The inoculated production flasks were incubated on an orbital shaker (as above) at 28°C and 220 rpm. These flasks were then sampled periodically for M139603 production (see below) until maximum production was achieved.

In these experiments various carbon sources were tested, these were:

Glucose	(Supplied by: Meritose, Tunnel Refineries)
Starch	(" "	Laing National, Trafford Park)
Cellobiose	(" "	BDH Biochemicals)
Maltose hydrate	(" "	Sigma)
Alpha-Lactose	(" "	Sigma)
Fructose	(" "	Sigma)
Sucrose	(" "	Tate and Lyle, "Silver Spoon")

These carbon sources were tested at three different initial concentrations, which were: 1, 5 and 10% (w/v). Except in the cases of cellobiose and maltose where only the 10% (w/v) concentrations were tested. In all the experiments duplicate flasks were tested.

3. Fermenter Experiment

i) Inoculum development.

The production of M 139603 in the fermenter was based on a two-step inoculum procedure, developed by Willis and Hood (unpublished data).

Inoculum flasks were prepared as above, however incubation proceeds for 72 hours, after which the whole flask is used to inoculate a laboratory scale fermenter containing 2.5 litres of inoculum medium. The same inoculum medium as above.

The culture conditions of the inoculum fermenter were as follows:

Temperature	28° C
Aeration	0.5 v/v/m
Agitation	500 rpm
pH was maintained at 7.0, via the addition of 50% NH ₄ OH (v/v) and 2M H ₂ SO ₄	
Dissolved Oxygen Tension was not controlled.	

The inoculum fermenter was a L.H. Engineering 5l glass vessel of the stirred tank design, with a double bladed impellor. In this fermenter growth was monitored via CO₂ production which was measured by a V.G. Micromass Mass Spectrometer.

Inoculum production continues for approximately 24 hours, after which a 10% (v/v) sample is used to inoculate the production fermenter. This occurs when the CPR (Carbon dioxide production rate) reached a level of approximately 8.0 mMl⁻¹h⁻¹. It should be noted that prior to inoculation of the production fermenter, purity tests were performed (as above).

ii) Production Fermenter

The production fermenter was a modified Braun biostat 4l glass vessel stirred tank. This fermenter contained 2.5 litre of the production medium.

a) Medium Composition

The medium constituents were - 10% (w/v) Glucose
 3% (w/v) Milou Pro Bio
 0.4% (w/v) Chalk
 0.4% (w/v) Minor Elements
 0.1% (v/v) Polypropylene Glycol.

b) Medium Sterilization

Both inoculum fermenter and production fermenter media are produced in 10l aliquots contained in 20 litre aspirators and sterilized at 121° C for 60 minutes via a normal cooling cycle of a British Sterilizer Motorclave

S.F.A. - W.J. (the temperature probe being placed at the base of a similar aspirator containing 20 litre inoculum medium).

However, due to heat lability of glucose, a solution of glucose is prepared at approximately 500 gl^{-1} in 10 litre aliquots (in a 10 litre aspirator) and sterilized at 121 C for 30 minutes.

In the production fermenter sufficient glucose solution is added to the general medium to give a concentration of 10% (w/v). This solution was also used to feed glucose into the culture broth at periods after inoculation (see table 5).

c) Culture Conditions

The production fermenter culture conditions were:

Temperature	28°C
Aeration	0.5 v/v/m
Agitation	500 rpm.
pH was maintained at 7.0 via the addition of 50% NH_4OH (v/v) and 2M H_2SO_4	
Dissolved Oxygen Tension was not controlled.	

Daily samples of the production broth (about 30 mls) were removed and analysed. (see below).

The fermentation proceeded until maximum production of ML39603 was achieved.

4. Analysis of Culture Broth

It should be noted that pH analysis and purity checks were only performed for the fermenter run.

i) pH Analysis

The pH of the broth was measured (using a Corning pH meter) as a check of the pH monitoring system in the fermenter.

ii) Residual Glucose

1 ml aliquots of the culture broth were centrifuged (using a Micro Centaur). The supernatant was removed and when appropriate diluted (to bring the glucose concentration within the range of the analyser) with double de-ionised water. The resultant solution was measured for

glucose using a Beckman glucose analyser.

iii) Purity checks

A Gram stain of the culture broth was prepared. This allowed a rapid examination of the broth for contaminants and also allowed the morphology of the culture to be determined. The prepared slides were also photographed (see results: Culture morphology).

Streak plates were also prepared from the culture broth one being incubated at 28°C and the other at 37°C.

iv) Determination of M 139603 Concentration.

A 1 ml sample of the culture broth was added to an appropriate volume of HPLC grade methanol, (the dilution factor being chosen to bring the sample concentration into the range covered by the standard solutions) contained in a 30 mls Sterilin disposable universal. The methanol/culture broth mix was then agitated for 30 minutes using a Griffin flask shaker. After agitation the sample was filtered (using a 0.45 µm Acrodisc disposable filter assembly, Acrodisc C.R. Gelman Sciences), and 1 ml of the filtrate was placed in an autosampler vial was measured against three standard concentrations of I.C.I. 139603 (in methanol). The standard concentrations were: 0.110, 0.250 and 0.505 g l^{-1} . Standards of each concentration were run before and after each batch of unknown samples were measured.

Measurement of M 139603 was by means of a Kontron HPLC system, which

consisted of the following:

- Uvikon 740 LC detector
- LC pump 410
- HPLC autosampler MS1660
- Reodyne 7010 injection valve pneumatic actuator, 6 port.
- Servoscribe chart recorder.
- Column: Spherisorb 5 OD52 - 125 x 5 mm

The chromatographic conditions employed were:

- Eluent: HPLC Methanol (90%) and 0.01M Potassium dihydrogen orthophosphate (in H₂O, buffered to pH 7)(10%)
- Wavelength: U/V absorption at 254 nm
- Flow rate: 2 ml/min
- Pressure: about 50 bar. Sample loop: 20 µl
- M 139603 retention time: about 3 minutes.

Table 5 Glucose Utilization in Fed-batch Fermenter Run

Fermentation Time (Days)	Residual Glucose Before Addition (gl^{-1})	Residual Glucose After Addition (gl^{-1})
6	0.12	40
10	0.40	28
11	5.30	20
12	0.00	27
13	4.00	38.3

Total Glucose added = 143.48 gl^{-1}

Initial Glucose concentration (at inoculation) = 98 gl^{-1}

Total Glucose used = 241.48 gl^{-1}

Results

- a) Graphs of M139603 production (figures 1 to 8)
(For shake flask results, points represent
an average figure from duplicate flasks)
- b) Table 6, Table of Maximum Yield of M139603
- c) Colony Morphology (plates 1 to 12)
- d) Description of Plates

Fig.1 Graph of M139603 Production Versus Time .

Carbon Source : Cellobiose

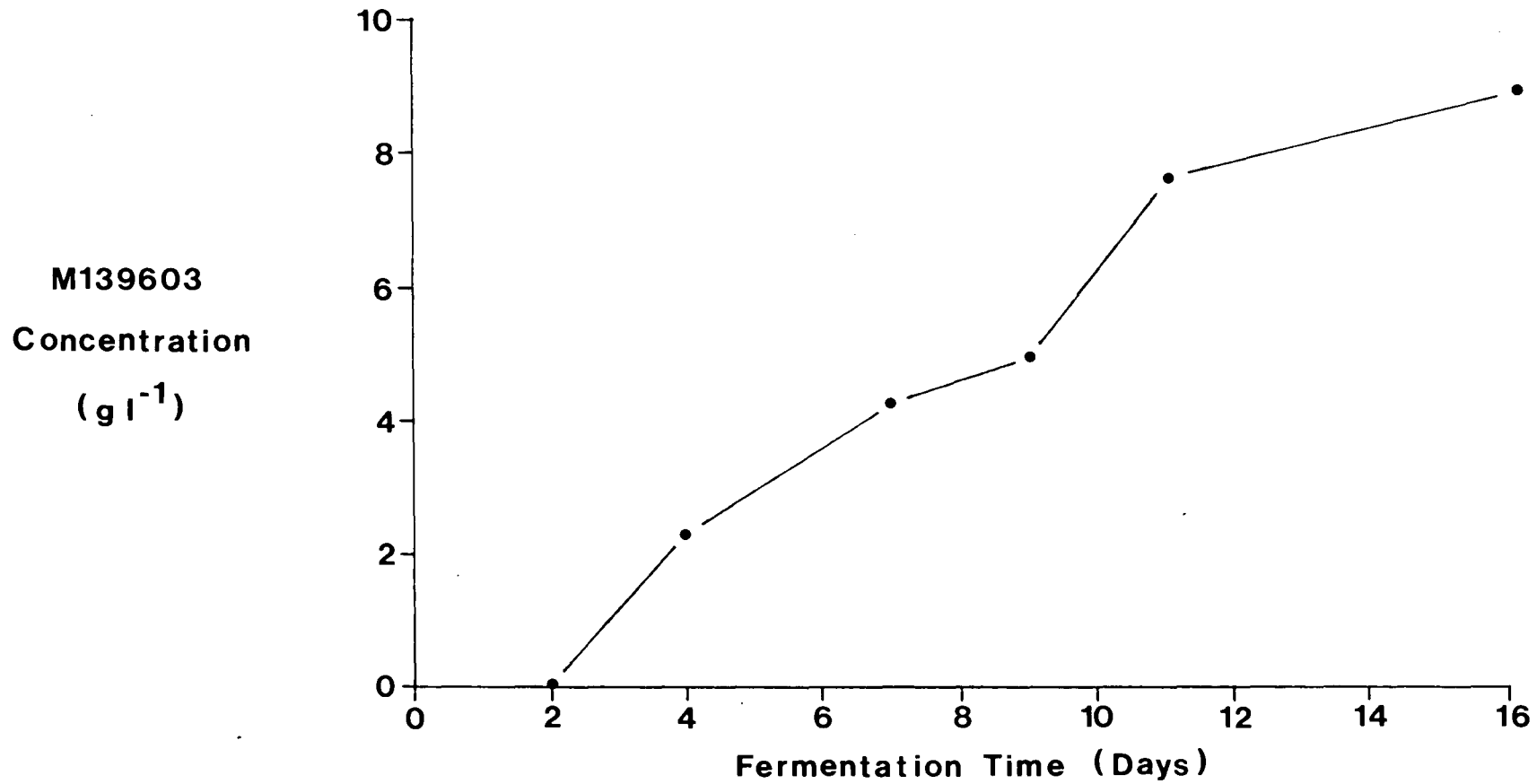


Fig.2 Graph of M139603 Production Versus Time.

Carbon Source : Glucose

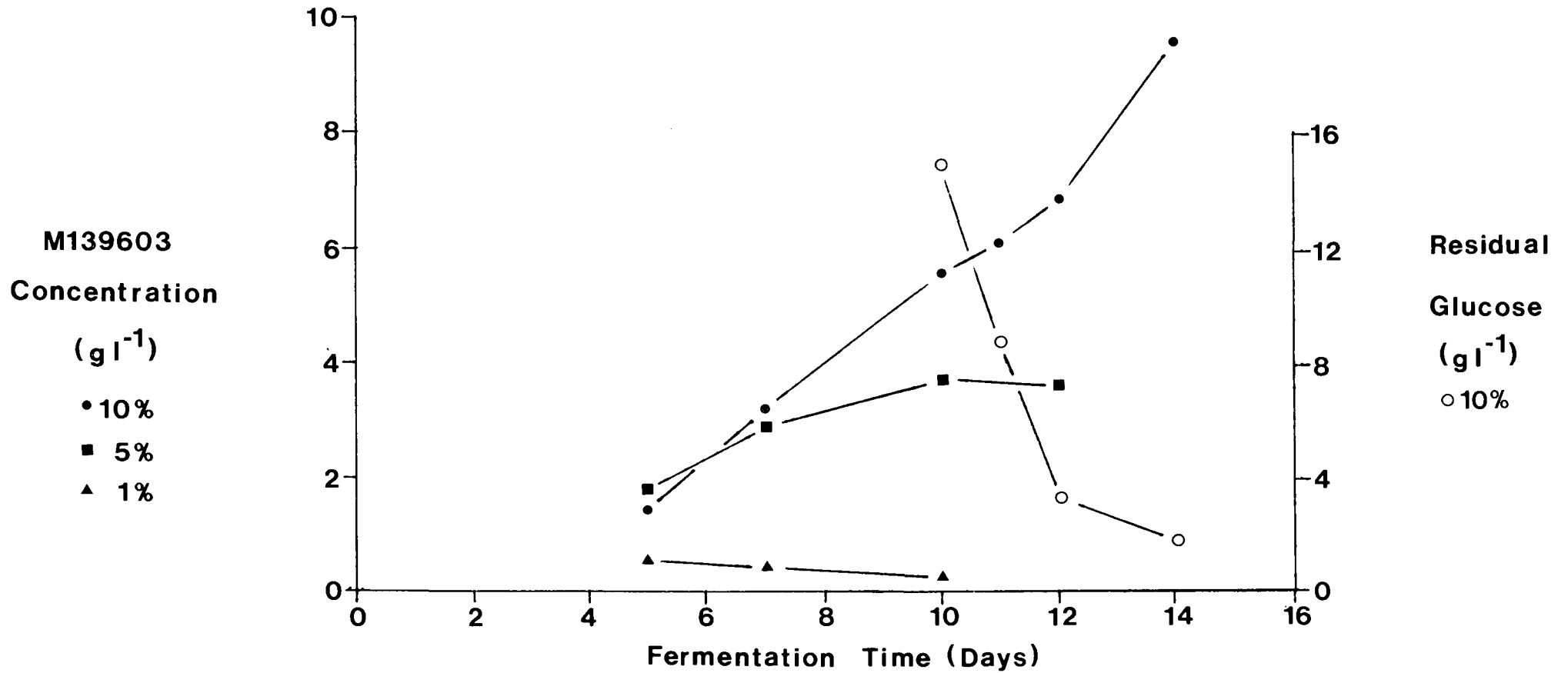


Fig.3 Graph of M139603 Production Versus Time.

Carbon Source : Maltose

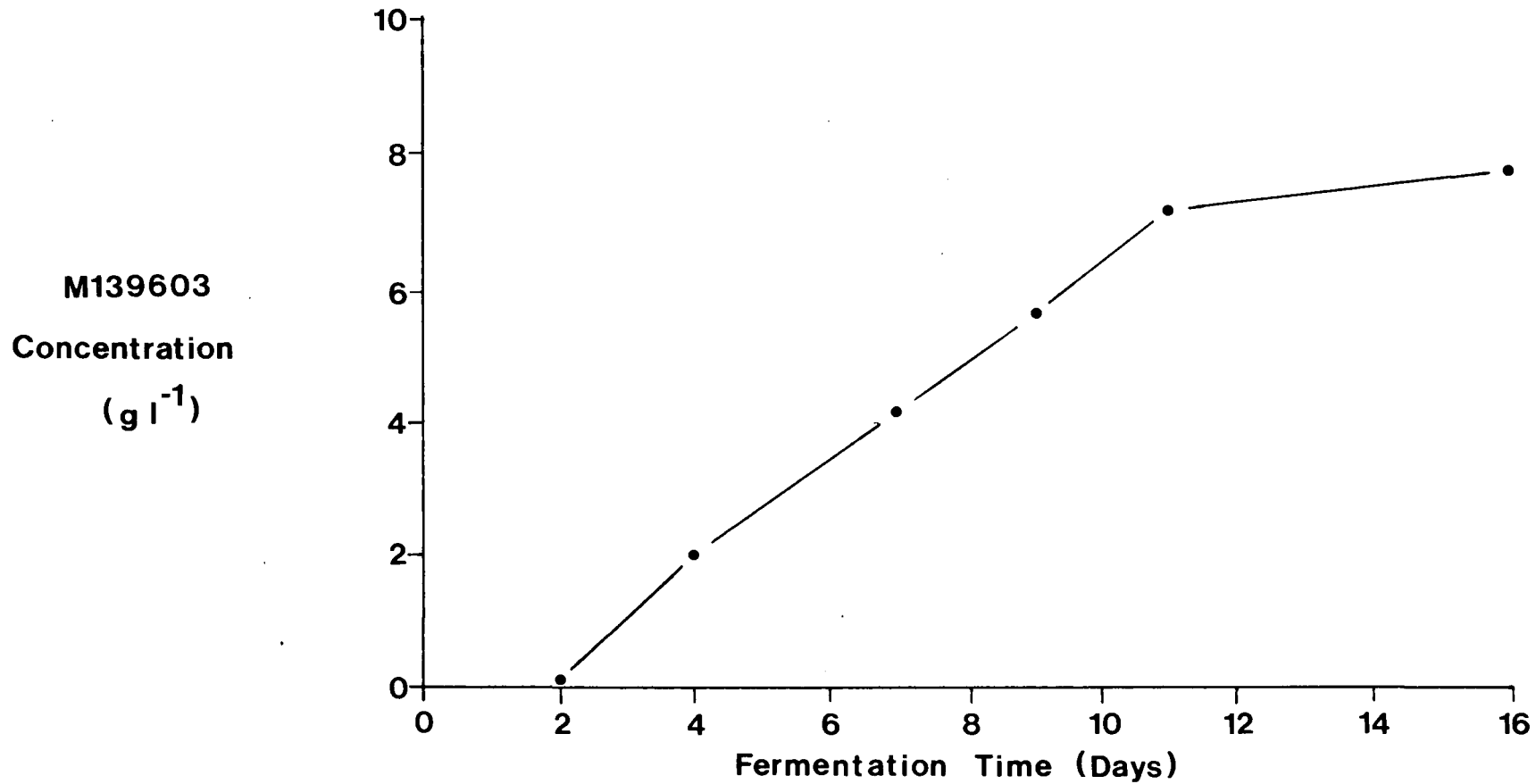


Fig.4 Graph of M139603 Production Versus Time.

Carbon Source : Starch

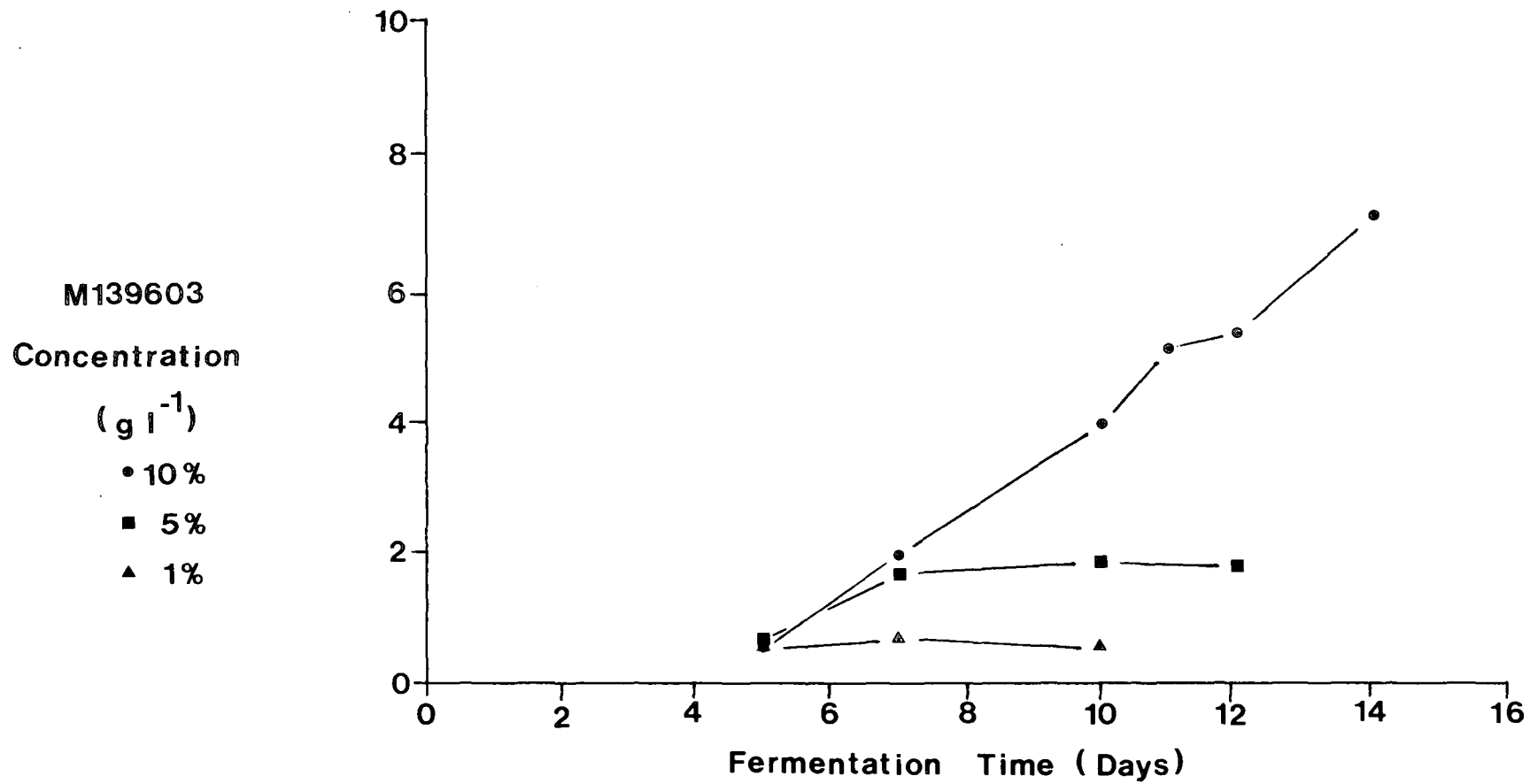


Fig.5 Graph of M139603 Production Versus Time.

Carbon Source : Sucrose

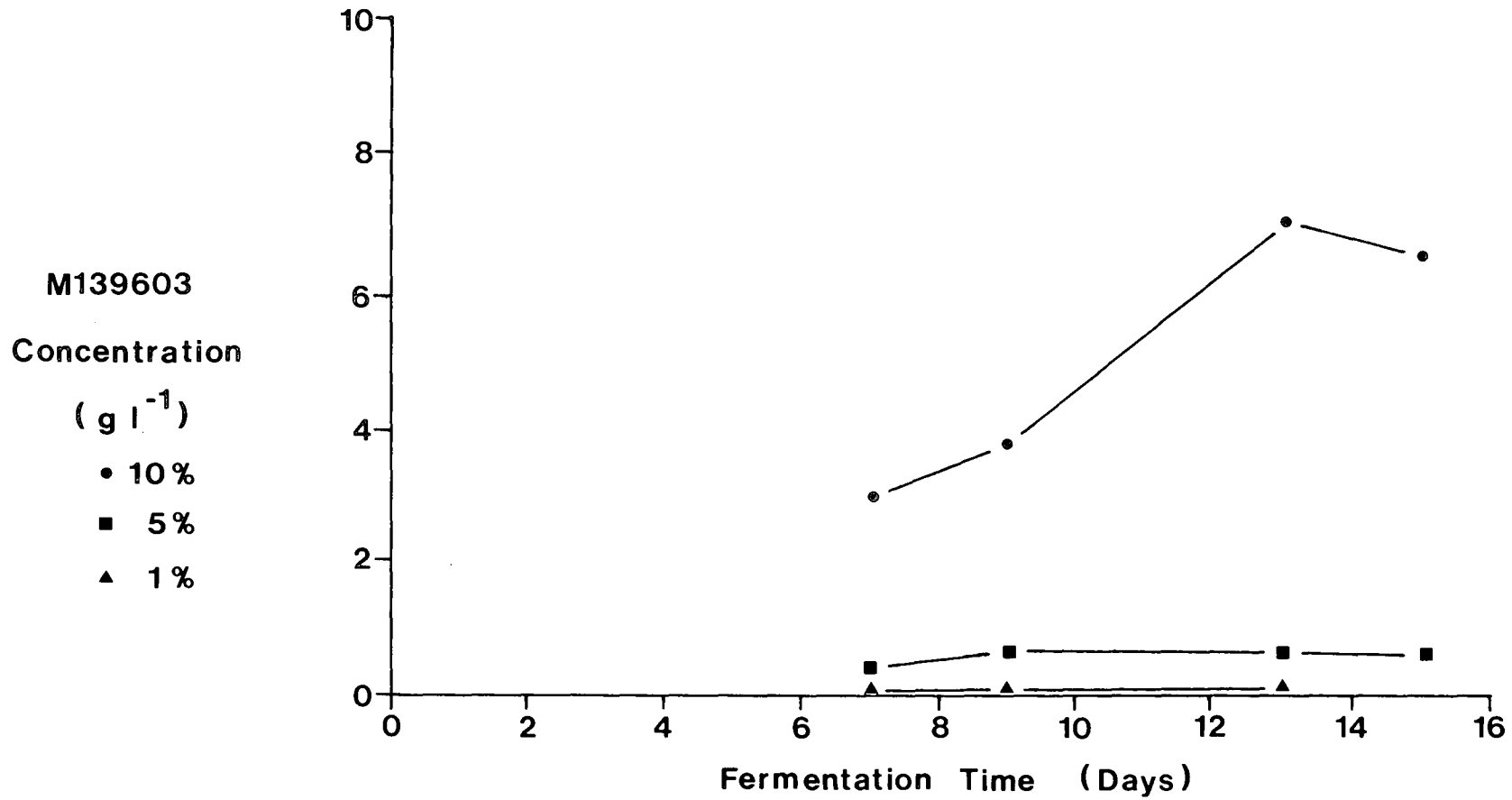


Fig.6 Graph of M139603 Production Versus Time.

Carbon Source : Lactose

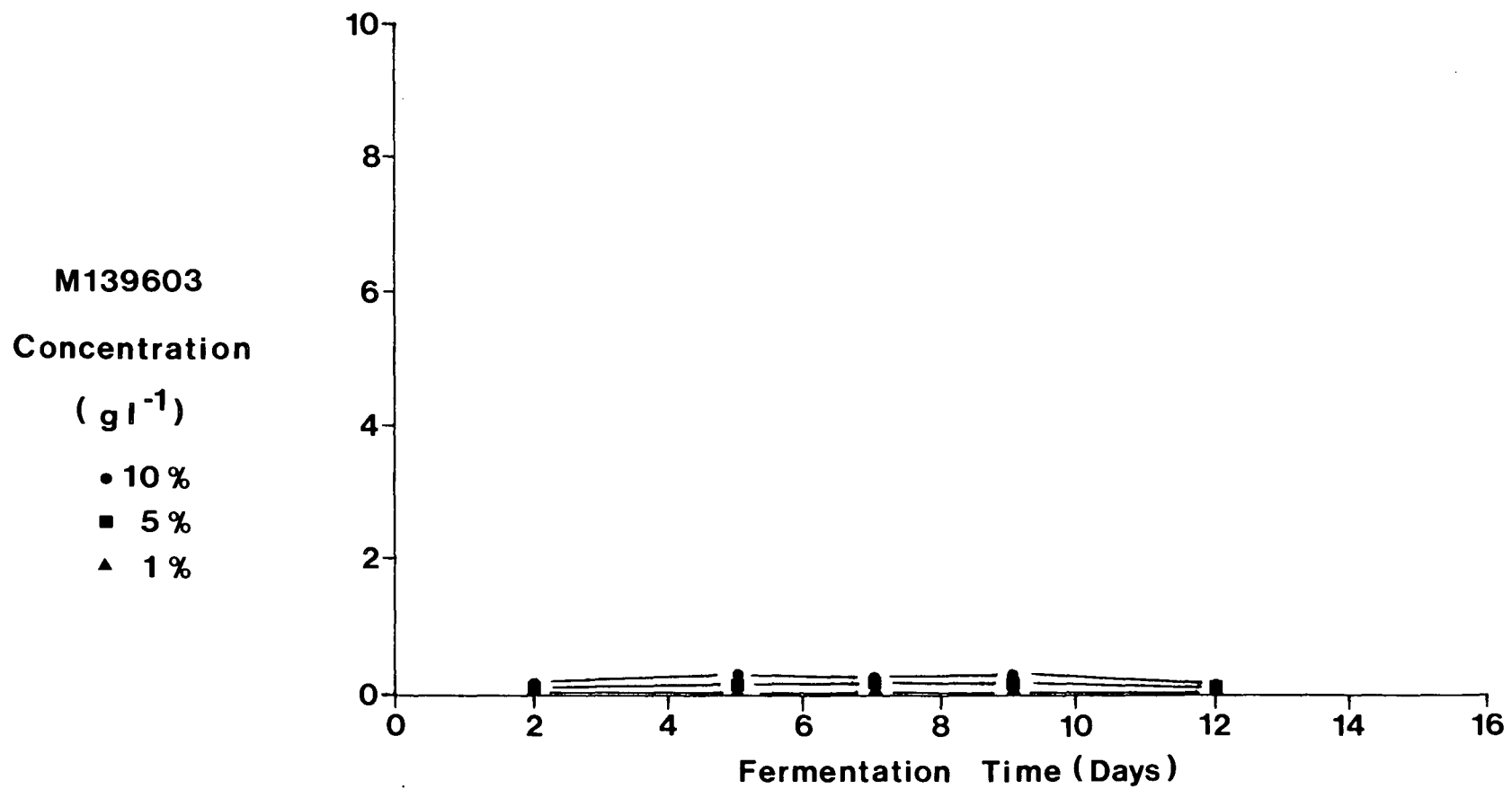


Fig. 7 Graph of M139603 Production Versus Time.

Carbon Source : Fructose

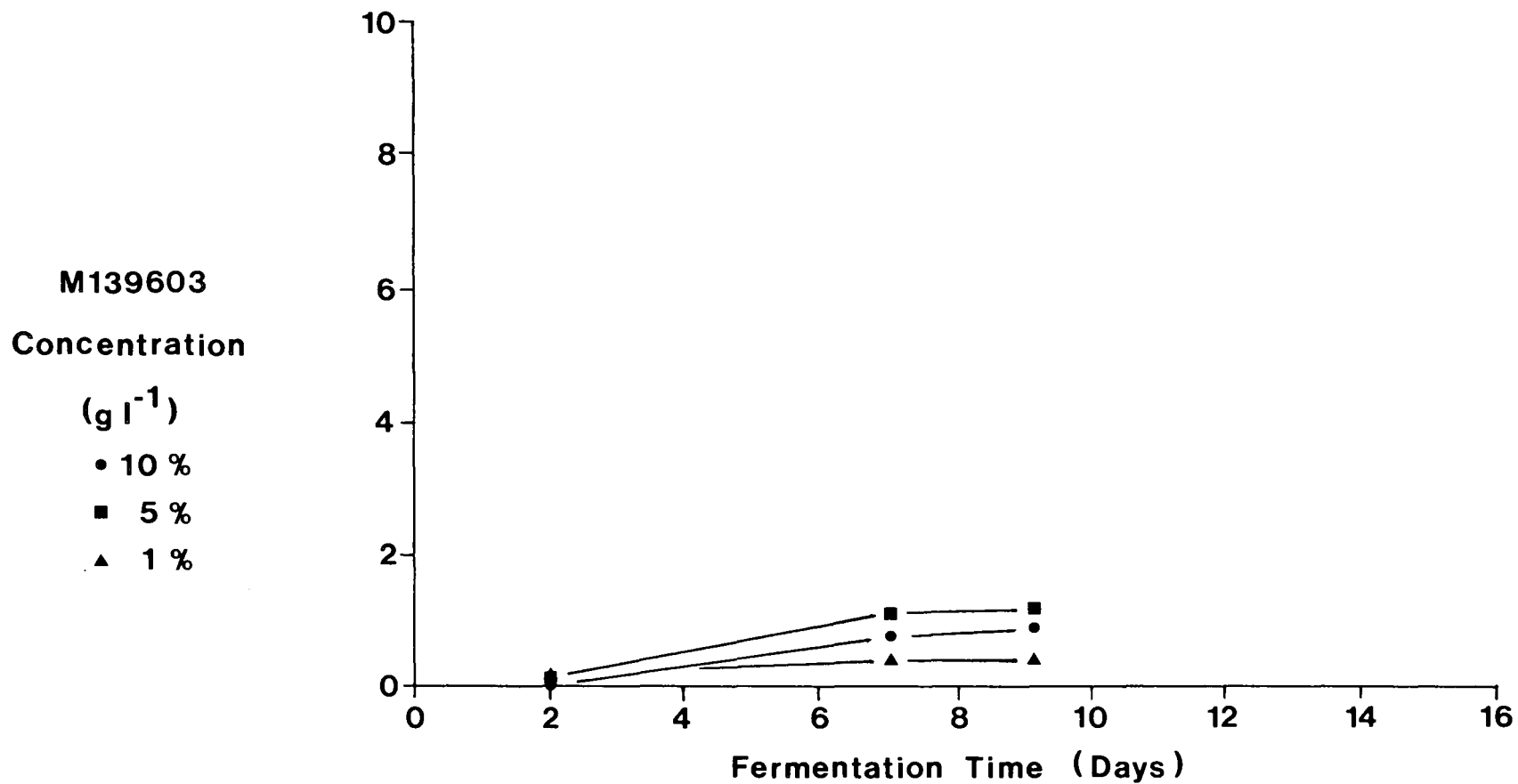


Fig.8 Graph of M139603 Production Versus Time
for a Fed-Batch Fermentation.

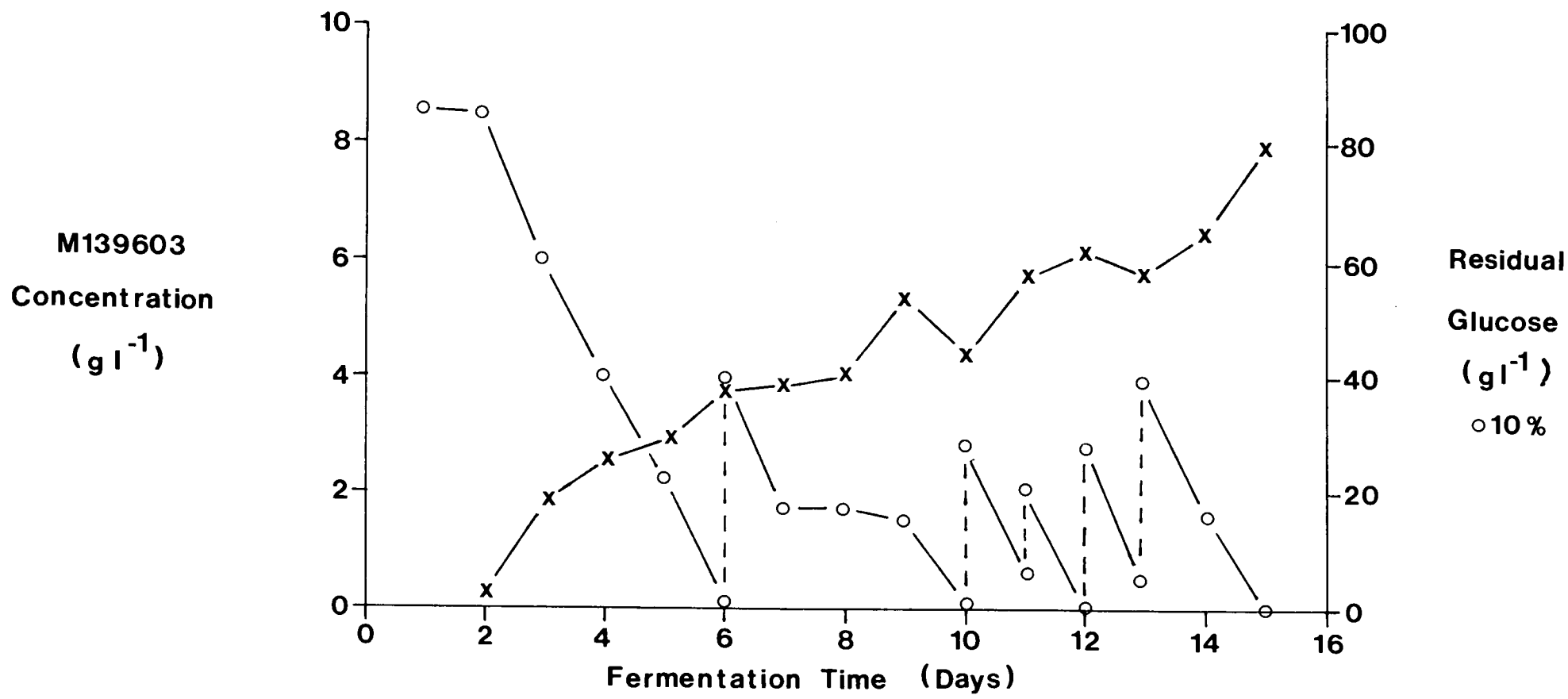


Table 6 Table of Maximum Yield of M 139603

Carbon Source	Concentration of Carbon Source % w/v gl^{-1}	Concentration of Highest Yield of M139603 gl^{-1}
Cellobiose	10	9.13
Glucose	1	0.55
	5	3.75
	10	9.65
Maltose	10	7.83
Starch	1	0.62
	5	1.88
	10	7.25
Sucrose	1	0.08
	5	0.63
	10	7.13
Lactose	1	0.10
	5	0.18
	10	0.29
Fructose	1	0.54
	5	1.23
	10	0.99
Fed-batch Fermentation (Glucose)	10	8.00

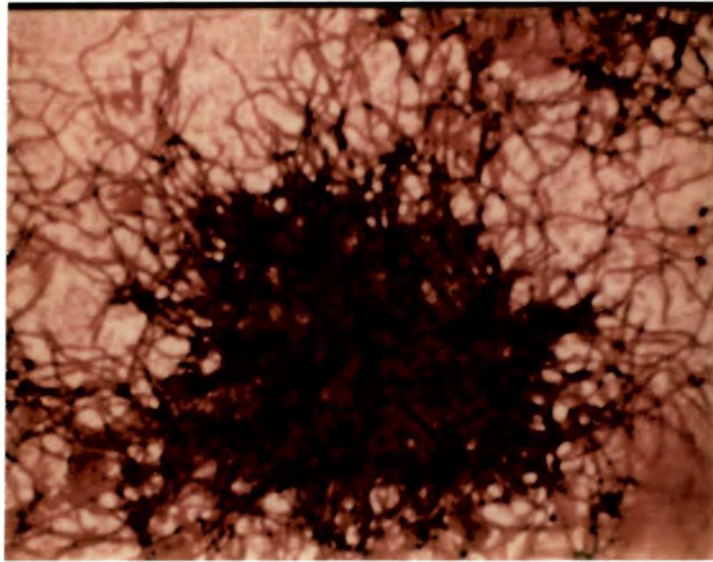


Plate 1

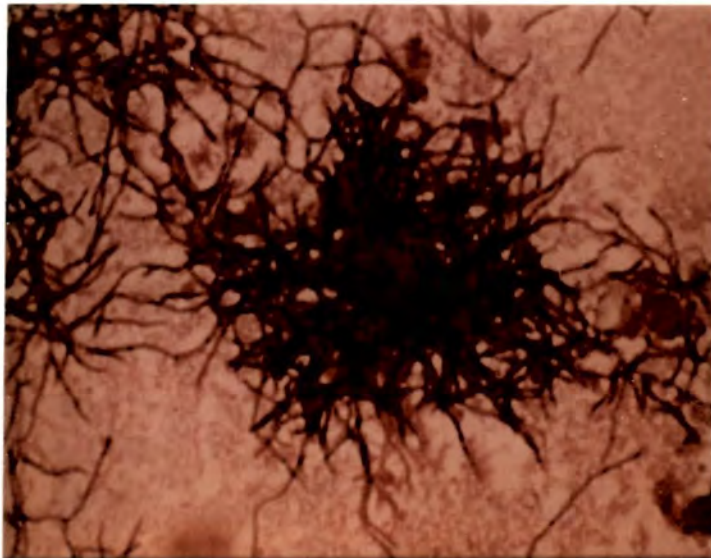


Plate 2

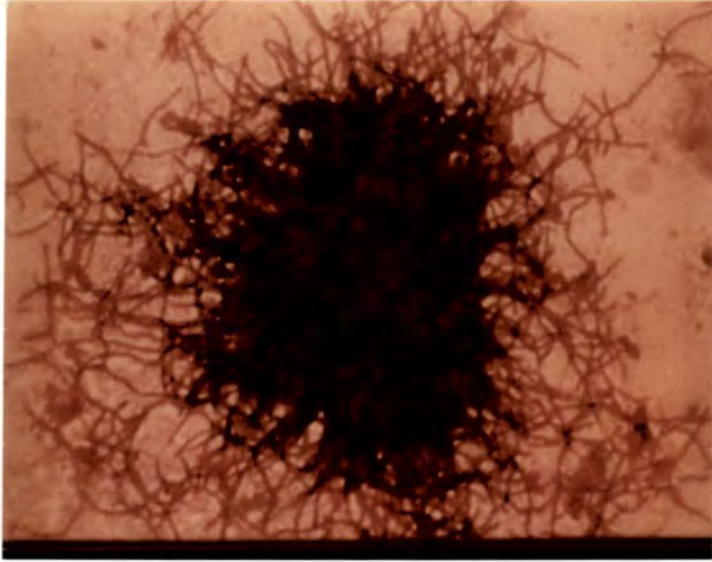


Plate 3

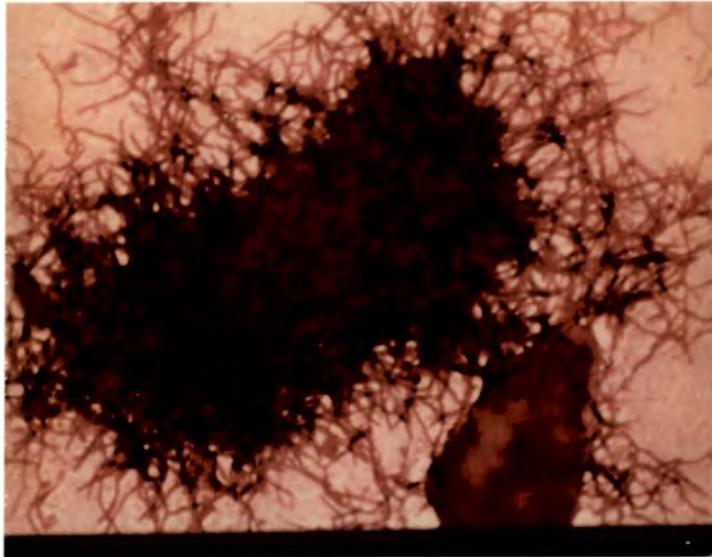


Plate 4

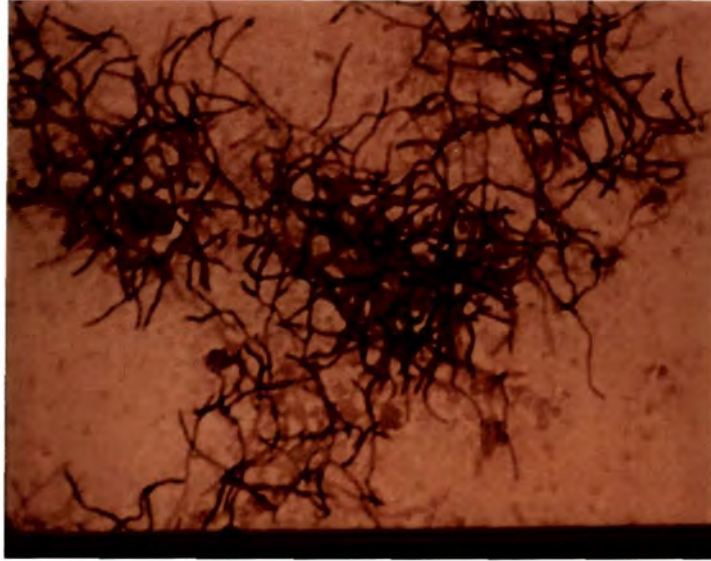


Plate 5

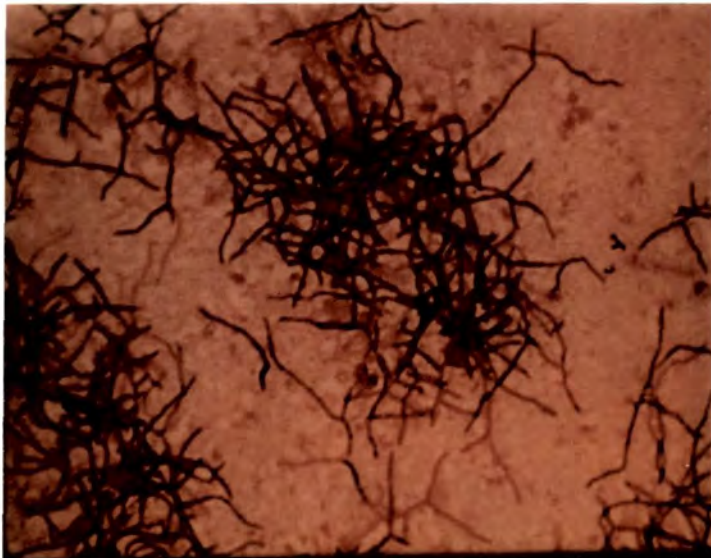


Plate 6

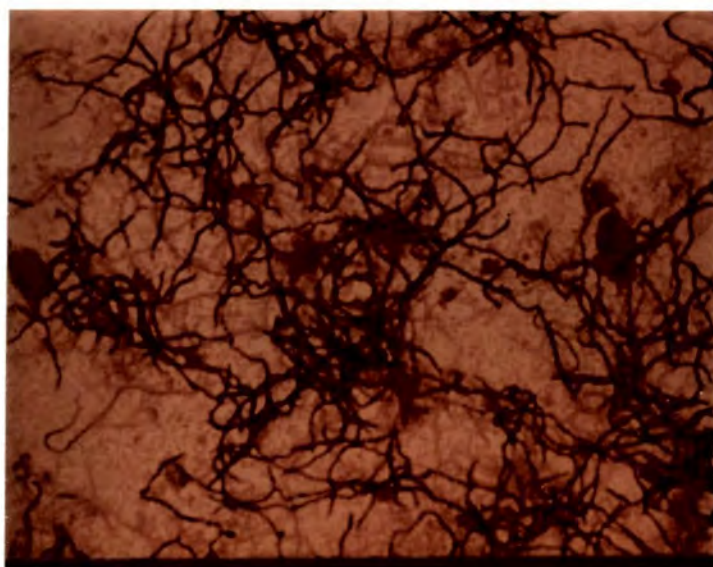


Plate 7

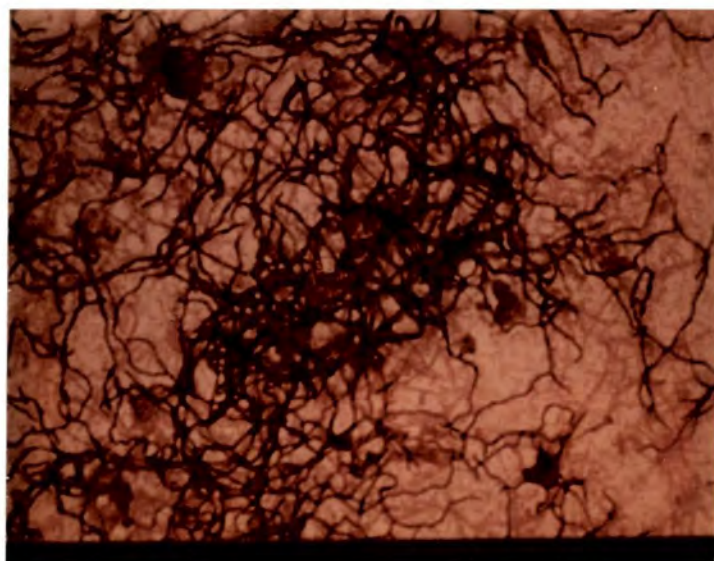


Plate 8

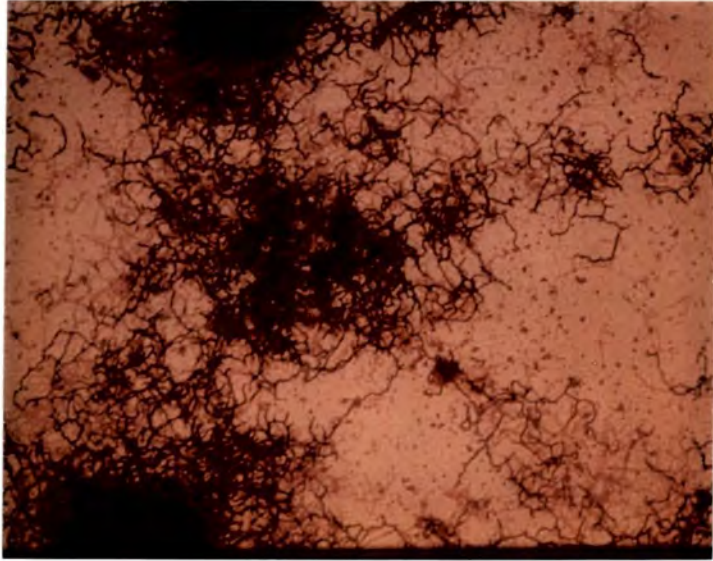


Plate 9

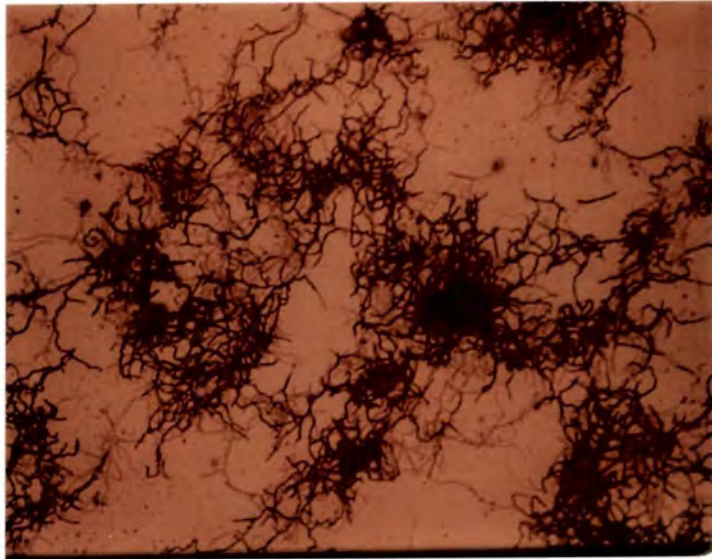


Plate 10

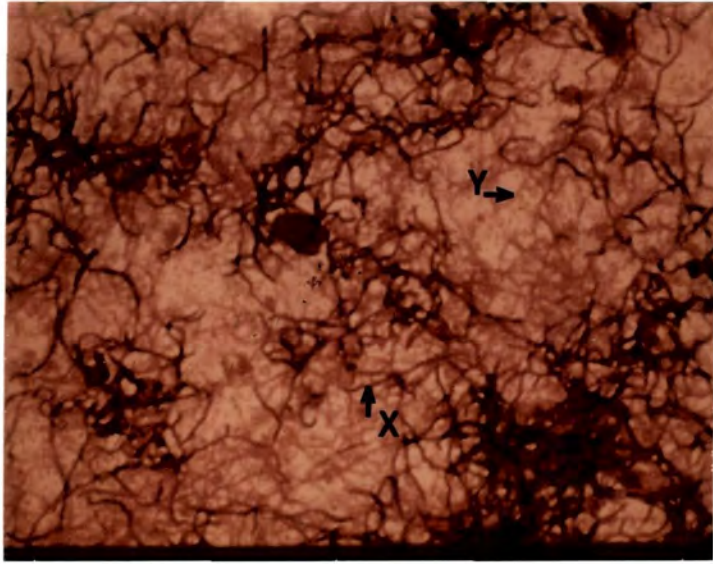


Plate 11

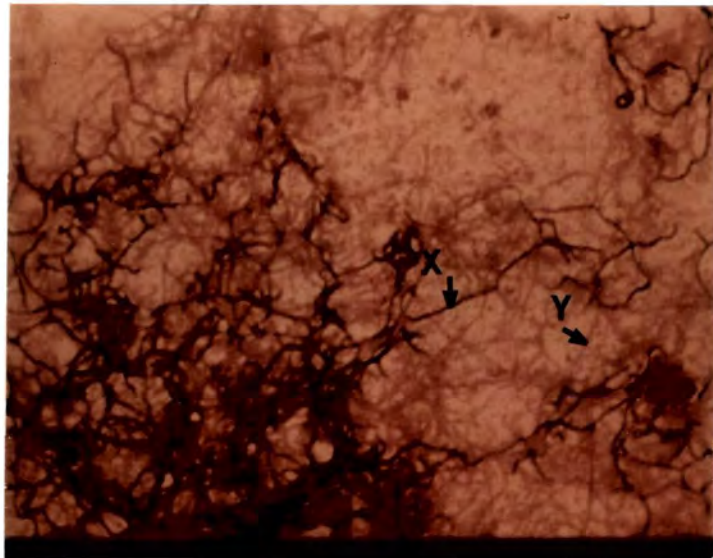


Plate 12

d) Description of Plates

Plates 1 and 2 (examined under oil using a X 100 objective lens).

The plates show the culture morphology with a fermentation running time of 4 days. The culture forms dense mycelial pellets with the hyphae staining gram positive.

Plates 3 and 4 (examined under oil, X 1 00 objective lens).

After 5 days as with plates 1 and 2, the mycelium is in the form of dense pellets, staining gram positive.

Plates 5 and 6 (examined under oil, X 100 objective lens).

After 8 days the mycelial pellets are becoming dispersed. Hyphae are stained gram positive.

Plates 7 and 8 (examined under oil, X 100 objective lens).

After 10 days the culture mycelium has become even more dispersed. Hyphae are stained gram positive.

Plates 9 and 10 (examined using X 40 objective lens).

After 11 days the culture mycelium has become very dispersed. The plate has been taken at lower magnification in order to show a wider field of view. The mycelium is stained gram positive.

Plates 11 and 12 (examined under oil, X 100 objective lens).

After 12 days the mycelium has become very dispersed, and has also become gram variable in staining characteristics with some hyphae staining gram positive (X) and some gram negative (Y).

Discussion

From the shake flask results it can be seen that the highest yields of M139603 were obtained from flasks containing an initial carbon content of 10% Glucose, 10% Cellibiose and 10% Maltose; with figures of 9.65, 9.13 and 7.83 gl^{-1} respectively. It can also be seen that the flasks containing 10% Starch and 10% Sucrose also gave reasonable yields of M139603 (7.25 and 7.13 gl^{-1} respectively), whilst Fructose gave a maximum yield of 1.23 gl^{-1} (5% initial carbon concentration) and Lactose (10%) gave a very poor yield of 0.29 gl^{-1} . It should be noted that in all cases, except Fructose, the highest yields were obtained from flasks containing an initial carbon content of 10%, and that yields of M139603 decrease with decreasing initial carbon concentration.

It would appear from these results that carbon catabolite repression, mediated by an excess of glucose in the medium, is not a factor involved in the regulation of enzymes responsible for M139603 synthesis.

This conclusion may be drawn since the highest yield of M139603 was obtained when the medium used contained a high concentration of glucose, a carbon source known to inhibit the production of a wide range of antibiotics and other secondary metabolites, whilst much lower yields were obtained on carbon sources which have commonly been used to circumvent carbon catabolite repression, for example, Lactose, Sucrose, Fructose and Starch (Demain et al, 1979; Martin and Demain, 1980).

It should be noted that glucose may not elicit a repressive response because it is not the carbon source preferred by S. longiscoroflavus for growth. Citrate (S. niveus, Kominek, 1972) and glycerol (S. Clavuligerus, Aharonowitz and Demain, 1978) have both been reported as being the favoured carbon source, whilst Chatterjee and

Vining (1981) report repression of α -glucosidase by Krebs cycle acids in S. venezuelae, despite the ineffectiveness of glucose, mannose, and other carbon sources known to support a fast rate of growth.

However, care should be taken when drawing comparisons between the repression of an inducible catabolic enzyme and control of antibiotics synthesis, since Chatterjee and Vining (1982,b) showed that whilst glucose depressed induction of β glucosidase by cellobiose, cultures of S. venezuelae using glucose, cellobiose or a mixture of the two saccharides, as their carbon source produced chloramphenicol during growth. Therefore in contrast with its repressive effect on β - glucosidase induction, glucose did not suppress chloramphenicol production, indicating that the control mechanisms that establish carbon source preferences are not linked to those that regulate antibiotic biosynthesis in S. venezuelae, Chatterjee and Vining, 1982 b.

Further evidence in support of the conclusion drawn from the maximum yields of ML39603, comes from examination of the graphs for ML39603 production (Figures 1 to 7).

Firstly if one considers the curves for flasks containing an initial carbon concentration of 10%, an indication of the rate of ML39603 production can be obtained by measuring the slopes of each curve between two standard points. Thus the slopes for 10% Glucose, Cellobiose, Maltose and Starch (between 2 and 7 gl^{-1} , ML39603 concentration) and Sucrose (3 and 7 gl^{-1} ML39603 are 0.735, 0.735, 0.746, 0.751 and 0.666 respectively (calculations not shown).

As can be seen these values are very close, indicating that the rate of production of ML39603 between 2 and 7 gl^{-1} (3 and 7 gl^{-1} , for Sucrose) is similar for each carbon source. Differences in the maximum yield come about due to differences in the rate, and duration of antibiotic

synthesis before and after the period of examination. If carbon catabolite repression was involved in the control of M139603 biosynthesis, then the rate of production of M139603 in media containing more complex carbon sources (for example, Starch and Cellobiose) would be higher than that for Glucose. This evidently is not the case since not only did the 10% glucose flasks produce the highest titre of antibiotic, but they also reached their maximum yield 48 hours before the 10% Cellobiose flasks, which were responsible for the second highest titre of M139603.

It should be noted however that the differences between the titres obtained from the 10% glucose, 10% Cellobiose, and possibly the 10% Maltose flasks could be accounted for by differences in the inoculum used to inoculate each batch of flasks. Since growth of S. longisporoflavus in shake flasks is based on a single stage shake flask inoculum procedure using a complex medium (see Materials and Methods), standardisation of the inoculum in terms of physiological state, growth rate and dry weight is impossible.

This problem is largely overcome in the inoculum development procedure used in fermentor studies (see Materials and Methods) since it is possible to measure growth rate by analysing CO₂ evolution from the seed fermentor, and hence transfer an inoculum to the production fermentor, when the optimum growth rate is achieved.

The rationale behind using three concentrations of each carbon source (except Cellobiose and Maltose, which could not be tested at the lower concentrations at the time of experimentation) is that if carbon catabolite repression is involved in the regulation of M139603 synthesis then lower concentrations of the repressive substrate(s) would result in higher yields and faster production rates, as is the case with chloramphenicol production by S. clavuligerus,

when grown on a range of media containing 0, 0.2, 0.4, 0.6 and 1.0% v/v glycerol and 0.4% L asparagine, 0.4% v/v glycerol gave the best volumetric titre of chloramphenicol by achieving a balance between growth rate (to obtain a critical level of biomass) and specific rate of chloramphenicol production. Higher concentrations of glycerol, yielded a higher level of biomass but much reduced specific antibiotic production and hence lower volumetric titre (actual concentration of antibiotic) whereas the converse was true for lower concentrations of glycerol (Demain et al 1979).

From figures 1 to 7 it can be seen that the only sugar to produce higher levels of M139603 production in flasks containing lower initial carbon concentrations, was Fructose with figures of 1.23 (5%) and 0.99 gl^{-1} (10%).

However, other observations on the state of the culture broth (data not shown), in that the mycelium quickly formed large pellets (in excess of 2 mm diameter, commonly an indication of stress in Streptomyces, P. Willis personal communication), whilst the milou pro component of the medium became solubilised and changed colour (indicating that this latter component was being degraded), coupled with the very low titre in all three sets of Fructose flasks leads me to believe that the figures obtained are not as a result of carbon catabolite repression, merely a reflection of the inherent variability of shake flask culture. The large variation in productivity, in respect to M139603 synthesis between the different carbon sources, cannot at this point be explained, beyond saying that the differences come about due to variations in the ability of S. longisporoflavus to utilize each carbon source for growth. However, no information is available on the growth rate of S. longisporoflavus during any of the above experiments, due to the inability to measure mycelial dry weight

throughout the experiments because of the high levels of suspended solids in the medium. Consequently this is an area of work which needs to be studied in more detail. Measurement of growth rate could be achieved by measurement of total DNA in the culture broth. Unfortunately such analysis was not possible at the time of experimentation. Likewise, nutrient utilisation within the culture broth requires more detailed analysis in order to more fully understand the control of M139603 synthesis. Again analysis of all carbon source utilisation for all the carbon sources other than glucose required detailed biochemical analysis, hence the lack of data on residual carbon in the medium except in the case of glucose.

A point of particular interest from the above shake flask experiments was the very poor showing of both Lactose and Fructose as a sole carbon source for S. longisporoflavus.

Lactose metabolism in Streptomyces has been studied by a number of workers (Sanchez and Hardisson, 1979; Chatterjee and Vining 1982 a).

As is the case with other organisms, for example E coli, β - galactosidase in those Streptomyces species studied is an inducible enzyme, however differences occur in the mode of induction. For example, in S. griseus β -galactosidase is induced by lactose but not D. galactose or some other galactosides, Dan and Szabo, 1973; Vitalis and Szabo, 1978.

Sanchez and Hardisson (1979) report that in S. violaceus galactose, in concentrations higher than 2% w/v, forms the best inducer for β -galactosidase. For Lactose to act as an efficient inducer of β -galactosidase in S. violaceus, requires pre culturing in lactose containing medium. If like S. violaceus, S. longisporoflavus requires pre-culturing on lactose before synthesis of β galactosidase can occur, then this would explain the poor results obtained from glucose.

The apparent inability of S. longisporoflavus to metabolise fructose

(as mentioned above) efficiently poses the question of why this should be. Metabolism of glucose and fructose in S. aureofaciens has been studied by Novotna and Hostalek (1985). Sugar phosphates, intermediates of carbohydrate metabolism, arise in micro organisms either by the action of hexokinases after the entry of the sugar into the cells or during transport by action of the Phosphoenolpyruvate: sugar phosphotransferase system (PTS).

However, PTS has been reported as missing in S. aureofaciens (Novotna and Hostalek, 1985) and S. violaceoruber (Sabater et al, 1972)

Consequently, entry of Fructose into metabolism requires the action of specific Fructokinase as has been reported in Sabater et al, 1972 and now Novotna and Hostalek 1985. It seems possible that the apparent inability of S. longisporoflavus to grow on fructose is due to this organism lacking a specific fructokinase, hence fructose cannot enter metabolism, as this requires a phosphorylated intermediate.

Analysis of the hypothesis could be carried out by utilising the methods of Novotna and Hostalek (1985) to prepare cell free extracts of S. longisporoflavus and determine their ability to produce fructose - 6 - phosphate, the product of fructokinase-phosphoenol pyruvate phosphorylation system.

The above conclusions drawn from the shake flask experiments, with regard to the apparent lack of involvement of carbon catabolite repression in M139603 biosynthesis are tentative conclusions based upon the results obtained from the above experiments. Positive proof of the involvement of carbon catabolite repression can only be determined by further experimentation, such that, it can be shown that addition of the repressive substrate to a medium prevents biosynthesis of M139603 until the concentration of that substrate is no longer inhibitive, and that secondly, addition of the substrate to a culture already producing M139603 prevents further M139603 production by preventing the de novo synthesis

of the key enzyme(s) of M139603 biosynthesis.

From the lab scale fermenter experiments it can be seen that the maximum yield of M139603 was 8 gl^{-1} . This result is disappointing when one considers the yield obtained from the same medium in shake flask culture and if one compares the yield to that obtained by the standard ICI production medium, of 15 gl^{-1} .

No conclusions may be drawn from this experiment due to a lack of data to compare against this result.

However, one can try and draw comparisons between the yields obtained from medium containing 10% glucose in shake flask and lab scale fermenter culture. As stated above, with the methods used, (see Materials and Methods) experiments at the lab scale fermenter level should involve less variation due to the inoculum used, when compared to experiments using shake flask culture. This is because the inoculum development procedure used in lab scale fermenter experiments ensures, assuming a standard volume of inoculum is transferred, that the inoculum used is as reproducible as possible since inoculum transfer can occur with, the inoculum cultures possessing the same (or very similar) growth rate as measured by volumetric CO_2 production rate. Conversely shake flask experiments utilize a single stage, shake flask inoculum procedure, with no control over inoculum transfer timing other than the 48 hour deadline.

As a consequence of the above comments it must be assumed that the factors affecting the yield of M139603 in the fermenter experiment occur once the fermentor has been inoculated and that given the same fermentation conditions the yield would be of the same order as that achieved, i.e. 8 gl^{-1} . However the shake flask experiments if repeated could yet achieve a higher yield of M139603 if the condition of the inoculum used is closer to the (as yet unknown) optimum.

Therefore it must be assumed that the failure to achieve the same level

of production in the lab scale fermenter as in the shake flask results, comes about due to differences in culture conditions between the fermenter and shake flasks.

Such differences include, different concentrations of Milou pro in the media, (2% Milou pro in shake flask media, and 3% in the fermenter medium, see materials and methods), different Sterilisation procedures, and differences in the physical environment. (for example optimum pH is maintained in the fermenter, and differences occur in agitation and aeration).

The concentrations of milou pro used in shake flask and fermenter have previously been determined as optimum by ICI workers (un published data). However, the carbon sources used were different to those used above and so it is possible that the concentration of milou pro may have to be altered in order to achieve higher yields with glucose as a carbon source.

As can be seen from materials and methods, the sterilisation procedure are completely different, with the shake flasks being autoclaved for half an hour, whereas medium for fermenters is autoclaved for 1 hour. Also because the production medium is sterilised in 10 L aliquots and so high temperatures are maintained for longer in the fermenter medium than the shake flasks. Consequently heat labile components of milou pro may be broken down under the fermenter medium sterilisation conditions. Again the sterilisation procedures have been determined previously. However, again the previous work may need to be re examined as the standard ICI production medium also contains 10% starch, whereas the fermenter medium used in the above experiment had its carbon content sterilised seperately. It could well be that the presence of starch in the medium absorbs a proportion of the heat used to sterilise the medium, and that in the absence of starch this heat goes to the milou pro, hence the composition of the milou pro after sterilisation

in the ICI standard production medium the fermenter medium used above, and the shake flask medium is chemically different, hence the differences in yield between the shake flask and fermenter experiments.

Differences in the physical environment between the shake flask and fermenter experiments for two reasons. Firstly no control of medium pH was used in shake flasks, whilst the fermenter maintained medium pH at 7.00. Secondly differences in the physical geometries between the shake flasks and fermenter pot, coupled with different modes of agitation and aeration mean that no comparison may be drawn. Finally, if the conclusions drawn above are held to be true then one must ask what mechanism does control ML39603 biosynthesis. To consider this one must draw comparisons with the control mechanisms known to regulate the production of other polyether and macrolide antibiotics.

This would seem to be a reasonable comparison since it is known that macrolide and polyether antibiotics are both manufactured by assembly of C_2 and C_4 acids using biochemical pathways that are analogous to the fermentation of fatty acids, Hutchinson, 1983.

In batch culture the factor that controls the onset of antibiotic biosynthesis is probably the deficiency of one or more nutritional growth limiting components, Martin and Demain 1980.

Control of initiation of antibiotic production often occurs at the level of transcription of antibiotic synthetase genes or by interference with translation of messenger RNA, Martin and Demain, 1980.

In the case of candicidin (whose production characteristics closely parallel ML39603, P. Willis personal communication) there is some evidence that repression is exerted at the level of **transcription** (Lirass et al 1977, Martin and Demain 1980) However, not all of the regulatory mechanisms controlling antibiotic biosynthesis are exerted at a single level (repression or inhibition). Again in the case of candicidin production, phosphate exerts a control at two levels, since candicidin

production is repressed until phosphate in the medium is depleted and secondly candicidin synthesis is inhibited by phosphate after the synthetase has been formed, Martin and Demain, 1976. To determine whether ML39603 is under similar control it is first necessary to establish a defined medium which would maintain ML39603 production, and then vary the concentration of phosphate in the medium. However, given the nutritional complexity of ML39603 production medium, coupled with a lack of knowledge as to the biochemistry of S. longisporoflavus the above would seem a difficult task.

As to carbon catabolite regulation, given the conclusions drawn above, carbon catabolite regulation could still be a factor in the regulation of ML39603 production, and much more work is required before a decision can finally be made. Again using candicidin production as an example, glucose exerts carbon catabolite regulation on candicidin production, which is alleviated by feeding glucose into the fermentation broth at a slow rate. This method of bypassing carbon catabolite regulation since the molecular mechanism of carbon catabolite regulation would seem to be associated with growth rate control of antibiotic synthesis. Hence feeding glucose at a rate which maintains a growth rate non inhibitive to antibiotic production may yet prove to be a method for increasing ML39603 production as is the case with Candicidin and Candihexin, Martin and Demain, 1980. The nitrogen content of ML39603 production medium is also known to have an effect on yield. Few studies have dealt solely with nitrogen metabolite regulation of antibiotic synthesis (Martin and Demain, 1980) but, several reports in the literature indicate that antibiotic biosynthesis may be regulated by ammonia and other readily utilised nitrogen sources, Martin and Demain 1980; Brana et al 1986. Again because of the nutritional complexity of milou pro, determination of the exact method of control of ML39603 synthesis by nitrogen will require further detailed study.

Finally, one other mode of regulation of antibiotic synthesis, feed back regulation, would also seem a likely control mechanism for M139603 production.

Feed back regulation comes about due to the antibiotic molecules themselves interacting allosterically with key enzymes in antibiotic synthesis.

Examples of antibiotics controlled in this manner, include cycloheximide, Nystatin and Candihexin (Martin and Demain, 1980).

It would seem most likely however, that control of M139603 production is not mediated by a single example of the above mechanisms, but more likely by a complex interaction of several of the above.

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