A Study on the Relationships Between
Growth, Antibiotic Production and Morphology in
Saccharopolyspora erythraea (Streptomyces erythraeus).

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Summary

The growth, productivity and morphology of *Saccharopolyspora erythraea* cultures grown in a defined medium were examined in a number of fermentation systems. In batch culture, the specific production rate of erythromycin was strongly linked to specific growth rate in both glucose- and nitrate-limited media, although it was difficult to determine whether the link was with growth rate *per se* or with some factor that also influenced the growth rate. In continuous culture, there was a clear separation in production kinetics with growth-linked production in glucose-limited chemostat but growth-dissociated production in nitrate-limited chemostat.

A simple filtration technique was used to resolve cultures into fractions with differing hyphal size distributions. During batch growth the relative proportions of these fractions changed. In the early growth phase, smaller mycelial fragments were predominant whereas larger fragments predominated during the later antibiotic production phase. This observation, together with some previous work performed in this group, indicated a link between morphology and production. This link was confirmed by studying the specific productivities of the subpopulations obtained after filtration. The subpopulation with smaller fragments (filtrate) grew faster, but were less capable of producing erythromycin than the subpopulation with the larger fragments (retentate). The use of these subpopulations as a source of inoculum resulted in different kinetics of growth and production in batch culture.

Attempts to manipulate the morphology of continuous cultures to achieve an increase in productivity gave mixed results. When a column separator was used to continuously enrich a nitrogen-limited chemostat with
the larger fragments, the result was a drop in productivity when compared with a control in which no enrichment occurred. It was suggested that the observations made in this system were due more to the starvation and subsequent shift-down occurring in the separator than to any effects upon morphology. Thus, enrichment of continuous cultures using an external vessel was not deemed suitable for the detection of the more subtle increases in productivity achieved with morphological manipulation.

When the morphology was altered by changing the stirrer speed (750rpm and 1500rpm), in nitrogen-limited chemostat, the results obtained reflected our expectations based on earlier batch culture studies. Higher biomass and erythromycin yields were obtained with the lower stirrer speed at three different dilution rates. The difference in productivity between the different stirrer speeds was more pronounced at the lower dilution rates. Using these results, a physiological model was proposed that spatially separated growth and secondary metabolism along the hyphal length. The consideration of the inherent heterogeneity of filamentous cultures remained a theme throughout these studies.
This thesis is dedicated, with love, to my mother and my sister

"He who would study organic existence
First drives out the soul with rigid persistence;
Then the parts in his hand may hold and class,
But the spiritual link is lost, alas!"

Johann Wolfgang von Goethe (Faust)
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Chapter 1. Introduction

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1.2 Metabolic control of secondary metabolism
1.3 Mechanisms of growth & hyphal elongation
1.4 Kinetics of growth in submerged culture
1.5 Relationship between morphology & production
1.6 Erythromycin production in *Saccharopolyspora erythraea*
1.1 Secondary metabolism in actinomycetes

1.1.1 Discovery, diversity, and exploitation.

Secondary metabolic products, including antibiotics, represent one of the largest and most diverse groups of natural products, and may be considered among the most industrially important of microbial metabolites. Although the majority of these compounds are produced by microorganisms, they may also be detected in a wide variety of organisms such as higher plants and animals. The relative importance of the actinomycetes as a source of antibiotics is illustrated below in table 1.

Table 1. The number of known antibiotics in 1984, and the relative importance of the various taxa that produce them (from Berdy, 1985).

<table>
<thead>
<tr>
<th>Type of Producing organism</th>
<th>Approximate number</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria</td>
<td>950</td>
<td>9</td>
</tr>
<tr>
<td>Actinomycetes</td>
<td>4600</td>
<td>43</td>
</tr>
<tr>
<td>Fungi</td>
<td>1600</td>
<td>15</td>
</tr>
<tr>
<td><strong>All microorganisms</strong></td>
<td><strong>7150</strong></td>
<td><strong>67</strong></td>
</tr>
<tr>
<td>Lichens</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Algae</td>
<td>250</td>
<td>2</td>
</tr>
<tr>
<td>Higher plants</td>
<td>2500</td>
<td>23</td>
</tr>
<tr>
<td>Animal organisms</td>
<td>700</td>
<td>7</td>
</tr>
<tr>
<td><strong>All 'higher' organisms</strong></td>
<td><strong>3550</strong></td>
<td><strong>33</strong></td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>10700</strong></td>
<td><strong>100</strong></td>
</tr>
</tbody>
</table>

The actinomycetes produce a large proportion of the antibiotics discovered to date (table 1). Interest in the isolation and screening of actinomycetes for novel, active secondary metabolites was initially stimulated by the discovery
of the antibiotic actinomycin (Waksman & Woodruff, 1940). During the following three decades, a multitude of screening programs were developed for the rapid analysis of naturally isolated actinomycetes, and the numbers of newly discovered, novel, bioactive compounds rose dramatically. The early screening programs concentrated on organisms that could be readily isolated on existing media, i.e. primarily members of the genera *Streptomyces*, *Micromonospora*, and *Nocardia*, which grew rapidly on the classical media and competed successfully with the more unusual groups of actinomycetes.

The search for novelty, however, is becoming increasingly more difficult, and although the capacity of the genus *Streptomyces* to produce antibiotics remains unsurpassed, it has become necessary to screen a far wider range of organisms in order to continue to supply new compounds for future use against drug-resistant bacteria, recalcitrant diseases, or for chemical modification. This, in turn, has led to a need to depart from the classical methods of isolation and screening and to encourage the isolation of actinomycetes on more selective, defined media, and their subsequent screening in more specific and sensitive screening protocols.

Table 2. Table showing the number of antibiotics produced by selected actinomycete genera (Berdy, 1984)

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Streptomyces</em></td>
<td>1934</td>
<td>3477</td>
<td><em>Streptosporangium</em></td>
<td>7</td>
<td>26</td>
</tr>
<tr>
<td><em>Micromonospora</em></td>
<td>41</td>
<td>269</td>
<td><em>Actinomyces</em></td>
<td>0</td>
<td>19</td>
</tr>
<tr>
<td><em>Nocardia</em></td>
<td>45</td>
<td>107</td>
<td><em>Pseudonocardia</em></td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td><em>Actinomadura</em></td>
<td>0</td>
<td>51</td>
<td><em>Streptoalloteichus</em></td>
<td>-</td>
<td>14</td>
</tr>
<tr>
<td><em>Actinoplanes</em></td>
<td>6</td>
<td>95</td>
<td><em>Micropolyspora</em></td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td><em>Streptoverticillium</em></td>
<td>19</td>
<td>64</td>
<td><em>Microbispora</em></td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td><em>Saccharopolyspora</em></td>
<td>-</td>
<td>33</td>
<td><em>Thermomonospora</em></td>
<td>1</td>
<td>4</td>
</tr>
</tbody>
</table>
The evidence of the increasing attention shown to the rarer, infrequently isolated genera is illustrated by table 2, where it can be seen that in 1974 about 94% of the antibiotics produced by actinomycetes had been produced by streptomycetes. Ten years later this figure had dropped to 83%. As screening protocols have become more selective and sensitive, the range of structures and activities of natural compounds that can be detected by these systems has been steadily increasing. An example of the diversity that natural products exhibit is shown below in table 3.

Table 3. Table showing the diversity of structure and bioactivity of microbial metabolites (modified from Berdy, 1985).

<table>
<thead>
<tr>
<th>Chemical features:</th>
<th>Bioactivities:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saccharides</td>
<td>Antimicrobial</td>
</tr>
<tr>
<td>Lactones, lactams</td>
<td>Antiviral</td>
</tr>
<tr>
<td>Pigments (Quinones, anthrocyans)</td>
<td>Antitumour (cytotoxic)</td>
</tr>
<tr>
<td>Peptides</td>
<td>Insecticide, miticide, etc.</td>
</tr>
<tr>
<td>Nucleotides</td>
<td>Growth regulators, hormones</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>Pesticides, herbicides</td>
</tr>
<tr>
<td>Aromatics</td>
<td>Immunoregulators</td>
</tr>
<tr>
<td><strong>Unusual structures:</strong></td>
<td></td>
</tr>
<tr>
<td>β-lactams, monobactams, macrocycles,</td>
<td></td>
</tr>
<tr>
<td>polyenes, polyines, multicondensed</td>
<td>Physiological (cholesterol</td>
</tr>
<tr>
<td>aromatics</td>
<td>lowering, hypotensive etc.)</td>
</tr>
<tr>
<td></td>
<td>activities</td>
</tr>
<tr>
<td></td>
<td>Enzymes, enzyme inhibitors</td>
</tr>
</tbody>
</table>

The key to the discovery of novel metabolites lies in the effectiveness (specificity and sensitivity) of the screening system. It had long been suspected that many active metabolites were being missed in screening programs due to a lack of sensitivity in the detection system. A classic example of this is illustrated by the detection of clavulanic acid in the culture
filtrate of *Streptomyces clavuligerus* by the Beecham group (Brown et al, 1976). This organism had previously been extensively studied and used by the Lilly group as a source of deacetoxycephalosporin C (Nagarajan et al, 1974). The field of antibiotic discovery has been characterised by a series of long quiescent periods in search and discovery programmes, interspersed with some spectacular jumps. An example of this was the development of organisms that were 'super sensitive' to ß-lactams which subsequently led to the discovery of new groups of antibiotics, e.g. the nocardicins and particularly the monobactams (Nolan, 1986).

For more than 50 years the actinomycetes have been exploited for their ability to produce a diverse range of active metabolites. This exploitation has, for the large part, been conducted empirically, driven by the need for novelty and higher yields. At the beginning of the century, microbiology was dominated largely by academic workers interested in the pathogenesis and biochemistry of the organisms that they studied. Through their dedicated investigation, the fundamental physiology of the unicellular bacteria and the fungi was unravelled. The knowledge of this basic physiology paralleled the discovery of the potential usefulness of such metabolic capabilities. Now, at the end of this century, the drive for profit, led by the pharmaceutical companies, has meant that comparatively little is understood about the basic physiology of the actinomycetes whereas their metabolic capabilities have been exploited in the extreme. Hopwood, in an article on genetic recombination and strain improvement (1977), stated that:

> the end products of long periods of natural evolution, have been taken and subjected to a rapid process of [mutation and selection] to produce varieties that are very different from anything that has been found before and from anything that would occur in nature in the future. Selection has often been for a single character, yield of grain, wool, or breast muscle, to produce a kind of “monster” that can survive only when cultured under highly artificial conditions. Exactly the same principles apply to microorganisms. In the frantic environment of large fermenters making [natural products], wild strains are out of place; genotypes

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able to produce comparatively huge yields of a single product are needed, just as in many branches of agriculture.

Vanek & Blumauerova (1986) reflected that "considerations within microbial ecosystems of the microbial world, or of the physiology, or of the functional significance of the synthesised compounds for the producing microorganisms are no longer the bases for elaborating a new screening method or test". It is the huge gaps in our knowledge of the fundamental physiology of these organisms that many researchers are currently trying to fill. The monoculture philosophy, ingrained in microbiologists since the advent of Koch's postulates, is slowly passing and it seems that the next century will be concerned more with the study of microbes in their natural environments. In such habitats the mixed culture is the rule and the pure culture the exception. The study of mixed microbial communities is already receiving considerable attention (see Bushell & Slater, 1981). The potential importance of microbial interactions is emphasised by papers such as that by Ohshima et al (1983), who described an 2.5-fold increase in the production of neopurpuratin in Streptomyces propurpuratus when grown as a mixed culture with a Bacillus sp.

1.1.2 The role of secondary metabolites

The question of the functional role in nature of secondary metabolites is fundamental and one which occurs to most microbiologists. The term secondary metabolite was first introduced to describe shunt products by Foster (1949) and further developed by Bu'lock (1961, 1974, 1975). There have been many publications on proposed roles for secondary metabolites, usually the assumptions about these compounds differed in line with the authors' expertise, and thus each has its merits. Broadly they can all be resolved into two groups:
(1) Those which suggest that it is not the *products* of secondary metabolism which are important, rather it is the *process* of secondary metabolism itself that is of importance to the organism:

- Possibly the best known of these proposals is the 'games room' theory (Zahner 1978; 1979). This theory suggests that secondary metabolism represents an evolutionary playground where the organism can experiment with mutations of its metabolism to see if they are beneficial without having to mutate primary pathways (which could be potentially lethal). This theory has much to recommend it, not least of which is the implicit assumption that the processes of primary and secondary metabolism are separated either temporally or spatially.

- Woodruff (1966), Weinberg (1971) and Neijssel & Tempest (1979) proposed that secondary metabolism was the result of normal enzymes of intermediary metabolism operating on abnormally high pools of primary metabolites. These abnormal pools were assumed to accumulate when active growth is no longer possible due to nutrient deficiency.

- Bu'lock (1961) suggested the maintenance hypothesis, whereby secondary metabolism served to maintain mechanisms essential to cell multiplication in operative order during phases when cell multiplication was no longer possible. Thus, during restricted growth, the intermediates of primary metabolism could be shunted along the secondary metabolic pathways, preventing the build up of large pools of intermediates and keeping the primary pathways active.

- Dhar & Khan (1971) and Luckner (1972) proposed a detoxification model where less toxic secondary metabolites are formed from more toxic intermediates. The former studied the conversion of phenylacetic acid to benzyl penicillin, which fits in with this idea but the majority of secondary
metabolites are actually less toxic, to the producing organism at least, than their precursors.

(2) The second group of ideas assume that it is the products of secondary metabolism, rather than the process, that is important to the producing organism.

- The best known of these is the competition hypothesis proposed by Brian (1957), where secondary metabolic products have a role in controlling the proliferation of competing organisms. Thus, the producing organism senses the presence of a competitor (perhaps through a decrease in its substrate uptake rate) and launches a biological attack, which may take the form of an antibiotic, a sporulation factor, or a number of other bioactive compounds.

- There have been a number of reports concerning the role of secondary metabolites as autoregulatory factors, governing the expression or repression of genes depending upon the prevailing conditions. For instance, some antibiotics have been shown to have a potential role in the sporulation process of unicellular and filamentous bacteria (Sarker & Paulus, 1972; Hodgson, 1971; Demain & Piret, 1979).

- Finally, there are those who see secondary metabolism linked closely with growth and view it as part of the natural process of maturation of the producing organisms (Vanek et al, 1973; Stouthamer, 1979).

The problem with formulating a hypothesis about the role of these compounds in situ is that virtually nothing is known about the physiology of the producing organisms in their natural environment. All of the current theories are extrapolations of phenomena that have been observed under conditions that probably never occur in nature, i.e. in screens, fermenters, enriched soils, sterilised soils, etc. Thus, in an antibiotic screen for example,
we may detect that an actinomycete produces a compound with antimicrobial activity against a range of organisms. We can determine its structure and also its mode of action against susceptible organisms. The extrapolation occurs when it is assumed that the active metabolite is not only produced in the soil, but that it is produced in an active form and in sufficient quantity to have an effect on a potential competitor.

This last point is one that can be used to negate the competition hypothesis, as in the soil environment it would be difficult to envisage the maintenance of a sufficiently high concentration of active material unless it were bound in some way to the cell wall of the producer and/or the target. However, these assumptions have been influenced largely by the 'golden age' of antibiotics (Vanek & Blumauerova, 1986) where a sufficiently high concentration of material was measured in terms of the microgram quantities used to evaluate its activity in screening media. The use of more sensitive methodologies has shown that compounds possessing antibiotic activity in screens possess more interesting and subtle activities in the producer at concentrations many orders of magnitude lower than those used to initially detect its presence. Examples include avermectins which possess antihelminthic activity at attomolar \(10^{-18}\) concentrations, and many small molecule messengers such as cAMP which are active at the picomolar \(10^{-12}\) level (Vanek & Blumauerova, 1986). Further examples include the production of polyether antibiotics with excellent coccidiostatic properties but whose role in the producing organism is probably connected with metal ion translocation (Westley et al, 1977). These compounds are part of a larger group of structurally related compounds referred to as the siderochromes which carry complexed \(\text{Fe}^{3+}\) ions and are produced by a large variety of microorganisms.

The fact that an isolated organism can produce an active secondary metabolite is important, not necessarily because of the mode of action that allowed it to be detected, but rather for the fact that the producer has the
ability to produce such a compound. In its natural environment this active compound might have evolved to serve a function wholly different from that exhibited under laboratory conditions, as illustrated in the example of the siderochromes.

Although some reports hold to the idea that secondary metabolism is an artefact of laboratory cultivation methods (Weinberg, 1971), it still seems likely that these compounds are produced in the soil, and other natural environments, for two main reasons. Firstly, there are elaborate control mechanisms governing the expression of the relevant genes responsible for the synthesis of secondary metabolites, which will be discussed in the next section (1.2). The majority of the literature concerning the physiology of actinomycetes examines the effects that nutritional and biochemical changes have on the ability of the organism to produce, and they demonstrate that the regulation of the production of these metabolites is indeed a complex, highly regulated process (Martin & Demain, 1980; Aharonowitz, 1980; Martin & Liras, 1989). Complexity of this type carries the fingerprint of intense natural selection, and implies that the production of these compounds in the natural environment is not just useful to the producing organism, but that it is essential for their survival.

The second reason to think that secondary metabolites are produced in the soil and other habitats, is the presence of resistance determinants in both the producer and in organisms that do not produce the bioactive compound. In *Saccharopolyspora erythraea* this resistance determinant is the ermE gene which codes for a protein that dimethylates a specific adenine residue within the 23s ribosomal subunit (Skinner & Cundliffe, 1982; Cundliffe, 1989). The presence of such resistant determinants can only be rationalised if one assumes that the host organism produces itself, or might encounter naturally, the compound to which it is resistant (Stone & Williams, 1992). Thus to summarise, it does seem likely that compounds such as
antibiotics are produced in the natural environment, and their production forms an integral part of the producing organism's natural physiology. Therefore, although it seems likely that these compounds are produced in natural environments, it does not explain why they should be produced.

When one examines which groups of organisms produce secondary metabolites, it becomes obvious that a large proportion of them are produced by filamentous organisms (see table 1). This is not to say that unicellular organisms are incapable of producing them, indeed many *Bacillus* spp. produce a range of secondary metabolites (e.g. bacitracin, gramicidin S, and tyrocidine), but rather it is a reflection that unicellular organisms do not produce the diverse range of compounds that the filamentous ones can. It would appear that there is some basic phenomenon underlying the ability of filamentous organisms to produce more secondary metabolic compounds than unicellular species, and it also seems likely that it is the nature of the filamentous lifestyle itself that is responsible for this.

Filamentous organisms have a great advantage over unicellular organisms in that, potentially at least, they can simultaneously undergo primary and secondary metabolic processes in the same 'cell'. At the tip the organism is growing rapidly; synthesising new cell wall material, DNA, RNA, etc., but further back along the hypha the primary metabolic pathways may be shut down and the secondary metabolic ones become active. Thus, the actinomycete filament has a primitive form of compartmentalisation that enables it to function as a simple 'proto-eukaryote' by spatially separating potentially incompatible processes along its hyphal length. These ideas fit well with Zahner's games room theory which requires that the processes of primary and secondary metabolism be spatially or temporally separated. It is because actinomycetes can simultaneously undergo primary and secondary metabolism that allows them to experiment more freely with the latter. As to
the specific function of secondary metabolites, the rationale behind their production may lie in the production of molecular diversity for its own sake.

An analogous situation is seen in the human immune system, where the body produces millions of different cell clones (B-cells) each recognising a single epitope. The vast majority of these clones will never encounter the epitope to their specific molecular recognition structure and if analysed individually (for instance if their structure showed activity in a particular screen) their functionality might be elusive. The role of these clones can, however, be determined when one considers the whole population. The body produces a large pool of diverse chemical structures 'anticipating' that the antigens of a potential pathogen will be recognised and subsequently neutralised. Thus individual products of secondary metabolism do not necessarily have to have a specific role of their own. The producing organism has no way of knowing what the future environment will be like (lack of substrate, potential competition, etc.) and therefore produces a range of structures 'anticipating' that one of these structures will provide some selective benefit in the future. This idea of 'contingency' in nature was recognised by Darwin, who saw specific individuals (animal species in this case but could equally be molecular structures) as a detail not a purpose or embodiment of the whole. In this scheme "..we are observing a contingent detail, and may yet hope for purpose, or at least neutrality, from the universe in general.." (see Gould, 1991 pp277-291).

Another great advantage that many filamentous organisms have over many unicellular ones is the ability to form spores at the end of the growth cycle. A unicellular organism may only be able to undergo secondary metabolism at the end of the growth cycle, when it has exhausted most of the available nutrients, and therefore any useful mutations that occur during this period are unlikely to be passed on to its progeny, as the organism will only enter the reproduction phase if nutritional conditions improve. With the
actinomycetes and the fungi, the situation is much more different as they are capable of producing spores at the end of the growth cycle. Thus any mutations that have occurred during secondary metabolism would be conserved in succeeding generations. It is of interest to note that one of the most successful unicellular bacteria, in terms of its ability to produce secondary metabolic products, *Bacillus*, is also capable of sporulation at the end of the growth cycle.

Not all actinomycetes are capable of producing spores, and many do not produce true filaments, but they are still capable of producing secondary metabolites. They generally do not produce them to the same extent as the actinomycetes that do both, e.g. *Streptomyces* spp. Perhaps then, it is no coincidence that the most frequently isolated actinomycete genus is the one which produces more than 80% of all antibiotics discovered in actinomycetes (see Table 2). It may be that this fact serves to illustrate the selective advantage that the production of molecular diversity through secondary metabolism can have on organisms in natural environments.
1.2 Metabolic Control of Secondary Metabolism

1.2.1. Carbon Catabolite Regulation

In many cultures, the presence of a rapidly utilisable carbon source such as glucose can result in the repression of antibiotic synthesis. Examples of this include actinomycin production by *Streptomyces antibioticus* (Gallo & Katz, 1972), candidin and candihexin production in *Streptomyces griseus* (Martin and McDaniel, 1974), puromycin synthesis in *Streptomyces alboniger* (Sankaran & Pogell, 1975) and the production of erythromycin by *Saccharopolyspora erythraea* (Escalante et al, 1982). Glucose is not the only carbon source to elicit this effect. Novobiocin production in *Streptomyces niveus* is suppressed by citrate, which is favoured as a substrate over glucose (Kominek, 1972). Glycerol and starch were reported to repress the synthesis of cephamycin C in *Streptomyces clavuligerus* (Lebrihi et al, 1988). It has been suggested that the molecular mechanisms for carbon catabolite regulation may be related to growth rate control of antibiotic synthesis (Bu'lock, 1974), such that the slow feeding of the interfering substance alleviates repression and may even stimulate the production of secondary metabolites. For example, slow feeding glucose to the *Streptomyces griseus* fermentation led to a stimulation of the production of the polyene macrolide antibiotics candidin and candihexin (Martin & McDaniel, 1974).

In some antibiotic cultures, glucose has been shown to repress a key enzyme in the biosynthesis of the antibiotic. Thus, in actinomycin production glucose represses phenoxyazinone synthase which is involved in the formation of the phenoxyazinone ring of this antibiotic (Gallo & Katz, 1972). In streptomycin biosynthesis, glucose affects the enzyme mannosidostreptomycinase which converts mannostreptomycin into the more active streptomycin (Inamine et al, 1969). Lebrihi et al (1988) demonstrated that carbon substrate repression of cephamycin C synthesis was mediated by phosphorylated intermediates of the glycolytic pathway inhibiting the activity
of expandase. Mechanisms other than catabolite repression have been reported as being involved in the interference of antibiotic synthesis by carbon sources. For example, the repressive action of glucose in the bacitracin fermentation is thought to be due to the accumulation of organic acids in the medium which cause a decrease in the pH (Haavik 1974a; Haavik 1974b).

Carbon catabolite repression of inducible catabolic enzymes in *E. coli* involves cyclic 3,5-monophosphate (cAMP) as a positive effector. It has been reported that cAMP relieved glucose repression of kanamycin production (Satoh et al, 1976), and that high tylosin producing mutants of *Streptomyces fradiae* possessed higher cAMP levels than the wild-type strain (Colombo et al, 1982). This evidence for cAMP as an effector of carbon catabolite repression in actinomycetes is largely circumstantial, and it may be that these effects are more closely linked to phosphate regulation than to carbon regulation (Martin & Demain, 1980). Mutants of streptomycetes deficient in glucokinase have been shown to be less sensitive to carbon source repression (Seno & Chater, 1983), and this has led Demain (1986) to propose a role for glucokinase in carbon regulation of antibiotic synthesis in actinomycetes.

### 1.2.2. Nitrogen Metabolite Regulation

It is now well known that some form of 'ammonium repression' of antibiotic production occurs in many fermentations where ammonium is supplied in excess. This effect is seen in the production of leucomycin (Tanaka et al, 1981), tylosin (Tanaka et al, 1986), cephalexin (Castro et al, 1985) and in erythromycin (Flores & Sanchez, 1985). It is not yet clear exactly how ammonium affects this repression. In tylosin production it appears to interfere with the synthesis of precursors by repressing the activity of two key enzymes, valine dehydrogenase (Omura & Tanaka, 1985) and threonine dehydratase (Lee & Lee, 1991). In *Streptomyces lactamdurans,*
ammonium appears to regulate the biosynthetic pathway of cephamycin in a co-ordinated manner by affecting the activity of the pathway at a number of sites (Castro et al, 1985).

Some authors have implicated a role for glutamine synthetase (GS) and alanine dehydrogenase (ADH) in ammonium repression (Aharonowitz, 1980; Martin & Demain, 1980). For instance, it has been observed that the highest specific production rates of cephalosporin were obtained under those growth conditions that led to high GS activity (Aharonowitz, 1979). Recently however, a number of studies have shown that the links may not be as close as previous experiments have suggested. Bascaran et al (1989) isolated auxotrophic mutants (GS-, GOGAT-, and ADH-) of Streptomyces clavuligerus which were still sensitive to nitrogen regulation of cephalosporin biosynthesis. A similar result has been demonstrated recently for erythromycin biosynthesis, where mutants of Saccharopolyspora erythraea were isolated that were insensitive to ammonium repression. When the GS and ADH levels of the mutants were compared with those of the wild-type it was found that there was no difference (Flores 1991). It thus seems likely that the regulatory region of this system that responds to the levels of nitrogen has not yet been discovered.

1.2.3 Phosphate regulation and the Stringent Response

High phosphate levels have been shown to inhibit the production of many antibiotics and antibiotic classes, for example: candicidin (Martin & Demain 1976); vancomycin (Metz & Doolin 1973); streptomycin (Miller & Walker 1970); and neomycin (Majumdar & Majumdar 1972). Consequently, the number of mechanisms proposed to explain this phenomenon is large (reviewed by Demain 1986). A few of the proposed mechanisms are discussed in more detail below.

In candicidin production, adenosine triphosphate (ATP) has been postulated as an effector in phosphate repression. It was found that the
addition of 10mM phosphate to antibiotic producing cells of *Streptomyces griseus* caused a rapid doubling of the intracellular ATP level just prior to the inhibition of antibiotic synthesis. There was only a small increase in the energy charge (0.80 to 0.85) observed during this repression, thus leading the authors to propose a role for ATP itself rather than for the adenylate energy charge in the cell (Martin & Demain 1976).

Many microbial phosphatases, which cleave phosphorylated intermediates, are often regulated via feedback inhibition or repression by inorganic phosphate. These enzymes are required in the biosynthetic pathways of some antibiotics. For instance, streptomycin biosynthesis involves at least three phosphate-cleaving steps in the formation of the streptidine moiety, and it is thought that high inorganic phosphate levels interfere with these steps leading to the observed repression (Miller & Walker 1970).

In *E.coli*, depletion of amino acids leads to the stringent response (reviewed Cashel & Rudd 1987), which is characterised by an immediate reduction in the overall rate of RNA synthesis apparently mediated by guanosine 5'-diphosphate 3'-diphosphate (ppGpp). During the stringent response complex changes in the pattern of gene expression occur; transcription of stable RNA genes is reduced with the simultaneous increase in the expression of other biosynthetic genes (e.g. amino acid biosynthetic genes; Shand *et al.*, 1989). There is also evidence that ppGpp plays a central role in growth rate control (Sarubbi *et al.* 1988). Several mutations can reduce ppGpp formation and result in the so-called 'relaxed' phenotype, where RNA synthesis continues during amino acid starvation (Strauch *et al.* 1991).

The stringent response and ppGpp formation has been observed for various *Streptomyces* species (Riesenberg *et al.* 1984; Ochi 1986; Ochi 1987; Ochi 1988). This latter author reported that antibiotic production, which began after nutritional shift-down, was preceded by increased levels of
ppGpp. Previous work presented conflicting accounts: a correlation was found in *Streptomyces aureofaciens* (Simuth et al. 1979), but not in *Streptomyces griseus* (An & Vining 1978). Ochi (1990) was also able to isolate relaxed mutants, analogous to the *relC* mutants found with *E. coli*, that produced decreased amounts of ppGpp under nutritional shift-down, and also produced less antibiotic. This naturally led many researchers to propose a role for the stringent response in secondary metabolism and for ppGpp as an effector in switching on antibiotic synthesis and indirectly in promoting morphological differentiation. Recently however work in *Streptomyces clavuligerus* has shown that ppGpp levels remained relatively stable prior to and during cephalosporin production (Bascaran et al. 1991). This study also showed that mutants with a reduced ppGpp-forming ability gave normal yields of antibiotic. The authors concluded that there is no obligatory relationship between initiation of secondary metabolism and the stringent response.

The interactions between the organism and its environment, and between primary and secondary metabolism, are extremely complex with many intricate and interdependent mechanisms. It is clear from the few reports regarding the regulation of antibiotic synthesis cited above, that the underlying mechanisms governing the control of secondary metabolism have still not been unravelled. The filamentous nature of the streptomycetes in particular provides a degree of culture heterogeneity far in excess of that witnessed for any of the well studied unicellular organisms. The processes of primary and secondary metabolism may well be occurring within the same culture at the same time, confusing any attempt to pinpoint the timing of events such as the initiation of antibiotic production. The heterogeneity of filamentous cultures will be examined in more detail in a later section, and it will remain a central theme throughout this report.
1.3 Mechanisms of hyphal elongation and growth

1.3.1 Hyphal elongation

On solid media, the kinetics of growth and branching in the vegetative hyphae of actinomycetes are similar to those of the filamentous fungi (Allen & Prosser 1983), where individual hyphae extend at a constant linear rate while total mycelial length increases exponentially. An exponential increase in the biomass is achieved by exponentially increasing the number of growing tips through branching. The mechanism by which individual hyphae extend has been the subject of a number of studies and was recently reviewed in detail by Prosser & Tough (1991).

The first studies on the mechanisms of growth in actinomycetes were based on micromorphological observations of developing mycelia. Gottlieb (1953) proposed that the extension of streptomycete hyphae occurred by tip growth which was supported by subapical regions which were static but viable. A similar technique was used by Schumann & Bergter (1976), who suggested that cytoplasmic synthesis and nuclear division were restricted to a 20µm region at the tip of the hypha. They also observed that branches were formed 80 to 100µm from the tip with no intercalary growth of the main hypha. Locci & Schaal (1980) used immunofluorescence to study apical growth, and found uniform labelling of growing mycelia. Subsequent growth of these mycelia in the absence of label showed that unlabelled regions were located at the tips of hyphae and at the poles of fragmented cells, indicating apical growth.

These early observations have since been followed up by a number of radiolabelling studies where hyphae were incubated in the presence of a tritiated precursor of cell wall peptidoglycan synthesis, N-acetyl-D-glucosamine (GlcNAc). In pulse labelling studies on Streptomyces antibioticus, it was found that incorporation of label occurred at the tips of
main and branch hyphae. Following a 1 minute incubation, 32% of the labelled GlcNAc was incorporated into the apical 1µm and 44% in the apical 2µm. In pulse-chase experiments, label was located sub-apically but not at the tip (Brana et al 1982a; Hardisson et al 1984). This group also carried out further studies to establish the source of label (almost 70%) which was located in regions beyond the apical 1µm (Miguelez et al 1988). They rejected metabolism, turnover and reincorporation of the label as possible sources, and suggested instead a multizone model where extension occurred in discrete zones linked to the cell growth cycle. Incorporation of new cell wall material therefore occurred in a series of zones behind the hyphal apex separated by non-growing regions.

Gray et al (1990) used similar radiolabelling techniques but achieved greater resolution using an electron microscope to study the autoradiographs obtained when Streptomyces coelicolor mycelia were incubated with tritiated GlcNAc. They found that following a 1 minute pulse labelling, 57%, 75%, and 87% of the label were incorporated into the apical 0.5µm, 1µm, and 1.5µm respectively. These workers also examined the effects of β-lactams on hyphal morphology, which affect the final stages of cell wall synthesis i.e. the rigidification process. They observed swelling only at the tips of hyphae rather than the beaded appearance required by the multizone model, and concluded that streptomycete hypha elongate solely by extension at the tip.

More recently, Miguelez et al (1993) reported that Streptomyces antibioticus was capable of growing on a minimal medium with GlcNAc as the only source of carbon, indicating that the cell wall precursor can be degraded and thus produce non-specific labelling. They found, using lysozyme digestion, that 86.5% of the label was incorporated into cell wall peptidoglycan, and that the majority (80%) of the lysozyme-resistant fraction was probably degraded and incorporated into protein. They also reaffirmed the presence of labelled cell wall material in regions up to 10µm from the tip,
which reinforced their earlier ideas that cell wall synthesis was not restricted to the tip but occurs at other sites distributed along the hypha. This explanation is unsatisfactory in the light of the earlier β-lactam studies of Gray et al (1990), and it seems more likely that the presence of label in regions of the cell wall removed from the tip may be due to the incorporation of the label into wall polymers other than peptidoglycan (e.g. in complexes with teichoic acids). Thus, in summary, it seems likely that hyphal elongation occurs solely at the tip of actinomycete hyphae, and that the presence of label in regions removed from the tip may be due to the addition of secondary wall polymers.

1.3.2 Macromolecular distribution

The macromolecular composition of individual hyphae cannot be determined directly, due to their small size, but have been determined by a combination of microautoradiographical, electron microscopic and histological studies. Electron microscopic studies indicate that the DNA exists as a fibrillar matrix extending continuously along the central longitudinal axis of the hypha which links continuously with the DNA in branch hyphae (Hopwood & Glauert, 1960; Gray, 1987). Discrete nuclear bodies, referred to as nucleoids, are often seen in stained preparations and are the result of condensation of the nuclear material (Prosser & Tough, 1991). It is thought that nucleoids of the smallest size may represent the condensation of discrete genomes. The distribution and size of these nucleoids has provided the basis for much of the work characterising the duplication cycle in actinomycetes.

The distribution of DNA in hyphae and the sites of active production of this macromolecule have been examined using Giemsa and other DNA stains. In young vegetative hyphae of Streptomyces hygroscopicus, Schumann & Bergter (1976) found a single nucleus was associated with a hyphal length of 1.4 to 1.6μm when grown in a complex medium, and 1.7 to
1.9 µm in minimal medium. Gray (1987) found a similar nucleoid ratio of 1.9µm in *Streptomyces coelicolor*, with no nucleoids present in the apical 2 to 5µm corresponding to the site of active cell wall synthesis. Nucleoids varied in size and shape, with dumbbell shapes thought to represent dividing nucleoids, and large aggregates arising from the suppression of nuclear segregation following replication. Similar variation in nucleoid shape has been reported by Kretschmer & Kummer (1987) in *Streptomyces granaticolor*, where nucleoid size increased with distance from the tip. Kretschmer (1987) found smaller nucleoids in the apical 35µm of fast growing hyphae, but these were restricted to the apical 15µm in slower growing hyphae indicating that the length in which DNA segregation occurs varies with specific growth rate.

It was also found that larger nucleoid aggregates were associated with the sites of branch formation, with evidence that segregation occurred at these points and the smaller nucleoids migrated into the newly formed branch.

The sites of nuclear synthesis have been studied using microautoradiography following pulse labelling with tritiated thymidine. Kummer & Kretschmer (1986) reported uniform labelling of hyphae up to 80µm from the hyphal tip, and concluded that DNA replication was not restricted to the apices or branches of actinomycete hyphae. They also calculated, using labelling pulses of varying length, that nucleoid segregation only occurred in the apical 30µm. The mycelia used in these studies however had been cultured for 12 hours on a medium supplemented with 125µg/ml of uridine, cytidine, adenosine, and guanosine. Supplementing the culture in this way with nucleosides may well have artificially elevated the regions of active nuclear synthesis. The studies by Gray (1987) are in accord with this idea as they found that the apical 20 to 30µm of *Streptomyces coelicolor* hyphae possessed a high rate of DNA replication which reduced in subapical and older hyphae. RNA synthesis, studied in a similar way by radiolabelled
incorporation of tritiated uridine, was uniform throughout the hyphae of young mycelia up to about 100\(\mu\text{m}\) from the tip.

In summary, it appears that actinomycete hyphae elongate solely by growth at the apex. Nuclear material is distributed uniformly throughout individual hyphae except in the apical 2 to 5\(\mu\text{m}\), where active cell wall synthesis is occurring. Nuclear replication is greatest 15 to 25\(\mu\text{m}\) behind this region. In regions further removed from the tip, DNA synthesis occurs at a reduced rate and larger nuclear aggregates are formed. Branches form only at the sites of these larger aggregates, and branch initiation is followed by segregation of nucleoids which migrate into the branch hypha (Prosser & Tough, 1991).

Riesenberg & Bergter (1979) studied the relationship between macromolecular composition and specific growth rate in *Streptomyces hygroscopicus*. They found that the specific DNA content of the biomass remained constant with changes in \(\mu\), specific protein content was also nearly independent of \(\mu\), but the specific RNA content increased considerably with increasing \(\mu\). They concluded that the mycelium of *Streptomyces hygroscopicus* consisted of hyphal regions with different synthetic activity with respect to DNA, RNA, and protein. In mycelial regions with high synthetic activity (i.e. the tips), the macromolecular composition was found to be quantitatively the same as that found in *E.coli* and *Aerobacter aerogenes* (two unicellular prokaryotes) which led them to propose that macromolecular synthesis in these regions is regulated in a similar fashion to unicellular bacteria. In older mycelial regions (i.e. further from the hyphal tip) the %DNA, %RNA, and %protein were found to be lower than in unicellular bacteria, leading to a considerable heterogeneity in macromolecular composition along the hyphal length. This was explained by decay of DNA, RNA, and protein synthesis and/or the synthesis of other substances such as cell wall constituents, storage products, etc. occurring in these regions to an
increased extent. They also concluded that the proportion of mycelial regions with high macromolecular synthetic activity rises with increasing specific growth rate.

1.3.3 The relationship between growth rate and morphology

In order to study the relationship between the specific growth rate of a filamentous microorganism and its morphology, it is first necessary to establish a number of parameters that provide some sort of qualitative and quantitative description of the morphological state of the mycelium. These parameters, referred to by Metz et al (1981) as 'morphology indices', were originally established to characterise the morphology of fungi. The most frequently used morphology index is the length of the hyphal growth unit (G or L/N), which is defined as the length of a particular mycelium divided by the number of tips it possesses (Caldwell & Trinci, 1973). Other parameters that are often used in the quantitative analysis of filamentous organisms include the length of the main hypha, the total hyphal length, the interbranch distance, the hyphal growth unit volume (Gv), hyphal radius and diameter, and many more. In mycelial organisms, an increase in μ must be linked to an increase in the apical growth rate (α), the mean branching rate (β), or the hyphal diameter. In organisms where the hyphal diameter remains constant the morphology of the mycelia will thus be determined by α and β, quantified by G (Riesenberg & Bergter, 1979).

There have been a number of studies on the relationship between growth rate and morphology in filamentous organisms, primarily in fungi. Thus, Trinci (1973) found that G in Neurospora crassa spco 1 was independent of μ. In Aspergillus nidulans (Katz et al, 1972) and Penicillium chrysogenum (Morrison & Righelato, 1974) G was found to decrease with increasing μ. In fast growing cultures of Streptomyces hygroscopicus Schumann & Bergter (1976) found that G increased with increasing μ. These studies were all performed on solid media where there are negligible shear
forces. In submerged culture there will be considerable shear due to the stirring action of the impeller which may cause fragmentation of the hyphae affecting morphological parameters such as main hyphal length, number of branches and thus the value of $G$.

The effects of cultivation conditions in submerged culture have been examined by Metz et al (1981) and vanSuijdam & Metz (1981), who found that the length of the main hypha decreased with increasing stirrer speed during continuous cultivation of *Penicillium chrysogenum*. They also examined the change in main hyphal length during batch culture and found an increase in the early stages due to growth, this was followed by a period where the main hyphal length remained relatively constant, and a subsequent decrease was observed which the authors concluded must be linked to breakage of hyphae. This conclusion may not be strictly correct however, as it must be remembered that the process of breakage is constantly occurring in the vessel, although not necessarily at the same rate, which is counteracted by growth and branching. Thus a dynamic equilibrium exists where an observed increase in main hyphal length means that growth is occurring more rapidly than the process of breakage. A decrease in main hyphal length might be due to an increase in fragmentation, but it is just as likely to occur when the growth rate slows down. This latter explanation seems more likely as the culture was in the process of switching to a more slowly assimilated carbon source (lactose) which would result in the required drop in growth rate. The results presented in this paper suffer from a problem that the majority of quantitative morphological studies share, and this is a large degree in variation of the measured morphological parameters. A variation of ±50% in the calculated mean for main hyphal length was frequently observed, in data where the means themselves varied by about 20% (i.e. a straight line parallel to the x-axis could be joined through all the error bars indicating with considerable confidence that there was no variation.
The reasons for this are linked to the fact that only small sample sizes can be analysed in detail, as even with automated image analysis, sample sizes in excess of a hundred require a large amount of time for data processing. In a population of $10^8$ or greater mycelial fragments per ml of culture (e.g. *Saccharopolyspora erythraea* culture at 5g/l; unpublished observation) a sample of a few hundred fragments represents a very small fraction. This is one of the reasons why this project used filtration techniques, as well as direct micromorphological characterisation, to yield information about the morphological status of the culture.

In continuous culture, where there is prolonged growth, new centres of growth must be created if washout is to be prevented. This presents no problem for unicellular organisms as it is achieved by cell separation following binary fission. In filamentous organisms it requires breakage or fragmentation of mycelia which is normally achieved through the action of the stirrer. The requirement for hyphal breakage and reseeding makes the establishment of steady states for filamentous cultures more difficult. Despite this, continuous culture has been used to study the physiology of growth and morphology in both fungi and actinomycetes.

Riesenberg and Bergter (1979) studied the effect of changing growth rate on the observed morphology in glucose-limited cultures of *Streptomyces hygroscopicus*. They reported that as specific growth rate increased from 0.05 to 0.32 hr$^{-1}$, $G$ decreased along with the mean interbranch distance and the distance from the hyphal tip to the first branch, but increased as the $\mu$ increased further from 0.32 to 0.42hr$^{-1}$. Kretschmer (1985) reported a more complex relationship when the culture was nitrogen-limited by casamino acids. Increases in $\mu$ led to an increase in $G$ for *Streptomyces hygroscopicus* and *Streptomyces granaticolor*, and interseptal distance was less than for glucose-limited cultures. In the latter organism, $\alpha$ and $\beta$ were shown to increase with increasing $\mu$ under nitrogen-limitation, but the reverse was
found in glucose-limited cultures. It was concluded that morphology and branching patterns of streptomycete mycelia therefore depend upon specific growth rate, but also upon the nature of the limiting substrate and its effect on the particular species being studied. The work published by both Riesenberg & Bergter and by Kretschmer was based on a similar peculiar methodology for cultivation of the streptomycetes in a chemostat. The working volume of their vessels was 192ml and 'about 200ml', respectively. It is difficult to see how a reproducible steady state could be achieved in such a small vessel, particularly with an aeration rate of between 1.3 and 1.5 vvm.

More recently, Tarbuck et al (1985) reported that batch fermentations of *Streptomyces clavuligerus* showed a dramatic decrease in clavulanic acid production when the stirrer speed was increased from 375 to 500rpm, although the link with morphology was not discussed. Belmar-Beiny & Thomas (1991) also examined the effect of stirrer speed on clavulanic acid production. They found that although the stirrer speed altered the morphology, they detected no increase in clavulanic acid titre and concluded that there was no direct link between morphology and productivity. The results they presented in this paper however did contain evidence that morphology and productivity are linked. The data from their paper is re-evaluated in chapter 9, which deals with the effect of stirrer speed on the morphology and production of erythromycin in continuous culture.
1.4 Kinetics of growth in submerged culture

"The study of the growth of bacterial cultures does not constitute a specialised subject or branch of research; it is the basic method of microbiology."

(Monod, 1949)

If we consider a unit volume of a growing culture, the rate of increase in cell numbers \( \frac{dN}{dt} \) is a function of the total number of cells already present \( N \) such that;

\[
\frac{dN}{dt} = \mu N
\]

(1)

where \( \mu \) is a constant and referred to as the specific growth rate. Rearranging equation (1) yields;

\[
\mu = \frac{1}{N} \cdot \frac{dN}{dt}
\]

(2)

which upon integration becomes;

\[
\mu t = \ln N_t - \ln N_0
\]

(3)

this equation is typically rearranged to give the exponential growth equation;

\[
N_t = N_0 \cdot e^{\mu t}
\]

(4)

Since the biomass of the cells in the population is easier to measure than concentration of the cells, then the number of cells \( N \) can be replaced by the concentration of biomass \( x \). Thus equation (2) becomes

\[
\mu = \frac{dx}{dt} \cdot \frac{1}{x}
\]

(5)

and equation (3) becomes

\[
\ln x_t = \ln x_0 + \mu t
\]

(6)

It can be seen that a plot of \( \ln x \) against \( t \) will yield a straight line during non-limited exponential growth, where the slope equals the specific growth rate. The relationship between \( \mu \) and the doubling time \( t_d \) of the culture is as follows;

\[
\mu = \frac{\ln 2}{t_d} = \frac{0.693}{t_d}
\]

(7)
1.4.1 Batch culture

The original studies on bacterial growth kinetics (example used here from Monod, 1949) reported that the typical batch fermentation consisted of a number of phases following the inoculation of a small population of bacteria into a suitable growth medium:

1. **Lag Phase.** When a batch of fresh medium is inoculated with cells taken from a culture that has been grown into the stationary phase there is a significant period of biochemical readjustment before the cells resume growth. If the cells are taken from a culture in the exponential phase, this period may be comparatively short or non-existent. The length of this lag phase can be influenced by many factors which impede the process of adaptation or of growth, or both. For example, the lag period is usually prolonged when organisms are transferred from a nutritionally rich environment to a relatively poor one. This is because biosynthetic pathways that were repressed in the rich medium have to be regenerated before growth can resume. During this period the specific growth rate is zero.

2. **Increasing Exponential Phase.** The culture starts to grow at an increasing rate.

3. **Constant Exponential Phase.** The specific growth rate is not an inherent and invariant property of the organism, but it is influenced markedly by the biochemical and physical conditions in which the process of growth takes place. In general, heterotrophic bacteria grow faster in media containing a mixture of complex organic nutrients (e.g. amino acids, purines, pyrimidines and vitamins) than they do in simple salts media. Further, with these simple, defined salts media some carbon substrates promote faster growth than others. Glucose particularly causes faster growth rates in many bacteria. The use of nitrate as a sole source of nitrogen, whose assimilation requires extra energetic demands upon the organisms, generally results in a lower growth rate than in the presence of ammonia, which requires less
energy input to facilitate assimilation. Many other factors affect the growth rate such as temperature and oxygen tension (Pritchard & Tempest, 1982). Thus, in this phase often just called the 'exponential phase', the growth rate of the organism reaches a maximum ($\mu_{\text{max}}$) governed by the maximum velocity of the various reactions involved in the synthesis of new biomass. The growth rate remains at this level until the concentration of some limiting factor approaches a critical level. The factors most commonly found to be limiting can be classified into three groups: (a) exhaustion of nutrients; (b) accumulation of toxic metabolic products; (c) changes in ion equilibrium, especially pH.

4. Decreasing Exponential Phase. As the limiting substrate is further consumed the growth rate slows down until, following exhaustion of the substrate, the growth rate reaches zero. It is during this phase that many secondary metabolites are produced (see later). For organisms growing on defined salts media the transition from exponential phase to stationary phase is abrupt. In complex media this phase can be prolonged as various other ancillary carbon substrates (especially amino acids) are sequentially utilised giving rise to slower and slower growth rates.

5. Stationary Phase. The growth rate is zero and thus the biomass concentration remains constant. This does not imply however that biosynthetic activity has ceased, as often this phase is accompanied by extensive changes in physiology particularly in spore-forming bacteria, fungi and actinomycetes. Some of the changes that occur in Saccharopolyspora erythraea during the stationary phase of growth are examined in Chapter 3.

6. Decline Phase. Without an adequate supply of all nutrients the culture starts to die, therefore the growth rate is negative. This is particularly the case when the source of carbon has been exhausted, where the onset of lysis is rapid resulting in a short stationary phase.
This is a very generalised view of bacterial growth, and any of these phases may be absent. Under suitable conditions, the lag and acceleration phases may often be suppressed, and the retardation and stationary phases are frequently so short as to be imperceptible (Monod, 1949).

Monod showed that the specific growth rate of an organism could be related to the concentration of a single limiting nutrient using an equation similar to the Michaelis equation describing the rate of an enzyme reaction in relation to the concentration of its substrate \( s \), such that:

\[
\mu = \mu_{\text{max}} \left( \frac{s}{K_s + s} \right)
\]

where \( K_s \) is a saturation constant related to the dissociation constant of the enzyme involved in the first step in the assimilation of the limiting substrate, it is numerically equal to the concentration of substrate required to allow growth to proceed at one-half its maximum rate.

1.4.2 Chemostat culture

If a constant stream of nutrients is fed to a batch culture, and the volume is kept constant by means of an overflow device, the resulting system is termed a chemostat. In this system the culture remains growing at an exponential but submaximal rate determined by the rate of addition of new substrate. More precisely, the growth rate is equal to the dilution rate \( D \) which is equal to the flow rate of fresh medium divided by the volume \( V \);

\[
D = \frac{F}{V}
\]

The changes in biomass concentration depend upon the relationship between the growth and the washout. Thus;

\[
\frac{dx}{dt} = \mu \cdot x - D x
\]

Substituting for \( \mu \) from equation (8) yields;

\[
\frac{dx}{dt} = x \left[ \mu_{\text{max}} \left( \frac{s}{K_s + s} \right) - D \right]
\]
When a culture is in steady state \( \frac{dx}{dt} = 0 \) and a unique value exists for the biomass concentration. This is referred to as the steady-state concentration and identified by the symbol \( \bar{x} \). The value of \( \bar{x} \) is dependent upon the concentration of the growth-limiting substrate in the feed \( (S_r) \) such that

\[
\bar{x} = Y(S_r - \bar{s})
\]

where \( Y \) is called the yield value expressed as grams of organism formed per unit (moles or grams) substrate. The value \( \bar{s} \) is called the residual substrate concentration and is the concentration of growth-limiting substrate in the vessel at steady state. This is usually very low and notoriously difficult to measure experimentally, but can be calculated from the expression

\[
\bar{s} = K_s \left( \frac{D}{\mu_{max} - D} \right)
\]

substituting equation (13) into equation (12) we get an expression that describes the steady state biomass concentration achieved (theoretically) with a particular substrate concentration;

\[
\bar{x} = Y \left[ S_r - K_s \left( \frac{D}{\mu_{max} - D} \right) \right]
\]

These kinetics have been used to accurately model the growth of many bacterial cultures, but it should be remembered that they have been derived largely empirically and consider the biomass to be homogeneous. Deviations from predicted kinetics are frequently observed at very low and very high growth rates which may be due to a number of factors particularly deviations in the composition of biomass leading to variation in the yield \( Y \) (Stouthamer, 1984).

### 1.4.3 Antibiotic productivity

In batch culture, the specific rate of antibiotic production \( (q_p) \) is calculated from the following equation (Pirt, 1975);

\[
q_p = \frac{dp}{dt} \cdot \frac{1}{x}
\]
where $dp/dt$ is the volumetric rate of production of the product. In our laboratory this is calculated as the slope of the curve obtained when the concentration of antibiotic ($p$) is plotted against $t$ using a partial cubic spline analysis. This method is discussed in more detail in chapter 2.5.

In continuous culture, the specific antibiotic production rate is related to the steady state antibiotic concentration ($\bar{p}$), the steady state biomass concentration, and to the dilution rate by the following equation:

$$q_p = \frac{\bar{p}.D}{\bar{\chi}}$$  \hspace{1cm} (16)
1.5 The relationship between morphology and production

Previous work in this group examined the source of the variability in activity observed when primary screening isolates giving active results on solid media failed to reproduce this in liquid media (Pickup et al, 1993). This lack of reproducibility was suspected to be linked with morphology in some way as it was often noted that these non-producers possessed a fragmented unicellular form in submerged culture (see figure 1).

Figure 1. These scanning electron micrographs, of the same streptomycete, illustrate the importance of morphology on the ability of the organism to produce secondary metabolic products. The micrograph on the left shows the highly fragmented (mean hyphal length 12.5µm), nocardia-like morphology which was associated with a loss in antibiotic productivity. The micrograph on the right shows the more usual morphology, these longer hyphal fragments were capable of antibiotic production (from Pickup et al, 1993).

In both fungi and actinomycetes it has long been known that prolonged continuous culture gives rise to morphological variants that gradually replace the parent strain. Generally the parent possesses a relatively sparsely branched form, whereas the variant (referred to as a colonial variant) possesses a more highly branched form. This is a particular problem in the case of Fusarium graminearum A 3/5, which is cultivated continuously for the
production of Quorn mycoprotein, where the appearance of colonial variants necessitates premature process termination, resulting in considerable economic losses. One of these colonial variants (C 106) has been characterised in detail. The values of $\mu_{\text{max}}$ in various growth systems did not differ markedly, but differences in the length of the hyphal growth unit were considerable; 223$\mu$m and 83 $\mu$m for the parent and colonial variant, respectively. Thus it appears that the mutation alters the spatial distribution of the biomass and not its rate of production (Trinci et al 1990).

When *Penicillium chrysogenum* was continuously cultivated under sucrose-limitation the appearance of a colonial variant led to a decrease in penicillin titre (Righelato 1976). Interestingly, the penicillin titre was maintained under nitrogen-, phosphate-, and sulphate-limitation and the colonial variant did not appear, suggesting that the type of nutrient limitation has an effect on the selection of these colonial variants. The lack of stability of antibiotic production during continuous cultivation has been demonstrated for a number of actinomycetes and fungi (table 4). The prolonged cultivation in continuous culture does not, however, always lead to a loss of culture productivity for all organisms.

**Table 4. Stability of antibiotic production by filamentous fungi and streptomycetes in continuous culture (from Trinci et al 1990)**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Antibiotic produced</th>
<th>Reduction in antibiotic production with prolonged cultivation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Streptomyces niveus</em></td>
<td>novobiocin</td>
<td>yes</td>
</tr>
<tr>
<td><em>Streptomyces aureofaciens</em></td>
<td>chlortetracycline</td>
<td>yes</td>
</tr>
<tr>
<td><em>Penicillium chrysogenum</em></td>
<td>penicillin</td>
<td>yes</td>
</tr>
<tr>
<td><em>Streptomyces griseus</em></td>
<td>streptomycin</td>
<td>no</td>
</tr>
<tr>
<td><em>Acremonium chrysogenum</em></td>
<td>cephalosporin</td>
<td>no</td>
</tr>
</tbody>
</table>
In any culture of an antibiotic-producing organism there will exist spontaneous mutants that have different morphological and general cultural features. A study of the antibiotic-producing capabilities of a number of classes of these variants in various *Streptomyces spp.* has been published recently and a selection of the data is shown below in table 5.

**Table 5. The antibiotic activity of spontaneous variants of streptomycetes producing different antibiotics (Kuznetzov *et al* 1992)**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Variants</th>
<th>Activity, % of Parent</th>
<th>Antibiotic produced</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. griseus</em></td>
<td>O</td>
<td>65.6</td>
<td>streptomycin</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>25.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><em>S. kanamyceticus</em></td>
<td>O</td>
<td>84.1</td>
<td>kanamycin</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>16.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><em>S. fradiae</em></td>
<td>O</td>
<td>81.1</td>
<td>neomycin</td>
</tr>
<tr>
<td></td>
<td>W</td>
<td>34.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><em>S. parvulus</em></td>
<td>O</td>
<td>83.6</td>
<td>actinomycin</td>
</tr>
<tr>
<td></td>
<td>W</td>
<td>80.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><em>S. antibioticus</em></td>
<td>O</td>
<td>68.7</td>
<td>oleandomycin</td>
</tr>
<tr>
<td></td>
<td>W</td>
<td>59.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><em>S. warraensis</em></td>
<td>O</td>
<td>89.0</td>
<td>nonactin</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>56.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>


The authors grouped these spontaneous variants into a number of classes based on their colonial appearance; *Oligosporic* - aerial mycelium was scant, *Asporogenous* - No aerial mycelium, *White* - aerial mycelium is white, and *Nocardia-like* - No aerial mycelium, colonies plicate, soft with the substrate mycelium separated into rod-like or coccoid elements. It can be seen that morphological changes were almost always accompanied by a reduction in antibiotic activity. Of more interest is the observation that the nocardia-like variant invariably produced no antibiotic at all, indicating that the filamentous form itself may be linked in some way to the production of these antibiotics. This would certainly be in agreement with the observations
made earlier about loss of activity in screening programs. It is the investigation of the link between growth, morphology and antibiotic production that forms the main aim of this thesis.

**1.6 Erythromycin production in *Saccharopolyspora erythraea***

**1.6.1 Introduction**

The first strains of this organism were isolated from soil in the Philippines and classified in the genus *Streptomyces*. Subsequent chemotaxonomic studies on the type strain of *Streptomyces erythraeus* (NRRL 2338) revealed that this strain was not a representative of the genus because its cell walls contained meso-diaminopimelic acid, arabinose, and galactose (Streptomycete cell walls characteristically contain *LL*-diaminopimelic acid). Thus the type strain was reclassified as *Saccharopolyspora erythraea* (Labeleda 1987). The specific epithet *erythraea* originates from the Greek 'erythros' meaning red, a reference perhaps to the appearance of the substrate mycelium and a diffusible pigment produced under certain cultivation conditions. The genus name *Saccharopolyspora* derived from 'saccharo-', is a reference to the fact that the majority of the strains in this genus were isolated from sugar-cane bagasse.

The importance of this organism lies in the fact that it produces a single class of clinically useful non-polyene macrolide antibiotics called the erythromycins. Erythromycin A was first isolated from *Saccharopolyspora erythraea* cultures by McGuire *et al* (1952). This antibiotic is now one of the most widely prescribed due to its very low toxicity and its particular success in treating group A streptococcal, staphylococcal, and pneumococcal infections (Oleinick 1975). The erythromycins are a group of structurally related compounds described in more detail in the next section.
1.6.2 Structure and mode of action

A common feature of all erythromycins is the highly substituted macrocyclic ring containing 1 oxygen and 13 carbon atoms, glycosidically linked with a neutral and an amino-deoxy sugar (desosamine and cladinose). The structure of this 14-membered ring (erythronilide) is shown in figure 2. Erythromycin works by inhibiting protein synthesis in susceptible organisms. More specifically, it inhibits protein synthesis by binding to the 50s subunit of bacterial ribosomes. The site of action is located on protein L4; the so-called 'streptomycin region'. The binding of the antibiotic to the protein causes conformational changes in the 50s subunit which affects the peptidyl transferase centre. Thus erythromycin does not directly inhibit peptide-bond formation, rather it is an effector acting on the donor site of peptidyl transferase thereby altering the velocity of the enzyme reaction. This in turn affects the rate of protein synthesis.

Figure 2: The Structure of Erythromycin A

The intact 14-membered lactone ring can be isolated from blocked mutants or by chemical hydrolysis of the parent antibiotic. The lactone ring appears to possess no antibacterial activity. Thus both the sugars and the lactone ring
are necessary for the antibiotic to be active, although different sugar moieties linked to the lactone produce a different spectrum of activity (Oleinick 1975). Several modifications to the erythromycin molecule have been achieved using blocked mutants or biotransformation, some of which have been patented as drugs. For example, Martin et al (1975) produced erythromycin E, a new antibacterial antibiotic, by a biotransformation of erythromycin A using Saccharopolyspora erythraea. Erythronilide B has been used as a hypocholesterolemic agent, and mycarosylhydroxy erythronilide B has been used as an antipyretic agent.

Saccharopolyspora erythraea is highly resistant to erythromycin and also to other members of the 'MLS' (macrolide, lincosamide, and streptogramin B) group of antibiotics (Graham and Weisblum, 1979). Resistance results from the dimethylation of a single adenine residue in the organism's 23s rRNA (Skinner & Cundliffe, 1982). The genes coding for this resistance factor (erm E) are located within a 35kb fragment of the genome which also contains the genes responsible for the synthesis of the antibiotic.

1.6.3 Synthesis of erythromycin

About 30 enzymatic steps are required for the biosynthesis of erythromycin A. The first 13 are carried out by a single enzyme complex, erythronilide synthetase, which is involved in biosynthesis of the lactone ring (Martin & Liras, 1989). Erythronilide is synthesised exclusively from condensation reactions between activated propionate and 2-methylmalonate precursors, similar to those involved in fatty acid synthesis (Rossi & Corcoran, 1973; Corcoran 1974). Propionate and propanol are more effective precursors than methylmalonate in stimulating erythromycin synthesis. This was interpreted as meaning that the primer 3C unit was of greater significance than the extending units. However, cell permeability to the different precursors and the utilisation of endogenous rather than exogenous precursors could also explain this observation. Propionate is activated by the
formation of Co-enzyme A esters, and it has been suggested that this activation is the rate-limiting step in erythromycin biosynthesis, although it should be noted that there are several ways in which propionyl CoA can be formed other than the ATP-requiring activation of free propionyl (Corcoran, 1974).
Chapter 2. Materials & Methods

2.1 Maintenance of microorganisms and preparation of inoculum
2.2 Defined media composition
2.3 Fermentation conditions
2.4 Sample analysis
2.5 Partial cubic spline analysis
2.1 Maintenance of microorganisms and preparation of inoculum.

There were two strains of *Saccharopolyspora erythraea* used in these studies. The strain primarily used was strain MG10534 obtained from the culture collection of Shell Research, U.K. which was originally supplied as the wild type NRRL 2338. The second strain was CA340 obtained from Abbott Laboratories, Chicago, USA, which had undergone some mutation from the parent strain. The organisms were stored long term, as a spore suspension, at -70°C using the Protect™ system (T.S.C. Ltd). From this working master culture a bead was removed and placed in a 250ml baffled Ehrlenmeyer flask containing 25ml of nutrient broth. The flask was incubated at 30°C on an orbital shaker set at 220rpm. When grown (usually about four days) this broth culture was used to make spread plates on Tomato Puree Oatmeal Agar (10g/l tomato puree, 10g/l oatmeal/baby food, 20g/l agar). The TPA spread plates were incubated at 30°C until grey spores were visible covering the surface of the agar (usually 7 days). These plates were stored at 4°C until required for experiments, up to a maximum of four months. One plate was used for each experiment.

Primary broth cultures were obtained by removing a large loopful of spores from a mature TPA spread plate and inoculating into a 250ml baffled Ehrlenmeyer flask containing 25ml of nutrient broth. The flask was incubated at 30°C on an orbital shaker set at 220rpm. This primary culture took about 48 hours to grow to a well dispersed broth of suitable density whereupon it was used to inoculate the secondary broth culture. 2ml of primary broth culture was used to inoculate 23ml of the secondary stage medium in a 250 ml baffled flask. The medium used in this secondary stage varied but was always the same as that used in the relevant experiment. These secondary
broth cultures were incubated for 48 hours at 30°C on an orbital shaker set at 220rpm. Each flask was then checked microscopically to ensure abundant dispersed growth, and no obvious contamination, prior to its use as an inoculum for an experiment.

2.2 Defined Media

The media used in this report are shown in the tables below. Table 7 shows the media used in the earlier studies in batch and chemostat culture. These gave biomass levels of between 4 and 5 g/l. For the later experiments lower biomass titres were required to make the techniques effective, and the media used to achieve this are shown in table 8. The nitrate and phosphate buffer (basal salts medium) were autoclaved together. The glucose was sterilised separately and added aseptically to the salts post-autoclaving. The trace elements were made up as shown below in Table 6 and filter sterilised. They were added aseptically to the basal salts medium post-autoclaving.

Table 6. Composition of the trace elements concentrate. Typically made up at 100x working concentration (i.e. 10ml added to each litre). 1M HCl was added dropwise to dissolve all the compounds.

<table>
<thead>
<tr>
<th>AnalalR Compound</th>
<th>concentration (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgSO₄.7H₂O</td>
<td>25.00</td>
</tr>
<tr>
<td>FeSO₄.7H₂O</td>
<td>2.50</td>
</tr>
<tr>
<td>CuCl₂</td>
<td>0.053</td>
</tr>
<tr>
<td>CoCl₂</td>
<td>0.055</td>
</tr>
<tr>
<td>CaCl₂.2H₂O</td>
<td>1.38</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>1.04</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>0.62</td>
</tr>
<tr>
<td>Na₂MoO₄</td>
<td>0.03</td>
</tr>
</tbody>
</table>
### Table 7. Media composition used for batch and continuous culture in chapters 3 to 7

<table>
<thead>
<tr>
<th>Analar compound</th>
<th>Carbon-limited (g/l)</th>
<th>Nitrogen-limited (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>15.0</td>
<td>30.0*</td>
</tr>
<tr>
<td>NaNO₃</td>
<td>11.12</td>
<td>3.52</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>7.0</td>
<td>7.0</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>1% FMT 30 (Antifoam)</td>
<td>10ml</td>
<td>10ml</td>
</tr>
<tr>
<td>Trace Elements</td>
<td>10ml</td>
<td>10ml</td>
</tr>
</tbody>
</table>

* In section 3.2.4 the effect of an excess of glucose is assessed, and thus the glucose concentration was increased to 60 g/l.

### Table 8. Media composition used for the selective recycle and later chemostat studies (Chapters 8 & 9). Note that the two media are nitrogen-limited.

<table>
<thead>
<tr>
<th>Analar compound</th>
<th>Recycle Expts (g/l)</th>
<th>Chemostat (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>7.5</td>
<td>15</td>
</tr>
<tr>
<td>NaNO₃</td>
<td>0.7</td>
<td>1.19</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>7.0</td>
<td>7.0</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>1% FMT 30 (Antifoam)**</td>
<td>10ml</td>
<td>10ml</td>
</tr>
<tr>
<td>Trace Elements</td>
<td>10ml</td>
<td>10ml</td>
</tr>
</tbody>
</table>

**Breox FMT 30 Antifoam is available from Water Management & Gamlen, Desalis Court, Hampton Lovitt, Droitwich, Worcester.

### 2.3 Fermentation conditions

#### 2.3.1 Batch Culture

Batch fermentations were performed in a 10l vessel (LH-2000 series instrumentation) with a working volume of 7l (see figure 3). There was no control of pH or DOT although these were monitored throughout the run. The stirrer speed was set to 500rpm and the culture stirred by two Rushton turbine impellors. Air was pumped into the vessel at a constant rate of 0.8vvm. Temperature was maintained at 30°C.
Figure 3. Photograph of the 10 litre LH 2000-series fermenter used in the batch culture studies.
2.3.2 Chemostat Culture

Chemostat culture was performed in a 2l vessel (LH 500 series instrumentation) with a working volume of 1.37l maintained by an overflow weir (see figure 4). The culture was stirred at 1000rpm using two impellers mounted on the stirrer shaft. The lower of the two was a Rushton turbine impeller situated just above the sparge line in order to maximise bubble breakage and dispersion. The top impeller was a low shear vortexing marine impeller, and was situated just below the liquid level in the vessel. The use of this low shear impeller at the top served to decrease splashing (and reduce subsequent wall growth), and also the vortexing action facilitated a more stable liquid level ensuring greater accuracy in maintaining a constant culture volume.

The pH of the culture was kept between 6.5 and 7.5 by the addition of 1M sodium hydroxide or 1M hydrochloric acid although very little acid or alkali was needed due to the buffering action of the feed. The dissolved oxygen tension was monitored but not controlled, and remained above 60% at all times. The flow of air into the vessel was kept at 1vvm. Temperature was maintained at 30°C.

Medium was fed into the vessel from a 20l reservoir using a Minipuls 3 pump (Gilson). The flow rate was checked regularly using an in-line calibrated glass pipette into which medium could be sucked and then removed using the pump. The medium reservoir was continuously stirred at 200rpm to ensure good mixing of the components because some of the trace elements precipitate and the antifoam is present as a suspension. Foaming was found to be adequately controlled (although foaming was non-existent except at lower dilution rates) using Breox FMT 30 antifoam (Water Management & Gamlen) added to the feed (0.01%v/v, see media recipes).
Figure 4. Photograph showing the fermenter set-up used for the continuous culture studies. The use of a vortexing impellor just below the liquid surface reduced splashing and facilitated a more stable maintenance of the culture volume.
2.4 Sample Analysis

2.4.1 Dry Weight

Routinely 10ml of culture broth was pipetted onto a predried, preweighed filter (Whatman 0.45µm). The biomass retained on the filter was washed with 3 x 10ml of reverse osmosis (RO) water. The filter was then dried in a microwave oven at maximum power for 6 minutes and left to cool overnight in a desiccator. (N.B. with biomass concentrations in excess of 4g/l, the filter must be left in the desiccator for up to 48 hours to ensure constant mass). Filters were reweighed after drying to constant mass on a four figure balance. A minimum of three replicates were weighed for dry weight determination and the sample deviation (σn-1) ranged from 2 to 5% of the sample mean.

2.4.2 Erythromycin Determination.

The antibiotic concentration was determined for culture supernatants using the cup-plate method with *Arthrobacter citreus* as the challenge organism. Cultures of *A. citreus* were grown overnight in nutrient both at 30°C. 5ml of this culture was then used to seed 250ml of molten nutrient agar at 45°C. The bacterial suspension was then poured into a 250ml Nunc bioassay dish and allowed to cool in a sterile air cabinet. When the agar had set, wells were formed by the removal of 8mm agar plugs. A set of standards were made using erythromycin A (sigma E6376). Stock erythromycin at 20mg/l was made up in phosphate buffer (K₂HPO₄ 7g/l & KH₂PO₄ 3g/l in RO water) and frozen in 5ml aliquots at -20°C. When required, samples were thawed and diluted in RO water to give a range from 4 to 20mg/l. Samples were diluted in RO water to bring them within the range of the standards (typically a 1 in 5 or 1 in 10 dilution was used). 100ml of diluted sample or standard was added to each well. Plates were incubated at 30°C for 48 hours and then left on the bench overnight (this latter step
improved the definition of the zones). The diameter of the zones of inhibition were measured using a set of Vernier calipers. Samples were routinely tested in triplicate with the sample deviation ($\sigma_{n-1}$) ranging from 2 to 10% of the sample mean (generally higher at higher concentrations). Confirmation of the identity of the antibiotic produced by this organism as erythromycin A was obtained using HPLC.

2.4.3 Glucose Determination

Glucose concentration in culture supernatants was determined using the glucose oxidase method (Trinder, 1969) with Sigma kit number 315-100 (Sigma Chemical Co. Ltd., Poole, U.K.). Samples were measured in triplicate against a standard glucose solution (Sigma Cat. No. 16-300) at 505nm.

2.4.4 Nitrate Determination

The concentration of nitrate in culture supernatants was determined using the nitrate reductase UV method (Beutler & Wurst, 1986) with Boehringer kit number 905-658. Samples were routinely tested in duplicate at 340nm.

2.4.5 DNA determination

The DNA content of the biomass was determined using the modification to the diphenylamine method described by Burton (1968).

Reagents:

Aqueous acetaldehyde (1.6%). Acetaldehyde is cooled and 1ml is transferred in a cooled pipette into 50 ml of RO water.

Diphenylamine reagent. 1.5g of diphenylamine is dissolved in 100ml of glacial acetic acid, and then 1.5ml of conc. sulphuric acid is added. Just before use, add 0.5ml of the 1.6% aqueous acetaldehyde.

Deoxyribose standard. Stock deoxyribose was diluted in 0.5N perchloric acid to give a range between 0 and 500 µM.
**Nucleic acid extraction:**

10ml of culture is centrifuged (3000g, 15 mins) and the supernatant removed. The biomass pellet is resuspended in 100mM MOPS buffer and spun down again. The buffer is discarded and the biomass pellet resuspended in 5ml 0.5N perchloric acid. The sample is then heated at 70°C for 15 minutes, agitating occasionally. The supernatant is collected and the pellet resuspended in a further 5ml aliquot of 0.5N perchloric acid. Again the sample is treated at 70°C for 15 minutes. The supernatant is collected and pooled with that from the first extraction and then frozen at -20°C until required for analysis.

### 2.5 Partial Cubic Spline Analysis

Parameters such as specific growth rate and specific rate of product formation are difficult to determine with any degree of accuracy for batch fermentations. This is because most of the techniques used to determine these parameters rely unduly upon each data point. The data obtained from batch fermentations is typically termed 'noisy', where the noise is caused by the heterogeneity in sampling and the various errors in the assays. It is important to understand why the partial cubic spline spline offers an advantage over more traditional methods of calculating specific rates, and therefore an example of two differing analyses for the determination of the specific growth rate is shown below.

Figure 5 shows a plot of the biomass obtained in a nitrogen-limited batch run (data from section 3.2.1) together with the natural logarithm of the biomass. The specific growth rate is often calculated from the following equation:

\[ x_i = x_ie^{\mu t} \quad (1) \]
where $x_0$ and $x_t$ are the biomass at time 0 and $t$ hours respectively, which rearranges to the following form:

$$\ln x_t = \ln x_0 + \mu t$$

(2)

Thus a plot of $\ln x_t$ versus $t$ yields a graph where the slope is $\mu$.

![Figure 5. Graph showing the biomass and the natural logarithm of the biomass (data obtained from nitrogen-limited batch fermentation). The points used for the determination of the specific growth rate are labelled 1 to 7.](image)

Table 9, overleaf, shows the various values returned for the growth rate depending upon the points used to calculate it. Using the first 7 data points suggests that the specific growth rate was constant at 0.078hr$^{-1}$ between 0 and 37.5 hours. Using points 2 to 5 suggests that $\mu$ was constant at 0.098hr$^{-1}$ between 13 and 24 hours; obviously both of the figures cannot be correct. Generally, it can be seen that the smaller the time period between which the growth rate is determined, the greater the correlation coefficient,
and the higher the specific growth rate obtained. If the data is noisy however, the straight line between the points will emphasise any error, particularly if the number of points is small. An interesting comparison of this and other techniques for calculating the peak specific growth rate of cultures has been examined by Mc.Dermott (1991).

Table 9. Using the points shown in figure 5 it is possible to calculate various values for the specific growth rate of the organism, each of which has a relatively high correlation coefficient.

<table>
<thead>
<tr>
<th>Points used to calculate the specific growth rate</th>
<th>Correlation coefficient</th>
<th>Slope, or specific growth rate (hr⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 to 7</td>
<td>0.927</td>
<td>0.078</td>
</tr>
<tr>
<td>2 to 7</td>
<td>0.991</td>
<td>0.078</td>
</tr>
<tr>
<td>2 to 6</td>
<td>0.998</td>
<td>0.091</td>
</tr>
<tr>
<td>2 to 5</td>
<td>0.999</td>
<td>0.098</td>
</tr>
</tbody>
</table>

As the cubic spline function generates a curve with a small time interval it is suitable for use in calculating rates of change, e.g. for biomass, as the curve is plotted, the program also calculates the slope of the curve which gives the volumetric growth rate. Division of this volumetric growth rate by the biomass gives a good approximation of the specific growth rate. Using a method to calculate the specific growth rate which uses large time intervals invalidates the expression:

$$\mu = \frac{dx}{dt} \frac{1}{x}$$  \hspace{1cm} (3)

which only applies when the time interval is infinitely small (i.e. as \( t \to 0 \)). The use of the cubic spline approaches these conditions more closely than the raw data and thus the spline gives greater accuracy and reveals more information than the logarithmic method illustrated above. The partial cubic spline analysis for the fermentation data shown above is shown in the results.
section 3.2.1, where it can be seen that the value of the specific growth rate follows a curve with a peak of $0.10\,\text{hr}^{-1}$ after 18 hours. Oner et al (1986) used spline functions to smooth batch fermentation data for the estimation of specific rates. More recently Bushell has used this technique for the analysis of both actinomycete (McDermott et al, 1993) and animal cell (Bushell et al, 1993) batch fermentations.

The partial cubic spline program is in BASIC (contact Dr. M. E. Bushell, University of Surrey, Guildford, GU2 5XH, U.K. for information on program listing) and calculates an equation of the form:

$$y = ax^b + cx^{b-1} + dx^{b-2}$$

(4)

where $b$ is the minimum value providing the minimum standard deviation between real and calculated values without cycling (Mc. Dermott, 1991). Thus the program fits an equation through data points 1, 2, and 3, and then links this to an equation through points 2, 3, and 4 and so on until a continuous curve covering all of the data is obtained. The 'smoothness' of the spline function, which is based on the $\chi^2$ 'goodness of fit' (Thornhill et al, 1989) may be traded against the accuracy with which the derived curve passes through the data points. Therefore the partial cubic spline allows an interpolated line to be calculated that expresses the trend in the data taking into account the sample error (routinely a 95% partial cubic spline is used which allows for a 5% error). The raw output from this program is not suitable for inclusion in this thesis, and so the curves obtained for the various specific and volumetric rates have been traced as accurately as possible onto more presentable axes prepared on a spreadsheet.
Chapter 3. Batch Fermentation

3.1 Introduction
3.2 Nitrogen-limited batch growth
3.3 Carbon-limited batch growth
3.4 Conclusions
3.1 Introduction

Batch cultures have been used extensively in the investigation of the regulation of secondary metabolite production, probably due to the ease of their execution and their widespread use in industrial processes. The kinetics that have been obtained in batch culture generally relate the onset or cessation of some metabolic activity (e.g. growth and secondary metabolite production) to time dependent changes in the cultivation environment. The timing of the accumulation of secondary metabolites in batch culture has been used frequently as a criterion for the relation between biomass growth and product formation. The knowledge about such relationships is of importance because it allows for greater control and optimisation of the physiological process of secondary metabolite production (Bushell, 1989). The effects of various nutritional factors on the production of secondary metabolites has been discussed earlier in chapter 1.2, and thus only a small selection of papers dealing specifically with production kinetics in batch culture will be discussed here.

A pattern frequently seen in batch cultures is the occurrence of two phases referred to as the trophophase and idiophase (Bu'lock et al, 1965; Bu'lock, 1974), where the production of biomass occurs in the former and the accumulation of secondary metabolic products occurs in the latter. As growth was assumed to have ceased in the idiophase, the secondary metabolites produced in this phase were naturally classified as growth-dissociated products. This segregation of primary and secondary metabolism seemed reasonable from the teleological viewpoint of the organism's metabolic economy. Thus, a well adjusted organism might be 'expected' to utilise the available resources for primary functions such as growth, and only when limitation occurred would the organism switch to secondary metabolism (Trilli, 1990).
This idea may well be too simplistic, especially with the observation that the production of secondary metabolites often occurs during the exponential phase of growth, particularly in defined media where the growth rates are generally lower. Demain (1986) for example, proposed that the terms 'growth-associated' and 'growth-dissociated' should not be adopted to describe the temporal relationship between growth and secondary metabolite production, due to the central role that nutritional factors play in the production kinetics in batch culture. Despite this, these terms continue to be used frequently.

Although *Saccharopolyspora erythraea* produces only a single class of antibiotics, the erythromycins, there are many actinomycetes that can produce a range of different secondary metabolic products. For example, *Streptomyces clavuligerus* produces at least 21 different antibiotics including penicillin N, cephamycin C, clavulanic acid, holomycin, and tunicamycin. These antibiotics may be produced simultaneously or sequentially as various nutritional factors induce the various synthetic pathways. An example of this is illustrated by the growth of *Streptomyces cattleya* in a defined medium, where the sequential exhaustion of glucose, ammonia and phosphate led to the sequential production of the antibiotics cyclopentenedione, cephamycin C, and thienamycin, respectively (Bushell & Fryday, 1983). All of these products were produced while the growth rate was in decline.

A switch from growth-dissociated to growth-associated production kinetics can often be achieved by switching from a complex medium to a defined one. Examples have been reported for the production of penicillin G by *Penicillium chrysogenum* (Pirt & Righelato, 1967) and the production of chloramphenicol by *Streptomyces venezuelae* (Malik & Vining, 1970). As mentioned earlier, the slower growth obtained in chemically-defined media has been suggested as the cause of the loss of the distinction between trophophase and idiophase (Demain, 1986).
There are numerous methods for determining growth, such as dry weight, protein, DNA, and various techniques based on optical density. Each of these methods provides useful information, but it is important to realise that they are not necessarily related nor do they mean the same thing. Obviously it is important to have an appropriate definition of growth when trying to link this to other physiological processes, and this was demonstrated by the work of Martin & McDaniel (1975). The authors showed that candicidin formation, in batch cultures of *Streptomyces griseus*, paralleled the increase in dry cell weight, suggesting growth-associated kinetics. However, when the DNA content of the culture was determined this revealed a clear separation of the growth and candicidin production phases.

Hobbs *et al* (1990) studied the formation of two pigmented antibiotics during the batch cultivation of *Streptomyces coelicolor* in a defined medium. They reported that the production of undecylprodigiosin in glucose-limited cultures paralleled the accumulation of biomass, whereas actinorhodin accumulation occurred after the growth had slowed. Their work concentrated primarily on the nutritional factors affecting the production kinetics of actinorhodin rather than undecylprodigiosin whose kinetics "...did not conform to those usually displayed by a secondary metabolite.". This view is typical of much of the work performed in defined media where the classical temporal separation of trophophase and idiophase has almost become law, such that any deviation from this traditional idea is viewed with suspicion and subsequently ignored.

The following batch fermentations examine the relationship between growth and erythromycin formation when *Saccharopolyspora erythraea* is grown under various nutritional conditions. The advantages and the limitations of batch culture as a tool for studying growth-product relationships will be discussed after the results have been presented.
3.2 Nitrogen-limited Batch Growth

3.2.1 Biomass and Erythromycin Data

During a nitrogen-limited batch culture the biomass concentration increased rapidly in the first 36 hours until it reached 4g/l, whereupon it then appeared to increase linearly for the next 24 hours (figure 6).

![Figure 6. Biomass and erythromycin data obtained during a typical nitrogen-limited batch fermentation.](image)

During this linear phase the biomass concentration increased to its maximum of about 5g/l. After peak biomass concentration was achieved, the culture started to lyse and the dry weight decreased.

The erythromycin concentration changed very little during the first 21 hours, but from 21 to 47 hours it increased rapidly. After 47 hours, further accumulation of erythromycin occurred, but at a lower rate.

Thus it can be seen that the period of greatest antibiotic accumulation (between 21 and 47 hours) occurred at the same time as the period of rapid
biomass accumulation. This simple analysis would in itself suggest that the production of erythromycin under nitrogen-limitation was growth-linked in some way. The partial cubic spline analysis of the data shows that the link is not perhaps as strong as at first suggested. Figure 7, shows that the specific growth rate (µ) increased following the inoculation into the vessel. This increase only continued for the first 18 hours however, after which point it started to fall. Therefore erythromycin was only produced in quantity in this culture when the growth rate had begun to fall, leading us to the conclusion that its production kinetics were growth-dissociated.

Figure 7. Partial Cubic Spline Analysis Showing the Specific Growth Rate and the Specific Erythromycin Production Rate Obtained During a Nitrogen-limited Batch Fermentation.

A further complication to this argument is provided by the spline analysis of the erythromycin data to yield the specific rate of production (qp) (figure 7). This analysis provides more detail to the early part of the fermentation when the erythromycin titre was increasing by small amounts. It shows that during the period of increasing growth rate the specific antibiotic production rate was also increasing, thus indicating that the drop in growth
rate itself was not the sole factor inducing the production of antibiotic. The peak rate of growth occurred after 18 hours, whereas the peak rate of antibiotic production occurred some 12 hours later. It appears that the production of antibiotic may be growth-linked in some way, but that it might lag behind the production of biomass by about 12 hours (in this case). It may be that the displacement of these two peak rates is due to a cell "maturation" effect. This concept of a cell maturation time was introduced by Blanch & Rogers (1971) to quantitatively model the kinetics of gramicidin S production in batch and continuous cultures of *Bacillus brevis*. The idea was later adapted for the growth of *Streptomyces* spp. by Brown & Vass (1973) and Martin & McDaniel (1975). These authors suggested that the cells in a culture could be divided into two groups termed "mature" (capable of synthesising the product) and "immature" cells, and that formation of secondary metabolites could be considered as a function of cell age. Good agreement was found between the production profiles predicted by the maturation model and those observed experimentally.

Thus a maturation time of 12 hours was observed for chloramphenicol production in *Streptomyces venezuelae* (Brown & Vass, 1973). Maturation times of 14, 17.5, and 18 hours were calculated for the production of the non-polyene macrolides candidin, candidicidin, and candihexin (Martin & McDaniel, 1975). The displacement by 12 hours of the peak rates of growth and antibiotic production shown in figure 5 would agree quantitatively with these reports. However, in similar experiments in this nitrogen-limited medium, using strains that had been left on an agar plate too long prior to use as an inoculum, the relationship between these peak rates was seen to vary from both occurring simultaneously, to the example cited here where they were separated by 12 hours (data not shown). This would suggest that 'hidden' factors other than the type of limiting nutrient, or a specific maturation time, may also be responsible for the relationship between growth
and antibiotic production (e.g. inoculum, as will be examined and discussed in chapter 5.4).

The next section discusses ideas raised from observations made in batch culture and from reports in the literature. The proposals discussed below have not been experimentally examined in detail and thus much of it is speculation. It is included however, as it could form the basis for a number of interesting future experiments.

### 3.2.2 The Concept of 'Morphological Memory'

The growth rate in nitrogen-limited culture increased steadily for the first 18 hours (figure 7). As the inoculum was still growing rapidly one might expect the growth rate to rise quickly, following inoculation, to a maximum (often referred to as \( \mu_{\text{max}} \)) and remain at this level until the substrate concentration fell sufficiently to inhibit the culture's growth. It seems likely that the culture never reached \( \mu_{\text{max}} \) or that if it did the growth rate fell again very rapidly. The reasons for this slow response to a new environment are unknown but may be linked in some way to the morphological and physiological status of the inoculum. It certainly seems likely that a filamentous culture will retain, in the mature regions of its hyphae at least, a morphological state that reflects an earlier physiological status. Thus, when examining the morphology of a culture, only the most recently formed regions will represent the current physiological and biochemical conditions prevailing in the culture's environment.

It is thought that an actinomycete hypha cannot produce growing tips along its complete length. The work of Kretschmer (1987) has revealed that new branches in Streptomyces granaticolor rarely occurred within 40\( \mu \text{m} \) of a growing tip, and that the average distance between neighbouring branches on the main hypha was 7\( \mu \text{m} \). This suggests that a filamentous organism only has a limited number of sites from which it can grow or produce a branch. Such sites have been referred to as e-sites.
(for elongation sites) and have been investigated in Streptomyces spp. (Kretschmer, 1985). The frequency of e-sites is inversely expressed by the hyphal growth unit (Caldwell & Trinci, 1973), this latter is equal to the mycelium length (L) divided by the number of branches (N). The dependence of L / N upon different growth rates has been studied in various actinomycetes (Riesenberg & Bergter, 1979; Kretschmer 1981; Kretschmer, 1985) with the conclusion that the length of the hyphal growth unit was not determined by \( \mu \) per se, but by the specificities of the nutrient dependent metabolism.

It has also been suggested that the appearance of new branches might be linked with the accumulation of nuclear material at certain points along the hypha, (Kretschmer & Kummer, 1987; Kretschmer, 1987). If these ideas are valid, then it seems likely that, at any point in the fermentation of an actinomycete, the culture only has a particular number of possible e-sites. In a culture growing slowly, one would expect this number to be lower than in a culture which was growing rapidly. Thus, when a slowly growing culture is introduced into an environment where nutrients are suddenly in excess it has a period when there are insufficient e-sites to realise a rapid increase in growth rate despite the fact that the relevant biochemical pathways required for rapid growth may be active. The culture therefore, effectively possesses a 'morphological memory' of its previous physiological state that may interfere with the rapid response to current environmental conditions, and it can be seen that this would play a large part in the early kinetics of subsequent growth.

The importance of the early stages of a fermentation (termed the preparatory phase) has been stressed by a number of authors (Calam, 1980; Vanek & Blumauerova, 1986), where it is seen as a crucial stage for the priming and development of suitable metabolic pathways which will be activated at a later time. In unicellular bacteria there is no 'morphological memory' because theoretically there are as many e-sites as there are bacterial cells (i.e. each bacterial cell is capable of producing another cell by binary fission). The lag phase and increasing exponential phase in these unicellular organisms is thus usually quite short. In
filamentous actinomycetes however, the growth may be restricted due the lack of e-sites resulting in an extended increasing exponential phase such as that seen in figure 7.

It is, perhaps, difficult to comprehend the concept of 'memory' reflected in the morphology of an actinomycete. There are however, analogous examples of a similar kind in the biology of higher organisms. One example is found in trees, where the impression of previous growing seasons leaves an imprint on the rings of the tree - enabling botanists to interpret the prevailing weather conditions in the past by examining these tree rings. Another example, of marine coral systems, provides a closer analogy to the branching of filamentous organisms. In this case, if we consider a coral which is old and quite extensive, it is easy to see how the current year's growth covers the surface of the coral, and that the morphology of the coral as a whole is the sum of the current and all of the previous years' growth. This previous growth (determined by conditions at that time) has effectively left an imprint on the morphology of the coral. Thus the coral effectively has a 'memory', imprinted upon its morphology, of conditions that occurred at some time in the past. The same argument applies to filamentous actinomycetes except that the time-scale involved reduces from years to hours.

If this idea is correct then it has a number of implications for systems which rely on rapid growth rate responses. An example is cyclic fed batch fermentation, where at the end of a growth cycle, when the growth rate is low, a portion of the culture is removed and fresh nutrients added at a constant rate, which causes an initial shift up in the growth rate. For a unicellular organism there would only be a short transient phase before the quasi-steady state is achieved and the growth rate is in step with the dilution rate. In filamentous bacteria this transient phase might take considerable time, so that the quasi-steady state is only achieved towards the end of a cycle. This would particularly be the case when the amplitude of the dilution rate (and thus the growth rate) change was large.
Such a situation could be readily identified in cyclic fed batch culture of a filamentous organism because there would be a drop in the biomass concentration (which theoretically remains constant) at the beginning of each cycle. Recent work in this laboratory has indeed identified this drop in the biomass during the cyclic fed batch culture of Saccharopolyspora erythraea (Lynch & Bushell, 1993). This would seem to confirm that this organism is unable to respond quickly to growth rate changes (at least in the defined media used for these experiments). To emphasise the importance of morphology in this phenomenon, these workers have repeated these experiments with a streptomycete that grows as a unicell, and found that in this case the response to up-shift in growth rate was very much more rapid (2 hours compared with 6-12 hours with Saccharopolyspora erythraea; personal communication).
3.2.3 Glucose and Nitrate Data

During nitrogen-limited batch cultures, glucose remained in excess following nitrate exhaustion at 30 hours, and continued to be assimilated until 70 hours, at which point it too became exhausted (figure 8).

![Figure 8. Glucose and Nitrate Concentrations During a Nitrogen-limited Batch Fermentation](image)

It is important to determine what physiological parameters were responsible for the various phases of growth observed during the batch fermentation. For example, what fermentation parameter had caused the growth rate to drop after 18 hours? There was very little biochemical change in the culture at this point in the fermentation: the dry weight had increased from 0.21 to 0.74g/l; the erythromycin concentration had doubled from 4 to 8 mg/l; and the glucose concentration had fallen slightly from 31 to 27 g/l. The pH and the dissolved oxygen tension remained relatively stable at 7.2 and 90% respectively. The nitrate concentration however had dropped sharply to about 20% of its original value and it is thus likely that this parameter controls...
the growth rate in the early part of the fermentation (this is of course to be expected for a medium designed to be limiting for nitrogen).

The spline analysis for the nitrate and glucose data is shown in Figure 9. As expected, the uptake rate of nitrate paralleled the growth rate in the first 30 hours and the two peaks coincided at 18 hours. Thus, the onset of the linear phase seems to be determined by the exhaustion of the nitrogen source and the cessation of true growth. This phase of storage material accumulation lasts for about 24 hours, during which the antibiotic synthesis rates are decreasing. The end of the linear phase coincides with the exhaustion of glucose from the medium, but whether these two factors are linked will be discussed in more detail below.
3.2.4 DNA Studies

The exhaustion of nitrate in the medium typically coincides with the start of the linear increase in biomass. How can the culture continue 'growing' in the absence of a nitrogen source? It has already been discussed (section 3.1) that culture biomass concentration is not by itself a particularly good measure of true growth in a culture, and it is generally accepted that the most reliable measure of growth is the DNA content (Martin & McDaniel, 1975). Thus, in later repeat experiments, the DNA content of the culture was measured as a reflection of the true growth of the culture in order to compare it to the increase in biomass. In this experiment it can be seen that the synthesis of this macromolecule stopped when the nitrate became exhausted (45 hours, point A on figure 10), whereas the culture biomass concentration continues to increase at a slow linear rate beyond this point.

Figure 10. Graph showing the increase in biomass and DNA during a nitrogen limited batch culture. Nitrate exhaustion in this fermentation is marked as point A
Thus the increase in dry weight observed during the linear phase is not true growth, but probably due to some other factor e.g. the accumulation of storage materials. A possible candidate for this storage material would be glycogen, as the accumulation of glycogen in the substrate mycelium of many microorganisms may be triggered by the exhaustion of the nitrogen source (Dawes & Senior, 1973). Glycogen is thought to play a role in the sporulation process in actinomycetes (Brana et al, 1980; Brana et al 1982b; Brana et al, 1986), which agrees with further observations made in this culture. It can also be seen in figure 10 that after 45 hours the concentration of DNA falls before increasing once again towards the end of the fermentation, a situation which has been reported previously for Streptomyces coelicolor A3(2) (Grannozzi et al, 1990). This increase in DNA at the end of nitrogen-limited batch growth has also been confirmed in this laboratory using radiolabelled incorporation studies (Wilson, 1993).

In unicellular bacteria, when growth is restricted by lack of an exogenous nitrogen source, the intermediates needed for RNA and protein synthesis cannot be made de novo. Under starvation conditions, these intermediates are provided by the hydrolysis of non-essential RNA and proteins, mediated by intracellular nucleases and proteinases, the synthesis or activation of which follows the onset of starvation (Pritchard & Tempest, 1982). It thus seems likely that, after exhaustion of the nitrate, Saccharopolyspora erythraea is unable to synthesise DNA de novo and therefore breaks down some of its non-essential nucleic acids and rebuilds them into new DNA. This would explain the observed drop and subsequent increase in the DNA content of the culture. Wilson (1993) investigated this phenomenon and concluded that the organism was undergoing sporulation, agreeing with the observations made by Brana et al. Although mature spores were not observed by light microscopy, sonication resistant structures were detected, suggesting the presence of spores.
Another point of interest about the nitrogen-limited batch culture of *Saccharopolyspora erythraea* was the fact that the volumetric production rate of erythromycin (not shown, figure 7 shows the specific rate) followed the glucose uptake rate (figure 9) and the two peak rates coincided. Thus there appears to be some role for glucose in the latter stages of this fermentation. A similar relationship between glucose uptake and methylenomycin accumulation has been reported for the batch fermentation of *Streptomyces coelicolor* A3(2) in a defined medium (Hobbs et al., 1992). Growth in these studies was determined by DNA content (the presence of Junlon in the medium prevented the use of dry weight as a measure of biomass in this case) and thus it is not known whether an increase in biomass was observed in the latter stages of the culture similar to that observed in the nitrogen-limited cultures reported here.

3.2.4 Nitrogen-limited Batch Growth with Excess Glucose

It was thought that the exhaustion of glucose from the nitrogen-limited medium might have been affecting the kinetics in either of two ways. Firstly, the exhaustion of glucose may have been causing the culture to lyse, thereby prematurely ending the linear phase and any subsequent stationary phase. Secondly, the fall in the glucose concentration itself could have been linked to the drop in erythromycin synthesis. It was decided to investigate the effect of an excess of glucose in this medium by repeating the experiment with an initial glucose concentration of 60 g/l compared with the 30g/l used previously.
The first 60 hours of this culture were virtually the same as that observed with the lower glucose concentration, i.e. a rapid increase in biomass for 30 hours followed by a linear phase lasting about 24 hours (figure 11). Surprisingly, the length of this linear phase remained unaltered by the availability of glucose (which even after 180 hours remained above 20g/l). Therefore it seemed likely that the length of this phase of storage material accumulation was determined by some intracellular parameter, perhaps linked to the ability of the organism to recycle its intracellular nitrogen reserves.

The main influence that the extra glucose had on the culture was that there was an extended stationary phase that was absent in the lower glucose medium. This phase lasted for almost 80 hours during which there was no appreciable drop in biomass (figure 11); glucose was still being utilised at a high rate (data not shown); dissolved oxygen tension remained below 100%
(indicating a substantial oxygen demand); and perhaps most interestingly, erythromycin continued to be produced. Almost 50% more antibiotic was formed in this higher glucose medium with the final titre, still rising at the end of the experiment, reaching about 200mg/l.

The spline analysis of the data shown in figure 11 indicated that the peak rates of growth and specific antibiotic production overlap, with the peak rates separated by about 8 hours (figure 12). As mentioned previously (chapter 3.2.1) it was not unusual for the peak rates to be separated in this manner in the lower glucose medium also. Another point of interest is that the peak growth rate was slightly higher in the higher glucose medium (0.13hr⁻¹ compared with 0.10hr⁻¹) and that the peak specific antibiotic production rate is similarly higher (2mg/g/hr compared with 1.6mg/g/hr), again suggesting a link between the growth and the erythromycin production of the organism.

![Figure 12. Partial cubic spline analysis showing growth rate and specific antibiotic production rate during a nitrogen-limited batch fermentation in which glucose did not become exhausted](image)

It can also be seen that the two phases of erythromycin production are separated by a short period, between 45 and 80 hours, where the
concentration of antibiotic remains constant (figure 9). The reason why the organism should terminate production and then reinstate it 35 hours later is unknown, but this repression occurred while the organism was in the linear phase and these two may be connected. Whatever the reason, if this 'shut down' actually occurs it would provide the ideal phase for studying the metabolic switches involved in erythromycin synthesis. This experiment was not repeated, however, so that it is impossible to say how reproducible this phenomenon is.
3.3 Carbon-limited batch growth

3.3.1 Biomass and erythromycin data and spline analysis

The dry weight and antibiotic data obtained during a culture in which glucose was the limiting substrate are shown below in figure 13. The partial cubic spline analysis of this data is shown in figure 14. It can be seen that the duration of the biomass accumulation phase was relatively short with the majority of the biomass and erythromycin being produced in the first 28 hours. After 28 hours, there was a short stationary phase, which lasted for about 20 hours, during which erythromycin continued to be produced although at a low rate. After 50 hours the dry weight and antibiotic titres began to fall as the culture underwent lysis.

The spline analysis shows that $\mu$ and $q_p$ were increasing at the same time and that the two peaks coincided (figure 14). The peak specific growth rate of 0.15 hr$^{-1}$ was slightly higher than that obtained for a nitrogen-limited
batch culture (usually about 0.13 hr\(^{-1}\)) and the peak \(q_p\) was similarly higher (2.25 mg/g/hr compared with 2 mg/g/hr). This data again suggested a distinct link between growth and the formation of erythromycin in this organism. Hobbs et al (1990) reported similar growth and production kinetics for undecylprodigiosin production in glucose-limited cultures of *Streptomyces coelicolor* A3(2). In glycerol-limited cultures of *Streptomyces clavuligerus*, however, the production of cephamycin C occurred when the growth rate was slowing down (Lebrihi et al, 1988), demonstrating that carbon-limitation per se does not produce growth linked production.

![Figure 14. Partial cubic spline analysis showing the growth rate and specific antibiotic production rate observed during a carbon-limited batch fermentation](image)

3.3.2 Glucose and nitrate data and spline analysis

The glucose became exhausted after about 30 hours, which coincided with the start of lysis in the culture (figure 15). The concentration of nitrate also fell rapidly in the early stages of the fermentation but its uptake stopped when the glucose was exhausted.
It can be seen that the peaks in volumetric production rates occurred almost simultaneously with those of the growth rate and the specific antibiotic production (fig 16).
The kinetics of production in carbon-limited medium thus appear to be strongly growth-linked. This is contrary to the observation made by McDermott (1991) where the growth of Saccharopolyspora erythraea in the same carbon-limited medium resulted in growth-dissociated production. Changes in the growth-associated or dissociated nature of antibiotic production in particular organisms have been reported before. As mentioned earlier, the transition from growth-dissociated to growth-associated kinetics can frequently be achieved by changing the growth medium from a complex one to one that is defined. Examples of the latter include bacitracin production by Bacillus licheniformis (Haavik, 1974a). It is also possible to achieve a change in kinetics with different strains, which will be illustrated below.

3.3.3 Glucose-limited batch growth of strain CA340

Figure 17. Biomass and erythromycin data obtained during a carbon-limited batch fermentation of CA340
The peak rates of growth and specific antibiotic production described in this chapter have occurred almost simultaneously or separated by up to 12 hours, with peak $q_p$ occurring after the peak growth rate is achieved. This situation can be reversed. Another strain of *Saccharopolyspora erythraea*, CA340 (Abbott Laboratories, USA) when grown in the carbon-limited medium gave unexpected results.

The biomass and antibiotic data for the growth of CA340 in the glucose-limited medium is shown in figure 17, and the spline analysis is shown below in figure 18. It can be seen that the peak $q_p$ precedes the peak growth rate in this example.

![Figure 18. Partial cubic spline analysis showing the growth rate and specific antibiotic production rate observed during the carbon-limited batch fermentation of CA340](image)

The regulatory mechanisms of bacterial cells usually ensure efficient use of nutrients. Hence, one might expect organisms to restrict to a minimum those compounds derived from the growth-limiting nutrient (Pritchard & Tempest, 1982). Carbon substrate limitation has been characterised by a
high carbon conversion efficiency where conversion of the substrate carbon into non-growth associated products is minimised (Tempest & Wouters, 1981). Under the conditions of carbon-limitation one might expect particularly low rates of production of erythromycin (which has a very high carbon content). The results presented here show that this is not the case, with peak specific rates for both nitrogen- and carbon-limited batch growth more or less similar, with $q_p$ values between 1.6 and 2 mg/g/hr. This suggested that erythromycin production in this organism was not just a response to physiological stress imposed by nutrient limitation, but possessed a role more directly linked with growth.

### 3.4 Conclusions

In this chapter it has been shown that the timing of the peak rates of growth and antibiotic production are not determined solely by the type of nutrient limitation that the organism is cultured in, but that other factors, not immediately apparent, can contribute to this relationship. The temporal relationship between the production of biomass and the production of secondary metabolites has been extensively used to classify the relationship between $\mu$ and $q_p$. However, the batch culture process possesses a number of features that complicate any conclusions drawn from such data. If we consider the kinetics of antibiotic production in nitrogen and the carbon limited fermentations, we can see that, although there is evidence that a strong link between $\mu$ and $q_p$ exists in *Saccharopolyspora erythraea*, it is not easy to determine whether the link is with growth rate *per se* or with some factor which also acts upon the growth rate.

The batch system provides an environment where physico-chemical parameters are constantly changing. The response of the organism to any environmental changes will not be instantaneous, and furthermore the abilities of the various metabolic control mechanisms to respond to change
differs (Savageau, 1976). In batch culture the constant change in culture conditions means that a non-equilibrium system exists intracellularly where the physiological state of the organism reflects some state that occurred earlier in its environment (Trilli, 1990). This non-equilibrium status of the metabolism of the organism will be accentuated by the inherent aging and heterogeneity of filamentous cultures.

Some of the problems encountered with batch culture can, theoretically at least, be overcome by using continuous culture. This type of fermentation allows the separate manipulation of nutritional factors and growth rate, and will be examined in the next chapter. However, the importance of batch culture as a tool for studying the physiology of microorganisms should not be lightly dismissed. There are positive and negative aspects to all fermentation systems, and each has something potentially useful to offer the microbial physiologist in terms of the response of an organism's metabolism to various nutritional and growth conditions.
Chapter 4. Continuous Culture

4.1 Introduction
4.2 Glucose-limited chemostat culture
4.3 Nitrate-limited chemostat culture
4.4 Equilibrium and heterogeneity in continuous culture
4.5 Conclusions
4.1 Introduction

There have been many reports published on the growth-productivity relationships of secondary metabolites in continuous culture, and these present a variety of different behaviours. For example, Sikyta et al (1961) found that the production of chlortetracycline by *Streptomyces aureofaciens* was growth linked in both carbon- and nitrogen-limited chemostat culture. The production of erythromycin by *Saccharopolyspora erythraea* was positively correlated with growth in both phosphate- and nitrogen-limited chemostat cultures (Trilli, 1990). The production of oxytetracycline by *Streptomyces rimosus* was also found to be growth-associated (Rhodes, 1984). Conversely, the production of thienamycin and cephamycin C was negatively correlated with growth in *Streptomyces cattleya* (Lilley *et al*, 1981), as was the production of gibberellins and bikaverins in *Gibberella fujikuroi* (Bu'lock *et al*, 1974). Lebrihi *et al* (1988) reported that the production of cephamycin C in *Streptomyces clavuligerus* was growth-dissociated in nitrogen-, carbon-, and phosphorus-limited cultures. The production of cephalosporin C by *Cephalosporium acremonium* is of interest because two different reports describe the production kinetics as growth-associated in one case (Kuenzi & Auden, 1983) and growth-dissociated in the other (Matsumura *et al*, 1978), indicating that differences in cultivation and/or strain of an organism can affect the $q_p$ versus $D$ relationship.

The relationship between growth and productivity is not always a straightforward one, with many papers reporting differing kinetics in different growth ranges. Perhaps the best documented of these is the biphasic relationship between growth rate and penicillin G production in *Penicillium chrysogenum* (Pirt & Righelato, 1967; Pirt, 1987). In this example, $q_p$ was seen to increase with increasing dilution rate between the range 0.1 to 0.15hr$^{-1}$, but to remain constant at higher dilution rates.
### 4.2 Glucose Limited Chemostat Culture

Four steady states at a range of dilution rates were achieved in the glucose limited chemostat culture, and the steady state biomass and erythromycin concentrations achieved with each are shown below in Figure 19. At all points glucose was undetectable and nitrate was in excess (data not shown).

**Figure 19. Steady state values for biomass and erythromycin concentration during growth of *S. erythraea* in a carbon-limited chemostat.**

![Graph showing steady state values for biomass and erythromycin concentration](image)

It can be seen that the biomass remained relatively constant at about 4.5g/l over the whole range of dilution rates, whereas the antibiotic concentration decreased with increased growth rate. The relationship between the dilution rate and the specific rate of antibiotic production is shown below in Figure 20. The general trend was an increase in $q_p$ with increasing growth rate. This suggested that there was some degree of linkage between erythromycin production and the growth rate, a finding that agreed qualitatively with the results obtained for batch growth under glucose limitation. This finding is also in agreement with other published work.
For example, the \( q_p \) of chlortetracycline by *Streptomyces aureofaciens* increased with increasing dilution rate under both carbon- and nitrogen-limitation (Sikyta *et al.*, 1961). Interestingly, work has also been published on the growth of *Saccharopolyspora erythraea* in continuous culture (Trilli *et al.*, 1987; & Trilli, 1990), which also concluded that there was a strong linkage between the \( q_p \) of erythromycin and the growth rate. The authors studied this relationship in a phosphate-limited medium at three dilution rates (0.03, 0.05, and 0.1 hr\(^{-1}\)) and found a good straight line agreement between increasing dilution rate and increasing \( q_p \). There has been some question over whether the medium used for these experiments was actually limiting for phosphate or for some other nutrient. McDermott (1991) suggested that the medium might be nitrogen-limited as she found that the kinetics of erythromycin production were strongly growth-dissociated under phosphate-limitation in batch culture. It appears more likely however that the medium used by Trilli was carbon-limited, as it is reported in the paper that “glucose was undetectable at all times indicating that in practice its uptake rate equalled the addition rate”. If
this medium was indeed glucose limited then the results presented here are in agreement with those found by Trilli et al (1987). A later publication (Trilli, 1990) provided data for a nitrogen-limited chemostat of Saccharopolyspora erythraea which also showed the same strong growth-linkage, but as the data presented was from 'unpublished results', the formulation of the medium for this fermentation was not included. Another factor to consider about these studies was that the wild type strain was not used, instead the authors used a mutant selected for its stability in continuous culture, thus perhaps it is not surprising that the production of erythromycin was growth-associated. If the product had been growth-dissociated one might have suspected this phenotype to be unstable in prolonged continuous culture (see chapter 1.5).
4.3 Nitrate-limited Chemostat Culture

Seven steady states were achieved in a nitrogen-limited chemostat and the steady state biomass and antibiotic concentrations are shown in figure 21. At all points the presence of nitrate was undetectable and glucose was in excess (see figure 22). It can be seen that there are a number of differences between the trends obtained under carbon-limitation and those presented below.

![Figure 21. Steady state values for biomass and erythromycin concentration during growth of S.erythraea in a nitrogen-limited chemostat.](image)

The steady state biomass was about 5g/l when the dilution rate was 0.025 hr\(^{-1}\), and that it decreased with increasing dilution rate until it reached about 3.5g/l at 0.07 hr\(^{-1}\) at which point it remained relatively constant with further increases in growth rate (figure 21). The steady state biomass concentration in continuous culture is determined by the concentration of the growth-limiting substrate in the feed. Assuming that the yield of biomass per unit substrate is constant and independent of growth rate, one would expect
the biomass concentration to remain constant except at low growth rates, where maintenance requirements become important, and when the culture exceeds \( \mu_{\text{max}} \) where the culture begins to wash out. If this increase in biomass reflected a true increase in replicatory growth we would have to assume that the yield constant for this organism growing on nitrate had increased, i.e. the organism can utilise its nitrogen source more effectively at lower growth rates than it does at high growth rates.

Changes in the steady state biomass concentration \((\bar{x})\) have been reported before. For example, Lebrihi et al (1988) studied the growth and cephamycin C production in continuous cultures of *Streptomyces clavuligerus*. When the growth was glycerol- or phosphate-limited, \(\bar{x}\) decreased at low dilution rates, which was attributed to the scarcity of ATP and other phosphorylated molecules. In ammonium-limited cultures however, \(\bar{x}\) remained relatively constant.

It is possible in this case however, that the increase in biomass was not a reflection of true replicatory growth but rather an indication that the organism was accumulating storage materials such as glycogen. When the organism was growing rapidly it was unable to lay down such reserves and thus the steady state biomass remained relatively constant. As the growth rate decreased, and the organism's metabolic activity slowed down, storage materials would start to accumulate. As glycogen is the storage form of glucose, one might expect the steady state glucose concentration to fall at lower growth rates if glycogen was being accumulated in the cell. Figure 22 shows that the glucose concentration did fall at dilution rates below 0.07hr\(^{-1}\). A similar increase in biomass, apparently due to non-replicatory growth at low growth rates, was also observed in batch culture under nitrogen limitation (see section 3.2).
A decrease in the steady state biomass with increasing dilution rate during nitrogen-limited chemostat culture of *Saccharopolyspora erythraea* has been reported before by McDermott (1993). It was proposed that the growth of the organism was inhibited by the formation of erythromycin, but this seems unlikely as the resistance determinant (the ermE gene) is expressed constitutively (Cundliffe, 1989). The medium used in those studies was the same as that used here, but the reported biomass level was about 10% of that shown in figure 19. McDermott also reported that steady state nitrate concentrations were very high, indicating that the chemostat was unlikely to be nitrogen-limited (the proposed product inhibition kinetics would lead to high \( \tilde{s} \), but as mentioned above inhibition by erythromycin seems unlikely). If, as reported, the complete medium was filtered into the feed reservoir, this may have led to the removal of some trace elements which are present in the medium as a cloudy precipitate. Trace element limitation would certainly account for the low biomass and erythromycin concentrations, and
the high residual nitrate levels. This removal was avoided in our experiments by the aseptic addition of sterile trace element concentrate to the feed reservoir after the filtration of the basal medium. It has also been suggested that the different kinetics observed in chemostat culture may be due to differences between strains of *Saccharopolyspora erythraea*, e.g. slight changes in substrate affinities and/or the erythromycin resistance/production genes (Bushell, private communication).

The concentration of erythromycin observed in the chemostat decreased with increasing growth rate (figure 21). This implied that the processes of growth and antibiotic production in nitrogen-limited continuous culture could be separated despite the high degree of growth linkage observed in batch culture. Hobbs *et al* (1990) reported a similar situation for the production of the pigmented antibiotic undecylprodigiosin which paralleled the increase in biomass in batch culture. In continuous culture however, the steady state concentration of this antibiotic was found to decrease with increases in growth rate. This result was not discussed in any detail.

Peak steady state antibiotic levels of 120mg/l were observed at a dilution rate of 0.04 hr⁻¹. At growth rates above 0.07 hr⁻¹ the concentration of antibiotic remained virtually constant at about 20mg/l. The specific rate of antibiotic production did not increase with increasing dilution rate (figure 22), suggesting that the production of erythromycin under nitrogen-limited conditions was not growth-linked, as was the case under carbon-limitation. Thus there appears to be a distinct difference between the kinetics of production between the two types of limitation.

The next section discusses the idea of an equilibrium of morphological and physiological states, and the spatial separation of growth and secondary metabolism within the actinomycete hyphae. These ideas, although they lack adequate experimental evidence at this point, are presented here because
they follow on more logically from the work already presented in this chapter than from later chapters. They also formed the basic hypothesis around which subsequent experiments were designed (see chapters 5 and 6).

4.4 Equilibrium and heterogeneity in continuous culture

The reason why the antibiotic concentration should fall at higher growth rates can be explained if we consider the culture in the chemostat as two physiological states in a loose equilibrium. The growing tips or e-sites are undergoing primary metabolism which, through the natural processes of growth and elongation, give rise to mature regions which may be undergoing secondary metabolism. If a filamentous organism can only grow by linear extension at a growing tip (as is the case in most actinomycetes), it follows that in order to grow faster the organism must produce more e-sites. In a chemostat, where the biomass concentration remains relatively constant, a proportion of the biomass will exist as growing tips, and a portion will exist in the mature phase - possibly producing antibiotics, storage materials and other secondary metabolites. In continuous culture we can visualise these portions of biomass with differing physiological states as being in an equilibrium. In order to grow quickly the organism must alter the equilibrium to favour growth, i.e. the proportion of the culture engaged in growth is increased at the expense of the proportion of the biomass engaged in other functions, therefore antibiotic production might be expected to drop. At low dilution rates the proportion of the culture involved in primary metabolism is low and thus the equilibrium tends towards the mature fraction and an increase in secondary metabolism.

When the dilution rate was at its lowest (0.012hr⁻¹) there was a drop in the antibiotic concentration that the previous hypothesis does not yet explain. In order to explain this observation it is necessary to extend the physiological
model a step further and consider the culture as being composed of more than just 'growing' and 'mature' fractions. It has been discussed earlier that growth occurs in filamentous actinomycetes by elongation. It has been shown that only the first 2\(\mu\)m are involved in cell wall synthesis. Beyond this there is a region of DNA production and segregation of daughter nuclei which is known to extend about 25 to 30\(\mu\)m from the tip (Schumann & Bergter, 1976; Kummer & Kretschmer, 1986; Kretschmer, 1987). Thus true growth and primary metabolism in an actinomycete hypha probably occurs primarily in the first 30\(\mu\)m. This length agrees closely with the figure obtained for the width of the peripheral growth zone (Trinci, 1971) for actinomycete colonies growing on solid media. Kretschmer (1988) estimated the width of the peripheral growth zone to be 25\(\mu\)m for Streptomyces granaticolor, and Allan & Prosser (1983) measured values between 40 and 50\(\mu\)m.

Thus in an actively growing mycelial fragment the portion of its biomass in the growth phase will be the sum of the biomass occurring within 30\(\mu\)m of a tip. This idea would suggest that the faster an organism grows, the more branched it must become, and thus the length of the hyphal growth unit would be expected to decrease (if we ignore other influences on the morphology such as fragmentation due to agitation). There have been various reports on the effect of growth rate on the hyphal growth unit length of filamentous organisms. For example, Reisenberg & Bergter (1979) observed a decrease in the length of the hyphal growth unit with increased growth rate in glucose-limited cultures of Streptomyces hygroscopicus. The situation for growth under nitrogen-limitation for two Streptomyces spp was found to be more complex (Kretschmer, 1985). A point worth noting is that these studies used chemostats with a working volume of only 200ml, and that only 30 to 50 mycelial fragments were examined at each dilution rate. These techniques may have led to considerable errors in maintenance of a steady state and reliable estimation of the hyphal growth unit, respectively, which are not
elaborated upon by the authors. The conclusions to be drawn from these reports is that the morphology and branching patterns of streptomycete mycelia depend upon growth rate and also the nature of the limiting nutrient, the particular species being studied, and the culture conditions (Prosser & Tough, 1991).

In the regions further back from the tip, the 'mature' regions, a different physiology is observed. Radiolabelled incorporation studies have shown that DNA synthesis is still occurring, although at a lower rate than in the first 30µm (Gray, 1987). It seems likely that the regions occurring more than 30µm from the tip are those responsible for secondary metabolism. Beyond 100µm the hypha becomes less metabolically active. The reason why this should be so is not known, but may be due to the fact that the uptake in nutrients is restricted as the hypha becomes more mature (possibly due to alterations in the cell wall). This would suggest that there may be a concentration gradient of essential nutrients along the length of a hypha, and that this gradient of nutrients is ultimately responsible for switching on the appropriate secondary metabolic pathways. Which of the many primary and secondary metabolic pathways is active in a particular section of hypha will thus depend upon its distance from the tip (and thus its 'age'). Therefore one could envision a
situation where there would be a sequential expression of all the pathways passing from those associated with the synthesis of new biomass at the tip, to those associated with antibiotic production, storage material accumulation and other secondary metabolic functions, and finally ending with the shut down of metabolism and stasis in the oldest regions of the hypha.

The ideas discussed above can be better visualised in the form of a diagram as shown in figure 23. From this we can see that various dilution rates favour the equilibrium in different ways and that certain metabolic functions will have an optimum growth rate value. For example, at high growth rates the equilibrium will shift towards the growth phase and there would be very little in the antibiotic production phase and virtually none in the phases of storage material accumulation and stasis. Thus at high growth rates the steady state biomass would remain relatively constant, and this is indeed seen in figure 22. (N.B. in carbon-limited chemostat no storage materials are synthesised so the steady state biomass remains constant at all the growth rates examined). Figure 22 also suggests that the optimum growth rate for erythromycin production occurs at aout 0.04hr⁻¹. At this point we have obtained the optimum proportion of biomass in the antibiotic production phase.

4.5 Conclusions

The kinetics of production of erythromycin in chemostat culture are profoundly affected by the type of nutrient that is limiting the growth. In glucose-limited chemostat the production of erythromycin was positively correlated with specific growth rate, whereas in nitrogen-limited chemostat culture the production kinetics were growth-dissociated. This clear difference in kinetics could not be predicted from the batch culture results, giving an
indication that the conditions imposed upon the physiology of the organism in these two systems is markedly different. It is thus dangerous to extrapolate observations of physiological responses made in one type of fermentation to expectations of what should occur in the other. Batch and chemostat culture impose their own quite different physiological stresses upon the organism in addition to nutritional and other factors, and it seems quite likely that these differences will be further confused or accentuated by the heterogeneity of the filamentous lifestyle together with the specificities of the individual metabolism of the producer.
Chapter 5.
Heterogeneity and Hyphal Subpopulations

5.1 Introduction
5.2 Resolution of a culture into two subpopulations
5.3 Variation in subpopulations during nitrogen-limited batch growth
5.4 Comparison of subpopulations as inocula for batch growth
5.5 Conclusions
5.1 Introduction

In chapters 3 and 4 it was proposed that the processes of growth and antibiotic production were spatially separated along the length of the actinomycete hypha. Ideally, this could best be proven by directly visualising the site of antibiotic production in growing hyphae. Unfortunately there are currently no techniques that could be used to unequivocably demonstrate that an antibiotic was being produced at a certain point along the hypha. For example, monoclonal antibodies could be raised to certain epitopes on the enzyme complex erythronilide synthetase, however, the presence of such a label in hyphal preparations would not indicate that the enzyme complex was actually active only that it was present. During the course of these studies the direct visualisation of erythromycin synthesis using Raman Infra-red Spectroscopy was briefly examined (data not shown). Although we were able to detect the presence of erythromycin in *Saccharopolyspora erythraea* cultures, the maximum resolution of the system was limited to about 10µm, making precise measurements, such as those required to scan individual hyphae, impossible.

Thus, in the absence of direct methods, more indirect methods of demonstrating the heterogeneity of filamentous cultures had to be used. This chapter outlines the use of a simple filtration to separate the culture into two subpopulations with differing fragment size distributions. This technique was used to monitor the changes in the relative amounts of the two subpopulations during batch growth and also to provide an idea of the ability of each to produce erythromycin.

5.2 Resolution of a culture into two subpopulations

The variation in the hyphal subpopulations was examined during a nitrogen-limited batch fermentation. The resolution of the culture into two sub-populations based on fragment size was achieved by filtration across a
muslin filter. This technique was developed from some earlier work by Pickup et al (1993) in this group and is described below.

The muslin (coarse) filter was made up of four layers of muslin, each of which had an average pore size of 264µm by 214µm (Pickup et al, 1993). The coarse filter was dried by heating in a microwave oven (maximum power for 6 minutes) and allowed to cool to constant mass under desiccation and then weighed. At each sample point 10ml of culture broth was pipetted onto the coarse filter and the biomass washed with 3x10ml aliquots of RO water. The biomass in the filtrate was then collected on a predried, preweighed Whatman filter with a pore size of 0.45µm and washed with 3x10ml aliquots of RO water. The coarse and fine filters were then dried to constant weight and reweighed to yield the dry weights of the retentate and the filtrate, respectively. This technique was performed in quadruplicate at each sample point with the sample deviation generally less than 10% of the sample mean.

5.3 Variation in subpopulations during nitrogen-limited batch growth

The results obtained when the technique described above was used throughout a typical batch fermentation are shown in figure 24. It can be seen that the dry weight of both the filtrate and retentate increased in the first 25 hours. After this time however, the level of biomass in the filtrate fraction remained almost constant at about 1.8 to 2.0g/l. These data can be better presented with the biomass in each fraction expressed as a percentage of the total dry weight as is shown below in figure 25.
If we make the assumption (which will be confirmed in chapter 7) that the biomass which passed through the coarse muslin filter, (i.e. the filtrate), possessed on average a smaller fragment size than that retained on the coarse filter (retentate), it can be seen that there was an increase in these smaller fragments in the early part of the culture. This was thought to reflect
fragmentation of the biomass. A similar period of fragmentation was reported in batch cultures of *Streptomyces clavuligerus* grown in a complex medium (Belmar-Beiny & Thomas, 1991). In repeat experiments, the increase in the percentage biomass in the filtrate was not as pronounced as that shown above, but the period of fragmentation was always observed. This period of fragmentation coincided with the increase in growth rate of the culture and may have a role in the acceleration of growth (possibly through increasing the possible number of e-sites, see section 3.2.2.). Reisenberg & Bergter (1979) came to a similar conclusion from their studies on the specific growth rate and morphology of *Streptomyces hylgroscopicus*.

They observed that in older regions of the hyphae the proportions of DNA, RNA, and protein of total cell dry weight were considerably less than that observed in young regions of the hyphae or in unicellular bacteria. It was suggested that this reduction in macromolecular content might be due to decay of the macromolecular synthetic pathways and/or the synthesis of substances such as cell wall constituents and storage materials. An increase in the stirrer speed at which the organism was cultivated (430rpm) resulted in an increase in fragmentation, and these fragments did not show the low level of macromolecules observed at the lower stirrer speed (60rpm). They suggested that fragmentation counteracted the continual aging process that occurred during mycelial growth by allowing new growing points to arise in older regions of the hyphae.

This idea fits in well with the observations and discussion made in an earlier section on the slow increase in specific growth rate during nitrogen-limited batch growth (see section 3.2). It was suggested that the inoculum possessed a 'morphological memory', of its previous physiological state, that prevented the growth rate of the culture from responding rapidly to a shift-up in nutritional conditions. The observations of Reisenberg and Bergter indicated that the biochemical basis of such a 'morphological memory' was
linked to the aging process in older regions of hyphae, and that the lower proportions of cell macromolecules observed in these regions may be the reason why they were not as capable of producing new e-sites as the younger regions. It seems likely therefore that the slow increase in the growth rate could be accelerated by an increase in the stirrer speed, with a subsequent increase in the fragmentation, during this early phase of growth.

Just as the predominance of smaller fragments occurred simultaneously with the increase in growth rate, the predominance of the larger fragments coincided with the production of erythromycin. Point B in figure 24 shows that the peak volumetric production of antibiotic in this fermentation occurred after 30 hours as the proportion of larger fragments in the culture is increasing. This suggested that, for nitrogen-limited cultures at least, there might possibly be some link between antibiotic production and the morphology of the culture (this link was investigated and the results are discussed in subsequent sections).

5.4 Comparison of subpopulations as inocula for batch growth

It has already been discussed that in replicate experiments of batch fermentations some variation existed in the relationship between the peak specific rates of growth and antibiotic production despite efforts to standardise the technique. This variation, it was thought, might be due to 'hidden factors' such as the status of the inoculum. Exactly what these factors could be has not yet been addressed, but the results shown in figure 25 indicate that one possible source of variation may be the relative proportions of hyphal subpopulations in the inoculum. For instance, if one assumes that the biomass in the retentate fraction has a higher specific rate of production then it can be seen that if one harvested the inoculum after 19 hours the proportion of more productive biomass (i.e. retentate fraction) would be 42%, whereas after 48 hours this proportion increases to 69%. It seems likely in
the context of previous discussion on 'morphological memory' that an inoculum that was 48 hours old would possess more regions of its mycelium per unit biomass where macromolecular synthesis had slowed. It would also suggest that this inoculum would be less able than one of 19 hours to respond rapidly to a shift-up in nutritional conditions due to a lower potential to form e-sites. In order to investigate this it was decided to perform an experiment where a culture would be separated into two subpopulations and these, together with a suitable control, would be used to inoculate a series of shake flasks. The biomass and antibiotic titres obtained would then be analysed using the partial cubic spline analysis.

**Method**

A standard glucose-limited batch fermentation (see Methods) was set up in a 2L LH 500-series vessel (working volume 1.75L) which was harvested after 48 hours to provide enough biomass for separation into the two hyphal subpopulations. A portion of the culture was separated using the coarse and fine filters as described previously. Each of the two subpopulations was then resuspended in glucose limited medium and 25ml aliquots of this suspension used to inoculate 16 x 250ml non-baffled* Erlenmeyer flasks. A portion of whole culture was collected onto fine filters and resuspended in the glucose-limited medium to provide a control for this experiment. Again 16 flasks were inoculated with 25ml of the control suspension. The 48 flasks were then incubated at 30°C on an orbital shaker shaking at 220rpm. At each of eight sample points two flasks from the filtrate, retentate and control series were harvested for dry weight and antibiotic determination (i.e. determinations were in duplicate). The results of this experiment are shown figure 26.

*N.B. non-baffled flasks were used to avoid unnecessary shear of the mycelial morphology

**Results**

The distribution of biomass existing in the two subpopulations for the control was determined and was found to be approximately equal (i.e. when
the control culture was passed through the coarse filter 49.7% of the biomass passed into the filtrate). Thus we might expect the behaviour of the control flasks to lie between that observed for each of the subpopulation.

The data shown in figure 26 appears at first sight to be quite similar, but the partial cubic spline analysis of this data shown in figure 27 shows that there are differences in the kinetics of growth and antibiotic production. The separation of the peak rates by about 12 hours for the filtrate indicated that these smaller particles required a greater time to reach maturation. The peak rates overlapped for the filtrate showing that a greater proportion of the biomass was already in the mature phase. A summary of the peak rates achieved in each set of flasks is shown below in table 10.

Table 10. Summary of the peak specific rates obtained through the partial cubic spline analysis of the data contained in fig 26.

<table>
<thead>
<tr>
<th>Source of Inoculum</th>
<th>Peak $q_{ab}$ (mg/g/hr)</th>
<th>Peak $\mu$ (hr$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filtrate (smaller fragments)</td>
<td>1.355</td>
<td>0.136</td>
</tr>
<tr>
<td>Whole Culture (Control)</td>
<td>1.406</td>
<td>0.123</td>
</tr>
<tr>
<td>Retentate (larger fragments)</td>
<td>1.570</td>
<td>0.119</td>
</tr>
</tbody>
</table>

From this table it is clear that the culture inoculated with biomass derived from the filtrate was able to grow faster than those of the control or the retentate, but they were not able to produce as much antibiotic. The peak rates of growth and erythromycin production of the control culture lie between those obtained for the two subpopulations, as one might expect if the kinetics of growth of a mycelial culture were determined by the sum of the various heterogeneous components of which it is composed.
Figure 26 The biomass and erythromycin data obtained when total culture is resolved into two subpopulations (filtrate and retentate) by a simple filtration technique and used to inoculate a series of shake flasks containing a glucose limited medium. The legend shown in fig26c applies to 26a and 26b.

Figure 27 Partial cubic spline analysis of the data shown in figure 26. It can be seen that the position of the peak rates of growth and specific antibiotic production are not constant, but are influenced by the morphology of the inoculum.
5.5 Conclusions

A growing culture of a filamentous organism was successfully split into two fractions using a simple filtration technique. The relative proportions of the two fractions or subpopulations was examined during a nitrogen-limited batch run. The growth phase of the culture was characterised by a high proportion of smaller fragments. The antibiotic production phase was characterised by an increase in the hyphal fragment size and the predominance of larger mycelia. An indirect link between size of the mycelial fragments and the ability to produce antibiotic was thus established.

In batch culture, earlier experiments showed a variable separation of the peak rates of growth and antibiotic production (chapter 3.2). The source of this variability was unknown but was suspected to be due, in part at least, to the status of the inoculum. The experiments in this chapter have shown that the temporal relationship between the peak rates can be influenced by the morphology of the inoculum. When fresh medium was inoculated with shorter fragments the culture grew rapidly but its ability to produce antibiotic was reduced. When larger fragments were used as a source of inoculum the trend was reversed with low growth rates but higher antibiotic productivity. These results again suggested a spatial separation of growth and antibiotic production.
Chapter 6.
Specific Erythromycin Production Rates of Hyphal Subpopulations.

6.1 Introduction
6.2 Resolution using sintered glass filters
6.3 Shake flask studies on erythromycin production
6.4 Variation in specific productivities
6.5 Conclusions
6.1 Introduction

In previous chapters it has been demonstrated that during batch culture of *Saccharopolyspora erythraea* the total culture may be resolved into subpopulations by a simple filtration technique, and that the proportions of these subpopulations vary throughout the culture. It has also been shown that the use of these subpopulations as an inoculum for batch growth can influence the kinetics of growth and production. The link between size and antibiotic productivity in these experiments, however, may have been largely circumstantial, or even an artifact of the filtration technique, and thus it was important to establish more directly the kinetics of the subpopulations. To do this it was necessary to isolate the fractions and to monitor the increase in antibiotic titre and biomass for each of them over a time course. This next chapter outlines the various techniques used to achieve reliable data in order to assess the specific antibiotic productivities of the filtrate and the retentate.

6.2 Resolution using sintered glass filters

The previous technique used to isolate the subpopulations, which involved passing the culture through a muslin filter, had a number of negative aspects that made it unsuitable for use in these experiments. The most important factor was the lack of homogeneity of the pore size, which could range from 30 to 250µm. It was thus decided to change the filtration system to one that would provide a more regular pore size. The ideal choice was a porosity 1 sintered glass filter with a pore size ranging from 100 to 120µm (BDH). These sintered glass filters offered a number of additional advantages over the muslin type filters in that they were easily cleaned and prepared, and they could be mounted in glass and plastic holders (BDH) and sterilised together facilitating maintained asepsis of the subpopulations. A photograph of the sintered glass filter and the holder is shown in figure 28.
Figure 28. Photograph showing the sintered glass filter and the filter holder used in these studies to resolve the culture into two fractions, the filtrate and the retentate.

A batch culture of *Saccharopolyspora erythraea* was grown in a 2L LH 500-series fermenter, containing a defined nitrogen-limited medium (glucose at 35g/l). The whole vessel was harvested 24 hours after inoculation. The
dry weight of biomass in the vessel was 1.77g/l at this time. 400ml of this culture was spun down (1000 x g, 15mins) and the supernatant harvested for later use. To prevent the blockage of the pores of the sintered glass filter the biomass was diluted 1 in 4 in 100mM MOPS buffer. 50ml aliquots of this suspension were passed onto the filter and rinsed with 3 x 10mM MOPS. The filtrate was then transferred to a sterile 1L Duran. The filter holder was inverted and the filter backflushed with 50ml 100mM MOPS. The backflushed retentate was also transferred to a separate sterile 1L Duran. This filtration and backflushing procedure was repeated until about 250mg of filtrate and retentate had been obtained. The dry weight of the filtrate, retentate, and the control (i.e. original culture harvested from the vessel) were then measured. Aliquots of each, sufficient to provide 30mg of biomass, were then collected onto sterile membrane filters (Whatman 0.45µm) and used to inoculate a non-baffled 250ml Erlenmeyer flask containing 30ml of the original culture supernatant.

6.3 Shake flask studies on erythromycin production

Thus the culture had been harvested, resolved into two fractions, and together with an unresolved control reinoculated at 1g/l into the same medium from which it was obtained. The use of the original culture supernatant ensures a minimum of physiological disturbance to the culture. A further control was added which only contained the harvested supernatant. Triplicate flasks were prepared for the filtrate, retentate, and the unresolved control at each sample point. Flasks were incubated on a shaking platform at 220rpm at 30°C, and harvested at intervals over 24 hours. The data obtained from this experiment is shown in figure 29 below.
For the first 10 hours the antibiotic production of the filtrate, retentate and control were separated. The retentate produced more erythromycin in this time than the total, which produced more than the filtrate. After 10 hours this relationship broke down, possibly due to growth and maturation which may have masked the morphological status initially imposed by the filtration. This idea would also account for the observations made in earlier experiments of this type performed in baffled flasks where the period during which there was a difference in productivities was reduced to less than 3 hours (data not shown). This observation would also agree with the suggestions of Riesenberg & Bergter (1979) that fragmentation offsets the maturation process by allowing new growing points to arise from old hyphal regions. The detail of the first 10 hours of this experiment is shown below in figure 30.
There was virtually no increase in the antibiotic titre with the filtrate subpopulation in the first 8 hours, compared with a 80% increase for the retentate and a 47% increase for the total. No biomass data was obtained in this experiment, although all the flasks were inoculated at the same concentration (1g/l), so it is difficult to make accurate estimates of the $q_p$ values for the various populations.

6.4 Variation in specific productivities

In later repeat experiments both the antibiotic and biomass concentrations were examined and the specific antibiotic productivities were calculated. The change in antibiotic titre obtained during the 12 hours together with the calculated specific antibiotic productivities of the subpopulations and the control is shown in table 11. The figures obtained for the peak $q_p$ in the previous chapter, where the muslin filter was used to resolve the filtrate and retentate, are included for comparison.
Table 11. The change in antibiotic titre obtained after incubating the various populations for 12 hours together with the calculated specific erythromycin production rates. The values from table 10 are included for comparison.

<table>
<thead>
<tr>
<th></th>
<th>Δ antibiotic (mg in 12 hrs)</th>
<th>q_p (mg/g/hr)</th>
<th>q_p (from table 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filtrate</td>
<td>50.78</td>
<td>1.286</td>
<td>1.355</td>
</tr>
<tr>
<td>Control</td>
<td>59.82</td>
<td>1.466</td>
<td>1.406</td>
</tr>
<tr>
<td>Retentate</td>
<td>67.02</td>
<td>1.740</td>
<td>1.570</td>
</tr>
</tbody>
</table>

The values obtained in this latest experiment are shown below in figure 31, where it can be seen that there is a clear separation between the specific productivities. The retentate produces 32% more antibiotic in 12 hours than the filtrate, which compares well with the figure of 38% seen in figure 30.
6.5 Conclusions

The use of sintered glass filters to resolve the biomass into filtrate and retentate gave more reliable and reproducible results than the muslin filters used in previous experiments. The degree of separation of the biomass was also increased, demonstrated by the larger separation in the $q_p$ values for the filtrate and retentate (table 12).

Using techniques designed to minimise the disturbance to the morphological status imposed by the filtration step (i.e. inoculation into the medium from which the biomass was obtained and the use of non-baffled flasks), we were able to show a clear separation in the antibiotic productivities over a 12 hour period (figure 31). When baffled flasks were used to grow the subpopulations the separation in productivities was reduced to 3 hours or less, indicating that fragmentation due to hydrodynamic forces played an important role in the kinetics of growth and antibiotic production.
Chapter 7.
Morphological analysis of subpopulations

7.1 Introduction
7.2 Methods
7.3 Population distribution of hyphal fragments
7.4 Theoretical biomass distribution
7.5 Calculation of a minimum length for production
7.1 Introduction

In chapter 6 it was demonstrated that the filtrate and the retentate possessed different growth and production kinetics. It has been proposed that the difference between the subpopulations is connected with the differing morphologies of the two fractions. The assumption that the filtrate contained fragments with a lower average size than those in the retentate will be examined in detail in this chapter. The morphology will be determined quantitatively by analysis of photographs taken of stained mycelia.

7.2 Methods

7.2.1 Preparation of material for staining

A modification of the technique of Packer and Thomas (1990) was used to prepare slides for morphological analysis. Samples of *Saccharopolyspora erythraea* were diluted 50 fold in 100mM MOPS buffer and agitated by hand for a few minutes to disperse the mycelial fragments in the suspension. Glass slides were held in a bunsen flame for a few seconds to remove moisture and grease and allowed to cool. This procedure allowed an even dispersion of mycelial suspension over the glass surface. 40µl aliquots from the suspension were spread over the surface of the slide and air dried. When dried, the slides were stained with methylene blue (0.3g methylene blue, 30ml 95%ethyl alcohol, in 100ml water) for 1 minute. The slides were then rinsed in RO water for 2 minutes and allowed to air dry for 2 hours. For each sample 6 slides were prepared.

Prepared slides were examined and photographed using a Leitz Orthoplan system. Phase contrast optics and a magnification of 200 were routinely used. To avoid operator bias, between 250 and 500 mycelial fragments were photographed for each sample. This compares favourably to previous studies of this type. Riesenberg and Bergter (1979) studied 20 mycelial trees of *Streptomyces hygroscopicus* at each sample point;
Kretschmer (1985) studied 30 to 50 *S. hygroscopicus* and *S. granaticolor* mycelial trees at each sample; Packer and Thomas (1990) analysed about 100 fragments, and a similar number were examined by Wiebe and Trinci (1991). Fields were chosen on the basis of clear, well dispersed fragments, avoiding areas where high concentrations led to the overlap of fragments, an example of a typical field is shown in figure 33. Photographic films (Ilford PanF, iso 50) were developed and printed by hand.

![Figure 33. Photograph showing a typical field of well dispersed mycelial fragments suitable for the analysis used in these studies (the scale is 1cm = 50μm).](image)

7.2.2 Morphological Analysis of Photographs

The maximum diameter of the fragments was determined simply by overlaying the photographs of the mycelial fragments with a transparent stencil which had circles of varying sizes drawn upon it. The circle diameters
ranged from 6 to 42mm with an interval of 2mm (i.e. 6, 8, 10...40, and 42mm). The scale was such that 20mm on the photographs equalled a length of 100µm on the original slide. The maximum fragment diameter was defined as the smallest circle on the stencil into which a fragment could be completely fitted. This is illustrated in figure 34.

7.2.3 Statistical Analysis

One of the most frequently used statistical tests in biology is to discover whether or not two sets of data are 'significantly' different. The statistical analysis used in this report is based on the Fisher-Behrens test (Campbell, 1974).

\[
C = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\left(\frac{\sigma_1^2}{n_1} + \frac{\sigma_2^2}{n_2}\right)}} \tag{1}
\]

Where \( \bar{x}_1, \sigma_1, \) and \( n_1 \) are the mean, sample deviation, and sample size respectively, of sample 1 and \( \bar{x}_2, \sigma_2, \) and \( n_2 \) are the mean, sample deviation,
and sample size respectively, of sample 2. This test can be accurately performed if the two samples, of possibly unequal size, are reasonably large (greater than 30) and it is assumed that the sample means are normally distributed about the population mean. The null hypothesis is that there is no significant difference between the two means of two large samples. The value of C is calculated and compared to the appropriate value of the standardised normal deviate (extract is shown below in Table 12). The interpretation of this test is that if the calculated value of C is greater than the tabulated value then the null hypothesis should be rejected and we can conclude that there is a significant difference between the sample means. The level of significance routinely used for such large samples is 99% (p=0.01).

Table 12. Extract of the t-table (Campbell, 1974) for use with the C-value.

<table>
<thead>
<tr>
<th>P=</th>
<th>0.10</th>
<th>0.05</th>
<th>0.01</th>
<th>0.002</th>
<th>0.001</th>
</tr>
</thead>
<tbody>
<tr>
<td>confidence %</td>
<td>90</td>
<td>95</td>
<td>99</td>
<td>99.8</td>
<td>99.9</td>
</tr>
<tr>
<td>C=</td>
<td>1.645</td>
<td>1.960</td>
<td>2.326</td>
<td>3.090</td>
<td>3.291</td>
</tr>
</tbody>
</table>

7.3 Population distribution of hyphal fragments

The raw data for the analysis of the two subpopulations derived from a nitrogen-limited batch fermentation is shown overleaf in table 13. The fermenter was harvested after 24 hours and resolved through a sintered glass filter with a pore size of 100 to 120µm as described in chapter 6.2. Slides of the culture were prepared, as described earlier, and photographed. These photographs then formed the basis for the following analysis, where it should be noted that the hyphal sizes are shown in millimetres. This is the scale of the photographs and not the actual sizes that would have been observed under the microscope (actual magnification = x200).
Table 13. Raw data obtained from morphological analysis of the filtrate and retentate obtained after resolution of an *S. erythraea* culture through a sintered glass filter.

<table>
<thead>
<tr>
<th>hyphal size mm</th>
<th>filtrate counts</th>
<th>retentate counts</th>
<th>hyphal size mm</th>
<th>filtrate counts</th>
<th>retentate counts</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>7</td>
<td>1</td>
<td>26</td>
<td>5</td>
<td>18</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>4</td>
<td>28</td>
<td>4</td>
<td>13</td>
</tr>
<tr>
<td>12</td>
<td>22</td>
<td>12</td>
<td>30</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>14</td>
<td>42</td>
<td>23</td>
<td>32</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>16</td>
<td>51</td>
<td>31</td>
<td>34</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>18</td>
<td>66</td>
<td>41</td>
<td>36</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>20</td>
<td>45</td>
<td>59</td>
<td>38</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>22</td>
<td>34</td>
<td>46</td>
<td>40</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>24</td>
<td>12</td>
<td>18</td>
<td>42</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total</td>
<td>300</td>
<td>284</td>
</tr>
</tbody>
</table>

7.3.1 Population distribution diagram

The raw data counts themselves are not directly comparable because of the unequal sample sizes (300 and 284), therefore the data was converted into a form where the two samples could be compared directly.

![Figure 35. Population histogram showing the distribution of hyphal fragments in the filtrate and the retentate after resolution through a sintered glass filter.](image)
Each of the counts was thus expressed as a percentage of the total observations. This converted data can be better visualised in the form of a population histogram as shown in figure 35. From this histogram it can be seen that there is a difference between the two subpopulations in terms of the distribution of hyphal fragments.

7.3.2 Statistical analysis of unweighted data

The results of the statistical analysis of the two populations is shown in table 14. The values of the sample deviation, mean, and variance are in millimetres (which reflects the sizes of the mycelial particles when measured from the photographs), but the C-statistic remains unchanged if we convert the scale measurements to those actually observed on the slide (i.e. $\mu m$).

Table 14. Values returned from the statistical analysis of the raw data contained in table 13. The values shown are in scale mm, but the conversion to $\mu m$ is shown in brackets for the means

<table>
<thead>
<tr>
<th>Statistic</th>
<th>Filtrate</th>
<th>Retentate</th>
</tr>
</thead>
<tbody>
<tr>
<td>sample deviation</td>
<td>4.15</td>
<td>5.38</td>
</tr>
<tr>
<td>sample mean</td>
<td>17.51 (=88$\mu m$)</td>
<td>20.44 (=102$\mu m$)</td>
</tr>
<tr>
<td>variance</td>
<td>17.23</td>
<td>28.99</td>
</tr>
<tr>
<td>sample count</td>
<td>300</td>
<td>284</td>
</tr>
<tr>
<td>C=7.354</td>
<td>Null Hypothesis: REJECT ($p=0.01$)</td>
<td></td>
</tr>
</tbody>
</table>

It can be seen that the value of $C$ is greater than the value of 2.326 shown in table 12, therefore we reject the null hypothesis and state that there is a significant difference between the two samples. The filtration method of resolving the culture obviously splits the hyphal fragments according to size, but it is by no means a perfect separation. There is a reasonable proportion of fragments in the filtrate that possess a diameter greater than the pore size of the filter, and similarly there are many fragments in the retentate below the
pore size that should have passed through the filter. An explanation of the presence of fragments in the filtrate that have a greater diameter than the pore size (100 to 120 µm) is that this technique only examines the longest portion of the mycelial tree. It is easy to see that a fragment may be long but relatively thin and thus its fate will be dependent upon its orientation as it passed across the filter. With the retentate, the pores of the filter may be blocked by large fragments thereby trapping smaller fragments, or entanglement may occur also preventing small fragments from passing through the filter.

7.4 Theoretical biomass distribution

The statistical analysis used so far has considered only the raw data, and thus considers the presence of a small fragment in the sample as important as the presence of a larger one. If we consider the amount of biomass associated with a particular fragment it quickly becomes apparent that on average there is very much more biomass associated with a fragment diameter of 200µm than one of 40µm. In order to consider what influence the size of the various fragments in a particular sample is having on its growth and productivity kinetics it is necessary to weight our raw data in some way to account for this difference in biomass. The ideal method to determine this would be to measure the biomass associated with each mycelial fragment using some form of image analysis. The equipment required for this analysis was not available, and thus a theoretical weighting was used. The theory behind this weighting is discussed in the next section.
7.4.1 Calculation of theoretical biomass

Assuming that the 2-dimensional mycelial forms observed on a slide are indicative of a 3-dimensional structure in submerged culture then it seems reasonable to assume that a cubic relationship may exist such that:

\[ x_d \propto d^3 \]  

(2)

where \( x_d \) = the theoretical biomass associated with a fragment of diameter \( d \).

There have been few studies examining the relationship between \( x_d \) and \( d \) in filamentous organisms, but recently Cox and Thomas (1992) used automated image analysis techniques to examine the relationship between pellet volume and diameter for *Aspergillus niger*. They reported the following relationship;

\[ V_c = \frac{\pi}{6} d_c^3 \]  

(3)

where \( V_c \) is the pellet core volume, and \( d_c \) its equivalent diameter. Thus if we assume that there is constant amount of biomass per unit volume, it seems likely that the relationship in (2) is entirely reasonable. The weighting factor used in the following analysis is based around the equation relating the diameter of a sphere to its volume;

\[ x_d = \frac{4}{3} \pi \left( \frac{d}{2} \right)^3 \]  

(4)

where \( x_d \) is the theoretical biomass associated with a mycelial fragment of maximum diameter \( d \).

7.4.2 Biomass distribution histogram

The theoretical biomass distribution obtained when this weighting factor is applied to the data in table 13 is shown in figure 36.
7.4.3 Statistical analysis of weighted data

The statistical analysis of the weighted data is shown in table 6. The values of the sample mean, sample deviation, and the variance are in theoretical biomass units (i.e. $x_d$ in equation 4). This is easily converted back to units reflecting the fragment diameter (i.e. µm) and this has been done for the sample means.

**Table 15. Values returned from the statistical analysis following the weighting of the raw data contained in table 13. The figures are in theoretical biomass units, the conversion of this into the fragment diameter is shown in brackets for the means.**

<table>
<thead>
<tr>
<th>Statistic</th>
<th>Filtrate</th>
<th>Retentate</th>
</tr>
</thead>
<tbody>
<tr>
<td>sample deviation</td>
<td>$2.9 \times 10^5$</td>
<td>$6.3 \times 10^5$</td>
</tr>
<tr>
<td>sample mean</td>
<td>$4.1 \times 10^5$ (=112 µm)</td>
<td>$6.8 \times 10^5$ (=132 µm)</td>
</tr>
<tr>
<td>variance</td>
<td>$8.5 \times 10^{10}$</td>
<td>$4.0 \times 10^{11}$</td>
</tr>
<tr>
<td>sample count</td>
<td>300</td>
<td>284</td>
</tr>
</tbody>
</table>

| C=9.55          | Null Hypothesis:  | REJECT (p=0.01)  |

Using the non-weighted data (relating solely to the size of the mycelial fragments) values of 88 and 102 µm were obtained for the mean particle
diameter for the filtrate and retentate respectively. Using the weighted data, i.e. when the theoretical biomass associated with each mycelial fragment is averaged for each subpopulation, the average theoretical biomass has a diameter of 112 µm and 132 µm for the filtrate and retentate, respectively. The necessity of using the weighted analysis to give an indication of biomass, rather than size, is important and will be discussed in the next section. The statistical analysis has shown that for both weighted or unweighted data there is a significant difference between the two subpopulations.

The previous chapter dealt with the fact that there appears to be a significant difference between the specific antibiotic productivities of the filtrate and the retentate. How the hyphal fragment size affects the antibiotic productivity of the culture may be linked in some way to the heterogeneous nature of the hypha. If the model suggested in figure 23 (chapter 4.4) has any validity, then one might expect that the retentate subpopulation was capable of producing more antibiotic because the fragments were larger and thus had a higher specific quantity of biomass in the mature productive phase. Therefore in an actinomycete culture, which contains a variety of fragments of different sizes, it seems likely that the larger fragments are capable of producing more erythromycin than the smaller fragments. This might be due to the spatial separation of primary and secondary metabolic pathways. The growing tips where primary metabolism is at its peak would represent a larger fraction of the biomass in a small fragment than in a larger fragment.

7.5 Calculation of a minimum length for production

If the ideas explained above are extended further, it becomes evident that in a growing culture a hyphal fragment must exceed a certain size before it reaches maturity and becomes capable of producing antibiotic. Given the data for the productivities of the two subpopulations, together with the
theoretical biomass distribution of the various hyphal fragments, it should be possible to calculate the diameter that a fragment must reach before it starts to make a significant contribution to the erythromycin concentration in the fermentation. To calculate this length we have to compare the biomass distributions in some way. Firstly, the cumulative frequency of the data is calculated for the percentage biomass distribution shown in figure 36. If we plot this data on axes it is commonly referred to as an ogive, however it is more useful for this analysis to plot (100% - cumulative frequency) on the y-axis. This will be referred to as the reflected ogive, as the plot is essentially the ogive reflected in the x-axis. This plot is shown in figure 37.

Figure 37 Reflected Ogive plot. The y-axis represents the proportion of the subpopulation possessing a fragment size greater than that shown on the x-axis.

The y-axis represents the proportion of the biomass possessing a fragment diameter greater than the value shown on the x-axis. For example, the graph shows us that 60% of the biomass in the filtrate has a fragment length greater than 90\(\mu m\), whereas more than 80% of the biomass in the retentate has a fragment length greater than 90\(\mu m\). At this diameter the ratio
of filtrate to retentate biomass distributions is 60% divided by 80% or 0.75, at 120µm the ratio becomes 0.22. In comparing the proportions of biomass, the ratio of 0.75 means that the filtrate only possesses 75% as much biomass above 90µm as the retentate. Thus we are able to compare the ratios of the biomass distribution by dividing the reflected ogive of the filtrate by that for the retentate. Figure 38 shows the comparison over the whole range of fragment diameters.

This plot is particularly useful because it allows us to compare the ratio of the biomass distribution with the ratio obtained for the specific antibiotic productivities. The \( q_p \) values for the filtrate and the retentate were 1.286 mg/g/hr and 1.740 mg/g/hr, respectively. The ratio of these two values yields the figure 0.739. This figure can then be compared with the biomass ratio to give a fragment diameter at which the ratio is the same as that of the
erythromycin productivities. The logic behind this comparison can be validated algebraically:

Assuming that the specific yield of product per gram of producing biomass \(x\) is the same in the retentate and the filtrate (thus the different production capability of the two subpopulations is simply a reflection of their different proportions of producing biomass), then

Let \(A\) = amount of producing biomass in 1g of filtrate,
Let \(B\) = amount of non-producing biomass in 1g filtrate,
Let \(C\) = amount of producing biomass in 1g of retentate, and
Let \(D\) = amount of non-producing biomass in 1g of retentate.

The specific antibiotic production of the filtrate

\[ q_p(\text{filtrate}) = \frac{Ax}{A+B} \]  

(1)

The specific antibiotic production of the retentate

\[ q_p(\text{retentate}) = \frac{Cx}{C+D} \]  

(2)

It can thus be seen that the ratios of the productivities

\[ \frac{q_p(\text{filtrate})}{q_p(\text{retentate})} = \frac{Ax}{Cx} \left( \frac{A+B}{C+D} \right) \]  

(3)

From the data in table 11, it can be seen that numerically this ratio is equal to

\[ \frac{1.355}{1.750} = 0.739 \]

In figure 37, at the minimum fragment diameter for production \(d_{\text{min}}\), assuming that there is such a thing, the ratio of the filtrate biomass greater than \(d_{\text{min}}\) to the retentate biomass greater than \(d_{\text{min}}\) is equal to

\[ \frac{A}{C} \]  

(4)

Thus figure 38 shows all the values of this ratio for values of \(d_{\text{min}}\) between 40\(\mu\)m and 220\(\mu\)m. Further, it can be seen that in equation (3)

\[ [A+B] = [C+D] = 1g \]
Therefore equations (3) and (4) are, in fact, numerically equal. Thus at $d_{\text{min}}$ the ratio of the filtrate to retentate biomass equals the ratio of the antibiotic productivities which equals 0.739. If we know the ratio of biomass at $d_{\text{min}}$ we can use this to calculate the actual value of $d_{\text{min}}$ itself.

This analysis is shown in figure 38, and the value of $d_{\text{min}}$ obtained for a ratio of 0.739 is 88µm. Therefore, at the time of sampling, it seems likely that the fragments with diameters less than 88µm were making no significant contribution to the antibiotic production in the culture. It should be noted that this diameter will probably vary with growth rate and various nutritional parameters throughout a batch run, but it does provide further indirect evidence for maturation and the spatial separation of growth and antibiotic production.

7.6 Conclusions

Morphological analysis has confirmed the earlier assumption that there was a significant difference in the size distributions of the two subpopulations. The biomass in the filtrate contained mycelial fragments that had a lower mean diameter than those in the retentate (88µm compared with 102µm). In the previous chapter it was demonstrated that the biomass in the filtrate had a lower $q_p$ value than the retentate. Together these results indicate that there is a link between morphology and antibiotic production.

By weighting the raw data to account for the exponential relationship between a fragment's size and its relative biomass, it was possible to construct a theoretical biomass distribution for each of the subpopulations. Using this data, and the specific antibiotic productivities measured in chapter 6, it was possible to calculate a minimum length below which the mycelial fragments were making no significant contribution to the antibiotic production in the culture.

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Chapter 8. Continuous Selective Recycle Studies

8.1 Introduction
8.2 Method
8.3 Selective biomass recycle
8.4 Non-selective biomass recycle
8.5 Conclusions
8.1 Introduction

In previous chapters indirect evidence has been presented to link the morphology of *Saccharopolyspora erythraea* with the production of erythromycin. The experiments in this chapter were designed to attempt to exploit the difference in specific productivities of larger and smaller hyphal fragments, by continuously enriching the culture with the supposedly higher producing fraction. The separation of the biomass into two fractions had previously been achieved by filtration, but the extension of this technique to the continuous filtration across a membrane with a suitable pore size (120µm) presents considerable logistic problems such as blockage, growth on the filter, maintenance of sterility, as well as the general design and construction of such a piece of equipment. A simpler option was to use a gravitational sedimentation system and attempt to achieve some selectivity in terms of hyphal size through the action of gentle mixing and settling-out. Gravitational sedimentation has been used during continuous alcoholic fermentation to achieve efficient cellular recycling (Maia & Nelson, 1993), although no selectivity was required in this application.

The aim of these studies was to set up a nitrogen-limited chemostat where a high proportion of the culture would be removed continuously and transferred to a separating device. The separator would split the culture into two fractions, based on hyphal size, and the fraction containing the larger fragments would be fed back into the vessel. The other fraction, containing the lower producing fragments, would be transferred to the waste vessel. The method used to achieve this continuous recycling is discussed in detail in the next section. The results contain not only the data for biomass and erythromycin titres obtained, but also the results of a detailed investigation into the morphology (based on the techniques developed in chapter 7) of the fractions yielded by the separator and by a non-selective control vessel.
8.2 Method

8.2.1 Fermenter set-up.

The configuration of the vessel itself was the same as that for a normal nitrogen-limited chemostat (see chapter 2.3.2), with the addition of a tube below the culture surface. A continuous stream of culture was therefore removed from the vessel and transferred to a separator, where the biomass was selectively split into two streams. One stream (referred to as the 'dilute' stream) was enriched with smaller, less productive biomass and transferred to waste. There was also a return-line, from the separator back to the vessel, which contained the higher producing mycelia. A simplified diagram of the system is shown in figure 39 below, and a photograph of the system is shown overleaf in figure 40.

![Diagram of apparatus](image-url)

Figure 39. Diagram of the apparatus used for continuous selective recycle. Culture is continuously removed from the vessel and pumped to the column separator. The biomass is split into two fractions by the combined action of gentle mixing by small air-bubbles travelling up the side of the column and sedimentation due to gravity. The fraction leaving the base of the separator, containing the larger fragments, is returned to the vessel.
Figure 40. Photograph of the apparatus used to in the continuous selective recycle experiments. Culture is continuously removed from the vessel and pumped into the column separator (on the right) where it is split into two fractions with differing hyphal size distributions.
The total flow \( (F) \) into the vessel is the sum of the medium flow rate \( (F_1) \) and the flow rate of the return stream \( (F_4) \). The flow out of the system is equal to the flow rate of the dilute stream \( (F_3) \), therefore the dilution rate \( (D) \) in this system can be calculated from:

\[
D = \frac{F_3}{V_v + V_s}
\]

where \( V_v \) and \( V_s \) equal the volume of the culture in the vessel and the separator, respectively. Using the equations suggested by Pirt (1975) for continuous biomass recycle it is possible to calculate the specific growth rate of the organism, but this was not seen as necessary for this particular study. However, it should be noted that the specific growth rate will be lower than the dilution rate at steady state, and the ratios of the two will depend upon the degree of concentration of the biomass in the return stream \( (F_4) \). As the concentration of the biomass was minimal the growth rate and the dilution rates were relatively similar (e.g. when \( D = 0.054 \text{hr}^{-1} \) in the non-selective recycle control, \( \mu = 0.049 \text{hr}^{-1} \)).

8.2.2 Selective column separator

A photograph of the separator is shown overleaf in figure 41. The biomass from the vessel entered the column through the side-arm located in the middle of the column. A significant amount of air was also carried along with the biomass which, as the bubbles rose to the surface, added a gentle mixing action to the upper portion of the separator. The action of gravity on the fragments caused them to settle out to the bottom of the separator, and thus the flow of biomass into the separator was kept high to prevent too much settling out. This also minimised the effects of nutrient starvation and oxygen limitation. Mean residence times were between 15 and 80 minutes depending upon the dilution rate at which the chemostat was operated.
Figure 41. Photograph showing the column separator used in these studies to continuously split the biomass into two fractions each containing a different hyphal fragment distribution. Biomass enters through the centre of the column and is returned to the vessel from the bottom. The dilute stream, containing fragments of smaller size, is removed from the top side of the separator and pumped to a calibrated waste vessel.
8.2.3 Non-selective control vessel

A set of control experiments were performed to establish whether any change in antibiotic titre was actually due to manipulation of the morphology of the biomass, or due to other factors such as the nutrient shift-down, oxygen limitation, etc. that must occur in the separator. Thus, a stirred aspirator, calibrated to the same volume as the separator, was used instead of the column separator in the control experiments. It was expected that the two streams of biomass leaving this control vessel would not have any difference in their fragment population distributions.

8.3 Selective biomass recycle

8.3.1 Biomass and antibiotic productivities

Steady states were achieved at two dilution rates (0.054hr\(^{-1}\) and 0.112hr\(^{-1}\)) and the resulting steady state biomass antibiotic and erythromycin concentrations were calculated and are shown in table 16. In repeat experiments similar values and the same trends were observed. For comparison the corresponding values obtained during nitrogen-limited chemostat culture are included. Increasing the dilution rate to 0.15hr\(^{-1}\) resulted in the slow washout of the culture and thus no data is presented from that fermentation.

<table>
<thead>
<tr>
<th></th>
<th>D=0.054</th>
<th>D=0.112</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steady state biomass concentration (g/l)</td>
<td>1.44</td>
<td>1.14</td>
</tr>
<tr>
<td>Steady state antibiotic concentration (mg/l)</td>
<td>21.8</td>
<td>9.83</td>
</tr>
<tr>
<td>Specific antibiotic productivity (mg/g/hr)</td>
<td>0.818 (1.00)</td>
<td>0.966 (0.60)</td>
</tr>
</tbody>
</table>
As expected, the biomass concentration increased at low dilution rates for the reasons discussed earlier (chapter 4). The steady state erythromycin concentration decreased with increasing dilution rate as it did in unenriched chemostat culture. Interestingly however, the highest $q_p$ value was obtained at the highest dilution rate, contrary to the trend shown in previous unenriched chemostat culture. The morphological analysis below may offer some explanation for this observation.

8.3.2 Population and theoretical biomass distributions

The population histogram in figure 42 shows the unweighted data obtained at the lower dilution rate using the morphological analysis described in chapter 7.

![Figure 42. Fragment size distribution histogram for selective biomass recycle at D = 0.054 hr⁻¹](image)

It can be seen that there is some difference between the two populations, where the percentage total count is higher for the filtrate than the vessel below 20mm, above this figure the reverse is the case. Whether this visible
difference is statistically significant will be shown later. The calculated theoretical biomass distribution is shown in figure 43, again there is a visible difference between the two distributions. This would indicate that the separator was having a significant selective effect on the fragments which are returned to the vessel.

The fragment size distribution obtained for the higher dilution rate, in figure 44, showed a much clearer separation than that obtained for the low dilution rate. This may have been due to the differing residence times for the two systems which were 80 and 14 minutes for the low and high dilution rates, respectively. To investigate this, the lower dilution rate was repeated with a residence time of only 40 minutes. In this case the steady state biomass was lower (1.34g/l compared with 1.44g/l), as was the steady state antibiotic concentration (16.0mg/l compared with 21.8mg/l) giving an overall lowering of the q_p value (0.693 compared with 0.818mg/g/hr). Therefore decreasing the residence time per se does not necessarily lead to an
increase in antibiotic titre. The theoretical biomass distribution calculated from the higher dilution rate is shown in figure 45.

**Figure 44. Fragment size distribution histogram for selective biomass recycle at D = 0.112 hr⁻¹**

![Fragment size distribution histogram](image)

**Figure 45. Theoretical biomass distribution for the hyphal populations in the dilute stream and the vessel at D = 0.112 hr⁻¹**

![Theoretical biomass distribution](image)
In comparison with the distributions shown for the lower dilution rate (figures 42 and 43) it can be seen that there is a much clearer separation of the two biomass populations. However, the degree of separation is best detected through statistical analysis, and this will be shown below.

8.3.3 Statistical analysis

The analysis was performed on both the unweighted and weighted data, i.e. to assess whether there was a significant difference in the populations of the dilute stream and the vessel both in terms of the fragments observed and the theoretical biomass associated with them (tables 17 and 18, respectively). The null hypothesis was that there was no significant difference between the means of the two populations, therefore rejecting this indicated that there was a significant difference.

Table 17. Statistical analysis of morphological data obtained during a nitrogen-limited continuous selective recycle fermentation at $D=0.054\text{hr}^{-1}$.

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Dilute stream</th>
<th>Vessel</th>
<th>Dilute stream</th>
<th>Vessel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(unweighted)</td>
<td>unweighted</td>
<td>weighted</td>
<td>weighted</td>
</tr>
<tr>
<td>Count</td>
<td>423</td>
<td>340</td>
<td>423</td>
<td>340</td>
</tr>
<tr>
<td>Skew</td>
<td>0.35</td>
<td>-0.03</td>
<td>2.3</td>
<td>1.6</td>
</tr>
<tr>
<td>Mean</td>
<td>17.69mm</td>
<td>20.14mm</td>
<td>4.7x10$^5$</td>
<td>6.6x10$^5$</td>
</tr>
<tr>
<td></td>
<td>(88$\mu$m)</td>
<td>(101$\mu$m)</td>
<td>(96$\mu$m)</td>
<td>(108$\mu$m)</td>
</tr>
<tr>
<td>Sample comparison</td>
<td>C-statistic =</td>
<td>6.008</td>
<td>Null hypothesis</td>
<td>Reject</td>
</tr>
<tr>
<td></td>
<td>Null hypothesis</td>
<td></td>
<td>Reject</td>
<td></td>
</tr>
</tbody>
</table>

Table 18. Statistical analysis of morphological data obtained during a nitrogen-limited continuous selective recycle fermentation at $D=0.112\text{hr}^{-1}$.

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Dilute stream</th>
<th>Vessel</th>
<th>Dilute stream</th>
<th>Vessel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(unweighted)</td>
<td>unweighted</td>
<td>weighted</td>
<td>weighted</td>
</tr>
<tr>
<td>Count</td>
<td>497</td>
<td>494</td>
<td>497</td>
<td>494</td>
</tr>
<tr>
<td>Skew</td>
<td>0.11</td>
<td>0.02</td>
<td>1.7</td>
<td>1.2</td>
</tr>
<tr>
<td>Mean</td>
<td>17.88mm</td>
<td>23.07mm</td>
<td>4.5x10$^5$</td>
<td>9.4x10$^5$</td>
</tr>
<tr>
<td></td>
<td>(89$\mu$m)</td>
<td>(115$\mu$m)</td>
<td>(95$\mu$m)</td>
<td>(122$\mu$m)</td>
</tr>
<tr>
<td>Sample comparison</td>
<td>C-statistic =</td>
<td>14.412</td>
<td>Null hypothesis</td>
<td>Reject</td>
</tr>
<tr>
<td></td>
<td>Null hypothesis</td>
<td></td>
<td>Reject</td>
<td></td>
</tr>
</tbody>
</table>
The statistical analysis showed that there was a significant difference (p=0.01) in the distribution of the hyphal fragments and theoretical biomass at both dilution rates. The column separator was therefore achieving the desired aim of separating the biomass, and enriching the return stream with fragments of a larger size. This separation was better (i.e. a higher C-statistic was observed) at the higher dilution rate, perhaps owing to the decreased residence time, and resulted in a specific productivity higher than that observed for chemostat culture at a similar dilution rate (see table 16). Whether this increase in productivity was due to the morphology or some other aspect of the recycle system can only be determined by comparison with the non-selective recycle experiment. It should also be noted that the skew of the data is generally positive, an observation also reported for studies on the distribution of the main hyphal length of *Streptomyces clavuligerus* in batch culture (Belmar-Beiny & Thomas 1991). Another interesting observation is that the mean fragment diameter of the dilute streams were virtually identical at 88µm and 89µm for the low and high dilution rates, respectively.

### 8.3.4 Calculation of percentage producing biomass

Using the data obtained by calculating the theoretical biomass it is possible to make a comparison of the relative potential of the biomass in the vessel and dilute stream to produce erythromycin. In chapter 7, it was calculated that the minimum size that a hyphal fragment had to reach before it made a significant contribution to the antibiotic production in the culture was 88µm. This figure can be used to calculate the percentage of the biomass in a particular population that is producing erythromycin, assuming that all fragments below this size do not make a contribution.
Table 19. Calculation of percentage producing biomass made by assuming that all biomass below 88µm is making no significant contribution to antibiotic concentration in the vessel.

<table>
<thead>
<tr>
<th></th>
<th>Dilute stream</th>
<th>Vessel</th>
</tr>
</thead>
<tbody>
<tr>
<td>D=0.054hr⁻¹</td>
<td>84.2%</td>
<td>92.4%</td>
</tr>
<tr>
<td>D=0.112hr⁻¹</td>
<td>87.3%</td>
<td>97.2%</td>
</tr>
</tbody>
</table>

The use of the 88µm minimum length is not really applicable to the lower dilution rate as it was calculated in chapter 7 at a high specific growth rate, but it is included as a guideline for comparative purposes. To summarise, the separating column was able to split the culture into two fractions with significantly different hyphal fragment distributions. However, when the fraction with the larger mean fragment diameter was fed back into the vessel, conflicting results were obtained. At low dilution rates, the specific productivity was lower than that achieved in chemostat. At the higher dilution rate the q_p value was much higher than that achieved in chemostat at the same dilution rate. It was suspected that the lower residence time in the separator might be responsible for the higher yields of antibiotic at the high dilution rate, possibly by increasing the separation between the fractions resulting in a greater enrichment in larger fragments in the vessel. Repeat experiments however, showed that decreasing the residence time did not lead to an increase in specific antibiotic production, indicating that the residence time per se was not responsible for the observed differences in antibiotic yield.

8.4 Non-selective biomass recycle

The increase in productivity observed at the higher dilution rate in the selective biomass recycle might have been due to the enrichment of the culture with larger, more productive fragments or it might equally have been
due to other factors related to the shift-down that may occur in the separator. This section reports the use of a stirred aspirator in place of the separating column. If enriching the culture with larger, more productive mycelial fragments led to an increased production of antibiotic one would predict that with non-selective recycle the yields should be lower than those observed previously.

8.3.1 Biomass and antibiotic productivities

As in the selective recycle experiments, two steady states were obtained at 0.053 and 0.107hr⁻¹ (table 20). The same trend found in the selective recycle of decreased steady state biomass and antibiotic concentrations with increased specific growth rate was observed. In repeated experiments similar values were obtained for $q_p$ at the high and low dilution rates.

<table>
<thead>
<tr>
<th></th>
<th>$D = 0.053$</th>
<th>$D = 0.107$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steady state biomass</td>
<td>1.22 (1.44)</td>
<td>1.17 (1.14)</td>
</tr>
<tr>
<td>concentration (g/l)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Steady state antibiotic</td>
<td>21.0 (21.8)</td>
<td>17.0 (9.7)</td>
</tr>
<tr>
<td>concentration (mg/l)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Specific antibiotic</td>
<td>0.912 (0.818)</td>
<td>1.555 (0.966)</td>
</tr>
<tr>
<td>productivity (mg/g/hr)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Contrary to our expectations, the specific productivities were significantly higher than those obtained using selective recycle. If the morphological analysis shows that there was no difference in the fragment distributions of the dilute stream and the vessel, it would indicate that the morphology of the culture was not playing a significant role in antibiotic production in this system.
8.3.2. Population and theoretical biomass distributions

Figure 46. Fragment size distribution histogram for non-selective biomass recycle at $D = 0.053$ hr$^{-1}$

Figure 47. Theoretical biomass distribution for non-selective biomass recycle at $D = 0.053$ hr$^{-1}$

It can be seen that there is virtually no difference in the distributions in the dilute stream and in the vessel when the stirred aspirator is used in place of the column separator.
When the data in figures 43 and 44 was compared with data obtained for the selective recycle fermentations at this dilution rate (figures 39 and 40) it could be seen that the separation of the biomass into two streams with
differing hyphal populations had not occurred to the extent that was observed with the separator. Thus, the use of the stirred aspirator to yield two identical fractions of biomass has been successful.

8.3.3 Statistical analysis

The data from the low and high dilution rates using the non-selective recycle is analysed and tabulated below. The mean particle sizes for the dilute stream and the vessel are much closer than that observed in the selective recycle experiments (tables 17 and 18), e.g. at the high dilution rate the weighted means for non-selective recycle were 107 and 105µm for the dilute stream and vessel, respectively, compared with 95 and 122µm for the selective recycle. The C-statistics for the non-selective recycle are all lower than those observed with the selective recycle demonstrating that there was less separation of biomass based on size in the former. The was some separation in the non-selective recycle, however, which was demonstrated by the rejection of the null hypothesis for the low dilution rate (table 21).

Table 21. Statistical analysis of morphological data obtained during a nitrogen-limited continuous non-selective recycle fermentation at D=0.053hr⁻¹.

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Dilute stream</th>
<th>Vessel</th>
<th>Dilute stream</th>
<th>Vessel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(unweighted)</td>
<td>unweighted</td>
<td>weighted</td>
<td>weighted</td>
</tr>
<tr>
<td>Count</td>
<td>446</td>
<td>456</td>
<td>446</td>
<td>456</td>
</tr>
<tr>
<td>Skew</td>
<td>0.13</td>
<td>0.34</td>
<td>1.7</td>
<td>1.7</td>
</tr>
<tr>
<td>Mean</td>
<td>18.03mm</td>
<td>19.00mm</td>
<td>4.9x10⁵</td>
<td>5.7x10⁵</td>
</tr>
<tr>
<td></td>
<td>(90µm)</td>
<td>(95µm)</td>
<td>(98µm)</td>
<td>(103µm)</td>
</tr>
<tr>
<td>Sample comparison</td>
<td>C-statistic = 2.609</td>
<td>Null hypothesis Reject</td>
<td>C-statistic = 2.60</td>
<td>Null hypothesis Reject</td>
</tr>
</tbody>
</table>
8.4.4 Calculation of percentage producing biomass

The theoretical percentage producing biomass (table 23) was lower, as expected, for the control experiments. This decrease in productive biomass was not accompanied by the predicted decrease in antibiotic production.

Table 23. Calculation of percentage producing biomass made by assuming that all biomass below 88µm is making no significant contribution to antibiotic concentration in the vessel.

<table>
<thead>
<tr>
<th>Dilute stream</th>
<th>Vessel</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>D=0.054hr⁻¹</strong></td>
<td></td>
</tr>
<tr>
<td>85.95% (84.2)</td>
<td>87.79% (92.4)</td>
</tr>
<tr>
<td><strong>D=0.112hr⁻¹</strong></td>
<td></td>
</tr>
<tr>
<td>91.15% (87.3)</td>
<td>90.15% (97.2)</td>
</tr>
</tbody>
</table>

The results indicate that the enrichment of the chemostat with larger fragments was, if anything, having a deleterious effect on the antibiotic production of the culture. However, it is difficult to separate other physiological effects, such as nutrient and oxygen limitation, that the column separator and the control were having on the biomass. Interestingly, the control vessel at the high dilution rate had a higher specific antibiotic
productivity than the nitrogen-limited chemostat at its most productive. This indicated that the action of continuously removing a portion of the culture to a separate vessel, where there was no feed of nutrients, had a beneficial effect on the culture's antibiotic productivity. This may have been due to the shift-down that would have occurred in the absence of nutrients. The recycle system used here may be acting more as a two stage chemostat (with recycle from stage 2 back into stage 1) rather than an ideal single stage chemostat with recycle. As this system appeared to be potentially very productive, it would be of interest to base future investigations around the kinetics of a two stage system.

8.5 Conclusions

The use of the column separator was a success in terms of splitting the culture into two fractions with differing hyphal size distributions. The enrichment of a nitrogen-limited chemostat with larger, supposedly more productive fragments, did not result in an increase in the specific productivity. The reasons for this were unknown but may be linked with the different physiological state existing in cultures at steady state compared with that observed in batch culture (see chapters 3 and 4).

The use of a stirred, control vessel, which gave little separation of fragments in the two fractions, gave high specific productivities when compared with both the selective recycle studies and the earlier chemostat studies. This may be an indication that the use of this method of recycle imposed a shift-down on the culture and that the effects of this may have swamped any effects due to morphology.
Chapter 9.
The effect of stirrer speed on morphology and production in chemostat culture

9.1 Introduction
9.2 Specific antibiotic production rates
9.3 Morphological analysis
9.4 Conclusions
9.1 Introduction

It was concluded in the previous chapter that the use of an external vessel to manipulate morphology and detect subsequent changes in productivity, was being influenced more by nutritional aspects, associated with the removal of the culture from the vessel, than by any morphological changes. It was thus decided that any manipulation of the morphology must occur within the chemostat, where nutritional conditions could be controlled to a greater extent. These experiments report the use of a high and low stirrer speed at a range of dilution rates to achieve a different population distribution at steady state.

There has been little work published on the effects of stirrer speed on the growth and productivity of actinomycetes. Tarbuck et al (1985) reported that batch fermentations of Streptomyces clavuligerus showed a dramatic decrease in clavulanic acid production when the stirrer speed was increased from 375 to 500rpm. More recently, Belmar-Beiny & Thomas (1991) also examined the effect of stirrer speed on clavulanic acid production. They found that although the stirrer speed altered the morphology, they detected no increase in clavulanic acid titre and concluded that there was no direct link between morphology and productivity. The results they presented in this paper however, do contain evidence that morphology and productivity were linked. The data from two (of three) replicate batch runs (referred to here as Run 1 and Run 2) under the same conditions is re-evaluated below in figures 50 and 51 overleaf.

It can be seen that the biomass levels in the two batch fermentations are approximately equal (figure 50). The clavulanic acid titres obtained from the two runs are very different however, with Run 2 producing only 56% as much as Run 1. Both of these fermentations were inoculated with spores from a single master stock, and both were run under the same fermentation
conditions, but the biomass in batch fermentation 1 is producing almost twice as much clavulanic acid as the biomass in batch fermentation 2.

Figure 50. Biomass and clavulanic acid titres obtained in two separate batch fermentations stirred at 990rpm (Data from Belmar-Beiny & Thomas, 1991)

Figure 51. The length of the main hypha measured during two duplicate batch fermentations (Data from Belmar-Beiny & Thomas, 1991)
This would indicate that there was something different about the biomass in the two batch fermentations. An indication of the difference was shown by their data on the length of the main hypha measured during each fermentation (figure 51). It can be seen that the main hyphal length in Run 1 at about 50 hours was almost twice that observed in Run 2, and it remained higher for many hours after this point. This would indicate that there was quite possibly some link between morphology and production in this fermentation. These results also indicated that, contrary to the authors' assumption, the use of a spore inoculum did not lead to a more reproducible morphological or physiological status in the subsequent culture. In this laboratory we have found that the use of an actively growing vegetative culture provided a much more reliable source of inoculum.

The papers of Metz et al (discussed earlier in chapter 1.3.3) and Belmar-Beiny & Thomas indicate that stirrer speed itself might not be having the effect on morphology that the authors have suggested. Murase & Kendrick (1986) showed that only under extreme conditions of shear, such as those produced in a Waring blender, does agitation result in hyphal damage of fungi. As actinomycete hyphae are an order of magnitude smaller, they would be more resistant to the shear stresses imposed by impellers. Much of the literature on the effects of stirrer speed on morphology fail to distinguish between effects caused by hydrodynamic stresses and those caused by nutritional conditions (Prosser & Tough, 1991). Certainly in the examples cited above, some of the conclusions that the authors have drawn can be interpreted in a different manner.

The studies in this chapter examined the effect that different stirrer speeds had upon the morphology of the culture and upon the antibiotic productivity. It should be noted that the alteration in stirrer speed resulted in a different steady state culture volume. The medium flow rate was therefore altered appropriately to maintain a constant dilution rate.
9.2 Specific antibiotic production rates

The results for steady state biomass and antibiotic, together with the calculated $q_p$ values are shown below in table 24. The effect of stirrer speed was assessed at three dilution rates, and at each of these a low (650rpm) and high (1500rpm) stirrer speed were used. Steady states were assumed after 4 volume changes.

Table 24. Data obtained during nitrogen-limited chemostat culture where the stirrer speed was altered.

<table>
<thead>
<tr>
<th>Steady state</th>
<th>D = 0.08</th>
<th>D = 0.06</th>
<th>D = 0.04</th>
</tr>
</thead>
<tbody>
<tr>
<td>Values</td>
<td>750rpm</td>
<td>1500rpm</td>
<td>750rpm</td>
</tr>
<tr>
<td>Biomass (g/l)</td>
<td>2.04</td>
<td>1.92</td>
<td>2.47</td>
</tr>
<tr>
<td>Antibiotic (mg/l)</td>
<td>23.3</td>
<td>20.8</td>
<td>37.6</td>
</tr>
<tr>
<td>$q_p$ (mg/g/hr)</td>
<td>0.913</td>
<td>0.867</td>
<td>0.913</td>
</tr>
</tbody>
</table>

Overall, there was a general increase in the steady state biomass with decreased specific growth rate, a trend reported earlier (chapter 4.3). At each of the three dilution rates, the steady state biomass was lower at the higher stirrer speed, with the effect more accentuated at the lower dilution rates. As discussed earlier, it has been suggested that fragmentation counteracts the ageing process in the hypha (Reisenberg & Bergter, 1979). The data presented here appears to be in agreement with this. At low stirrer speeds, where shear stresses are lower, fragmentation of mycelia occurred at a reduced rate, and therefore more of the biomass existed further from a growing tip. Using the idea of equilibrium discussed in chapter 4 (see particularly figure 23 on page 89), we can see that the lower rate of fragmentation has shifted the equilibrium in favour of the storage material phase, and a subsequent increase in the steady state dry weight. At higher
stirrer speeds, fragmentation offset ageing, which in turn reduced the biomass capable of producing storage materials, hence the lower steady state biomass.

The specific productivity did not show such a distinct trend due to the complicating factor of the variation in steady state biomass. As this variation could be due to the increased synthesis of storage materials it is possible that it did not represent an increase in true growth. Thus, in this instance, the use of the steady state erythromycin concentration as an indicator of the productivity of the system appears to be more useful and appropriate.

The same trend observed with the steady state biomass was repeated for the steady state antibiotic concentration. Thus an overall increase in erythromycin concentration was observed at the lower dilution rates, with the lower stirrer speeds at each dilution rate giving the highest titres of erythromycin. These results can also be explained, as for the increased biomass, by the equilibrium model suggested in figure 23. If statistical analysis of the morphological data confirms the supposition that higher stirrer speeds has led to an increased fragmentation, then it adds more indirect evidence for the link between morphology and productivity, and also to the idea that secondary metabolism in filamentous actinomycetes is spatially separated along the hypha from the point of growth (i.e. the tip).

9.3 Morphological analysis

The statistical analysis results were only performed on one dilution rate (D=0.08hr-1), but microscopic observation of the cultures at the lower dilution rates showed the same results as for the highest dilution rate. Photographs of typical fields at 750rpm and 1500rpm are shown in figures 52 and 53, respectively. They illustrate the large disparity between the hyphal morphologies achieved by manipulating stirrer speed.
Figure 52. Photograph showing the morphology typically observed at the various dilution rates when the stirrer speed was 750rpm. (Scale: 1cm = 50\,\mu m)
Figure 53. Photograph showing the morphology typically observed at the various dilution rates when the stirrer speed was 1500rpm. (Scale: 1cm = 50µm)
As in previous chapters, the population distributions for the raw and weighted data are included below in figures 54 and 55.

**Figure 54. Population histogram for a nitrogen-limited chemostat using two stirrer speeds**

**Figure 55. Theoretical biomass distribution during a nitrogen-limited chemostat culture performed at two stirrer speeds**
The distributions show a clear separation of the two populations, indicating that stirrer speed has had a profound effect upon the morphology of the culture. This was confirmed by the statistical analysis shown below in table 25. The C-statistic was the largest achieved by attempting to manipulate the morphology of the culture, showing that this technique has been the most successful in achieving different morphological status in a culture under the same nutritional and physiological conditions. Thus, in these experiments, there can be little doubt that the changes in antibiotic production have occurred as a result of a change in morphology.

Table 25. Statistical analysis of morphological data obtained during a nitrogen-limited chemostat at D=0.08hr⁻¹ using two different stirrer speeds.

<table>
<thead>
<tr>
<th>Analysis</th>
<th>750rpm unweighted</th>
<th>1500rpm unweighted</th>
<th>750rpm weighted</th>
<th>1500rpm weighted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Count</td>
<td>371</td>
<td>361</td>
<td>371</td>
<td>361</td>
</tr>
<tr>
<td>Skew</td>
<td>0.03</td>
<td>0.20</td>
<td>0.95</td>
<td>1.9</td>
</tr>
<tr>
<td>Mean</td>
<td>24.74mm (124μm)</td>
<td>14.02mm (70μm)</td>
<td>1.1x10⁶ (128μm)</td>
<td>2.2x10⁵ (75μm)</td>
</tr>
<tr>
<td>Sample comparison</td>
<td>C-statistic = 30.315</td>
<td>Null hypothesis</td>
<td>Reject</td>
<td>C-statistic = 23.47</td>
</tr>
</tbody>
</table>

9.4 Conclusions

When different stirrer speeds were used during continuous culture, the morphology of *Saccharopolyspora erythraea* was strongly affected. When the stirrer speed was low (750rpm), a clumped morphology was observed (figure 52) together with an increased steady state biomass concentration, attributed to an increased synthesis of storage materials. At the higher stirrer speed, the mean fragment diameter was only 56% of that observed at the lower speed (figure 53). The production of erythromycin was reduced in these smaller fragments, particularly at low dilution rates, confirming earlier results in batch culture where the biomass in the filtrate also possessed a smaller
mean fragment diameter than those of the retentate and produced lower quantities of antibiotic. The trends observed in these experiments could be explained using the physiological model, based on the spatial separation of primary and secondary metabolism along the hypha, discussed earlier in chapter 4.
Chapter 10. Overall Conclusions
Chapter 10

The growth, productivity and morphology of *Saccharopolyspora erythraea* cultures grown in a defined medium was examined in a number of fermentation systems. Partial cubic spline analysis of measured fermentation parameters allowed the calculation of volumetric and specific rates of production. In batch culture, the specific erythromycin production rate was strongly linked to the specific rate of growth in both nitrogen- and carbon-limited media, although it was difficult to determine whether the link was with growth rate per se or with some factor that also influenced the growth rate. Continuous culture was thus used to further examine the growth/productivity relationships in this organism, and this revealed a clear separation in production kinetics between the two media. Growth-linked production of erythromycin was observed in a carbon-limited medium, whereas the kinetics were growth-dissociated using a nitrogen-limited medium.

A simple filtration technique was used to resolve cultures into fractions with differing hyphal size distributions. The biomass passing through the filter (filtrate) possessed a lower mean fragment diameter than the biomass remaining on the filter (retentate). During batch growth, the relative proportions of these fractions in the culture was observed to change with time. In the early growth phase, smaller mycelial fragments were predominant in the culture, whereas, during the later antibiotic production phase of batch growth larger fragments predominated. This observation, together with some previous work performed in this group, indicated that there may be a link between morphology and production.

This link was further enhanced by the observation that the use of filtrate or retentate biomass as an inoculum for subsequent batch growth gave rise to differences in production kinetics. The biomass in the filtrate fraction had a lower specific erythromycin production rate than the retentate biomass, but a higher specific growth rate. The peak rates of growth and production using the filtrate biomass were offset by 12 hours, whereas they
occurred simultaneously when the retentate biomass was used. Thus, the kinetics of growth and production were not determined solely by the type of nutrient limitation that the culture was grown under, but also to some extent by the status of the inoculum.

Using techniques designed to minimise the disturbance to the morphological status imposed by the filtration step, it was possible to directly measure the specific productivities of the isolated fractions. The smaller (filtrate) biomass possessed a specific antibiotic production rate of 1.286 mg/g/hr compared with 1.74 mg/g/hr measured for the larger (retentate) biomass, thus confirming the link between hyphal size and antibiotic production.

Attempts to manipulate the morphology of continuous cultures to achieve an increase in productivity gave mixed results. The use of an external column separator was effective in continuously enriching a nitrogen-limited chemostat with the larger, potentially more productive, mycelial fragments. When compared with a typical unenriched chemostat this system was found to be more productive, indicating that morphological manipulation may have led to an increase in erythromycin titre. To further examine this hypothesis the experiments were repeated with an external (control) vessel in which no enrichment of larger fragments occurred. This system gave higher specific productivities than the column separator. It was suggested that the observations made in these experiments were due more to the nutrient starvation and subsequent shift-down occurring in the external separator/control vessel than to any effects upon morphology. Thus, although the use of the external vessel gave excellent productivities, it was not deemed a suitable tool for the detection of the more subtle increases in productivity achieved with morphological manipulation. It was further suggested that this system, due to its high productivity, might be a good
candidate to study using the kinetics of a two-stage chemostat with recycle between stage 1 and stage 2.

When the morphology of the culture was altered by changing the stirrer speed between 750 and 1500rpm, in nitrogen-limited chemostat, the results obtained reflected our expectations based on earlier batch culture studies. The use of the lower stirrer speed gave rise to larger mycelial fragments, higher steady state biomass and erythromycin yields at each of three dilution rates. This difference in productivity between the two stirrer speeds was more pronounced at the lower dilution rates. This observation could be explained using the physiological model, proposed in earlier chapters, which spatially separated the processes of hyphal growth and secondary metabolism along the hyphal length.

The inherent heterogeneity of filamentous cultures has remained a theme throughout these studies. The cause of this heterogeneity is linked to the nature of hyphal extension where growth occurs at the tip and gives rise to successively more mature regions further back along the hypha. It is suggested that, in *Saccharopolyspora erythraea* cultures grown in defined media at least, the processes of primary and secondary metabolism are separated along the length of the hypha (illustrated diagrammatically in figure 56 overleaf), and that this knowledge can be used to optimise a process so as to achieve an increase in product titre.
Figure 56. Diagrammatic illustration (not to any scale) of the physiological model for growth and antibiotic production proposed for *Saccharopolyspora erythraea* growing in defined medium. The processes of primary and secondary metabolism are separated spatially along the growing hypha. At the tip hyphal elongation occurs which gives rise to successively more mature regions further back from the tip. It is in these mature regions that secondary metabolic pathways become induced and erythromycin synthesis, glycogen accumulation, etc. can occur.
References


