The effect of oxygen limitation on antibiotic production and its relevance to screening for novel secondary metabolites

Gavin John Clark  B.Sc. (Hons)

Thesis presented for the degree of Doctor of Philosophy to the School of Biological Sciences, University of Surrey

September 1993
CORRIGENDA

page 6, line 19; should read ‘single’ instead of ‘sole’
page 17, line 4; should read ‘strategies’ instead of ‘stategies’
page 19, line 2; should read ‘relies’ instead of ‘relys’
page 20, line 22; should read ‘manageable’ instead of ‘managable’
page 28, line 20; should read ‘hyphae’ instead of ‘mycelium’
page 110, line 9; should read ‘independently’ instead of ‘independantly’
page 115, line 12; should read ‘experimental’ instead of ‘experiment’
page 120, line 7; should read ‘independently’ instead of ‘independantly’
page 122, line 6; should read ‘pigment’ instead of ‘piment’
If a choice had to be made between screening natural organisms for something new and exciting versus doing genetic engineering, I would go with the screening

van Brunt and Klausner, 1987
ABSTRACT

High throughput in screening programs is achieved by using small scale cultures. An unfortunate consequence of using small scale cultures is the inability to measure oxygen status. It is therefore not known how often oxygen limitation occurs in screens, nor what effect it might have on natural product diversity. This study investigates the effect of oxygen limitation on antibiotic production and assesses its relevance to screening for novel secondary metabolites.

Dissolved oxygen tension was measured in shaken flasks and tubes with the novel use of a microprobe. Shake flask cultures of *Saccharopolyspora erythraea* and *Amycolatopsis orientalis* indicated that oxygen limitation has a place in screens provided that other nutrient limitations are also present. This finding was supported by a screen of 320 unknown actinomycetes performed in shaken tubes.

Oxygen-limited batch fermenter cultures of *S. erythraea* and *A. orientalis* suggested that the stimulus for antibiotic production may be down-regulation of growth. This suggestion was consistent with the low levels of erythromycin production in oxygen-limited chemostat cultures of *S. erythraea*. ATP was identified as a possible intracellular negative effector of antibiotic production. These findings suggest an antagonistic role for antibiotics in nature, and are a step towards rationalising screen designs.
(C) Gavin John Clark 1993
CONTENTS

1) INTRODUCTION

1.1) Microorganisms as sources of new drugs

1.2) Detecting novel microbial metabolites

   1.2.1) Isolation of microorganisms
   1.2.2) Generating biosynthetic novelty
   1.2.3) Culture systems used in screens
   1.2.4) Stimulating metabolite production

      1.2.4.1) The role of secondary metabolites in the
               life cycle of the producing organisms
      1.2.4.2) The regulation of secondary metabolism

   1.2.5) Assays to detect bioactivity
   1.2.6) Chemical characterisation and identification of bioactives

1.3) Oxygen limitation and screening

   1.3.1) Does oxygen limitation occur in screens?
   1.3.2) How would oxygen limitation affect a screen?
   1.3.3) Does oxygen limitation have a place in screens?

1.4) Mechanisms by which oxygen limitation
     influences secondary metabolism

1.5) Factors influencing aeration studies

   1.5.1) Method of culture

      1.5.1.1) Batch culture
      1.5.1.2) Continuous culture

   1.5.2) Method used to vary aeration efficiency

      1.5.2.1) Stirrer speed and air flow rate (shaker speed and flask type)

         1.5.2.1.1) The effect of hydrodynamic stress on antibiotic production
         1.5.2.1.2) The effect of carbon dioxide on antibiotic production

      1.5.2.2) Changing the composition of the input gas at constant total flow

   1.5.3) Culture medium

1.6) Objectives of this project

1.7) Chapter outline

page

   1
   2
   3
   3
   7
   8
   9
   9
   11
   17
   20
   21
   21
   22
   23
   23
   25
   25
   27
   28
   28
   28
   28
   29
   31
   31
   33
   33
2) MATERIALS AND METHODS 34
2.1) Organisms and culture conditions 35
2.2) Media 35
2.3) Inoculum preparation 36
2.4) Dry weight determination 36
2.5) Culture liquid collection 36
2.6) Erythromycin and vancomycin assays 37
2.7) Glucose assay 38
  2.7.1) Assay principle 38
  2.7.2) Assay details 38
2.8) Nitrate assay 39
  2.8.1) Assay principle 39
  2.8.2) Assay details 39
2.9) Phosphate assay 39
2.10) Adenine nucleotide assay 40
  2.10.1) Assay principle 40
  2.10.2) Making cell extracts 41
  2.10.3) Assay details 41
2.11) Measuring dissolved oxygen in small scale cultures (shake flasks and tubes) 41

3) SHAKE FLASK EXPERIMENTS IN DEFINED MEDIA 46
3.1) Objective 47
3.2) Summary 47
3.3) Experiment outline 48
3.4) Results 50
  3.4.1) Nutrient limitations and growth dynamics 50
  3.4.2) Secondary metabolite production under different nutrient limitations 60
    3.4.2.1) S. erythraea 60
    3.4.2.1) A. orientalis 62
    3.4.2.3) Antibiotic productivity 64
3.5) Conclusions and Discussion 66
6) CHEMOSTAT EXPERIMENTS IN DEFINED MEDIA  
6.1) Objective  
6.2) Summary  
6.3) Experiment outline  
6.4) Results  
   6.4.1) N-ltd medium  
   6.4.2) RICH medium  
6.5) Conclusions and discussion

7) REVIEW AND FUTURE WORK  
7.1) Measuring the oxygen status of small scale cultures  
7.2) The potential of oxygen limitation for  
   stimulating secondary metabolism  
7.3) Mechanisms by which oxygen limitation  
   influences secondary metabolism  
7.4) The role of secondary metabolites in the  
   life cycle of the producing organisms  
   7.4.1) Erythromycin and vancomycin  
   7.4.2) Red pigment

8) REFERENCES
LIST OF TABLES

Table 1: Defined medium compositions 35
Table 2: Antibiotic activities detected in a screen of 320 actinomycetes 95
Table 3: The influence of culture oxygen status on antibiotic responses 98

LIST OF FIGURES

Figure 1: The compression fitting used to seal the oxygen microprobe in small scale culture vessels 42
Figure 2: The use of a dissolved oxygen microprobe in a flask 43
Figure 3: The use of a dissolved oxygen microprobe in a tube 44
Figure 4: Method Summary (shake flask experiments in defined media) 49
Figure 5: The effect of different types of nutrient limitation on secondary metabolite production by S. erythraea 61
Figure 6: The effect of different types of nutrient limitation on vancomycin production by A. orientalis 63
Figure 7: A Chi-squared test of the Null hypothesis “SM14 and SM97 gave identical ratios of anti-S. aureus and anti-C. albicans antibiotics” 96
Figure 8: Chemostat culture of S. erythraea in N-ltd medium 106
Figure 9: Chemostat culture of S. erythraea in RICH medium 108

LIST OF PHOTOGRAPHS

Photograph 1: The appearance of flask cultures of S. erythraea in O-ltd, P-ltd, N-ltd and C-ltd media after 96 hours incubation 60
Photograph 2: The morphology of A. orientalis in fermenter culture 78
Photograph 3: The morphology of S. erythraea in fermenter culture 79
LIST OF GRAPHS

Graph 1: Flask culture of *S. erythraea* in RICH medium
(dry weight and dissolved oxygen tension versus time) 51

Graph 2: Flask culture of *A. orientalis* in RICH medium
(dry weight and dissolved oxygen tension versus time) 51

Graph 3: Flask culture of *S. erythraea* in P-ltd medium
(dry weight and phosphate concentration versus time) 53

Graph 4: Flask culture of *A. orientalis* in P-ltd medium
(dry weight and phosphate concentration versus time) 53

Graph 5: Flask culture of *S. erythraea* in P-ltd medium
(dry weight and dissolved oxygen tension versus time) 54

Graph 6: Flask culture of *A. orientalis* in P-ltd medium
(dry weight and dissolved oxygen tension versus time) 54

Graph 7: Flask culture of *S. erythraea* in N-ltd medium
(dry weight and nitrate concentration versus time) 55

Graph 8: Flask culture of *A. orientalis* in N-ltd medium
(dry weight and nitrate concentration versus time) 55

Graph 9: Flask culture of *S. erythraea* in N-ltd medium
(dry weight and dissolved oxygen tension versus time) 56

Graph 10: Flask culture of *A. orientalis* in N-ltd medium
(dry weight and dissolved oxygen tension versus time) 56

Graph 11: Flask culture of *S. erythraea* in C-ltd medium
(dry weight and glucose concentration versus time) 57

Graph 12: Flask culture of *A. orientalis* in C-ltd medium
(dry weight and glucose concentration versus time) 57

Graph 13: Flask culture of *S. erythraea* in C-ltd medium
(dry weight and dissolved oxygen tension versus time) 58

Graph 14: Flask culture of *A. orientalis* in C-ltd medium
(dry weight and dissolved oxygen tension versus time) 58
Graph 15: An oxygen-sufficient fermenter culture of *A. orientalis* in RICH medium (dry weight and glucose concentration versus time) 62

Graph 16: Erythromycin productivity under different nutrient limitations 65

Graph 17: Vancomycin productivity under different nutrient limitations 65

Graph 18: Fermenter culture of *S. erythraea* in RICH medium (dry weight and dissolved oxygen tension versus time) 71

Graph 19: Fermenter culture of *A. orientalis* in RICH medium (dry weight and dissolved oxygen tension versus time) 71

Graph 20: Fermenter culture of *S. erythraea* in RICH medium (dry weight and glucose concentration versus time) 72

Graph 21: Fermenter culture of *A. orientalis* in RICH medium (dry weight and glucose concentration versus time) 72

Graph 22: Fermenter culture of *S. erythraea* in RICH medium (dry weight and nitrate concentration versus time) 73

Graph 23: Fermenter culture of *A. orientalis* in RICH medium (dry weight and nitrate concentration versus time) 73

Graph 24: Fermenter culture of *S. erythraea* in RICH medium (dry weight and specific growth rate versus time) 76

Graph 25: Fermenter culture of *A. orientalis* in RICH medium (dry weight and specific growth rate versus time) 76

Graph 26: Growth rates of *S. erythraea* and *A. orientalis* in oxygen-limited fermenter cultures 77

Graph 27: Growth and production rates of *S. erythraea* in oxygen-limited fermenter culture 82

Graph 28: Fermenter culture of *S. erythraea* in RICH medium (dry weight and adenylate energy charge versus time) 84

Graph 29: Fermenter culture of *A. orientalis* in RICH medium (dry weight and adenylate energy charge versus time) 84
Graph 30 : Fermenter culture of *S. erythraea* in RICH medium  
(dry weight and nucleotide concentration versus time) 85

Graph 31 : Fermenter culture of *A. orientalis* in RICH medium  
(dry weight and nucleotide concentration versus time) 85

Graph 32 : Tube culture of *S. erythraea* in SM14 medium  
(dry weight and dissolved oxygen tension versus time) 91

Graph 33 : Tube culture of *A. orientalis* in SM14 medium  
(dry weight and dissolved oxygen tension versus time) 91

Graph 34 : Tube culture of *S. erythraea* in SM97 medium  
(dry weight and dissolved oxygen tension versus time) 92

Graph 35 : Tube culture of *A. orientalis* in SM97 medium  
(dry weight and dissolved oxygen tension versus time) 92
ABBREVIATIONS

D.O. - dissolved oxygen tension
r.p.m. - revolutions per minute
H.P.L.C. - high performance liquid chromatography
v/v/m - volumes of air supplied per volume of medium per minute
AEC - adenylate energy charge
ATP - adenosine-5' triphosphate
ADP - adenosine-5'-diphosphate
AMP - adenosine-5’-monophosphate
µ - specific growth rate
Q_{ery} - specific erythromycin production rate
EDTA - ethylene diamine tetraaminoacetate
P.D.I. - product diversity index
I am indebted to Dr Michael Bushell of The University of Surrey and Dr David Langley of Glaxo Group Research for their advice and encouragement. I would also like to mention my colleagues in the laboratory with whom it has been a great pleasure to work: Giles Wilson, Steve Martin, Jon Smith, Jo Moore, Hilary Lynch, David Venables, Paul Ives, Kieran O’Donovan and Di Simpson. I would also like to thank my wife Joanne for her support during the preparation of this thesis.
1) INTRODUCTION
1.1) MICROORGANISMS AS SOURCES OF NEW DRUGS

The search for new drugs has increasingly been directed towards the rational design of synthetic molecules bearing a structural relationship to the effectors, substrates, intermediates or products of biochemical reactions (Wang and Walsh, 1978). However, where there is a lack of understanding of a reaction mechanism or if the synthetic approach has failed, then a screen of microbial metabolites could provide an unexpected lead molecule (Nisbet, 1982).

Microorganisms are capable of producing a vast number of compounds which are not readily synthesised, and their diversity ranges from simple amino acid analogues to much larger macrolides, glycopeptides and glycolipids (Berdy, 1985). The biological activities of these compounds is equally diverse, including antibacterial-, antifungal- and antitumour-antibiotics, enzyme inhibitors, microbial regulators, antihypertensives, pesticides, herbicides and immunomodulators (Franco and Coutinho, 1991). Such diversity has vindicated the efforts devoted to microbial screening. Moreover, it is clear that the microbial resource is largely untapped because high rates of discovery of new compounds continue (Harada, 1986) in spite of the large arsenal of compounds that already exists (Laskin and Lechevalier, 1988). With new drugs being sought in all areas of human medicine and agriculture, the search for novel microbial metabolites will continue to be a major activity of the pharmaceutical industry.
1.2) DETECTING NOVEL MICROBIAL METABOLITES

Novel microbial metabolites are detected through screening programs, which are inter-disciplinary tasks generally involving the following steps (Nisbet, 1982; Franco and Coutinho, 1991);
1) isolation / generation of microorganisms
2) cultivation in liquid media (‘fermentation’) to promote metabolite formation
3) assays to detect bioactivity
4) chemical characterisation and identification of bioactive metabolites.

Innovative modification at any or all of these key steps can result in the production, detection and identification of novel compounds, and an increase in throughput statistically increases the chances of success (Okami and Hotta, 1988). This section examines how the needs of screening programs can be satisfied and highlights some important developments reported in the last decade.

1.2.1) Isolation of microorganisms

Even modern isolation programs are often limited to the examination of aerobic, heterotrophic organisms that grow well at neutral pH and ambient temperature (Goodfellow and O’Donnell, 1989). These organisms represent only a tiny fraction of the gene pool found in natural habitats (Williams et. al., 1984). Thus, a large number of well-known genera and species are isolated and screened in the hope that new compounds will be discovered. Although this random approach continues to yield new
compounds, it also results in costly re-discovery of known compounds. It is apparent that discoveries of novel compounds will be increasingly dependent upon the development of objective strategies for the isolation and characterisation of novel and rare microorganisms (Nolan and Cross, 1988).

**The microbial resource**

Some kinds of microorganisms have been more fruitful sources of metabolites than others - actinomycetes are by far the most prolific producers of antibiotics followed by fungi, eubacteria and algae (Franco and Coutinho, 1991). This information enables modern screening programs to maximise efficiency by focusing on likely metabolite producers. Among actinomycetes *Streptomyces* have been the dominant producers (Goodfellow and O’Donnell, 1989), but this probably reflects the relative ease of isolation of this genus (Bushell, 1988). Rare actinomycetes are being isolated in greater numbers and are proving to be an excellent source of new antibiotics, with a capacity to produce diverse structural classes comparable to *Streptomyces* (Okami and Hotta, 1988). Like actinomycetes, fungi are believed to be an under-exploited source of novel compounds (Dreyfuss, 1989). The importance of fungi has been underlined by the recent demonstration that fungal fruiting bodies are sources of new metabolites (Moriguchi *et. al.*, 1987; Bernillon *et. al.*, 1989).

**Distribution of species**

Ecological studies have shown that species are not universally distributed in soils (Keast *et. al.*, 1984a, b), and thus emphasize the need to isolate organisms from new and unusual substrates. New species have been
isolated from soils from remote islands (Hotta et. al., 1980), from marine (Mynderse and Crandall, 1989), acid (Flowers and Williams, 1977) and alkaline (Sato et. al., 1985) environments, and from hot sites such as compost heaps (Patel et. al., 1989).

**Sample pretreatments**

Novel organisms are usually isolated from environmental samples by applying appropriate selection pressures at various stages of the dilution plate procedure (Goodfellow and O'Donnell, 1989). The treatment of a soil sample prior to plating is an important factor in selective isolation. Air-drying or heating of soils is commonly applied to favour organisms which form spores resistant to dessication and heat. It may be possible to establish taxon-specific heat treatments as there is evidence that actinomycetes have different heat sensitivity profiles (Goodfellow and Simpson, 1987). Rehydration of soil samples has been used to encourage the release of zoospores into the aqueous phase, so facilitating the isolation of strains belonging to all of the actinoplanete genera (Nolan and Cross, 1988). It has recently been demonstrated that storage of soil samples can cause a loss of viability of up to 50% of the isolates, especially organisms that spore poorly (Woodruff, 1986). This indicates that novel organisms may be isolated if soils are plated at or near the sampling site.

An ingenious addition to pretreatment methods is the addition of a cocktail of polyvalent phage to soil suspensions in order to reduce the growth of *Streptomyces* on isolation plates (Williams and Vickers, 1988). The method is simple and phage have the advantage of being specific to
organisms with the same wall chemotype (Prauser, 1984).

**Isolation medium and conditions**

The selectivity of the isolation medium is a function of its nutrient composition, pH, the addition of selective inhibitors and incubation conditions. The importance of medium ingredients is exemplified by the isolation of the new and rare actinomycete genus *Rhodococcus* on media containing n-alkanes (Nesterenko and Kuasnikov, 1986). The incorporation of antibiotics in culture media has become a routine method for isolating a large number of other rare actinomycetes (Goodfellow and O'Donnell, 1989). Preselection of candidate producer organisms can be attempted by using a rational approach: it is well known that producing organisms are resistant to their own antibiotics. Hence, the antibiotic produced by a rare genus should be useful in its selective isolation. This rationale was demonstrated by the use of vancomycin to isolate *Amycolatopsis* (Lechevalier et al., 1986).

Numerical taxonomic studies can reveal information on the nutritional requirements and selective agents for rare organisms (Goodfellow and O’Donnell, 1989). For example, the growth responses of *Streptomyces* to sole carbon and/or nitrogen sources were used to formulate media to favour the growth of selected species (Williams et al., 1983a, b). Numerical taxonomy is likely to become increasingly important as automated data acquisition systems facilitate the generation of high quality data bases.

Incubation times for isolation plates are usually from 7 to 14 days, when most of the *Streptomyces* will have produced colonies visible to the
naked eye. Longer incubation times have often been disregarded because of the argument that slow-growing cultures would be unsuitable candidates for economic fermentations. However, the early growth of some organisms may modify the environment of the isolation plate by supplying growth factors or reducing the concentration of toxic components. Tardy strains do eventually appear which show normal growth characters when isolated (Nolan and Cross, 1988). Prolonged incubation was one of the factors contributing to the isolation of the slow-growing endophyte *Frankia* (Lechevalier, 1981).

An unusual approach to selective isolation was the use of an elegant membrane filter-stripping method for removing non-mycelial colonies from the surface of the isolation plate (Hirsch and Christenson, 1983; Hanka *et al*., 1985).

**Colony selection**

In most screening programs candidate colonies are selected for further study by using a stereomicroscope. Colonies can be selected at random or chosen on the basis of some morphological feature, but very few taxa can be recognised with any degree of certainty on a primary isolation plate (Nolan and Cross, 1988). Chromogenic or fluorogenic substrates which indicate the elaboration of genus specific enzymes (O'Donnell, 1988) may help in the isolation of a higher proportion of particular taxa provided a suitable group of organisms can be chosen for study.

**1.2.2) Generating biosynthetic novelty**

In addition to the natural variation among microorganisms, genomes of divergent biosynthetic capabilities may be constructed by laboratory gene
manipulation (Okami and Hotta, 1988). The potential exists to modify known antibiotics by feeding precursor analogues into blocked pathways (Delzer et. al., 1984), to cross strains to obtain hybrids capable of making hybrid products (Yamashita et. al., 1985) or to introduce specific enzymic or regulatory modifications (Hopwood et. al., 1985). The success of these approaches so far has been limited to the generation of analogues of known metabolites.

1.2.3) Culture systems used in screens

Having obtained a variety of putative producer strains it is necessary to cultivate them in order to promote metabolite formation. Culture methods comprise two basic types - surface cultures which use static liquid media or agar-solidified media, and submerged cultures which use shaken liquid media. Most screening groups have investigated both methods and have resorted to submerged culture mainly because the physical heterogeneity of macrocolonies leads to reproducibility problems on scaling up to submerged homogeneous cultures (Nisbet, 1982). The antibiotics fumaramidmycin (Maruyama et. al., 1975) and SF1993 (Shomura et. al., 1979) were reported to be produced only by agar-grown cultures. In the former case the antibiotic was apparently inactivated in submerged culture and in the latter it was subsequently shown to be produced by filamentous forms in diluted liquid media. Nisbet (1982) suggested that oxygen limitation may account for both effects and stressed the importance of using shaken cultures with high oxygen transfer rates. The potential impact of oxygen limitation on liquid culture screening programs is considered later following a review of
strategies for the cultivation of microorganisms to promote metabolite formation.

1.2.4) Stimulating metabolite production

1.2.4.1) The role of secondary metabolites in the life cycle of the producing organisms

The rational stimulation of microbial metabolite production must follow an understanding of why microorganisms produce the metabolites in the first place. Unfortunately, little is known about the role of many metabolites in the life cycle of the producing organisms (Hunter, 1992). Indeed this lack of knowledge is reflected in the broad classification of many compounds as 'secondary metabolites' because they do not appear to be essential for the vegetative growth of the producing organism in pure culture (Demain et al., 1983). An obvious role of antibiotics is as weapons against competitors in the environment (Katz and Demain, 1977), but matters have been complicated by suggestions that some antibiotics have other functions in the producing organism. For example, the peptide antibiotics seem to be involved in the sporulation process (Katz and Demain, 1977) and the antibiotic methylenomycin has been implicated in the formation of aerial mycelium (Wright and Hopwood, 1976). These studies suggest that the primary function of these secondary metabolites is as regulators of differentiation, and antibiotic activity is purely serendipitous.

Zahner (1979) has suggested that secondary metabolism functions as a playground for evolution. In this scheme enzymes of low specificity form new compounds from available intermediary metabolites. Some of these new
compounds may eventually find a use, either inside the cell or in the external environment. Zahner therefore predicts that many secondary metabolites will have no function at all, while others may have more than one.

Several authors have suggested that the process of secondary metabolism may be far more important to the producing organism than the final products. Dhar and Khan (1971) proposed that secondary metabolites are formed in order to reduce the accumulation of a more toxic intermediate (the ‘detoxification hypothesis’). Bu’Lock (1961) proposed that secondary metabolism maintains growth mechanisms in operative order when growth is not possible (the ‘metabolic maintenance hypothesis’). Woodruff (1966) proposed that secondary metabolism maintains a reasonable balance of primary metabolites when conditions cannot support balanced growth (the ‘unbalanced growth hypothesis’). The unbalanced growth hypothesis suggests that metabolic intermediates accumulate when a nutrient becomes growth-limiting through failure of control mechanisms which adjust flow through pathways. Instead of being excreted directly, accumulated metabolites are acted upon by enzymes of low substrate specificity. A succession of reactions leads to the formation of a complex metabolite termed a ‘shunt’ product which is excreted. All propositions that the process of secondary metabolism is of importance do not preclude the possibility that secondary metabolites have subsequently acquired a functional role.

Until now the study of secondary metabolism has been mainly descriptive. Compared with primary metabolism, the enzymology and regulation of pathways is poorly understood (Hunter, 1992). Recent
advances in molecular biology may increase our knowledge of how gene expression is controlled which in turn may illuminate the role of secondary metabolites for the producing organism. Indeed, the recent demonstration that antibiotic biosynthesis, regulation and resistance genes are clustered (Hopwood, 1988) has implied an antagonistic function in nature (Stone and Williams, 1992).

1.2.4.2) The regulation of secondary metabolism

It is evident from the foregoing discussion that secondary metabolites are likely to serve a number of different roles in different microorganisms. Varied roles of secondary metabolites imply that there will be considerable variation in the mechanisms controlling their biosynthesis. It follows that there can be no 'ideal' production medium for secondary metabolites, and culture media with varied compositions are essential in screening programs. The design of culture media for a screen can be rationalised to a degree by assessing the major factors influencing secondary metabolism in a variety of microorganisms. These factors include carbon-, nitrogen-, and phosphate-regulation, growth rate, trace elements and unusual media and culture conditions (Iwai and Omura, 1982; Nisbet, 1982; Demain, 1986). Each of these factors will now be considered along with the consequences that such factors have for medium design.

Carbon regulation

Carbon regulation is the term used when secondary metabolism is suppressed by the presence of high concentrations of rapidly-used carbon sources, such as glucose and glycerol. Interfering carbon sources act by repressing
synthesis and inhibiting activity of synthases of secondary metabolism (Demain, 1986). Antibiotics sensitive to carbon regulation include actinomycin (Brown et. al., 1983), cephalosporins (Aharonowitz and Demain, 1978), cephamycin C (Cortes et. al., 1984), puromycin (Sankaran and Pogell, 1973), streptomycin (Inamine et. al., 1969) and tylosin (Sprinkmeyer and Pape, 1978). Production media for such secondary metabolites generally combine rapidly-used and slowly-used carbon sources in the same medium (Demain, 1986). The rapidly-used carbon source is usually used first in a rapid growth phase which does not support secondary metabolism. After the favoured carbon source is depleted, growth proceeds more slowly on the second carbon source and secondary metabolite production is stimulated. Such production media are often described as being ‘carbon-limited’ because the carbon source is the nutrient limiting growth.

**Nitrogen regulation**

Nitrogen regulation is the term used when secondary metabolism is suppressed by the presence of high concentrations of ammonium and certain amino acids. Interfering nitrogen sources usually act by repressing synthesis and inhibiting activity of synthases of secondary metabolism (Demain, 1986). Antibiotics sensitive to this form of nitrogen regulation include cephamycin C (Aharonowitz and Demain, 1979), actinomycin (Katz et. al., 1984), tetracycline (Behal et. al., 1983) and tylosin (Omura et. al., 1984). Another type of nitrogen regulation occurs when an amino acid and a secondary metabolite are derived from the same pathway. Feedback inhibition and / or repression of the early common pathway by the amino
acid interferes with the flux into the secondary metabolite branch. Secondary metabolites subject to this form of nitrogen regulation include the antibiotic candididin which is suppressed by aromatic amino acids (Martin, 1983), and the antitumour agent macebin which is suppressed by tryptophan, p-aminobenzoic acid and anthranilic acid (Tanida et. al., 1980). Production media for secondary metabolites sensitive to nitrogen regulation classically use soybean meal because its slow breakdown prevents large accumulations of ammonium or amino acids (Demain, 1986). Other suitable nitrogen sources include nitrate and slowly metabolised amino acids. Alternatively, nitrogen regulation is sometimes overcome by adding ammonium-trapping agents to culture media, such as powdered tribasic magnesium phosphate and natural zeolites. Media designed to overcome nitrogen regulation are often described as being ‘nitrogen-limited’ because the nitrogen source is the nutrient limiting growth.

**Phosphate regulation**

Phosphate regulation is the term used when secondary metabolism is suppressed by high concentrations of phosphate. There appear to be at least two mechanisms for the phosphate effect (Martin, 1977). In the first phosphate inhibits and / or represses phosphatases involved in secondary metabolite biosynthesis. Such is the case for streptomycin biosynthesis in which phosphate inhibits the phosphatase responsible for converting streptomycin phosphate to streptomycin (Walker and Walker, 1971). This type of phosphate interference may be expected whenever the biosynthetic pathway includes phosphorylated intermediates.
The second mechanism is a more general one in which phosphate or an intracellular effector produced from extracellular phosphate inhibits and/or represses synthases other than phosphatases. Possible effectors are ATP and ADP because concentrations drop suddenly just before antibiotic production phases in candicidin-producing *Streptomyces griseus* (Liras et. al., 1977), tylosin-producing *Streptomyces fradiae* (Vu-Trong et. al., 1980), tetracycline-producing *Streptomyces aureofaciens* (Janglova et. al., 1969) and levorin-producing *Streptomyces levoris* (Zyuzina et. al., 1981). With resting cells of *S.griseus*, the addition of phosphate leads within 5 minutes to a 2-to-3-fold increase in intracellular ATP followed 10 minutes later by a decreased rate of candicidin production (Martin and Demain, 1976). Furthermore, in *S.aureofaciens* and *S.fradiae*, the intracellular ATP content is much higher in ancestral low-producing strains than in higher producing mutants (Janglova et. al., 1969; Hanel et. al., 1984). Martin (1977) has suggested that Adenylate Energy Charge (AEC) may be the effector for secondary metabolism rather than ATP or ADP. AEC is a linear measure of the metabolic energy stored in the adenine nucleotide pool, and is calculated as the mole fraction of ATP plus half the mole fraction of ADP (Atkinson and Walton, 1967);

\[
AEC = \frac{\text{ATP} + 0.5 \text{ADP}}{\text{ATP} + \text{ADP} + \text{AMP}}
\]

However, AEC seems an unlikely effector in the light of reports that antibiotic production is affected by large changes in the total pool of
adenylate phosphates which do not significantly affect AEC (Hostalek et. al., 1974; Curdova et. al., 1976; Martin et. al., 1978; Vu-Trong et. al., 1981; Dietzler et. al., 1974).

Production media for secondary metabolites sensitive to phosphate regulation contain low phosphate concentrations or phosphate-trapping agents such as alaphosphane or kanamatsuchi (Demain, 1986). Media designed to overcome phosphate regulation are often described as being 'phosphate-limited' because the phosphate source is the nutrient limiting growth.

**Growth rate**

Since many secondary metabolites are formed only at low growth rates, the question arises as to whether carbon, nitrogen or phosphate limitation merely acts by lowering growth rate to a value which stimulates secondary metabolism. Often higher-producing mutants display lower growth rates, suggesting that growth rate might be the key factor (Kralovkova and Vanek, 1979). A study of *Streptomyces cattleya* has indicated that some secondary processes are stimulated by low growth rates irrespective of the type of nutrient limiting growth, while others require a specific type of nutrient limitation (Lilley et. al., 1981). Thus, a greater variety of metabolites can be detected in a screen by cultivating each test organism in a variety of media designed to have different growth-limiting nutrients. Nisbet (1982) has suggested using different forms of both the growth-limiting and the growth-sufficient nutrients in order to obtain a wide variety of growth rates.
Trace elements

Trace element limitation is important for optimal production of metabolites that facilitate accumulation of the depleted element - for example, iron-chelating siderochromes are produced under conditions of iron limitation (Neilands, 1974). However, metal cations may also act as stimulants to product formation when they are required as cofactors. Thus, cobalt ions stimulate the production of aminoglycosides (Yamamoto et. al., 1977) and beta-lactams (Butterworth et. al., 1979). Screening programs must allow for these possibilities by including media containing different quantities of a variety of trace elements.

Unusual culture media and culture conditions

Given the diverse control mechanisms for antibiotic production it is not surprising that some organisms produce novel metabolites in unusual culture media or culture conditions (Okami and Hotta, 1988; Franco and Coutinho, 1991). The scope for manipulating fermentation media and conditions is limited only by the imagination of the microbial physiologist. Some successful approaches include the use of media with high phosphate concentrations (Miyoshi et. al., 1972), poor nutrition (Okami et.al., 1976), high temperatures (Reusser, 1985), low temperatures (Yoshida et. al., 1972), controlled aeration (Kunze et. al., 1987), high aeration (Arai et. al., 1976, 1977), inhibitors of biosynthetic enzymes (Schulman et. al., 1985), mixed cultures (Imanaka, 1986), regulators of product patterns which may be incorporated into the antibiotic molecule (Cassinelli, 1990) and the use of adsorbent resins to increase the stability and yield of highly unstable
Compromises in screen designs

The previous discussion has outlined an enormous number of potential strategies for the cultivation of microorganisms to promote metabolite formation. It is clear that a screen design must be a compromise between the need for a variety of culture conditions and practical and economic limitations. The choice of culture conditions is likely to become increasingly rationalised with advances in our understanding of conditions favouring the production of specific types of metabolites.

1.2.5) Assays to detect bioactivity

The isolation and culture of microorganisms is a time-consuming and expensive process. It is therefore important to subject test culture broths to the greatest possible number of assays to detect bioactivity. Usually the detection assays are specially developed to be compatible with culture broths since this source presents a number of problems (Nolan and Cross, 1988). Even at the start, culture media are generally a complex mixture of poorly defined components and, once growth has taken place, various substrates have been depleted, products have been excreted into the medium, lytic activity may have started, and the situation is totally undefinable. Furthermore, any desired bioactive metabolites are likely to be present at very low concentrations. An ideal detection assay should therefore be sensitive, selective and specific, yet robust enough to withstand interference from culture broth constituents. An additional desired quality is a rapid response time.
In the early years of screening, detection assays for antibiotics depended directly on the desired biological activity. This approach is non-selective and is little used today in screening programs which face ever increasing problems of rediscovery of known metabolites. Detection assays are now highly target-directed, encompassing a wide range of biochemical tests, enzyme inhibitor models, receptor-binding assays, *in vivo* assays, and combinations of several individual assays (Franco and Coutinho, 1991). These modern approaches are exemplified by assays for antitumour and immunomodulatory agents.

**Antitumour assays**

If clinically useful antitumour antibiotics are to be found, culture broths need to be tested to show their activity in an animal tumour model such as murine leukaemia (Okami and Hotta, 1988). However, for initial screening of culture broths the *in vivo* animal model is not practical because it not only has limited sensitivity and selectivity, but is also costly and time-consuming. Consequently, various *in vitro* test systems have been devised as prescreens. Among these prescreens are the Plate Agar Diffusion Assay and the Biochemical Induction Assay.

The Plate Agar Diffusion Assay is the mammalian cell analogue of the classical bacterial agar diffusion / zone of inhibition assay (Garretson *et. al.*, 1981). Plates containing agar to which P388 murine leukaemia cells have been added are prepared, and paper discs impregnated with the test broth are applied and incubated for 36 hours. The paper discs are then removed and the plate flooded with sodium 2,6-dichloroindophenolate. Activity is
indicated by the formation of blue zones.

The Biochemical Induction Assay relies on the fact that compounds which damage DNA are potential antitumour agents (DNA replication is more rapid in tumour cells than in normal cells). This assay exploits a genetically-engineered lysogenic *Eschericia coli* strain (BR513) which produces β-galactosidase instead of phage in response to DNA-damaging compounds (Elespuru and White, 1983). The induced β-galactosidase is visualised by adding the chromogenic substrate 6-bromo-2-napthyl-β-galactopyranoside. This reagent turns bright red on hydrolysis. Thus, a plate containing *E.coli* BR513 is spotted with drops of the test broths, the induction response is elicited and the plate is overlaid with a layer of agar containing the colourless substrate. Active samples are easily seen as red spots or zones. Being DNA-target linked the Biochemical Induction Assay was shown to be much more selective than the Plate Agar Diffusion Assay (Garretson et. al., 1981).

**Assays for immunomodulators**

The search for immunomodulators was stimulated by the discovery that the antifungal cyclosporin A is also a powerful immunosuppressors (Borel, 1986). Cyclosporin A is now used to prevent rejection of tissue and organ grafts. The search for safer, more efficient compounds has been aided by the Mixed Lymphocyte Reaction (Goto et. al., 1982). This assay mixes responder and stimulator lymphocytes from rat spleens with culture filtrates. Following a 3 day incubation period, the resulting cell proliferation is monitored by pulsing the culture for 4 hours with radiolabelled thymidine.
A number of immunosuppressors have been discovered using the Mixed Lymphocyte Reaction, including FK506, a macrolactone which is more potent than cyclosporin A (Kino et al., 1987a, b). The binding proteins for a number of immunosuppressive agents have recently been characterised (Rosen et al., 1990), opening the way for the development of receptor-binding assays.

1.2.6) Chemical characterisation and identification of bioactives

The drug discovery process is incomplete until the bioactive metabolites detected in assays are isolated in pure form in sufficient quantity to enable chemical structures to be determined. The success of chemists in this respect is borne out by the rarity of reports of bioactive metabolites whose structure has not been determined.

The typical unknown bioactive metabolite coexists in a fermentation mass in minute quantities along with several other compounds and often as a component of a complex of similar compounds. To make matters worse the fantastic biological activity (usually initially claimed) might turn out to be unstable to a variety of conditions, such as heat, light and pH.

Isolation involves a series of steps beginning with the recovery of the bioactive metabolite from the fermentation mass with the use of solvent extraction, or adsorption on resins, ion exchangers or charcoal (Franco and Coutinho, 1991). The primary objective is to concentrate the bioactive metabolite into a manageable form and quantity for further purification, which can then be achieved by a series of chromatographic procedures. The isolation process needs to be monitored by an efficient assay capable of
qualitative and quantitative assessment of the target moiety as well as the associated impurities. This is relatively straightforward in the case of antibiotics with the use of Thin Layer Chromatography (T.L.C.), High Performance Liquid Chromatography (H.P.L.C.), bioautography, and an in vitro evaluation on agar plates. Targets having a pharmacological, enzyme, or time-consuming assay need to be correlated with a T.L.C. spot or H.P.L.C. peak in order to facilitate their isolation.

Purification procedures can involve liquid-solid, liquid-liquid, or affinity chromatography with a variety of gel filtration techniques being implemented at various stages.

The identification of a molecule logically requires two steps (Franco and Coutinho, 1991);
1) comparison of the metabolite with those known by using a combination of H.P.L.C., mass spectrometry and T.L.C.
2) assignment of a chemical structure by using a combination of nuclear magnetic resonance and mass spectrometry.

1.3) OXYGEN LIMITATION AND SCREENING
1.3.1) Does oxygen limitation occur in screens?

Screening programs require a high throughput of organisms in order to increase the chances of discovering new metabolites. Fermentation is therefore carried out in small scale culture vessels such as shaken flasks or tubes. An unfortunate consequence of using small scale culture vessels is the inability to monitor dissolved oxygen tension. The likelihood of oxygen limitation is impossible to assess without dissolved oxygen data because it is a
function of vessel aeration efficiency, medium composition and the specific oxygen uptake capabilities of the organisms. Thus, while shake flasks and tubes can have aeration efficiencies comparable with stirred, aerated fermenters (Tunac, 1989; Freedman, 1970; McDaniel and Bailey, 1969; Morimoto et al., 1979), even high aeration efficiencies may be offset by the use of culture media which give rise to high oxygen demand rates. The oxygen status of screen cultures will be known only by developing a method for measuring dissolved oxygen tension in small scale cultures.

1.3.2) How would oxygen limitation affect a screen?

Most screens are designed so that each test organism is grown in a variety of media formulated to have different growth-limiting nutrients. This range of growth-limiting nutrients maximises the chances of expressing the product-forming potential of each organism in the screen. It has been suggested that oxygen limitation could eclipse effects of other nutrient limitations and so rob the screen of the potential benefit of having a variety of media (Bushell, 1989). This suggestion is supported by a small number of reports which demonstrate that oxygen limitation does not merely delay effects of subsequent nutrient limitations, but exerts its own influence on secondary metabolism. Thus oxygen limitation has been shown to stimulate the production of Gramicidin S (Vandamme et al., 1981), but suppress the production of cephamycin C (Rollins et al., 1988, 1990), tylosin (Chen and Wilde, 1991) and rifamycin (Virgilio et al., 1964). Given that oxygen limitation might critically undermine a screen, there is an urgent need to develop a means of assessing the oxygen status of small scale cultures.
1.3.3) Does oxygen limitation have a place in screens?

Oxygen limitation can stimulate the production of Gramicidin S (Vandamme et al., 1981). This suggests that oxygen limitation may deserve equal status with other nutrient limitations as a potential means of stimulating antibiotic production. Oxygen limitation could be designed either by formulating a medium to have high concentrations of all other nutrients, or by reducing the aeration efficiency of culture vessels by lowering shaker speed, increasing culture volume or changing vessel design. Such deliberate inclusion of oxygen limitation in a screening program must follow the development of a method for measuring the oxygen status of small scale cultures because it is only worth including oxygen-limited cultures if all other cultures are proven to be oxygen-sufficient.

In view of this discussion, two objectives of this project are:

1) To develop a method for measuring the oxygen status of small scale cultures
2) To assess the potential of oxygen limitation as a means of stimulating secondary metabolism in screening programs.

1.4) MECHANISMS BY WHICH OXYGEN LIMITATION INFLUENCES SECONDARY METABOLISM

Oxygen limitation has rarely been reported in secondary metabolite fermentations, and consequently little is known about the mechanisms by which oxygen limitation influences secondary metabolism. Two reports indicate that oxygen limitation may directly affect antibiotic biosynthetic enzymes;
1) Rollins et. al. (1990) demonstrated that oxygen derepression of deacetoxycephalosporin C synthase is an important regulatory mechanism in cephамycin C production by *Streptomyces clavuligerus*. 

2) Vandamme et. al. (1981) suggested that the positive effect of oxygen limitation on gramicidin S production by *Bacillus brevis* is due to reduced inactivation of gramicidin synthetases which are known to be oxygen labile (Friebel and Demain, 1977a, b).

In *Saccharomyces cerevisiae* there is a class of genes encoding oxygen-dependent functions such as cytochrome subunits, oxidases and saturases which are induced at low oxygen tension. The expression of these hypoxic genes presumably allows the cell to utilise limiting oxygen more efficiently (Zitomer and Lowry, 1992). If similar genes also occur in antibiotic-producing organisms then the effects of oxygen limitation on secondary metabolism may be a complex function of effects on primary and secondary metabolism. It has recently been demonstrated that the respiratory chains of *Escherichia coli* and *Azotobacter vinelandii* are modified under conditions of oxygen starvation (Poole, 1993). An increasing knowledge of the respiratory chains of *Saccharopolyspora erythraea* (Scott et. al., 1989, 1992) and *Streptomyces clavuligerus* (Scott et. al., 1992; Scott and Poole, 1988) has opened the way for similar studies with antibiotic-producing actinomycetes.

A better knowledge of the mechanisms by which oxygen limitation influences secondary metabolism could lead not only to the discovery of new compounds in screens, but also to improved yields in production scale
fermenters in which cultures are subject to cyclic variations in dissolved oxygen tension (Vardar and Lilley, 1982). An objective of this project is to investigate the mechanisms by which oxygen limitation influences secondary metabolism.

1.5) FACTORS INFLUENCING AERATION STUDIES

The two preceding sections have outlined the objectives of this project and have thus defined it as 'a study of oxygen limitation and its relevance to screening for novel secondary metabolites'. A large number of aeration studies have not yet been considered because they concern aspects of aeration not directly relevant to oxygen limitation and screening. However, these reports deserve consideration because they provide insight into a number of factors which influence aeration studies. These factors will now be considered under the following headings:

1) method of culture
2) method of varying aeration efficiency
3) culture medium.

1.5.1) METHOD OF CULTURE

1.5.1.1) Batch culture

A batch culture can be considered to be a 'closed system'. In this form of culture, a sterilised nutrient solution is inoculated with microorganisms and growth then proceeds during an incubation period under suitable physiological conditions. Nothing is added in the course of the fermentation except air, an antifoam agent and acid or base to control the pH. Microbial metabolism causes constant changes in the composition of the culture
medium, the biomass concentration and the secondary metabolite concentration. Batch cultures have been employed extensively for the investigation of the regulation of secondary metabolism. The closed nature of batch cultures mean that any effects of the aeration condition on secondary metabolism are likely to be time-dependent. Indeed, studies with *Streptomyces fradiae* (Chen and Wilde, 1991), *Streptomyces clavuligerus* (Yegneswaran and Gray, 1991) and *Penicillium chrysogenum* (Rolinson, 1952) have demonstrated that high antibiotic productivity demands high oxygen availability only during the growth phase.

The oxygen transfer capability of a fermenter may continuously decrease during batch culture due to:

1) increasing viscosity caused by increasing mycelial concentration (Banks, 1977; Deindoerfer and Gaden, 1955; Carilli et al., 1961).

2) antifoam addition (Aiba et al., 1973; Hall et al., 1973).

Such continuous changes in system oxygen transfer capability further complicate aeration studies performed in batch culture.

Shake flask culture is a form of small scale batch culture which differs from fermenter batch culture in that:

1) there may be a significant concentration of culture liquid during a fermentation due to water loss through evaporation (evaporation is minimised in fermenters by the use of condensers at air outflow points). Hajny (1964) has compensated for this by making up flask volumes with water prior to analysis, but this assumes that concentration of the culture
liquid has no effect on cellular metabolism. Loss of water by evaporation can be minimised by incubating flasks in an atmosphere with high humidity.

2) foam may accumulate because antifoam is not added during a fermentation. Foam may have an adverse effect on the oxygen transfer rate by preventing adequate mixing and increasing the residence time of bubbles (Hall et. al., 1973).

The worth of flask culture in aeration studies is restricted by the inability to measure dissolved oxygen tension. However, aeration studies carried out in flasks are important because results directly relate to screening programs.

1.5.1.2) Continuous culture

Continuous cultures are open systems in which sterile nutrient solution is added to the bioreactor continuously while an equivalent amount of converted nutrient solution with microorganisms is simultaneously removed. The most commonly used form of continuous culture is the chemostat in which cell growth is controlled by adjusting the concentration of one medium nutrient. Any nutrient can be used to limit growth, including carbon sources, nitrogen sources, salts and oxygen. Chemostat cultures have great simplifying power in physiological studies because they;

1) allow the separate manipulation of nutritional factors and growth rate
2) eliminate transient effects by the achievement of steady states.

In the light of the great simplifying power of chemostat culture, it is surprising how little it has been used in the study of secondary metabolism. With respect to studies of aeration and secondary metabolism, one important
deficiency is oxygen-limited chemostat cultures. However, carbon-source-limited chemostats have been used to study the influence of oxygen tension on phenazine production (Messenger and Turner, 1983) and carbon dioxide tension on penicillin production (Pirt and Mancini, 1975).

1.5.2) METHOD USED TO VARY AERATION EFFICIENCY

1.5.2.1) Stirrer speed and air flow rate (shaker speed and flask type)

Aeration efficiency may be varied in a fermenter by changing air flow rate and/or stirrer speed, or in a flask by changing flask type (size or presence/absence of baffles), culture volume, shaker speed or shaker type (reciprocating/rotary). Aeration studies which use these strategies to vary aeration efficiency do not separate the variables of oxygen supply, hydrodynamic stress and carbon dioxide flushing, a serious limitation in view of reports indicating that both hydrodynamic stress and carbon dioxide may influence antibiotic production. These reports will now be considered.

1.5.2.1.1) The effect of hydrodynamic stress on antibiotic production

For antibiotic-producing fungi increased stirrer speed has been reported to:

1) change mycelial morphology: mycelia are shorter and thicker with a higher frequency of branching (Dion et. al., 1954; Camposano et. al., 1961). It has been suggested that increased branching rates lead to increased resistance to damage (Mitard and Riba, 1988).

2) cause nucleotide leakage. The rate of nucleotide leakage was found to be
directly related to impeller velocity (Tanaka and Ueda, 1975; Tanaka et. al., 1975b).

3) decrease growth rate (Tanaka et. al., 1975a, b).

4) decrease antibiotic yield (Pitt and Bull, 1982; Smith et. al., 1990).

The influence of increased stirrer speed on growth rate and antibiotic yield is complicated by its other effects on mixing and oxygen transfer. For example, although there may be increased mycelial damage as stirrer speed is increased, improved oxygen transfer may result in increased growth rate and antibiotic productivity. In such fermentations there will be an optimum stirrer speed. Optimal stirrer speeds have been demonstrated for the production of citric acid by Aspergillus niger (Clark and Lentz, 1963; Ujcova et. al.,1980) and penicillin by Penicillium chrysogenum (Dion et. al.,1954; Vardar and Lilly, 1982; Konig et. al., 1981).

Few reports have addressed the effect of hydrodynamic stress on actinomycetes. Leakage of intracellular nucleotides at high stirrer speeds has been noted for a single Streptomyces spp. (Tanaka et. al., 1975b). Clavulanic acid production has been reported to be adversely affected by high stirrer speeds (Tarbuck et. al., 1985), but this has been disputed by Belmar-Beiny and Thomas (1990).

1.5.2.1.2) The effect of carbon dioxide on antibiotic production

Carbon dioxide has been found to inhibit the production of macrocin (Chen and Wilde, 1991), erythromycin (Nash, 1974) and penicillin (Pirt and Mancini, 1975; Ho et. al., 1987). Inhibition of antibiotic production may occur either with (Chen and Wilde, 1991; Ho et. al., 1987) or without
(Nash, 1974; Pirt and Mancini, 1975) inhibition of growth. The fact that penicillin production has been inhibited by carbon dioxide both with and without growth inhibition indicates that the effect of carbon dioxide is dependent on growth conditions (Ho et. al., 1987; Pirt and Mancini, 1975). Ho et. al. (1987) reported that carbon dioxide increases mycelial branching frequency in *Penicillium chrysogenum*. They suggested that the inhibition of growth by carbon dioxide might be offset by an increased branching frequency. No study has determined the mechanism by which carbon dioxide inhibits antibiotic production, although Nash (1974) and Chen and Wilde (1991) have suggested that carbon dioxide affects antibiotic biosynthetic enzymes.

Sherstobitova *et. al.* (1976) have reported that tetracycline production by *Actinomyces aureofaciens* in defined medium is stimulated by low levels of carbon dioxide but inhibited by higher levels. It is possible that the stimulatory effect of carbon dioxide is caused by a need for carbon dioxide for the production of essential compounds - for example oxaloacetate can be formed by the carboxylation of pyruvate in five different ways. This possibility is supported by work on *Pseudomonas fluorescens* (Gill and Tan, 1979) which indicates that carbon dioxide is required for biosynthesis during growth on minimal medium.
1.5.2.2) Changing the composition of the input gas at constant total flow

The variable of hydrodynamic stress may be eliminated from aeration studies if aeration efficiency is varied by changing the composition of the input gas at constant total flow. Thus aeration efficiency has been increased by increasing the proportion of oxygen in the input gas and decreased by increasing the proportion of nitrogen in the input gas. A study of *Streptomyces fradiae* (Flickinger and Perlman, 1980) demonstrated that pulsed oxygen additions to the inlet gas stream can suppress respiration. The possibility that sudden pulses of oxygen affect cellular metabolism raises questions about the studies of Rollins et. al. (1989, 1990) on *Streptomyces clavuligerus* because here manual regulation of oxygen flow rates resulted in deviations in dissolved oxygen tension of up to 10% on either side of set points.

1.5.3) CULTURE MEDIUM

Culture medium has been demonstrated to regulate the effect of aeration efficiency on antibiotic production in batch culture;

1) *Streptomyces clavuligerus* has been reported by the same workers to have specific cephemycin C production increased by oxygen saturation in complex medium (Rollins et. al., 1988, 1990) but decreased by oxygen saturation in defined medium (Rollins et. al., 1989).

2) *Bacillus brevis* requires a much lower aeration efficiency for high specific rates of gramicidin S production in defined medium than in complex medium (Vandamme et. al., 1981).

4) Tetracycline production by *Streptomyces rimosus* is less dependent on aeration efficiency in defined medium than in complex medium (Rose, 1979; Oblozhko *et al*., 1977).

Any of the following hypotheses may explain why culture medium is able to regulate the effect of aeration on antibiotic production:

1) Oxygen demand is different in different media, and therefore the degree of oxygen satisfaction varies in different media aerated at the same rate. For example, in a given culture system media containing high concentrations of rapidly-used nutrients would lead to high oxygen demand rates and oxygen limitation, while media containing slowly-assimilated nutrients would lead to lower oxygen demand rates and oxygen sufficiency.

2) Oxygen transfer is hampered as medium viscosity increases with nutrient concentration (Vandamme *et al*., 1981).

3) Different media have different dissolved oxygen and carbon dioxide concentrations (Schumpe *et al*., 1982) and antibiotic production is sensitive to these differences.
1.6) OBJECTIVES OF THIS PROJECT

This project seeks to determine the effect of oxygen limitation on antibiotic production and assess its relevance to screening for novel secondary metabolites. Satisfaction of this overall objective demands;  
1) the development of a method for measuring the oxygen status of small scale cultures  
2) an assessment of the potential of oxygen limitation as a means of stimulating secondary metabolism  
3) an investigation of the mechanisms by which oxygen limitation influences secondary metabolism

1.7) CHAPTER OUTLINE

Chapter 2 describes the materials and methods appropriate to all results chapters, including the details of a novel method for measuring dissolved oxygen tension in small scale culture vessels. Chapters 3 to 6 form the experimental results section. In Chapter 3, flask experiments with *Saccharopolyspora erythraea* and *Amycolatopsis orientalis* are used to test the potential of oxygen limitation for stimulating secondary metabolism. Observations are consolidated with appropriate batch fermenter cultures in Chapter 4 and chemostat cultures in Chapter 5. Chapter 6 investigates the effect of oxygen limitation on a screen for actinomycetes and thus compliments experiments with known antibiotic-producing organisms. In Chapter 7 findings from all previous chapters are summarised as an integrated discussion, and areas for future work are identified.
2) MATERIALS AND METHODS
2.1) ORGANISMS AND CULTURE CONDITIONS

The organisms used in this study were *Saccharopolyspora erythraea* ATCC MG10534 and *Amycolatopsis orientalis* ATCC 19795. All cultures were incubated at 30°C.

2.2) MEDIA

Table 1: Defined medium compositions

<table>
<thead>
<tr>
<th>ingredient</th>
<th>C-ltd</th>
<th>P-ltd</th>
<th>N-ltd</th>
<th>RICH</th>
</tr>
</thead>
<tbody>
<tr>
<td>glucose (g/l)</td>
<td>15</td>
<td>30</td>
<td>30</td>
<td>50</td>
</tr>
<tr>
<td>NaNO₃ (g/l)</td>
<td>11.12</td>
<td>11.12</td>
<td>2.38</td>
<td>18.53</td>
</tr>
<tr>
<td>KH₂PO₄ (g/l)</td>
<td>3</td>
<td>0.1</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>K₂HPO₄ (g/l)</td>
<td>7</td>
<td>-</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>MOPS* (g/l)</td>
<td>-</td>
<td>21</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>trace sol'n (ml/l)</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>PPG** (ml/l)</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

*3-[N-morpholino]propanesulphonic acid

**polypropylene glycol antifoam (used only in shake flasks: 0.01% Breox FMT30 (Water Management and Gamlen) was used in fermenters)

Trace element solution was concentrated in order to give the following final concentrations (g/l): MgSO₄.7H₂O 0.25, FeSO₄.7H₂O 0.025, CoCl₂ 0.00055, CuCl₂ 0.00053, CaCl₂.2H₂O 0.0138, ZnCl₂ 0.0104, MnCl₂ 0.0062, Na₂MoO₄ 0.0003. The pH of all media was adjusted to 7.0. Media were filter sterilised rather than autoclaved in order to avoid caramelisation. It was necessary to filter sterilise the trace elements separately from other
medium components as a concentrate at pH 2 in order to prevent precipitation and subsequent removal during filtering.

2.3) INOCULUM PREPARATION

Nutrient Agar spread plates of S. erythraea and A. orientalis were prepared from freeze-dried stocks via Nutrient Broth culture in baffled shake flasks (4-5 days at 220 r.p.m.). Spread plates were stored at 4°C for up to 2 months.

Four colonies from a Nutrient Agar plate were mashed up using a wire loop and used to prepare a Nutrient Broth culture in a baffled shake flask (3-5 days at 220 r.p.m.). 2 ml of Nutrient Broth culture was then used to prepare an inoculum culture in 48 ml of experiment medium in a baffled flask (48 hours at 220 r.p.m.). Inoculum cultures were always grown in the experiment medium in order to avoid carry-over of nutrients from Nutrient Broth. Inocula were added at 4% of culture volume in all shake flask and batch fermenter experiments.

2.4) DRY WEIGHT DETERMINATION

Membrane filters were dried in a microwave oven, dessicated overnight, and weighed. Weighed membrane filters were washed in 0.01% (v/v) Tween 80, placed in a filter unit, and rinsed with 20 ml reverse osmosis water. 10 ml of shaken sample was filtered, rinsed with 30 ml reverse osmosis water, dried in the microwave, dessicated overnight, and weighed.

2.5) CULTURE LIQUID COLLECTION

Culture liquid was collected by centrifuging culture samples at 3000 r.p.m. for 10 minutes and removing the supernatant.
2.6) ERYTHROMYCIN AND VANCOMYCIN ASSAYS

Erythromycin and vancomycin concentrations were determined by agar bioassay as follows. *A. citreus* was grown overnight in Nutrient Broth at 30°C. 250 ml of molten Nutrient Agar at approximately 40°C was seeded with 2 ml of neat overnight culture and poured into a large bioassay plate (Intermed Nunc). When the agar had set, 7mm wells were formed using a hollow tube attached to a suction pump. 100 µl volumes of culture liquid / standards were then applied to each well (standards were used at 100, 80, 60, 40 and 20 µg/ml). Plates were incubated at 30°C for 24 hours to enable growth of *A. citreus* and diffusion of antibiotic. A further 24 hour incubation period at room temperature served to clarify inhibition zone borders. The diameters of the inhibition zones were then measured to the nearest 0.5mm.

The use of bioassay for measuring antibiotic concentration suffers from a lack of specificity and accuracy. With respect to specificity, the growth of the challenge organism may be affected by;

a) a secondary metabolite other than that under study

b) medium nutrients

c) sample pH.

The accuracy of bioassay is restricted by the lack of clarity of many inhibition zone borders. High performance liquid chromatography (H.P.L.C.) would be a much more specific and accurate method of measuring antibiotic concentration. However, H.P.L.C. was not available in this study.
Subsequent work in the laboratory using an H.P.L.C. assay (Hilary Lynch, personal communication) confirmed that the bioassay was providing an accurate estimate of erythromycin concentration.

2.7) GLUCOSE ASSAY

Glucose concentrations were determined by the method of Trinder (1969) using an assay kit (Sigma; Cat. No. 315-500).

2.7.1) Assay principle

The enzymic reactions involved in the assay are as follows;

\[
\text{glucose} + \text{H}_2\text{O} + \text{O}_2 \xrightarrow{\text{glucose oxidase}} \text{gluconic acid} + \text{H}_2\text{O}_2
\]

\[
\text{H}_2\text{O}_2 + 4\text{-aminoantipyrine} + \text{p-hydroxybenzene sulphonate} \xrightarrow{\text{peroxidase}} \text{quinoneimine dye} + \text{H}_2\text{O}
\]

Glucose is first oxidised to gluconic acid and hydrogen peroxide, in the reaction catalysed by glucose oxidase. The hydrogen peroxide formed reacts in the presence of peroxidase with 4-aminoantipyrine and p-hydroxybenzene sulphonate to form a quinoneimine dye, with an absorbance maximum at 505 nm. The intensity of the colour produced is directly proportional to the glucose concentration in the sample.

2.7.2) Assay details

In order to bring glucose concentration into the range of the assay, culture liquid from RICH, N-ltd and P-ltd was diluted by a factor of 50, while that from C-ltd was diluted by a factor of 25. Any tests negative for
glucose were repeated using neat culture liquid. Standard solutions were used at 750, 600, 450, 300, 150 and 0 µg/ml glucose.

2.8) NITRATE ASSAY

Nitrate concentrations were determined by the method of Beutler and Wurst (1986) using an assay kit (Boehringer Mannheim; Cat. No. 905 658).

2.8.1) Assay principle

Nitrate is reduced by nicotinamide-adenine dinucleotide phosphate (NADPH) to nitrite in the presence of the enzyme nitrate reductase;

\[
\text{nitrate} + \text{NADPH} + \text{H}^+ \xrightarrow{\text{nitrreductase}} \text{nitrite} + \text{NADP}^+ + \text{H}_2\text{O}
\]

The amount of NADPH oxidised during the reaction is stoichiometric with the amount of nitrate. The decrease in NADPH is measured by means of its absorbance at 340 nm.

2.8.2) Assay details

In order to bring nitrate concentration into the range of the assay, culture liquid from RICH was diluted by a factor of 100, while that from P-ltd and C-ltd was diluted by a factor of 10. Culture liquid from N-ltd was used undiluted.

2.9) PHOSPHATE ASSAY

Phosphate concentrations were determined using the colorimetric method of McDermott (1991);

Acetone, 2.5M sulphuric acid and 10mM ammonium molybdate ((NH₄)₆Mo₇O₄·4H₂O) were mixed in the ratio 2:1:1 respectively to give ASA solution. 4 ml ASA solution was added to 0.5 ml culture liquid and then
0.4 ml of 1M citric acid was immediately added. The intensity of the colour change is measured at 355 nm and is directly proportional to the phosphate concentration in the sample.

In order to bring phosphate concentration into the range of the assay, culture liquid from RICH, N-ltd and C-ltd was diluted by a factor of 100. Culture liquid from P-ltd was used undiluted. Standard solutions were used at 100, 80, 60, 40, 20 and 0 µg/ml phosphate (added as KH₂PO₄).

2.10) ADENINE NUCLEOTIDE ASSAY

Adenine nucleotide concentrations were measured by the method of Davison and Fynn (1974) using the Lumac AEC Kit (Sonco ltd., Cat. No. 9281-0).

2.10.1) Assay principle

ATP is assayed by bioluminescence using the firefly assay according to the following reaction:

\[
luciferin + luciferase + ATP \rightarrow (luciferin-luciferase-AMP) + PPi
\]

\[
(luciferin-luciferase-AMP) + O_2
\]

\[
\rightarrow oxyluciferin + luciferase + CO_2 + AMP + LIGHT
\]

The light emitted is measured in a sensitive photometer and is proportional to the quantity of ATP present. ADP and AMP are measured after enzymic conversion to ATP as follows;

\[
ADP + \text{phosphoenol pyruvate} \xrightarrow{\text{pyruvate kinase}} \text{ATP} + \text{pyruvate}
\]
AMP + ATP \text{ adenylate kinase } \rightarrow 2 \text{ADP}

2.10.2) Making cell extracts

Culture samples were filtered through a 0.45 µm filter and the biomass rinsed with 10ml Modified Hepes Buffer (mM : Hepes 25, MgSO$_4$ 7.5, EDTA 1). The filter and biomass were then shaken for 1 minute in 10 ml 0.1% Benzethonium chloride in Modified Hepes Buffer (BEC). This extraction procedure was performed in the shortest possible time (2 minutes) in order to minimise changes to nucleotide levels during processing. In order to ensure that nucleotide concentrations were similar in all extracts the volume of sample filtered varied from 100 ml to 10 ml during a batch run. BEC extracts were stored at -18°C.

2.10.3) Assay details

For assay, BEC extracts were diluted 1 in 100 using BEC. Standard ATP solutions were used at 0.01, 0.001 and 0.0001 µg/ml and were measured in a cuvette containing duplicates of a BEC extract (“internal standardisation”) in order to account for any assay interference by cell components. Bioluminescence was measured in a Biocounter / 3M 2010A.

2.11) MEASURING DISSOLVED OXYGEN TENSION IN SMALL SCALE CULTURES (SHAKE FLASKS AND TUBES)

Dissolved oxygen tension (D.O.) was measured in shake flasks and tubes using the Neonatal Oxygen Catheter (size 5Fr) made by Biomedical Sensors Ltd. This microprobe is marketed as part of an Umbilical Artery Oxygen Monitoring System for neonates, but has here found a new
application in measuring D.O. in shaken small scale microbial cultures. The fine sensing tip of these microprobes (1.75 mm diameter) makes them ideal for measuring D.O. in small scale cultures. The microprobes were supplied sterile and could not be autoclaved. Insertion of the microprobe into a sterile small scale culture vessel demanded the use of a compression fitting which could form an air-tight seal (Figure 1).

**Figure 1 : The compression fitting used to seal the oxygen microprobe in small scale culture vessels**

The compression fitting was inserted into a glass side-arm at the base of the shake flask or test tube using a silicon-based glue (Figures 2 and 3). In order to minimise changes to fluid dynamics within the culture vessel, only the 2mm-long sensing tip of the microprobe was extended into the vessels.
Figure 2: The use of a dissolved oxygen microprobe in a flask
Figure 3: The use of a dissolved oxygen microprobe in a tube
Once the microprobe was secured inside the flask or tube, sterile medium was added (50ml to shake flasks and 10ml to tubes) and the microprobe was connected to a reader (Neocath 1000; Biomedical Sensors Ltd.) and chart recorder. The microprobe membrane then required a hydration period which was carried out under operating conditions of rotary shaking (tubes were angled at 30° from vertical during shaking, and both flasks and tubes were shaken at 250 r.p.m.). The hydration period was deemed to be complete when the microprobe signal stabilised (after about 30 minutes). The microprobe reading was then calibrated to 100% saturation. The effective zero of the microprobe was determined by bubbling nitrogen gas through the medium. The effective zero varied from 5% to 14% for different microprobes: re-scaling of values was required to obtain true values of dissolved oxygen tension. 100% saturation of the medium was re-established prior to inoculation. The microprobe signal was found to be highly stable during rotary shaking (dissolved oxygen readings fluctuated by only 2% over several hours prior to inoculation).
3) SHAKE FLASK EXPERIMENTS
IN DEFINED MEDIA
3.1) OBJECTIVE

To investigate the potential of oxygen limitation as a means of stimulating antibiotic production in screening programs.

3.2) SUMMARY

Using defined media, two known antibiotic producers were subjected to a variety of nutrient limitations, including oxygen, glucose, nitrate and phosphate. This experiment design enabled oxygen limitation to be compared with other nutrient limitations which are commonly used in screening programs. All fermentations were performed in flasks rather than fermenters in order to simulate screens as closely as possible.

Oxygen limitation was effective at down-regulating growth, and was able to stimulate the production of some secondary metabolites produced under other types of nutrient limitation. This finding indicated that oxygen limitation deserves equal status with other types of nutrient limitation as a potential means of stimulating secondary metabolite production in screens.
3.3) EXPERIMENT OUTLINE

Time-course shake flask fermentations were performed using *S. erythraea* and *A. orientalis* in P-ltd, N-ltd, C-ltd and RICH media. Each medium was designed to down-regulate culture growth by a specific nutrient limitation: P-ltd, N-ltd and C-ltd media were formulated to have very low concentrations of phosphate, nitrate and glucose, respectively, while RICH medium was formulated to have relatively high concentrations of all these nutrients in order that oxygen became growth-limiting. Oxygen limitation in RICH medium was ensured by using flask cultures of low aeration efficiency (unbaffled 250ml flasks containing 50ml culture volumes and incubated at 150 r.p.m). Confirmation of growth-limiting nutrients was facilitated by using fully defined media containing single nutrient sources which could be assayed. Sample flasks were taken at 6-14 hour intervals and dry weight, antibiotic, glucose, nitrate and phosphate concentrations were determined. Duplicate flasks were taken at each sample point in order to compensate for flask-to-flask variations. The oxygen status of each culture was monitored continuously in a single flask using a dissolved oxygen microprobe.

This experiment design is summarised in Figure 4.
Figure 4: Method Summary

- S. erythraea
- A. orientalis

SHAKE FLASK FERMENTATIONS

DEFINED MEDIA

- C-ltd
- N-ltd
- P-ltd
- RICH
3.4) RESULTS

3.4.1) Nutrient limitations and growth dynamics

The intended nutrient limitations were achieved in each medium, and both *S. erythraea* and *A. orientalis* showed similar growth dynamics under each type of nutrient limitation.

These findings will now be illustrated for each medium in turn.
**RICH medium (Graphs 1 and 2)**

In RICH medium, dissolved oxygen tension (D.O.) fell to zero when dry weight reached about 3.5 g/l. From this point D.O. remained at zero while growth proceeded at a reduced rate for at least 40 hours. Since no other medium nutrients were exhausted over the duration of the fermentations, culture growth was unequivocally oxygen limited.

*Graph 1: Flask culture of *S. erythraea* in RICH medium*

*Graph 2: Flask culture of *A. orientalis* in RICH medium*

*nutrients not exhausted not shown*
Although oxygen limitation has been reported in other actinomycetes (Rollins et. al., 1988, 1990; Chen and Wilde, 1991; Virgilio et. al., 1964), growth responses were unclear in these studies because periods of oxygen limitation were very short.

The ability to continue growing after D.O. has reached zero may be important for survival and competition in the natural environment (soil) when conditions become microaerophilic. We can speculate that microaerophilic habitats may occur in soil which becomes waterlogged or compacted. Microaerophilic growth has been reported in several genera of actinomycetes, including *Agromyces, Arachnia, Oerskova* and *Rothia* (Lechevalier and Lechevalier, 1981).
P-ltd medium

In P-ltd medium, phosphate was the first medium nutrient to become exhausted, and thus culture growth was phosphate-limited (Graphs 3 and 4). From the point of phosphate exhaustion growth proceeded at a reduced rate for 30 to 50 hours until lysis occurred when a second medium nutrient - glucose - was exhausted.

Graph 3: Flask culture of *S. erythraea* in P-ltd medium

Graph 4: Flask culture of *A. orientalis* in P-ltd medium

*arrows mark points of glucose exhaustion: nutrients not exhausted not shown*
D.O. remained above 82% over the duration of cultures in P-ltd medium (Graphs 5 and 6), and thus oxygen did not become growth-limiting.
In N-ltd medium, nitrate was the first medium nutrient to become exhausted, and thus culture growth was nitrate-limited (Graphs 7 and 8). From the point of nitrate exhaustion growth proceeded at a reduced rate for about 60 hours until lysis occurred when a second medium nutrient - glucose - was exhausted.
D.O. remained above 75% over the duration of cultures in N-ltd medium (Graphs 9 and 10), and thus oxygen did not become growth-limiting.
C-ltd medium

In C-ltd medium glucose was the first and only nutrient to become exhausted because lysis occurred at the point of glucose exhaustion (Graphs 11 and 12). Thus culture growth was glucose-limited.

*nutrients not exhausted not shown
D.O. remained above 79% over the duration of cultures in C-ltd medium (Graphs 13 and 14), and thus oxygen did not become growth-limiting.
Lysis occurred at the point of glucose exhaustion not only in C-ltd medium, but also in N-ltd and P-ltd media (lysis did not occur in RICH medium because glucose was not exhausted over the duration of cultures). Thus, under no growth condition examined did *S. erythraea* or *A. orientalis* make intracellular storage compounds from excess medium glucose. This indicates that the vegetative cells of these organisms are unable to survive even brief exposure to conditions of carbon-source exhaustion, a finding which is consistent with observations in some other actinomycetes (Grootwassink, 1976). In contrast with responses to glucose exhaustion, both *S. erythraea* and *A. orientalis* were able to survive and grow following the exhaustion of medium phosphate or nitrate. This indicated that phosphorus- and nitrogen- sources can be recycled within the cell following their exhaustion from the culture medium. *We can speculate that S. erythraea and A. orientalis are rarely subject to carbon-source exhaustion in the natural environment, and so have not evolved mechanisms for storing carbon sources. In contrast, phosphorus- and nitrogen- source exhaustions may be common in the natural environment, and this may account for the evolution of mechanisms for intracellular recycling of these nutrients.* This hypothesis is tenable because nutritional insufficiency is the most common extreme of the natural environment encountered by microorganisms (Neissel and Tempest, 1979; Mateju *et. al.*, 1985).
3.4.2) Secondary metabolite production under different nutrient limitations

3.4.2.1) S. erythraea

*S. erythraea* produced erythromycin under all 4 types of nutrient limitation - oxygen, phosphate, nitrate and glucose (erythromycin productivities are discussed later in section 3.4.2.3).

The culture liquid of oxygen- and phosphate- limited fermentations turned red, indicating that a red pigment was produced in addition to erythromycin. No red pigment was evident in the culture liquid of nitrate- and glucose- limited fermentations. Photograph 1 illustrates these findings.

Photograph 1: The appearance of flask cultures of *S. erythraea* in O-ltd, P-ltd, N-ltd and C-ltd media after 96 hours incubation.
The response of *S. erythraea* to the different types of nutrient limitation is summarised in Figure 5.

**Figure 5**: The effect of different types of nutrient limitation on secondary metabolite production by *Saccharopolyspora erythraea*. 
3.4.2.2 A. orientalis

A. orientalis produced vancomycin under 3 types of nutrient limitation - phosphate, nitrate and glucose (vancomycin productivities are discussed later in section 3.4.2.3). No vancomycin was produced under oxygen limitation. It was necessary to check that the lack of vancomycin production in RICH medium was a consequence of oxygen limitation rather than medium composition (the high nutrient concentrations of RICH medium may have repressed vancomycin production). To this end an oxygen-sufficient fermenter run was performed in RICH medium by using a high aeration rate. This culture was glucose-limited, and vancomycin was produced (Graph 15).

Graph 15 : An oxygen-sufficient fermenter culture of A. orientalis in RICH medium*

* A 3L LH2000 series fermenter was used with an air flow rate of 1.3 v/v/m and a stirrer speed of 500 r.p.m. An arrow marks the point of glucose exhaustion. Nutrients not exhausted not shown.
Thus, the lack of vancomycin production in flask culture was proven to be a consequence of oxygen limitation. The response of *A. orientalis* to different types of nutrient limitation is summarised in Figure 6.

**Figure 6:** The effect of different types of nutrient limitation on vancomycin production by *Amycolatopsis orientalis*. 

---

63
3.4.2.3) Antibiotic productivity

In order to compare antibiotic productivity under different types of nutrient limitation it was necessary to normalise antibiotic concentrations with respect to biomass. This was effected by calculating the specific antibiotic yield on biomass at the point of maximum pre-lysis antibiotic concentration.

Both erythromycin and vancomycin productivities were at least 2.5 times higher under phosphate limitation than under any other type of nutrient limitation (Graphs 16 and 17).
This finding was consistent with reports that excess phosphate inhibits the biosynthesis of many antibiotics, including vancomycin (Martin, 1977; Mertz and Doolin, 1973).

It is noteworthy that oxygen limitation was almost as effective at stimulating erythromycin production as nitrate- and glucose- limitations.
3.5) CONCLUSIONS AND DISCUSSION

1) The potential of oxygen limitation in screening programs

Oxygen limitation was effective at down-regulating growth and was also able to stimulate the production of some secondary metabolites produced under other types of nutrient limitation. These findings indicated that oxygen limitation deserves equal status with the other types of nutrient limitation as a potential means of stimulating secondary metabolite production in screens.

The potential of oxygen limitation for stimulating secondary metabolite production is measured in an actinomycete screen in Chapter 5.

2) Anomalous responses of model organisms to oxygen limitation

Both *S. erythraea* and *A. orientalis* were able to produce their respective antibiotics when growth was phosphate-, nitrate- or glucose-limited. In contrast, these two organisms responded differently to oxygen limitation: *S. erythraea* was able to produce erythromycin, but *A. orientalis* was not able to produce vancomycin. It was surprising that erythromycin can be produced under oxygen limitation because the biosynthesis of erythromycin requires the direct incorporation of oxygen via an oxygenase (Corcoran, 1981). The fact that erythromycin is produced under oxygen limitation indicates that the stimulus for antibiotic production does not necessarily bear any relation to the specific requirements of biosynthetic pathways. Chapter 4 describes experiments aimed at increasing our understanding of the physiological basis for the different responses of *S. erythraea* and *A. orientalis* to oxygen limitation.
3) A model for the physiology of red pigment production

*S. erythraea* produced a red pigment when growth was down-regulated by phosphate- and oxygen- limitations, but not when growth was down-regulated by nitrate- or glucose- limitations. The following 'unbalanced growth model' for the physiology of red pigment production is proposed: *When nitrate or glucose become growth-limiting, both energy generation and biosynthesis are impeded from the first metabolic step. Under these conditions no accumulation ('pooling') of intracellular metabolites occurs and no red pigment is produced. However, when oxygen or phosphate become growth-limiting, energy generation is impeded from concluding or near-concluding metabolic steps (in the case of oxygen the terminal transfer of electrons in oxidative phosphorylation is affected, while in the case of phosphate the generation of ATP from ADP and phosphate is affected). Under these conditions intracellular metabolites accumulate transiently in traffic-jam-backlog style. This accumulation of metabolites causes an increase in the energy required for preserving intracellular solute concentrations ('cell maintenance energy') at a time when the rate of energy generation is decreasing. *S. erythraea* excretes these pooled metabolites as red pigment in order to reduce metabolite pool sizes and maintenance energy and so prolong survival. The red pigment may thus be considered to be a 'shunt' or 'overflow' metabolite (Woodruff, 1966), although it may also have other functions. Some indirect evidence for the 'unbalanced growth model' is provided by the chemostat studies of Chapter 6.
4) BATCH FERMENTER EXPERIMENTS IN DEFINED MEDIA
4.1) OBJECTIVE

Shake flask experiments of Chapter 3 demonstrated that two known antibiotic-producers respond differently to oxygen limitation: *S. erythraea* was able to produce erythromycin, but *A. orientalis* was not able to produce vancomycin. It was surprising that erythromycin could be produced under oxygen limitation because the biosynthesis of erythromycin requires the direct incorporation of oxygen via an oxygenase (Corcoran, 1981). The fact that erythromycin was produced under oxygen limitation indicated that the stimulus for antibiotic production does not necessarily bear any relation to the specific requirements of biosynthetic pathways.

This chapter uses batch fermenter cultures to investigate the physiological basis for the different responses of *S. erythraea* and *A. orientalis* to oxygen limitation.

4.2) SUMMARY

Using a fermenter, cultures of *S. erythraea* and *A. orientalis* were oxygen limited at an early stage of growth. Responses to oxygen limitation in fermenter culture were consistent with responses observed previously in flask cultures - oxygen limitation stimulated erythromycin production by *S. erythraea*, but inhibited vancomycin production by *A. orientalis*. However, oxygen limitation was a less effective means of reducing growth rate in *A. orientalis* than in *S. erythraea*. This indicated that *A. orientalis* is better able to adapt to oxygen limitation than *S. erythraea*, and suggested that the stimulus for antibiotic production may be down-regulation of growth rate. A
low ATP concentration in early growth stages was identified as a possible intracellular effector of antibiotic production.

4.3) EXPERIMENT OUTLINE

Batch fermenter cultures of *S. erythraea* and *A. orientalis* were made in the fully defined RICH medium using an LH2000 series 3l fermenter. Oxygen-limitation was ensured by using low aeration and agitation rates: an air flow rate of 0.7 v/v/m, and a stirrer speed of 300 r.p.m. pH was not controlled in order to simulate culture conditions in the shake flask. Dissolved oxygen was monitored using an Ingold polarographic dissolved oxygen electrode. Foaming was eliminated by including 0.1% Breox FMT30 (Water Management and Gamlen) in the medium. Samples were taken at 6-10 hour intervals and dry weight, antibiotic, glucose, nitrate, phosphate and nucleotide concentrations were determined.
4.4) RESULTS AND DISCUSSION

4.4.1) OXYGEN LIMITATION AND GROWTH DYNAMICS

Oxygen was the first nutrient to become exhausted in cultures of both organisms (Graphs 18 and 19). The point of oxygen limitation was evident as a sudden reduction in culture growth rate as dissolved oxygen tension (D.O.) approached zero. D.O. remained at zero for the remaining culture time which consisted of a 30-40 hour phase of near-linear growth (i.e. oxygen-limited growth) and a prolonged stationary phase at about 3 g/l dry weight.
Stationary phases were characterised by:

1) a constant rate of carbon source consumption (Graphs 20 and 21) - this reflected the constant rate of supply of oxygen, the growth-limiting nutrient.
2) a lack of nitrate consumption (Graphs 22 and 23).

**Graph 22** : Fermenter culture of *S. erythraea* in RICH medium

- **Graph 23** : Fermenter culture of *A. orientalis* in RICH medium

3) production of a red pigment

**NOTE** : The production of red pigment by *A. orientalis* was inconsistent with oxygen-limited shake flask cultures of this organism (Chapter 3) which did not produce red pigment. The discrepancy may be explained by differences in the severity of oxygen limitation - flask cultures were oxygen-limited at about 3.5 g/l dry weight whereas fermenter cultures were oxygen-limited at under 2 g/l dry weight.
The large sample volumes required for assays meant that fermentations could not be accurately followed to lysis. However, a repeat fermentation with *S. erythraea* indicated that stationary phases would continue until all medium glucose is exhausted (data not shown).

For both organisms the growth and nutrient concentration dynamics indicate that oxygen-limited growth (consuming both glucose and nitrate) continues for a finite period after which only maintenance energy requirements (consuming only glucose) can be met. The metabolic switch from growth to maintenance may be precipitated by;

1) the exhaustion of a precursor or cofactor for protein synthesis
2) a detrimental accumulation of intracellular metabolites.

The latter explanation is consistent with the 'unbalanced growth hypothesis' (proposed in Chapter 3) which suggests that red pigment is produced as a shunt or overflow metabolite.
4.4.2) SPECIFIC GROWTH RATES

The error margins of dry weight measurement were so low that error bars were smaller than the plotted data points. These low error margins permitted the accurate calculation of specific growth rates from computer-calculated partial cubic spline fits to dry weight data points using the method of McDermott et. al. (1993).

Graphs 24 and 25 show the profiles of specific growth rate plotted with the partial cubic spline curves from which they were calculated.
For both organisms specific growth rate rapidly rises to reach a maximum value just before D.O. fell to zero. From this point growth rate quickly fell to a stable value during the 30-40 hour period of linear, oxygen-limited growth. Growth rate finally falls to zero as oxygen-limited growth gives way to prolonged stationary phases.
Although the two organisms showed similar profiles of specific growth rate, actual growth rates were higher in *A. orientalis* both before and after the onset of oxygen limitation (Graph 26).

Prior to the onset of oxygen limitation the growth rate of *A. orientalis* reached a maximum of 0.101 /h in while that of *S. erythraea* reached a maximum of only 0.072 /h. Following oxygen limitation the growth rate of *A. orientalis* stabilised at 0.051 /h while that of *S. erythraea* stabilised at only 0.022 /h. The differences in growth rate of cultures of *S. erythraea* and *A. orientalis* can be explained by taking into account the different morphologies of these organisms and by considering that culture
morphology can influence the proportion of growing biomass. The following discussion elaborates this argument: actinomycete hyphae grow only at the tips (Prosser and Tough, 1991), and therefore growth rate is influenced by the number of hyphal tips per unit biomass, i.e. the proportion of growing biomass. When *A.orientalis* grows in liquid culture a mycelium is not formed because hyphae immediately fragment into numerous very short, unbranched lengths (Photograph 2).

**Photograph 2: The morphology of *A.orientalis* in fermenter culture***

* A sample was taken from an oxygen-limited fermenter culture in RICH medium after 25 hours of growth and a heat-fixed slide preparation was stained with crystal violet, then photographed at 400X magnification. Samples taken at other time-points showed similar morphology.
This hyphal fragmentation means that the culture of *A. orientalis* has a large number of hyphal tips per unit biomass, and thus can support high growth rates.

When *S. erythraea* grows in liquid culture hyphae elongate and branch, maintaining their integrity to form mycelial clumps (Photograph 3).

**Photograph 3 : The morphology of *S. erythraea* in fermenter culture**

* A sample was taken from an oxygen-limited fermenter culture in RICH medium after 25 hours of growth and a heat-fixed slide preparation was stained with crystal violet, then photographed at 400X magnification. Samples taken at other time-points showed similar morphology.
This hyphal elongation means that the culture of *S. erythraea* has fewer hyphal tips per unit biomass than the culture of *A. orientalis*, and thus cannot grow as fast as *A. orientalis*.

It is important to note that the percentage reduction in specific growth rate imposed by oxygen limitation was significantly greater for *S. erythraea* than for *A. orientalis* - the growth rate of *S. erythraea* was reduced by 70% when oxygen limitation occurred while that of *A. orientalis* was reduced by only 50%. This indicates that *A. orientalis* was better able to adapt to a limited oxygen supply than *S. erythraea*. This adaptation may involve increasing or modifying the content of respiratory enzymes or cytochromes, a possibility which is supported by reports that respiratory chains of *Escherichia coli* and *Azotobacter vinelandii* are modified under conditions of oxygen starvation (Poole, 1993).
4.4.3) ANTIBIOTIC PRODUCTION

Oxygen limitation stimulated erythromycin production by *S. erythraea*, but inhibited vancomycin production by *A. orientalis*. Thus responses to oxygen limitation in fermenter culture were consistent with those responses observed previously in flask cultures (Chapter 3). The finding that oxygen limitation was a less effective means of down-regulating the growth of *A. orientalis* than *S. erythraea* (section 4.4.2) suggests that the stimulus for antibiotic production may be down-regulation of growth rate, i.e. under oxygen limitation *S. erythraea* produced erythromycin because there was a large step-down in growth rate, but *A. orientalis* did not produce vancomycin because there was not a large enough step-down in growth rate.

4.4.4) KINETICS OF ERYTHROMYCIN PRODUCTION

The kinetics of erythromycin production were investigated in order to test the hypothesis (proposed in section 4.4.3) that down-regulation of growth stimulates erythromycin production in *S. erythraea*. Production kinetics were evaluated by comparing the profiles of specific growth rate (µ) and specific erythromycin production rate (Q_{ery}). Values of µ and Q_{ery} were derived from computer-calculated partial cubic spline fits to data points using the method of McDermott *et. al.* (1993), and Graph 27 shows the profiles so obtained.
Growth rate reached a maximum value about 10 hours before erythromycin production rate reached a maximum. This offset of peak growth and production rates was consistent with growth-dissociated production kinetics for erythromycin production. Erythromycin production rate steadily fell as growth rate stabilised during oxygen-limited growth, but a second increase in erythromycin production rate occurred as growth rate finally fell to zero. This second increase in erythromycin production rate provided further support for the idea that down-regulation of growth is the stimulus for erythromycin production.

It is evident from Graph 27 that some erythromycin production occurred before growth rate was down-regulated. This early erythromycin
production may be explained by the constant presence of a small proportion of senescing biomass in the culture: A physiological gradient exists along hyphae, with rapidly-growing apical regions giving way to senescing distal regions (Prosser and Tough, 1991). Thus, at any time-point in batch culture there will be some old and senescing biomass. During senescence, growth processes are down-regulated and erythromycin production is stimulated. The physiological gradient along hyphae means that absolute correlations between down-regulation of growth and erythromycin production cannot be obtained. However, comparisons of peak growth and production rates are still valid because they represent the synchronous responses of most of the culture biomass to the down-regulation of growth imposed by sudden oxygen limitation.

4.4.5) Adenylate energy charge and adenine nucleotide concentrations

In section 4.4.2 down-regulation of growth was identified as a possible explanation for the different antibiotic-responses of S. erythraea and A. orientalis to oxygen limitation. In order to consolidate this explanation, it was necessary to identify the intracellular effector of antibiotic production. Reduction in ATP concentration or Adenylate Energy Charge have been identified as possible effectors of antibiotic production in other actinomycetes (Martin, 1977) and thus nucleotide concentrations were investigated here.
Profiles of Adenylate Energy Charge (AEC) were similar for cultures of *S. erythraea* and *A. orientalis* (Graphs 28 and 29) - AEC therefore did not account for the different patterns of growth and antibiotic production observed in these organisms. AEC was low on inoculation (0.2-0.4), but increased to over 0.9 as D.O. fell to zero. AEC remained high (above 0.75) throughout the period of oxygen-limited growth and the prolonged stationary phase. Thus, oxygen-limitation did not impose any sudden change on AEC.

*Graph 28: Fermenter culture of *S. erythraea* in RICH medium*

*Graph 29: Fermenter culture of *A. orientalis* in RICH medium*

*arrows mark points at which dissolved oxygen tension reached zero*
Profiles of ADP and AMP concentration were similar for both organisms, concentrations remaining low throughout culture (Graphs 30 and 31). Although profiles of ATP concentration were similar for most of the culture time, significant differences were apparent during the early growth stages prior to oxygen-limitation: ATP concentration reached 750 nM/g in *S. erythraea*, but 1600 nM/g in *A. orientalis*.

*Graph 30: Fermenter culture of *S. erythraea* in RICH medium*

*Graph 31: Fermenter culture of *A. orientalis* in RICH medium*

*arrows mark points at which dissolved oxygen tension reached zero*
The different ATP concentrations during early growth stages may account for the different antibiotic-responses of *S. erythraea* and *A. orientalis*. In *A. orientalis* the high ATP concentration may inhibit or repress the vancomycin biosynthetic enzymes. Such repression or inhibition of antibiotic biosynthetic enzymes may not occur in *S. erythraea* which has a lower ATP concentration. We can speculate that ATP may repress antibiotic synthesis in a control mechanism which resembles the lactose operon in *Escherichia coli* (Jacob and Monod, 1961): ATP may bind to a repressor protein which in turn binds to a regulatory gene or promoter site for biosynthetic enzymes. Inhibition of pre-formed antibiotic biosynthetic enzymes could occur by direct, reversible binding of ATP. Atkinson and Walton (1967) have provided evidence that ATP binding is a regulatory mechanism for some enzymes of primary metabolism. Any hypothesis must assume that ATP concentrations in early growth stages can affect antibiotic production for the remaining culture time, an assumption which is supported by observations that growth conditions in early growth stages can affect later antibiotic-production phases (Mateju et. al., 1986).

Despite the differences in intracellular ATP concentration, profiles of AEC were similar for the two organisms. This finding was consistent with observations in other actinomycetes (Hostalek et. al., 1974; Curdova et. al., 1976; Martin et. al., 1978; Vu-Trong et. al., 1981; Dietzler et. al., 1974), and indicated that AEC was not an intracellular effector of antibiotic production.
4.5) CONCLUSIONS

1) In fermenter cultures of *S. erythraea* and *A. orientalis* oxygen limitation at early stages induced formation of a red pigment, and a stationary phase during which glucose consumption and dry weight increase were uncoupled.

2) Oxygen limitation in fermenter culture stimulated erythromycin production by *S. erythraea* but inhibited vancomycin production by *A. orientalis*. This confirmed responses to oxygen limitation observed previously in flask cultures (Chapter 3).

3) *S. erythraea* had lower growth rates than *A. orientalis*. A hypothesis accounting for this phenomenon was proposed which takes into account morphological differences.

4) Oxygen limitation was a less effective means of reducing growth rate in *S. erythraea* than in *A. orientalis*. This indicated that *A. orientalis* is better able to adapt to oxygen-limitation than *S. erythraea*, and suggested that the stimulus for antibiotic production may be down-regulation of growth rate. The kinetics of erythromycin production were consistent with this suggestion.

5) A low ATP concentration in early growth stages was identified as a possible intracellular effector of antibiotic production.
5) THE EFFECT OF OXYGEN LIMITATION ON A SCREEN FOR ANTIBIOTICS
5.1) OBJECTIVE

To investigate the potential of oxygen limitation as a means of stimulating antibiotic production in an actinomycete screen.

5.2) SUMMARY

A dissolved oxygen microprobe was used to assess the oxygen status of tube cultures of *S. erythraea* and *A. orientalis* in dilutions of a complex medium. This information was used to establish presumptive oxygen-sufficient and oxygen-limited cultures in a screen of 320 actinomycetes at Glaxo Group Research. The responses of *S. erythraea* and *A. orientalis* indicated that any osmolarity and viscosity variables imposed by medium dilution were insignificant compared with differences in culture oxygen status.

Oxygen limitation caused a 5.7% reduction in the number of antibiotics detected. However, 8.8% of the organisms produced antibiotics only when oxygen-limited. These findings demonstrated that oxygen limitation has potential as a means of stimulating secondary metabolite production in screens.
5.3) EXPERIMENT OUTLINE

5.3.1) Establishing oxygen-limited and oxygen-sufficient cultures for the screen

5.3.1.1) Varying the oxygen status of screen cultures

Screen cultures were made in test tubes angled at 30° from vertical on a rotary shaker at 250 r.p.m. This culture configuration formed part of Glaxo's routine screening operation and therefore could not be varied. Culture volumes were restricted to 10ml for this study - larger volumes caused excessive splash, while smaller volumes were below the limit for microprobe measurement of oxygen status. Thus, the aeration efficiency of cultures was not amenable to control and the oxygen status of cultures could only be varied by changing medium nutrient concentration ('medium dilution'). The 'medium dilution' method of varying culture oxygen status introduced additional unwanted variables of osmolarity and viscosity.

5.3.1.2) Choice of culture medium

The choice of medium for this study was restricted by the need to use a Glaxo complex medium (SM14) known to favour secondary product formation (SM14 had the following composition (g/l): glucose 10, soypeptone (Labem) 20, LabLemco 5, NaCl 5, ZnSO₄ 0.01; the pH was adjusted to 7.0 with 5M KOH).

5.3.1.3) Assessing culture oxygen status

Having decided on a suitable growth medium for this study, it was necessary to establish dilutions of this medium which would give oxygen-limited and oxygen-sufficient cultures. To this end, time-course studies of
S. erythraea and A. orientalis were performed in tube cultures in SM14, and dissolved oxygen status was assessed using a dissolved oxygen microprobe as described in Chapter 2.

In SM14, cultures of both organisms became oxygen limited for at least 20 hours (Graphs 32 and 33). If we assume that other actinomycetes have similar oxygen requirements, then tube cultures in SM14 are presumptively oxygen limited.
In order to establish oxygen-sufficient cultures, time-course studies of *S. erythraea* and *A. orientalis* were performed in tube cultures in a half-strength dilution of SM14 (named SM97). In SM97, cultures of both organisms were oxygen-sufficient (Graphs 34 and 35). If we assume that other actinomycetes have similar oxygen requirements, then tube cultures in SM97 are presumptively oxygen-sufficient.
It is noteworthy that the responses of the two model organisms to oxygen limitation in SM14 matched responses to oxygen limitation previously observed in defined medium (Chapters 3 and 4), i.e. under oxygen limitation *S. erythraea* produced erythromycin, but *A. orientalis* did not produce vancomycin (data not shown). This finding indicates that the influence of osmolarity and viscosity variables on antibiotic formation is insignificant when compared with the influence of oxygen limitation.

5.3.2) Screening

5.3.2.1) Growth of isolates and extraction of antibiotics

320 actinomycetes were taken from Glaxo's culture collection. Seed cultures were prepared by incubating frozen agar plugs for 4 days at 30°C in tube cultures of Growth Medium shaken at 250 r.p.m. (Growth Medium had the following composition (g/l): glucose 15, glycerol 15, soypeptone (Labem) 15, NaCl 3, CaCO₃ 1; the pH was adjusted to 7 with 5M KOH prior to autoclaving). Tubes were angled at 30° from vertical during shaking in order to enhance mixing and gas transfer. Several glass beads were included in each tube in order to facilitate homogenisation of the agar plugs.

1ml of each seed culture was used to inoculate each of 2 'production' cultures - SM14 and SM97 (full- and half- strength media, respectively). Production cultures were made in tubes angled at 30° from vertical, but glass beads were not included. Cultures were incubated at 30°C for 6 days on a rotary shaker at 250 r.p.m. Antibiotic extracts were prepared from production cultures by adding an equal volume of methanol to the broth, leaving for 1 hour, then filtering.
5.3.2.2) Agar bioassay

Antibiotics were detected in each methanol extract by agar bioassay against two challenge organisms: *Staphylococcus aureus* ATCC864 and *Candida albicans* ATCC316. Challenge organisms were grown overnight in Malt Yeast Glucose Peptone Agar (MYPG) at 37°C. MYPG had the following composition (g/l): malt extract 3, glucose 10, peptone (Oxoid) 5, yeast extract (Difco) 3. 300ml of molten Nutrient Agar at approximately 40°C was seeded with challenge organisms and poured into a large bioassay plate (Intermed Nunc). For *S. aureus* plates the agar seed consisted of 2ml of a 1 in 100 dilution of the overnight MYPG culture plus 0.8ml of tetrazoleum (the tetrazoleum served to clarify inhibition zones by staining live organisms pink). For *C. albicans* plates the agar seed consisted of 2ml of neat overnight MYPG culture. When the agar had set, 7mm wells were formed by punching holes in the agar with a hollow tube and removing the agar plug so formed. 50µl volumes of methanol extracts were then applied to each well. Standard antibiotic solutions were also included on each plate in order to monitor plate-to-plate variations. For *S. aureus* plates the standard was a 1 µg/ml solution of chlortetracycline, while for *C. albicans* plates the standard was a 0.1 mg/ml solution of nystatin. All plates were incubated at 37°C for 24 hours to enable growth of the challenge organisms and diffusion of antibiotics.

Antibiotic activity was scored simply as presence or absence of inhibition zones.
5.4) RESULTS

The number and type of antibiotic activities detected in the screen are given in Table 2.

**Table 2 : Antibiotic activities detected in a screen of 320 actinomycetes**

<table>
<thead>
<tr>
<th>challenge organism</th>
<th>number of organisms showing antibiotic activity in SM14 (oxygen-limited)</th>
<th>number of organisms showing antibiotic activity in SM97 (oxygen-sufficient)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>142</td>
<td>150</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>56</td>
<td>60</td>
</tr>
<tr>
<td><strong>TOTALS</strong></td>
<td><strong>198</strong></td>
<td><strong>210</strong></td>
</tr>
</tbody>
</table>

The pattern of antibiotic activities was broadly similar in oxygen-limited (SM14) and oxygen-sufficient (SM97) cultures, with anti-*S.aureus* activities being between 2 and 3 times more common than anti-*C.albicans* activities. A Chi-squared test on the ratios of antibiotic types confirmed that culture medium (and therefore oxygen status) did not influence the ratio of broad classes of antibiotics detected (Figure 7). The total number of antibiotics detected was also similar in the 2 media, with oxygen-limited cultures (SM14) giving 5.7% fewer actives than oxygen-sufficient cultures (SM97).
Figure 7: A Chi-squared test of the Null hypothesis "SM14 and SM97 gave identical ratios of anti-\textit{S. aureus} and anti-\textit{C. albicans} antibiotics".

<table>
<thead>
<tr>
<th></th>
<th>Anti-\textit{S. aureus} activity</th>
<th>Anti-\textit{C. albicans} activity</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>observed (O)</td>
<td>142 / 320 = 0.444</td>
<td>56 / 320 = 0.175</td>
<td>0.619</td>
</tr>
<tr>
<td>expected (E)</td>
<td>[0.619 \times \frac{150}{320} / (\frac{150}{320} + \frac{60}{320})] = 0.442</td>
<td>[0.619 \times \frac{60}{320} / (\frac{150}{320} + \frac{60}{320})] = 0.177</td>
<td>0.619</td>
</tr>
<tr>
<td>deviation (O-E)</td>
<td>0.002</td>
<td>-0.002</td>
<td>0</td>
</tr>
<tr>
<td>(O-E)^2</td>
<td>[2 \times 10^{-6}]</td>
<td>[2 \times 10^{-6}]</td>
<td></td>
</tr>
<tr>
<td>(O-E)^2 /E</td>
<td>[4.52 \times 10^{-6}]</td>
<td>[4.52 \times 10^{-6}]</td>
<td>[\text{Chi}^2 = 1.58 \times 10^{-5}] with 1 degree of freedom</td>
</tr>
</tbody>
</table>
A commonly-used parameter for assessing screen data is the Product Diversity Index (P.D.I.), which is calculated as follows;

\[
P.D.I. = \frac{\text{number of detection assay positives} \times \text{number of detection assays}}{\text{number of test organisms} \times \text{number of test organisms}}
\]

The similar numbers of antibiotics detected in SM14 and SM97 were reflected in similar Product Diversity Indices for these media;

SM14 (oxygen-limited), P.D.I. = \(\frac{142 + 56}{320 \times 2} = 0.31\)

SM97 (oxygen-sufficient), P.D.I. = \(\frac{150 + 60}{320 \times 2} = 0.33\)
A consideration of the total numbers of antibiotics detected in the screen gives no information about the responses of individual organisms. Table 3 reveals that the 5.7% reduction in the number of antibiotic activities detected in oxygen-limited cultures (SM14) is not simply the result of non-production by a few organisms, but rather is the net effect of non-production by 8.8% of the organisms under oxygen-sufficiency (SM97) and 12.5% of the organisms under oxygen limitation (SM14).

Table 3: The influence of culture oxygen status on antibiotic responses

<table>
<thead>
<tr>
<th>GROUP OF ACTINOMYCETES</th>
<th>percentage of the 320 actinomycetes screened</th>
</tr>
</thead>
<tbody>
<tr>
<td>able to produce antibiotics in both oxygen-sufficient (SM97) and oxygen-limited (SM14) cultures</td>
<td>53.1 %</td>
</tr>
<tr>
<td>able to produce antibiotics only in oxygen-sufficient cultures (SM97)</td>
<td>12.5 %</td>
</tr>
<tr>
<td>able to produce antibiotics only in oxygen-limited cultures (SM14)</td>
<td>8.8 %</td>
</tr>
</tbody>
</table>

Thus, oxygen limitation stimulated the production of some antibiotics which were not produced in oxygen-sufficient cultures. This finding was consistent with results from confidential receptor-based screens (data not shown).
5.5) CONCLUSIONS / DISCUSSION

Oxygen limitation caused a 5.7% reduction in the number of antibiotics detected. However, 8.8% of the organisms produced antibiotics only when oxygen limited. These findings demonstrated that oxygen limitation has potential as a means of stimulating secondary metabolite production in screens.

In this study we have demonstrated that oxygen limitation exerts its own specific influence on secondary metabolism in the complex medium SM14 - in other words, in SM14 oxygen limitation does not merely delay effects of subsequent (here unidentified) nutrient limitations. We can speculate that oxygen limitation would also have specific effects on secondary metabolism in other culture media regardless of the types of subsequent nutrient limitations. In this scenario, oxygen limitation would eclipse effects of other nutrient limitations and so rob the screen of the potential benefit of having a variety of culture media. Further studies are required to validate this hypothesis.
6) CHEMOSTAT EXPERIMENTS IN DEFINED MEDIA
6.1) OBJECTIVE

Oxygen-limited batch fermenter cultures of Chapter 4 indicated that erythromycin production by *S. erythraea* is stimulated by down-regulation of growth. This definition of the growth-biosynthesis relationship rests on two major assumptions:

1) that the production of erythromycin is stimulated by a decline in growth rate rather than oxygen limitation *per se*

2) that the biosynthetic activity of the culture is in equilibrium with the composition of the environment, i.e. that adaptation to a changing environment is instantaneous.

These assumptions are tested in this chapter by using chemostat cultures which eliminate transient effects and enable the separate manipulation of oxygen supply and growth rate.
6.2) SUMMARY

Using defined media, chemostat cultures of *S. erythraea* were established under both oxygen- and nitrate- limited conditions. New oxygen-limited steady states were created by reducing fermenter air flow rates. Culture behaviour was monitored at all steady states, and also during the transient phases between steady states.

Very little erythromycin was produced in any oxygen-limited steady state (maximum concentrations were about 10 µg/ml). This finding was consistent with the idea that erythromycin production is stimulated by down-regulation of growth rather than oxygen limitation *per se*. No increase in erythromycin production was observed during the transient phases to new oxygen-limited steady states, but this was probably because reducing air flow rates caused only a small down-regulation of culture growth rate.

In contrast to oxygen-limited steady states, substantial erythromycin production occurred at nitrate-limited steady state. This indicated that nitrate limitation *per se* is able to stimulate erythromycin production independantly of any down-regulation of growth rate. Thus, chemostat studies revealed that erythromycin production in batch cultures may be the result of the combined influence of nutritional factors and down-regulation of growth.
6.3) EXPERIMENT OUTLINE

Chemostat cultures were performed using a LH500 series 1.5l fermenter stirred at 750 r.p.m. Experiments were performed in 2 media as follows;

a) N-ltd medium

A nitrate-limited steady state was established in N-ltd medium at a growth rate of 0.055 /h, a pH of 7.5, and an air flow rate of 1.5 v/v/m. An oxygen-limited steady state was then established by reducing the air flow rate to 0.025 v/v/m with all other parameters kept constant.

b) RICH medium

An oxygen-limited steady state was established in RICH medium at a growth rate of 0.066 /h, a pH of 7.7, and an air flow rate of 2 v/v/m. A different oxygen-limited steady state was then established by reducing the air flow rate to 0.75 v/v/m with all other parameters kept constant.

Samples were taken at regular intervals throughout both experiments, and dry weight, erythromycin, glucose, nitrate and phosphate concentrations were determined. Steady states were confirmed by taking 3 samples over a 24 hour period following a minimum of 3.5 fermenter volume changes of medium.
6.4) RESULTS

6.4.1) N-ltd medium (refer to Figure 8)

At nitrate-limited steady state, dry weight was 3.9 g/l and erythromycin concentration was 40 µg/ml. Evidence that growth was nitrate-limited was provided by relative concentrations of nutrients: nitrate was barely detectable in the culture medium (nitrate concentration was below 0.05 g/l), while all other measurable nutrient concentrations remained high (dissolved oxygen tension [D.O.] was above 90% saturation, glucose concentration was 23 g/l, and phosphate concentration fluctuated widely above 3 g/l [phosphate data not shown]). Oxygen became growth-limiting when the air flow rate was reduced, as indicated by the drop in D.O. to below 20% saturation. A transient phase from nitrate-limited to oxygen-limited steady states was characterised by:

a) culture wash-out and subsequent partial recovery to the oxygen-limited steady state biomass concentration (nutrient concentration changes mirrored changes dry weight concentrations, particularly in the case of nitrate).

b) a steady drop in erythromycin concentration.

At oxygen-limited steady state, dry weight was 3.5 g/l while erythromycin concentration dropped to about 10 µg/ml. Evidence that growth was oxygen limited is provided by relative concentrations of nutrients: D.O. was below 10% saturation, while all other measurable nutrient concentrations remained high (nitrate concentration was above 0.05 g/l and therefore higher than the nitrate-limited steady state concentration,
glucose concentration was 26 g/l, and phosphate concentration fluctuated widely above 3 g/l (phosphate data not shown).

No red pigment was observed at any point in the experiment.
The 3 graphs show different parameters of the same culture. Nitrate- and oxygen- limited steady states are shown with the transient phase between them. The time from 90 to 115 hours shows the progression to a nitrate-limited steady state. At 115 hours the air flow rate was reduced. The time from 115 hours shows the progression to an oxygen-limited steady state.
6.4.2) RICH medium (refer to Figure 9)

At the first oxygen-limited steady state, dry weight was 5.9 g/l and erythromycin concentration was about 10 µg/ml. Evidence that growth was oxygen limited was provided by relative concentrations of nutrients: D.O. was below 10% saturation, while all other measurable nutrient concentrations remained high (glucose concentration was 33 g/l, nitrate concentration was 10 g/l, and phosphate concentration fluctuated widely above 3 g/l [phosphate data not shown]). When the air flow rate was reduced, a transient phase to a second oxygen-limited steady state was characterised by culture wash-out and subsequent partial recovery to the new oxygen-limited steady state biomass concentration. No changes in nutrient concentrations were observed during the transient phase, but this was probably because the magnitude of such changes were smaller than the nutrient assay error margins. Erythromycin concentration remained at about 10 µg/ml throughout the transient phase. At the new oxygen-limited steady state dry weight was 5.6 g/l while erythromycin concentration remained at 10 µg/ml.

No red pigment was observed at any point in the experiment.
**Figure 9:** Chemostat culture of *S. erythraea* in RICH medium*

The 3 graphs show different parameters of the same culture. Two oxygen-limited steady states are shown with the transient phase between them. The time from 180 to 260 hours shows the progression to an oxygen-limited steady state. At 260 hours the air flow rate was reduced. The time from 260 hours shows the progression to a second oxygen-limited steady state.

*The 3 graphs show different parameters of the same culture. Two oxygen-limited steady states are shown with the transient phase between them. The time from 180 to 260 hours shows the progression to an oxygen-limited steady state. At 260 hours the air flow rate was reduced. The time from 260 hours shows the progression to a second oxygen-limited steady state.*
6.5) CONCLUSIONS AND DISCUSSION

1) The very low erythromycin concentrations at oxygen-limited steady states indicated that erythromycin production in oxygen-limited batch cultures was probably stimulated by down-regulation of growth rather than oxygen limitation per se.

Very little erythromycin was produced in any oxygen-limited steady state - maximum concentrations were about 10 µg/ml. This very low level of production is inconsistent with the idea that oxygen limitation per se stimulates erythromycin production. The low concentrations of erythromycin are more likely to reflect the constant presence of a small proportion of senescing biomass in the culture: *A physiological gradient exists along hyphae, with rapidly-growing apical regions giving way to senescing distal regions (Prosser and Tough, 1991).* Thus, at any time-point in chemostat culture there will be some old and senescing biomass. During senescence, growth processes are down-regulated and erythromycin production is stimulated.

2) The lack of increased erythromycin production during transient phases between steady states may not be significant because the transient phases may not have accurately simulated the down-regulation of growth observed in oxygen-limited batch culture.

The reduction in air flow rates was able to induce only a small down-regulation of growth, as indicated by small decreases in steady state biomass.
concentrations (0.4 g/l from nitrate-limited to oxygen-limited steady state in N-ltd medium, and 0.3 g/l between the two oxygen limited steady states in RICH medium). Thus, the lack of erythromycin production in transient phases was possibly because growth could not be down-regulated sufficiently by reducing air flow rate.

3) The moderate erythromycin concentration at nitrate limited steady state (40 µg/ml) indicated that nitrate limitation stimulated erythromycin production independently of any down-regulation of growth rate.

4) The lack of red pigment production at oxygen- and nitrate- limited steady states was consistent with the 'unbalanced growth hypothesis' for red pigment production (proposed in Chapter 3).

The unbalanced growth hypothesis states that a transient accumulation of intracellular metabolites is required for red pigment production. By definition, at steady state no transient accumulation of metabolites can be occurring and therefore no red pigment can be formed.

5) The lack of red pigment production during the transient phases between steady states may not be considered significant because the transient phases represented only a small down-regulation of growth.

Red pigment production was not stimulated during the transient phases to new oxygen-limited steady states which were imposed by reducing air flow rates. This seems to contradict the idea that red pigment production is
stimulated by a transient accumulation of intracellular metabolites because such an accumulation would surely occur during transient phases. However, the reduction in air flow rates was able to induce only a small down-regulation of growth, as indicated by small decreases in steady state biomass concentrations (0.4 g/l from nitrate-limited to oxygen-limited steady state in N-ltd medium, and 0.3 g/l between the two oxygen-limited steady states in RICH medium). Thus, the lack of red pigment production in transient phases was probably because the extent of intracellular metabolite accumulation was likely to have been insufficient for red pigment production.
7) REVIEW AND FUTURE WORK
This project sought to determine the effect of oxygen limitation on antibiotic production and assess its relevance to screening for novel secondary metabolites. Satisfaction of this overall objective demanded;
1) the development of a method for measuring the oxygen status of small scale cultures
2) an assessment of the potential of oxygen limitation as a means of stimulating secondary metabolism
3) an investigation of the mechanisms by which oxygen limitation influences secondary metabolism.

The following discussion describes how these objectives have been satisfied and identifies avenues of potential future work.

7.1) MEASURING THE OXYGEN STATUS OF SMALL SCALE CULTURES

Oxygen limitation was demonstrated in both shaken flasks (Chapter 3) and shaken tubes (Chapter 5) with the novel use of a dissolved oxygen microprobe. This confirmed that oxygen limitation is a potential threat to screening programs. The likelihood of oxygen limitation depended on the composition of the growth medium - media with high nutrient concentrations became oxygen limited while media with lower nutrient concentrations remained oxygen sufficient. The dissolved oxygen microprobe has potential in screening programs as an aid to medium design because it enables culture oxygen status to be
guaranteed. While it is neither cost effective nor practical to use the microprobe in every screen culture, the microprobe could be used to assess the oxygen status of a screen by monitoring cultures of appropriately selected 'model' organisms. Reasonable accuracy would be expected if the model organisms spanned the broad taxonomic groups taking part in the screen - for example a fungus, an actinomycete and a unicellular bacterium. Such use of microprobes in screens will probably only become routine practice when cheaper, purpose-built microprobes are available - the microprobes used in this study were commercially available for monitoring the blood of neonates and as such were expensive (£60 each) and could be used only twice. One restriction for the use of microprobes in screening is the culture volume limit for microprobe operation - at low culture volumes the microprobe may not be sufficiently immersed during shaking to give accurate readings. In this study the microprobe was found to give accurate readings in two types of small scale culture;

1) unbaffled shake flasks with 50ml culture volume shaken at 150 r.p.m.
2) tubes angled at 30° from vertical with 10ml culture volume shaken at 250 r.p.m.

Culture volume limits for microprobe operation were not established.
7.2) THE POTENTIAL OF OXYGEN LIMITATION FOR STIMULATING SECONDARY METABOLISM

The potential of oxygen limitation as a means of stimulating secondary metabolism was assessed in two ways;
1) shake flask experiments with 2 known antibiotic-producing organisms grown in defined media (Chapter 3)
2) a screen of unknown actinomycetes grown in complex media (Chapter 5).

In shake flask experiments, defined media were used to subject the erythromycin producer *Saccharopolyspora erythraea* and the vancomycin producer *Amycolatopsis orientalis* to a variety of nutrient limitations, including oxygen-, glucose-, nitrate- and phosphate- limitations, respectively. This experiment design enabled oxygen limitation to be compared with other nutrient limitations which are commonly used in screening programs. Both *S. erythraea* and *A. orientalis* were able to produce their respective antibiotics when growth was glucose-, nitrate- or phosphate- limited. In contrast, the two organisms responded differently to oxygen limitation - *S. erythraea* was able to produce erythromycin, but *A. orientalis* was not able to produce vancomycin. These findings indicated that product diversity in a screen would be reduced if oxygen limitation occurred in all screen cultures. The information provided by flask experiments was increased by the discovery that *S. erythraea* produced a red pigment under oxygen- and phosphate- limitations, respectively. The red pigment was clearly a secondary metabolite subject to regulatory mechanisms different from those of erythromycin and vancomycin. The fact
that both red pigment and erythromycin were produced under oxygen limitation suggested that there may be some secondary metabolites which are only produced under oxygen limitation. Thus, shake flask studies with known antibiotic-producers indicated that oxygen limitation deserves a place in screening provided that other nutrient limitations are also present.

In the screen of unknown actinomycetes, oxygen-sufficient and oxygen-limited tube cultures were compared. The oxygen status of the cultures was designed by using dilutions of the same complex medium, and was confirmed using the dissolved oxygen microprobe in cultures of 2 ‘model’ actinomycetes. The responses of the model organisms indicated that any osmolarity and viscosity variables imposed by medium dilution were insignificant compared with differences in culture oxygen status. Although oxygen limitation caused a 5.7% reduction in the number of antibiotics detected, 8.8% of the organisms produced antibiotics only when oxygen-limited. This finding demonstrated that oxygen limitation has potential as a means of stimulating secondary metabolite production in screens.

To summarise, findings in shake flask experiments with known antibiotic-producers were consistent with findings in the screen of unknown actinomycetes. Collectively these two studies demonstrated that oxygen limitation deserves equal status with other types of nutrient limitation as a potential means of stimulating secondary metabolite production.
7.3) MECHANISMS BY WHICH OXYGEN LIMITATION INFLUENCES SECONDARY METABOLISM

*S. erythraea* and *A. orientalis* responded differently to oxygen limitation in shake flask culture (Chapter 3) - *S. erythraea* produced antibiotic but *A. orientalis* did not. These different responses made these two organisms an ideal base on which to study the mechanisms by which oxygen limitation influences secondary metabolism.

Oxygen-limited batch fermenter experiments of each organism were performed in order to investigate the physiological basis for the different responses to oxygen limitation (Chapter 4). Adenine nucleotide concentrations and Adenylate Energy Charge (AEC) have been identified as possible intracellular effectors of antibiotic production in other actinomycetes (Martin, 1977), and so these parameters were investigated here. Responses to oxygen limitation in fermenter culture were consistent with responses observed previously in shake flask cultures - oxygen limitation stimulated erythromycin production by *S. erythraea* but inhibited vancomycin production by *A. orientalis*. It was noted that oxygen limitation was a less effective means of reducing growth rate in *A. orientalis* than in *S. erythraea*. This indicated that *A. orientalis* is better able to adapt to oxygen limitation than *S. erythraea*, and suggested that the stimulus for antibiotic production may be down-regulation of growth rate. Although profiles of intracellular nucleotide concentration were similar for the two organisms for most of the culture time, during early growth stages ATP concentration was 50% higher in *A. orientalis* than in *S. erythraea*. 
These findings suggested that a low ATP concentration in the early growth stages was the intracellular effector of antibiotic production. We can speculate that ATP may repress antibiotic synthesis in a control mechanism which resembles the lactose operon in *Escherichia coli* (Jacob and Monod, 1961): ATP may bind to a repressor protein which in turn binds to a regulatory gene or promoter site for biosynthetic enzymes. Alternatively, inhibition of pre-formed antibiotic biosynthetic enzymes could occur by direct, reversible binding of ATP. Atkinson and Walton (1967) have provided evidence that ATP binding is a regulatory mechanism for some enzymes of primary metabolism. Any hypothesis must assume that ATP concentrations in early growth stages can affect antibiotic production for the remaining culture time, an assumption which is supported by observations that growth conditions in early growth stages can affect later antibiotic-production phases (Mateju *et. al.*, 1986). Despite the differences in intracellular ATP concentration, profiles of AEC were similar for the two organisms. This finding was consistent with observations in other actinomycetes (Hostalek *et. al.*, 1974; Curdova *et. al.*, 1976; Martin *et. al.*, 1978; Vu-Trong *et. al.*, 1981; Dietzler *et. al.*, 1974), and indicated that AEC was not an intracellular effector of antibiotic production.
The suggestion that erythromycin production by *S. erythraea* is stimulated by down-regulation of growth rests on two major assumptions:

1) that the production of erythromycin is stimulated by a decline in growth rate rather than oxygen limitation *per se*

2) that the biosynthetic activity of the culture is in equilibrium with the composition of the environment, i.e. that adaptation to a changing environment is instantaneous.

These assumptions were tested in Chapter 5 by using chemostat cultures which eliminate transient effects and enable the separate manipulation of oxygen supply and growth rate. Using defined media, chemostat cultures of *S. erythraea* were established under both oxygen- and nitrate- limited conditions. New oxygen-limited steady states were created by reducing fermenter air flow rates. Culture behaviour was monitored at all steady states, and also during the transient phases between steady states. Very little erythromycin was produced in any oxygen-limited steady state (maximum concentrations were about 10 μg/ml). This finding was consistent with the idea that erythromycin production is stimulated by down-regulation of growth rather than oxygen limitation *per se*. The low concentrations of erythromycin at oxygen-limited steady state are likely to reflect the constant presence of a small proportion of senescing biomass in the culture: *A physiological gradient exists along hyphae, with rapidly-growing apical regions giving way to senescing distal regions* (Prosser and Tough, 1991). Thus, at any time-point in chemostat culture there will be some old and senescing biomass. During senescence, growth processes are down-
regulated and erythromycin production is stimulated. No increase in erythromycin production was observed during the transient phases to new oxygen-limited steady states, but this was probably because reducing air flow rates caused only a small down-regulation of culture growth rate. In contrast to oxygen-limited steady states, substantial erythromycin production occurred at nitrate-limited steady state. This indicated that nitrate limitation per se is able to stimulate erythromycin production independently of any down-regulation of growth rate. Thus, chemostat studies revealed that erythromycin production in batch cultures may be the result of the combined influence of nutritional factors and down-regulation of growth.

It is clear from this study that chemostats have major potential for elucidating the mechanisms of secondary metabolite production. Some areas of future work relevant to aeration include;

1) the investigation of a wide range of growth rates at oxygen-limited steady state (in this study with S. erythraea, only growth rates of 0.055 /h and 0.066 /h were investigated)

2) an assessment of the effect of carbon dioxide tension on antibiotic production - this could be achieved by varying gas compositions in a chemostat in which growth is limited by any nutrient, including oxygen. Carbon dioxide tension is a culture variable which merits further study because a number of reports have indicated that it can affect antibiotic production (Chen and Wilde, 1991; Nash, 1974; Pirt and Mancini, 1975; Ho et al., 1987).
7.4) THE ROLE OF SECONDARY METABOLITES IN THE LIFE CYCLE OF THE PRODUCING ORGANISMS

A knowledge of the mechanisms by which oxygen limitation influences secondary metabolism can stimulate hypotheses that might explain why microorganisms produce the secondary metabolites in the first place. Such hypotheses might aid the rational design of screening programs and so lead to the discovery of new products.

7.4.1) Erythromycin and vancomycin

This study has indicated that the stimulus for erythromycin and vancomycin production may be down-regulation of growth. This finding is consistent with an antagonistic role of these antibiotics in nature. The natural environment for these antibiotic-producing organisms is soil. We can imagine that a sudden change in the availability of nutrients would occur when two organisms growing through soil meet and begin to compete for the same substrate. Organism A would have been assimilating nutrients at a rate appropriate for its growth rate. The arrival of organism B, feeding on the same substrate, would decrease the availability of nutrients to organism A. The decreased nutrient availability would down-regulate growth, which in turn would stimulate antibiotic production to slow down or eliminate the competitor. This seems an efficient self-defence mechanism because the organism takes on the additional metabolic load needed to make antibiotics only when it is in direct competition for exactly the same substrate, and only when that substrate is the nutrient currently limiting its rate of growth.
7.4.2) Red pigment

*S. erythraea* produced a red pigment when growth was down-regulated by phosphate- and oxygen- limitations, but not when growth was down-regulated by nitrate- or glucose- limitations. The lack of bioactivity of red pigment (personal communication - J. Dewitt, T. Paulus, Abbott Laboratories, Chicago) suggests that red pigment does not have an antagonistic role in nature. However, the production conditions for red pigment indicate that it may be a 'shunt' metabolite produced by the action of enzymes of low substrate specificity on a transient accumulation of metabolic intermediates (the 'unbalanced growth hypothesis' proposed by Woodruff, 1966): *When nitrate or glucose become growth-limiting, both energy generation and biosynthesis are impeded from the first metabolic step. Under these conditions no accumulation ('pooling') of intracellular metabolites occurs and no red pigment is produced. However, when oxygen or phosphate become growth-limiting, energy generation is impeded from concluding or near-concluding metabolic steps (in the case of oxygen the terminal transfer of electrons in oxidative phosphorylation is affected, while in the case of phosphate the generation of ATP from ADP and phosphate is affected). Under these conditions intracellular metabolites accumulate transiently in traffic-jam-backlog style. This accumulation of metabolites causes an increase in the energy required for preserving intracellular solute concentrations ('cell maintenance energy') at a time when the rate of energy generation is decreasing. S. erythraea excretes these pooled metabolites as red pigment in order to reduce metabolite pool sizes and maintenance energy.*
and so prolong survival. The suggestion that red pigment is a ‘shunt’ or ‘overflow’ metabolite does not preclude the possibility that it has evolved other functions.
8) REFERENCES


125


136


