https://theses.gla.ac.uk/

Theses Digitisation:

https://www.gla.ac.uk/myglasgow/research/enlighten/theses/digitisation/

This is a digitised version of the original print thesis.

Copyright and moral rights for this work are retained by the author

A copy can be downloaded for personal non-commercial research or study, without prior permission or charge

This work cannot be reproduced or quoted extensively from without first obtaining permission in writing from the author

The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the author

When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given

Enlighten: Theses
https://theses.gla.ac.uk/
research-enlighten@glasgow.ac.uk

Fermentation Development of *Streptomyces thermonitrificans* ISP5579

by

Frances Mary Burke

A thesis submitted in accordance with the requirements for the degree of

Doctor of Philosophy

April 1991

Institute of Genetics

The University of Glasgow

ProQuest Number: 11008001

All rights reserved

INFORMATION TO ALL USERS
The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript
and there are missing pages, these will be noted. Also, if material had to be removed,
a note will indicate the deletion.

ProQuest 11008001

Published by ProQuest LLC (2018). Copyright of the Dissertation is held by the Author.

All rights reserved.
This work is protected against unauthorized copying under Title 17, United States Code
Microform Edition © ProQuest LLC.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Summary

Conventional antibiotic fermentations are operated in the temperature range 25-30^{0}C, with large cooling requirements due to the generation of metabolic heat and the high agitation requirements of viscous, mycelial fermentation broths. If the fermentations could be carried out at an elevated temperature, the energy requirement for cooling would be reduced dramatically. In addition, the faster reaction rates would decrease fermentation times. This project investigated the feasibility of expressing cloned heterologous antibiotic pathways, in a thermophilic streptomycete host chosen for a good fermentation performance.

S. thermonitrificans was the chosen species. A defined medium, defined inocula and analytical techniques for the fermentation and analysis of growth were developed for *S. thermonitrificans* . Fermentation data analyses by carbon balancing were carried out with a carbon recovery of 93% or better. The growth energetics were investigated in glucose-limited chemostat culture, to give approximate values for the maintenance coefficient of 0.41 g biomass.(mole glucose)$^{-1}$.hr^{-1}, and a maximum growth yield, of 95.4 g biomass.(mole glucose)$^{-1}$. This compares favourably with the growth energetics of common mesophilic prokaryotes and suggests that *S. thermonitrificans* is well-adapted to growth at elevated temperatures.

A recombinant strain of *S. thermonitrificans* was investigated. It contained the plasmid pBROC139 which is based on a high-copy number streptomycete plasmid, with a DNA insert from *S. clavuligerus,* expected to encode genes for the production of the yellow antibiotic, holomycin. *S. clavuligerus* has an optimum growth temperature of 26^{0}C, and a maximum of 30^{0}C. However the yellow product was expressed in *S. thermonitrificans* in defined medium at temperatures up to 50^{0}C. The product has been shown to be insert-specific, and similar in properties to the product formed in a range of heterologous hosts, containing the same plasmid. The rate of production in *S. thermonitrificans* pBROC139 (judged qualitatively) was considerably more rapid at 50^{0}C compared to 30^{0}C. The product was shown not to be holomycin, by comparison with an accurate standard, and a preliminary purification of the yellow product has been carried out. Mass spectral analysis implied that the partially-purified product might be composed of two homologues of the N-methyl pyrrothine class of antibiotics.

An attempt was made to express oxytetracycline in *S. thermonitrificans* by plasmid transformation with plasmid pFZ163, containing an insert encoding the pathway for oxytetracycline. In addition, conjugation of *S. thermonitrificans*

with a conjugative plasmid containing the same insert was attempted. Both were unsuccessful, possibly due to the combined influences of large plasmid sizes and a restriction barrier which exists between *S. thermonitrificans* and other *Streptomyces.*

This project has provided a thermophilic streptomycete host-vector system, amenable to precise physiological study by the method of materials balancing, which has a good fermentation performance in defined and complex media. In addition *S. thermonitrificans* has growth characteristics which imply that it grows efficiently at an elevated temperature (45^0C).

The work presented in this thesis is my own unless otherwise acknowledged. No part of this thesis has been previously submitted for examination leading to the award of a degree.

F. M. Burke

April 1991

Acknowledgements

I wish to thank my supervisor, Dr Iain Hunter for proofreading this thesis, and to Drs Lloyd Czaplewski, John Clutterbuck, Les Fixter and Marshall Stark for portions of the same task.

I would also like to thank Lloyd and Marshall for constructive discussions, Dr Bob Hill and Professor Charles Brookes for helpful advice and Drs Ritchie, Ryecroft and Foster for analyses. Dr David Mousedale must also be thanked profusely for his technical expertise, advice and supervision on purification matters. Thanks also to Dr Martin Gilpin and Mr S. Spear of SmithklineBeecham, for help in the purification and identification of the product from plasmid pBROC139.

Thankyou to Marion, for everything, and to the Strep group (and honourary members) for all the fun times. The Media ladies must be complimented on the excellent job they do, and particularly their cheerfulness in the face of medium pots cooling in the autoclave at 8.15 am. I would also like to thank Eileen, Margaret and Anne O'Dowd for their welcome in C26, whilst I was working there, and Bioflux, for the use of their equipment.

I acknowledge reciept of funding from the Wolfson Foundation.

CONTENTS

Section — Page

Summary....ii
Declaration....iv
Acknowledgements.... v
Contents....vi
Abbreviations....xvi

Chapter 1 - Introduction to The Fermentation Developtment of *S. thermonitrificans* ISP5579.

1.0 General Introduction....1

Streptomyces Development
1.1 Germination....2
1.2 Vegetative growth....3
  1.2.1 Actinomycete Vegetative Cell Structure....3
  1.2.2 Growth on solid media....3
  1.2.3 Growth in Submerged Culture....3
    1.2.3a Branched mycelial growth....3
    1.2.3b Mycelial Growth in the Form of Pellets....5
1.3 Sporulation....6
1.4 Regulation of Development- Current Knowledge....7
1.5 Secondary Metabolism....7
1.6 Autoregulation and The Role of Secondary Metabolites in the Natural Life Cycle of *Streptomyces*....8
1.7 Regulation of Secondary Metabolism at the Molecular Level....10
1.8 Secondary Metabolite Production in Submerged Culture....12
1.9 Nutritional Regulation of Secondary Metabolism....12
  1.9.1 Carbon Catabolite Repression....12
  1.9.2 Phosphate Repression....14
    1.9.2a Physiological Observations....14
    1.9.2b Observations at the Molecular-Genetic Level....15
  1.9.3 Nitrogen Regulation....16
1.10 The Influence of Dissolved Carbon Dioxide and Oxygen on Production of Secondary Metabolites....16
1.11 Growth Rate Regulation of Secondary Metabolism....17
1.12 Process Design for Secondary Metabolite Production....17
1.13 The Molecular Genetics of *Streptomyces* Secondary Metabolism....21

1.14 Thermophilic *Streptomyces spp.* 21
1.14.1 *S. thermonitrificans* ISP 5579 21
1.14.2 Adaptations to Growth at Elevated Temperature 21
1.14.3 Studies of the Physiology and Molecular Biology of Thermophilic *Streptomyces*. 22
1.15 Economic Advantages of Thermophilic Fermentations 23
1.15.1 Heat Production in Large Scale Fermentations 23
1.15.2 Reaction Rate, Productivity and Broth Rheology 25
1.15.3 Disadvantages of Fermentation at Elevated Temperature - and Potential Methods for their Alleviation 26
1.16 The Glasgow Project 27
1.17 The Scope of This Work 27

Chapter 2 - Materials and Methods

2.1 Introduction 29
2.2 Chemicals and Consumables 29
2.3 Strains 30
2.4 Growth Media 30
2.5 Sterilisation 31
2.6 *S. thermonitrificans* Strain Preservation 31
2.6.1 Preparation of Spore Suspensions of *Streptomyces* 31
2.6.2 Preparation of Master Cultures 32
2.7 Cultivation 32
2.7.1 Shake Flask Culture 32
2.7.2 Solid Substrate Cultivation 32
2.7.3 Spore Pre-germination 33
2.7.4 Homogenisation of mycelium 33
2.7.5 Determination of Spore Concentration 33
2.7.6 Determination of The Number of Colony Forming Units 34
2.8 Assay Techniques 34
2.8.1 Dry Weight 34
2.8.2 Determination of Glucose Concentration 34
2.8.3 Determination of Ammonium Ion Concentration 35
2.8.4 Determination of Phosphorous Concentration 35
2.8.5 Determination of Total Organic Carbon Concentration 36
2.8.6 Elemental Analysis of Mycelium 36
2.9 Photomicrography 36
2.10 Fermenters 36
2.10.1. Associated Services 36
2.10.2 Fermenter Configurations 37

2.10.3 Specification and Methods used with the Bioengineering Fermenter....37
2.10.3 a Sampling....37
2.10.3 b Agitation....38
2.10.3 c Temperature....38
2.10.3 d pH control....38
2.10.3 e Dissolved oxygen concentration....38
2.10.4 Specification and Methods used with the LH Fermenters...39
2.10.4 a Agitation....39
2.10.4 b Temperature control....39
2.10.4 c Air supply....39
2.10.4 d pH Monitoring and Control....39
2.10.4 e Inoculation....40
2.10.4 f Sampling....40
2.10.5 Modifications and Techniques used in the Establishment of Continuous Culture for *S. thermonitrificans*....40
2.10.6 On-line Analysis of Carbon Dioxide....42
2.10.6 a Infra-red detection and analysis....42
2.10.6 b Precautions Required for Accurate Cumulative Determination of CO_2.....43

2.11 Growth Media for *In vitro* and *In vivo* Gene-transfer Manipulations in *Streptomyces*....44
2.11.1 Solutions for Plasmid Transformation of *S. thermonitrificans*....45
2.11.2 Solutions for the Manipulation and Visualisation of Plasmid DNA....45
2.11.3 Antibiotics for Plasmid Selection....45
2.11.4 Preparation of Plasmid DNA....45
2.11.5 Digestion of Plasmid DNA with Restriction Enzymes....46
2.11.6 Gel Electrophoresis....47
2.11.7 Photography of Agarose Gels....47
2.12 Streptomyces *in vitro* Gene Transfer Techniques....47
2.12.1 Growth of *Streptomyces* mycelium for the Preparation of DNA....47
2.12.2 Growth of *Streptomyces* mycelium for the preparation of protoplasts....47
2.12.3 Transformation with plasmid DNA....47
2.12.4 Preparation of Protoplasts....47
2.12.5 Transformation of Protoplasts....48
2.12.6 Regeneration of Transformed Protoplasts....48
2.12.7 Plasmid Selection in *Streptomyces*....48

2.13 Streptomyces *in vivo* Gene Transfer Manipulations 48
2.13.1 Replica Plating 48
2.14 Bioassay of Oxytetracycline 49
2.15 Bioassay for Holomycin 49
2.16 Discontinuous Fed Batch Cultivation of *S. thermonitrificans* pBROC139 49
2.17 Solvent extraction 50
2.18 Rotary evaporation 50
2.19 Small scale evaporation 50
2.20 Thin layer chromatography 49
2.21 Visualisation of TLC 50
2.22 Chromatography and Analysis 50
2.22 a Column Chromatography 50
2.22 b HPLC 50
2.22 c FPLC 51
2.22 d Nuclear Magnetic Resonance Analysis 51
2.22 e Mass Spectrometric Analysis 51

Chapter 3 Fermentation Development of *S. thermonitrificans* ISP5579

3.1 Introduction 52
3.1.1 Development of a Fermentation For The Expression of a Recombinant Product from *S. thermonitrificans* in a Materially-Balanced System 52
3.1.2 Growth Kinetics of Filamentous Organisms 53
3.1.3 Retrospective Experimental Strategy 54
3.2 Preliminary Growth Studies 54
3.2.1 Growth curves for *S. thermonitrificans* on Complex Medium, in Flask Experiments 54
3.2.2 Growth Curves for *S. thermonitrificans* on Semi-Defined Medium in Flask Experiments 57
3.2.3 Investigation of the Growth of *S. thermonitrificans* in a 9 Litre Bioengineering Fermenter in Semi-Defined LMM 59
3.4.0 Medium Development 60
3.4.1 To Investigate and Alleviate a Possible Nutrient Limitation in Semi-Defined LMM 62
3.4.2 The Effect of Phosphate on Growth in Semi-Defined Medium (LMM) 64
3.4.3 The Influence of Shear Stress and the Polymer Additive, Junlon, on Growth of *S. thermonitrificans* 65
3.5 Summary Conclusions from Preliminary Experiments and the Subsequent Experimental Strategy 68

3.6 Optimisation of the Growth Conditions for *S. thermonitrificans* on Evans Medium 68
3.6.1. The Effect of Variation in Medium Component Concentration on Growth in Evans Medium 69
3.6.2 Development of *S. thermonitrificans* Minimal Medium (579 MM) 71
3.7 Development of Defined Inocula 71
3.7.1 Pre-germination of Spores of *S. thermonitrificans* for Subsequent Growth on 579MM 72
3.8 Optimisation of Growth in 579MM using Defined Inocula 73
3.8.1 The Influence of Glucose Concentrations in 579MM on Growth of *S. thermonitrificans* 73
3.8.2 The Differences in Carbon Conversion Efficiencies Between Different Growth Conditions 74
3.8.3 The Influence of Inoculum Spore Concentration on Final Biomass Concentration in 579MM Containing 6 $g.l^{-1}$ Glucose 77
3.9 Fermenter Growth using 579MM and Defined Inoculum 79
3.9.1 Aeration, Agitation and Shear in Fermenter Cultivation 79
3.9.2 Aeration of *Streptomyces* Cultures 81
3.10 A Modified Inoculum Development Procedure for Use on a Fermenter Scale and In Large Matrix Experiments 83
3.10.1 Optimisation of the Fragmented Mycelial Inoculum Concentration 84
3.11 Final conclusions for this Chapter 87

Chapter 4 - Investigation into the Growth Energetics of *S. thermonitrificans* using a Chemostat.

4.1 Introduction 88
4.2 Qualitative Description of Batch and Continuous Microbial Culture 88
4.2.1 Batch Culture 88
4.2.2 Continuous Culture 91
4.3 Disadvantages relating to the use of Continuous Culture 91
4.4 The Reason for the Investigation into the Growth Energetics of *S. thermonitrificans* 93
4.5 Energetic Consequences of Growth at Elevated Temperature 94
4.6 Continuous Cultivation of *S. thermonitrificans* 95
4.7 Mutation in Continuous Culture 98
4.8 Experimental Determination of Maximum Growth Yield and Maintenance Energy 100
4.9 Implications of the Experimentally-Determined Value for the

Maximum Growth Yield with respect to Glucose 103
4.10 Implications of the Experimentally Determined Value for the Maintenance Coefficient 104
4.11 Further Use of Data from Chemostat Culture 106
4.12 The Influence of Carbon Source on Growth in Defined Medium in Flask Cultures 108
4.13 Investigation of the Influence of Nitrogen Source on Submerged Mycelial Morphology of *S. thermonitrificans* in Flask Cultures 111
4.14 Final Conclusions for this Chapter 112

Chapter 5 - Fermentation of *S. thermonitrificans* in a 7 litre Stirred Tank Bioreactor and Data Analysis using Material Balances

5.1 Introduction 114
5.1.1 Fermentation Data Analysis using Material Balances 114
5.2 Establishment of Fermentations 116
5.2.1 On-line Data 116
5.2.2 Microscopical Morphological Profile 116
5.2.3 Off-line Data 122
5.3 Elemental Analysis of Biomass 124
5.4.0 A Fermentation Balance Equation for *S. thermonitrificans* based on Stoichiometry 126
5.4.1 Determination of Stoichiometric Parameters for *Klebsiella aerogenes*, *Escherichia coli* and Roels' "average microorganism". 128
5.4.2 Comparison of the Stoichiometric Parameters for *S. cattleya*, *K. aerogenes*, *E. coli* and *S. thermonitrificans* 129
5.5 Fermentation Data Analysis 130
5.6 Discussion of the Experimental Results 136
5.7 Discussion and Comparison of Experimental and Stoichiometric Yield Values 136
5.8 Conclusions From this Chapter 137

Chapter 6 An Attempt to Construct a Recombinant strain of *S. thermonitrificans* containing a Low Copy-Number plasmid, pPFZ163 Encoding the Pathway for Oxytetracycline Biosynthesis.

6.0 Introduction 139
6.1 Planned Approach to the Study of a Recombinant Strain of *S. thermonitrificans* containing a Plasmid which Enabled the Formation of Oxytetracycline in Culture Broths 139
6.2 Gene Transfer in *Streptomyces* 140
6.3 Plasmid Transformation in *S. thermonitrificans* 141
6.4 Reasons for the proposed use of an alternative recombinant.... 142

6.5 Attempted Transformation of *S. thermonitrificans* with pFZ163..144
6.6 Conjugation and the Applicability of the Technique to this Study....145
6.7 Intraspecific Conjugation - the Construction of *S.lividans* TK64 pIJ940....150
6.8 Frequency of Transconjugation of pIJ940....151
6.9 Screening of Putative Transconjugants....152
6.10 Interspecific Conjugation - An Attempt to Construct *S. thermonitrificans* pGLW101....152
6.11 Conclusions, Discussion and Future Work....154

Chapter 7 - Expression, Isolation and Preliminary Purification and Identification of the Product from *S. thermonitrificans* pBROC139.

7.0 Introduction....156
7.1 Holomycin....156
7.2 A Brief Theory of the Techniques used in the Structural Elucidation of the Product....159
7.2.1 Ultraviolet and Visible Spectroscopy....159
7.2.2 Electron Impact Mass Spectrometry....159
7.3.0 Investigation into the Production of a Yellow Pigment at Elevated Temperature in *S. thermonitrificans* pBROC139 cultures....160
7.3.1 Investigation into the Influence of Temperature on Expression....160
7.3.2 The Influence of Temperature on the Apparent Rate of Production of the Yellow Pigment in Broths....163
7.4.0 Identification of the Yellow Product....164
7.4.1 Solvent Extraction using Butanol....164
7.4.2 Solvent Extraction using Ethyl Acetate....164
7.4.3 Solvent Extraction using Broths Produced at 30^0C and 50^0C and An Attempt to Develop a Simple Assay for Expression of a Yellow Colour....165
7.5 Purification of The Yellow Product....168
7.5.1 Discontinuous Fed Batch Cultivation for Reproducible, Rapid Production of High-Intensity-Yellow Broths....169
7.5.2 Normal Performance Liquid Chromatography....172
7.5.3 Thin Layer Chromatography of Fractions....172
7.5.4 Spectrophotometric Investigation of Fractions....173
7.5.5 Bioassay Development....173
7.6.0 High Performance Liquid Chromatography and Fast Protein Liquid Chromatography....175
7.6.1 Silica Gel Filtration Chromatography (HPLC-mode)....175

7.6.2 Ion Exchange Chromatography....178
7.6.3 Reverse Phase Chromatography....180
7.7.0 An Additional Purification....183
7.7.1 Comparison of 3-Dimensional Test Spectra (those of *S. thermonitrificans* pBROC139) with 3-Dimensional Control Spectra (those of *S. thermonitrificans* pIJ702).... 186
7.7.2 Options Available for Continuing the Identification....191
7.8.0 Interpretation of Structural Information....191
7.8.1 Interpretation of Mass Spectra....191
7.8.2 Further Investigation....195
7.9 Discussion and Future Work....197

CHAPTER 8- Final Discussion and Conclusions

8.1 The Glasgow Project....200
8.2 Future Potential of *S. thermonitrificans*....201
8.3 General Comments Concerning the Study of *Streptomyces*....201
8.4 Final Conclusion....203

Appendix
(Mass Spectra of Standards used for Work in Chapter7) 204

References....206

Tables

Table 3.1 63
Table 3.2 66
Table 3.3 66
Table 3.4 70
Table 3.5 75
Table 3.6 75
Table 3.7 78
Table 3.8 85
Table 3.9 86
Table 4.2.... 105
Table 4.3 110
Table 4.4 110
Table 5.1 118
Table 5.2 125
Table 5.3 126
Table 5.4 126

Table 5.5 131
Table 5.6 131
Table 5.7 135
Table 5.8 135
Table 5.9 135
Table 6.1 149
Table 7.1 161
Table 7.2 162
Table 7.3 166
Table 7.4 170
Table 7.5 174
Table 7.6 196

Figures

Figure 2.1 37
Figure 2.2 37
Figure 2.3 41
Figure 3.1 56
Figure 3.2 56
Figure 3.3 56
Figure 3.4 56
Figure 3.5 58
Figure 3.6 58
Figure 3.7 58
Figure 3.8 61
Figure 3.9 78
Figure 3.10 80
Figure 3.11 80
Figure 3.12 81
Figure 3.13 85
Figure 4.1 90
Figure 4.2 92
Figure 4.3 96
Figure 4.4 102
Figure 5.1 115
Figure 5.2 117
Figure 5.3 117
Figure 5.4 117
Figure 5.5 119
Figure 5.6 120
Figure 5.7 121

Figure 5.8 123
Figure 5.9 123
Figure 5.10 123
Figure 5.11 133
Figure 5.12 133
Figure 5.13 133
Figure 5.14 134
Figure 6.1 147
Figure 6.2 148
Figure 6.3 153
Figure 7.1 157
Figure 7.2 157
Figure 7.3 161
Figure 7.4 162
Figure 7.5a 166
Figure 7.5b 166
Figure 7.6 170
Figure 7.7. 171
Figure 7.8 174
Figure 7.9a 176
Figure 7.9b 177
Figure 7.11 179
Figure 7.12 181
Figure 7.13 182
Figure 7.14. 184
Figure 7.15. 185
Figure 7.16 187
Figure 7.17 188
Figure 7.18. 188
Figure 7.19. 190
Figure 7.20 192
Figure 7.21 193
Figure 7.22 193
Figure 7.23 196
Figure A.1 204
Figure A.2 205

Abbreviations

BDH	British Drug Houses
BRL	Boehringer Research Laboratories
TES	N-tris (hydroxymethyl) methyl-2-aminoethane sulphonic acid
TSB	Trypticase Soy Broth
LMM	Linda's Minimal Medium
579MM	579 Minimal Medium (for strain ISP5579)
ABTS	Di-ammonium 2,2'- azino-bis (3-ethylbenzothiazoline-6-sulfonate)
PEG	Polyethylene Glycol
dO_2	Dissolved oxygen concentration expressed as a % of fraction of the starting dissolved oxygen concentration.
cAMP	cyclic Adenosine Monophosphate
ccc DNA	Covalently closed circular DNA
NTA	Nitrilo-triacetic Acid
EDTA	Ethylene diamine tetraacetic acid
579	*S. thermonitrificans* ISP5579
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
C.P.U.	Central processing unit
NMR	Nuclear magnetic resonance spectroscopy
MS	Mass spectrometry
TLC	Thin layer chromatogram
amu	Atomic mass units
rpm	Revolutions per minute
psi	Pounds inch^{-2}
vvm	Volume of air.volume of medium^{-1}min^{-1}(air flow rate)
bar	Barometric pressure
v/v	Volume per volume
w/v	Weight per volume
CFU	Colony forming unit
ppm	Parts per million
h	hour

1.0 General Introduction

Streptomyces are filamentous, chemoheterotrophic, aerobic, spore-forming eubacteria. They stain positive with Gram stain, are highly oxidative and form cell walls containing L-L-diaminopimelic acid and glycine. Their characteristic DNA base composition has a high G-C bias of between 69 and 73% on a molar basis, and their genome is between 6-9 megabases of DNA based on pulse-field gel electrophoresis data (Woese, 1987: Hopwood and Kieser, 1990). They possess a complex life cycle of morphological and physiological development supremely adapted to colonising relatively hostile, intemperate environments.

Streptomyces occupy a characteristic niche in the soil ecosystem, aiding the recycling of biomass by producing a wide range of cellulolytic, ligninolytic and xylanolytic degradative enzymes (McCarthy, 1987). At least one *Streptomyces* enzyme, dehydroquinase from *S. coelicolor,* is highly homologous at the amino acid level and in functionality with the catabolic enzyme from *Neurospora crassa* and may reflect either convergent evolution or gene transfer (White *et al.,* 1990; P. J. White, personal communication). Based on amino acid sequence identity of cloned genes in two streptomycetes and three filamentous fungi, two genes of the ß-lactam pathway (encoding isopenicillin N synthetase and deacetoxycephalosporin C synthetase) appear to have been transferred horizontally from streptomycetes to fungi (Weigel *et al.*, 1988; Miller and Ingolia, 1989a and b). The morphology, growth and formation of metabolites from *Streptomyces* under certain nutrient conditions also bears resemblance to the soil fungi, and it may be more pertinent to compare *Streptomyces* to soil fungi rather than to enteric bacteria such as *Escherichia coli*.

Typically, following an environmental stimulus, spore outgrowth is followed by apical extension and branching, enabling colonization of solid or particulate substrates with vegetative mycelia. At the air-substrate interface, hydrophobic aerial mycelia form, which differentiate to produce long chains of spores with a species-characteristic phenotype. The formation of aerial mycelia is typically associated with the lysis of substrate mycelium, often accompanied by the simultaneous production of antimicrobial compounds. A fresh pool of microbial metabolites is released during lysis which may be used to fuel the sporulation process (Mendez *et al.,* 1985b).

The genus *Streptomyces* has generated a large degree of both academic

and industrial interest. The complex life cycle of spatial and temporal differentiation and the ability to produce a wide range of non growth-associated metabolites has provided interest and funding for research and development, both inside and outside academia. These metabolites have uses in the fields of medicine, science and animal husbandry. The first commercial streptomycete fermentation was initiated in 1954, following the discovery of streptomycin in *Streptomyces griseus* (Schatz, Bugie and Waksman, 1943) and study of the genetics of the genus was initiated by the pioneering work of D.A. Hopwood in the 1950's.

Streptomyces species produce over 70% of all known antibiotics (Berdy, 1980); this includes 80% of all antibiotics commercially produced. As a result there is tremendous interest in exploiting the genus for products with novel uses. Industrial processes using *Streptomyces* are highly competitive, moderate-volume, high-value fermentations; these processes are developed by in-house research and remain as confidential, unpublished work. The systematics and taxonomy of the actinomycete genera are in a state of constant updating and renewal. As recently as 1987, *Streptomyces erythreus* was reassigned to the genus *Nocardia* and given the name *Saccharopolyspora erythrea* based on the presence of meso-diamino-pimelate in the cell wall in contrast to *Streptomyces* which possess L-L-diaminopimelate (Donadio *et al.,* 1989).

Streptomyces Development

1.1 Germination

Streptomyces undergo a complex life-cycle, one stage of which includes sporulation, generating spores for dispersal. On germination, the spores swell, lose refractility and germ tubes appear. The amino acid L-valine is commonly found to stimulate and L- glutamate to inhibit germination of spores, whilst germination appears to be dependent on the presence of sufficient concentrations of Ca^{2+} ions (Ensign, 1978; Salas *et al.*, 1983). Consequently germination is often poor in defined medium which is capable of supporting good post-germination mycelial growth. Trehalose is common in spores and is thought to act as an energy source (Ensign *et al.,* 1986). It has been postulated that energy reserves and spore-history (such as the nutrient environment at the time of sporulation) are extremely important in effecting efficient germination and subsequent mycelial growth (Vanek, 1988). Additionally, it has been suggested that antibiotics may play a role in maintaining dormancy and the timing of spore germination. This hypothesis is based on the identification of three antibiotics, monensin, lasalcolid and

roseofungin, in spore coats, although their role is unknown (Grafe, 1988).

1.2 Vegetative growth

1.2.1 Actinomycete Vegetative Cell Structure

Polyphosphate granules and lipid globules are the most common cytoplasmic inclusions in *Streptomyces*. The importance of polyphosphate is described in section 3.4.4. Lipid inclusion bodies represent sites of accumulation of neutral lipids, whilst storage of polyhydroxybutyrate has been observed in some species (Kannan and Rehacek, 1970). There is evidence that large quantities of spermine (a polyamine, widespread in bacteria and yeasts) may accumulate in thermotolerant (but not mesophilic) *Streptomyces* (Hamana and Matsuzaki, 1987). In *E. coli* it is known that spermine may stabilise DNA. It may have a similar role in thermotolerant *Streptomyces*.

The cell wall consists of peptidoglycan, together with teichoic acid and neutral polysaccharides. Cross walls form to delimit the individual hyphae (Locci and Sharples, 1984). Cell-cell contact is maintained between the compartments by microplasmodesmata-like pores (Caslavska *et al.,* 1978).

1.2.2 Growth on solid media

Following germination and spore outgrowth, streptomycetes produce hyphae which branch and spread radially, forming monocentric colonies when grown on solid media. Growth occurs by tip extension, demonstrated by pulse-chase labelling of N-acetyl-D-glucosamine and autoradiography in *S. antibioticus,* and *S. coelicolor* A3(2) (Brana, 1982; Gray *et al.,* 1990). Unlike filamentous fungi, germ tube hyphae have faster extension rates than hyphae which develop later (Allan and Prosser, 1985).

1.2.3 Growth in Submerged Culture

1.2.3a Branched mycelial growth

In studies of actinomycete mycelial growth in continuous culture with glucose as the limiting substrate, it was observed that the hyphal growth unit varies according to the strain and the dilution rate (Kretschmer *et al.*, 1981; Riesenberg and Bergter, 1979). The hyphal growth unit may be defined as the relationship between the hyphal length and number of hyphal tips (influenced by the degree of branching), a coefficient introduced by Trinci (1973). Actinomycetes with rapid growth kinetics were shown to have large values for the hyphal growth unit, which vary with growth rate. However

Nocardia, (which have very slow growth rates), were found to have a growth rate-independent hyphal growth unit. Kretschmer postulated that the mean apical growth rate is regulated by a growth rate-dependant mechanism. The change in mean apical growth rate with respect to branching rate allows for changes in mycelial morphology with respect to the nutrient quality of the environment. At low specific growth rates and poor nutrient environments, then the predominance of apical growth over branching provides a means for 'searching' for nutrients. At elevated growth rates and relatively better nutrient conditions, mycelial growth can be directed to optimising the utilization of nutrients in the immediate environment by branching frequently.

A novel unit has since been proposed; the hyphal growth unit volume. This is the hyphal growth unit with respect to hyphal volume (Trinci, 1990), and remains constant with variation in dilution rate in *S. hygroscopicus.* Trinci has proposed that mycelial organisms (both fungi and actinomycetes) can regulate branch initiation by monitoring biomass accretion and responding to growth conditions to maintain a constant hyphal growth unit volume. The biochemical basis for this mechanism is unknown (Trinci, 1990).

During mycelial growth in prolonged continuous culture, it is common for the parental strain to be supplanted by highly branched morphological variants, called friable or colonial variants, discussed in Chapter 4. Very little work in this field has been carried out with *Streptomyces,* and hence data for filamentous fungi are discussed. In the fungus *Neurospora crassa*, it has been shown that cAMP mediates in the control of mycelial branching, and that endogenous cAMP levels in wild type are greater than in colonial strains, correlating with the degree of mycelial branching (Scott *et al.,* 1973; Pall *et al.,* 1981). The variants are suggested to reflect aberrations in a putative control system mediated by cAMP. It is known that mechanical shear, rapid temperature changes and treatment with uncoupling agents all cause rapid, transient increases in the levels of intracellular cAMP in *Neurospora crassa.* It is postulated that a basal level of cAMP is required for normal growth; if this is varied, as part of a stress response, or due to exogenous supply, then a different set of responses is triggered (Trinci, 1990). In *S. coelicolor,* colonial variants arising in continuous culture were unable to make the antibiotics undecyl prodigiosin and actinorhodin (Hobbs, 1988).

Although cAMP is present in *Streptomyces,* the role it plays has only been studied with respect to secondary metabolism and nutrient repression. It does not appear to have a role in catabolite repression and it is regarded as having an unknown function (Aharonowitz, 1982). However during growth phase, intracellular cAMP concentrations for *S. hygroscopicus* have been shown to

be relatively high, decreasing prior to the onset of idiophase. This response has been suggested to be associated with differentiation processes unrelated to secondary metabolism (Martin, 1989). In general, in submerged culture of *Streptomyces* it is common for morphological changes to occur during cultivation; distinct phases of primary and secondary mycelium with changes in mycelial morphology have been reported for *S. rimosus*, and *S. griseus* in which the formation of secondary mycelium is correlated with antibiotic production (Bader,1986; Luedeking, 1967; Erdei *et al.*, 1973). By comparison with some filamentous fungi, it may be worthwhile to investigate whether cAMP in *Streptomyces* in submerged culture may mediate in responses (putatively stress- and/or differentiation-related), which are manifested as changes in mycelial morphology.

There is a fundamental lack of information and research into modelling and simple investigation of the basic growth kinetics and morphology of *Streptomyces* in submerged culture. In view of the economic importance of submerged culture of *Streptomyces* this is surprising and somewhat short-sighted, a sentiment echoed by Bushell in his review (1988). In addition to the reports by Kretshmer, and Riesenberger and Bergter (1979a; 1979b; 1981), discussed earlier, other reports include work on modelling the washout kinetics of *S. coelicolor* pellets and a comparison of image analysis with digitizing table analysis for analysing mycelial morphology (Tough, 1990 - unavailable for reference; Adams and Thomas, 1988). The tedious, unrewarding and time-consuming nature of quantitative analysis of mycelial morphology, together with the small dimensions of streptomycete hyphae must be partly to blame for the lack of interest in the subject. However, with the introduction of low-cost, high-powered microprocessors, and the availability of image analysis application software, semi-automated image analysis is now feasible. Such technology would remove the tedium inherent in quantitative analysis of mycelial morphology, and would free manpower for more immediately-satisfying, related areas of research within the same project.

1.2.3b Mycelial Growth in the Form of Pellets

For submerged culture of mycelial organisms, one of two gross morphological forms are adopted; either filamentous or pellet mycelial growth (Pirt, 1966). The formation of mycelial pellets in submerged cultures of filamentous organisms is related to the inoculum concentration, dissolved oxygen tension, culture shear in cultivation, pH and polymer additives (Byrne and Ward, 1989; Schugerl *et al.,* 1983: Braun and Lifshitz,1991). Pellet structure varies between species, from densely-packed mycelium where diffusion

through the pellet limits growth, to loosely-woven mycelium, where mass transfer may be effective in nutrient transport to the hyphae at the interior (Pirt, 1966).

Mycelial growth in the form of pellets is important due to the negative influence it can have on growth and product formation. Estimations of the distance a diffusing nutrient can penetrate cell tissue were made by Pirt (1966, using the Hill analysis, 1910). For glucose limited growth, the theoretical maximum distance for diffusion of glucose was 260 µm; and for dissolved oxygen, 40 µm. As pellets are usually larger than these distances (pellet diameters of up to 8 mm have been recorded for *S. thermonitrificans*), then the mycelium at the centre of a densely-packed pellet will be starved of nutrients. The growth rate for mycelium in the form of densely-packed pellets is, therefore, less than the maximum specific growth rate for mycelium in the filamentous growth form, due to this diffusional limit to growth. Pelletting also results in a heterogeneous population of mycelia, which may be detrimental to product formation, and secondary metabolite production may occur asynchronously. In pellets of *S. tendae*, four different regions of hyphal growth have been identified. An outer layer, within which active growth can occur, two partly-lysed middle layers, and a central fully-lysed zone (shown in Braun and Lifshitz, 1991). It has been postulated that the study of the influence of pellet morphology on the productivity of secondary metabolites may be a tool for directing the formation of specific metabolites which would be unavailable in free-cell systems, *e.g.* in the low-oxygen environments which occur at pellet centres, or as pseudo-immobilised cultures (Braun and Lifshitz, 1991). The growth kinetics of *Streptomyces* pellets are similar to those of agar colonies (Bushell, 1988).

1.3 Sporulation

It has been shown that lysis of the substrate mycelium fuels the development of aerial mycelium and pulse-chase labelling studies have shown that nutrient migration occurs from the base of the colony towards the upper regions for the development of aerial mycelium (Mendez *et al.,* 1985a and 1985b). Glycogen has been implicated as having a role in the process of sporulation; it is thought that the mobilisation of stored glycogen may induce turgor pressure changes and supply energy for the emergence of aerial mycelium (Chater, 1989).

Streptomycete spores are formed from the hyphal parent cell wall. They are produced either singly, in chains or spore vesicles and develop as a result of the septation of pre-existing hyphae. Following septation, the deposition of

additional wall material occurs and septum cleavage and condensation of cytoplasmic contents then take place to form the mature spore. A fibrous sheath surrounds the spores and gives the aerial mycelium its species-characteristic colour and appearance. The major role for spores in the life cycle of *Streptomyces* is in the colonization of new environments by dissemination in soil water.

1.4 Regulation of Development- Current Knowledge

Conditional mutants in aerial mycelium formation are readily isolated in *S. coelicolor*, one of these mutant classes, *bldA* has been studied in depth. The *bldA* gene product codes for a rare leucine tRNA (TTA codon), which recognises UUA codons. The model proposes that the *bldA* gene product is required to transcribe the regulatory molecules for the initiation of sporulation and antibiotic production. Secondly, the *whiG* gene in *S. coelicolor* encodes a specific sigma factor σ^{whiG}. Expression of *whiG* in submerged culture on a high copy number plasmid, leads to sporulation in submerged culture; and for cultivation on solid substrates, abundant premature sporulation. Inactivation of whiG leads to complete absence of sporulation. *Whi G* is therefore implicated in the regulation of sporulation, specifically in the decision for change in aerial mycelium extension to sporulation. These two areas of research are reviewed by Chater, in more detail than is applicable here (1989).

1.5 Secondary Metabolism

In 1961 the biphasic growth profile in submerged culture characteristic of non growth- associated product kinetics was recognised and defined (Bu'lock, 1961). The two phases are now called the growth, primary or tropho phase and the production, secondary or idio - phase. Metabolites produced in the idiophase are called idiolites, or secondary metabolites. Previously (and subsequently) there has been tremendous speculation about the role of secondary metabolites to the producing organism.

It was recognised in 1974 that "secondary metabolism is an aspect of the differentiation which limited growth usually implies", recognising that the production of secondary metabolites in submerged culture tends to occur in conditions of nutrient limitation (Bu'lock, 1974). Limited or unbalanced growth is not a condition restricted to organisms which produce the commonly-known secondary metabolites, such as the antibiotics and ergot alkaloids; the production of these non-essential metabolites is one manifestation of limited growth. Limited or unbalanced growth in *Klebsiella aerogenes* leads to the

accumulation of precursor molecules such as acetate (Neijssel and Tempest, 1975). In *Escherichia coli* B SPAO ethylene is produced in a secondary production phase, in cultures grown on L-methionine (Shipston and Bunch, 1989). In *E.coli* ML308 acetate excretion is observed in cultures with excess glucose, in a non-growth associated production phase (Holmes and Bennet, 1971). Following glucose depletion, the acetate is utilised as an energy source. In both cases of acetate excretion, acetate may be regarded as a secondary metabolite, which is not essential for growth but allows a condition of unbalanced growth to be maintained. Manipulation of the central metabolic pathway of carbon catabolism (supplying an additional plasmid-encoded copy of phosphoenol pyruvate carboxylase) enabled a reduction in acetate excretion and a concurrent increase in growth yield (Okungbawa, 1991). Hence it may be suggested, by extrapolation to *Streptomyces*, that if the role of antibiotic production in *Streptomyces* is merely to allow the continuation of unbalanced growth by catabolism through glycolysis, then increasing the throughput of primary precursors may not have the desired effect of increasing antibiotic titre. Instead it may enable efficient primary anabolic metabolism to continue by 'balancing" growth. It will therefore be interesting to observe the effect of influencing precursor supply pathways (by increasing the dosage of key primary metabolic pathway genes) on the kinetics and antibiotic production profile of *S. coelicolor* (work in progress in this laboratory). The potential for increased antibiotic titres in streptomycete strains manipulated in this manner may merely require further manipulation of the growth conditions.

Work on *S. coelicolor*, using proline transport mutants which are also blocked in proline degradation has shown that these mutants overproduce undecyl prodigiosin possibly due to the lack of proline degradation (the mutation does not stop proline transport, but allows transport which is not subject to feedback regulation). Undecyl prodigiosin is biosynthesised from proline (Feitelson *et al.,* 1985). This implies that undecyl prodigiosin production in *S. coelicolor* A3(2) is not feedback-regulated (Hodgson, 1990). It can therefore be postulated that the production of undecyl prodigiosin in *S. coelicolor* may be a mechanism for the continuation of unbalanced growth. The organisms that produce complex secondary metabolites are also developmentally complex; therefore these products may play a role in cell-cell communication and cytodifferentiation (NIsbet and Porter, 1989). Recent reviews of fact and speculation regarding the functions of secondary metabolites include those of Vining, (1990) and Davies, (1990).

1.6 Autoregulation and The Role of Secondary Metabolites in the Natural Life Cycle of *Streptomyces*

Autoregulation in *Streptomyces* has been described in detail (Grafe, 1989). A brief summary of some known functions for autoregulators is presented below in association with their perceived role and importance in the natural life cycle of *Streptomyces.*

A factor. A factor mutants of *S. griseus* have been shown to lose both the capacity for streptomycin biosynthesis and sporulation (Horinouchi and Beppu, 1988: Hara *et al.,* 1983). Exogenous supply of A factor restores sporulation and streptomycin biosynthesis and as a result, it is suggested that A factor acts as an intraspecific pheromone promoting cytodifferentiation, sporulation and antibiotic biosynthesis (Chater, 1989). Exogenous A factor may act as either a stimulator or inhibitor of sporulation in a range of *Streptomyces* species and may interact with a separate regulatory gene *AfsB* to achieve the same results. Of a total of 203 actinomycete strains investigated, 30 were found to elaborate A factor-like substances (Hara and Beppu, 1982)

Factor C. Factor C is a regulatory protein, important in the induction of sporulation, and is expressed in all 23 streptomycete strains which have been examined (Szeszak *et al.,* 1990). It is found in reproductive hyphae and induces the release of intracellular potassium with a concomitant increase in intra- and extracellular NAD glycohydrolase activity. Factor C has an antagonistic effect with factor A: potassium efflux stimulated by factor C is reduced by factor A. Similarly the increase in NAD glycohydrolase activity which occurs with factor C, may be reduced by factor A. Factor C acts synergistically with nicotinamide in the stimulation of potassium efflux. It has been speculated that factor C may exert its effects on sporulation by mediation through a membrane receptor molecule, possibly by ribosylation, which would explain the interaction with NAD glycohydrolase (Barabas, 1988)

Pamamycin. This stimulates aerial mycelium formation in *Amy*$^-$ mutants. The pamamycin-producing strain, *S. alboniger,* also produces two inhibitors of the formation of aerial mycelium which, in turn, reverse the effect of pamamycin. Pamamycin acts as an inhibitor of the respiratory-dependent uptake of inorganic phosphate by the host (Kondo *et al.*, 1988). This mode of action is interesting as phosphate regulation is known to be physiologically important in *Streptomyces* (see Section 1.8).

A Germination Inhibitor is known to be produced by *Streptomyces viridochromogenes* (Ensign *et al.*, 1976). It is a lipophilic antibiotic which inactivates Ca^{2+} - activated ATPase. Inhibition is removed as a result of binding Ca^{2+} ions to the inhibitor. The Ca^{2+} ions may be present in the medium, or in intraspore reserves; alternatively the inhibitor may be leached from the spores into the medium, so allowing germination to take place. It is postulated that the leaching of an inhibitor into the medium prevents germination of spores of other species and confers a selective advantage (Ensign *et al.*, 1980; Grund *et al.*, 1985).

Autoregulation, illustrated by the examples described above, is widespread in *Streptomyces* and may represent an important survival mechanism in the soil ecosystem. The competitive effectors and inhibitory properties of some of the secondary metabolites may hold the physiological state of a culture in a precise condition such that the existence of a regulation event is undetectable.

1.7 Regulation of Secondary Metabolism at the Molecular Level

The regulation of secondary metabolism is intuitively mediated by molecules or cascades which ultimately must result in an induction at the genetic level to allow formation of the metabolite. The regulation of the formation of a class of compounds as diverse as the secondary metabolites produced in *Streptomyces* is necessarily complex, and many reports suggest that regulatory responses are either species-specific or pathway-specific. Simple classification of regulatory systems is not feasible, and at most, either specific examples or only very general trends (*e.g.* nutrient repression, detailed in 1.8) can be stated. In addition, in some cases there appear to be a number of different means for achieving the same effect.

Streptomyces display the stringent response on nutrient "shift down" (where mycelium is transferred from casamino acid medium to synthetic medium), or on the addition of serine hydroxamate, a competitive inhibitor of seryl-tRNA synthestase (Riesenberg *et al.*, 1984; Ochi, 1986; Bibb and Strauch,1990: Strauch *et al.*, 1991). Nutritional shift-down leads to a reduction in the rate of RNA synthesis, the accumulation of intracellular ppGpp and pppGpp and reductions in growth rate, responses which are also observed in *E. coli* (Cashel and Rudd, 1987). Inducer accumulation in the stringent response in *Streptomyces* has been implicated as one level of regulation of secondary metabolism, although mechanistic coupling has not been demonstrated (Ochi, 1987b). More recently, it has been negated as a direct response associated with the production of antibiotics in *S. coelicolor* based on

comparisons between serine hydroxamate-treated cultures and those subjected to nutritional shiftdown (Strauch *et al.,* 1991). Cultures with serine hydroxamate added during exponential growth showed a continuation of exponential growth, accumulation of ppGpp, reduction in the rate of RNA synthesis and antibiotic production kinetics which followed a normal pattern (*i.e.* antibiotic production in stationary phase). However cultures subjected to nutrient shift -down at the same stage in growth as the serine hydroxamate-treated cultures showed a switch into antibiotic production within 1 hour (assessed by identiying a specific transcript asssociated with the production of the antibiotic actinorhodin). It was suggested that the precise role of the ppGpp increase was difficult to determine (the ppGpp increase was "necessary, but not sufficient" for the induction of actinorhodin production in *S. coelicolor*). It was noted that the observed influences of nutritional shift down were probably a result of the physiological trauma involved in amino-acid starvation, rather than a feature of the stringent response (Strauch *et al.*, 1991).

The stringent response may be induced automatically by growth on defined media. For *Streptomyces spp.* MA406-A-1, growth of cultures on defined medium resulted in a high intracellular concentration of ppGpp throughout growth. However antibiotic production occured in stationary phase, implying a level of control of antibiotic production superimposed or combined with the response to ppGpp accumulation (Ochi, 1986). The increase in GTP accumulation which occurs on induction of the stringent response is thought to mediate in the induction of morphogenesis and sporulation (Ochi, 1987)

It appears likely that *Streptomyces*, in a similar manner to *Bacillus* use different classes of RNA polymerase to sequentially express sets of genes during its developmental cycle, potentially allowing temporal control of gene expression. RNA polymerase exists as a holoenzyme, composed of 5 subunits (aßß'∂),of which the sigma factor is required for promoter recognition. Sigma factor heterogeneity has been observed in *Streptomyces* (Westpheling *et al.,* 1985) and at least 7 different forms have been identified in*S. coelicolor* alone. This is reviewed by Buttner (1989).

It has been proposed that differentiation and antibiotic biosynthesis are controlled by a cascade of physiological events triggered by a common effector, possibly ATP (Martin, 1988). This is discussed in more detail in section 1.10.2.

1.8 Secondary Metabolite Production in Submerged Culture

Antibiotics may be produced in non-ecological conditions, in large volumes of liquid media on production scales. The "signals" for production of antibiotics in liquid culture, and their role in the life cycle of the organism may be different to those used by the organism colonising solid substrates. In liquid culture, with the exception of one known strain, *S. griseus*, sporulation is suppressed, and yet in the correct nutrient and growth conditions antimicrobial products may be produced (Kendrick and Ensign, 1983). It has also been reported that the morphology of the strain in liquid culture is important for the formation of antibiotics (Luedeking, 1967). As previously explained, filamentous organisms are found to adopt either open mycelial or pelletted forms of growth in liquid culture.

Characteristically antibiotics are produced in a non growth-associated manner. In complex medium, antibiotic formation normally occurs during the stationary phase of growth when the growth rate slows due to a nutrient limitation, or due to build up of toxic products. In economic fermentations, the length of stationary phase may be prolonged by supplying additional carbon sources to supply energy for mycelial maintenance functions and to extend the period of antibiotic production. In contrast, it is common to see growth-associated antibiotic production in cells which are transferred from a rich to a defined medium (Vining and Doull, 1988).

Several reviews of the physiological regulation of secondary metabolism are available, discussing specifically the influences of carbon, nitrogen and phosphate repression on secondary metabolism, or dealing with the the wider question of physiological regulation (Shapiro, 1989; Bushell, 1989; Martin,1989a; Demain, 1989; Trilli 1990). Characteristically, these reviews consist of accounts of the responses shown by specific organisms, and the trends that can be inferred from the response. These reflect the complexity of the processes involved, the diversity of secondary products and the lack of definitively-identified common effector molecules. A summary of the importance of these nutrients in the control of antibiotic production in *Streptomyces* is given below.

1.9 Nutritional Regulation of Secondary Metabolism

1.9.1 Carbon Catabolite Repression

Generally when glucose is supplied to *Streptomyces* cultures, it is consumed rapidly but interferes with the biosynthesis of secondary metabolites (Vining,

1988). In addition, various enzymes which are not considered to be concerned with secondary metabolism are repressed by glucose *e.g.* glucose isomerase, ß-glucanase and α-amylase (Virolle, 1986; Lilley and Bull, 1974; Sanchez and Quinto, 1975). It is generally accepted that cAMP mediated carbon catabolite repression is not a mechanism which, in general, leads to de-repression of the genes for the biosynthesis of secondary metabolites (Aharonowitz, 1982; reviewed by Demain, 1989), although some conflicting evidence exists (Colombo *et al.*,1982).

Glucose, glycerol and citrate may exert some form of repression on antibiotic synthesis in different species. *S. clavuligerus* cannot utilise glucose, suspected to be due to the lack of a glucose transport system, and the preferred carbon source is glycerol. However glycerol exerts a repressive effect on secondary metabolism (Williams and McDonald, 1966; Aharonowitz and Demain, 1978). In *S. niveus,* carbon catabolism of citrate is preferred to that of glucose, and on a mixed carbon source a diauxic response is shown. After citrate is exhausted, there is a lag phase as the organism begins to utilise glucose, succeeded by the formation of novobiocin (Kominek, 1972). In *S. coelicolor,* glucose has a repressive effect on antibiotic biosynthesis and glucose kinase has been implicated as an important enzyme influencing this repressive effect. Mutants of *S. coelicolor* which were defective in glucose kinase (and were unaffected in glucose transport) were less sensitive to glucose repression of antibiotic synthesis than the parental strain (Hodgson, 1982; Seno and Chater, 1983). In *S. fradiae* and *Saccharopolyspora erythrea* carbon source repression of secondary biosynthesis is mediated by phosphorylated sugars, in agreement with the results for *S. coelicolor* (Madry *et al.,* 1979; Escalante *et al.,* 1982).

Specific enzymes of secondary metabolic pathways have also been shown to be repressed by the presence of glucose. Phenoxazinone synthase in the actinomycin pathway in *S. antibioticus* is suppressed by the presence of glucose. This repression has been shown to be exerted at the transcriptional level by mRNA studies (Jones, 1985). Similar effects on a several enzymes in the biosynthetic pathway are seen for cephalosporin biosynthesis and puromycin biosynthesis in *S. alboniger, S. parvullus* and *S. lactamdurans* (Katz, 1980; Cortes *et al.,* 1986; Sankaran and Pogell, 1975).

The role of carbon regulation is complex, as the concentration or type of carbon source may influence the growth rate of a producing organism. This influence may occur due to increased carbon source utilization upon supply, resulting in increased growth rates, or reduction in growth rate in cases of depletion. In addition, growth rate limitation may be conferred in the case of

slow-release carbon substrates such as starch, or by growth suppression in cases of growth inhibition. The role of the carbon supply is complicated by the inextricable linkage between carbon and energy supply to heterotrophic microorganisms. As a result, it can be postulated that effects linked with carbon metabolism may be mediated through precursor supply pathways, or through molecules involved in energy storage or supply.

1.9.2 Phosphate Repression

1.9.2a Physiological Observations

A wide range of secondary metabolic pathways are subject to regulation by phosphate. The aminoglycosides, tetracyclines, macrolides, polyenes and polyether biosynthetic pathways seem to be highly sensitive to the phosphate concentration in the environment. Inhibition is observed with phosphate concentrations in excess of 3-5mM phosphate in the organisms producing these antibiotics. In contrast, the peptide and ß-lactam antibiotic pathways are less sensitive to phosphate concentration; inhibition is observed with phosphate concentrations in excess of around 25mM (Aharonowitz and Demain, 1977).

Specific mechanisms of phosphate regulation have been identified, *e.g.* phosphatase inhibition in the streptomycin and neomycin biosynthetic pathways and the direct inhibition of antibiotic synthetases (Miller and Walker, 1969; Majumdar *et al.,* 1979 . Direct inhibition may be observed with anhydrotetracycline oxygenase in the tetracycline biosynthetic pathway (Behal *et al.,* 1979; Behal *et al.,* 1982), PABA synthase in the candicidin biosynthetic pathway (Gil *et al.*, 1985) and at least four synthetase enzymes in the production and conversion of protylonolide in the biosynthesis of tylosin (Madry, 1982). As described in section 1.9.1, there is also some evidence that phosphorylated sugar molecules may exert a regulatory effect on the biosynthesis of antibiotics in *S.coelicolor, S. fradiae* and *Saccharopolyspora erythrea.*

There are a battery of phosphorylated molecules which may mediate in response to environmental conditions. These include cAMP, ATP (and the 'Energy Charge'), GTP, ppGpp, and pppGpp. The role of GTP, ppGpp and pppGpp have been described in association with the stringent response. The evidence regarding carbon catabolite repression mediated by cAMP has been reviewed by Demain (1989). Reports suggest that cAMP does not mediate in the responses shown by *Streptomyces* to phosphate repression (Martin, 1989). For phosphate-limited cultures of *S. griseus,* the phosphate

inhibition, and relief of the inhibition by addition of exogenous phosphate has no influence on cAMP concentration (Terry and Springham, 1981). However, ATP has been shown to be a potential mediator, as intracellular levels of ATP show a rapid increase in response to exogenously-added phosphate (Effenberger *et al.*, 1983). In addition intracellular ATP levels in *S. aureofaciens* have been shown to vary between high and low titre producing organisms (Janglova *et al.*, 1969). Phosphate limitation has been shown to uncouple respiration in turimycin producing cultures, leading to a rapid decrease in ATP formation rate (Effenberger *et al.*, 1983). The concentration of adenylates in *S. fradiae* was found to correlate with growth phase; high concentrations of adenylates occurred during trophophase and low concentrations during idiophase (Vu-trong *et al.*, 1980). The energy charge, a parameter useful for considering energy-linked metabolic control, has been suggested to relate to the regulation of secondary metabolism (Atkinson, 1969). The energy charge is a numerical value which quantifies the relationship and concentration of the various phosphorylated adenosine molecules within a culture. However in *S. griseus* and *S. fradiae,* the energy charge remained constant throughout growth and antibiotic production (Martin *et al.,* 1978; Vu-trong *et al.,* 1980: Vu-trong *et al.,* 1981). Martin has proposed that it is the concentration of ATP rather than the energy charge which mediates in phosphate control (Martin, 1989).

1.9.2b Observations at the Molecular-Genetic Level

A specific DNA sequence (a "phosphate control sequence") has been isolated from a 1Kb fragment, located upstream of the *pabS* gene in *S. griseus.* A 113bp fragment has been isolated from this fragment, which is recognized by *S. lividans* RNA polymerase (Rebollo *et al.,* 1989). Genes fused to this fragment, became subject to phosphate repression, and a functional analogy between the operator sequences recognised by cAMP receptor protein (CRP) in the lactose and galactose operons has been made (Martin, 1989).

Studies of phosphate deregulated mutants have been made in *S. noursei, S. aureofaciens* and *S. griseus* (Novotna *et al.,* 1984; Hanel *et al.,* 1984). Phosphate deregulated mutants of *S. noursei, S. aureofaciens* and *S. griseus* produced high titres of antibiotic in normally antibiotic-repressing phosphate concentrations. In addition mutants of *S. noursei* contain high intracellular ATP concentrations. Martin has proposed that phosphate-deregulated mutants of *Streptomyces* are impaired in energy metabolism, suggesting a role for ATP in the mediation of secondary metabolism, and the "cascade hypothesis", where secondary metabolism and differentiation

process are postulated to be controlled by "Master Genes" which trigger these responses in cascades (Martin, 1989). Phosphate control sequences, and their importance in control has been reviewed (Llras *et. al,* 1990).

1.9.3 Nitrogen Regulation

Traditionally, complex sources of nitrogen have been used as principal nitrogen sources in commercial fermentations. These include residues from the processes of soya oil production (soybean meal), corn flour production (corn-steep liquor) or brewing (distiller's solubles). These nitrogen sources empirically were found to give favourable yields in production processes due to the 'slow release' of metabolisable nitrogen as the fermentation progressed (G. T. Banks, personal communication). In general it is recognised that an excess of ammoniacal nitrogen at the onset of secondary metabolite production can either delay the secondary growth phase, and/or reduce final titres of product (Shapiro, 1989).

The presence of ammonium ions in culture broth typically represses the synthesis of many secondary metabolites, *e.g.* the production of cephalosporins by *S. clavuligerus*; erythromycin by *Saccharopolyspora erythrea*; chloramphenicol by *S. venzuelae* ; tylosin by *S. fradiae* and lincomycin by *S. lincolnensis* (Aharonowitz and Demain, 1979; Brana *et al.,* 1985; Flores and Sanchez, 1985; Westlake *et al.,* 1963; Shapiro and Vining, 1983; Masuma *et al.,* 1983; Young *et al.,* 1985). Glutamate dehydrogenase, alanine dehydrogenase and glutamine synthetase are implicated in the regulation of secondary metabolism, however conflicting patterns and relationships exist between the activity of these enzymes and the pathway effects which are observed. In *S. clavuligerus* a correlation between high glutamine synthetase activity and cephalosporin titre has been shown, whereas in *S. noursei*, the opposite effect has been shown (Aharonowitz and Demain, 1979; Grafe, 1977). The review by Shapiro (1989), provides a very detailed account of the nitrogen regulatory effects reported for each class of antibiotics.

1.10 The Influence of Dissolved Carbon Dioxide and Oxygen on The Production of Secondary Metabolites

Carbon dioxide has been found to be toxic to the growth of some filamentous organisms, and results in decreases in productivity particularly in fungi (I. Hunter, personal communication). However there are no reports that this is true for *Streptomyces*. For commercial*Streptomyces* production processes, using broths with high biomass densities, growth inhibition due to high

dissolved carbon dioxide concentrations can occur, and has deleterious consequences on antibiotic titres (K. Dixon, personal communication).

In *S. rimosus* and *S. aureofaciens*, a doubling in productivity was observed for a 6.2 fold increase in oxygen partial pressure for wild type strains, achieved by increasing the total pressure (Liefke *et al.,* 1990). High yielding mutant strains were more sensitive to pressure than the wild type strains, and productivity decreased with increasing total pressure in these strains. A general tendency for growth inhibition was observed for all strains at the elevated pressures. This report is important as the hydrostatic pressure experienced at the base of a large production-scale fermenter is considerable; and if not taken into account in process design and strain selection, can result in failure in scale-up procedures.

1.11 Growth Rate Regulation of Secondary Metabolism

Secondary metabolism tends to occur at low specific growth rates for the producing organism in submerged culture (Bu'lock, 1975). The nutrient-mediated effects described in section 1.9 may, therefore, be the result of growth rate effects which are observed as a consequence of the varying nutrient concentration. In order to rigorously investigate the physiology of secondary metabolite production, the nutrient variables must be varied independently of growth rate in chemostat culture. The data in the following section have been obtained from growth in chemostat culture.

In many cases there is an inverse relationship between the optimum production of a metabolite and growth rate, as expected. Inverse relationships for specific productivity of antibiotic with growth rate occur for the production of thienamycin and cephamycin by *S. cattleya* in phosphate, carbon and nitrogen-limited cultures (Lilley *et al.*, 1981); and the production of tylosin in *S. fradiae* in a range of nutrient limited environments (Gray and Bhuwapanthanapun, 1980). However, direct relationships exist for growth rate and antibiotic production by *S. aureofaciens* under either carbon or nitrogen-limited conditions (Sikyta *et al.,* 1961),*Saccharopolyspora erythrea* under phosphate and nitrogen-limited conditions (Trilli *et al.,* 1987; Trilli, 1990), and *S. rimosus* in phosphate, dual phosphate-carbon and phosphate-nitrogen limitations (Rhodes, 1984).

1.12 Process Design for Secondary Metabolite Production

Optimisation of fermentations for the production of secondary metabolites must fulfil three requirements;

i) the fermentations must enable accumulation of high biomass concentrations with high molar growth yields in a relatively short time,
ii) a transition into a high productivity stage of secondary metabolite production must be achieved and
iii) the secondary metabolite production phase should be sustained.

The concentration of metabolite in the culture broth is very important economically, as up to 50% of production costs may be in the recovery stage (Stowell, 1983). Plant required for solvent extraction is extremely expensive owing to the requirement for a spark-free environment, whilst solvents and their recovery are costly and often inefficient processes. Hence, although productivity may be enhanced, in economic terms the concentration of metabolite is of major importance. Most industrial process development is highly competetive, and unreported. A review of some aspects of this subject has been given by Bushell (1988). Wherever possible, examples from the streptomycete literature are given, however in some cases, the use of non-streptomycete reports is unavoidable.

Strain selection: multivariate analysis has been shown to be of use in the selection of mutants with high antibiotic titre; it can be used to design selective isolation procedures (Onadipe and Bushell, 1987; Bull *et al.*, 1990). Selection on the basis of a high specific productivity at a low growth rate, has been suggested in screening programs for the isolation of strains with improved metabolite production, with the aim of achieving efficient carbon conversion (Stouthamer and van Verseveld, 1987).

Batch Culture: Most commercial processes are operated in batch mode, often due more to historical reasons and plant availability than those of productivity. However, batch culture is probably a 'safe' cultivation method for highly genetically unstable strains, which include the high-titre antibiotic production strains. In the design of a novel process, the cost structure of the project will dictate process priorities,*e.g.* the optimisation of specific yield with respect to substrate, in cases where substrate cost is high. In general, product recovery and plant costs will be dominant features in the cost-breakdown of a process for production of a secondary metabolite.

Continuous culture: although volumetric productivity is enhanced in continuous culture over batch culture (Bailey and Ollis, 1986), product concentration is lower, due to the short residence time of broth in the culture vessel. In addition, production strains are generally highly mutated, subject to instability, and prone to decreased antibiotic titres following prolonged cultivation. However the use of continuous culture as an investigative tool is

without doubt, a very powerful technique, useful in the optimisation of a process (described in more detail in Section 4.2).

Fed batch; this would appear to be a compromise between the two culture systems (batch and continuous) described above for large scale production. It is useful for extending the period of productivity of a culture by supplying a carbon source, either intermittently or continuously, to fuel cell maintenance functions and product formation in non-repressing conditions. The technique can be made more sophisticated by the inclusion of a feedback circuit to control medium flow *e.g*. the pH auxostat (Agrawal and Lim, 1984). In this cultivation system medium inflow is controlled according to the pH in the vessel. It can be further modified with independent flows of medium and base to give the "Bistat" (Minkevich *et al.*, 1989; Larsson *et al.*, 1990).

A variation on simple fed batch culture is to remove part of the culture at regular intervals, replacing it with fresh medium, a method known as cyclic fed batch culture. Extreme changes in cultivation conditions occur in this technique, but can be minimised by suitable attention to cycle control in relation to the growth and production kinetics (Bushell, 1988). This technique has been successfully used experimentally with the production of tylosin from *S. fradiae.* By variation in the feed volume (amplitude) and time between feeds (period), the specific productivity of the culture with respect to tylosin was maintained continuously for 10 days (Gray and Vu-trong, 1987).

Immobilisation: this offers many advantages as a method of producing non growth- associated products. Growth and culture degradation do not occur, and providing efficient immobilisation processes are used which do not employ large diffusional limitations, productivity may be high and rapid. However, on production scale, unconventional plant are required (unconventional that is for the production of secondary metabolites) such as air-lift or bubble column fermenters. Repeated batch operation of *S. cattleya* and *S. clavuligerus* using mycelia immobilised on cotton cloth showed constant yield of antibiotics over twenty three-day fermentations (Joshi and Yamazaki, 1987). Actinomycin D was produced by *S. parvullus*, cultivated in batch and fed batch mode in a stirred tank bioreactor and immobilised in calcium alginate and fermentation carried out in an air-lift bioreactor (Dalili and Chau, 1988). The use of synthetic starvation medium in all experiments with a carbon and nitrogen derepression was one feature which enabled high productivities to be achieved in these experiments. Providing a low dilution rate was used with the continuous, immobilised process, productivity of actinomycin D was 1.14 $\mu g.ml^{-1}.h^{-1}$ compared to 0.49 $\mu g.ml^{-1}h^{-1}$ for the fed batch process and 0.15 $\mu g.ml^{-1}.h^{-1}$ for the batch culture. Loss of viability

occurred with the immobilised culture after 9 days of continuous operation, and 11 days for the fed-batch culture.

In an economic and productivity comparison of immobilization of actinomycetes for use in bubble column reactors with conventional methodology, the immobilised processes were potentially considerably advantageous (Buckland *et al.,* 1985)

On-line product removal: many primary and secondary biosynthetic processes are subject to feedback inhibition. Product precipitation and alteration of pH have been used in the past to crudely alleviate feedback inhibition. Conventionally, the reduction or removal of feedback regulation may be attained by mutation and selection for overproduction. On line product removal would be an alternative simple strategy for the alleviation of a regulatory feedback. Dykstra *et al.,* (1988) have described a *S. griseus* fermentation for the production of cycloheximide with an associated, on-line step incorporating an adsorption resin immobilised in hydrogel beads for product removal. A continuous passage of cell-free broth over the resin and back to the fermenter both efficiently removed the antibiotic, preventing feedback inhibition, and rendered product concentration and purification simultaneously. Adsorption resins, which remove organic solutes from polar solvents, are relatively insensitive to fouling (*e.g.* by broth proteins), and possess high loading capacities.

On-line monitoring and control; may represent the most realistic process development tool, accepting that high productivity in a defined plant, with a defined organism and strain may be determined empirically. In this case, precise monitoring and control allows reproducibility between fermentations to be attained. Computer control, gas analysis (using solution phase and gas phase mass spectrometry) for analysis of all dissolved and gaseous gases together with HPLC product analysis is the ideal specification.

Mass balancing, described in detail in chapter 5 is a useful non-invasive tool in the investigation of the macroscopic parameters of a fermentation in a defined process. It is also an essential technique in physiological state variable analysis (or estimation, in the case of incomplete mass balance) for fermentation process control (Konstantinov and Yoshida, 1989; Liao, 1989). It has been used admirably as a tool for the investigation of secondary metabolite formation by *S. cattleya* (Fryday and Bushell, 1983). In this study the RQ value was found to be characteristic of the physiological state of the culture and the type of product synthesised. In addition these changes were found to coincide with distinct nutrient limitations. When a specific nutrient

reached a low value, formation of a specified metabolite was initiated. Information about the efficiency of the process was also determined, based on the reductance of nutrients and biomass. This report is discussed in greater detail in Chapter 5.

1.13 The Molecular Genetics of *Streptomyces* Secondary Metabolism

This has been reviewed by Hunter and Baumberg, 1989 and Martin and Liras, 1989. Information concerning the available cloned pathways for this project have been summarised (Czaplewski,1989), and therefore will not be discussed further.

1.14 Thermophilic *Streptomyces spp.*

1.14.1 *S. thermonitrificans ISP 5579*

The strain chosen for this project was originally isolated from Indian soil (Desai and Dhala, 1967), and was selected for this project on the basis of a good "growth performance" in shake flask cultures of TSB, ATCC and L broth (L. Harvey personal communication).

Moderate thermophiles tend to span the temperature boundary 55-60^oC, whereas extreme thermophiles cannot grow below 55^0C (Weigel, 1990). In contrast to most *Streptomyces* which labour in their growth above 30^oC, *S. thermonitrificans* has an optimum growth temperature in the range 40-50^oC, with a maximum growth temperature of 55^0C and a minimum of 30^0C.

1.14.2 Adaptations to Growth at Elevated Temperature

One of the most important adaptations to growth at elevated temperature is an increased saturation in membrane lipids in order to maintain an effective delimiting and selective barrier membrane with a liquid crystalline structure. It is documented in species other than *Streptomyces*, that lipid composition is adapted both genotypically and phenotypically to growth at high temperature (Russell and Fukunaga, 1990; Pond and Langworthy, 1987). In *Streptomyces* a study of the fatty acid composition of a thermophilic strain (an unspecified strain) has shown that cells grown at different temperatures show relatively small alterations in the saturation of fatty acids. Lipid saturation and branching were found to increase with temperature (Heinen and Lauwers, 1983).

Growth at an elevated temperature must elicit efficient enzyme functioning at these temperatures. This may be due either to enzymic

thermostability or to a rapid turnover and resynthesis. In general, extreme thermophiles have enzymes stabilized structurally and by substrate interactions so that they have higher temperature optima compared to enzymes from mesophiles (Hartley and Payton, 1982). However in a single study of a facultative thermophilic streptomycete (unspecified species) a thermal 'switching' phenomenon has been observed. For growth below 50°C five central metabolic enzymes have been shown to have increasing thermostability with growth temperature. However above 50°C thermostable enzymes were produced (Heinen and Lauwers, 1983). In addition, high levels of polyamines have been implicated as a feature in enhancing enzyme stability of *Streptomyces* at elevated temperatures (Hamana and Matsuzaki, 1987).

The respiratory chain of *S. thermonitrificans* has been shown to have thermostable substrate-directed respiratory activity, remaining 50% higher than that of *S. thermoviolaceous* at 55°C. These results were obtained from membrane preparations at growth temperatures between 37°C and 55°C (Edwards and Ball, 1987). It has also been shown that *S. thermonitrificans* possesses a *d*- type cytochrome, a *b*-type, *c*-type, *a*-type and possibly an *aa3* -type cytochrome.

1.14.3 Studies of the Physiology and Molecular Biology of Thermophilic *Streptomyces*

In the commercial arena, a mutant *S. thermotolerans* was used for the bioconversion of macrolide antibiotics into a range of ester derivatives, although it is no longer a commercial process (J. Turner, personal communication). The advantage of using *S. thermotolerans* for the acylation reaction is that acylation is faster and gives better yield of product compared to alternative mesophilic strains. (Shreve *et al.,* 1985). The advantage of this particular mutant is that it has a consistent performance in transformation in contrast to the type strain which produces a macrolide antibiotic, carbomycin, and hence a mixture of products consisting of carbomycin together with the specific product of the bioconversion (Kirst *et al.*, 1986).

S. thermotolerans has been the subject of an investigation into the molecular genetics of carbomycin biosynthesis. The resistance genes, *carA* and *carB,* have been cloned and sequenced (Epp *et al.*, 1987). *CarB* has a greater than 50% similarity with the RNA methylase in *Saccharopolyspora erythrea* and is thought to act as an RNA methylase on 23S rRNA, conferring resistance to macrolides. The resistance genes have been used to probe libraries to isolate flanking genes. Genes required for lactone formation, for addition of sugar molecules to the lactone ring and for acylase activity have also been isolated (*carC, carD* and *carE).* Subclones containing the genes

conferring acylase activity (*carE*) on *S. thermotolerans* were found to enable production of a hybrid antibiotic, isovaleryl spiramycin, when transformed into *S ambofaciens*, and when transformed into *S. lividans* supplied with exogenous spiramycin (Richardson, 1989; Epp *et al.*,1989).

Another thermomophilic streptomycete, *S. thermoviolaceous* has been the subject of a physiological study. The influence of carbon source and pH on granaticin production have been investigated (James and Edwards, 1988). Granaticin was found to be synthesised in a growth-associated manner on defined medium; it was not limited by nitrogen when glutamate was used as a sole source of carbon but was prevented with glucose as a carbon source. The repressive influence of glucose could be relieved by the use of a medium low in nitrogen. Cytochrome spectra were found to change with growth conditions, and a *d* type cytochrome was identified in glucose-grown cells.

The enzyme, ß l-4 glucanase has been studied in defined medium in an unspecified thermophilic streptomycete, with the aim of investigating the regulation of the extracellular polysaccharase of the strain (Lilley and Bull, 1974). Maximum production of the enzyme was found to occur during stationary phase following the addition of an inducer late in the fermentation.

1.15 Economic Advantages of Thermophilic Fermentations

Thermophilic fermentations are of commercial interest for a number of reasons;-

Firstly, extreme thermophiles are known to produce thermostable enzymes, of interest for use in large scale applications such as lipases and proteases in detergents and for industrial transformation processes (Brock, 1986; Kristjansson, 1989). Thermophilic enzymes are also known to tolerate more extreme environments than their mesophilic counterparts and are of interest for use in dual solvent systems. Secondly, thermophilic processes are more efficient to run on a large scale because less energy is required for cooling processes. In addition the increased reaction rate reduces turnaround times, enhancing the productivity from a particular plant. Process disadvantages for fermentation at elevated temperature are discussed in section 1.15.2, together with possible methods for their alleviation.

1.15.1 Heat Production in Large Scale Fermentations

In any fermentation there are two major sources of heat evolution; mechanical energy from both aeration and agitation, and endogenous heat evolution from mycelial growth. In shake flasks and small fermenters, energy input from

mechanical means is small and the heat evolved by respiring mycelia in small volumes of culture is negligible compared to evaporation and radiation. However, for production scales, heat evolution by mechanical and physiological means is large with respect to evaporation. In any production fermentation, a large proportion of energy consumption is directed towards cooling the process.

In physical terms an appreciation of the amount of energy consumed by cooling may be obtained if we consider the numbers involved.

In a production fermentation:-

Typical power input = 1 horsepower per 400 litres
$= 641\ kcal.hr^{-1}.400^{-1}$
$= 1.50\ kcal.hr^{-1}.l^{-1}$

This would tend to raise the medium temperature by 1.5 ^0C per hour (as the specific heat capacity of water is 1 kcal per litre per ^0C), based solely on mechanical energy input directly delivered to the medium (figures adapted from Bader, 1986).

Incorporating endogenous heat evolution into the expression; as the heat of respiration of glucose per mole O_2 consumed is typically 112 kcal. mol O_2^{-1}, and for oil, 104 kcal. mol O_2^{-1}, then for a typical mesophilic fermentation with a typical oxygen uptake rate of 30mmol $O_2.\ l^{-1}.hr^{-1}$;

heat produced = $(30\ x10^{-3})$ x 110 kcal. $mol0_2^{-1}$
$= 3.3\ kcal.l^{-1}.\ hr^{-1}$ (Adapted from Bader, 1986)

This effect would tend to raise the medium temperature by 3.3^0C per hour. Hence combined input energies would tend to raise the broth temperature by around 5^0C per hour. For microorganisms growing at more rapid rates than mesophiles the heat of respiration per unit volume of broth would be greater and the increase in temperature more pronounced. Under normal conditions, energy is required to remove this excess heat.

Heat is removed from a fermentation using the temperature gradient across a heat transfer surface delivered through a water jacket or through a transfer loop. The source of the coolant is important, and cooling requirements in tropical climates are far greater than those in temperate zones, due to the shallower temperature gradient between the fermenter and cooling water. Large volumes of cold water or refrigeration systems with closed cooling loop

circuits are required to supply coolant. Water must be at normal temperatures before disposal into natural water courses; therefore both systems require the input of cooling energy (otherwise the warm water is a potential pollutant and heavy discharge levys are enforced). It has been calculated that a saving of £10,000 *p.a.* could result for every degree celsius increase in fermentation temperature for a typical antibiotic process (Stowell, 1982). These data are calculated solely in terms of cooling energy reductions, and do not take into account any increases in the rate of fermentation. Energy balances for fermentations with cooling requirements both for mesophilic and thermophilic processes have been established (Aiba *et al.,* 1983).

1.15.2 Reaction Rate, Productivity and Broth Rheology

According to the Arrhenius relationship, catalytic activity increases with temperature. Hence metabolic fluxes and growth rates also increase with temperature, providing the optimal growth rate of the organism is not exceeded.

Faster growth and flux rates would reduce the time taken for both growth and production stages in a fermentation. In a fed-batch process, following a growth stage, the conversion rate of carbon source to product would be faster than a comparable mesophilic fermentation. This would increase plant productivity as a result of faster turnaround times: more fermentations could be run in a particular piece of plant per annum, or more profitably, less plant would be required. This does not take into the account the effect of temperature on product stability. Inactivation kinetics for tyrosinase produced by a recombinant streptomycete containing pIJ702 have, unsurprisingly, been found to follow an Arrhenius relationship (Gardner and Cadman, 1990). However, for any product inactivation, a shorter fermenter residence time would reduce the influence of inactivation. Hence, a faster inactivation rate as a result of operation at an elevated temperature may be equivalent, or less, in total quantity of inactivated product, compared to that of a mesophilic fermentation (which must be continued for a longer length of time).

Streptomycete production fermentations characteristically have very viscous broths due to high biomass concentrations and the pseudoplastic response to shear of filamentous organisms (Banks, 1977). In practice shear response varies during the fermentation, but towards the end of a fermentation large inputs of energy to agitation are required in order to maintain high mass transfer rates (Bushell, 1988). These tend to increase the broth temperature still further, requiring cooling energy. Viscosity is inversely related to temperature and for an increase in temperature from 30 to 45^{0}C the viscosity

of water decreases by 25% (Weast *et al.*, 1972). The lowered viscosity of fermentation broths at elevated temperatures would contribute a useful energy saving during the later stages of a fermentation. In addition, downstream operations may be facilitated if the product is sufficiently stable, so cooling would not be required. Additionally, filtration, sedimentation and centrifugation would be made easier with low viscosity broths. It is also thought that fermentations at elevated temperature carry less risk of adventitious contamination compared to conventional mesophilic fermentations (Hartley and Payton, 1982: Sonnleitner and Fiechter,1983). However on production scales, stringent asepsis must be maintained at all times from economic and safety perspectives.

1.15.3 Disadvantages of Fermentation at Elevated Temperature - and Potential Methods for their Alleviation

Work at elevated temperature would require a very efficient aeration regime as gaseous solubility decreases with temperature. The solubility of oxygen in water decreases by 20% for a temperature change from 30^{0}C to 45^{0}C. Conventionally, oxygen limitation has been alleviated by the use of modified plant such as the use of modified impellers and baffles (Atkinson and Mavituna, 1983).
Alternatively the reduced oxygen solubility which occurs at elevated temperature could be alleviated by the use of emulsified oxygen-vectors (Rols *et al.*, 1990). For *Aerobacter aerogenes* the use of *n* -dodecane as an auxiliary liquid resulted in a 3.5 fold increase in the K_La value (the volumetric oxygen transfer coefficient). This increase was the result of the increase in the diffusion gradient for solution of gas from the oxygen-vector to the medium, in contrast to the very shallow gradient which exists between gaseous and dissolved oxygen. Hence, the efficiency of the major rate-limiting step in gaseous transfer was increased.

A further alternative for the alleviation of the reduced oxygen solubility which occurs with elevated temperature would be the heterologous expression of an intracellular bacterial haemoglobin. This soluble haemoglobin-like molecule is synthesised in *Vitreoscilla,* in response to hypoxic environments. It has been cloned and heterologously expressed in *E. coli* (Khosla and Bailey, 1988b) and in *S. coelicolor* and *S. lividans* (Magnolo *et al.,* in press). In *E. coli,* it has been shown to improve the efficiency of growth and the productivity of heterologously expressed proteins at low dissolved oxygen tensions (Khosla and Bailey, 1988a). It has been deduced that this increased growth efficiency is the result of an improvement in the efficiency of ATP formation, or energetic coupling in oxygen-limited conditions, as kinetic

processes remain unaffected (Khosla and Bailey, 1990). In *Streptomyces* it has been shown to result in increased antibiotic yields and reduction in the sensitivity of secondary metabolism to aeration conditions.

1.16 The Glasgow Project

The aim of this project was to assess the feasibility of producing antibiotics at elevated temperature in a strain and growth conditions suitable for rigorous physiological study. A number of strategies were available to achieve this aim which are described in the thesis of Czaplewski (1989). A strategy was chosen based on a thermophilic streptomycete strain which had the potential for good fermenter performance, lacking an endogenous antimicrobial product (selection is described in more detail in Chapter 3). Techniques to enable gene transfer were developed (Czaplewski, 1989).

Six criteria were to be fulfilled:
1. Strain selection (work of L. Harvey, unpublished).
2. Development of a transformation system (Czaplewski, 1989).
3. Construction of a recombinant strain (Czaplewski, 1989).
4. Heterologous expression of the plasmid-encoded pathway at 45^{0}C, purification and assay development of the product (described in this thesis)
5. Fermentation development for materials balancing (described in this thesis)
6. Carbon balanced fermentations (described in this thesis)

Several interesting features have developed in the course of this project, particularly with respect to the molecular genetics of the strain. This work, by Czaplewski, is summarised below.

Transformation methods for *S. thermonitrificans* with plasmid DNA were developed. This allowed introduction of plasmid pIJ702 into *S. thermonitrificans* at frequencies of $1x10^3$ per µg of pIJ702. A recombinant strain *S. thermonitrificans* pBROC139 was constructed. The plasmid pBROC139 is a pIJ702-based plasmid constructed by Beecham, containing DNA from a total digest of *S. clavuligerus*, and thought to encode the antibiotic holomycin. A thermotolerant actinophage TA, and a host range mutant TAmt1 were isolated and characterised. In addition, a plasmidogenic conjugative transposable element (STP1) was isolated and aspects of its molecular biology were investigated.

1.17 The Scope of This Work

The work described in this thesis includes the fermentation development of

the wild type strain for growth in a 7 litre Bioengineering fermenter under defined growth conditions in minimal media (Chapter 3). Preliminary investigations into the physiology and growth energetics of the wild-type strain in flask and continuous culture under defined conditions were carried out (Chapter 4), together with carbon balanced and stoichiometric descriptions of the fermentation in defined medium (Chapter 5). This thesis also describes an attempt to transform *S. thermonitrificans* with a large 50Kb single copy plasmid encoding the pathway for oxytetracycline production (Binnie *et al,* 1989), and *in-vivo* manipulations in an attempt to achieve the same result (Chapter 6). Finally the purification and preliminary identification of the previously uncharacterised product from pBROC139 is described (Chapter 7).

MATERIALS AND METHODS

2.1 Introduction

Sections 2.3-2.10 describe the strain preservation and culture techniques which were used in the maintenance and growth of *Streptomyces*, and more specifically *S. thermonitrificans*. This section also describes the quantitative techniques used in study of the strain and the preparation of fermenters for microbial cultivation in both batch and continuous modes. Methods and equipment developed specifically for the requirements of the project are described in a subsection; Development of Methods.

Section 2.11-2.14 describe the additional features for preservation and cultivation of plasmid-containing *Streptomyces* strains, the methods of preparation and transformation of protoplasts for *S. thermonitrificans* and the methods of plasmid DNA preparation, restriction and visualisation by agarose gel electrophoresis. In addition the bioassay for oxytetracycline is described.

Section 2.15-2.21 describes the materials and methods used in the purification and identification of the antimicrobial product expressed by *S. thermonitrificans* pBROC139.

2.2 Chemicals and Consumables

	Source
General and AnalaR Chemicals and AnalaR and HPLC grade solvents	BDH, , Koch-Light Laboratories, May and Baker, Formachem.
TLC Media	BDH
Growth Media	Difco, Oxoid.
General Biochemicals	Sigma, Pharmacia, BRL.
Agarose	BRL
Antibiotics	Sigma, Squibb and Sons, Pfizer
JunIon	Honeywill and Stein
Antifoams	BDH, Sigma
Assay Kits;	
Glucose Perid	BDH
Phosphorous	Sigma
Ammonia	Sigma
Filter and glass fibre papers	Whatman
Calibrating gases	British Oxygen Company, Special Gas Division.

2.3 Strains

Strain		Reference
S. thermonitrificans		Desai and Dhala, 1967
S. thermonitrificans pBROC139		Czaplewski, 1989
S. thermonitrificans pIJ702		Czaplewski, 1989
S. thermonitrificans pIJ916		Czaplewski, 1989
S. albus [G153 (r^- m^+)]		Hunter, personal communication
S. albus pBROC139		Czaplewski, 1989
S. albus pIJ702		Czaplewski, 1989
S. albus pIJ916		Czaplewski, 1989
S. albus pFZ163		Binnie *et al.,* 1989
S. lividans pBROC139		M. Burnham, personal communication
S. lividans pIJ702		Czaplewski, 1989
S lividans TK54	*his*-2, *leu*-2, *spc*-1	Hopwood *et al.,* 1985
S. lividans TK64	*pro*-2, *str*-6	Hopwood *et al.,* 1985
S. albus pFZ163		Binnie *et al.,* 1989
S. lividans TK64 pFZ163		Section 6.1.3, this work
S. lividans TK54 pIJ940		I.Hunter, personal communication
S. lividans TK64 pIJ940		Section 6.1.5, this work
S. lividans TK64 pGLW101		I.Hunter (personal communication)

All plasmids contained the *tsr* gene from *S. azureus* and their prescence was maintained by selection for thiostrepton resistance.

2.4 Growth Media

Trypticase Soy Broth (TSB): 15 g BBL trypticase soya broth powder per litre.
Mannitol-Soya Agar: 20g Mannitol, 20g soybean flour, 16g agar, 1 litre tap water.
Emersons agar: Emersons agar was purchased as a powder from Difco and was used as directed by the manufacturers.
LMM (Semi-defined); NH_4NO_3 2.0g, $MgSO_4.6H_20$0.9g, tap water 900 ml, pH adjusted to 7.0 prior to sterilization. Glucose 15 g and K_2HPO_4 0.99g, KH_2PO_4 0.59g were sterilized separately in a volume of 100ml, following pH adjustment to 7.0, at 112^{0}C for 10 minutes and added aseptically to the remainder of the medium, to a final volume of 1litre. In media for flask cultivation, 20g TES buffer pH7 (Sigma).
LMM (Fully-defined); NH_4NO_3 2.0g, $MgSO_4.6H_20$ 0.9g, KCl 0.50g, $ZnSO_4.7H_2O$ 0.20g, $NH_4Fe(SO_4)_2.12H_20$ 0.10g, $MnSO_4.4H_2O$ 0.05g, $CuSO_4.5H_20$ 0.005g,$Na_2MoO_4.2H_2O$ 0.005g, $CoCl_2.6H_20$, 1 litre distilled water, pH was adjusted to pH7 prior to sterilization. In media for flask

cultivation, 20g TES buffer pH7 (Sigma).
Glucose and phosphate were sterilized separately and added aseptically as described for semi-defined LMM.

Evans Minimal Medium (Evans, 1970) NH_4Cl 5.34g, KCl 0.75g, $Na_2SO_4.10H_2O$ 0.64g, Citric acid 0.42g, $MgCl_2$ 0.12g, $CaCl_2$ 0.002g, Na_2MoO_4 0.0002g, trace metal solution (described below), 5ml, 1 litre distilled water. In media for flask cultivation, 20g TES buffer pH7 (Sigma).

S. thermonitrificans Minimal Medium (579 M M); NH_4Cl 2.67g, KCl 0.75g, $Na_2SO_4.10H_2O$ 0.64g, Citric acid 0.42g, $MgCl_2$ 0.12g, $CaCl_2$ 0.002g, trace metal solution (described below) 5ml, 1litre distilled water. In media for flask cultivation, 20g TES buffer pH7 (Sigma).

Trace metal solution; Concentrated HCl 10ml, ZnO 0.4g, $FeCl_3.6H_2O$ 5.4g, $MnCl_2.4H_2O$ 2g, $CuCl_2.2H_2O$ 0.17g, $CoCl_2$.2H2O 0.47g, H_3BO_4 0.06g, 1 litre distilled water.

Pre-germination Medium (Hopwood *et al.* 1985): (double strength) 10g Difco yeast extract, 10g Difco casamino acids, 0.01M $CaCl_2$ (autoclaved separately), made up to 1 litre with distilled water. 1x pre-germination medium was made by the mixing of equal parts of the above and TES buffer (0.05M, pH8)

2.5 Sterilisation

All glassware, solid media and liquid growth media for flask cultivation were sterilised by autoclaving at 121°C for 15 minutes; glucose and phosphate were sterilized at 5 p.s.i. for 10 mins. Antibiotics were sterilized by filtration through 0.22μm filters (Millipore). The LH fermenter was sterilised for 30minutes at 121°C for 30 minutes, 20 litre medium pots at 121°C for 1 hour, and the 9 litre Bioengineering fermenter was sterilised *in situ,* according to the manual accompanying the fermenter.

2.6 *S. thermonitrificans* Strain Preservation

2.6.1 Preparation of spore suspensions of *Streptomyces* ; A concentrated spore suspension was required for inoculating liquid cultures of *Streptomyces spp.* The protocol described by Hopwood *et al.,* (1985) was followed with minor modifications.

A boiling tube containing a slant of Emersons agar (produced by pouring 15ml of molten agar into the tube and allowing it to solidify with the tube placed 5° to the horizontal) was inoculated with 10^7 spores of *S. thermonitrificans* and incubated at 37°C. After four days incubation, the culture was covered in a dark grey mass of spores. The slant was frozen at -20°C. Spores could then be harvested immediately or stored indefinitely.

Spores were harvested by rubbing the agar surface with a 10 ml pipette containing 5 ml of sterile water. When the surface of the slant was scraped clean of spores the 5 ml of water was used to wash the slant and resuspend the spores. The spore suspension thus produced was dark grey in colour and contained little agar or mycelial fragments. The spore suspension was filtered through a cotton wool filter as described in Hopwood *et al.*, (1985). The filtered spore suspension was aliquoted and frozen at -20^{0}C; the aliquots were thawed and mixed on a vortex mixer prior to use. The titre of the spore suspension was determined after storage at -20^{0}C overnight and was found to be constant for at least six months (Czaplewski, 1989).

2.6.2 Preparation of Master Cultures A spore sample from the original slope of *S. thermonitrificans* from the Williams *Streptomyces* culture collection (ISP5579) was streaked on Emerson agar to form single colonies. Following growth at 37^{0}C for 7 days, a type-typical colony was selected and inoculated onto a 5ml agar slope. This was incubated and the spores allowed to germinate, grow and sporulate (slope A). Five 5ml slopes were inoculated with the spores from slope A and allowed to grow and sporulate (slopes B). These 5 replicate mini-slopes were stored at -20^{0}C, as master cultures (B) together with the original slope (A) from the original single colony isolate. Slant slopes (15 ml) were prepared from the master cultures (B) when required (around 20 slopes were prepared per master culture). Master cultures were prepared whenever required from slope A. Hence spores for flask cultivation or pregermination were not more than 2 slope cultivations from the original single colony isolate slope (A).

In general, mini slopes were used for long term spore storage and 10 ml slopes for medium term storage. For short term storage, spores were harvested from slopes and stored in water.

2.7 Cultivation

2.7.1 Shake Flask Culture ;Liquid cultures of *S. thermonitrificans* were prepared by inoculation of liquid media with a known concentration of spores (or mycelium) and grown at a specified temperature for a specific length of time. For all studies at 45^{0}C, the shaking incubator used was constant, set at 225 rpm with a base plate throw of 5 cm. For studies at 37^{0}C, a shaker set at 200 rpm was used. For all experiments in liquid culture, temperature was maintained at 45^{0}C unless otherwise stated.

2.7.2 Solid Substrate Cultivation All routine solid substrate cultivation was carried out at 37^{0}C unless otherwise stated.

2.7.3 Spore Pre-germination ; The method described by Hopwood *et al*, (1985) was used to pre-germinate spores of *S. thermonitrificans* (described in Section 3.7). This method was generally carried out in replicates of 4, preparing spores from 40 agar slopes and subdividing these. Around 10^{10} spores, collected from 10 slopes of Emerson agar were suspended in 10ml of TES buffer, and heat shocked for 10 minutes at 50°C. They were then cooled under a cold water tap. An equal volume of double strength pre-germination medium was added and the suspension was incubated for 3 hours at 30°C with orbital shaking at 250 rpm. The pre-germinated spores were recovered by centrifugation (12000g for 10 min) and resuspended in 2ml of sterile water. Spores were then stored for up to six months at -20°C.

2.7.4 Homogenisation of mycelium Two forms of homogenisation were used. The first involved the use of a hand homogeniser, commonly used for macerating plant or animal tissue. It may be described as a thick-walled test tube with a rough inner surface into which an oval-shaped roughened bulb fits snugly. Homogenisation of tissue occurs due to pressure shock as the bulb is passed through the liquid over the rough surface of the bulb and inner surface of the test tube. The advantage to the use of this equipment over a pestle and mortar is that the enclosed nature of the tube enables asepsis to be maintained without too much difficulty. The apparatus is sterilized by soaking in ethanol for several hours followed by rinsing with sterile distilled water in a sterile air flow and air drying in the same sterile air flow. Mycelium fragmented by this technique was used immediately.

A second form of homogenisation, described in Chapter 3, entailed using 1g of glass beads for every 100ml of medium, and resuspending mycelium in 10th of the original volume. The mycelial paste and glass beads were mixed on a vortex mixer for 1 minute sessions (cooling on ice between sessions) for a total of 20 minutes, in a sealed plastic tube. This method was found experimentally to give an optimum degree of fragmentation for mycelium grown on 579 minimal medium and destined for subsequent use as mycelial inoculum. Fragmented mycelium was stored for short term use (within one month) in water at -70°C.

2.7.5 Determination of Spore Concentration A suitable dilution of a spore suspension in distilled water was pipetted into a petri dish (10 µl per plate). Molten Emersons agar at a temperature of 55°C was then poured into the dish, mixed by swirling and allowed to set. Plates were incubated at 42°C for 24 hours. The spore concentration was determined by counting the number of streptomycete colonies in 10µl of the spore suspension, then multiplying this by the dilution value, to give a number for the spore concentration per ml water.

It was not necessary to heat the filter papers for a second period of time, as a reasonably constant dry weight was attained with a single heating step.

2.7.6 Determination of The Number of Colony Forming Units This assay was carried out as for the determination of spore concentration, with the exception that serial dilutions were prepared using micropipette tips cut with a heated spatula to provide a broad tip. The streptomycete colonies formed after 24 hours of growth at 45^{0}C were used to give a value for the number of colony-forming units per ml of the original broth sample.

2.8 Assay Techniques

2.8.1 Dry Weight As a result of the mycelial (and often pelletted) growth of *S. thermonitrificans,* accurate estimation of growth by assay of optical density was not feasible. Dry weights were determined according to the method described by L Harvey (personal communication). Whatman Number 1 filter paper discs (4.25cm) or glass fibre discs (GF/C) were numbered in pencil and dried for 10 minutes in a 600W microwave oven set on defrost. The papers were allowed to cool in a dessicator in the prescence of silica gel, then rapidly weighed, and the mass recorded to 3 decimal places on an accurate microbalance. The appropriate filter was then placed on a sintered glass filtration unit (50mm diameter, Whatman), 10ml samples of broth pipetted onto the tared filter and filtered through the unit under vacuum, followed by two washes with distilled water. The filter was then dried in the microwave oven as previously described, allowed to cool and weighed. The differential mass of original and final filter papers multiplied by 100 provided the value for dry weight concentration in g.l^{-1} (10ml samples were used). Sampling was carried out in triplicate (or greater) and the mean value determined.

2.8.2 Determination of Glucose Concentration A glucose assay kit (GOD-Perid) was used, based on the technique of Werner (Werner *et al.*, 1970). The assay is dependent on the following reactions;

$$\text{Glucose} + O_2 + H_2O \leftrightarrow \text{gluconate} + H_2O_2$$
(in the presence of glucose oxidase)

$$H_2O_2 + \text{ABTS (pale yellow)} \leftrightarrow \text{coloured complex (green)} + H_2O$$
(in the presence of peroxidase)

The assay is extremely sensitive, and was found to be difficult to use with high concentrations of glucose (15 g.l^{-1} or greater), owing to the use of large dilutions which introduced systematic errors into the data. At glucose concentrations of around 15 g.l^{-1}, dilutions were determined gravimetrically to 4 decimal places using a Sartorius microbalance accurate to 4 decimal places. Culture broth samples were stored frozen and, following a defrost stage,

mycelium was pelletted by centrifugation at 12,000g for 5 minutes and the supernatant used for analysis. All samples and reagents were equilibrated at room temperature for at least 30 minutes before use. Dilutions were used to bring the glucose concentration to a level at which the final spectrophotometric reading was less than 0.3 absorbance units (a 1 in 500 to a 1 in 100 dilution) by the use of a combination of gravimetric techniques and positive displacement micropipettes (Finnpipettes). Diluted samples and reagent were then added to disposable 1cm path-length cuvettes using micropipettes (Gilson, Pipetteman). These were incubated at room temperature for 45 minutes, after which no increase in absorbance occurred, and the reaction was complete (determined experimentally). The coloured complex was stable for a maximum of 1-2 hours. The optical density of the mixture was measured at 610nm and glucose concentration determined according to a standard curve (absorbance is proportional to glucose concentration over the range 0-30μg).

2.8.3 Determination of Ammonium Ion Concentration (Fawcett and Scott, 1960: Chaney and Marback, 1962). A Sigma urea nitrogen assay kit was used in which ammonium concentration can be estimated based on the indophenol blue method, following the procedure described by the manufacturer. The method relies on the reaction between alkaline hypochlorite and phenol to form indophenol in the prescence of nitroprusside as catalyst. The order of addition of reactants to the assay mix must be strictly according to that described by Sigma, in order to maintain the action of the catalyst. The mix was then incubated at room temperature for 40 minutes. Indophenol blue was measured at 510 nm and was proportional to ammonium concentration over the range 0-3.6 μg. Actual values for ammonium concentration were determined by calibration against a standard curve which was checked for each set of assays. The spectrophotometric reading was taken within 20 minutes following the termination of incubation, as the coloured complex formed was unstable after this time. Dilutions for this assay were the same as those for assay of glucose (dilutions of 1 in 5 to 1 in 20).

2.8.4 Determination of Phosphorous Concentration (Fiske and Subbarow,1925) The assay depends on a complex reaction between phosphomolybdate, bisulphite, sulphite and 1-amino-2-naphthol-sulphonic acid to give a blue complex. Absorbance was monitored at 660nm. The colour developed in 10 minutes and was stable for only 10 minutes. Absorbance was proportional to phosphorous concentration over the range 0-0.2mg and was determined by reference to a standard curve which was checked for each set of assays. Dilutions for this assay were the same as for the glucose assay (a zero to 1 in 10 dilution).

2.8.5 Determination of Total Organic Carbon Concentration A Shimadzu TOC50 instrument was used according to the manufacturers instructions. A test sample size of 10µl was used in replicates of 5, with correction for the inorganic carbon present in each sample whenever necessary.

2.8.6 Elemental Analysis of Mycelium Biomass in mid exponential phase was prepared by inoculating 3 x 200ml TSB with a concentration of 10^6 spores.ml^{-1} medium and grown at 45^0C for 24 hours. Each flask was harvested separately. Biomass was extensively washed and the cell paste subdivided to give a total of 6 samples for freeze drying. Following freeze drying the samples were further subdivided to give a total of 12 samples, 4 replicates in triplicate. The mycelium was then analysed by the Microanalysis Section, Department of Chemistry, University of Glasgow (Carlo-Erba Elemental Analyser Model 1106), by total combustion in the presence of catalysts followed by chromatographic separation of the gaseous products and analysis by conductivity.

2.9 Photomicrography: A Leitz Orthoplan universal large-field microscope was used together with a PHACO x25 objective for phase contrast microscopy to final magnifications of 250 and 312x, and a Vario-Orthomat camera. Fresh specimens were prepared immediately prior to use. Premium quality microscope slides and cover-slips of grade 1 standard, were used together with Fuji Velvia positive film (ASA 50). Film was used rapidly, stored at 4^0C until developed by to the E21 developing process.

2.10 Fermenters

Bioengineering Type L1523 LH Type CC1500

2.10.1. Associated Services;

Bioengineering fermenter (9 litre volume); steam at a pressure of 40 p.s.i. and mains cold water were used for temperature control of the *in-situ* sterilizable Bioengineering fermenter. Air supply to the 9 litre Bioengineering fermenter was by means of a separate heavy duty compressor. Routine air flow was calibrated using a rotameter (10 litre per minute maximum flow rate). Accurate air flow rate measurement and control was carried out using a mass flow controller.

LH fermenters (2.5 litre volume); the LH fermenters required a supply of mains cold water for the condenser and a single 13 amp socket. The fermenter was not issued with a condenser and it was necessary to improvise (described in

section 2.10.4). Air supply for the LH fermenters was by means of an internal compressor (3 litre per minute maximum flow rate); air flow rate was monitored by a rotameter on the front of the control housing.

2.10.2 Fermenter Configurations

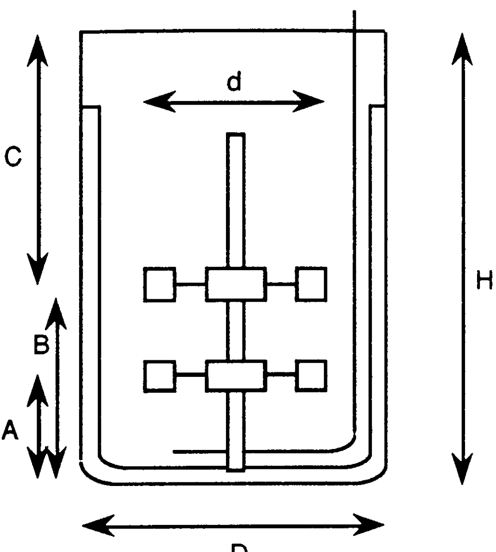


Figure 2.1 Diagrammatic representation of Bioengineering 7 litre working volume, *in-situ* sterilizable fermenter.

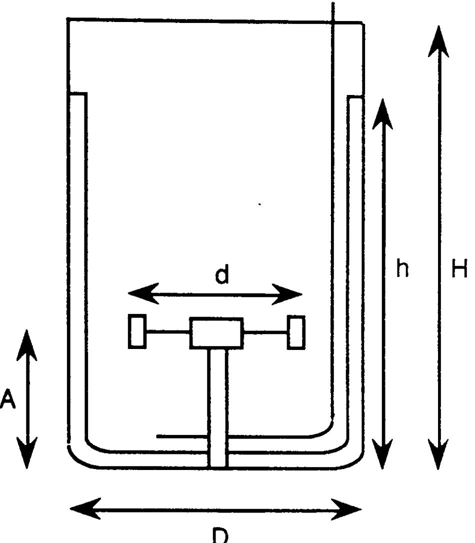


Figure 2.2 Diagrammatic representation of LH 2.5 litre working volume fermenter

Key

H = height of fermenter = 51.7 cm
D = diameter of fermenter = 15.5 cm
d = impeller diameter = 8.4 cm
A = height to first impeller = 8.4 cm
B = height to second impeller = 16.8 cm
C = height from second impeller = 26.5 cm
w = baffle width = 1.0 cm

H = 20.0 cm
D = 15.0 cm
d = 7.0 cm
A = 5.0 cm
h = height of baffles = 15.0 cm

2.10.3 Specification and Methods used with the Bioengineering Fermenter

2.10.3a Sampling The fermenter was equipped with a steam-sterilizable

delivery valve at the base of the fermenter. This was used for sampling, steaming for 15 minutes prior to sampling, allowing the line to cool, and re-steaming the line for at least 15 minutes following sampling. Samples were taken ideally at six hourly intervals, with shorter intervals around turning points; sample volumes were less than 1% of the initial volume.

2.10.3 b Agitation; The motor for driving the double turbine impeller shaft was situated in the base housing of the fermenter, external to the sterilizable vessel with a complex mechanical seal to maintain sterility.

2.10.3c Temperature A platinum resistance thermometer submerged in medium was used to monitor temperature. This probe was accurate to within 1^0C (checked experimentally) at 45^0C. The temperature control circuit in general maintained temperature constant to within 0.3^0C, providing steam and cooling water were available at the required pressures.

2.10.3d pH control An Ingold steam-sterilizable pH probe with a housing which could be pressurised, was fitted to the fermenter. The probe was calibrated immediately prior to sterilization, against buffers at pH7.0 and 9.2, and following sterilisation, by sampling the fermenter and comparing the medium pH given by the Bioengineering monitor, with the medium pH given by an off-line pH meter (calibrated with buffers at pH7 and pH9.2). During sterilisation, the probe was pressurised at 1bar above the fermenter pressure. This prevented movement of medium components across the permeable glass membrane into the internal electrolyte, and prevented fracture of the glass membrane.

Calibration was carried out as follows: the pH probe was submerged in buffer at pH7.0, and the pH set to 7.0. The probe was washed, wiped dry and submerged in buffer at pH9.2 The slope of the potentiometric control module was then adjusted until the value given was that of pH9.2. The pH reading in buffer of pH7 was then re-checked. When not in use, probes were stored upright in 3M KCl solution. Control of pH was effected using an internal feedback circuit (proportional-integral-differential response), with sensitivity controls set to to less than 0.3 pH unit, and pump T_{on} and T_{off} settings to a suitable value (dependant on the fermentation). A single W atson Marlow constant speed peristaltic pump was linked to the control unit and used to pump alkali (1M NaOH) into the fermenter.

2.10.3e Dissolved oxygen concentration; An Ingold steam-sterilizable polarographic electrode was used. It required a polarising voltage to be applied at all times during the life of the electrode membrane. The electrode was stored in water beneath the fermenter when not in use with a power supply at all times.

Calibration of dissolved oxygen concentration was carried out following sterilization. The probe was zeroed electrically, by turning the scale switch to the zero position. This electrical zero had been checked off-line by passing oxygen-free nitrogen over the tip of the probe for a few minutes; the zero reading was found to be true. The maximum point was set after the fermenter was equilibrated for at least 10 minutes (at the required temperature and air flow rate). The scale setting was then adjusted to a suitable setting for calibration to 100% and minor adjustments made to bring the displayed concentration to this value.

2.10.4 Specification and Methods used with the LH Fermenters

2.10.4a Agitation; This was achieved by means of an external motor, driving the turbine within the fermenter electromagnetically. In the newer of the LH fermenters, specific fermenter bearings situated under the turbine housing, must be used, greased with heat-stable high-viscosity lubricant. Lack of attention to these bearings can cause either seizure of the agitation system, or rusting, which disturbs the fermentation and interferes with microbial growth. The older version of the same fermenter does not require these bearings, and as a result is simpler to use.

2.10.4b Temperature control; Heating was carried out by a conventional rod heating element inserted through the top plate, as described previously. Temperature control was carried out using an immersed thermometer and a feedback control circuit. Cooling was not required due to the elevated cultivation temperature of 45^0C.

2.10.4c Air supply This has been discussed in the section on services. However a fermenter modification was necessary to use the fermenter as a chemostat for operation at 45^0C, owing to the lack of a condenser on the exit air line. Fragile glass condensers were unsuitable; instead a condenser from a 9 litre Chemap vessel was used. This was attached to the 2.5 litre LH fermenter using silicone tubing, improvising an extended U tube to minimise the chance of fermenter contamination. Originally the effluent from this U tube was passed through a drechsel bottle containing the antimicrobial detergent, Hibitane. However excessive foaming occurred and it was found that it was more suitable to maintain the exit free of restrictions. The U tube was terminated by a short stretch of silicone tubing which could be clipped off to restrict the exit air flow in order to increase the pressure inside the vessel for sampling.

2.10.4d pH Monitoring and Control Ingold pH probes were used for pH monitoring and control, and LH peristaltic pumps for addition of alkali. Calibration of the pH probe was carried out in accordance with the method described for the Bioengineering fermenter 2.10.3. The use of 1M NaOH for pH

control was adequate. If acid was required for pH adjustment, this could be added manually through the inoculation port.

2.10.4e Inoculation Inoculation and addition of phosphate and glucose following sterilisation were achieved through an inoculation port in the top plate. A blind silicone plug 4.0cm diameter x 2cm depth, was drilled with 12 mm diameter hole into which a silicone suba-seal of the same diameter was inserted. The suba seal was replaced for each experiment. Inoculation was achieved using a sterile hypodermic syringe through the suba seal using absolute ethanol to ensure maintenance of sterility within the fermenter.

2.10.4f Sampling This was carried out using a sampling hood. A clip isolating the sampling hood from the fermenter was released and air passed down the tube through the air filter in the hood to clear the sampling tube of any resident mycelium Air flow from the fermenter was then restricted causing a pressure build up in the fermenter and consequent emission of vessel contents into the sampling vial via the connecting tube. Release of the restriction in the exit air line terminated the flow of broth into the sampling vial. The hood-fermenter line was then cleared as previously described and clipped off. The filled vial was removed by unscrewing from the hood and replaced with a fresh vial using a combination of ethanol and flame asepsis.

2.10.5 Modifications and Techniques used in the Establishment of Continuous Culture for *S. thermonitrificans*

Cultivation of the a batch fermentation for 24 hours was allowed prior to the initiation of continuous culture. The arrangement of equipment is represented diagrammatically in Figure 2.3.

A W atson Marlow variable-speed peristaltic pump (501 series) was used for medium influx (with fresh tubing of either 1.6 mm or 3.2 mm internal diameter and 1.6mm wall diameter in each case). The pump was pre-calibrated with tubing in these sizes in order to estimate flow rates during operation. Effluent pumps were set to a value greater than two times the inlet rate and constant volume maintained by a weir device. Accurate flow was determined by measurement of the rate of effluent flow from the fermenter. Providing that the tubing passing through the inlet pump was not deformed the flow was stable. However minor adjustments due to the decrease in hydrostatic pressure in the medium vessel were required on average every 48-72 hours.

Small sample volumes (10-15ml) were taken to minimise the disruption from steady state, and at least 5 generations were allowed to elapse at each dilution

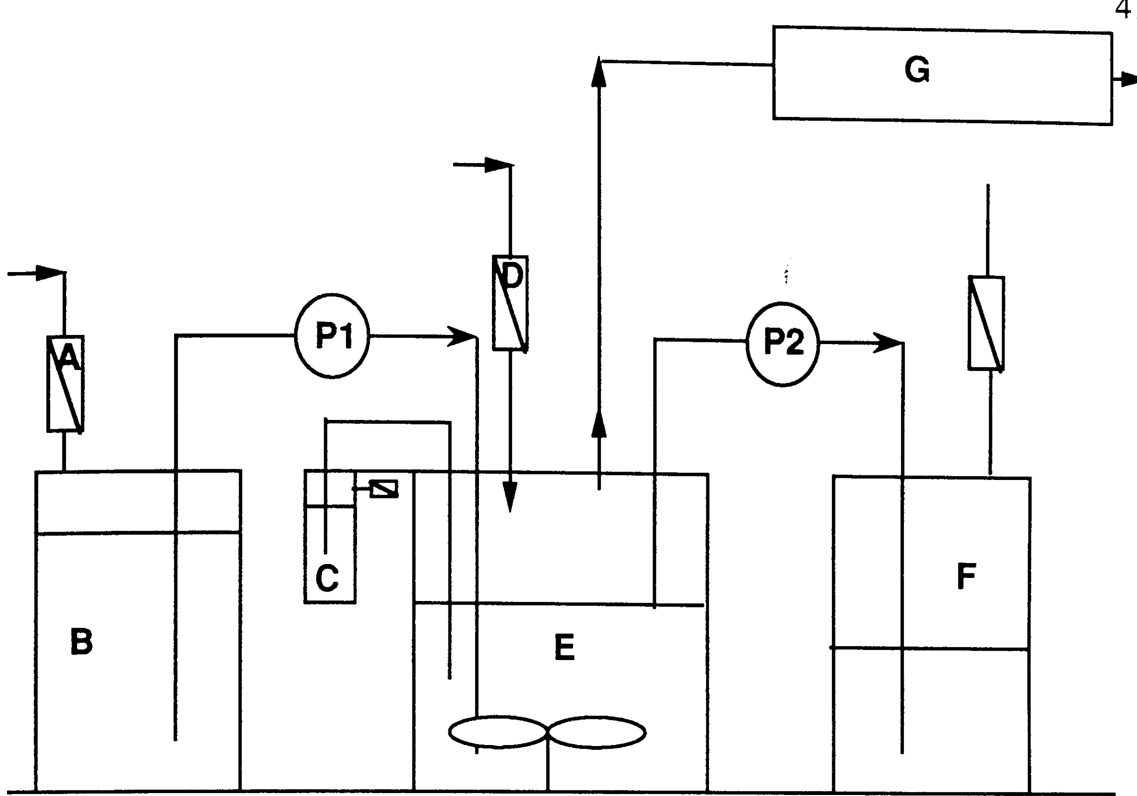
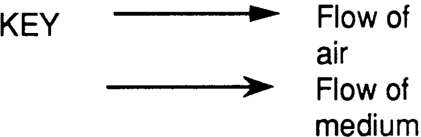


Figure 2.3 Diagrammatic Representation of the Arrangement for the Chemostat

KEY



Flow of air

Flow of medium

A Inlet air filter to reservoir medium pot

B Reservoir medium pot

C Sampling device

D Inlet air filter to chemostat

E Constant volume chemostat (1.60 litres)

F Effluent reservoir

G Infra-red carbon dioxide analyser

P1 Inlet pump - rate of flow throgh this pump determines the flow rate of medium to the fermenter (F/1.60 = D)

P2 Effluent pump - rate is set to a value twice that of P1

A correction value for the dissolved carbon dioxide concentration was not incorporated into the expression. However, given the rapid gas flow rates, and the negligible inorganic carbon concentration determined by analysis, then the errors introduced by this omission would be expected to be very small and within experimental errors.

rate. Assuming perfect mixing and a homogeneous culture, in one dilution approximately 50 % of mycelia will be that from the previous steady state. Following a further dilution 25% of the population will be mycelia from the previous steady state. However, following 5 dilutions, then less than 4% of the cell population will be remaining from the preceding steady state (this error was below the sensitivity of biomass assays). The on-line performance of the chemostat was monitored using an infra-red carbon dioxide analyser. The analyser was used as an on-line indicator of the condition of the chemostat. Within two dilutions following a change in dilution rate, the output of carbon dioxide was observed to be stable, and was indicative of potential steady-state conditions. A steady value for carbon dioxide output is a sensitive confirmation of the achievement of steady state in a chemostat culture (Sinclair, 1987). Following the harvest of mycelium at steady state (after 5 dilutions), 20ml of a glucose solution (200g.l $^{-1}$) was added to the fermenter . The increase in CO_2 output observed indicated that glucose was a limiting nutrient. In addition, the dilution rate was altered, and if necessary, the medium pots were changed over at this stage.

2.10.6 On-line Analysis of Carbon Dioxide

2.10.6 a Infra-red detection and analysis Organic and inorganic molecules generally absorb energy in the infra-red region of the electromagnetic spectrum due to vibrational stretching and bending of bonds. These absorptions have characteristic wavelengths and can be used to identify a specific molecule. In the ADC carbon dioxide analyser (Type 877) a split beam detection system for carbon dioxide was used, relying on a continuous passage of air through the machine, over a column of soda lime to remove CO_2, for base-line comparison. A constant gas flow through the test cell was maintained for all analyses. The output (%CO2) may be converted into units of millimoles of carbon dioxide using the following equation derived by Hamilton (1976) based on the Ideal Gas Laws;

$$CO_2 \text{ production} = \frac{b - F.298.\ 10^{-2}.\ 79.04}{(100-b).273.\ 22.4}$$

where b = corrected reading on CO_2 analyser in %

F = air flow rate through fermenter in litres. min^{-1}

$$= \text{mmol CO2. culture volume}^{-1}\text{.minute}^{-1} \quad \text{(at r.t.p)}$$

pH was maintained at pH 7.0 throughout the fermentations . Cumulative CO_2 values were obtained by plotting the corrected on-line data graphically, enlarging the graph to 4 times its original size and determining the area beneath the curve

Calibration of the mass flow controller (at the premises of Brooks Instruments) was carried out at 23.5 ^{0}C and 1018 bar atmospheric pressure. A correction factor should therefore have been applied to correct flow to r.t.p. However, the difference between recorded and corrected flow would have been minor, and within the limits of experimental error.

and segments of the curve, by mass and by counting squares. The area and mass of a square of originally 5 cm by 5 cm was used to calibrate the values obtained, by determining the amount of CO_2 that this area represented according to the above equation. The area and mass values for the experimental curve for CO_2 production were then converted into units of CO_2 in millimoles. The mean values for determination by mass and by area were used in subsequent calculations.

2.10.6 b Precautions Required for Accurate Cumulative Determination of CO_2.
To quantitate accurately the amount of gaseous carbon released during a fermentation, a number of features of gas analysis must be addressed. Firstly gas flow must be determined accurately and secondly, the sensitivity of the detector must be suitable for the fermentation under analysis. A Brooks Mass Flow controller was used (Models 5850 and 5878) to monitor and control air flow into the fermenter, with a maximum flow rate of 15 litres min^{-1}. The controller was calibrated prior to delivery, and checked prior to use for the mass balanced fermentations described in Chapter 5 at the premises of Brookes Instruments, Stockport. The controller was accurate to within 1% at the flow rates used experimentally.

According to the thermodynamic properties of gases, there is a relationship between mass flow and the heat capacity of a gas. The mass flow controller essentially consists of two temperature sensors, one situated downstream of the other, and a heating element. The gas passing through the controller is heated, and the temperature difference between the 2 sensors (downstream of the heating element) is proportional to the actual mass flow rate of the gas. The controller also generates a proportional -integral-differential response (PID), controlling a valve downstream of the sensor to alter mass flow to the required level. Using this controller, gas flow to the fermenter was maintained at one volume of air per volume of medium per minute.

A second problem for analysis of CO_2 is the sensitivity of the machine. The ADC analyser was equipped to monitor at a maximum limit of 1% or 5% CO_2 in the effluent stream. Given the normal values for CO_2 output during a fermentation of *S. thermonitrificans* (up to 0.4% on the 1% channel), the 1% channel was adequate for use. A suitable calibration gas was required and a choice was made of a mixture of 0.8% CO_2 in oxygen-free nitrogen. This gas was mixed and supplied to order by the British Oxygen Company, with a certified concentration given to 2 decimal places and accurate to within 1%. Oxygen-free nitrogen was used to zero the analyser. The CO_2 concentration of air passing through the fermenter prior to inoculation was checked for each fermentation. The basal level of 0.04% CO_2 remained constant for all

analyses, and this value was used to determine the corrected CO_2 values, ie the actual % CO_2 value produced by growth of *S. thermonitrificans*, by subtraction from values obtained experimentally. During a fermentation the zero and span calibrations for the analyser were checked every 24 hours.

The composition of the effluent gas was maintained constant by the use of gas-impermeable tubing. The analyser was sited adjacent to the fermenter. This reduced length of connecting tubing between fermenter and analyser and minimised dead space and potential sites for loss of CO_2. The accurate response of the analyser to CO_2 concentrations at a range of CO_2 concentrations could not be checked, owing to the lack of equipment for mixing gases to a flow rate of 1litre/minute. However the manufacturers reported the response to be linear (ADC manual).

2.11 Growth Media for *In vitro* and *In vivo* Gene-transfer Manipulations in *Streptomyces;*

R2 Medium (for the cultivation and regeneration of protoplasts of *S. lividans*):
R2A- 44g agar, 0.5g K_2SO_4, 20.2g $MgCl_2.6H_20$, 5.9g $CaCl_2.2H_2O$, 20g glucose, 6g proline, 0.2g casamino acids, 4ml trace elements solution (Hopwood et al., 1985), made up to 1 litre in distilled water.
R2B- 11.5g MOPS, 10g yeast extract, 203g sucrose, adjusted to pH 7.4 with NaOH, made up to 1 litre in distilled water.
Equal volumes of R2A (melted and cooled to 55^oC) and R2B plus 1ml of 1% (w/v) KH_2PO_4 were combined prior to use.
Antibiotic Medium 2 (Oxoid); used for the bioassay of oxytetracycline. This medium is low in transition metal ions which can chelate with oxytetracycline and remove biological activity. The medium is supplied as a powder and reconstituted according to the manufacturers instructions.
R579: Made up in two parts, A and B, for the regeneration of protoplasts of *S. thermonitrificans.* R579/A;0.5g K_2SO_4, 6.0g proline, 0.2g casamino acids (Difco), 4.0ml trace elements (described in Hopwood *et al.*, 1985), 20.0g glucose, 5.0g NaCl, 40.0g Difco-bacto agar made up to a volume of 900ml and dispensed into 90ml aliquots.
R579/B; 11.5g TES buffer (Sigma) pH7.2 with NaOH, 45.5g Mannitol, 2.0g yeast extract (Difco), 8.0g beef extract (Difco), 8.0g peptone (Difco), made up to a total volume of 900 ml and dispensed in 90ml aliquots for sterilization. Part A was melted and part B heated to 55^oC, the two components were mixed and 1ml of 1% KH_2PO_4, 3.75ml of 0.8M $MgCl_2$, 6.25ml of 3.2M $CaCl_2$ and 10ml of water were added. The agar was poured and dried according to the technique described in the section on preparation of protoplasts (Section 2.14).

2.11.1 Solutions for Plasmid Transformation of *S. thermonitrificans*

Transformation mix (T-mix): 12.5g sucrose, 7.35g $CaCl_2.2H_2O$, 0.218g K_2SO_4, 1 ml trace elements, 2.9g maleic acid, adjust to pH 8.0 with 1M Tris, 500ml distilled water. Add 25% (w/v) PEG 1000 before use.
Medium P: 5.73g TES, 103g sucrose, 2.93g $MgCl_2.7H_2O$, 0.5g K_2SO_4, 3.68g $CaCl_2.2H_2O$, 2ml trace element solution. Adjust to pH 7.4 with NaOH and make up to 1 litre in distilled water.
PEG Solution: 20% (w/v) PEG 8000. 2.5M NaCl.

2.11.2 Solutions for the Manipulation and Visualisation of Plasmid DNA

10X TBE Buffer pH 8.3: 109g Tris, 55g boric acid, 9.3g $Na_2EDTA.2H_2O$ made up to 1 litre in distilled water, pH to 8.3.
Agarose gel loading buffer: 0.025% (w/v) bromophenol blue, 0.025% (w/v) xylene cyanol, 25% (w/v) ficoll, 0.5% (w/v) SDS, 50mM EDTA.
10x React 3 buffer: 50mM Tris-HCL pH8.0, 10mM $MgCl_2$, 100mM NaCl.
TE Buffer: 10mM Tris-HCl, 1mM EDTA, pH 8.0.
Lysozyme solution; 50 mg/ml lysozyme in 0.01M Tris pH8.
Sucrose (34% w/v) in TE (10mM Tris-HCl, 1mM EDTA, pH 8.0)
Ethidium bromide 15 $mg.ml^{-1}$
Saturated caesium chloride in TE buffer.

2.11.3 Antibiotics for Plasmid Selection: The antibiotic concentrations used throughout for both liquid and plate selection were as follows:

Name	Selective Concentration	Storage Concentration	Storage Temperature
Thiostrepton	25 $\mu g.ml^{-1}$	10$mg.ml^{-1}$ (DMSO)	4^oC
Spectinomycin	100 $\mu g.ml^{-1}$	40$mg.ml^{-1}$	4^oC
Streptomycin	25 $\mu g.ml^{-1}$	20$mg.ml^{-1}$	-20^oC
Tetracycline	25 $\mu g.ml^{-1}$	4$mg.ml^{-1}$ (in 2M HCl)	-20^oC
Hygromycin	200 $\mu g.ml^{-1}$	50$mg.ml^{-1}$	4^oC

Stock solutions were added to molten agar, pre-cooled to 55^oC.

2.11.4 Preparation of Plasmid DNA; the neutral lysis materials and method described by Hopwood *et al,* (1985) were used, with modifications : 200ml cultures of 24 hour-old mycelium were harvested by centrifugation (12000g, 5 minutes at 4^oC). The pellet was resuspended in 50ml TE with 34% (w/v) sucrose and 10ml 0.25M EDTA pH8.0. 5ml of lysozyme solution was added and the mix incubated at 37^oC for 10-40 minutes until lysis occurred. The advantage of the incorporation of sucrose into the medium is that prolonged

incubation times may be used with large amounts of mycelium, without DNA degradation at this step due to the hypertonicity of the lysozyme solution (34% sucrose). The solution was cooled by the addition of ice-cold TE and sucrose (50ml), mixed and incubated on ice. All successive steps were carried out with pre-cooled apparatus in a cold room.

10% (w/v) SDS solution at $60^{0}C$ (21.6 ml) was added to the solution and stirred gently with a glass rod. At this stage the cells were lysed and the mix became extremely viscous. 43.2 ml of ice-cold 5M NaCl was then added to the solution which was stirred gently. The salt precipitated proteins and some macromolecular components and the viscosity decreased; following overnight incubation at $4^{0}C$, the precipitate was pelletted by centrifugation in 6 oak-ridge tubes at 31,000g in a Beckman JA20 rotor for 30 minutes. A half volume of cold 30% (w/v) PEG 8000 was added to the supernate to give a final concentration of 10% PEG. The supernate was allowed to stand for 4 hours. The resulting precipitate was collected by centrifugation at 2455g in a Beckman JA14 rotor for 1 hour and dissolved in 10ml of a saturated caesium chloride solution prepared in TE. Ethidium bromide was added to a final concentration of 600 $\mu g.ml^{-1}$, and the density of the solution carefully adjusted to 1.58g/ml. The solution was centrifuged in a Beckman VTi65 vertical rotor at 289,000g for 5 hours at $20^{0}C$. Two bands were visible with UV illumination. The lower band represented supercoiled ccc plasmid DNA and the upper, chromosomal and relaxed plasmid DNA. The lower band was recovered using a 1ml syringe and the ethidium bromide removed by repeated butanol extractions (using water-saturated butanol). After dialysis with at least 3 changes in a 100 fold excess of TE, the plasmid DNA was precipitated by the addition of 1/10th volume of 5M NaCl and 2 times volume of absolute ethanol. The precipitated plasmid DNA was pelleted by centrifugation (27000g, $4^{0}C$, 15 minutes) and washed twice with 70% (v/v) ethanol. The pellet was then dried briefly in a vacuum dessicator before it was redissolved in 100 µl TE. This preparation yielded large amounts of pure plasmid DNA suitable for all *in vitro* manipulations, and gave good yield of pure plasmid DNA from large low-copy plasmids, provided all manipulations are carried out gently and with attention to features which give may shear DNA, such as mixing, trituration, (note the absence of the normal step of dissolving crystals of caesium chloride in the DNA/TE solution), and restricting shear in hypodermic needles to a minimum.

2.11.5 Digestion of Plasmid DNA with Restriction Enzymes: Restriction enzyme digests using the enzyme *EcoR1* were performed as directed by the supplier. They were used to quantitate DNA with respect to a known concentration of standard DNA and to check the accurate restriction pattern for the plasmid pFZ163 with this enzyme.

2.11.6 Gel Electrophoresis. DNA was visualized on horizontal neutral agarose gels using 0.8% (w/v) gels. BRL model H6 gel kits were used for rapid analysis of DNA digested with restriction enzymes or following plasmid DNA purification. 0.16g agarose was added to 20ml of TBE, boiled then cooled to 60°C. Ethidium bromide was added to a final concentration of 200ng.ml^{-1} and the molten agarose poured into a 7.6cm X 5.1cm gel caster with an 8 well slot former (4.1 X 0.8mm wells). After the gel had set, the slot former was removed and the gel placed in the tank with 500ml of TBE. The gel was electrophoresed for 30-45 mins with an applied voltage between 2-10V cm^{-1} depending on the time available and the level of resolution required.

2.11.7 Photography of Agarose Gels; Gels stained with ethidium bromide were viewed on a 302nm UV transilluminator and photographed using a Polaroid Land camera fitted with a Kodak Wratten filter (No. 23A) and Polaroid type 67 land film.

2.12 Streptomyces *in vitro* Gene Transfer Techniques

2.12.1 Growth of *Streptomyces* mycelium for the preparation of DNA Plasmid-containing strains of *S. albus* were grown in TSB at 34^{0}C and 250rpm with thiostrepton for maintenance of plasmid.

2.12.2 Growth of *Streptomyces* mycelium for the preparation of protoplasts For the formation of protoplasts, spores of *S. thermonitrificans* at a concentration of 10^{6}. ml^{-1} were inoculated into TSB and grown at 37^{0}C at 200rpm shaker speed. A volume of 25 ml in a 250ml erlenmeyer flasks was used. The culture was harvested after 22-24 hours of growth.

2.12.3 Transformation with plasmid DNA; Plasmids were introduced into *Streptomyces* by genetic transformation using the PEG-mediated method described by Hunter (1985) and modifications described by Czaplewski (1989).

2.12.4 Preparation of Protoplasts; 25ml cultures of *S. thermonitrificans* were grown at 37°C. The mycelium was pelleted at 12000g for 10 minutes and washed twice in 10.3% (w/v) sucrose, then resuspended in 5ml of medium P (containing 0.25 mg ml^{-1} lysozyme) and incubated at 37°C for 40 minutes. Formation of protoplasts was monitored microscopically and the reaction terminated by addition of 5ml of P medium, trituration and quenching on ice for a few minutes. Protoplasts were then filtered through cotton wool, pelleted at 12000g for 10 minutes and washed twice in P medium. Finally they were resuspended in 2ml of medium P, dispensed into 100µl aliquots and used immediately.

2.12.5 Transformation of protoplasts. DNA was added in a volume of less than 10µl and the mixture was incubated on ice for 10 seconds, then 0.5ml of PEG solution was added and the solution returned to incubate on ice for 1 minute. 0.5ml of medium P was then added and subsequent dilutions were made in medium P.

2.12.6 Regeneration of transformed protoplasts. The method of preparation of regeneration medium for protoplasts was standardised. The media were stored in two parts (A and B); the former solid and the latter liquid. Both were steamed until portion A melted. They were incubated at 50°C for about 30 minutes and the B portion was poured into A and mixed by swirling. 1ml of a 1% (w/v) KH_2PO_4 was then added. The mixture was swirled and poured into 9 cm diameter petri dishes. 200 ml of medium was sufficient for 8 plates. The plates were left half open to the air in a laminar flow hood for 45 minutes when they were turned 180° and their position in the hood altered so that those at the front were positioned toward the back of hood. After a further 45 minutes the plates ready for use.

2.12.7 Plasmid selection in *Streptomyces* was carried out with thiostrepton (provided by E. R. Squibb, New Jersey, U.S.A.) dissolved in DMSO to make a 1% (w/v) stock solution. Transformed protoplasts of *S. thermonitrificans* were selected after 16-22 hours non-selective growth at 30°C by overlaying the protoplast regeneration plates with 4ml of a 55 µg ml^{-1} thiostrepton solution in a 10.3% (w/v) sucrose solution.

Protoplasts of *S. lividans* were regenerated on R2 agar plates using 1ml sucrose solution containing thiostrepton at 220µg.ml^{-1}. Protoplasts of *S lividans* were frozen stocks provided by K.J.Linton, prepared according to the method of Hunter (1985).

2.13 *Streptomyces in vivo* Gene Transfer Manipulations (Conjugation)

Spores of the required strains were mixed together in eppendorf tubes on a vortex mixer. 1ml aliquots were then applied to dry solid agar plates and spread using a flamed glass spreader. Following growth, the spores were harvested by loosening spores from the surface of agar with a wire loop and the addition of 1ml water. When a dense spore suspension had been obtained it was removed using a micropipette and added to an empty eppendorf tube. Serial dilutions were then made for subsequent analysis.

2.13.1 Replica Plating This is described in the manual by Hopwood *et al.*, 1985.

2.14 Bioassay for Oxytetracycline: 1cm diameter plugs were taken from 40ml plates of Emerson agar and either inoculated with spores of a test colony of *Streptomyces* or between 1-10µl of 1mg.ml^{-1} oxytetracycline (1-10µg per plug). The plugs were incubated in a water saturated environment at either 37^{0}C for *S. thermonitrificans* , or 30^{0}C for *S. lividans*, until growth and sporulation of the test colony had occurred. Control and tetracycline plugs were incubated overnight at 30^{0}C to allow diffusion of the antibiotic throughout the plug. A culture of *Bacillus subtilis* EME105 was grown to stationary phase, diluted 1in 100 in L broth and 100µl of the diluted culture added to 100ml of molten antibiotic medium 2 at 55^{0}C. The agar was poured and allowed to set. The plugs of test colonies and calibration plugs were placed on the surface of the agar, round the perimeter, 8 plugs per petri dish. The test plates were then incubated overnight at 37^{0}C. Zones of clearing of *B. subtilis* corresponded to presence of a growth inhibitor (predicted to be an antibiotic) - the diameter of the zone of growth inhibition is logarithmically related to the concentration of antibiotic.

2.15 Bioassay for Holomycin; *Flavobacterium spp.* is an extremely sensitive organism for the assay of holomycin. However it is a Class II pathogen, and was therefore used with containment standards suitable for this class of organisms.

Liquid cultures were grown for 24 hours without shaking at 30^{0}C, and added to molten L-agar (55^{0}C) at a concentr ation of either 1 µl, 0.1 µl or 0.01µl of *Flavobacterium* culture per ml agar. This seeded agar was then used to overlay TLC plates, the product of separation experiments, in which one of the tracks contained a holomycin standard. The agar plate was allowed to solidify and incubated overnight at 30^{0}C. Comparison of test and holomycin tracks was made with the test tracks following incubation. A zone of growth inhibition would indicate the presence of a factor preventing growth of *Flavobacterium spp.*, predicted to be due to antibiotic activity.

2.16 Discontinuous Fed Batch Cultivation of *S. thermonitrificans* pBROC139; TSB containing thiostrepton was inoculated with spores (minimum 10^6 spores.ml^{-1}) of *S. thermonitrificans* pBROC139 in a volume of 50ml. The medium was added to 500ml erlenmeyer flasks (ie with a resulting medium/flask volume ratio half the normal) and allowed to grow for 20 hours at a temperature of 45^{0}C. After this time 80ml of 579MM (15g.l^{-1} glucose, 3mM phosphate, containing thiostrepton) was added to the flask, and the flask returned to incubate. Every 24 hours for a total of 4 days a further 80ml of 579MM was added to the flask. Following cultivation for 5 days in total, the flasks were harvested, mycelium pelletted by centrifugation and supernates

collected for use in the purification of the yellow product.

2.17 Solvent extraction This was carried out using 1 litre or 500ml separating funnels, filled with 1/3 volume of test mixture and 1/3 volume of solvent. The pH was corrected as necessary, and extractions were repeated 3 times. The lower layer was drained from the base of the funnel, and the upper layer collected for evaporation

2.18 Rotary evaporation this was carried out using a Buchi rotary evaporator and vacuum generated using either a water pump, or a vacuum pump, without heating.

2.19 Small scale evaporation This was accomplished using a Speedivac centrifugal vacuum apparatus, in accordance with the manufacturer's instructions.

2.20 Thin layer chromatography Mini - TLC plates were prepared by subdividing prepared polythene-backed silica TLC plates (18cm x 18cm) into 6 smaller plates. A pencil line was drawn lcm from the base of the plate, onto which spots of the test mixture were placed, allowing the damp spots to dry in a stream of air prior to subsequent application of sample. Plates were developed in a small jar where the atmosphere was pre-equilibrated with solvent. Plates were lowered into the jar and developed for 20-40 minutes. Larger scale preparative TLC was carried out using glass-backed TLC plates (prepared with Kieselgur, BDH), running the plates in a tank for 1-2 hours.

2.21 Visualisation of TLC Coloured spots were marked. Plates were viewed with long wavelength and medium wavelength UV, or after spraying with iodine solution, to locate colourless spots.

2.22 Chromatography and Analysis

2.22.aColumn Chromatography This is described in section 7.5.2. Amberlite XAD-4 resin (Sigma), a hydrophobic adsorption resin was used for isolation of the yellow pigment. It is conventionally used for adsorption of organic molecules from polar solvents.

2.22.b HPLC A Phillips PU4100 Programmable Liquid Chromatograph was used together with a Multichannel Detector (Model PU4021) with a Phillips P3202 C.P.U. working in the Microsoft Windows environment. They were used in accordance with the manufacturer's manual. The columns and matrices used are described in Section 7.6.7.

2.22 c FPLC A Pharmacia system was used in accordance with the manufacturers instructions. The matrices used experimentally, are described in Section 7.6.7.

2.22.d Nuclear Magnetic Resonance Analysis This was carried out by Dr Ryecroft, Department of Chemistry, University of Glasgow, and Dr Julia Foster, University of Leeds.

2.22.e Mass Spectrometric Analysis; this was carried out by Dr Tony Ritchie, Department of Chemistry, University of Glasgow.

CHAPTER 3

Fermentation Development of *S. thermonitrificans*

3.1 Introduction

An initial screen of fourteen thermotolerant *Streptomyces* was made by Dr Linda Harvey, and scored against the range of factors listed below :-

1. Maximum biomass production at elevated temperatures (between 40 to 60°C)
2. Absence of extracellular polysaccharide production; extracellular polysaccharide would produce viscous culture broths and act as a drain on the carbon supply.
3. Absence of detectable, host-encoded antimicrobial product; to simplify the purification of a potential heterologous antimicrobial product.
4. Absence of mycelial growth in the form of pellets in the complex media, Trypticase soy broth (TSB), Luria broth (L broth) and ATTC broth. Mycelial growth in the form of pellets is frequently detrimental to product formation, and complicates physiology studies by forming heterogeneous cell populations..

On the basis of these results *S. thermonitrificans* was chosen for the project. A defined medium, LMM, in fully and semi-defined versions was devised (L. Harvey, personal communication described in Materials and Methods).

3.1.1 Development of a Fermentation For The Expression of a Recombinant Product from *S. thermonitrificans* in a Mass-Balanced System

It is common for the products of recombinant *Streptomyces* to be expressed on a small scale in shake flask culture. However, the aim of this project was to take a different approach. The strategy was to investigate quantitatively the growth and expression of a product by a recombinant streptomycete in a stirred tank reactor, in a system that could be analysed simply and comprehensively.

A defined medium for growth of *S. thermonitrificans* was required, to gain precise control over the nutrient environment. Growth conditions were needed which could be manipulated and measured accurately, combined with the development and implementation of relevant assay techniques. As the project progressed, it was necessary to assess the results with respect to the project requirements and proceed accordingly, re-optimising growth conditions when required.

3.1.2 Growth Kinetics of Filamentous Organisms

Exponential growth of mycelial organisms in submerged culture is the result of apical branching and fragmentation: each individual hypha grows only at a linear rate (Trinci, 1969). In studies of mycelial morphology in continuous culture, dilution rate was found to influence morphology in *S. hygroscopicus* (Trinci, 1990; Kretschmer, 1981, detailed in Chapter 1). For mycelial organisms, dense filamentous growth lowers the apparent mass transfer coefficient by increasing the viscosity of broths. However, growth in the form of pellets may occur for cultivation conditions stimulating a high frequency of branching, (Bushell, 1988). The pelleted form of mycelial growth contrasts with the filamentous growth form, producing low viscosity broths which are composed of mycelia in clumps. The pellet centre is composed of early-grown hyphae whilst later growth is primarily situated on the outer edge of the pellet as the hyphae grow and branch out. Although growth in the form of pellets results in broths of low viscosity, large mass transfer gradients exist across the pellet.

It has been shown experimentally that various fungi may grow according to the exponential law:

$$x_t = x_o e^{\mu_m t}$$ (Pirt and Callow, 1960)

where x_t = biomass at time t ($g.l^{-1}$)
x_o = biomass at time 0 ($g.l^{-1}$)
μ_m = maximum specific growth rate (h^{-1})

Exponential growth in mycelial organisms is compatible with apical extension providing branching of hyphae occurs. However a cube root relationship (given below) has been found experimentally to apply to submerged growth for some fungi and actinomycetes at specific stages in their growth (Marshall and Alexander, 1960)

$$x_t = x_o^{1/3} + kt$$ (Pirt, 1966) where k = constant

The growth kinetics of pellets may not adhere rigidly to the cube root theorem (Pirt, 1966). For intra-pellet hyphae that are interwoven loosely, turbulence may allow penetration of medium, resulting in partially exponential or exponential growth. Hence there is an apparent anomaly - growth rate influences morphology, and conversely morphology influences growth rate. Under normal conditions morphology can be optimised for the requirements of the process, by adjusting medium composition or growth conditions (Bailey and Ollis, 1986; Braun and Lifshitz, 1991).

3.1.3 Retrospective Experimental Strategy

Preliminary growth studies on complex and semi-defined media were carried out. Biomass concentrations were low, reproducibility was poor, and growth was consistently in the form of dense mycelial pellets. A growth limitation was suspected to be in operation, but could not be alleviated during media trials. A fully-defined medium was therefore devised, based on a previously reported general-purpose medium. This medium (579MM) was subsequently optimised by adjusting the glucose concentration to a suitable level for both efficient carbon conversion, and accurate determination of glucose and biomass concentration. Inoculum development procedures were then devised, suitable for adaptation of *S. thermonitrificans* for growth on the defined medium 579MM. These procedures were adapted for use in large-scale experiments and for fermenter-scale cultivation. A modified aeration method, enabled filamentous growth of *S. thermonitrificans* on the minimal medium 579MM in a 9 litre Bioengineering fermenter (working volume 7 litres).

3.2 Preliminary Growth Studies

3.2.1 Growth curves for *S. thermonitrificans* on Complex Medium, in Flask Experiments

Growth curves of the organism on TSB (a complex medium rich in organic nitrogen sources and phosphate) at 37^{0}C and 45^{0}C are shown in Figure 3.1 and Figure 3.2.

Inoculum was prepared by germinating spores and growing in TSB overnight at the required growth temperature, using a concentration of 10^7 spores ml^{-1} medium. The mycelium was washed twice and resuspended in the original culture volume. This was used to inoculate flasks to a final mycelium concentration of 2% (v/v). Flasks were grown in duplicate, pooled and samples taken in triplicate. The minimum requirements for all growth studies are listed below

1. Experimental variables were set up in triplicate
2. Samples were taken in triplicate wherever possible
3. All experiments were repeated at least once
4. Wherever possible standard deviations are given.

The curves for growth of *S. thermonitrificans* in TSB at 37^{0}C and 45^{0}C gave final biomass concentrations of 5 and 4.5 g.l^{-1} at 37^0 C and 45^{0}C (Figures 3.1 and 3.2). Errors in the last data point for each curve were ascribed to mycelial

lysis. During the initial stages of growth the mycelium formed miniscule pellets which later dispersed around 8 hours into the cultivation. From 8 hours to termination, growth was in the form of dispersed mycelial mats. This change from pellet to dispersed mycelial morphology during the initial stages of growth is reported to occur for many antibiotic fermentations (Bader, 1986; Luedeking, 1967).

The growth curves shown in Figures 3.1 and 3.2 illustrate the response in growth rates and final biomass concentrations by *S. thermonitrificans* to different growth temperatures. Maximum biomass levels at 45°C were less than those at 37°C (5 g.l^{-1} in comparison with 4.5 g.l^{-1}) whereas the maximum (approximate) growth rate was higher at elevated temperature, estimated to be 0.30 g biomass.h^{-1} at 45^{0}C in contrast to 0.23g.h^{-1} at 37^{0}C (estimated by taking tangents to the curve Figure 3.1 and 3.2). Because of the undefined kinetics of these growth curves, the growth rates are culture growth rates, and are not specific growth rates (which rely on exponential growth).

For 3.1 Slope = $\frac{4.2 - 1.4}{20 - 8.0}$ = 0.23 g biomass.h^{-1} (37^{0}C)

For 3.2 Slope = $\frac{4.0 - 1.0}{14 - 4.0}$ = 0.30 g biomass.h^{-1} (45^{0}C)

The differences in final biomass concentrations were attributed to a higher maintenance requirement at the higher temperature, which is a general trend for microorganisms (Wallace and Holmes, 1986). The concept of the maintenance requirement and its determination are discussed in detail in Chapter 4.

The growth kinetics were difficult to determine. It is normally acknowledged that filamentous organisms are capable of exponential growth in liquid culture due to mycelial fragmentation providing a potential increase in the number of growing points. The filamentous morphology shown by broths of *S. thermonitrificans* in these experiments would suggest that these type of growth kinetics would predominate. The growth curves of *S. thermonitrificans* on TSB roughly approximated to a sigmoidal shape on a non-exponential plot, with a rapid death phase.Therfore, possibly the growth curves could be explained more meaningfully as intermediate relationships between exponential, cube root and linear growth kinetics. It has been observed by Bushell in his review (1988) that non-exponential biomass accumulation kinetics are observed for most published streptomycete growth curves.

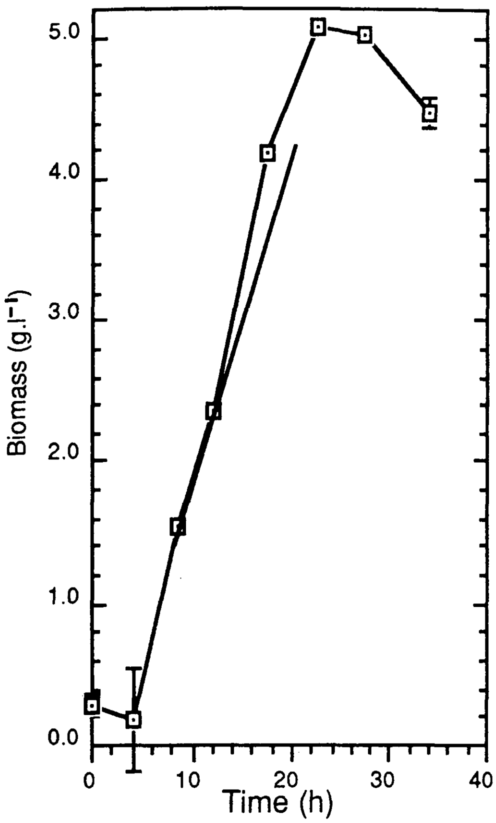


Figure 3.1 Growth curve of *S. thermonitrificans* in TSB at 37 ℃

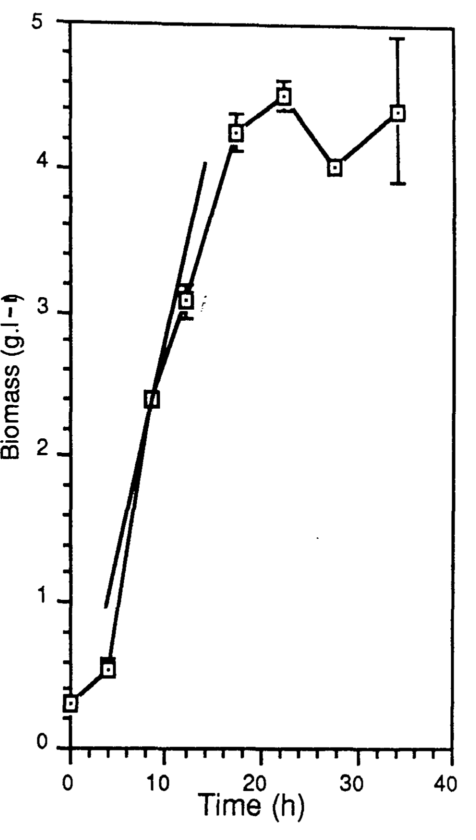


Figure 3.2 Growth curve of *S. thermonitrificans* in TSB at 45 °C

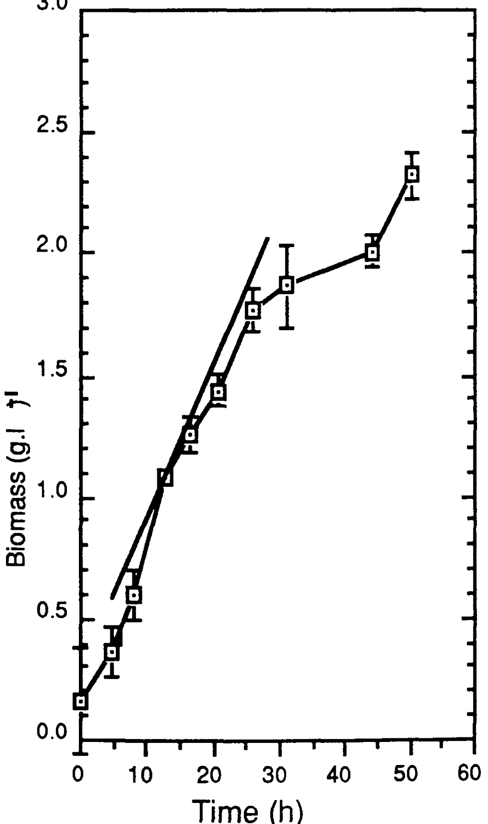


Figure 3.3 Growth curve of *S. thermonitrificans* in Semi-defined LMM at 37 °C

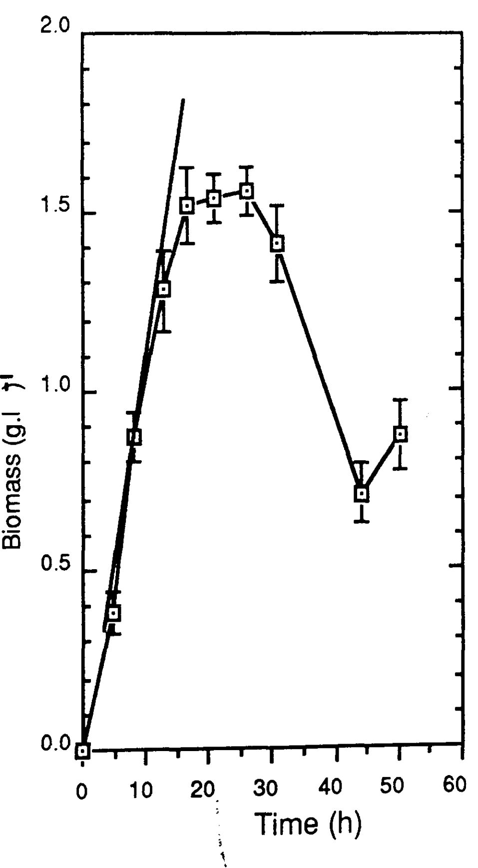


Figure 3.4 Growth curve of *S. thermonitrificans* in Semi-defined LMM at 45 ℃

3.2.2 Growth Curves for *S. thermonitrificans* on Semi-Defined Medium in Flask Experiments

The final biomass concentrations for *S. thermonitrificans* were very low for growth on semi-defined LMM, in the initial stages of the project. Final biomass concentrations of 0.37 +/- 0.21 $g.l^{-1}$ were achieved in 48 hours with a spore inoculum of 2 x 10^3 per ml LMM. This result was confirmed, using a larger spore concentration of 5 x 10^8 (see 3.7.0, development of defined inocula). An inhibition of germination in semi-defined medium was suspected to account for the low final biomass concentrations. Inhibition of germination in the absence of high concentrations of calcium and complex amino acid sources is a common occurrence (Ensign, 1978)

The use of stationary phase inocula pre-grown on TSB at a concentration of 10% (v/v) in semi-defined LMM resulted in increased final biomass concentrations toward 1.03 +/- 0.26 gl^{-1} at 45^0C. Further manipulations and experimentation, such as varying the degree of shear stress during the preparation of inocula, by vortex mixing, ultimately improved growth to enable final biomass concentrations of between 1 and 2 $g.l^{-1}$ to be achieved. These were in reasonable agreement with those previously recorded for growth of *S. thermonitrificans* in semi defined LMM in flask culture with 15$g.l^{-1}$ glucose (2.3 $g.l^{-1}$, L. Harvey, personal communication). A growth experiment was carried out using sheared mycelial inoculum with semi-defined LMM at 37^0C. Spores were pre-grown on TSB for 48 h at 37^0C, harvested and sheared by vortex mixing. The sheared mycelium was inoculated into semi defined LMM with a 10% (v/v) inoculum ratio. The growth curve was repeated at 45^0C (Figures 3.3 and 3.4).

By taking tangents to each curve, values which represent the approximate maximum growth rate for *S. thermonitrificans*, at both 37^0C and 45^0C were obtained. Given the undetermined kinetics of these growth curves, treatment assuming exponential growth and Monod kinetics was considered erroneous. Instead approximate maximum linear growth rates are presented, to show the variation in growth rate with temperature. The data indicate that the rate of biomass accumulation is greater at 45^0C than at 37^0C.

For 3.3 $\quad \text{Slope} = \dfrac{2.0 - 0.6}{27 - 5} = 0.06$ g $biomass.h^{-1}$ (37^0C)

For 3.4 $\quad \text{Slope} = \dfrac{1.5 - 0.4}{14 - 4} = 0.11$ g $biomass.h^{-1}$ (45^0C)

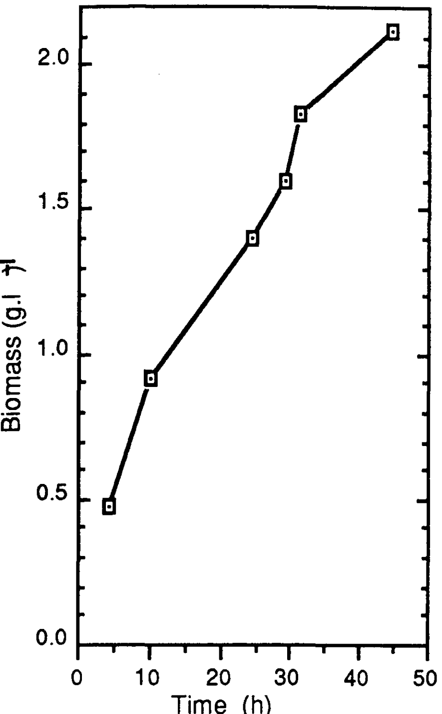


Figure 3.5a Growth Curve for *S. thermonitrificans* cultivated in a Stirred Tank Reactor

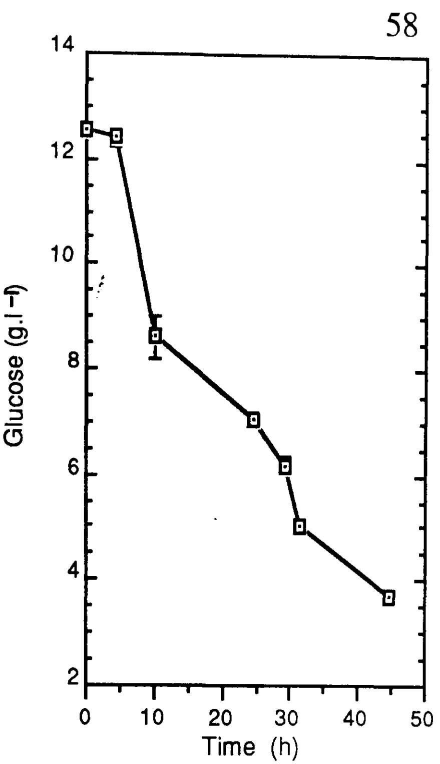


Figure 3.5b Curve of Glucose Utilisation for *S. thermonitrificans* cultivated in a Stirred Tank Reactor

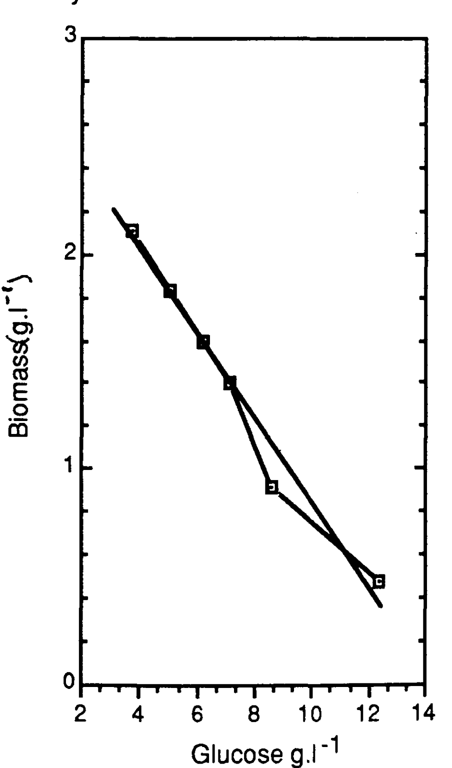


Figure 3.6 Regressed Data for the Determination of a Yield Coefficient for the Data shown in Figures 3.5a and b

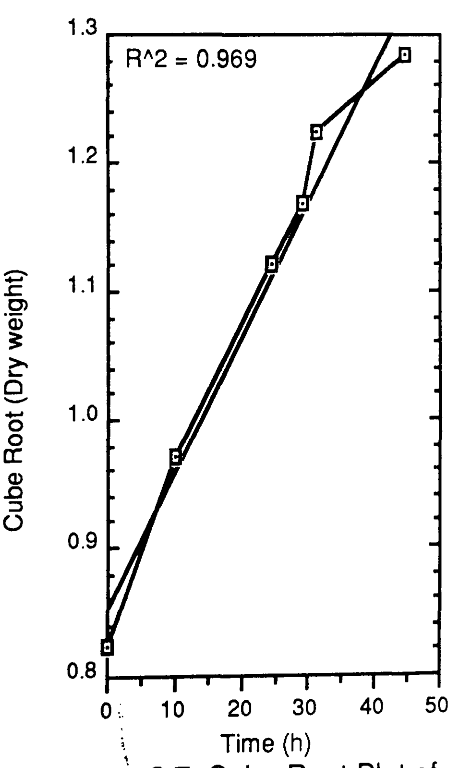


Figure 3.7 Cube Root Plot of Dry Weight for Data Shown in Figure 3.5a (subjected to Linear Regression).

It was noted that lysis and a death phase occurred under these conditions at 45^{0}C, following 30 hours of growth in semi defined LMM, but was not studied further. A heterogeneous mixture of pellets of many different sizes, were observed by visual examination of biomass samples, and not the filamentous mycelial growth observed with *S. thermonitrificans* grown on TSB.

3.2.3 Investigation of the Growth of *S. thermonitrificans* in a 9 Litre Bioengineering Fermenter in Semi Defined LMM

A 7 litre fermentation in semi-defined LMM in the Bioengineering fermenter was established, using a10% (v/v) seed stage inoculum (prepared from a 48h culture at 45^{0}C, with a spore concentration of 10^6 spores per ml TSB medium). The glucose concentration was maintained at 83 mM and the phosphate concentration at 10mM. Agitation and aeration were set at 400 r.p.m. and 1v.v.m., temperature at 45^{0}C and pH maintained at pH7.0. Fermenter cultivation was carried out for 48 hours. The curves for growth and glucose utilisation are presented in Figure 3.5.

Final biomass concentrations reached a value of 2.2 g.l^{-1} after 45 hours for this experiment. Of particular concern was the observation that growth was in the form of small, tight pellets, 2-3 mm in size, which could not be observed under the microscope without sectioning. Microscopical observation indicated that, as expected, intra-pellet growth was extremely dense. This fermentation was repeated five times; in four cases the dissolved oxygen concentration did not drop below 79%, and in one instance a minimum dissolved oxygen concentration of 40% was achieved. This implied that the mycelial pellets had a very slow rate of oxygen uptake. Final biomass concentrations were between 1.5 and 2.2 g.l^{-1} for up to 80 hours of growth. For individual fermentations, growth curves were of varying shape and yield coefficients with respect to glucose utilisation were inconsistent. One explanation for the inconsistency in the determination of yield coefficients could be due to errors in the dilutions used to estimate the glucose concentration. However, by checking the dilutions gravimetrically, this was discounted. A yield with respect to glucose of 0.19 g biomass.g^{-1} glucose for growth at 45^{0}C was obtained for the data shown in Figure 3.5 (from the slope of the regressed biomass v glucose curve Figure 3.6). The growth data from Figure 3.5 could be fitted to a cube root curve (Figure 3.7), which may indicate that pellet-growth kinetics predominated in the stirred tank bioreactor. Each fermentation carried out under these conditions gave growth curves which approximated to cube root kinetics (not shown), implying that growth was diffusion-limited. Cube root plots of flask growth curves did not give straight lines (not shown).

The theoretical maximum diffusion distance for oxygen is 40 μm (Pirt, 1966; from Hill, 1928). As the pellets formed in the stirred tank fermentations were considerably larger than this value and pellet growth very dense, it can be postulated that only the hyphae at the periphery of the pellet were respiring. This hypothesis would explain the low oxygen demand by the culture. If this was the case, then only a proportion of the culture was capable of active growth and the variation in pellet diameter could explain some of the kinetic variability observed. These slow and inefficient growth kinetics were considered unsuitable for the requirements of the project, and methods to overcome both the pellet morphology and poor kinetics were sought.

3.4.0 Medium Development

Initially it was thought that a medium limitation preventing growth beyond around 2 $g.l^{-1}$ was the reason for the low yields of biomass with respect to glucose in semi-defined LMM. A strategy to identify and alleviate a potential nutrient limitation was therefore adopted. However no specific nutrient limitation could be identified. A large portion of this work, some of which is described in 3.4, was carried out with cultures resulting in low, variable, final biomass concentrations and mycelial pellets of varying size and apparent hyphal density.

A fully defined version of LMM which allowed growth of *S. thermonitrificans* had previously been devised (see Materials and Methods), however it was found that medium components would precipitate prior to, and during, sterilization. This problem could not be alleviated by isolating specific components, assembling the medium after sterilization, and only the use of chelators prevented precipitation. Chelators, such as nitrilotriacetic acid should establish a dissociation equilibrium and small amounts of trace metal should dissociate from the chelator as the microorganism uses the dissolved sources. At a constant pH of 7.0, *S. thermonitrificans* was unable to compete with the chelators NTA and EDTA when used at concentrations required to fulfil their role in preventing the formation of precipitates. Figure 3.8 describes relevant work.

As a result of the inability to use fully defined LMM, shown in Figure 3.8, a strategy was adopted to identify a putative simple nutrient limitation by systematic supplementation of the non-precipitable, semi-defined medium, LMM. The information gained would be used to modify existing media, or to develop a novel medium. In addition, inoculum development techniques and ideas would progress using a medium in which growth, albeit kinetically unacceptable, could be observed. Semi-defined LMM was used as a control.

Figure 3.8 Summary of Work for the Use of Fully Defined LMM for Growth of *S. thermonitrificans*

Variable	Result	Conclusion
Phosphate and glucose removed from medium for sterilization, and added aseptically prior to inoculation.	Precipitation of unspecified medium components on addition of the trace metal solution.	Unacceptable
All medium components sterilized separately and assembled aseptically following sterilization.	Precipitation of unspecified medium components on addition of trace metals.	Unacceptable
Addition of EDTA and NTA to the point at which precipitates begin to dissolve (the minimum requirement), and also at 0.5 mM, 0.25mM and 0.05 mM final concentration	No growth of *S. thermonitrificans* at any concentration. No precipitation.	)Possibly the chelators)were toxic to *S.*)*thermonitrificans* or)have an affinity for trace)metals with which *S.*)*thermonitrificans* cannot)compete.
Supplementation of semi-defined LMM (which does not require trace metals and with which no components precipitate) with the 3 chelators together with 0.25 M magnesium chloride to a final concentration of 10mM (to 'mop up' chelation sites) at concentrations described above.	No precipitation of any medium component Normal growth	The chelators were not toxic to growth of *S. thermonitrificans,* but competed for trace elements with the streptomycete.

3.4.1 To Investigate and Alleviate a Possible Nutrient Limitation in Semi-Defined LMM

Three experiments are described (of many carried out) and the results are shown in Table 3.3. Duplicate cultures were harvested after 30 hours, sampled in triplicate and repeated;-

1. Semi-defined LMM was supplemented individually and in all combinations with magnesium, zinc and manganese salts used at the concentrations described for fully-defined LMM (in which component precipitation was a problem)

This experiment was an attempt to investigate whether components which are present in fully defined LMM were necessary to achieve final biomass concentrations greater than 2 $g.l^{-1}$. Semi-defined LMM medium containing magnesium, zinc and/or manganese salts, at the concentrations used in the fully-defined version of LMM, were added to the fermentation in an experiment encompassing all possible combinations of these 3 trace components, individually and together. Results are given in Table 3.1. In general any increase or decrease in biomass concentration in comparison with control flasks were minor. It was concluded that these metals were not the limiting factor for growth of *S. thermonitrificans* .

2 Semi defined LMM was supplemented with the mixed amino acid supplements used in Hopwood Complete Medium (Hopwood *et al.*, 1985) and, in a separate experiment, with a casamino acid supplement (at a final concentration of 20 $g.l^{-1}$). Results are shown in Table 3.1. Growth in all supplemented cultures showed increases over control cultures. This was considered the result of an increase in easily-utilised organic carbon and nitrogen sources. A relatively large biomass increase would have been expected if a nutrient or precursor limitation had been overcome, given the carbon source concentration in the medium (glucose 15$g.l^{-1}$). As a defined medium based on a single carbon source was the aim of this work, the result was not investigated further.

3. Supplementation with yeast extract. Yeast extract supplemented semi-defined LMM was prepared at the concentrations shown in Table 3.1. These supplemented media gave higher final biomass concentrations, increased growth rate and dispersed mycelial morphology, than the unsupplemented media, judged qualitatively. It was considered that removal of a growth

Table 3.1 Attempts to Alleviate a Potential Nutrient Limitation in Semi-Defined LMM

Variable	Final Biomass Concentration (g.l^{-1})	Results expressed as a % compared to Control
Experiment 1		
(final mineral concentrations as for fully defined LMM)		
Magnesium	1.61 +/- 0.09	90
Manganese	1.61 +/- 0.05	90
Zinc	1.77 +/- 0.04	99
Magnesium and Manganese	1.41 +/- 0.05	83
Magnesium and Zinc	1.63 +/- 0.11	91
Manganese and Zinc	1.67 +/- 0.16	94
Magnesium, Manganese and Zinc	1.70 +/- 0.26	95
Control	1.78 +/- 0.20	----
Experiment 2		
Amino Acid Supplements (Hopwood *et al.*, 1985)	1.04 +/- 0.02	136
Casamino Acids (20 ml of 1% solution.l^{-1})	1.21 +/- 0.04	159
Control	0.76 +/- 0.01	----
Experiment 3		
Yeast Extract (2.5 g.l^{-1})	2.60 +/- 0.07	226
Yeast extract (5.0 g.l^{-1})	3.62 +/- 0.06	314
Yeast Extract (10 g.l^{-1})	4.38 +/- 0.29	381
Control	1.15 +/- 0.06	---

limitation had possibly occurred. Yeast extract contains high levels of B vitamins, phosphate and small amounts of easily-assimilated organic carbon and nitrogen sources. The higher final biomass concentrations coincided with a switch from pellet to filamentous mycelial morphology. It was considered that the factors listed below could have had a role in the increase in biomass concentrations observed in this experiment.

i. supply of B vitamin sources
ii. high phosphate levels
iii. a growth factor, capable of causing a switch from pellet, to filamentous growth
iv. a growth rate suitable for filamentous mycelial growth in contrast to mycelial growth in the form of pellets , possibly as a result of the change in availability of organic nitrogen and carbon sources.

Further experiments were designed to test the potential effects of these four factors on growth. Variations in the levels of inorganic phosphate above 3mM were found to have little or no influence on growth in LMM (data not shown). Similarly the inclusion of a mixture of the B vitamins nicotinamide, pyrodoxine-HCl, biotin and riboflavin in media (at 0.1g of each per litre) had no detectable effect on growth (data not shown). In light of this it was evident that some unidentified factor was responsible for the enhanced growth of *S. thermonitrificans* in media supplemented with yeast extract . The 'unidentified factor' was suspected to be an increased growth rate. Growth rate increases are observed in minimal media supplemented with yeast extract for *Xanthamonas campestris* (Rye *et al.*, 1988) and *E.coli* (Hunter, personal communication).

3.4.2 <u>The Effect of Phosphate on Growth in Semi-Defined Medium (LMM)</u>

When polyphosphokinase is present, some *Streptomyces* may store reserves of phosphate as inorganic polyphosphate (Hostalek *et al.*, 1976). Polyphosphate is a store of activated phosphate, which is osmotically inert and can be accumulated to a high concentration. Polyphosphates are known to participate in the regulation of intracellular ATP and other nucleotide levels in some organisms. This enables some attainment toward independence of microbial metabolism from environmental conditions (Kulaev, 1985). The intracellular presence of polyphospoglucokinase enables polyphosphate to mimic the function of ATP (Szyona, 1962). Hence polyphosphate storage was considered a relevant factor to be taken into account in interpreting yield data for *S. thermonitrificans* pre-grown on complex medium. Accumulation of polyphosphate could occur when *S. thermonitrificans* was grown on TSB and

could be used as an energy source when the organism was grown on semi-defined LMM.

Inocula of varying age (20, 44 and 68 hours), grown in TSB were prepared and inoculated (10% v/v) into flasks containing or lacking exogenous phosphate (at 10mM concentration). Flasks were inoculated in duplicate, sampled in triplicate and the experiment repeated. The mean final biomass concentrations are presented in Table 3.2. From these results, it was concluded that no apparent growth of *S. thermonitrificans* occurs in minimal medium lacking an added phosphate source. The biomass levels detected in medium lacking phosphate were those of the initial inoculum (see table). Hence from these data, polyphosphate storage could be disregarded as an influence on the yield of biomass with respect to substrate in LMM. There may be some polyphosphate storage and mobilisation, but at a level which was considered negligible.

3.4.3 The Influence of Shear Stress and the Polymer Additive, Junlon, on Growth of *S. thermonitrificans*

Experiments were devised using a variety of shearing techniques at four different stages during inoculum preparation and cultivation on minimal medium. These are listed below.

- Shear on the spore suspension prior to inoculation (shear by vortex-mixing)
- Shear during germination of inoculum in TSB (use of baffled flasks, springs or glass beads)
- Shear during the wash for inoculation into semi defined medium (vortex shear, with and without glass beads)
- Shear during growth in semi defined medium (use of baffled flasks, springs or glass beads)

No increase in final biomass concentration, or alteration of pellet morphology occurred with any shear technique used. In contrast, some of the variations resulted in decreased biomass levels, which coincided with the occurrence of very small, tight-meshed pellets of mycelium. In addition the use of baffles or springs during growth stages resulted in mycelial lysis when observed microscopically.

It did not appear that a random increase in shear stress could result in increased final biomass concentration, or the promotion of filamentous morphology *per se*. It would have been relevant to investigate the effect of reducing shear stress, by increasing the medium viscosity during growth on

Table 3.2 Investigation into the Effect of an Absence of Phosphate on Growth of *S. thermonitrificans*

Inoculum Age	Final Biomass Concentration $g.l^{-1}$	
	With Phosphate (10 mM)	Lacking Phosphate
20 hours	1.91 +/- 0.03	0.27 +/- 0.12
44 hours	2.34 +/- 0.03	0.29 +/- 0.06
68 hours	1.00 +/- 0.04	0.24 +/- 0.18
Inoculum mass	0.35 ($g.l^{-1}$ final concentration)	

Table 3.3 Investigation into the Influence of Junlon in Semi Defined LMM for Growth of *S. thermonitrificans*

Junlon Concentration $g.l^{-1}$	Final Biomass Concentration (this also includes residual Junlon) $g.l^{-1}$	
0.5	1.45 +/- 0.21	
1.0	2.45 +/- 0.80	(small junlon residue observed)
1.5	5.35 +/- 1.10	(large junlon residue observed)
2.0	9.63 +/- 1.81	(")
2.5	8.72 +/- 0.64	(")
Control (lacking junlon)	1.91 +/- 0.21	

semi defined LMM. However, it is difficult to study the relationship of shear stress with growth, quantitatively and independently, of mass transfer variables. Reductions in bulk liquid mass transfer rates would accompany increased medium viscosity, and a lower growth rate would therefore be predicted. For *S. coelicolor*, the inclusion of polyethylene glycol at high concentrations in defined media resulted in slightly increased levels of biomass and a more open mycelial network, as a result of an increased medium viscosity (Hodgson, 1982; Hobbs *et al*, 1989). In addition, starch-based defined medium, with a high viscosity, has been reported to give filamentous growth of *S. coelicolor* (Vining and Doull, 1989).

For *S. coelicolor,* a defined medium incorporating a polyacrylic resin, conventionally used as a paint thickener, has been devised in order to promote a more dispersed morphology (Hobbs *et al.,* 1989). This polymer, junlon, was originally used to modify the submerged growth morphology of *Aspergillus spp.* in minimal media for turbidometric growth assay (Trinci, 1983). The mode of action of the polymer has not been determined definitively, however it is due to some extent on its electrostatic charge properties and is thought to act by coating growing cells with a negative charge. It has been shown that electrostatic repulsion of spores occurs in medium containing the resin (Jones *et al.,* 1988).

An experiment was established using LMM supplemented with Junlon at a range of concentrations from 0.5 $g.l^{-1}$ to 2.5 $g.l^{-1}$. Junlon was sterilized separately from other medium components at pH7 and added post-sterilization. The results are shown in Table 3.3. However the data are inaccurate and subject to large errors because the resin was retained on filter paper following filtration. In addition, as a result of the viscosity of the samples, high vacuum pressures and long filtration times were required (several hours). Junlon remains bound to mycelium on centrifugation in the same manner as during filtration and, therefore, could not be considered a viable alternative technique. Results are shown in Table 3.3.

As the resin is charged, then by altering pH it was thought possible to minimise the charge interactions sufficiently for the resin to be washed off mycelia. A dense white insoluble precipitate was formed on addition of acid to a pH of around 5.0, simultaneously with a decrease in viscosity; however the precipitate could not be easily separated from mycelium.

From the results shown in Table 3.3, Junlon was unsuitable in cases where dry weight determinations were required. Turbidometric assay was unsuitable as an alternative method of growth assay for *S. thermonitrificans*: the alleviation

of the normally dense, pelleted morphology of *S. thermonitrificans* in semi-defined LMM containing junlon was relatively minor. The concentration of some intracellular polymers, such as total DNA or total protein may have been suitable for analysing growth, however, calibrating the results against dry weight would have been problematic, given that junlon prevents the accurate determination of this parameter. In addition the use of junlon would have prevented the use of carbon balancing at a later stage in the project (carbon balancing is described in Chapter 5).

3.5 Summary Conclusions from Preliminary Experiments and the Subsequent Experimental Strategy

From the work described to date, low final concentrations of biomass correlated with the occurrence of pelleted broths, and high levels of biomass were only achieved with well-dispersed filamentous broths using media containing organic carbon and nitrogen sources. Simple experiments were insufficient to identify a nutrient limation. Therefore it was considered that work would be better directed to to identifying a fully- defined medium on which to improve the morphology of *S. thermonitrificans,* rather than developing a novel medium. A fully defined medium (Evans, 1967) was identified as a potential replacement medium for LMM. Maximum biomass concentrations of 2 $g.l^{-1}$ and yield values of around 0.20 $g.g^{-1}$ glucose were obtained in Evans medium with 15 $g.l^{-1}$ glucose.

3.6 Optimisation of the Growth Conditions for *S. thermonitrificans* on Evans Medium

Evans defined medium was considered preferable to both versions of LMM as a consequence of its fully defined nature. The potentially variable nature of tap water as a mineral source, and the precipitation of trace metals which occurred with fully- defined LMM were considered unsatisfactory. The use of semi-defined LMM formulated with distilled water, supplemented with nitrogen, carbon, phosphate and magnesium nutrient sources only, was considered unsuitable because the apparent growth limitation for *S. thermonitrificans* in minimal medium was unidentified. The choice of tap water as a medium component was originally based on large-scale process economics (which was also the reason for the initiation of the investigation - the potential of a process with a reduced cooling requirement). The decision to use distilled water-based media did not change the ultimate project aim. Instead it would broaden the investigation, allowing rigorous physiological investigation of the growth of *S. thermonitrificans*, with which a potential large-scale process could be optimised. At a later date, it is feasible that a tap water-based medium, or a

complex medium could be used in the efficient production of a specified recombinant product on a production scale.

3.6.1. The Effect of Variation in Medium Component Concentration on Growth in Evans Medium

Evans medium circumvents the precipitation of trace metal and medium components by the use of citric acid as a chelator and medium components which are compatible on sterilization. (In retrospect, the use of citric acid as a chelator for LMM may have alleviated component precipitation). According to data presented in Chapter 4 and the classification tables of Williams *et al.*, 1982, *S. thermonitrificans* is unable to utilise citric acid as a carbon source, and, if used, would not influence carbon conversion observations. It was also noted that mass balancing would be complicated by the background of residual carbon due to the chelator (12 millimoles, 6% of the carbon for a $6g.l^{-1}$ glucose medium). However, if necessary, analysis of culture supernatants by HPLC could supply data for this correction.

The effect on growth of a variation in the concentration of each component of Evans medium was investigated in a matrix experiment. Flasks were cultivated for 48 hours, using the following criteria;
i) absence of a component,
ii) half the final concentration described for a component in Evans medium
iii) final concentration described for a component in Evans medium (marked with an asterisk, *)

From the results presented in Table 3.4, it was concluded that sulphate, calcium, magnesium and potassium were apparently growth-limiting substrates. Phosphate and nitrogen were limiting for growth at concentrations below half that specified for Evans medium. Additionally, the absence of trace metals did not prevent growth, but their inclusion resulted in biomass concentrations which were greater than in their absence. This was postulated to be the result of contaminants in the stocks of other medium components which satisfied some of the trace metal requirements. Finally, molybdate was found to have no detectable influence on growth in these conditions.

It may be inferred that sulphate, calcium, magnesium, and potassium are limiting for growth at these concentrations. However experiments in which these specific components were supplied at concentrations twice and three times that specified for Evans medium surprisingly did not result in greater final concentrations of biomass.

Table 3.4 Influence of the Concentration of Individual Medium Components in Evans Defined Medium for Growth of *S. thermonitrificans* (15 g.l^{-1} glucose concentration).

Component	Concentration	Final Biomass Concentration (g.l^{-1})
Nitrogen (NH_4Cl)	Zero	0.21 +/- 0.08
"	50 mM	1.67 +/- 0.50
"	*100 mM	1.63 +/- 0.77
Phosphate (KH_2PO_4)	Zero	0.39 +/- 0.10
"	5 mM	1.60 +/- 0.34
"	*10 mM	1.66 +/- 0.10
Magnesium ($MgCl_2$)	Zero	0.37 +/- 0.05
"	0.6 mM	0.67 +/- 0.12
"	*1.2 mM	1.75 +/- 0.35
Potassium (KCl)	Zero	0.78 +/- 0.10
"	5 mM	1.03 +/- 0.08
"	*10 mM	1.89 +/- 0.18
Sulphate (Na_2SO_4)	Zero	0.77 +/- 0.14
"	1 mM	0.69 +/- 0.02
"	*2 mM	1.44 +/- 0.20
Calcium ($CaCl_2$)	Zero	0.61 +/- 0.17
"	0.01 mM	0.61 +/- 0.15
"	*0.02 mM	1.49 +/- 0.22
Trace Metals	Zero	0.90 +/- 0.08
(listed in Materials and Methods)	*5 ml.l^{-1}	1.20 +/- 0.13
Molybdate ($NaMoO_4$)	Zero	2.09 +/- 0.24
"	*0.1 nM	1.93 +/- 0.20

(final biomass concentrations of between 1.7 and 2.2 g.l^{-1} were observed following growth for 48 hours). The apparent insensitivity of the dry weight of *S. thermonitrificans* to greater concentrations of sulphate, calcium, magnesium and phosphate was suspected to be partially related to the pelleted broth morphology. It was therefore decided to adopt the concentrations of these components specified for use in Evans medium, with the option of re-optimising the medium composition at a later stage.

If this aspect of the project had been pursued, following the achievement of filamentous broths in a stirred tank bioreactor, further optimisation the medium composition could usefully have been carried out. The calcium, potassium, sulphate and magnesium concentrations could then have been varied and the effects observed independently of the pellet growth-form. However, the approach eventually adopted at a later date, was to optimise the glucose concentration for growth of *S. thermonitrificans* on the modified Evans medium (see 3.8.1).

3.6.2 Development of *S. thermonitrificans* Minimal Medium (579 MM)

From the results in 3.6., a defined *S. thermonitrificans* medium (579MM) was developed. Major variations from Evans medium were; a reduced nitrogen concentration (100 mM to 50 mM), potential reduction in phosphate concentration (10 mM to 3 mM if required) and absence of molybdate. A phosphate concentration of 3 mM was identified as the lowest concentration coincident with biomass concentrations of 2 g.l^{-1} (analysis showed that phosphate concentrations decreased by 2 mM with respect to initial concentrations following growth to a maximum biomass concentration of around 2 g.l^{-1}). Phosphate is implicated as a key regulatory nutrient in a wide range of pathways involved in secondary metabolism (Martin, 1989). For this reason it was considered important to be able to manipulate the medium to obtain low initial phosphate concentrations for later possible use in a system for expressing biosynthetic pathways of secondary metabolism. Assays of residual nitrogen and residual phosphate following a normal flask fermentation, showed that these nutrients were in excess of the requirements for growth. Glucose was retained as a carbon source at 15 g.l^{-1} (83 mM) this concentration was optimised at a later date.

3.7 Development of Defined Inocula

Following the identification of a defined medium, suitable for implementing the project strategy, the specific requirements for 7 litre fermenter-scale cultivation could be addressed. It was desirable to have mycelium which had not been

subjected to nutrient "shift-down" from a medium rich in amino acid sources, to one poor in these nitrogen sources. In addition it was desirable to have inoculum which had been adapted for growth on glucose as a carbon source.

"Shift-down" from rich medium to defined medium has been shown to induce the stringent response in *Streptomyces* (Ochi, 1986: Strauch *et al.*, 1991) and is discussed in more detail in Section 1.7. The avoidance of a nutrient "shift-down" was considered important during inoculum preparation. For *S. coelicolor*, an apparent growth-associated production of actinorhodin has been reported on defined medium (Vining and Doull, 1988). Inoculum for this work was initially developed on complex medium, then washed and transferred to semi-defined medium. Presumably a stress response which includes the stringent response is induced, together with actinorhodin production, by this shift down in nutrient quality. The work was repeated using pre-germinated spores and non-growth associated actinorhodin production kinetics were observed, illustrating the importance of the inoculum development procedure for subsequent growth and product formation (Doull and Vining, 1990).

3.7.1 Pre-germination of Spores of *S. thermonitrificans* for Subsequent Growth on 579MM

Spores of *S. thermonitrificans* failed to germinate in defined medium, possibly due to lack of sufficient calcium, or to the presence of a germination inhibitor (section 3.2.2). The spore pre-germination method developed by D. Hodgson was therefore tested (Hopwood *et al*, 1985). It was hoped that pre-germinated spores would continue growth on 579MM. The method involves an initial heat shock at 55^{0}C for 10 minutes and subsequent incubation in a yeast extract-based medium supplemented with calcium ions, for 2-3 hours. Pre-germination was carried out for two spore samples with an initial concentration of 10^8 spores per ml, incubating for 2 and 3 hours in a complex medium after heat shock. The spore numbers in both samples of pre-germinated spores were counted both before and after a freezing step, using the spore count method described in Section 2.7.5. This test of spore viability subsequent to storage at -20^{0}C, was to ensure that the pre-germination technique would be suitable for preparation of spores in advance of cultivation. Spores were inoculated into 579MM at a constant final spore concentration of $5x10^5$ per ml, whilst non-pregerminated spores were inoculated to a final concentration of $5x10^6$ per ml. Results are presented in Table 3.5.

Biomass concentrations of around 2g.l^{-1} were obtained using pre-germinated spores (Table 3.5). This spore pre-germination method was therefore suitable for use with *S. thermonitrificans.* Elevated levels of non-pregerminated spore

inocula did not overcome the postulated germination inhibition (see table). In cultures inoculated from non-pregerminated spores, probably a small number of spores germinate in an asynchronous manner, leading to a low inoculum concentration and hence unacceptable growth dynamics and low final biomass concentrations. After freezing, spore viability decreased only slightly, and therefore the viability of pre-germinated spores, following freezing, was adequate. A disadvantage of pre-germinated spores was that, in contrast to non-pregerminated spores, they had undergone a nutrient shift for two hours. However this was considered unavoidable.

3.8 Optimisation of Growth in 579MM using Defined Inocula

Defined inocula (3.7) and medium (3.6) had been developed. The optimisation of growth conditions using these techniques was therefore a natural progression. Initially this entailed investigating the influence of glucose and the concentration of pre-germinated spore inocula on growth.

3.8.1 The Influence of Glucose Concentrations in 579MM on Growth of *S. thermonitrificans*

Media were prepared with glucose concentrations listed in Table 3.6 and inoculated with a high pre-germinated spore inoculum (5 x 10^6 ml^{-1} final concentration). The experiment was terminated after 30 hours of growth, when there was no residual glucose in flasks which had initially contained 3 $g.l^{-1}$ glucose. Residual glucose concentrations before and after growth were determined using the glucose oxidase method in duplicate, and used to calculate the yield Y_{XS} (*i.e.* the ratio of biomass concentration to glucose depletion from the medium). Flasks were prepared in duplicate, sampled in triplicate, and the experiment repeated.

The results shown in Table 3.6 suggested that the growth efficiency might be influenced by the concentration of glucose. Initially it was thought most probable that high glucose concentrations resulted in a toxic influence on growth. However, it was also noted that flasks with relatively high glucose concentrations gave broths with pellets of much greater density and heterogeneity than those of lower glucose concentrations. Broths with the most loosley-packed, open-meshed pellets were those of 3 $g.l^{-1}$ glucose concentration.

Aspergillus nidulans colony growth rate on solid substrate is linear with respect to the log of the initial glucose concentration. The frequency of branching of the mycelium is also influenced by growth rate (Trinci ,1969). Growth rate in *S.*

hygroscopicus has been shown to influence the branching frequency and rate of apical extension in continuous culture (Riesenberg, 1979; Kretschmer, 1981). By extrapolation to *S. thermonitrificans,*, it is postulated that glucose, in potentially influencing the growth rate, may simultaneously influence mycelial morphology by increasing the mycelial branching frequency in conditions of high initial glucose concentrations. The lower initial growth rates which are postulated to occur with low initial glucose concentrations may contribute to more efficient carbon conversion efficiencies (possibly enhanced by more open-meshed pellets). This greater efficiency might occur by reducing the influence of cube root and diffusion-limited kinetics on overall growth kinetics. The increase in efficiency would occur as a result of the larger proportion of actively growing biomass and smaller proportion of mycelia which would not be contributing to *de novo* growth, but would require energy for the maintenance of viability. In order to test this hypothesis, methods for quantifying the growth rate of pellets early in their formation would be required together with image analysis techniques for the investigation of hyphal arrangement and quantitation of the branching frequency and values for the hyphal growth unit. An equally valid explanation for the results shown in table 3.6 could be that glucose was toxic to the growth of *S. thermonitrificans* at concentrations of $15 g.l^{-1}$ and above.

It had been noted previously that biomass limits were achieved which were independent of the residual glucose concentration in cultures grown on semi-defined LMM (3.2.3). In this case it was postulated that a biomass concentration limit was attained; due to either a limiting factor in the medium or mycelium, or due to the pelletted morphological growth form (3.4.0). The inefficiency is now postulated to be partly related to a high initial glucose concentration (described above). From the results presented in section 3.8.1, a glucose concentration of $6\ g.l^{-1}$ was chosen as a suitable concentration for general use in 579MM. For high inoculum concentrations (5×10^5 spores ml^{-1} final concentration) in flasks, then $6\ g.l^{-1}$ glucose may be utilized at a rate giving a reasonable carbon conversion efficiency. Final biomass concentrations with initial glucose concentrations of $6\ gl^{-1}$ are sufficient to enable the determination of relatively sensitive, accurate values for dry weight.

3.8.2 The Differences in Carbon Conversion Efficiencies Between Different Growth Conditions

Differences in carbon conversion efficiency were manifested as variations in yield of biomass per gram glucose substrate with differences in initial glucose concentrations (3.8.1). Possible reasons for these differences are;

Table 3.5 Pre-germination of spores of *S. thermonitrificans*, and the influence on growth in 579MM. (Means of six determinations, from duplicate experiments.)

Variable	Viable spores ml^{-1}
Defrosted spores no pre-germination	76 (+/- 3) x 10^6
Spores pre-germinated for 2 hours	83 (+/- 5) x 10^6
Spores pre-germinated for 3 hours	94 (+/- 17) x 10^6
Spores pre-germinated for 2 hours (frozen)	12 (+/- 2) x 10^6
Spores pre-germinated for 3 hours (frozen)	13 (+/- 5) x 10^6

Submerged Cultivation in 579MM	Biomass $g.l^{-1}$
Defrosted spores (no pre-germination 5 x 10^6/ml)	0.45 (+/- 0.15)
Spores pre-germinated for 2 hours (frozen)	1.95 (+/- 0.35)
Spores pre-germinated for 3 hours (frozen)	1.88 (+/- 0.20)

Table 3.6 The Influence of Initial Glucose Concentration on Yield. Mean values for glucose concentrations are presented. Initial biomass concentrations (pre-germinated spores) were negligible. 'S' is the concentration of glucose depleted from the medium to achieve the final biomass concentration.

Glucose Concentration ($g.l^{-1}$)				
Initial	Final	(S)	Final Biomass Concentration (x) ($g.l^{-1}$)	Yield (x/s) g.g $Glucose^{-1}$
3.0	0.0	3.0	1.47 (+/- 0.14)	0.49
7.5	1.4	6.1	2.99 (+/- 0.06)	0.49
15	6	9.0	3.25 (+/- 0.07)	0.36
30	21	9.0	3.46 (+/- 0.15)	0.38

1. Glucose could have been used as source of energy for conversion to storage molecules, such as polysaccharide or polyhydroxybutyrate (Kannan and Rehacek, 1970). This would lead to apparently inefficient growth. However, this possibility was easily negated. Biomass from a flask fermentation with an initial glucose concentration of 15 g.l^{-1}, following growth for 84 hours at 45^{0}C was washed and transferred to minimal medium lacking exogenous carbon and energy sources. No further detectable growth occurred, suggesting that energy storage on a large scale did not occur.

2. A form of shunt or "overflow" metabolism, as described by Neijssel and Tempest could have occurred, resulting in the excretion of carbon in the form of overflow products such as acetate (Neijssel and Tempest,1986). This would also result in apparently inefficient growth. However, this second possibility was negated following the completion of mass balanced fermentations (described in Chapter 5) for which all carbon could be accounted. Although only fermentations with initial glucose concentrations of 3 g.l^{-1} are presented in Chapter 5, duplicate fermentations with initial glucose concentrations of 6 g.l^{-1} and a single fermentation with initial glucose concentration of 12 g.l^{-1} were also carried out and all carbon successfully accounted.

3. There could be 2 or more active isoenzymes of glucose kinase having different K_m and efficiencies for glucose uptake. In this case it could be possible that the efficient uptake of glucose occurs at low glucose concentrations. This possibility could not be investigated easily, and detailed investigation was beyond the scope of the project. Very little research has been carried out on transport systems in *Streptomyces* .

4. Glucose in high concentrations may be partially toxic to growth of *S. thermonitrificans.* Unfortunately this possibility could not be tested, however, the avoidance of high glucose concentrations in media would pre-empt an influence of this type, on growth.

5. Glucose concentration might influence morphology and/or growth rate, so that higher total energy drains to maintenance would occur with high initial glucose concentrations, coincident with a longer fermentation.

It has been reported that in continuous culture of *Fusarium graminearum* and in *S. hygroscopicus* the hyphal growth unit and the branching frequency are influenced by growth rate (discussed in the Introduction, Trinci ,1990; Kretschmer, 1981). In addition work in Chapter 4 describes the determination of an approximate maintenance coefficient for *S. thermonitrificans.* The approximate value for the maintenance coefficient (0.47 millimoles glucose. g

dry weight $^{-1}$. h^{-1}) is compatible with an explanation incorporating energy drains for maintenance in *S. thermonitrificans* to explain inefficient carbon conversion following extended periods of growth.

3.8.3 The Influence of Inoculum Spore Concentration on Final Biomass Concentration in 579MM Containing 6 $g.l^{-1}$ Glucose

In designing this experiment, it was recognised that low spore concentrations could only be obtained by serial dilution to low concentrations and high spore concentrations would use a large proportion of the pre-germinated spore stocks. The former was known to give low final biomass concentrations, and the latter condition was impractical for general use. It was therefore conducted using a small number of closely related values for spore concentration, conditions which were experimentally applicable, results are shown in Table 3.7.

The results were plotted semi-logarithmically (Figure 3.9). They suggested that a logarithmic relationship between the spore concentration and final biomass concentration might exist for the spore concentrations used in this experiment. In addition, there were differences in the intra-pellet hyphal density between the higher inocula concentrations (with a greater quantity of pellets with an apparently more open hyphal network) and lower inocula concentrations, (a smaller quantity of larger pellets with greater apparent hyphal network density), judged visually and microscopically. It would appear that for 10^6 spores per ml medium, each spore contributes less to the final biomass concentration than for each spore at a concentration of 10^4 spores per ml. A possible explanation for this result may be that, as a result of the greater spore concentration at 10^6 ml, then the initial glucose depletion rates would be faster, and the lag phase would be shorter. Therefore, a limitation in carbon substrate would be achieved earlier into the fermentation. The faster fermentation (with a the higher spore concentration) could have a smaller energy drain for maintenance; alternatively the more-rapid glucose utilisation rate may mediate in morphological responses favourable for an efficient carbon conversion efficiency. Therefore, this result may reflect the influence of a specific ratio of inoculum to substrate which influences carbon conversion efficiency and/or the intra-pellet hyphal network density. A similar logarithmic relationship between spore inoculum and final biomass concentration was observed for *S. coelicolor* using spore concentrations between 10^4 and 10^7 spores ml^{-1} (Hobbs *et al.,* 1990).

The demand for spore inocula of this concentration for use in large-volume experiments could not be satisfied without massive stocks of pre-germinated spores. Therefore, an additional step was incorporated into the inoculum

Table 3.7 The Effect of Inoculum Spore Concentration on Final Biomass Concentration in 579MM containing 6 $g.l^{-1}$ glucose

Final Spore Concentration (ml medium)$^{-1}$	Final Biomass Concentration ($g.l^{1}$)
$6x10^{-4}$	0.84 (+/- 0.07)
$3x10^{-5}$	1.54 (+/- 0.28)
$6x10^{-5}$	1.86 (+/- 0.07)
$2x10^{-6}$	2.87 (+/- 0.07)

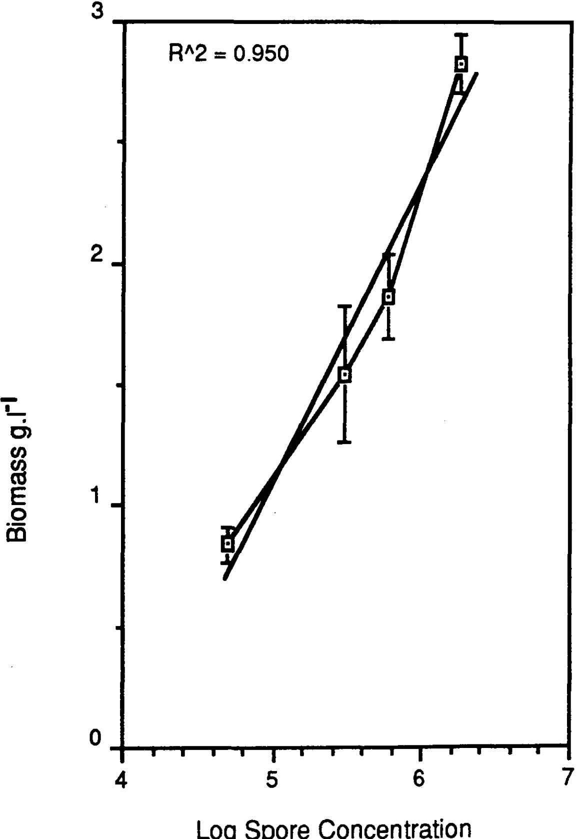


Figure 3.9 Semi-logarithmic Plot of Spore Inoculum Number against final Biomass Concentration subjected to LInear Regression

development procedure. This later step enabled the use of high concentrations of potential sources of apical hyphae in the form of fragmented mycelia (described in section 3.12.0).

3.9 Fermenter Growth using 579MM and Defined Inoculum

A fermentation in a 9 litre maximum volume Bioengineering fermenter with 7 litres of 579MM was sterilized *in situ* . Glucose, to 6 $g.l^{-1}$ (33 mM) and 10 mM phosphate was added post sterilization. The inoculum consisted of a 10% (v/v) ratio of mycelia, pre-grown in 579MM using pre-germinated spores. However final biomass concentrations were no higher than 1 $g.l^{-1}$ following 56 hours of cultivation. This did not compare favourably with results obtained in flask culture on the same medium containing 6 $g.l^{-1}$ glucose. Figure 3.10. In addition, the mycelial morphology was in the form of large, dense mycelial pellets, approximately 2-3 mm in diameter Figure 3.11.

The final biomass concentrations were consistent with those obtained previously in the Bioengineering fermenter using inoculum pre-germinated in TSB and grown in semi-defined LMM. For this previous fermentation, a yield of 0.19 g.g $glucose^{-1}$ (see Figure 3.6) was obtained.

For Figure 3.9;	Yield = $\frac{1.15}{6}$	= 0.19 g biomass.g $glucose^{-1}$

In the fermentation shown in Figure 3.10, the yield was consistent with the previous fermentation shown in Figure 3.6. Because mycelial inoculum was in the form of pellets initially formed by growth in flasks, the number of potential apical hyphae in the inoculum was low, and probably contributed to the slow, inefficient growth kinetics. These low yields of biomass with respect to glucose depletion were coincident with (apparently) high, intra-pellet hyphal network densities.

3.9.1 Aeration, Agitation and Shear in Fermenter Cultivation

Fermenter aeration using Rushton turbine impellers, baffles and sparger-aeration provides a high aeration efficiency with respect to power input, and is a preferred aeration method for highly aerophilic fermentations. However, airlift fermenters, or fermenters with propeller or modified impellers are more suited to shear-sensitive organisms. Under normal conditions, *S. thermonitrificans* would not be considered shear-sensitive, however the design of the Bioengineering fermenter is such that a long, small diameter vessel is fitted with 4 baffles with sharp edges. As a result of this aspect, the ratio of shear

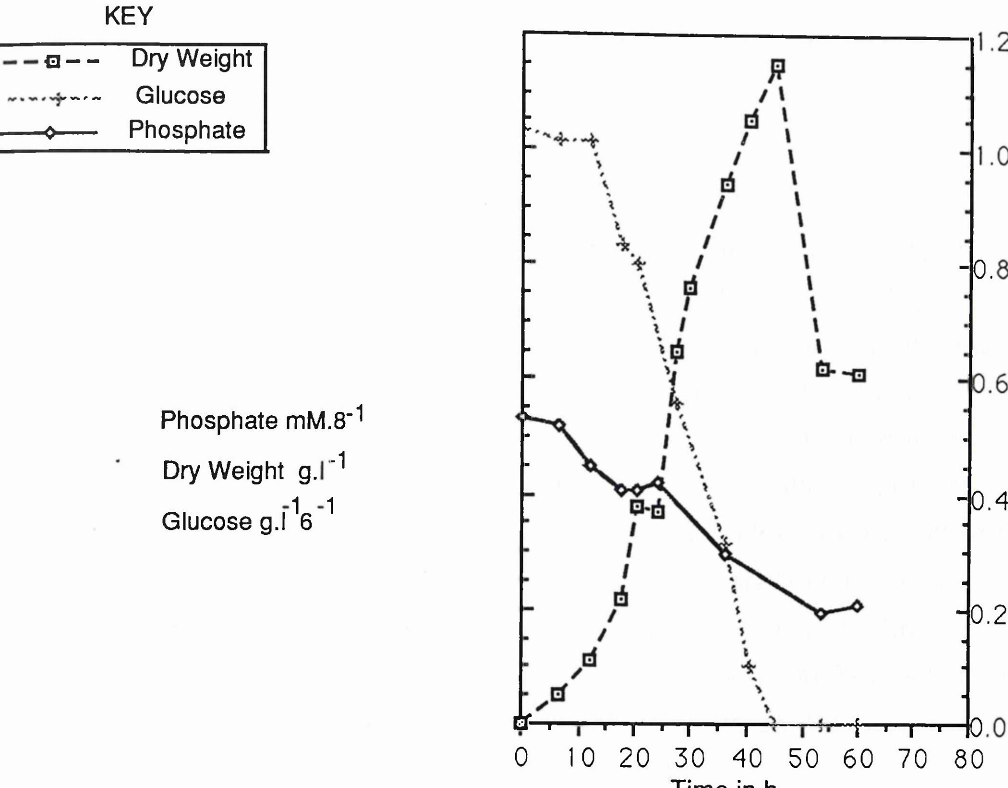


Figure 3.10 Growth Curve of Biomass Accretion, Glucose Utilisation, and Phosphate Utilisation for *S.thermonitrificans* grown with Sparged Aeration in the Bioengineering Fermenter.

Figure 3.11 Photograph of the periphery of a single pellet of *S.thermonitrificans* grown in the conditions described for Figure 3.10 for 30 hours.
(Phase Contrast Photomicrography, 312x Magnification)

stress to superficial air velocity (a specific term describing the aeration rate in terms of vessel configuration) is postulated to be high.

3.9.2 Aeration of *Streptomyces* Cultures

Scale-up according to impeller-tip speed has been proposed for cultures of *Streptomyces* and shear sensitive organisms (Steel and Maxon, 1978; Nlenow, 1990), in place of scale-up on the basis of a constant power input or aeration efficiency (for description of these principles see a Biochemical Engineering text such as Bailey and Ollis, 1986). This implies that consistency between growth conditions for *Streptomyces* is more easily attained on the basis of a constant shear stress rather than constant agitation or aeration rates. A modified impeller has been designed for cultures of *Streptomyces,* and provides even mixing and a relatively low shear stress in relation to the aeration efficiency (Steel and Maxon, described in the review by Bushell,1988). In section 3.4.4, it was suggested that excessive shear stress during growth was potentially deleterious to the growth of *S. thermonitrificans.* Therefore an approach was taken to lower the shear stress during growth by a change in the fermenter aeration regime. A change in the overall configuration of the Bioengineering fermenter for growth of *S. thermonitrificans* was not feasible; but vortex aeration was considered a suitable alternative.

This is a relatively low-shear cultivation method in comparison with conventional sparged aeration regimes (G. T. Banks, personal communication). It is a relatively inefficient mode of aeration (in terms of power input), relying on gaseous entrainment via the development of a vortex at a turbine impeller. For the Bioengineering vessel, it required the removal of the sparger and baffle cage, together with the supply of air to the head of the vessel. Agitation was increased to a value at which the vortex reached the first impeller in the vessel - 700 rpm. Growth curves depicting growth in the 9 l Bioengineering using vortex aeration are presented in Figure 3.12.

Final biomass concentrations using this aeration method reached 1.5 $g.l^{-1}$ at 700 rpm for a glucose concentration of 6 $g.l^{-1}$ and the overall morphology of mycelium was improved.

$$\text{Yield} = \frac{1.5\ \text{g biomass}}{6\ \text{g glucose}} = 0.25\ \text{g biomass.g glucose}^{-1}$$

Microscopic analysis of morphology after 24 hours of cultivation showed a form which was indistinguishable from the mycelial morphology observed with fermentations on the complex medium, TSB. Initially, in the vortex-aerated

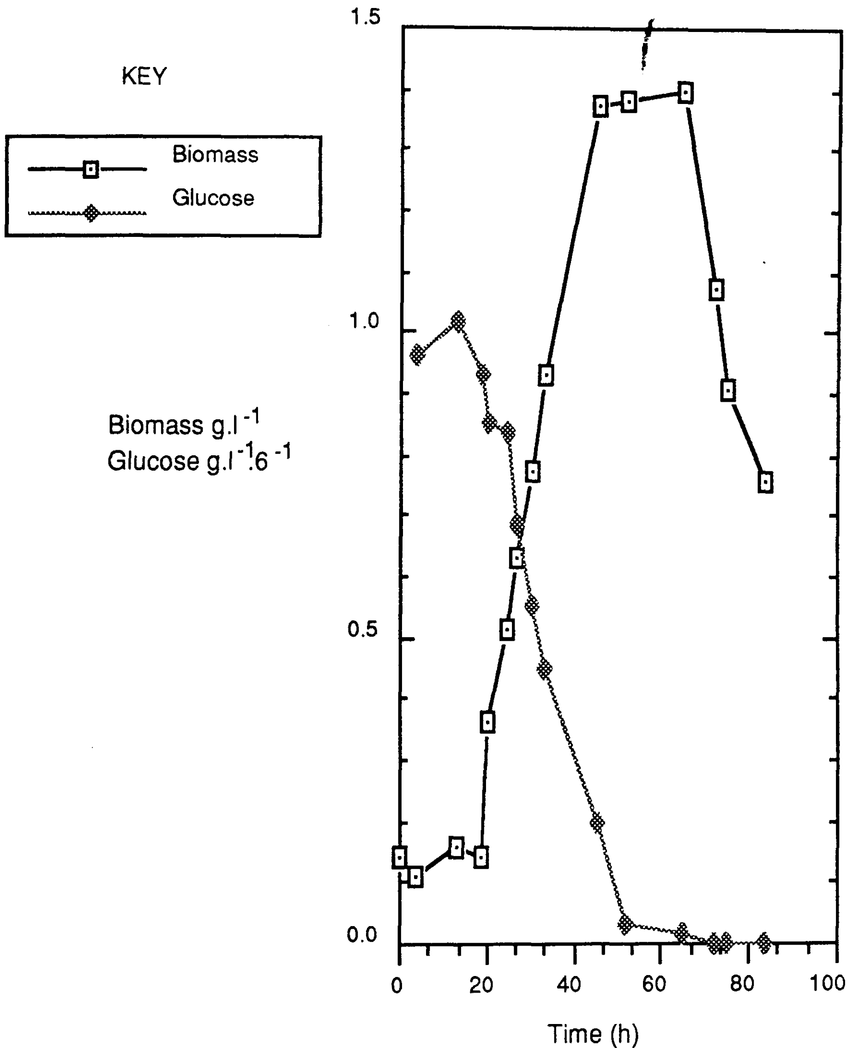


Figure 3.12 Growth Curve of Biomass Accretion and Glucose Utilisation for *S. thermonitrificans*, Grown With Vortex Aeration in a Stirred Tank Reactor.

fermentation using 579MM, the broth was composed of small pellets. However these had fragmented at 20 hours of cultivation, at which time the mycelium could be observed to be in the form of filamentous mats. In later fermentations using this aeration method the dissolved oxygen concentration was shown to drop to zero at about 24 hours post inoculation, contrasting with the fermentations described with sparger aeration (see 3.2.3). This suggested that the growth rate at this point in the fermentation was limited by the rate of supply of oxygen rather than due to an intra-hyphal diffusional resistance which was postulated to occur for the fermentations described in 3.2.3. In addition, the use of antifoams is not necessary in vortex-aerated fermentations, owing to the surface characteristics of the vortex, which disperse foam by entrainment. Foaming, during cultivation of *S. thermonitrificans* using sparger aeration, was found to be severe during the later stages of growth. Antifoam (polypropylene glycol) at a minimum of 0.1% (v/v) was essential in these conditions to prevent foam-over, and loss of the vessel contents through the exit air line. Water-immiscible antifoams result in heterogeneous media, which complicate analysis of total organic carbon in supernates. Therefore the absence of an antifoam requirement was an additional advantage to the vortex aeration method. It was used for all subsequent fermentations. Ideally the two aeration regimes (sparger and vortex aeration), should have been compared on the basis of the volumetric transfer coefficient K_La. However this was considered to be beyond the scope of the project.

For all experimental results and observations in cases of increased shear stress during cultivation, it was maintained that increase in shear stress in both flasks and the aeration regimes used in the fermenters would also increase the rate of oxygen transfer to the medium during cultivation. On the basis of a recent review, it is now thought that the increase in oxygen transfer rate as a result of cases of increased shear in cultivation is a major influence in the formation of pelletted broths of *S. thermonitrificans* , and not increased shear as had been postulated to this point in the project (Braun and Lifshitz, 1991). Hence the reduction in the rate of oxygen transfer, predicted to occur for vortex aeration at a specific agitation rate in comparison with sparger aeration, is now thought to be the major reason for the alleviation in pellet morphology, by a growth rate-mediated effect.

3.10 A Modified Inoculum Development Procedure for Use on a Fermenter Scale and In Large Matrix Experiments

The use of pre-germinated spores for 7 litre fermentations was considered impractical because of the excessively large numbers of spores required. Additionally, a 10% (v/v) mycelial inoculum pregrown in 579MM from pre-

germinated spores was unsuitable (see 3.11.0). The use of very high percentage inoculum ratios by using mycelial biomass concentrations greater than 10% (v/v), may have been appropriate, but fermentations with high concentrations of mycelial inocula have to be interpreted with care. If high biomass concentrations are used then the inoculum development procedures adopted may have a greater influence on subsequent growth, than the growth variables operable in the fermenter. Therefore, an alternative inoculum, which retains a high concentration of potential apical hyphae, with a low concentration of biomass was required.

Homogenisation of mycelial inoculum using a hand homogeniser was found to alleviate the combination of low final biomass concentrations and dense inter-pellet hyphal morphology when the fragmented mycelial inoculum was used in flask cultivation. The optimisation of mycelial fragmentation was therefore carried out. A washed, 30-hour culture of *S. thermonitrificans,* pre-grown on 579MM using pre-germinated spores was resuspended in a 10 % (v/v) ratio of 579MM with 10mM phosphate and 6 $g.l^{-1}$ glucose. The mycelial paste was aliquoted into two 30 ml centrifuge tubes and 5 g of 100 mesh glass beads added (1ml paste to 1g beads). The tubes were agitated using a vortex mixer (maximum setting) for a total of 30 minutes, cooling on ice for 20 seconds each minute. Samples were taken at discrete time points in triplicate, plated onto solid medium and incubated. A value for the colony forming unit ratio per ml original culture broth was calculated from the counts obtained per plate, shown in Table 3.8.

For increasing periods of vortex agitation, the number of viable colony forming units increased, with an agitation period beyond which units did not maintain viability. An inoculum development procedure, described in Figure 3.12 was adopted, based on these results. Inocula gave consistent results in flask trials and mycelial viability, following storage at -70^{0}C, was maintained for at least one month using this technique. For each series of fermentations, inocula were prepared in advance according to this procedure.

<u>3.10.1 Optimisation of the Fragmented Mycelial Inoculum Concentration</u>

It was necessary to identify a suitable concentration of mycelial inoculum for use in large-scale cultivation. Fragmented mycelium was used at the final concentrations listed in Table 3.9. In each experiment, flasks were inoculated in triplicate, pooled, 6-8 samples were taken for each variable and the experiment repeated. These results indicated that fragmented inoculum concentrations of around 1-2% (v/v) were adequate to achieve biomass concentrations of around 2 $g.l^{-1}$ in flask cultures. Fragmented inoculum

Table 3.8 The Response of *S. thermonitrificans* to Shear Stress Shown by the Viability of Colony forming Units ("cfu"s). Means of the duplicated, samples taken in triplicate are presented.

Vortex Time	Colony forming units/ml original culture volume
O seconds	Not determined
30 seconds	644 +/- 98
1 minute	640 +/- 52
2 minutes	696 +/- 84
3 minutes	652 +/- 64
4 minutes	676 +/- 40
8 minutes	668 +/- 168
10 minutes	1116 +/- 39
14 minutes	1044 +/- 160
20 minutes	2168 +/- 600
24 minutes	No growth
30 minutes	No growth

Figure 3.13 Mycelial Inoculum Development Procedure

Inoculate 400 ml of 579MM (6 gl^{-1} , 33 mM glucose, 10 or 3 mM phosphate) with pre-germinated spores at a concentration of 5 x 10^5 ml-1 final concentration of spores.

|

Harvest mycelium, wash and resuspend in 579MM, in 10% v/v of the original volume.

|

Add 1 g.ml-1 100 mesh glass beads, and agitate using a vortex mixer for 20 minutes, cooling on ice every 1-2 minutes. Sample and plate onto solid medium and into 3 ml L broth cultures to check for sterility.

|

Aliquot fragmented mycelium into 6 vials and freeze at -70^{0}C.

Table 3.9 The Influence of Mycelial Inoculum Concentration on Final Biomass Concentration. Means values are presented. The value for corrected biomass concentration is the final biomass concentration from each flask minus the mass of biomass introduced in the inoculum (fragmented mycelium at a concentration of 2 $g.l^{-1}$)

Inoculum Concentration (v/v)	Final Biomass Concentration ($g.l^{-1}$)	Corrected Final Biomass Concentration ($g.l^{-1}$)
0.5%	1.64 (+/- .20)	1.63
1%	2.29 (+/- 0.16)	2.27
2%	2.45 (+/- 0.12)	2.41
5%	2.10 (+/- 0.20)	2.00
10%	2.17 (+/- 0.64)	1.97) Lysis was apparent in these
50%	2.20 (+/- 0.31)	1.0) cultures
100%	Complete lysis of culture	

concentrations greater than 2% (v/v) did not appear to increase final biomass concentrations. Values higher than 2% (v/v) resulted in culture lysis within 48h, probably as a consequence of initially rapid growth rates, leading to a premature death phase. Lysis was detected easily by the refractory filtration shown by lysed cultures (glass fibre paper and high vacuum are required) and the gel layer which formed on the surface of glass fibre paper following filtration of these cultures.

Inoculum values of 0.5%(v/v) showed considerably reduced levels of biomass, suggesting that inoculum levels below 1%(v/v) were unsatisfactory to achieve maximum final biomass concentrations in flasks. This result also implies that a minimum level of point source inoculum is provided in a 1 to 2% (v/v) inoculum volume. Although initial growth rates may be increased (according to qualitative, visual examination during growth) using inoculum concentrations of 10% (v/v), the endpoint biomass concentration was relatively unchanged compared to lower inoculum concentrations. Biomass levels above 10% (v/v) would not be suitable for use as inocula due to the rapid lysis observed.

These results identified that a 1% (v/v) inoculum volume was adequate and would not preclude the attainment of maximum levels of biomass of around 2 $g.l^{-1}$ in flasks. The use of this mycelial inoculum concentration was therefore adopted for the final inoculum development strategy. This is described in the flow chart, Figure 3.13.

3.11 Final conclusions for this Chapter

This chapter has described some of the experiments which have been used to gain further insight into the growth characteristics of *S. thermonitrificans.* Inoculum development procedures, both for spore and mycelial inocula, have been developed which give reproducible, high concentrations of viable inocula. In addition, these inocula have not been subjected to nutrient shift down during development. The development of a fully-defined minimal medium, 579MM, based on a previously-reported medium has been described. This medium enables growth to suitable biomass concentrations (2 $g.l^{-1}$) both in flask and fermenter, and contains nitrogen and phosphate at non-limiting concentrations which are suitable for manipulation within a narrow range, for possible use in the expression of a product from a secondary metabolic biosynthetic pathway. Finally, optimisation of growth parameters for the requirements of the project, primarily inoculum and glucose concentration together with a fermenter modification permit filamentous mycelial growth of *S. thermonitrificans* in a 7 litre Bioengineering stirred tank reactor.

4.1 Introduction

Following the development of fermentation techniques which would allow an accurate assessment of microbial growth in defined conditions, it was considered desirable that a basic understanding of the physiology of *S. thermonitrificans* was developed. This would enable identification of areas and means by which a potential fermentation process could be optimised.

4.2 Qualitative Description of Batch and Continuous Microbial Culture

4.2.1 Batch Culture Microbial cells are commonly grown in batch culture; a semi-closed system in which mass may be exchanged freely by gaseous exchange, but solid and liquid component exchange is restricted. In simple batch culture the growth rate of exponentially-growing biomass tends towards zero as a nutrient limitation occurs. As a result, conditions in batch culture are continuously transient. The dynamic description of batch culture which follows below is a simplified, typical interpretation of a microbial fermentation. For a detailed account of the dynamics of microbial growth and cell composition in batch culture, a recent review is recommended (Wanner and Egli, 1990).

Lag phase. Following inoculation a period of slow growth is observed, commonly called the lag phase. This period of slow growth may be a result of the inoculum requiring to adapt to the new environment. It is particularly apparent if the inoculum has been prepared in medium with different composition to that of the growth medium, or has been chilled or frozen before use.

Exponential phase; this phase is defined as a period during which the growth rate of the population increases. Following adaptation to the new environment, cell growth rate increases to a maximum, when, under normal conditions, cell number, cell dry weight, intracellular DNA, RNA and protein are assumed to increase exponentially. All essential nutrients required for growth are present in excess such that the culture grows at the maximum specific growth rate. During this phase it is assumed that growth is governed by a factor intrinsic to the cell. Conditions are transient, as nutrients are depleted and products are produced and excreted, continually altering extracellular concentrations. It is common for species with high maximum specific growth rates and high oxygen requirements to reach a growth rate limit determined by the rate of oxygen supply to the medium. This is particularly evident in cultures with the potential to achieve high biomass concentrations, and as a consequence high oxygen

demand. In the case where the oxygen becomes limiting then the culture will continue to grow, but at a decreasing rate, determined by the method of oxygen supply. As the biomass concentration increases, the growth rate of the culture decreases. This is illustrated below:-

As $\frac{dx}{dt} = \mu x$ (assuming Monod growth kinetics where dx/dt is the rate of change of biomass concentration, x is the biomass concentration and μ the specific growth rate)

As the culture growth rate dx/dt is determined by the rate of oxygen supply, which is constant, then with increasing biomass concentration, the specific growth rate μ must decrease.

Stationary phase; This is defined as a period during which the proportion of actively growing species in the population declines. As mineral and carbon sources near exhaustion and products are continually excreted, then the culture enters stationary phase. The growth rate becomes limited by an exhausted nutrient, or growth inhibition occurs as a result of product accumulation. When an essential specific nutrient becomes limiting, the potential for *de novo* increase in cell material is absent. Viable cells in a non-carbon limited culture require energy for respiration. This is supplied as energy obtained by the catabolism of residual carbon substrate, or from the products of lysed, non-viable cells, whilst the limited nutrient may be considered to be recycled by lysis. No net growth is assumed to occur and the culture is described as being in stationary phase. Growth processes are balanced by death processes. Storage of energy reserves such as polyhydroxybutyrate may typically occur in stationary phase. This is observed in *Azotobacter* (Senior *et al.,* 1972).

Death phase; This is a period in which the proportion of non-viable cells increases to such an extent that death processes predominate over growth. In the case of a limiting energy source then continuous turnover of the energy source will result in a depletion in cell numbers and dry weight of the biomass as a result of both endogenous metabolism (cell turnover), and maintenance metabolism (described in detail in Section 4.5). The dynamic situation existing in a microbial culture is illustrated in Figure 4.l.

Nutrient limitation can result in conditions of unbalanced growth, typically coincident with exponential growth in chemostat culture, or stationary growth phase in batch culture. Examples of unbalanced growth are given by

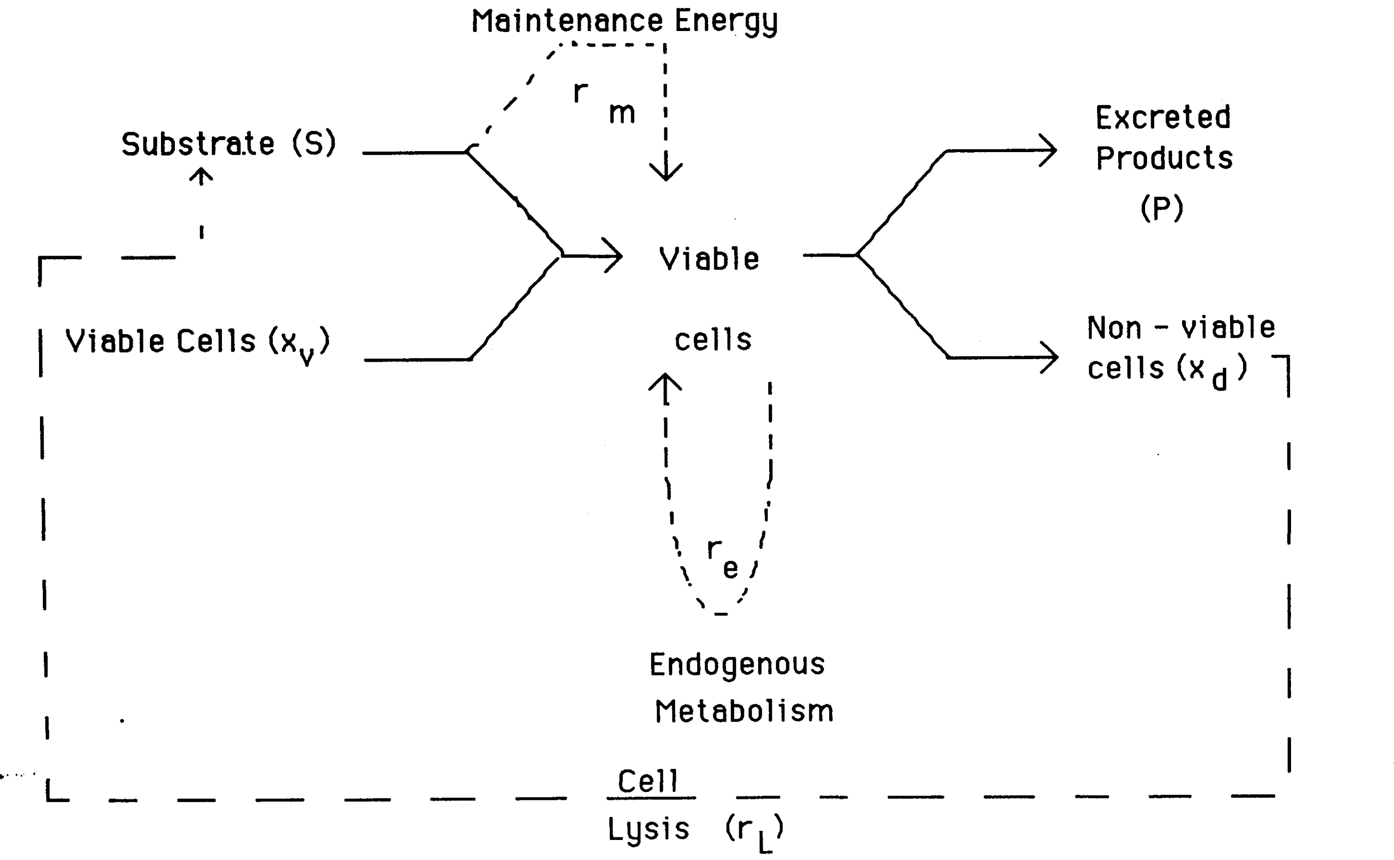


Figure 4.1 A Diagramatic Representation of the Energetic Interactions Existing in a Microbial Culture (adapted from Sinclair 1987).

Escherichia coli in carbon-limited batch culture, in which acetate excretion occurs (Holms and Bennett, 1971), *Klebsiella aerogenes* in potassium-limited chemostat culture in which acetate excretion occurs (Neijssel and Tempest, 1986) and *Erwinia herbicola* in carbon-limited chemostat culture in which exopolysaccharide production occurs (Linton *et al.*, 1988). In addition, nutrient limitation may trigger specific developmental changes in developmentally-complex bacteria such as *Bacillus* or *Streptomyces.* (Chater, 1984)

4.2.2 Continuous Culture

In contrast to closed (batch) systems of culture, exchange of medium and cells occurs in continuous (open) culture systems. Major advantages of continuous culture in comparison with batch culture are that growth rate is specified, nutrient conditions are constant and experimental variables may be manipulated independently. The reason for the defined nature of growth in continuous culture is illustrated mathematically by the biomass balance given in Figure 4.2.

The technique of continuous culture was proposed by Monod, and independently, by Novick and Szilard (all 1950). One system of continuous culture relies on maintaining a constant culture volume- the chemostat. This consists of a fermenter containing a microbial culture in exponential growth continuously fed with medium at a dilution rate less than the specific maximum growth rate of the strain. Regardless of the dilution rate of the chemostat, the culture is growing exponentially, and hence the Monod model for growth may be applied. The medium is supplied at a constant rate and contains all nutrients required for growth, of which one is limiting, and the remainder in excess. The volume of culture is maintained constant by harvesting culture at a rate equal to the influx of fresh medium. Following a transient phase of growth the culture enters a steady state, in which the rate of growth balances the medium dilution rate. Establishment of a chemostat system for continuous culture is described in Section 2.10.5. Biomass balances for both batch and continuous processes are described in Figure 4.2.

4.3 Disadvantages relating to the use of Continuous Culture

Aside from the difficulties experienced with filamentous organisms (described later), the study of any organism in continuous culture is subject to two major drawbacks. Firstly the data obtained and physiological parameters deduced are only applicable to the growth system used for the continuous culture studies, and cannot be directly translated to batch culture, or fed-batch culture (the favoured regimes for large scale production of most microbial products).

Figure 4.2 Setting Biomass Balances over both Open and Closed systems;

Batch

At any instant in time, then biomass concentration x in volume V, is V_x (g biomass. l^{-1}).

Therefore $\frac{d}{dt}(V_x) = V.r_x$

Where r_x is the rate of growth of x (g biomass.hr^{-1}).

As $r_x = x.\mu_x$

where μ_x is the specific growth rate (h^{-1})

and $\mu_x = \frac{\mu_m S}{K_s + S}$ (assuming Monod kinetics)

μ_{max} is the maximum specific growth rate (h^{-1}), S is the limiting substrate concentration ($g.l^{-1}$) and K_s is the saturation constant ($g.l^{-1}$),

$$\frac{dx}{dt} = \mu_m x$$

On integration, this gives

$$x = x_0 e^{\mu m t}$$

(Assuming the yield coefficient, Y_{xs} remains constant and $S >> K_s$)

The expression for the biomass balance incorporating post exponential growth phases results in an extremely complex balance equation, which may be integrated with difficulty;

$$\frac{dx}{dt} = \frac{\mu_m x [Y_{xs} S_{(0)} - x + x_{(0)}]}{K_s Y_{xs} + Y_{xs} S_{(0)} - x + x_{(0)}}$$

Continuous

Assuming steady state, then in an infinitessimally short time period, dt a culture with a specific growth rate μ (h^{-1}) in a volume V (litres) with an influx of medium at a rate F ($l.h^{-1}$), will grow according to the following relationship;

$$V.dx = \mu_x .dt. V - F.x\, dt$$

Dividing throughout by dt and V;

$$\frac{dx}{dt} = \mu .x - D. x$$

$$\left[\text{since } D = \frac{F}{V}\right]$$

At steady state, $\frac{dx}{dt} = 0$,

Therefore $\mu = D$

where D = dilution rate

This implies that the specific growth rate of the organism is defined by the dilution rate of the continuous culture system.

A range of basic physiological parameters for the organism in culture may be defined using mathematical transformations and experimental data, based on the biomass balance described above. The derivation of the kinetic parameters and constants, discussed in detail in Pirt, 1974, are very similar to those for the study of enzyme kinetics.

In contrast, studies undertaken in batch culture can be used to scale-up a process for operation on a larger scale. Nevertheless, the values and information obtained from continuous culture data are useful for approximation in alternative systems using the same organism and for comparison between strains. Knowledge based on experience with an organism in continuous culture may allow intuitive optimisation of a process using different growth conditions by a skilled operator. Continuous culture may be used to make a detailed analysis of aspects pertaining to the physiology of an organism, described in Pirt (1974) in more detail than is applicable here.

The material balances derived in Figure 4.2 rely on perfect mixing, and on the assumption of Monod kinetics.

4.4 The Reason for the Investigation into the Growth Energetics of *S. thermonitrificans*

It has been suggested that microbial energetics, from which the efficiency of growth may be judged, should be used to guide the selection of production strains (Stouthamer and van Verseveld, 1987). This would enable the prediction of maximum growth yields and provide some explanation for the influence of culture conditions on productivity. It was considered relatively important to have an appreciation of the growth energetics of *S. thermonitrificans* in order to assess whether the growth of this strain at elevated temperature was compatible with a potentially efficient antibiotic production process.

In an ideal situation, to study any aspect of the energetics of an organism, a system of continuous culture must be established. As has been described earlier, the physiological study of an organism in continuous culture is far preferable to its study during the transient, unsteady states existing in batch culture. It has been suggested by Bu'lock (1988) that the power of continuous culture in the investigation of key regulatory concepts is not being realised. This is probably due to the time-consuming nature of continuous culture experiments and due to the requirement for rigorous asepsis and genotypically-stable cultures. It was considered a high priority to attempt the establishment of a system of continuous culture for the study of some aspects of the physiology of wild-type *S. thermonitrificans*.

4.5 Energetic Consequences of Growth at Elevated Temperature

The requirement for maintenance energy to ensure the viability of organisms was predicted by Monod in 1942 and described by Pirt (1965). It is known that

the maintenance requirement is a function of temperature (Sonnleitner and Fiechter,1983: Wallace and Holmes, 1986). The value assigned to maintenance energy is commonly considered to include an endogenous metabolism term, although technically, maintenance and endogenous metabolism are distinct processes. The maintenance coefficient is correctly described as the energy required for processes other than growth, for example, maintenance of osmotic, pH and other ionic gradients, the maintenance of cell integrity in hostile environments and motility. It is derived from the oxidation of substrate. Endogenous metabolism is correctly described as the energy derived from the oxidation of cell substance to CO_2 (Tempest and Neijssel, 1984). However, maintenance energy and endogenous metabolism will be considered together, as their measurement cannot be divorced from each other, and the distinction may be regarded as theoretical rather than practical.

The observed increase in maintenance coefficient with temperature leads to reduced efficiencies of carbon conversion at elevated temperatures (Esener, 1983: Wallace and Holms, 1986). This may be simplistically explained; as rate processes increase with temperature, then degradative reaction rates increase proportionally, tending to damage the integrity of intact cells or hyphae. Replacement of cellular components at an increased rate is therefore necessary at elevated growth temperatures, to counteract the increased rate of degradation. In addition, passive processes such as diffusion are also faster at elevated temperatures and cellular transport processes therefore require an increased supply of energy to compete. However, thermophiles would be expected to be structurally adapted to growth at their optimum temperatures, and grow more efficiently than mesophiles at the elevated temperatures. Many enzymes from thermophilic species are used commercially as a result of their thermostability (particularly proteases in 'biological' washing powders).

As temperature has been documented to cause increases in the maintenance coefficient, the value of this coefficient would be important in assessing whether there would be greater economic advantage for the production of antibiotics at elevated temperature. It was realised that maintenance may be a large energy drain to the organism for growth at 45^{0}C. However, *S. thermonitrificans* was selected on the basis of an efficient growth performance (high final biomass concentration) in comparison with 13 other thermophilic *Streptomyces* from culture collections (3.1) cultured under identical conditions. Therefore, it was hoped that the maintenance requirement would be reasonably comparable with that of common streptomycete mesophiles. It was considered important to assess whether the strain is well-adapted, in terms of molar growth yield and the value for the maintenance coefficient for growth at these temperatures.

4.6 Continuous Cultivation of *S. thermonitrificans*

Continuous cultivation was established as described in Section 2.10.5 and a single experiment carried out. Conditions and methods for this system were established using batch culture and short periods of continuous culture (up to 48 hours) prior to this experiment. During continuous culture, samples for the determination of biomass and residual glucose concentration were collected and dilutions in water (from 10^{-2} to 10^{-9} with respect to the sample) of culture broth prepared for plating to single colony forming units. The morphology of these colony forming units was noted and any change in the population recorded. Fermenter broth samples were also visualised microscopically and photographed. Carbon dioxide output was used to confirm the attainment of steady state due to the extremely sensitive response to culture perturbations which may be observed using this on-line monitoring technique. Glucose concentration in the effluent stream was checked and confirmed to be zero for all samples. Data from continuous culture are presented in Table 4.1 and interpreted in Section 4.8.

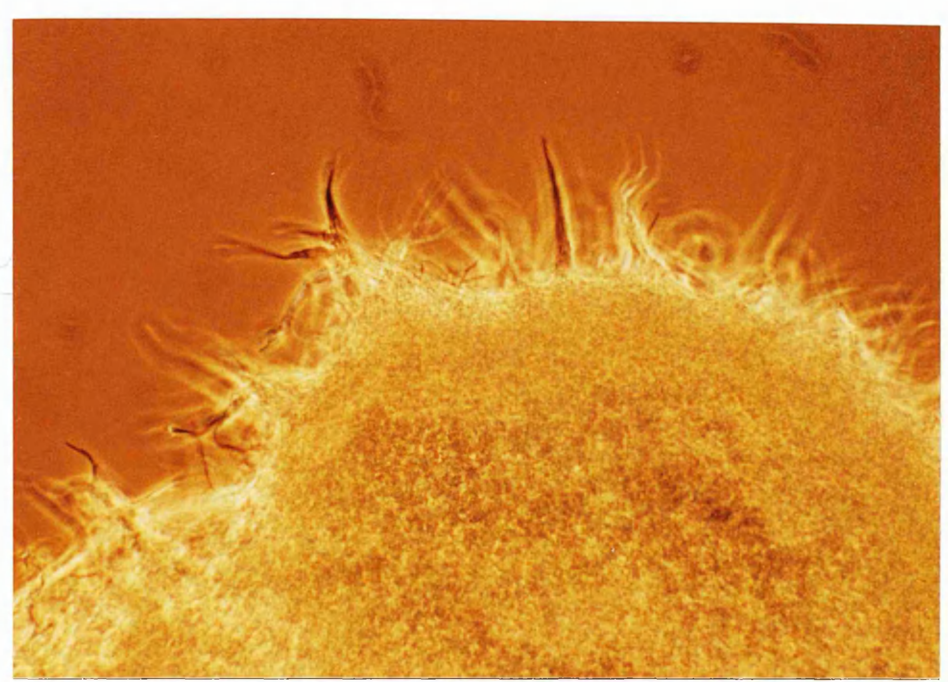


Figure 4.2 Mycelial Morphology for *S. thermonitrificans* in Batch Culture (18 hours) (Phase Contrast, 312x Magnification)

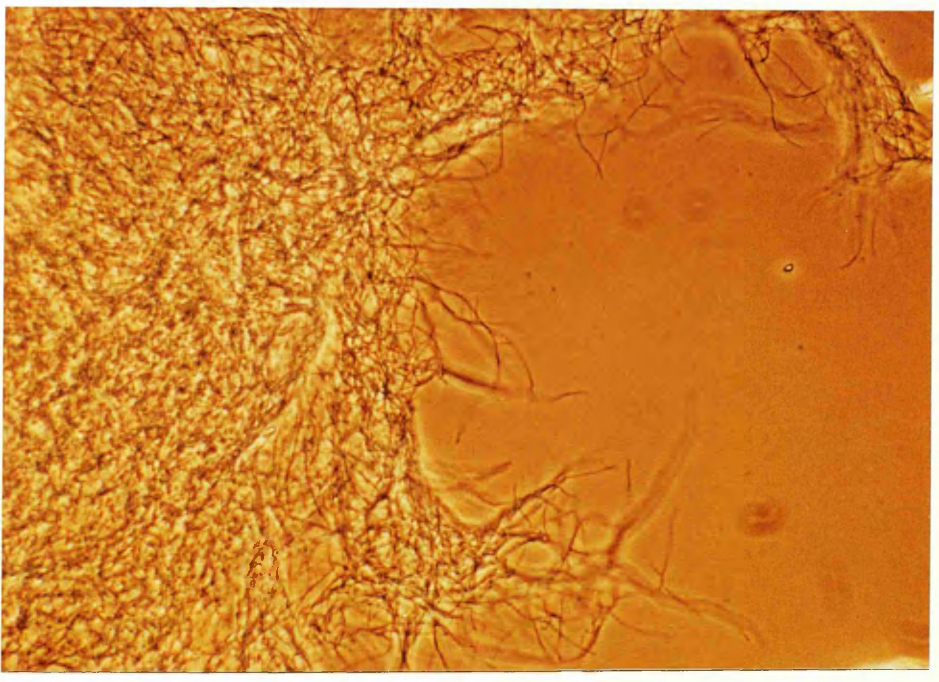


Figure 4.3 Mycelial Morphology for *S. thermonitrificans* in Continuous Culture (24 hours after initiation of inlet flow).
(Phase Contrast, 312x Magnification)

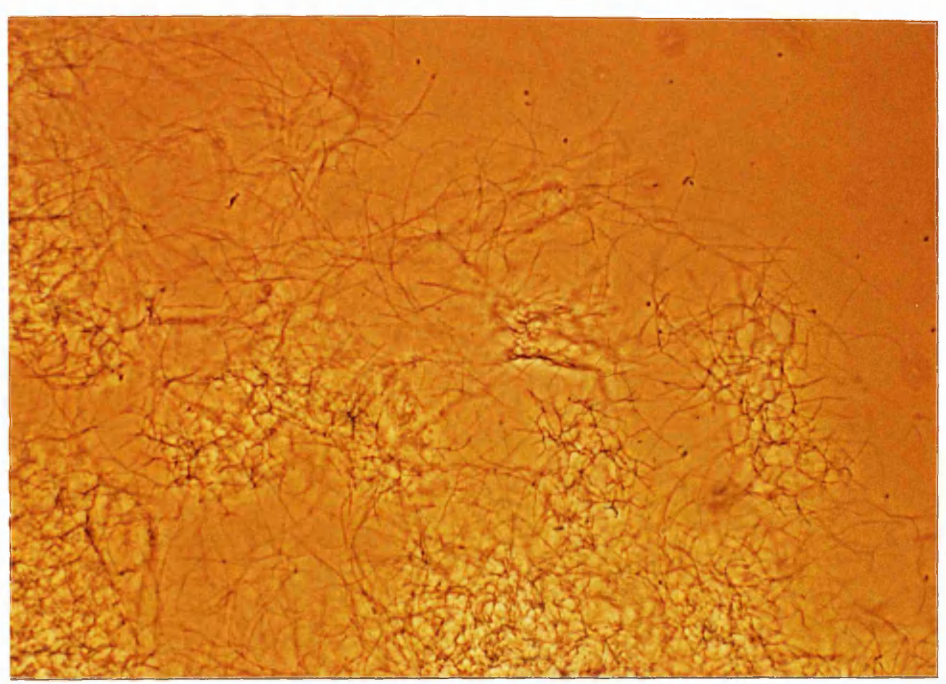


Figure 4.4 Mycelial Morphology for *S. thermonitrificans* in Continuous Culture (50 hours after initiation of inlet flow).
(Phase Contrast, 312x Magnification)

Apparent broth morphology; following inoculation, within the first 20 hours of fermentation, culture growth was in the form of small pellets up to 2-3mm in size. However, fragmentation of the pellets occurred around 20-21 hours, leading to a change in broth colour from colourless to slightly yellow and subsequent growth in the form of a mixture of large mycelial mats mixed with small pellets. In the past, attempts to establish continuous cultivation with pelleted cultures had led to washout of the culture even with extremely low dilution rates. This was probably due to a lack of mycelial fragmentation in these broths. Culture broths 24 hours after inoculation were similar in morphology to those found in flask-grown cultures on a complex medium, TSB, and were composed of large mycelial mats. Successful establishment of continuous culture could be attained using batch cultures after 28 hours of growth.

Within 24 hours of continuous culture the apparent broth morphology altered; the large mycelial mats were no longer present and the broth appeared uniform and pale cream in appearance. The viscosity of this turbid broth was increased over that of the original medium. When visualised through the microscope, a dense homogeneous suspension of loosely-branched mycelial mats could be seen.

Colony morphology; Diluted broth samples plated onto Emerson agar showed that some of the characteristics of the culture were changing as the fermentation progressed.

Firstly, in samples at 24 hours post-inoculation, during the period of batch culture preceding the switch to continuous culture, some colony forming units were identified as lacking the capacity to sporulate (postulated 'bald' variants). Bald colonies were characteristically of two types, large (up to 10 mm diameter) and small (2-3 mm). The large bald colonies disappeared rapidly following the switch to continuous culture, possibly due to fragmentation, and consequent decrease in colony size (see later. The smaller colony type became the major constituent until takeover by colonial variants after two generations. The rapid disappearance of large bald colony forming units following switch to continuous culture was mirrored by an apparent morphological change in the culture broth, from large dense mycelial mats, to a more fragmented, filamentous type of growth. The disappearance of the larger colonies could have been the result of a change in size of the initial mass of the colony-forming unit. Colonies of *Streptomyces* spread only a limited distance over the surface of agar, in contrast to those of fungi (Hopwood *et al.,* 1985) and a difference in inoculum mass would explain the difference in size of the colonies. All bald colonies were smooth-surfaced colonies raised above the surface of the agar and firm

when lifted using a wire loop or toothpick. The morphology of these postulated mutants remained constant following repeated transfer (2 times) to both Emerson and Soya agar, both media which normally support sporulation of *S. thermonitrificans.*

Following the switch to continuous culture, the appearance of a second morphological variant was identified at a low level in the total population of colony forming units (around 5%). These mutants were first identified 24 hours following the initiation of continuous culture at a dilution rate of $0.17h^{-1}$. The mutants were found to constitute 100% of the population at a 48 hour time point following initiation of continuous culture (*i.e.* 2 generations into continuous culture). From this time point the population contained only the second class of morphological variants (colonials). They were considerably flatter in appearance on the surface of agar compared to the bald colonies, with a 'pleated' surface morphology. They were generally capable of surface growth, with very little penetration of the agar, and spread over the surface of agar to a greater extent than the bald colonies and could be lifted easily with toothpick or wire loop. Sporulation of these colonies occurred infrequently, at a level of less than 1% of the total population of colonial variants. The morphological characteristics remained the same after two subcultures.

4.7 Mutation in Continuous Culture

Mutation to a colonial morphology is characteristic of filamentous organisms in continuous culture. The time of occurrence of these mutations in chemostat culture may be accurately predicted for given strains of fungi and *Streptomyces* (Trinci, 1990). These mutations are thought to occur as a result of the selective pressure for a fragmented mycelial morphology, which occurs when a chemostat is operating in the absence of oxygen limitation, with high agitation levels, and hence high levels of shear stress. Excessively high agitation levels of 900 rpm, predetermined in batch culture, were used in the LH fermenter in this study to ensure no oxygen limitation would occur in a culture of *S. thermonitrificans* containing 6 $g.l^{-1}$ glucose. This is probably the major factor implicated in the rapidity of take-over by colonial variants, together with the use of readily metabolisable carbon and nitrogen sources and an initial high rate of dilution.

In *S. coelicolor*, growth on a defined medium containing ammonia as nitrogen source and 2 $g.l^{-1}$ glucose enabled takeover by colonial mutants at around 20 generations, alternatively expressed as 20 dilutions (Hobbs, 1988 and 1989). However, the same medium containing sodium nitrate as nitrogen source, a less easily utilized nitrogen source, and the same glucose concentration could

be used to retard the takeover to about 60 generations. The colonial mutants produced during extended cultivation of *S. coelicolor*, in addition to losing the capacity for sporulation, produced an uncharacterised brown pigment and ceased the production of antibiotics normally produced by this strain, actinorhodin, methylenomycin and undecyl prodigiosin. It has been reported that adjustment of medium composition may extend the period before take-over occurs, presumably as a result of a change in selective pressure (Trinci, 1990).

Streptomyces are known to be subject to genetic instability at high frequencies (Birch *et al.*, 1990: Hopwood and Chater, 1990: Mathumathi *et al.*, 1990), and this inherent instability, in the presence of the selective pressures existing in a chemostat would lead to gross population changes. This is a problem with the use of continuous culture. However continuous culture may be suitable for the selection of mutants with improved morphology. Colonial or friable mutants are known to occur in *S. coelicolor* continuous culture under carbon limitation; in *S. rimosus* (I. Hunter personal communication); in *S. thermonitrificans* in continuous culture under glucose limitation (described above) and in *S. lividans*. These latter mutants, of *S. lividans*, occur in chemostat cultures under an unspecified limitation and the apparent morphology is not reported in detail. These mutants are described as 'slowly-sedimenting mycelium variants' (Grafe, 1990). Any morphological variants isolated from continuous culture would be required to be stable, but following the establishment of this prerequisite, then further development of the variant may be worthy of investigation. In production strains, where loss of host-encoded antibiotic production is disadvantageous, this approach to culture development may be inapplicable. However for a project in which *S. thermonitrificans* is merely a heterologous host, a novel approach may be to select for stable variants with enhanced morphology. Selection for enhanced growth rate and yield values in comparison with the parent strain could also be made using chemostats and turbidostats at high growth rates. A selected mutant could then be developed as a host for plasmid-encoded antibiotic pathways. Loss of antibiotic production capacity would be obviated by antibiotic selection for the retention of plasmid, although structural instability of the plasmid-encoded pathway could still occur.

Rapid mutation and take-over of a population of microorganisms in continuous culture is not a phenomenon unique to filamentous strains. In *Erwinia herbicola*, a plant pathogenic bacterium infecting plum fruit, a rapid mutation resulting in lack of exopolysaccharide production occurs in nitrogen-limited continuous culture and is selected in preference to the parental type (Linton *et al.*, 1988 and 1990). This mutation and selection occurs reproducibly between 17-19 generations at a dilution rate of $0.044h^{-1}$ and 10 generation times at a dilution rate of $0.23h^{-1}$. This results in a decrease in the rate of polysaccharide

production from 0.37 $g.g^{-1}.h^{-1}$ to $0.03g.g^{-1}.h^{-1}$. Loss of exopolysaccharide production does not appear to result in any increase in growth efficiency. In *Agrobacterium radiobacter*, exopolysaccharide mutants may be selected by prolonged chemostat culture and show almost identical growth energetics to the parental strain (Linton, 1990). The reason for mutation to deficiency in exopolysaccharide production cannot be adequately explained by microbial energetics. In addition, the viscous broths of highly branched *Streptomyces* cultures and the broths of exopolysaccharide producers have similar rheological responses to shear stress (predicted pseudoplastic responses, Banks, 1977 and personal communication). The mutation in both cases results in broths of low viscosity in conditions of high shear. However, the physical similarities of the resulting culture broths may be unrelated to the selective pressure and the similar result of mutation to broths of low viscosity in conditions of high shear may be a coincidence.

The apparent changes in broth morphology described during the establishment of continuous culture of *S. thermonitrificans* have also been described for chemostat culture in *S. tendae* ; with semi-defined medium and ammonia as the limiting nutrient (Lohr *et al.*, 1989).

4.8 Experimental Determination of Maximum Growth Yield and Maintenance Energy

Maintenance energy may be considered to be the energy required for the maintenance of viability. Substrate consumption is related to yield by $Y = x/S$ g biomass. g glucose^{-1}, therefore, $S = x/Y$, Y is the experimental yield, Y_{max} is the growth yield without a maintenance requirement)

Total rate of substrate consumption = Rate of consumption for Growth - rate of consumption for maintenance

$$\frac{\mu . x}{Y_{max}} = \frac{\mu . x}{Y} - mx$$

Therefore;

$$\frac{1}{Y} = \frac{1}{Y_{max}} + \frac{m}{\mu}$$

Where m = maintenance g glucose.g biomass.h^{-1}
μ = specific growth rate h^{-1}
Y = yield of biomass g dry weight.g glucose^{-1}
Y_{max} = Maximum growth yield (no m)

A double reciprocal plot of growth rate against yield with respect to substrate will give a line of slope m and intercept of $1/Y_{max}$ (Pirt, 1975)
The results have been interpreted in the light of the information introduced in

section 4.5 namely that after 2 generations a morphological variant took over the population in the chemostat of *S. thermonitrificans* and remained apparently stable. Hence the results are only presented as approximations for the purpose of discussion. In view of the results described for *S. coelicolor*, in which adjustment of the medium composition was required to ensure genotypically-stable populations, further work using continuous culture was not carried out (Hobbs *et al.*, 1988).

Ideally the genotypic stability of a culture in continuous culture should be confirmed under experimental conditions for at least 60 generations prior to implementing experiments. However, this is generally impracticable. To provide a complete set of data for the experiment described below, the range of growth rates ideally should have been increased, although dilution rates below $0.05h^{-1}$ are not generally accepted as compatible with a perfectly-mixed culture, and would not be considered (Stouthamer, 1990). In addition each experiment should establish a steady state twice at each dilution rate; first increasing dilution from low to high rates, and then reducing dilution rates to obtain the second set of values. Data for the two steady states corresponding to each dilution rate should correspond. The experiment would require repetition to confirm results. The experimental results for the determination of maintenance coefficient and maximum growth yield are given in Table 4.1 and Figure 4.5. Errors were determined by calculating 1/yield for each biomass value recorded, then calculating the standard deviations from these values.

Slope of line = $\frac{3.36 - 2.46}{18 - 6}$ = 0.075 g glucose.g biomass-1.hour-1

or 0.41 mmol glucose.$g^{-1}.h^{-1}$

The intercept on the Y axis (A) = 1.97

Therefore, maximum growth yield = 0.53 g biomass.g $glucose^{-1}$(inverse of A)

= 95.4 g biomass. mol $glucose^{-1}$

These values are presented as approximations and not rigorous determinations for reasons described previously.

Table 4.1 Continuous Culture Results for the Determination of Yield with respect to Dilution Rate.

Dilution rate	Biomass (g)	Yield (g cells.g glucose^{-1})
0.17	2.44 (+/- 0.14)	0.40
0.10	2.23 (+/- 0.18)	0.37
0.08	1.91 (+/- 0.09)	0.31
0.06	1.83 (+/- 0.14)	0.30

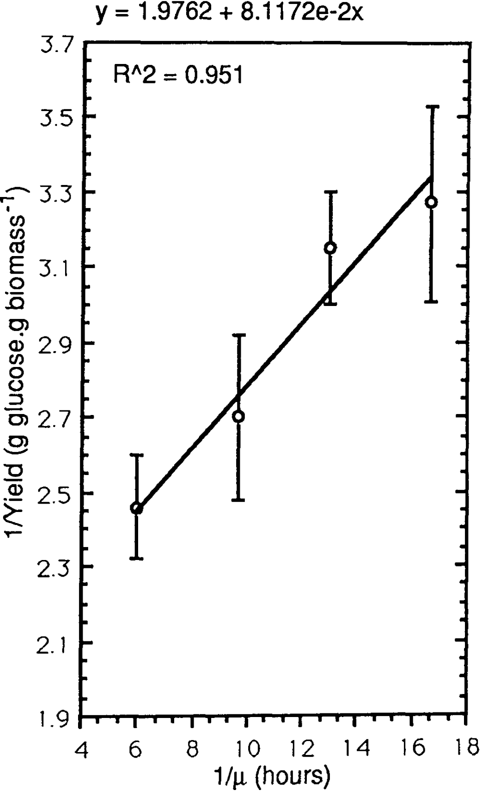


Figure 4.2 Plot of $1/\mu$ against 1/Yield for the Determination of the Value for the Maintenance Requirement and the Maximum Growth Yield

In terms of the total energy economy of the cell, substrate level phosphorylation is proposed to contribute an amount of energy that is of the same order of magnitude as that consumed for energy-requiring transport processes. Therefore, the combined influences of substrate-level phosphorylation and energy-requiring transport processes, being opposing, can be considered to be relatively small in comparison with the energy conserved during respiration (Jones, 1991).

4.9 Implications of the Experimentally-Determined (approximate) Value for the Maximum Growth Yield with respect to Glucose

Assuming a mean $Y^{ATP}/_{max}$ value of 13.5 g biomass per mole ATP for growth on glucose; and a value for $Y^{max}/_{O_2}$ of 60 g biomass per mole O_2 for an aerobic organism with a maximum growth yield of 100 g biomass.mole glucose^{-1}, (values taken from Jones, 1977) then the approximate ATP/O ratio for *S. thermonitrificans* (with a maximum growth yield of 95 g biomass.mole glucose^{-1}) - is given by;

$$\frac{ATP}{O} = \frac{Y_{max}\,O_2}{2.Y_{max}\,ATP} = \frac{60}{13.5 \times 2} = 2.2$$

(From Jones, 1988)

This relationship does not take into account any ATP generated by substrate level phosphorylation. However, in terms of the total energy economy of a bacterium, the energy conserved by substrate level phosphorylation is relatively small. It implies there are around 2 sites of respiratory chain energy conservation in the electron transport chain for *S. thermonitrificans*. Information concerning the respiratory chain composition of *S. thermonitrificans* from difference spectra suggests that it consists of *b* , *c* and an *aa-3* type (H^+-pumping oxidase) cytochromes together with a CO-reacting cytochrome oxidase *o* (Edwards and Ball, 1987). This is compatible with a respiratory chain containing up to 3 sites of energy conservation (Jones, 1977), and predicted higher growth yields than organisms lacking the cytochrome *c* and *aa-3* (Jones, 1988) The presence of both cytochrome *aa-3* and *c* are considerably significant in their influence on energy transduction, in relation to the efficiency of growth and product formation in industrial fermentations (Jones, 1991).

Respiratory chain composition and its applicability to yield studies is useful for discussion and comparison of the maximum potential of organisms. However, discrepencies between predicted and experimental values can occur, the result of physical deviations in membrane proton permeability, the presence of respiratory chains which may be branched, or futile cycles (Jones, 1988). Therefore, knowledge of the cytochrome content does not necessarily imply that the efficiency of energy transduction can be predicted. However, the value for the maximum growth yield for *S. thermonitrificans* (95g biomass. mole glucose^{-1}) would suggest that major deviations between predicted yield values (of 110g biomass mole^{-1} glucose) and experimentally-obtained maximum yield values do not occur. Therefore, futile cycles or branched respiratory chains are unlikely to be a major energy drain to this organism.

4.10 Implications of the Experimentally Determined (approximate) Value for the Maintenance Coefficient

The approximate maintenance requirement derived for *S. thermonitrificans* is large, but is comparable with some prokaryotic organisms grown in defined medium with glucose as the limiting nutrient. A comparison of some reported prokaryotic maintenance coefficients are given in Table 4.2 (from Stouthamer and van Verseveld, 1987). As a general rule prokaryotes have considerably larger maintenance coefficients than those of eukaryotes such as yeast and fungi (Stouthamer, 1990).

The large value for the approximate maintenance coefficient for *S. thermonitrificans,* 0.41, is comparable to that of *E. coli* (Baschnagel *et al.,* 1969) and *Bacillus licheniformis* in a recycling fermenter. This latter result illustrates the influence shown by the cultivation conditions on the maintenance value, alternating conditions of high and low glucose concentrations are known to result in high values for the maintenance coefficient. The data for *S. thermonitrificans* were obtained for growth with glucose at a concentration of $6.g.l^{-1}$ at an very high agitation rate of 900 rpm (to ensure dissolved oxygen was not limiting). In retrospect, it would have been more suitable to use a lower glucose concentration (and therefore a lower cell density), with a reduced agitation rate. Growth of microorganisms in high shear conditions results in high values for the maintenance requirement due to cell damage (Bayer, *et.al.,* 1990). Therefore the maintenance value for *S. thermonitrificans* cultivated in high shear conditions at a temperature of $45^{0}C$ is not unacceptably high, and it can be suggested that the maintenance drain for growth at $45^{0}C$ is not overly excessive. Ideally the maintenance value for *S. thermonitrificans* should be compared against the maintenance value for a commercial antibiotic-producing mesophilic streptomycete in continuous culture under glucose limitation. However, no maintenance coefficients for *Streptomyces* have been reported.

Although the maintenance requirement for *S. thermonitrificans* is relatively large, in commercial fermentations, the faster growth rate of a thermotolerant streptomycete may outweigh the influence of an increased maintenance requirement.

Table 4.2 Comparison of Some Published Values for the Maintenance Coefficient In Different Prokaryotic Organisms. All the data were derived from chemostat cultivation under glucose limitation.

Organism	Maintenance coefficient		Reference
	($mmol.g^{-1}.h^{-1}$)	($g.g^{-1}h.^{-1}$) *b*	
Escherichia coli	0.5 (calculated)	0.090	Baschnagel *et al.,* 1969
	0.4 (from *b*)	0.072	"
Klebsiella aerogenes	0.30		Stouthamer *et al.*, 1975
Bacillus licheniformis	0.24		Stouthamer *et al.*, 1975
Bacillus licheniformis	0.365 (grown in glucose - limited recycling fermenter)		Stouthamer *et al.*, 1975
S. thermonitrificans (approximately)	0.41	0.073	This work

It has been postulated by Neijssel and Tempest (1976) that, in contrast to the
mathematical derivation of Pirt, maintenance is a function of growth rate. A mathematical relationship for maintenance incorporating a growth rate dependent and a growth rate independent term has been postulated (Stouthamer, 1977: Chesbro, 1988). In addition, it has been shown that growth yield is influenced by the type of growth limiting nutrient. (Andrews, 1989). Further, in *Klebsiella aerogenes* and *E.coli,* the elemental composition of biomass has been shown to be influenced by the nutritional limitation employed in the chemostat and by the steady state specific growth rate (Esener and Roels, 1982: Bremer and Dennis, 1987). Overall, these reports suggest that the Monod model for growth of microorganisms is simplistic, and although it is found experimentally to be adequate, the derivation of more useful results may require the analysis of continuous culture data with a more complex growth model.

Many discrepancies exist in the literature with respect to the determination of the maintenance coefficient, both within species (which would be expected due to different cultivation regimes), but also within data sets for the same experiment. A method for establishing the consistency of published maintenance values and yields has been proposed (Solomon and Erickson, 1981), based on the use of the concept of 'available electrons' or reductance, true energetic yields and yield values for biomass with respect to oxygen, carbon dioxide and energy substrate in the same experiment. This method does not deal with cell composition differences; however, the small variations of biomass composition (a variation of 5-6% in *Klebsiella aerogenes*) may be disregarded providing extreme accuracy is not required (Esener and Roels, 1982). In addition the use of calorimetry in the estimation of maintenance values has been suggested, and used successfully with *Bacteroides* and *Selenomonas* (Russell,1986).

4.11 Further Use of Data from Chemostat Culture

A range of manipulations of chemostat data may be carried out to provide either more accurate or more detailed information concerning the physiology of a microbial culture. For a genetically-stable continuous culture of *S. thermonitrificans* these experiments and transformations would have been considered and are discussed below.

In a continuous culture at steady state the growth rate is equal to the dilution rate of an essential rate-limiting nutrient. If the dilution rate for a typical steady state culture is increased to a value beyond the maximum growth rate, then washout occurs. In this situation, the microbial culture can no longer grow at a

rate equivalent to the dilution rate and the rate of loss of biomass exceeds the growth rate, leading to washout. Washout may be used to derive the maximum specific growth rate. The microbial culture must grow at the maximum rate during washout, in view of the stress placed upon it. Therefore, the rate of growth determined under these conditions is the maximum specific growth rate. The rate may be determined from a plot of x (biomass concentration) against time at a dilution rate in excess of the maximum specific growth rate of the culture. The first order decay kinetics of this curve include terms for the dilution rate and the maximum growth rate, and as D is known, then μ_{max} can be determined. This is described mathematically below (Pirt and Callow, 1960; Pirt, 1975).

For $S >> K_s$ and $D > D_c$ where

D_c is the critical dilution rate ie the dilution rate at which steady state may just be maintained, when D ($l.min^{-1}.culture\ volume^{-1}$) = μ_{max} (h^{-1})
S is the reservoir limiting substrate concentration ($g.l^{-1}$)
K_S is the substrate saturation constant ($g.l^{-1}$)

then $$\mu = \mu_{max}$$

Therefore substituting μ_{max} for μ in the biomass balance under these conditions gives;

$$\ln x = (\mu_{max} - D)t + \ln x_{(0)}$$

A logarithmic plot of ln(x) against (t) would then give a curve with slope (μ_{max} - D). Dilution rate D is known, and hence μ_{max} may be deduced.

The washout determination of the maximum specific growth rate for *S. thermonitrificans* was not carried out, in view of the filamentous nature of streptomycete growth. This simple relationship between maximum specific growth rate and washout was considered inaccurate, as washout would be determined partly by the rate of culture growth, partly by morphology and by the morphological response to shear. Mycelial mats would be washed from the culture, and the total number of potential sources of apical hyphae would be reduced in contrast with unicellular cultures, when each cell is capable of further division. In a mycelial culture, only apical cells are capable of extension. Providing mycelial fragmentation occurred at a rapid frequency, then the number of potential sources of apical hyphae may be replenished at a rate in excess of the dilution rate. However if fragmentation does not replenish inocula at a suitable rate, then the relationship would not hold. Therefore, it was thought that an expression including a term related to morphology would be

required, and that simple experiments determining maximum growth rate from washout using the Monod relationship would be inaccurate. Determination of a model for washout of mycelial cultures was beyond the remit of this project.

The maximum specific growth rate is useful as a tool with which a process may be judged relative to alternative processes and a value which is useful in the optimisation of a process. An organism tends to grow in the most efficient mode close to the maximum growth rate, as the maintenance requirement is then at its lowest value (proportionally) with respect to biomass formation, and the specific processes which characteristically operate at low growth rates do not apply (Chesbro, 1988; Stouthamer, 1988 and 1990). These processes result in a lower maximum growth yield (Y_{max}) at slow growth rates. The consequence of slow growth is discussed in detail in the report by Stouthamer. It is therefore desirable that process development results in culture conditions in which the organism can grow at a value close to the maximum rate. In addition, rate and therefore productivity with respect to time are greater at higher growth rates. The potential of a strain for a high throughput process may be simplistically compared with alternative strains on the basis of μ_{max}.

In view of the genotypic instability of wild type chemostat cultures of *S. thermonitrificans*, further investigations of the physiology of the strain were conducted in flasks. These are described in Sections 4.11 and 4.12.

4.12 The Influence of Carbon Source on Growth in Defined Medium in Flask Cultures

Growth of an organism on a range of carbon sources may provide insight into the relative growth efficiencies on each source and into the presence and absence of specific catabolic pathways. Additionally, negative results may suggest transport limitations. In view of this, an investigation into the effect of various carbon sources was carried out, under identical conditions and concentrations with respect to carbon and inoculum.

A range of carbon sources (pH7), sterilised at 10 p.s.i. for 10 minutes at carbon concentrations equivalent to 6 $g.l^{-1}$ glucose (198 mg atom carbon) and 2 $g.l^{-1}$ (66 mg atom carbon) in defined media were prepared. These were inoculated with frozen mycelial inocula to a 2% (v/v) concentration (development of this inocula is described in 3.10.1). Two different concentrations of carbon source were used to observe any influence carbon source concentration might have on the quantities of biomass produced. Flasks were inspected daily and finally

Table 4.3 Investigation into the Influence of Carbon Source on Growth in Defined Medium

For concentration 198 mg atom Carbon

Carbon Source	Biomass
Glucose	1.26 $g.l^{-1}$ (+/- .04)
Lactose	0.03 $g.l^{-1}$ (+/- .01)
Galactose	0.07 $g.l^{-1}$ (+/- .03)
Fructose	0.12 $g.l^{-1}$ (+/- .03)
Xylose	0.06 $g.l^{-1}$ (+/- .00)
Mannose	0.07 $g.l^{-1}$ (+/- .02)
Mannitol	0.30 $g.l^{-1}$ (+/- .07)
Citric Acid	0.06 $g.l^{-1}$ (+/- .04)

Biomass values for all flasks except glucose at the 66 mg atom carbon were negligible and not recorded.

Qualitative Results from Secondary Flasks

Carbon Source 198 mg atm C	Quality of Growth
Glucose	Excellent growth
Lactose	Very poor growth
Galactose	Poor growth
Fructose	Good growth but large pellets
Xylose	Very poor growth
Mannose	Poor growth
Mannitol	Relatively good growth
Citric Acid	No growth

Table 4.4 Investigation Into the Effect of Nitrogen Source on Growth in 579MM

Nitrogen Source	Biomass
Ammonium nitrate	1.03 (+/- 0.13)
Sodium nitrate	0.70 (+/- .096)
Casamino acids	6.08 (+/- 0.19)
Sodium glutamate	1.31 (+/- 0.15)
Ammonium chloride (control, nitrogen source used in 579MM)	1.21 (+/- 0.18)

harvested after 120 hours of growth, when it was judged that all potential growth would have occurred regardless of the length of lag phase and growth rate. Flasks were inoculated with mycelial inocula in triplicate and the experiment repeated. Fragmented mycelial inocula were used in this experiment to enable a high concentration of potential apical hyphae to be provided for subsequent growth. The fragmentation of mycelial inoculum had been optimised (described in 3.10). Results are shown in Table 4.3.

It was recognised that the mycelial inoculum had undergone a pre-adaptation for growth on glucose. In order to indicate whether this inoculum adaptation had an excessive influence on results, inocula (10 ml) from each flask with carbon concentration at 198 mg atom carbon following growth for 120 hours was used to inoculate a secondary flask containing the same carbon source at the same concentration. The final biomass concentration following incubation for a further 72 hours in the secondary flask was similar to that demonstrated in the primary flasks (data not shown, qualitative description given). Hence any influence in this experiment due to adaptation to a different carbon source was considered negligible.

The lack of growth on citric acid was probably due to a transport limitation and lack of growth on citrate is confirmed for this strain in the tables of Williams *et al.*, 1982. The utilization of glucose, fructose and mannitol, all of which are substrates that feed into the glycolytic pathway, suggests that glycolysis is active. The less efficient utilization of mannitol is surprising, in view of the more reduced status of this carbon source, which would be expected to give greater yields than glucose. The non-utilization of lactose may be explained if extracellular ß-galactosidase is not produced by this strain, or no transport system exists for direct uptake of intact lactose molecules. In *E. coli*, lactose is specifically uptaken by lactose permease. The poor utilization of the hexoses, galactose and mannose is surprising, and may suggest that transport limitations for these sugars exist. The non-utilization of xylose, a pentose sugar which feeds into glycolysis may imply again that a transport limitation possibly exists, or that heat degradation of the carbon source has occurred (although unlikely). In contrast to *E. coli* , transport systems have not been subject to detailed study in *Streptomyces*. However it is known that a phosphotransferase system does not exist in *Streptomyces* (D. Hodgson, personal communication to I. Hunter: Ramono, 1970).

These results show unequivocally that glucose is a preferred simple carbon source for *S. thermonitrificans* under the conditions used. Suitable alternative carbon sources would be mannitol and fructose. Glucose is considered ideal as a carbon substrate because the majority of quantitative physiological data

have been obtained for growth on glucose, enabling comparison of data from *S. thermonitrificans* with a wide range of alternative systems. Glucose was considered adequate for the project in view of the ease of assay and the wide range of simple colo rimetric analysis techniqes available. In addition, glucose is conventionally used as one of many cost-effective carbon feedstocks in commercial fermentation processes and hence is particularly suitable for a project, in which comparison with conventional mesophilic streptomycete fermentations ideally should be made.

4.13 Investigation of The Influence of Nitrogen Source on Submerged Mycelial Morphology of *S. thermonitrificans* in Flask Cultures

A study of the influence of nitrogen source on growth was considered important for a number of reasons. Firstly, it was found that growth on defined media in Erlenmeyer flasks resulted in pelleting of *S. thermonitrificans,* whereas pellet ing was absent from growth in complex media, or in defined media supplemented with yeast extract (described in chapter 3). In *S. thermoviolaceous*, another thermophilic streptomycete, the use of glutamate as sole carbon source in a defined medium was found to alleviate pelleted growth to the extent that it did not occur (James, 1990). Inocula concentrations of 6% (v/v) were a prerequisite for dispersed growth on this glutamate medium. The glutamate defined medium for *S. thermoviolaceous* encouraged the production of pelleted broths when glucose was used as a carbon source or when lower inoculum concentrations were used. Secondly, it has been shown with *S. coelicolor* in continuous culture that the use of ammonium as a source of nitrogen exacerbates the problem of take-over of the chemostat with filamentous mutants, described in 4.7. The use of sodium nitrate has been found to retard this takeover (Hobbs *et.al.*, 1988). It was therefore important to consider alternative inorganic nitrogen sources and assess their potential for use in a minimal medium for *S. thermonitrificans.* The use of amino acids as a source of nitrogen was rejected as incompatible with the ultimate use of carbon balancing for fermentation analysis.

Flasks of each nitrogen source, listed in Table 4.4, at a concentration of 50 mM with respect to nitrogen at pH7.0 and glucose at a concentration of 33 mM were inoculated with 2% (v/v) mycelium in triplicate. Nitrogen sources were sterilised by filtration. Samples were taken after 84 hours and the experiment was repeated. Mycelial morphology was analysed in a qualitative manner and biomass results are presented for each experiment in Table 4.4.

Surprisingly, pelleted broths were found in all cases, and the relatively large errors in biomass determination reflect the heterogeneous nature of the

pelleted broths. Growth was considerably increased in casamino acid-containing medium. This could have been a result of the mixture of carbon sources and a consequent increase in growth rate, in addition to the effect of a mixed organic nitrogen source. It can be concluded that the pelleted broths observed in flasks of *S. thermonitrificans* on defined medium, and in the lag and early exponential phases of growth in stirred tank reactors are not solely due to the use of an inorganic nitrogen source. Final biomass concentrations were greater in medium with ammonium chloride as an inorganic nitrogen source in comparison with sodium nitrate. However, the latter compound could serve as a suitable replacement for developing media specifically for use in chemostat culture, to retard the take-over by morphological mutants.

4.14 Final Conclusions for this Chapter

The continuous system of culture is undoubtedly superior for investigating *Streptomyces* physiology for the reasons discussed in this chapter. Data from continuous culture, in view of the simplicity of the material balance at steady state, is amenable to simple transformation yielding a range of useful growth parameters . Although the filamentous morphology of *Streptomyces* in continuous culture is problematic, this is not insurmountable providing developmental work takes place

In contrast, results obtained in batch culture do not grapple with the basic growth characteristics of a specified strain, although data from flask batch experiments are useful, particularly in the investigation of a large number of variables. Flask experiments are ideal in developmental work (such as that described in Chapter 3), although it should be noted that results have to be interpreted with caution due to the large number of dependent variables.

Experiments described in this chapter have identified that rapid mutation to a filamentous morphology in a glucose-limited chemostat occurs within 2 generations of the switch to continuous culture in *S. thermonitrificans* in high agitation conditions. This variant has an approximate maintenance requirement of 0.41 mmoles glucose. g biomass^{-1}. h^{-1}, and an approximate maximum growth yield of 95 g biomass per mole glucose. Providing the variant is similar in energetic terms to the parent, then these energetic values suggest the strain may be may be well adapted to growth at elevated temperatures, as the maximum growth yield is comparable with other prokaryotic mesophiles. Discussion of the growth energetics of *S. thermonitrificans* is continued in Chapter 5.

The final conclusion has to be that *S. thermonitrificans* is an adequate host for

use in expression studies at elevated temperature with regard to the investigation of potential production efficiency in economic terms. Energetic performance is comparable with that of prokaryotic mesophilic bacteria. It grows at an optimum temperature of around $45^{0}C$, a temperature at which large savings in cooling requirements will be made on a production scale. Additionally, the strain is amenable to physiological study, using techniques developed and discussed in Chapter 3 and this Chapter, and has a maximum specific growth rate in excess of $0.17h^{-1}$, determined by continuous culture (Table 4.7). Successive work described in later chapters details 7 litre batch fermentations analysed by the techniques of materials balancing (Chapter 5), attempts at developing a recombinant *S. thermonitrificans* with the capacity to produce oxytetracycline (Chapter 6) and development of a purification scheme for an unknown antimicrobial product (Chapter 7).

CHAPTER 5

Fermentation in a 7 litre Stirred Tank Bioreactor and Data Analysis using Material Balances

5.1 Introduction

Following the development of techniques suitable for growth of *S. thermonitrificans* in a stirred tank reactor, it was planned to undertake the analysis of several fermentations. The final aim (in ideal circumstances) would have been to analyse fermentations with a recombinant *S. thermonitrificans* expressing an antibiotic pathway at fermentation temperatures of 37^0C and 45^0C; to compare the antibiotic productivity and energetics of the strain under the two growth conditions. This chapter describes the establishment of a system for such analyses and reports the microscopical mycelial morphology, on-line and off-line data for three replicate fermentations. A stoichiometric fermentation balance is presented and analysis of experimental data, using material balances, is shown. The mathematical treatment of physiological results could form the basis for a physiological (rather than a mathematical) model for growth of *S. thermonitrificans*; one way of achieving this is briefly described. Finally, the experimentally-derived yield values are compared with yield values obtained from the stoichiometric fermentation balance and the resultant deviations discussed.

5.1.1 Fermentation Data Analysis using Material Balances

For fermentation material balances, the macroscopic properties which relate to a population of organisms in a system bounded by the fermenter vessel are under investigation, in contrast to the properties relating to a single microbe, or metabolic pathway. This is the application of the formal principles of thermodynamics to a biological system. The microorganism is regarded as an open system in equilibrium with the conditions in the fermenter. Growth requires the availability of elements needed to form additional cell mass, and the free energy of substrates consumed should exceed the free energy of cells and metabolic products formed. A batch fermentation may be described as a semi-closed system in which energy may be exchanged freely with the universe, and mass may be exchanged freely by gaseous exchange, but solid and liquid component exchange is restricted following inoculation. Simple balances can easily be set over the system for each component , based on the first Law of Thermodynamics, illustrated in Figure 5.1 for an aerobic, heterotrophic microorganism. The inoculum acts as a catalyst for the formation of biomass.

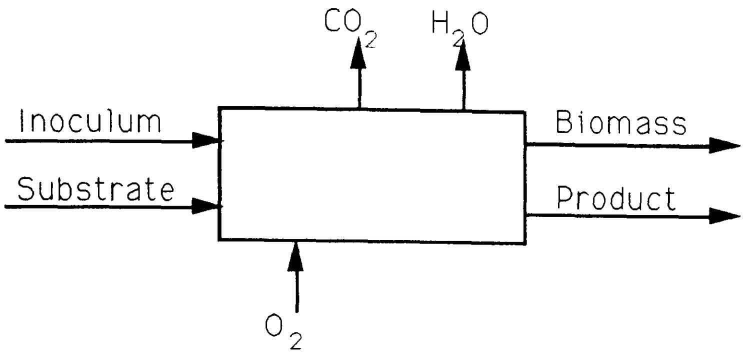


Figure 5.1 System and Flows for the Macroscopic Analysis of Growth and Product Formation in an Aerobic, Chemoheterotrophic Microorganism

Hence if the amount of an element contained in the system and the flows from the system is quantitated, it can be established whether fermentation data are consistent. If there is a apparent discrepancy in elemental recovery, then either a numerical error is present, or there is an additional source or flow that has been unaccounted. For repetitive fermentations of the same type, the conditions can be optimised, then analysed by the materials balance technique. The materials balance data can then be incorporated in a physiological model (Konstantinov and Yoshida, 1989). If the biomass elemental composition is known, then a stoichiometric fermentation balance for growth, or growth and product formation can be established for comparison with experimental balance data.

The theory of the application of the principles of macroscopic analysis and thermodynamics to biological systems is comprehensively reported by Roels (1980). Metabolic pathway analysis, based on the known biochemistry of a process would be an alternative method of achieving the same result as the method of material balancing presented in this chapter (Papoutsakis, 1984). However, the technique of material balancing is more suited to analysis of a process in a biochemically uncharacterised organism such as *S.thermonitrificans*. More recent papers include a comparisons of metabolic pathway analysis and material balancing, (Tsai and Lee, 1988 and 1989), physiological state control (Konstantinov and Yoshida, 1989) and state estimation in the presence of incomplete mass balance (Liao, 1989).

For carbon-balanced fermentations, the sum of CO_2 output, biomass and product accretion and residual substrate in common units of carbon should equal the input concentration of carbon substrate and inoculum. This simple balance was used to analyse fermentations of *S. thermonitrificans* grown in a 7 litre Bioengineering fermenter with vortex aeration and minimal medium (579MM) containing 3 $g.l^{-1}$ (33mM) glucose, 10mM phosphate and 50mM nitrogen (ammonium).

Fermentations and carbon dioxide exit gas analysis were established as described in 2.10.3 using media and techniques discussed in Chapter 3. Graphical representation of the on-line data for three replicate fermentations are presented in Figures 5.2 to 5.4. A discussion of this data is given in 5.2.2. A microscopical morphological profile of the fermentations is given in Figures 5.5, 5.6 and 5.7, described in Table 5.1. Off-line results are graphically presented in Figures 5.8 to 5.10, followed by a discussion of this data.

5.2.1 On-line Data

The Dissolved Oxygen Concentration. This is expressed as a proportion of the maximum dO_2 prior to inoculation (set at 100%). Following inoculation, a period of decrease in the dissolved oxygen concentration occurred from 10 hours to 30 hours; the rate of this decrease reaching a maximum during the period covering 20-30 hours (Figures 5.2-5.4). A minimum value for the dO_2 concentration was achieved around 30 hours for each fermentation followed by a very steep increase. This minimum dO_2 concentration coincided with the point of total glucose depletion (see Figure 5.6 to Figure 5.10). The sharp increase in dO_2 concentration following the minimum point is a result of the lack of glucose substrate and the consequential reduced requirement for oxygen as an electron acceptor.

Carbon Dioxide Output. On-line data presented in Figures 5.2-5.4 are expressed as a percentage of the corrected gas flow through the analyser (ie the carbon dioxide concentration in the fermenter effluent stream minus that in the inlet fermenter stream) Gas flows through the fermenter remained at one volume of air per volume of medium per minute (1vvm) and similarly, flow through the analyser remained constant. The on-line parameter is therefore a rate function and reflects the physiological state of the culture at a discrete point in time. Carbon dioxide output increased from around 10 hours after inoculation. Maximum rates of carbon dioxide output were achieved around 30 hours, followed by a sharp decrease. The curve for output of carbon dioxide may loosely be described as the reverse of the curve for decrease in dissolved oxygen concentration. In both cases the slopes reflected the inter-dependant influences of growth and substrate availability on gas exchange.

5.2.2 Microscopical Morphological Profile

In all fermentations carried out under these agitation conditions in minimal medium, small pellets formed early in the fermentation which fragmented by

Figure 5.2
On-line Data for
Fermentation F17

Figure 5.3
On-line Data for
Fermentation F20

Figure 5.4
On-line Data for
Fermentation F23

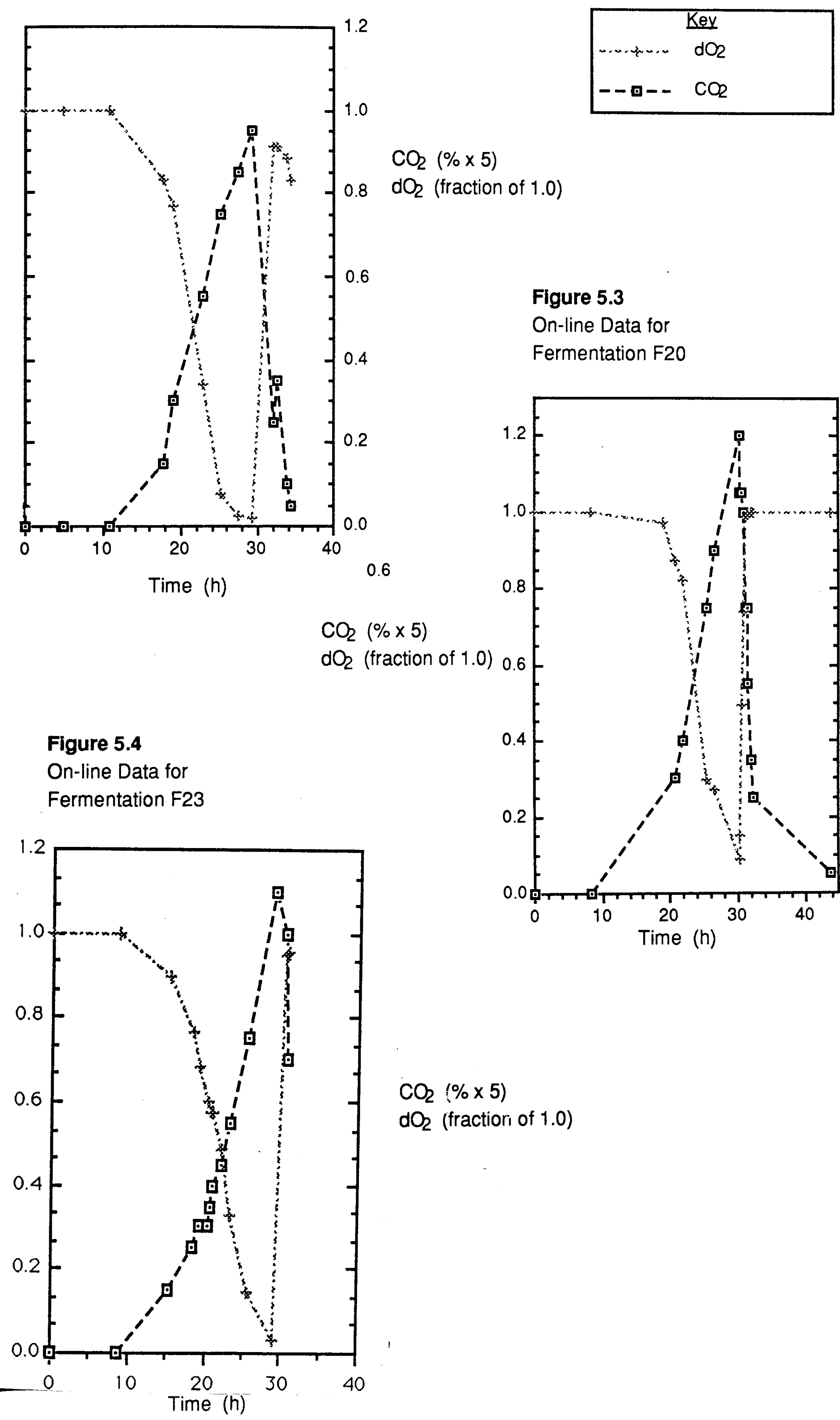


Table 5.1 Description of the Mycelial Morphology shown in Figures 5.5, 5.6, and 5.7

Frame	Harvest Time (inoculation at 0 hours)	Description
Figure 5.5		
17a	10.28	Small, discrete open-meshed pellets
17b, 17c	17.63 and 18.9	Small mats, groups of pellets
17d -17j	22.39 to 34.18	Mycelial mats
Figure 5.6		
20a	8.20	Small, discrete open-meshed pellets
20b, 20c	18.54	"
20d, 20e	20.28	Small mats, groups of pellets
20f, 20g	21.53-26.29	Mats of uneven hyphal density
20h-20j	30.09-43.54	Mycelial mats of even, open-meshed, hyphal density
Figure 5.7		
23a, 23b	8.30	Small, discrete open-meshed pellets
23c, 23d	15.12	Groups of pellets, single large dense pellet
23e	19.21	Mats of uneven hyphal density
23f-23l	21.0-30.48	Mats with near-even, open-meshed, hyphal density.

17a →

17b →

17c →

17d →

17e →

17f ←

17g ←

17h ←

17i ←

17j ←

Figure 5.5 Photographs of Mycelial Morphology for *S.thermonitrificans* during Fermentation F17 (Phase Contrast MIcroscopy, x312)

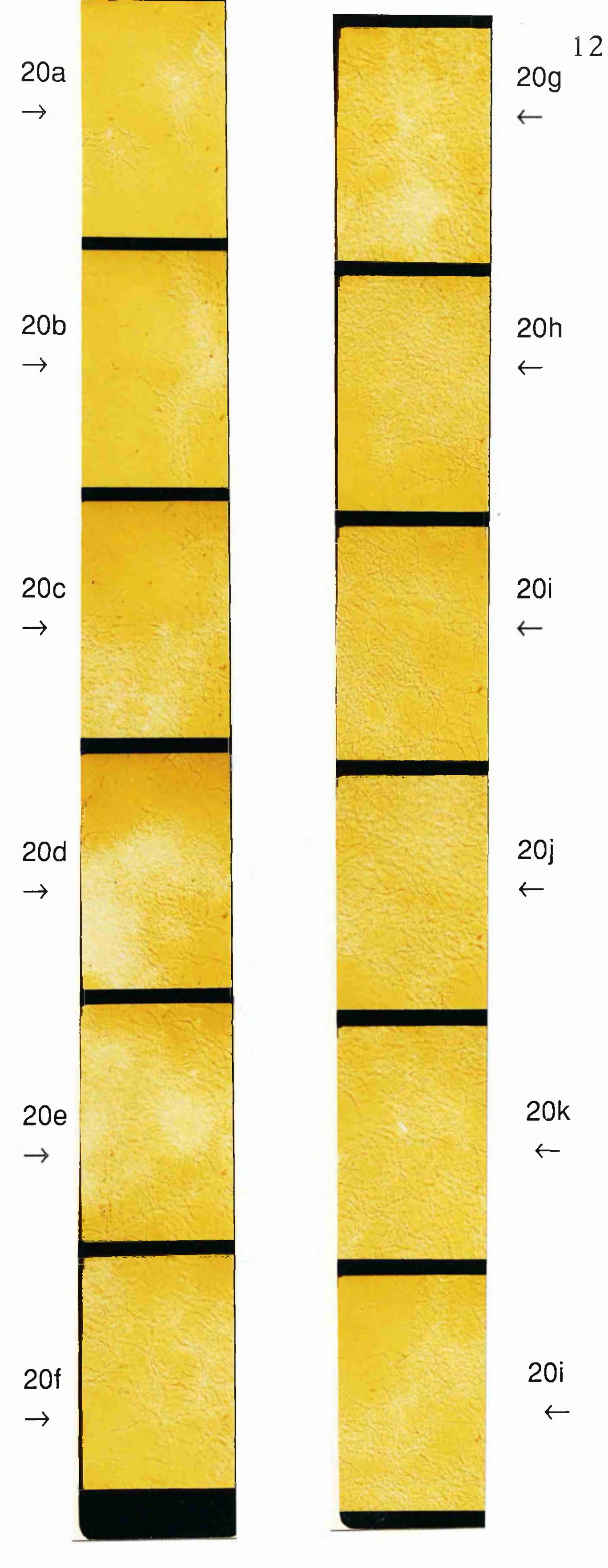


Figure 5.6 Photographs of Mycelial Morphology for *S.thermonitrificans* during Fermentation F20 (Phase Contrast MIcroscopy, x312)

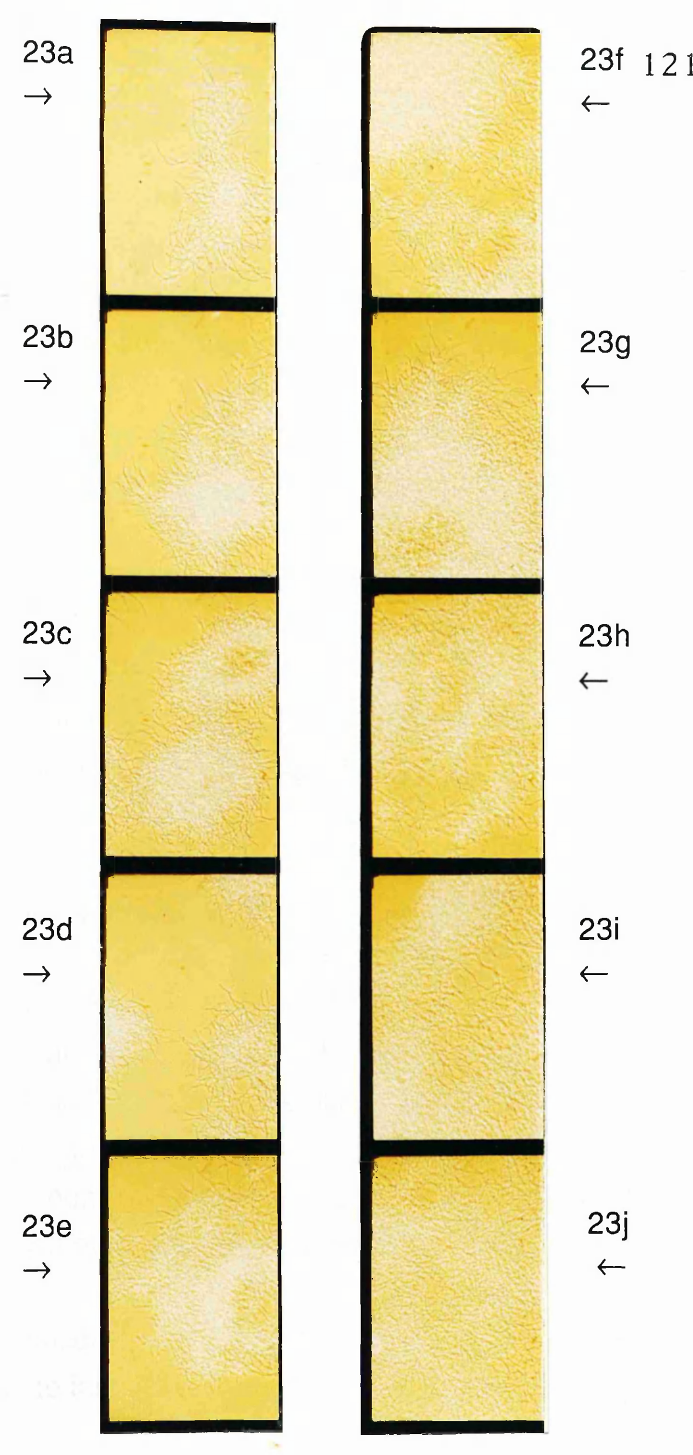


Figure 5.7 Photographs of Mycelial Morphology for *S.thermonitrificans* during Fermentation F23 (Phase Contrast MIcroscopy, x312)

about 20 hours at a dO_2 of around 50% (Figures 5.5, 5.6, 5.7). These small pellets were largely superposed by large wefts of open-meshed mycelia by 20 hours. The mycelial morphology then remained reasonably constant for the remainder of the fermentation. This pattern of mycelial morphology was consistent between fermentations under these conditions, and was almost indistinguishable from the morphology of *S. thermonitrificans* grown on the complex medium TSB. The mycelial morphology for *S. thermonitrificans* observed in these agitation conditions contrasted markedly with those of both flask cultures and stirred tank bioreactor cultivation (with the baffle cage in place, see 3.9). Details of harvest time and morphology descriptions are given in Table 5.1.

5.2.3 Off-line Data

Data for biomass accretion, cumulative CO_2 output, glucose depletion, phosphate depletion and ammonium depletion are presented in Figures 5.8 to 5.10

Biomass Accretion. Following inoculation, a phase of rapid growth with increasing growth rate and quasi-exponential kinetics occurred after 10 hours. In all cases a peak of biomass accumulation was attained after around 30 hours of growth, at a value of approximately 1 $g.l^{-1}$, which corresponded with the point of glucose depletion. A rapid decrease in the biomass concentration occurred after this point. This decrease was presumably the result of the endogenous metabolism of cell components to yield energy for maintenance of viability in an ever-decreasing proportion of the population.

Glucose Utilisation. The rate of glucose utilisation was initially very low. After 20 hours, the utilisation rate began to increase very rapidly until glucose exhaustion occurred at 30 hours.

Cumulative Carbon Dioxide Output. This is expressed in terms of millimoles and is the amount of gaseous carbon evolved during the fermentation. The curve for carbon dioxide output was apparently the image of the curve for glucose utilisation, for active growth of the culture (as would be expected). After 30 hours, in the absence of glucose, presumably cell material or extracellular products of lysis were consumed to fuel respiration processes. In this region, the slope for CO_2 output would be expected to be a function of the slope for biomass depletion.

Phosphate Utilisation. The curve for phosphate utilisation possessed a shallow slope in comparison with that for glucose. Following growth, phosphate

Figure 5.8
Off-line Data from
Fermentation F17

Key

- Dry Weight
- Phosphate
- Cumulative CO_2
- Glucose
- Nitrogen

1.2
1.0
0.8
0.6
0.4
0.2
0.0

0 10 20 30 40

Time (h)

Dry Weight ($g.l^{-1}$)
Phosphate ($mM.10^{-1}$)
Nitrogen ($mM.100^{-1}$)
Cumulative CO_2 ($mM.10^{-1}$)
Glucose ($mM.20^{-1}$)

Figure 5.9
Off-line Data from
Fermentation F20

1.2
1.0
0.8
0.6
0.4
0.2
0.0

0 10 20 30 40 50

Time (h)

Dry Weight ($g.l^{-1}$)
Phosphate ($mM.10^{-1}$)
Nitrogen ($mM.100^{-1}$)
Cumulative CO_2 ($mM.10^{-1}$)
Glucose ($mM.20^{-1}$)

Figure 5.10
Off-line Data from
Fermentation F23

1.2
1.0
0.8
0.6
0.4
0.2
0.0

0 10 20 30 40

Time (h)

Dry Weight ($g.l^{-1}$)
Phosphate ($mM.10^{-1}$)

Cumulative CO_2 ($mM.10^{-1}$)
Glucose ($mM.20^{-1}$)

was still in excess. In each fermentation, approximately 2.5 millimoles of phosphate were removed from the medium (per litre), leaving a final medium concentration for phosphate of 7.5mM.

Nitrogen Utilisation. Residual ammonium concentrations of 37 mM were found with all fermentations, in comparison to an initial concentration of 50mM. This illustrates that the inorganic nitrogen concentration of the medium is in excess of the requirements for growth. If it proved necessary to reduce the concentration of ammonium in the medium, a level of 25 mM would suffice for growth to final biomass concentrations of up to 2 $g.l^{-1}$.

5.3 Elemental Analysis of Biomass

To attempt a stoichiometric balance of the fermentation, an estimate of the proportion of specific elements in biomass was required. Elemental analysis was performed in the infancy of the project, prior to the development of 579MM. For this reason biomass was cultivated on TSB. An estimate of yield, reductance and respiratory quotient based on the elemental analysis of biomass is presented in the following section. Ideally, the elemental analysis should be repeated using mycelium cultivated in 579MM. The method is presented in Section 2.8.6.

Following the lyophilisation of 3 replicate samples, subdivided for lyophilisation, the 6 samples were subdivided again, to give a total of 12 samples; 3 replicates in quadruplicate. Results are tabulated in Table 5.2.

From the data shown in Table 5.2 an approximate value for the molecular formula of biomass may be obtained (by dividing each result by the atomic mass of the specific element so giving the number of gram-atoms and normalising to a carbon unit of 1.0). The ash content of the biomass (oxidised mineral components) was not determined, however, as phosphorous typically comprises 50% of the total ash (Bailey and Ollis, 1986), then an approximation for the residue can be made (proportion for phosphorous minus that of sulphur).

Analysis; C: 41.8%, H: 6.5%, N: 11.3%, P: 2.64%, S: 1.11 (estimate for ash 1.5%). By difference, O; 34.5%
(Number of g-atoms - C: 3.5, H: 6.5, N: 0.80, O: 2.15, P: 0.085, S: 0.034)
Empirical Formula; $CH_{1.85}O_{0.61}N_{0.22}P_{0.024}S_{0.010}$
Molecular Mass: 27.60g

The biomass elemental composition for *S. thermonitrificans* is in the same

Table 5.2 Elemental Analysis of Freeze-Dried Biomass of *S. thermonitrificans*

Flask Sample	Element	Proportion in Biomass (%)	Standard Deviation
Mean of A	Carbon	41.4	0.48
Mean of B	"	42.2	0.27
Mean of C	"	41.97	0.26
Mean of A, B and C	"	41.86	0.20
Mean of A	Hydrogen	6.58	0.08
Mean of B	"	6.53	0.06
Mean of C	"	6.50	0.07
Mean of A, B and C	"	6.53	0.004
Mean of A	Nitrogen	11.24	0.17
Mean of B	"	11.48	0.01
Mean of C	"	11.4	0.09
Mean of A, B and C	"	11.3	0.67
Mean of A	Phosphorous	2.35	0.04
Mean of B	"	3.11	0.01
Mean of C	"	2.46	0.12
Mean of A, B and C	"	2.64	0.81
Mean of A	Sulphur	1.23	0.35
Mean of B	"	1.09	0.15
Mean of C	"	1.00	0.10
Mean of A, B and C	"	1.11	0.12

Table 5.3 Reported Biomass Compositions for Organisms using Glucose and Ammonia as Sources of Carbon and Nitrogen.

Organism	Elemental Formula	Reference
"Average" microorganism	$CH_{1.79}O_{0.50}N_{0.20}$	Roels, 1980
Brevibacterium spp.	$CH_{1.625}O_{0.50}N_{0.125}$	Erickson *et al.,* 1979
Saccharomyces cerevisiae	$CH_{1.7}O_{0.5}N_{0.17}$	Wang *et al.,*1967
Saccharomyces cerevisiae	$CH_{1.64}O_{0.52}N_{0.16}$	Harrison, 1967
Saccharomyces cerevisiae	$CH_{1.83}O_{0.56}N_{0.17}$	de Kok and Roels,1980
Saccharomyces cerevisiae	$CH_{1.81}O_{0.51}N_{0.17}$	Wang *et al.,* 1976
Candida utilis	$CH_{1.83}O_{0.54}N_{0.17}$	Herbert 1976
Candida utilis	$CH_{1.87}O_{0.56}N_{0.20}$	"
Candida utilis	$CH_{1.83}O_{0.46}N_{0.19}$	"
Candida utilis	$CH_{1.87}O_{0.56}N_{0.20}$	"
Klebsiella aerogenes	$CH_{1.73}O_{0.43}N_{0.22}$	"
Klebsiella aerogenes	$CH_{1.73}O_{0.43}N_{0.24}$	"
Klebsiella aerogenes	$CH_{1.75}O_{0.437}N_{0.17}$	"
Escherichia coli	$CH_{1.77}O_{0.49}N_{0.24}$	Bauer and Ziv, 1976
S. cattleya	$CH_{1.6}O_{0.58}N_{0.17}$	Bushell and Fryday, 1983
S. thermonitrificans	$CH_{1.85}O_{0.61}N_{0.22}$	Present study

Table 5.4 A Summary of the Reported Stoichiometric Parameters for Some Organisms Derived from Analysis of Biomass Composition.
Underlined values are those determined by calculation (Section 5.5).

Organism	Yield (g.g glucose^{-1})	Reductance	RQ	Reference
"Average" microorganism (mean of 19 formulae)	0.68	4.19	1.33	Roels, 1980
Brevibacterium spp.	---	4.25	---	Erickson *et al.,* 1979
S. cerevisiae	0.50	4.19	1.04	Cooney *et al..,* Wang *et al.,* 1977
Candida utilis	0.54	4.12	1.16	Herbert, 1976
Candida utilis	---	4.27)	---	(Means from
Klebsiella aerogenes	0.66	4.20)	1.38	Roels 1980)
S. cerevisiae	---	4.20)	---	"
Escherichia coli	0.69	4.07	1.1	Bauer and Ziv, 1976
S. cattleya	0.78	3.93	0.8	Bushell and Fryday, 1983
S. thermonitrificans	0.70	3.97	1.0	Present study

range as the composition reported for other species, shown in Table 5.3. A detailed fermentation analysis of the stoichiometry suggested by the elemental composition is presented in Section 5.4.1. The proportion of carbon in biomass (41.8%) shown in Table 5.3 was used in the carbon balance analysis (Section 5.5).

5.4.0 A Fermentation Balance Equation for *S. thermonitrificans* based on Stoichiometry

A fermentation balance equation for biomass accretion based on stoichiometry may be calculated, based on the molecular formula for biomass of *S. thermonitrificans* ;

$$0.22\ C_6H_{12}O_6 + 0.22\ NH_3 + 0.30\ O_2 + 0.22P + 0.010S \rightarrow CH_{1.85}\,O_{0.61}\,N_{0.22}\,P_{0.022}\,S_{0.010} + 0.3\ CO_2 + 0.72\ H_2O$$

$$(0.22 \times 180\ \text{g glucose} \rightarrow 27.34\text{g biomass})$$

From this, a stoichiometric yield value (g cells/g substrate) may be calculated;

$$\text{Yield} = \frac{27.60}{0.22 \times 180}\ \text{g biomass. g glucose}^{-1}$$

$$= 0.70\ \text{g biomass g glucose}^{-1}$$

$$= 126\ \text{g biomass mole glucose}^{-1}$$

A comparison between this stoichiometric yield value and the experimentally obtained value is discussed in section 5.9. A value for the degree of reductance of the biomass may be derived based on the analysis of Erickson (1979). In this analysis the number of available electrons in one g atom of biomass is determined assuming carbon has a reductance of 4, hydrogen 1, oxygen -2 and nitrogen in biomass and ammonia -3 (nitrate has a value of -2).

$$\text{Reductance degree of biomass} = 4 + 1.85(1) + 0.61(-2) + 0.22(-3) = \underline{3.97}$$

A stoichiometric RQ value may be derived;

$$RQ = \frac{\text{Ratio of C02 output}}{\text{Input of O2}} = \frac{0.30}{0.30}$$

$$\text{Therefore RQ} = \underline{1.0}$$

These results are summarised in Table 5.4, in which the values are compared with available values for the same parameters from other organisms.

5.4.1 Determination of Stoichiometric Parameters for *Klebsiella aerogenes, Escherichia coli* and Roels "average microorganism"

A balance equation for biomass accretion based on stoichiometry may be calculated for *Klebsiella aerogenes* and *Escherichia coli* using the mean of the elemental composition reported by Herbert, 1966 and Bauer and Ziv, 1976;

For *K. aerogenes;*

$0.20\ C_6H_{12}O_6 + 0.22\ NH_3 + 0.145\ O_2 \rightarrow CH_{1.75}\ O_{0.44}\ N_{0.22} + 0.2\ CO_2 + 0.65\ H_2O$

(0.20 x 180 g glucose $\rightarrow$ 23.87g biomass)

Yield = 23.87/ (0.20 x 180) cells. g glucose^{-1} = 0.66 g biomass. g glucose^{-1}
= 119 g biomass. mole glucose^{-1}

$RQ = \frac{\text{Ratio of C02 output}}{\text{Input of O2}} = \frac{0.20}{0.145}$ RQ = 1.38

For *E. coli;*

$0.20\ C_6H_{12}O_6 + 0.24\ NH_3 + 0.18\ O_2 \rightarrow CH_{1.77}\ O_{0.49}\ N_{0.24} + 0.2\ CO_2 + 0.68\ H_2O$

(0.20 x 180 g glucose $\rightarrow$ 24.97g biomass)

Yield = 24.97/ (0.20 x 180) g cells g glucose^{-1} = 0.69 g biomass. g glucose^{-1}
= 124 g biomass. mole glucose^{-1}

Reductance degree of biomass = 4 + 1.77(1) + 0.49(-2) +0.24(-3) = 4.07

$RQ = \frac{\text{Ratio of C02 output}}{\text{Input of O2}} = \frac{0.20}{0.18}$ RQ = 1.1

For an "average microorganism";

$0.20\ C_6H_{12}O_6 + 0.20\ NH_3 + 0.15\ O_2 \rightarrow CH_{1.79}\ O_{0.50}\ N_{0.20} + 0.2\ CO_2 + 0.60\ H_2O$

(0.20 x 180 g glucose $\rightarrow$ 24.55g biomass)

Yield = 24.55/ (0.20 x 180) g cells g glucose^{-1} = 0.68 g biomass. g glucose^{-1}
= 122 g biomass. mole glucose^{-1}

$RQ = \frac{\text{Ratio of C02 output}}{\text{Input of O2}} = \frac{0.20}{0.15}$ RQ = 1.33

5.4.2 Comparison of the Stoichiometric Parameters for *S. cattleya, K. aerogenes, E. coli* and *S. thermonitrificans*

S. thermonitrificans in comparison with *S. cattleya,* had a lower theoretical yield value of 0.70 (compared to 0.78 for *S. cattleya*) and a slightly larger value for the reductance degree for biomass of 3.97 (3.93 for *S. cattleya*, Bushell and Fryday, 1983). The RQ for *S. cattleya* was considerably less than that for *S. thermonitrificans* (RQ of 0.8 for *S. cattleya* in comparison with 1.00 for *S. thermonitrificans*). The high yield value for *S. cattleya* was consistent with the oxidised state of the biomass (low value for the degree of reductance). In comparison with the calculated stoichiometric values for *K. aerogenes,* both streptomycetes showed yield values which were greater than the values for this organism; this would be expected from the reductance value for *K. aerogenes* (4.21). In addition, *K. aerogenes* was calculated to have a high RQ (1.33), as would be expected from the reduced state of the biomass. For *E. coli,* values for RQ, yield and reductance were close to those for *S. thermonitrificans* (degree of reductance of 4.07, yield of 0.69 and RQ of 1.1 for *E. coli,* in comparison with 4.00, 0.69 and 1.00 for *S. thermonitrificans*).

In the report by Bushell and Fryday the RQ for *S. cattleya* was markedly dissimilar to that of the microorganisms with which it was compared; a value of 0.8 in contrast to 1.04 for the eukaryotes *Saccharomyces cerevisiae* and 1.16 for *Candida utilis.* The addition of 3 further organisms for comparison appears to indicate a considerable stoichiometric difference between *S. cattleya* and other organisms, now including *E. coli, K. aerogenes* and *S. thermonitrificans.* Comparing the values for Roels "average microorganism" with *S. cattleya* and *S. thermonitrificans,* the RQ and reductance value for biomass of both streptomycetes tend towards a more oxidised state than the average. This may be a general trend for the condition of the biomass of *Streptomyces*, but would require a larger body of information for comparison, and statistical analysis.

It is relevant to note that the yields derived from stoichiometry are potential maximal yields. They may be relevant in the comparison of strains, but the stoichiometric relationship takes no account of inefficiencies in energy transduction and is therefore never achieved in experimental culture.

Hence the reductance and RQ of *S. thermonitrificans* is in the same range as that of *E. coli* and *S. cerevisiae,* whereas *S. thermonitrificans* differs from *S. cerevisiae* in terms of yield. In addition, the stoichiometric parameters for *S. thermonitrificans* differ from those of *K. aerogenes* and *C. utilis* as due to the reduced state of biomass for these two organisms, and from *S. cattleya* due to

the extremely oxidised state of the biomass of this streptomycete. However both *S. thermonitrificans* and *S. cattleya* tend to have biomass which is more oxidised than the "average microorganism" reported by Roels.

5.5 Fermentation Data Analysis

Based on the carbon balance shown in Figure 5.1, an experimental balance may be assessed by summation of the total organic carbon in the supernatant, the carbon in biomass and the cumulative gaseous carbon respired during the fermentation (inoculum concentration of 1%, $0.2 g.l^{-1}$ was assumed to be negligible). Total organic carbon analysis was performed using a Shimadzu TOC 50 analyser. The method is presented in Section 2.8.5. The fermentation balances are presented in Table 5.5.

The data show that all carbon can be accounted for. In addition, there was agreement between values for the total organic carbon in the supernatant and residual glucose (Table 5.6). This agreement suggests that extracellullar products were a minor carbon drain to the organism. The residual carbon levels (at 29 hours and onwards), which which were somewhat different from glucose concentration data, can be considered due to both cumulative errors and low concentrations of undefined extracellular products. These extracellular products could include lysis products.

Biomass accretion, glucose uptake and cumulative CO_2 excretion were converted into common units of carbon (millimoles of carbon atoms), and a plot of biomass against glucose and carbon dioxide in millimoles resulted in curves which intersected. They were subjected to linear regression (Figures 5.11, 5.12 and 5.13). By determination of the mean slope for the glucose utilisation curves (related to the yield with respect to glucose) and the mean slope of the CO_2 curves (related to the yield with respect to CO_2 output), a mean set of simultaneous equations were obtained. The data for determination of the mean values are presented in Table 5.7, from the equations presented below

For F17	$y = 116.58 - 2.40x$	Glucose utilisation
	$y = -6.35 + 1.31x$	CO_2 accumulation
For F20	$y = 122.16 - 2.69x$	Glucose utilisation
	$y = -3.43 + 1.49x$	CO_2 accumulation
For F23	$y = 129.84 - 3.16x$	Glucose utilisation
	$y = -5.26 + 1.84x$	CO_2 accumulation

The unit mMC should correctly be described as milligram-atoms of carbon. All references to this unit therefore should be interpreted as references to the unit milligram-atoms of carbon.

Table 5.5 Fermentation Carbon Balances (values of TOCA are corrected to allow 12 mM C due to citric acid as chelator in 579MM).

Time (hours)	Cumulative CO_2 (mMC)	Biomass (mMC)	Total Organic Carbon in Supernate (mMC)	Sum (mMC)	Fermentation Balance (%)
Fermentation 17 (Initial Glucose Concentration = 113 mMC)					
0-17.63h	3.0	12	98	113	100%
0-25.46h	22.5	29	54	105	93%
0-34.33h	73.1	24.8	12.3	110	97%
Fermentation 20 (Initial Glucose Concentration = 113 mMC)					
0-21.88h	13.0	8.7	94	116	103%
0-25.37h	22.0	18.2	77	117	103%
0-30.1h	51.3	40.0	18	109	97%
Fermentation 23 (Initial Glucose Concentration = 122 mMC)					
0-19.35h	11.0	13.0	96.4	120.0	98 %
0-21.02h	14.6	18.9	88.0	121.5	99%
0-29.13h	70.1	31.1	23.0	124.0	101.8%

Table 5.6 Comparison of Values for TOCA with Values for Residual Glucose Concentration for the same Samples.

Time	Glucose Concentration (mMC)	TOCA (mMC)
Fermentation 17		
17.63h	100	98.5
25.46h	60.0	54.6
34.33	0.0	12.3
Fermentation 20		
21.88h	93	94.6
25.37h	73	77
30.15h	20	18
Fermentation 23		
19.35h	93	96.4
21.02	91	88
29.13	14	23

The mean simultaneous equations are;

$y = 122.86 - 2.75x$ Glucose utilisation (a)

$y = -5.01 + 1.54x$ CO_2 accumulation (b)

Hence the mean yield with respect to glucose is 1/ slope of equation a), the curve for glucose utilisation with respect to biomass concentration

Slope of line described by equation a = 2.75

Therefore yield $= \frac{1}{2.75}$ g C in biomass. g C in glucose^{-1}

This value can be converted to g biomass. g glucose as shown below;

$$Y_{x/s} = \frac{1}{2.75} \times \frac{100}{41.8} \times \frac{72}{180}$$

Where carbon in biomass is 41.8% of dry weight (shown in Table 5.2)

$= 0.34$ g biomass. g glucose^{-1}

Therefore an accurate experimental yield value for growth of *S. thermonitrificans* with 3g.l^{-1} glucose in 7 litre stirred tank bioreactor (Bioengineering);

$Y_{x/s} = 0.34$ g cells.g glucose^{-1}

$= 61.2$ g cells.mole glucose^{-1}

This result compares favourably with the experimentally-obtained yield values for a range of mesophilic microorganisms, shown in Table 5.8, and with yield values obtained during fermentation development in which maximum yield values of 0.25 g biomass.g glucose (45 g biomass per mole glucose) were obtained, suggesting the fermentation yield has been improved, and the fermentation optimised to some extent.

If this approach had been continued (using line equations for the various carbon components), time course data could have been obtained, by curve fitting to the variable-time plots. The rate relationships for accumulation of biomass, carbon dioxide and glucose utilisation could be obtained (as equations of the curves) and subsequently analysed to determine the mean relationships. The yield and rate equations could then be combined to give a three dimensional mathematical description of the fermentation; a model derived from physiological parameters. Hence, in subsequent fermentations, measurement of carbon dioxide, with on-line data analysis would allow a

Figure 5.11
Regressed Carbon Data
for Fermentation F17

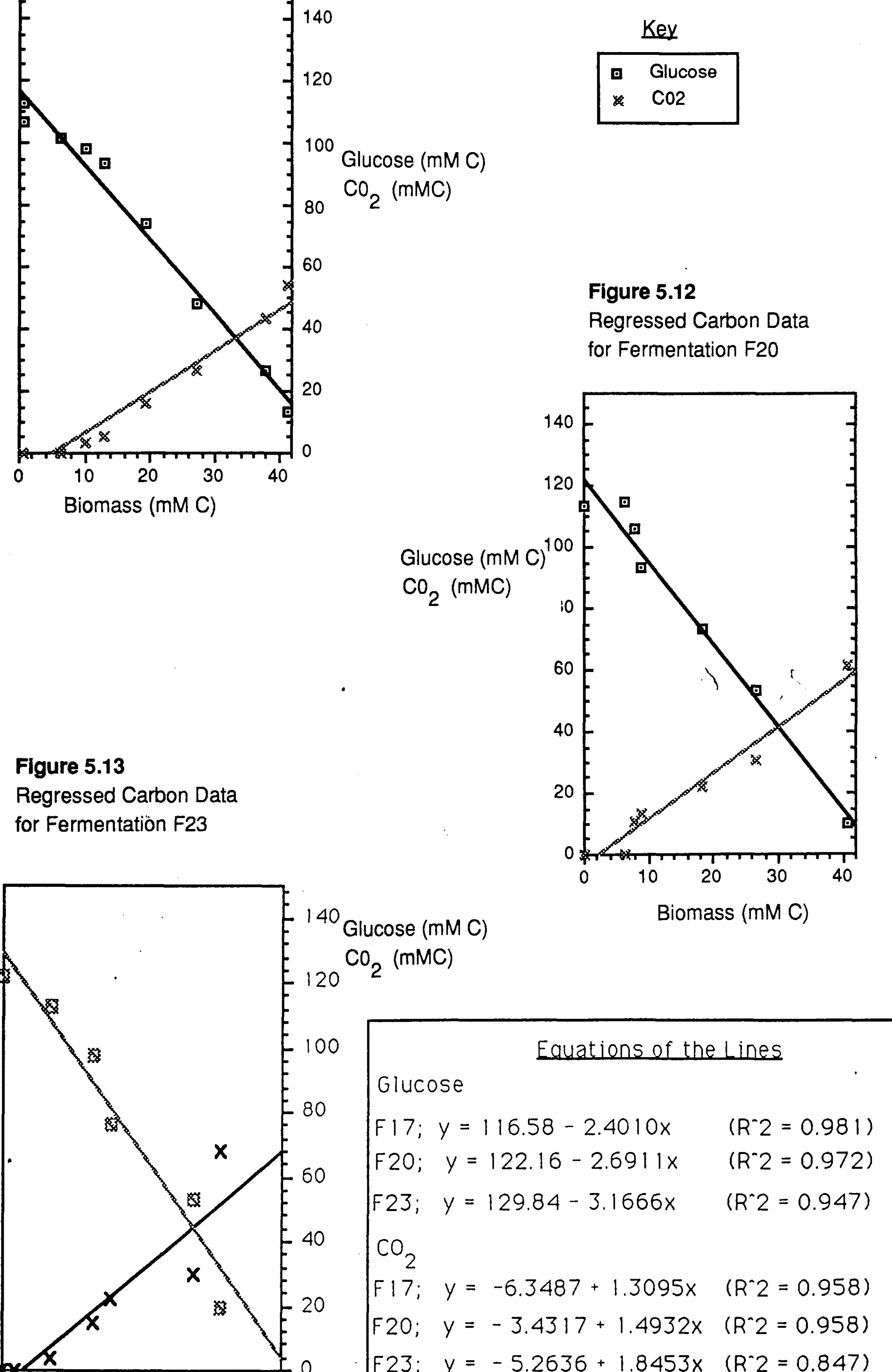


Figure 5.12
Regressed Carbon Data
for Fermentation F20

Figure 5.13
Regressed Carbon Data
for Fermentation F23

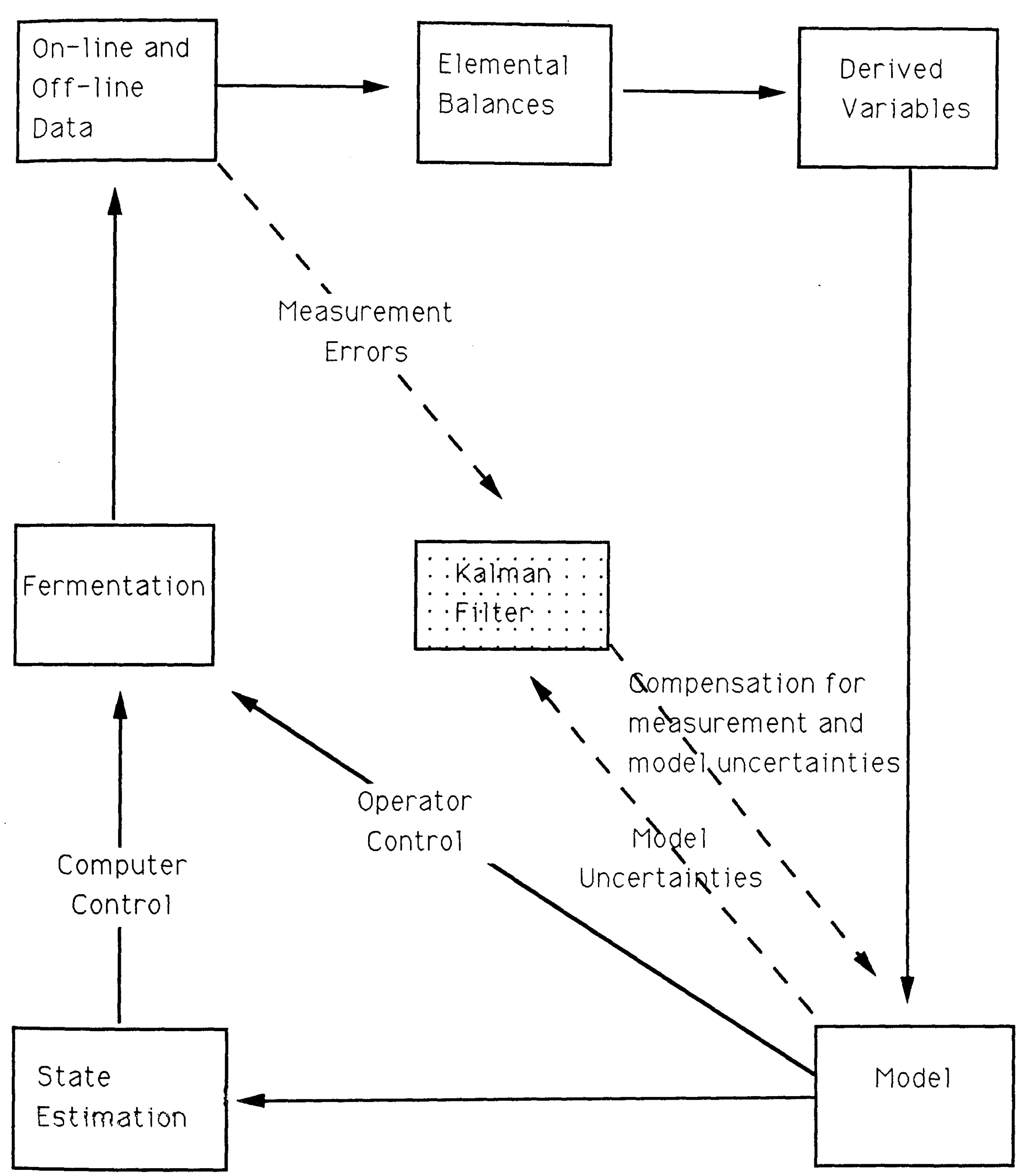


Figure 5.14 Flow sheet of the Potential Use of Mass-Balanced Fermentation Data

Table 5.7 Parameters Derived From the Regressed Simultaneous Equations for Carbon dioxide Production and Glucose Depletion in Relation to Biomass Concentration.

Data Set	Slopes of line (glucose)	Intercept	Slopes of line (CO_2)	Intercept
(F17)	-2.40	116.58	1.31	-6.35
(F20)	-2.69	122.16	1.49	-3.43
(F23)	-3.16	129.84	1.84	-5.26
Mean	-2.75	122.86	1.54	-5.01
Standard Deviation	0.38	6.6	0.26	1.47

Table 5.8 Yield factors for a Range of Microorganisms Grown on Minimal Media with Glucose as Substrate. (From Nagai, 1979).

Organism	Yield ($g.g^{-1}$)	Yield (g.mole)
Aerobacter aerogenes	0.40	72.7
Candida utilis	0.51	91.8
Penicillium chrysogenum	0.43	77.4
Rhodopseudomonas spheroides	0.45	81.0
Pseudomonas fluorescens	0.38	68.4
S. thermonitrificans	0.39	70.3

Table 5.9 Stoichiometric and Experimental Growth Parameters Discussed in Section 5.8.

Parameter	Location of Derivation	Type	Value (g biomass. mole $glucose^{-1}$)	Comment
Yield	Section 4.6	Theoretical	110	Cytochrome c in respiratory chain.
Yield	Section 5.4	Stoichiometric	126.0	Stoichiometric balance equation
Max growth yield	Section 4.5	Experimental	95.4	Intercept on line of $1/\mu$ against 1/yield
Yield	Section 4.5	Experimental	72.0	Yield at a dilution rate of $0.17h^{-1}$ (6 $g.l^{-1}$ glucose)
Yield	Section 5.6	Experimental	61.2	Mean yield value for batch growth on 3 $g.l^{-1}$ glucose.

prediction of the physiological state of the fermentation to be made. This potential use of the results of mass-balanced fermentations is illustrated in Figure 5.14.

Physiological models of this type can be used for accurate analysis and comparison of fermentations, and are obviously very powerful in process control. They allow on-line access into aspects of the process which cannot be observed from on-line measurements. For a fermentation without a complete mass balance, analysis of this type is still suitable by allowing the uncharacterised product to be accommodated in a 'product' reaction subspace, defined in terms of quantity of carbon (Liao, 1989).

5.6 Discussion of the Experimental Results

The mean experimental growth yield of 61.2 g biomass.mole glucose^{-1} is reasonably consistent with continuous culture data for the LH fermenter grown in high-agitation conditions at a dilution rate of 0.17h^{-1} (yield 72.0 g biomass. mole glucose^{-1}, see Section 4.7). In view of the differences between the two culture systems, values falling into similar ranges may be regarded as consistent, and would not be expected to be identical. If the experimental growth yield in batch culture is compared with the maximum growth yield for growth in the chemostat (95.4 g biomass. mole glucose^{-1}) it can be suggested that the total drain of carbon to maintenance was relatively high in this series of batch cultures and could account for up to 30% of dissipated carbon. However, the short fermentation time (30-40 hours) minimised the influence of maintenance on biomass formation.

5.7 Discussion and Comparison of Experimental and Stoichiometric Yield Values

The stoichiometric and experimental values discussed in this paragraph are summarised in Table 5.9. From the data presented in this table, it may be concluded that there is a large discrepancy between maximal yield values and experimentally-obtained values; this deviation has already been partially discussed in 4.6.

Values for yield determined by stoichiometry and by extrapolation of knowledge of the cytochrome components of *S. thermonitrificans* are both similar (110 and 126 g biomass per mole glucose). Similarly experimental values for growth yields at high growth rates, in partially-optimised batch culture and in chemostat, are both similar (around 60-70 g biomass per mole glucose).

From the respiratory chain composition of *S. thermonitrificans* on the basis of difference spectra, it may be suggested that there are potentially 3 sites of energy conservation in the electron transport chain (because cytochromes b, c and a are present, and a proton-pumping cytochrome oxidase, *aa*-3). Therefore, expected yield coefficients would be of the order of 110 g.mole^{-1} glucose (this has been discussed previously in section 4.6). The value for yield derived from the fermentation balance equation was 126 g biomass mole glucose^{-1}. However, predicted and stoichiometric values for yield neglect energy for maintenance; this is potentially a source of the difference between stoichiometric and experimental growth yield values. Therefore, comparison of predicted yield values are more accurately made with the experimentally-obtained maximum growth yield; a yield value without maintenance (95.4 g biomass.mole glucose^{-1} for *S. thermonitrificans*). In this comparison, the difference between experimental and stoichiometric values is lessened; a value of 95.4 g biomass. mole glucose^{-1} for the maximum growth yield, in contrast to the predicted values of around 110 -126g biomass. mole glucose^{-1}. Therefore the maximum growth yield may therefore be regarded as loosely consistent with the values predicted by stoichiometry and predicted P/O ratios, when the drawbacks of these latter two estimations are taken into account.

It can be concluded that optimisation of the growth of *S. thermonitrificans* requires the creation of conditions conducive to maximum growth rates (and therefore minimal drain of the energy supply for maintenance requirements).

The yield equations derived for growth of *S. thermonitrificans* on 3 g.l^{-1} glucose by regression analysis and determination of mean values, would be suitable for use as the basis of a model for growth in these conditions. It would be necessary to curve-fit the time course data from the off-line fermentation reports, and amalgamate the yield and rate relationships mathematically (by substitution and using the principles of Calculus) to give a three dimensional description of the fermentation. The physiological model would then be useful for testing out ideas, for formulating and planning experiments and for predicting the state of a fermentation from an on-line measurement. This approach could most usefully be applied in a dedicated microprocessor with software for fermenter control and data acquisition capable of operating in a multitasking mode; whereas the construction of the model could be carried out using advanced application software (such as Mathematica for the Macintosh).

5.8 Conclusions From this Chapter

This chapter has presented results both on-line, off-line, and microscopical, for fermentations of *S. thermonitrificans* using medium containing 3g.l^{-1} glucose.

These fermentations are consistent, relative to the variability which is characteristic of *Streptomyces* fermentations.

A theoretical analysis of the fermentation, based on stoichiometric relationships derived from the empirical formula for biomass has been presented. The theoretical values for reductance, RQ and yield in *S. thermonitrificans,* determined stoichiometrically, are reasonably similar to those of *Escherichia coli* and *Saccharomyces cerevisiae.* However they contrast markedly with those of *S. cattleya* and *Klebsiella aerogenes*, probably because of the oxidised nature of the biomass for *S. cattleya* and the reduced nature of the biomass for *K. aerogenes.*

Experimental results have been manipulated to give a relationship for biomass accretion, glucose utilisation and carbon dioxide output. From this relationship, an accurate experimentally-determined mean yield value has been obtained for growth in these conditions. This experimental yield value is reasonably consistent with yield values obtained at high growth rates in chemostat culture.

A comparison of the experimental and stoichiometric data has been made. The stoichiometric analysis and prediction (according to the respiratory chain composition) suggest that*S. thermonitrificans* may conserve energy at up to three sites in the electron transport chain. The maximum growth yield of 95.4g biomass per mole glucose (determined from chemostat culture) is consistent with this proposal. From these results an avenue for the optimisation of growth of *S. thermonitrificans* has been suggested. Optimisation of the growth of *S. thermonitrificans* would require the creation of conditions for growth at rapid growth rates, in which the carbon and energy dissipation as a result of maintenance processes is minimised with respect to the formation of biomass.

Fermentation data has been presented in this chapter with which data consistency can be assessed. The fermentation conditions developed for *S. thermonitrificans,* enable the implementation of reproducible fermentations on a minimal medium which give consistent growth data.

6.0 Introduction

The quantitative investigation of the physiology of a thermophilic streptomycete expressing a heterologous antimicrobial product, using the techniques discussed in the previous chapter, was the original project aim. However, a suitable recombinant strain of *S. thermonitrificans,* expressing a known heterologuous product, was not available at this stage in the project. Work described in this chapter explains the approaches taken to alleviate this; however it was unsuccessful. If the creation of a recombinant strain of *S. thermonitrificans*, producing oxytetracycline, had been achieved, it would have enabled the scheme of work described in Section 6.1 to have been carried out. Sections 6.2-6.4 describe attempts at plasmid transformation of *S. thermonitrificans* for the creation of a recombinant strain containing a plasmid with an insert encoding the oxytetracycline pathway. Sections 6.6 onwards describe the work involved in attempting to achieve the same end, using the technique of *in vivo* conjugation.

6.1 Planned Approach to the Study of a Recombinant Strain of *S. thermonitrificans* containing a Plasmid which Enabled the Formation of Oxytetracycline in Culture Broths

1. **Assessment of the temperature range for biosynthesis in *S. thermonitrificans.*** *S. rimosus* (the host for the DNA insert) has a maximum growth temperature of 34^{0}C, a temperature at which *S. thermonitrificans* will also grow, although slowly. Therefore potential expression in *S. thermonitrificans* could be monitored at growth temperatures between 30-34^{0}C. Growth and heterologous expression studies at 45^{0}C and 50^{0}C would identify whether oxytetracycline production at these temperatures would have been possible. If these growth temperatures were unsuitable for product formation then incremental increases in growth temperature from 34^{0}C would have enabled the identification of temperatures for both reasonable growth of *S.thermonitrificans* and product formation.

Product formation at the elevated growth temperatures would have been potentially difficult: any enzyme on the biosynthetic pathway could have been thermolabile at these temperatures, or the product could have been rapidly degraded. It has been shown that low phosphate conditions are required for oxytetracycline biosynthesis in *S. rimosus* (Rhodes, 1984). It was not envisaged that the identification of nutrient conditions required for formation of the product would be a problem using a defined medium in which

components can easily be varied (such as 579MM).

2. **Copy number determinations under similar growth conditions as those to be used in mass balanced fermentations** (using both *S. thermonitrificans* pIJ916, the control strain and *S. thermonitrificans* pPFZ163). The plasmid pIJ916 and its derivative pPFZ163 are described in Section 6.4. It was expected that the plasmids would have been stable and in low copy-number (1-2 per cell). If these results had confirmed this, then subsequent fermentations could have assumed that copy number was invariant. If the results had not suggested this was the case, then copy number determinations would have been required for all biomass samples, and results would have required correlation with copy number, in concert with experimental variables.

3. **Optimisation of fermentation parameters**. The growth conditions for mass balanced fermentations (described in chapter 5) would have required optimisation for the recombinant *S. thermonitrificans* pFZ163 and the control *S. thermonitrificans* pIJ916 cultures on a shake flask scale. Variation in the concentration of carbon source, phosphate concentration and nitrogen concentration in shake flask cultures would have been optimised, and the effect on productivity and efficiency of oxytetracycline biosynthesis assessed. The effect of feeding additional glucose during stationary phase and the influence on productivity of the culture would have also be assessed. A regime for fermenter scale cultivation would have been devised based on these results.

4. **Mass balanced fermentations with *S. thermonitrificans* pIJ916 and *S. thermonitrificans* pPFZ163 at 34^{0}C and 45^{0}C** (or alternative suitable temperatures identified in 1. above). Product analysis by HPLC (for oxytetracycline), would have been required in addition to the analyses described in Chapter 5. A quantitative description of the carbon balances for the recombinant fermentations could then have been made.

4. Comparison of the carbon balances of the fermentations at the two temperatures would have enabled the final assessment - **Can antibiotics be produced more economically and efficiently at elevated temperatures?**

6.2 Gene Transfer in *Streptomyces*

Streptomycetes are amenable to gene transfer using a number of approaches, plasmid transformation mediated by PEG (Bibb *et al.*, 1978), natural plasmid-mediated conjugation (Bibb *et al.*, 1977), phage-mediated transduction (Stuttard, 1979), protoplast fusion induced by PEG (Hopwood, 1977), transfection by phage DNA (Suarez and Chater, 1980a) and PEG-induced

transformation by chromosomal DNA in liposomes (Makins and Holt ,1981).
Plasmid transformation mediated by PEG and plasmid-mediated conjugation were used in this project and will be briefly described (6.3 and 6.5).

6.3 Plasmid Transformation in *S. thermonitrificans*

The development of techniques for plasmid transformation of *S. thermonitrificans* is described in detail (Czaplewski, 1989). A brief summary is given here (media and protocols are described in Materials and Methods).

The introduction of plasmids into streptomycetes by transformation requires that protoplasts can be formed, that a suitable compatible vector for propagation in the putative transformant exists, and that the protoplasts can be regenerated. In this project (work described in Czaplewski, 1989) novel protoplast formation and regeneration techniques suitable for *S. thermonitrificans* had to be developed, together with identification of vectors able to replicate at elevated temperatures. Initial transformation studies using DNA from *S. lividans* were unsuccessful, and efficient transformation was only achieved with DNA propagated in *S. albus*. It was postulated that this was due to a potential restriction barrier, based on experiments using a host-range actinophage mutant. The transformation protocol for *S. thermonitrificans* specifies that vector DNA is propagated in *S. albus* to circumvent any potential restriction barrier.

S. thermonitrificans was found to require specific conditions for protoplast formation. Mycelium was cultivated at 37^0C in TSB for 20 hours, followed by an incubation step in the presence of lysozyme. This incubation step caused cleavage of ß 1-4 linkages in peptidoglycan, enabling partial digestion of the cell wall to leave intact protoplasts. The concentration of lysozyme required for protoplast formation of *S. thermonitrificans* (0.25 mg lysozyme per ml of osmotically-stabilised buffer) is lower than that used for mesophilic *Streptomyces* (*e.g* 5 $mg.ml^{-1}$, Hunter, 1985; 1 $mg.ml^{-1}$, Hopwood *et al.,* 1985). Protoplasts were formed in 30-40 minutes at 30^0C, and could be visualised through the microscope as spheres subject to Brownian motion. Protoplasts had to be manipulated in isotonic buffers for successive steps to prevent hypotonic lysis or plasmolysis. DNA was introduced by plasmid transformation into protoplasts in the presence of high molecular-weight polyethylene glycol. The transformation mix was then plated onto solid regeneration medium. Regeneration medium was specifically designed for this strain, and contained a precise balance of divalent cations together with mannitol to act as an osmotic stabilizer. Mannitol replaced sucrose which is conventionally used as an osmotic stabilizer but is inhibitory to growth of *S. thermonitrificans.*

Thiostrepton was the predominant plasmid selection marker used in studies with *S. thermonitrificans.* Direct selection for resistance to thiostrepton on protoplasts is not possible, as resistance to thiostrepton requires methylation of ribosomal RNA. A period of time must elapse during which ribosomal methylation may occur, prior to selection on the basis of thiostrepton resistance. For *S. thermonitrificans* around 18-20 hours was found to be a suitable period of time for regeneration, and resistant colonies could then be positively selected using thiostrepton overlays to a final surface concentration of 10 µg per plate.

6.4 Reasons for the proposed use of an alternative recombinant *S. thermonitrificans* strain, based on a low copy-number vector, in preference to *S. thermonitrificans* pBROC139, which is based on a high copy-number vector

A strain of *S. thermonitrificans* containing the plasmid pBROC139 was constructed (Czaplewski, 1989). It became apparent that the use of this recombinant strain was complicated by the unidentified nature of the yellow product expressed from pBROC139 (described in detail in Chapter 7). In addition the use of a high copy-number vector on which the plasmid pBROC139 is based (pIJ702), is not ideal for this project, for reasons detailed below.

The use of high copy-number vectors complicates accurate study of expression from pathways encoded on the vectors for a number of reasons. In *E. coli,* specific growth rates decreased by up to 70% for increasing plasmid copy number, with high copy- number *Col E1* based vectors, in 2 litre stirred tank bioreactors (Betenbaugh *et al.,* 1989). In addition, a decline in recombinant cell viability was observed. Conversely, it has been shown that the plasmid copy number (for high copy-number plasmids in *E.coli)* decreases with increasing growth rate (Lee and Bailey, 1984). Intuitively, these results would be expected, as plasmid replication and pathway expression would represent a metabolic burden on the host, and a reduction in growth rate, culture viability, or plasmid copy-number may be considered to be a predicted outcome. By extrapolation to *Streptomyces*, similar changes in growth kinetics may be expected to occur. In addition, copy-number experiments in *S. lividans,* using pIJ303 , a plasmid based on the pIJ101 replicon, have shown that total plasmid copy number varied from 270 copies per cell at 9 hours in a specific *S. lividans* fermentation, to 650 copies per cell after 50 hours of cultivation (Richards and Ward, 1990). The use of high copy-number vectors in *Streptomyces,* such as those based on the pIJ101 replicon, further complicate studies because formation of product may reach a limit defined by the availability of intracellular

components such as substrate, precursors, RNA polymerase, ribosomes, and specific cofactors required for expression. Cloned-gene expression in *E. coli* has been shown to be limited by the cellular transcription capacity, which beyond a threshold, is independent of promoter strength of the specific gene, or of plasmid copy-number (Peretti and Bailey, 1987).

In contrast to high-copy number plasmids based on the pIJ101 replicon (on which pIJ702 is based), streptomycete low copy-number plasmids based on the SCP2 replicon have reported copy numbers of 1-2 per cell (Lydiate *et al.*, 1985). Titration of intracellular effector molecules is therefore less likely to influence production profiles from low-copy number plasmids in *Streptomyces*. Therefore a low copy-number vector was considered to be more suited to the ultimate implementation of the project , than a plasmid based on a high copy-vector such as pIJ702.

The biosynthetic pathway for oxytetracycline formation by *S. rimosus* was cloned and expressed in the heterologous hosts, *S. lividans* and *S. albus* on a low copy-number SCP2-based plasmid, pIJ916 (Binnie *et al.*, 1989). The Pfizer plasmid encoding the cluster for oxytetracycline biosynthesis on an SCP-2 based vector (plasmid pFZ163) was therefore a suitable candidate for attempted transformation into *S. thermonitrificans*. In addition, study of the expression of the oxytetracycline biosynthetic pathway was ideally suited to the project in view of the industrial and economic importance of the product; the academic relevance of polyketide antibiotics; the library of information concerning tetracycline biosynthesis (Behal *et al.*, 1987), and the ease of assay (HPLC analysis techniques developed at Pfizer were available).

Oxytetracycline is a modified tetracycline antibiotic of the polyketide class discovered in the 1950s. The tetracyclines possess similar, but slightly different activity spectra and differ only in substituents attached to the 4-ring structure. Oxytetracycline is the sole tetracycline antibiotic produced by natural strains of *S. rimosus,* and a postulated biosynthetic pathway derived from malonyl CoA subunits has been described (Binnie *et al.,* 1989). Isolation of mutants blocked in oxytetracycline biosynthesis is detailed in the paper by Rhodes *et al.,* (1981); a further paper describing the cloning of resistance genes and structure of the gene cluster involved in biosynthesis has been published (Butler *et al.,* 1989). In addition to HPLC analysis detailed above, a simple bioassay using *Bacillus subtilis* as the sensitive organism for quantitation of the antibiotic was available, described in Section 2.14.

The plasmid pFZ163 was made available for the project by Pfizer, Sandwich, Kent, supplied in the strain *S. albus* G153 (a restriction minus, modification

plus strain) Plasmid pFZ163 contains a DNA fragment 30 Kb in length (Binnie *et al.*, 1989), cloned into the unique *Eco* R1 restriction site of pIJ916. As described previously, this plasmid is a low copy-number vector and carries an antibiotic selection marker, *tsr* (the thiostrepton resistance gene) and the SCP2 origin of replication.

6.5 Attempted Transformation of *S. thermonitrificans* with pFZ163

Plasmid DNA was prepared from *S. albus* according to the neutral lysis method for SCP2-based plasmids (Hopwood *et al.*, 1985). This method was chosen as it did not involve a DNA denaturation step and large volumes of culture could be manipulated easily. As the plasmid was large (50 Kb) and susceptible to shear, the DNA was treated very gently. Using this method about 30µg of DNA could be obtained from 500 ml of harvested cells.

Introduction of plasmid pFZ163 by transformation was attempted in *S. thermonitrificans* using typically 300 ng–1µg of plasmid DNA. However, no transformants containing pFZ163 were obtained, reasons for which are discussed later in the chapter. Control transformations using 300 ng of pIJ916 and pIJ702 were used to measure and compare transformation efficiency of plasmids with SCP2 and pIJ101 replicons respectively. The DNA was initially passaged through *S. albus* prior to introduction into *S. thermonitrificans*, for reasons discussed previously (Section 6.1). Regeneration controls were run in parallel to the transformation experiments by plating putative protoplasts on regeneration media and media lacking osmotic support, to measure protoplast viability. Mock transformations, (lacking DNA) were also used, with and without selective overlays, to confirm the efficacy of the selection technique. Regeneration efficiency was normally between 10^7 and 10^9 per 100 µl of the protoplast mixture. Transformation efficiency with SCP2-based vectors (pIJ916) was known to be 100 fold lower compared to that of pIJ702 in *S. thermonitrificans* (Czaplewski, 1989). In this work, transformation efficiency with pIJ702 was reproducibly between 10^3 - 10^4 transformants per µg DNA, whilst that of pIJ916 was between 10^{-1} and 10^{-2} transformants per µg, transformation efficiencies which agree with those described in Czaplewski, 1989. In general, transformation efficiency was found to be partially-dependant on the quality of the protoplasts.

The plasmid pFZ163 (prepared in this work, from *S. albus)* was used to transform *S. lividans* TK54 and TK64 protoplasts to check the transformational capacity of the plasmid DNA. A relatively high transformation efficiency was achieved, in the order of 10^3 -10^4 protoplasts per µg DNA. Colonies of putative *S. lividans* transformants were patched onto selective agar containing

thiostrepton (at 10 μg/ml); resistance to oxytetracycline or tetracycline cannot be used to identify expression of the resistance genes from the oxytetracycline pathway as *S. lividans* is endogenously resistant to oxytetracycline. All transformants were found to be stably resistant to thiostrepton. The integrity of the DNA was checked by assessing expression of oxytetracycline using the bioassay described in Section 2.14. Oxytetracycline and a brown coloured pigment (a breakdown product of oxytetracycline produced in cultures which express oxytetracycline) were both expressed in all the *S. lividans* transformants. From the above it can be concluded that plasmid pFZ163 prepared during this work is both viable and functional in the heterologous host *S. lividans.*

The failure to introduce plasmid pFZ163 by transformation into*S. thermonitrificans* is unsurprising, although disappointing. The plasmid is 2.5 times the size of pIJ916, which itself has a low transformation efficiency (10^1 - 10^2 per μg DNA) in *S. thermonitrificans* and consequently results in 2.5 times fewer DNA molecules in the reaction mix compared with pIJ916 (on a μg basis). This could be circumvented by using large amounts of DNA; however, it was difficult to obtain such large amounts of the large, low copy-number plasmid. The large size of pFZ163 may also have increased the likelihood of host restriction-modification mechanisms leading to poor transformation efficiency (the larger the molecule the greater the likelihood of it containing a recognition site for a restriction endonuclease). A minor restriction barrier is thought to exist between *S. thermonitrificans* and *S. albus.* Consequently, it is possible that the large size of the plasmid reduces the chance of overcoming this. Difficulty with transformation of large plasmids has been described (Czaplewski, 1989: Leonardo *et al.*, 1990).

Hence transformation of *S. thermonitrificans* with pFZ163 was unsuccessful. The method of conjugation was identified as an alternative method for introduction of the oxytetracycline pathway.

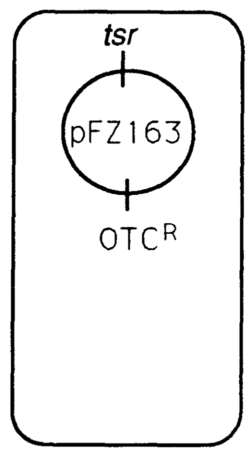
6.6 Conjugation and the Applicability of the Technique to this Study

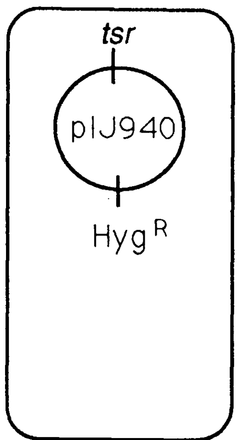
In view of the lack of success using *in-vitro* plasmid transfer, it was decided to adopt an alternative approach using *in-vivo* gene transfer methodology. Plasmid pIJ940 is a 24.9 kb streptomycete vector, containing the SCP2 origin of replication, the mobilisation functions encoded by *tra* and also the resistance determinants for hygromycin and thiostrepton (*hyg* and *tsr*). It was therefore identified as a suitable conjugative vector for use in this work. Conjugation in *Streptomyces* has not been characterised, and no information exists about either the formation of mating pairs, pili or DNA transfer.

Conjugative plasmids were originally identified in *Streptomyces coelicolor* A3(2) in 1957, when plasmids SCP1 and SCP2 were identified. SCP1 was found to exist in either an autonomous form or an integrated form (Hopwood, 1957; Vivian, 1971; Bibb 1977). SCP2 can act as an efficient sex-factor without stably-interacting with the host chromosome (Hopwood and Chater, 1986). Conjugation between *E. coli* and *S. lividans* has been described (Mazodier *et al.*, 1989); also between *Streptomyces* in soil (Wellington *et al.*, 1990) whilst interspecific mating is thought to be the major recombinational event occurring within the streptomycete soil population (Raffi *et al.*, 1989).

Transfer of genetic information by conjugation in *E. coli* relies on transfer of single stranded DNA via cell-cell contact from a donor to suitable host. This process is plasmid-encoded and includes the formation of conjugative pili, the nicking and initiation of DNA transfer at the *ori*T site, separation of the two DNA strands and strand transfer. In the recipient, complementary strand synthesis of the DNA occurs according to the methylation status of the recipient, using host-encoded rather than plasmid-encoded functions. In contrast, replacement strand synthesis in the donor follows the methylation status of the donor. Some mobilisation of the chromosome of the host may occur for integrated plasmids in *E.coli,* although chromosome mobilising ability is not well-understood (Willetts and Wilkins, 1984; Chater and Hopwood, 1989). The restriction of unmodified DNA relies on recognition of specific double stranded sequence; therefore transfer of DNA in a single-stranded form may be a mechanism which could be used to alleviate restriction barriers between species. Following conjugative strand synthesis in the host cell, the modified DNA is no longer a suitable substrate for restriction. The mechanism of DNA transfer by conjugation in *E. coli* (Gram positive) must necessarily be different to that in *Streptomyces* (Gram negative), due to differences in cell wall structure and membrane arrangement. However, the hypothesis for overcoming restriction barriers by transferring DNA in the single stranded form may still hold for inter-species transfer in *Streptomyces*, as intergeneric transfer between *Streptomyces* and *E. coli* has been shown (Mazodier *et al.*, 1989). It was therefore decided to attempt this strategy for transfer of the pathway for oxytetracycline production into *S. thermonitrificans.*

A conjugative plasmid (pGLW101) was constructed by allowing co-cultivation of *S. lividans* TK64: pFZ164 (control product from experiment described in 6.2) and *S. lividans* TK54: pIJ940 (a strain containing the conjugative *Streptomyces* plasmid described previously), see Figure 6.1. The construction of pGLW101 in *S. lividans* TK64 was the work of I. Hunter (personal communication). *S. lividans* transconjugants were selected on the basis of plasmid encoded hygromycin resistance and chromosomally-encoded streptomycin resistance.





X

TK64 *pro-2, str-6*

(proline auxotroph streptomycin resistant)

S. lividans TK64:pFZ163

TK54 *his-2, leu-2,spc-1*

(histidine and leucine auxotroph, spectinomycin resistant)

S. lividans TK54;pIJ940

Looking for transfer of the conjugative plasmid pIJ940 to *S.lividans* TK64, and homologous recombination at the thiostrepton resistance gene *tsr,* to give a conjugative plasmid containing the OTC pathway inserted in plasmid pFZ163.

↓

Select for streptomycin and hygromycin resistance - putative *S.lividans* TK64:pIJ940 transconjugants

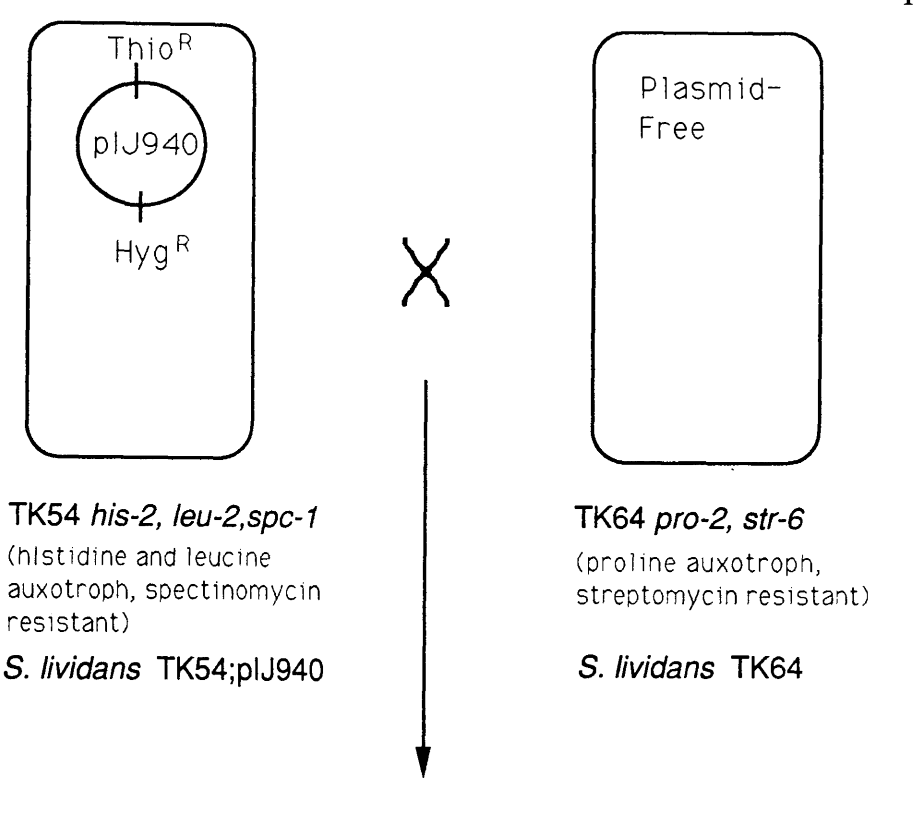
↓

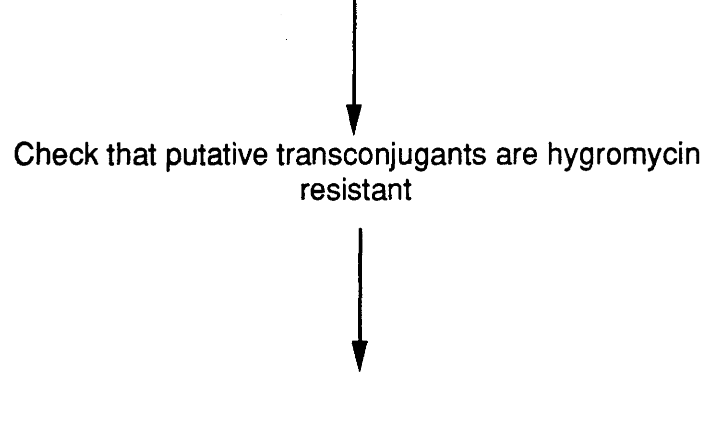
Screen for *pro* $^{-}$, *spc*^{S}, OTC ^{R} transconjugants - transconjugants which have undergone homologous recombination

↓

S. lividans TK64:pGLW101

Figure 6.1 Diagrammatic Representation of the Strategy employed in the construction of the Strain *S. lividans* TK64:pGLW101 (I. Hunter, personal communication).





S. lividans TK64;pIJ940

Figure 6.2 Diagrammatic Representation of the Strategy used in the Construction of the Strain *S. lividans* TK64:pIJ940

Table 6.1 Intraspecific Conjugation Results

Antibiotic Selection		CFU/ml	Comment
Streptomycin (ineffective in use)	(A)	3.6 (+/- 0.2) $\times 10^{10}$	Total population of *S.lividans* TK54 and TK64 with and without plasmid
Thiostrepton + Streptomycin (ineffective in use)	(B)	2.3 (+/- 0.1) $\times 10^{10}$	Total population of *S.lividans* TK54 and TK64 with plasmid-encoded thiostrepton resistance
Spectinomycin	(C)	2.6 (+/- 0.5) $\times 10^{8}$	Numbers of *S.lividans* TK54 in the total population (selection against *S.lividans* TK64)
Spectinomycin + Thiostrepton	(D)	2.7 (+/- 0.1) $\times 10^{8}$	Numbers of *S.lividans* TK54 pIJ940 in the population.

Putative transconjugants were then screened on the basis of proline requirement on minimal media (a secondary chromosomal marker), spectinomycin sensitivity (the alternative *S. lividans* strain, *S. lividans* TK54 is resistant to this antibiotic) and oxytetracycline resistance (encoded by the 30Kb pFZ163 insert). Subsequent physical isolation of the plasmid pGLW101 by M. Butler (Pfizer) has recently shown that the conjugal plasmid contains vector pIJ940 sequence with an insert corresponding to the insert in plasmid pFZ163 flanked by thiostrepton resistance determinants (I. Hunter, personal communication). The presence of two *tsr* sequences flanking the insert may have induced insert instability, but this was not quantitated.

6.7 Intraspecific Conjugation - the Construction of *S.lividans* TK64:pIJ940

The plasmid pGLW101 was available in *S. lividans* TK64. However the vector alone, pIJ940, the control plasmid for this experiment, was available only in *S. lividans* TK54. Therefore an intraspecific conjugation experiment was required to provide the donor strain, *S. lividans* TK64:pIJ940.

The strain construction is shown diagrammatically in Figure 6.2. Plasmid pIJ940 was constructed by mixing *S. lividans* TK54: pIJ940 spores with an excess of *S. lividans* TK64 spores. These were incubated at 30^{0}C for 10 days and the spores harvested. Dilutions of the spores from the mating mixture were plated in duplicate on soya-mannitol agar and were replica plated in triplicate to soya-mannitol agar containing spectinomycin to select for *S. lividans* TK54 (plasmid-free and plasmid-containing versions), spectinomycin and thiostrepton, to select for *S. lividans* TK54: pIJ940; and to soya-mannitol agar containing streptomycin and thiostrepton. Ordinarily, streptomycin would be used to specifically select the strain *S. lividans* TK64 (plasmid-free, and plasmid-containing versions) and medium containing spectinomycin and thiostrepton would be specifically selective for *S. lividans* TK64:pIJ940. However a selection strategy for *S. lividans* TK64 based on negative selection was employed for reasons discussed in the following paragraph.

Streptomycin resistance was found to be unsuitable for use as a marker in these experiments, possibly due to degradation, or biological inactivation of the antibiotic. Controls showed that *S. lividans* TK64 (the laboratory stock strain) was capable of growth on medium containing streptomycin. Streptomycin may easily be biologically inactivated (I. Hunter, personal communication) and in some laboratories solid streptomycin is added to media immediately prior to use, with no preliminary solution step, to prevent degradation and inactivation processes (C. Owen, personal communication). In retrospect, use of this method for the preparation of streptomycin selection

media might have alleviated the problem. Negative selection was used for the selection of *S.lividans* TK64 based on sensitivity to spectinomycin. All other selection strategies were checked prior to initiating the experiment, and were observed to function as predicted.

Colonies from plates containing streptomycin and thiostrepton were screened for spectinomycin sensitivity, by patching onto soya-manitol agar containing thiostrepton with and without spectinomycin. Colonies which were spectinomycin sensitive and thiostrepton resistant were identified as putative *S. lividans* TK64:pIJ940 transconjugants. The screening of transconjugants is described in 6.7.

6.8 Frequency of Transconjugation of pIJ940

The frequency of transconjugation may be determined by difference (see Table 6.1). The numerator, the total number of *S.lividans* TK64:pIJ940 transconjugants (B-D), is given by the number of plasmid containing colonies (of both *S. lividans* TK64:940 and *S. lividans* TK54:940), B , minus the number of plasmid containing colonies known to be *S. lividans* TK54:940 (resistant to spectinomycin and thiostrepton), D. The denominator (A-C) is the total population of *S. lividans* TK64 and TK54 (A) minus the numbers of *S. lividans* TK54 colonies, (resistant to spectinomycin), C.

Therefore; the frequency of transconjugants (per recipient) $= \frac{(B - D)}{(A - C)}$

= 0.63 transconjugants per recipient

This result is high, but may be explained by virtue of the large excess of *S. lividans* TK64 spores in the mating mix, the length of cultivation time (10 days), the filamentous form of growth in which spread within mycelium may occur (Wellington *et al.*, 1990) and the intraspecific nature of the conjugation.

The efficiency of transconjugation was checked by patching 100 colonies from a thiostrepton-containing plates (the plasmid-containing population) onto media with thiostrepton and spectinomycin (to select for *S. lividans* TK54), and thiostrepton alone. The results suggested that around 85% of the plasmid-containing population were *S. lividans* TK64 transconjugants in reasonable agreement with replica-plating data.

6.9 Screening of Putative Transconjugants

Ten colonies of the proposed transconjugants (from the colony patches described above) were cultivated on selection media containing thiostrepton and hygromycin, or containing thiostrepton, hygromycin and spectinomycin (together). All colonies were resistant to thiostrepton and hygromycin and sensitive to spectinomycin. It can be concluded that these colonies have arisen from *S. lividans* TK64 transconjugants. A colony was chosen for use in the interspecific mating to *S. thermonitrificans* and spore samples prepared.

6.10 Interspecific Conjugation - An Attempt to Construct *S. thermonitrificans* pGLW101

An attempt to construct *S. thermonitrificans* pGLW101 is described in this section, using the strains *S. thermonitrificans*, *S. lividans* TK64;pGLW101, with *S. lividans* TK64:pIJ940 as a control plasmid. The experimental strategy is described in Figure 6.3. However, plasmid transfer with the control plasmid did not occur.

It was necessary to have at least one marker to select for the transfer of plasmid, one to select for the donor strain and one for the recipient strain. In the conjugation experiment 4 factors existed on which discrimination could occur;

1. *S. thermonitrificans* is resistant to spectinomycin, in contrast to *S. livdans* TK64 which is sensitive,
2. Growth temperatures- *S. lividans* will not grow at 45^{0}C, whereas *S. thermonitrificans* grows optimally at this temperature. Both strains can be cultivated at 37^{0}C,
3. Thiostrepton resistance - encoded on plasmid pIJ940,
4. Hygromycin resistance - encoded on plasmid pIJ940.

S. lividans sporulates on soya medium, forming around10^{10} spores per plate, whereas *S. thermonitrificans* has a lower efficiency of sporulation on this medium (10^7 -10^8 per plate). Soya medium was used for co-cultivation (at 37^{0}C) as both strains sporulate on this medium, in contrast to alternative media. Spores from *S. lividans* TK64: pIJ940 and *S. thermonitrificans* were mixed in the ratios 1:1,10:1, 10^2:1, 10^3:1, 10^4:1, 10^5:1 (analysed by counting spore colony forming units as described in Materials and Methods), and plated onto non selective agar at 37^{0}C. Spores were collected after 5 days of co-cultivation. The spores were plated onto spectinomycin-containing soya mannitol agar and cultivated at 45^{0}C for selection of *S. thermonitrificans,* to

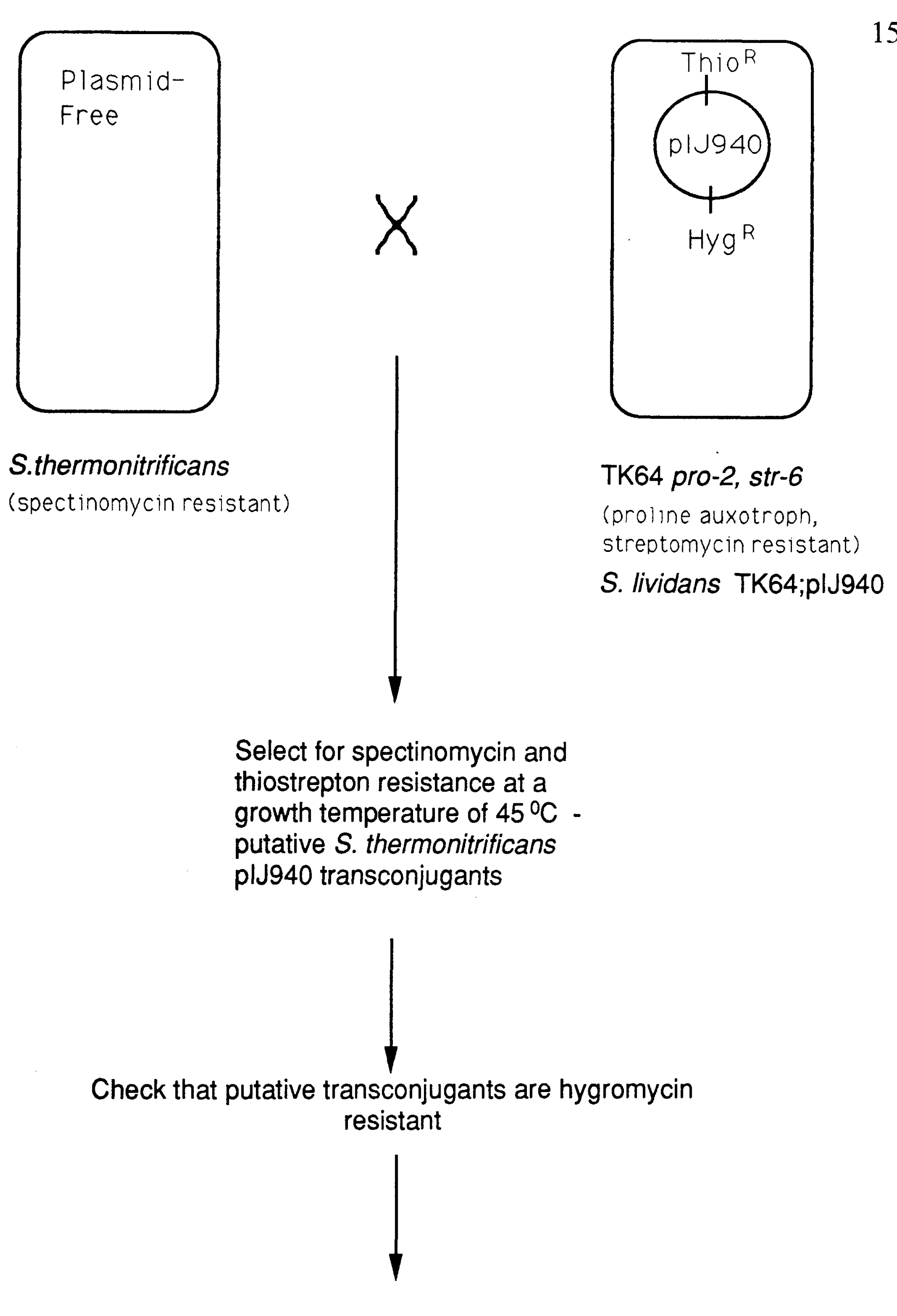


Figure 6.3 Diagrammatic Representation of the Strategy used in the attempted Conjugation of *S.lividans* TK64:pIJ940 with *S.thermonitrifcans.*

select *S. lividans* TK64:pIJ940, spores were plated onto medium lacking spectinomycin and incubated at 30^{0}C. Thiostrepton-containing plates were also used in each case for the selection of plasmid-containing strains.

In all experiments 5 x 10^{10} spores were obtained for *S. lividans* TK64: pIJ940 under the correct selective conditions (30^{0}C cultivation temperature). In only one experiment were *S. thermonitrificans* colonies recovered, using spore ratios of 1:1. *S. thermonitrificans* was recovered for spore ratios of 1:1 at a concentration of 4 x 10^2 per plate (alternatively expressed as per mating), a relatively low concentration compared to *S. lividans.* None of the colonies possessed resistance to either thiostrepton or hygromycin as determined by replica plating, or by direct antibiotic selection and it is concluded that plasmid transfer had not occurred.

The optimisation of spore concentration was not attempted. However, because because *S. thermonitrificans* was recovered as a very low proportion of the mixed population, the balance of spore concentration for the original experiment may have been far from optimum. It was also suggested that a microbial product of *S. lividans* could be inhibitory to growth of *S. thermonitrificans,* or that the growth of *S lividans* at 37^{0}C could out-compete *S. thermonitrificans* for vital nutrients, by growing more luxuriantly and sporulating more profusely. The presence of an inhibitory compound excreted from *S. lividans* was tested by streaking colonies of *S. thermonitrificans* in close proximity to *S. lividans.* No growth inhibition occurred, suggesting that co-cultivation of *S lividans* and *S. thermonitrificans* could occur. However, competition on the basis of growth fitness on the media used may be a factor in the low recovery of *S. thermonitrificans* from the mating mixture.

Further work in the laboratory identified that interspecific transfer of pIJ940 to *S. thermonitrificans* was possible in that thiostrepton and hygromycin resistant colonies were obtained from interspecific crosses with pIJ940 as the conjugal vector, but were not analysed (I. Hunter, personal communication). In conjugation experiments with *S. thermonitrificans* and *S. lividans* pGLW101 (construction described in 6.4) no transconjugants were recovered. The precise reasons for the lack of isolation of transconjugants using the large plasmid pGLW101 were not investigated.

6.11 Conclusions, Discussion and Future Work

Repeated experiments in transformation of pFZ163 to *S. thermonitrificans* yielded no transformants. This could be due to the large size of the plasmid, coupled with the low transformation frequency with SCP2-based plasmid

vectors in *S. thermonitrificans*. It has been suggested that development and optimisation of regeneration media and protoplasting techniques should be continuous, each new advance requiring re-optimisation of existing techniques (Czaplewski, 1989). Increases in transformation efficiency, by following this development strategy, may enable transformation of *S. thermonitrificans* with plasmid pFZ163 at a later date.

Optimisation of conjugation with control strains of *S. lividans* TK64: pIJ940 to give high transconjugation frequencies might be possible, in which case further attempts with pGLW101 may yield transconjugants. The use of *S. albus* as the donor species for conjugal plasmids may enable greater transconjugation frequencies to be achieved with pIJ940, or alternatively, a strain cured of the plasmid pIJ940 may be more efficient in subsequent experiments as a recipient. In either case, conjugation of either *S. albus* pGLW101 or *S. lividans* pGLW101 with *S. thermonitrificans* may be successful with further developmental work.

Expression, Isolation, and Preliminary Purification and Identification of the Product from *S. thermonitrificans* pBROC139.

7.0 Introduction

Plasmid pBROC139 contains a 5.3 kb DNA insert from a *Sau*IIIa digest of *S.clavuligerus* total DNA, inserted into the *Bgl* II site of the streptomycete high copy number vector pIJ702 (Katz *et al.*, 1983). It confers on *S. lividans,* the ability to produce a yellow compound and was originally suggested to encode the complete or partially complete pathway for the production of the antibiotic holomycin (M. Burnham, personal communication). A strain of *S. thermonitrificans* containing pBROC139 was constructed (Czaplewski, 1989) to assess the feasibility of producing antibiotics in *Streptomyces* at elevated temperatures.

This chapter describes the preliminary studies undertaken to assess the expression of a yellow product at temperatures up to 50^{0}C in *S. thermonitrificans* (Section 7.3). In addition, the attempted identification of the product by comparison with a holomycin standard will be discussed (Section 7.4). As a result of this investigation the product has been shown not to be holomycin. Attempts to purify the uncharacterised product are described in section 7.5 and 7.6, whilst the structural elucidation of the purified sample is described in section 7.8. It has been tentatively suggested that the pigmented broth formed as a result of growth of *S. thermonitrificans* pBROC139 might contain two pyrrothine antibiotics; aureothricin and isobutyropyrrothine.

7.1 Holomycin

Holomycin is a yellow, antimicrobial agent produced both by *S.griseus* NRRL2764 and by *S.clavuligerus* (Ettlinger *et al,* 1959: Kenig and Reading, 1979). It is a member of the pyrrothine class of antibiotics and is an inhibitor of a wide range of bacteria including *Sarcina lutea* NCTC 8340, *Corynebacterium diptheriae* and *Streptococcus pyogenes* (minimum inhibitory concentrations for the latter 2 strains are 1 $\mu g.ml^{-1}$, Gaumann, 1961). It is a particularly potent inhibitor of growth of *Flavobacterium spp.* C2116 (Kirby, 1978), and as such this bacterium is used in the bioassay of holomycin activity. The chemical structure of holomycin is given in Figure 7.1 and its chemical characteristics, in Figure 7.2. A sample of pure holomycin (1 mg) was provided by Pfizer for this investigation.

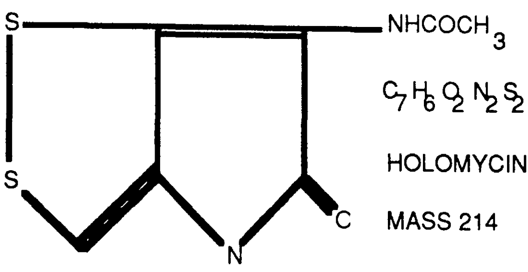


Figure 7.1 Structure of Holomycin

<u>Holomycin</u>

Isolation: extracted from culture filtrate with ethyl acetate
Nature: Neutral, orange-yellow rhombic
Melting point: 264-271^{0}C
Analysis(%): C 39.25, H 2.79, N 13.07, S, 29.77, CH_3CO 21.38, C-Me 7.0
UV max in unspecified solvent : 245, 302 and 390 nm
Theoretical Proportions (calculated according to formula $C_7H_6O_2N_2S_2$); C 39.2%, H 2.8%, N 13.0%, S 29.9%, O (by difference) 15%
UV max in methanol (e) ; 246 (4665), 301(2354) and 388 (7918) nm
^{1}H NMR spectrum (90MHz, DMSO-d_6) ∂: 1.98 (3H, s, CH_3CO-), 6.97 (1H, s, >C=CH), 9.75 (1H, s, -CONH-), 10.56 (1H, s, -CONH-).
Mass spectrum; m/e 214 (M^+), 172 and 43.

Figure 7.2 Chemical Characteristics of Holomycin (Data from Umezawa, 1967 and Kenig, 1979)

There are discrepancies in the literature regarding production of holomycin in *Streptomyces clavuligerus*, the donor strain for the pBROC139 insert. The wild type *S. clavuligerus* strain (NRRL 3585) was capable of producing holomycin when grown on sporulation agar and Jones Minimal Agar (Kirby, 1978). However, in another report (Kenig and Reading, 1978) holomycin production was not observed in the parental strain for their studies, but only in a mutant strain of *S. clavuligerus*. Strain IT1, the mutant strain of *S. clavuligerus* , could produce holomycin only when grown in a chemically-defined medium optimised for holomycin production; with glycerol and sucrose as carbon sources and in conditions which precluded the formation of cephem and ß-lactam antibiotics. The discrepancies between these two reports may be explained if the parental strain described in the Kenig and Reading paper is a production strain, and hence differs genotypically from the type strain.

A comparison between 10 mutants which have lost the ability to produce holomycin and the influence of this loss on the synthesis of "other antibiotics" has been made (Kirby, 1978). In one out of ten instances there was a correlation between holomycin production and the biosynthesis of cephem and ß-lactam antibiotics. It was inferred, on the basis of these results, that holomycin production is distinct from the synthesis of the major antibiotics produced by *S. clavuligerus*. Data and methods used to reach this conclusion were not described. However, in strain screening programmes, loss of holomycin production is loosely correlated with the development of *S. clavuligerus* strains with high titres for the production ß-lactam antibiotics. The improved yield of ß-lactam antibiotics in *S. clavuligerus* strains unable to produce holomycin (*hol*$^-$ mutants), is possibly due to an enrichment in the throughput of primary precursors to the biosynthesis of secondary products. Structurally holomycin is very similar to a putative structure formed by the condensation of two cysteines, or alternatively, to a structure derived from N-acetyl-penicillamine (described in Czaplewski, 1989). If the holomycin biosynthetic pathway is derived from either of these precursors, it would explain the apparent competition between the for ß-lactam biosynthesis and holomycin production. High cysteine requirements for the synthesis of acetyl cysteinyl valine are prerequisites for high titres of ß-lactam antibiotics. This strategy, which explains the putative mechanisms for the influence of holomycin on ß-lactam titres is explained in more detail in Czaplewski, (1989). ß-lactam antibiotics are not the commercial product formed by *S. clavuligerus*, and the construction of *hol* $^-$ mutants merely represents a historical step in screening and selection of production strains. *S. clavuligerus* is used for the production of the cephem, clavulanic acid at Smithkline Beecham. Prior to the studies described in this thesis and that of Czapleski, the product from the plasmid pBROC139 had not been characterised.

7.2 A Brief Theory of the Techniques used in the Structural Elucidation of the Product.

The structure of an unknown compound may be deduced by investigation of molecular spectroscopic patterns obtained from the interaction of the sample with radiation or bombardment with electrons. Ultraviolet and visible spectroscopy and mass spectrometry were used in structural elucidation and will be described in more detail. Elemental analysis using chemical and physical techniques would have been a suitable investigative tool; however the quantities required for this destructive technique were in excess of the quantities which could reasonably be purified. Infra-red spectroscopy may have yielded useful information, but continued structural investigation of the product was prematurely halted following NMR analysis at Leeds University.

7.2.1 Ultraviolet and visible spectroscopy If radiation is allowed to impinge on a substance, some is reflected, some absorbed and some transmitted. The transmitted radiation may be resolved into its constituent wavelengths and recorded to give an absorption spectrum. These spectra are useful as they arise from transitions between electronic energy levels accompanied by changes in vibrational and rotational states. Solution absorption spectra are not finely resolved because of solvent-solute interactions which smooth out the spectrum, but for a specified solvent, an absorption spectrum may be used in the identification of the solute.

It was noted at an early stage in the project that the synthesis of a yellow chromophore in chemical terms is more commonly observed than that of other chromophores (M. Stark, personal communication). Consequently the presence of a yellow chromophore was of little diagnostic value with respect to identification of the product, without a known, easily-identified λ_{max} absorption.

7.2.2 Electron Impact Mass spectrometry This technique is used in structural elucidation and identification of a compound, and relies on the separation of ions on the basis of charge to mass ratio. For this technique, the sample is vapourised and introduced into the ion source, where it is bombarded by electrons. As a result of the energy of the electron source (the potential energy is of a far greater magnitude than that of electrons in molecular orbitals), no bond movement occurs during ionisation, and specific fragmentation of the sample takes place. Molecular ions are those that reach the collector without decomposition, and the recorded fragment ions are those which are formed in the source; ions formed by fragmentation during

acceleration give weak signals ('metastables'). (H.M. Morris, personal. communication).

The ions are expelled, deflected, collected and their abundances are recorded as a function of their charge to mass ratios. Interpretation of a spectrum relies on basic chemical principles, knowlege of characteristics peculiar to the ionisation technique employed and a knowledge of basic interpretation rules given in most standard spectroscopy texts (for example Dyke *et al.*,1978: Freifelder, 1982).

The technique is very powerful and requires small quantities of material <100ng), although it has the disadvantage of destroying the sample. If vapourisation can be assessed, (*e.g.* with a coloured compound), vaporisation conditions may be selected to be specific for the molecule under investigation. However, as a result of the vaporisation of a mixture, the abundance report may then be biased towards the molecule under investigation.

7.3.0 Investigation into the Expression of a Yellow Pigment at Elevated Temperature in *S. thermonitrificans* pBROC139 cultures.

7.3.1 Investigation into the Influence of Temperature on Expression

Spores of *S. thermonitrificans* pBROC139 and pIJ702 were inoculated at high density into TSB medium at 30^{0}C and 45^{0}C and grown for a period of time. The length of cultivation for the seed stage inoculum was found to be important in influencing the final pigment intensity in broths cultivated at 30^{0}C. This is probably due to the slow growth at this temperature. The incubation time for seed inocula (48 hours at 45^{0}C and 5 days at 30^{0}C) was therefore the result of crude optimisation to enable growth and pigment production at both temperatures. Samples of 3 ml were taken from seed cultures at each temperature and used to inoculate 579MM (579 minimal medium) containing 3 mM phosphate and 82 mM glucose (15g.l^{-1}). These minimal medium cultures were then incubated - the procedure is summarised in Table 7.1. As stated in chapter 3, the minimum requirements for all growth experiments were that they were carried out in duplicate or triplicate and repeated at least once. A preliminary seed stage in TSB was required because spores of *S. thermonitrificans* did not germinate in minimal medium (see chapter 3 for further information).

Following incubation for the requisite period of time in minimal medium, the supernatants from broths inoculated with *S. thermonitrificans* containing the control plasmid pIJ702 did not show a yellow colour, whereas those of

Table 7.1 Experimental Procedure for An Investigation into the Effect of Temperature on Expression of the Yellow Colour in Broths of *S. thermonitrificans* pBROC139.

Temperature	Inoculum Type	Incubation in TSB	Inoculum Type	Incubation in 579MM
30^{0}C	Spore $>6x10^{6}$/ml	5 days	3ml TSB-grown mycelium	2 weeks
45^{0}C	Spore $>6x10^{6}$/ml	48 hours	3ml TSB-grown mycelium	48 hours

Figure 7.3: *S. thermonitrificans* broths cultivated under conditions suitable for formation of a yellow coloured broth at each temperature.

Table 7.2 Experimental Procedure for an Investigation into the Influence of Temperature on the Rate of Production of Yellow Broths from *S. thermonitrificans* pBROC139.

Temperature	Inoculum	Incubation in TSB	Inoculum	Incubation in 579MM
30^{0}C	Spore $>6x10^{6}$/ml	48 hours	3 ml TSB-grown mycelium	48 hours
45^{0}C	Spore $>6x10^{6}$/ml	48 hours	3 ml TSB-grown mycelium	48 hours

Figure 7.4 *S. thermonitrificans* cultures grown under similar conditions (identical apart from temperature), for 48h, illustrating variation in rate of production with temperature (by the differential intensities of broths).

1 pIJ702 at 30^{0}C
2 pBROC139 at 30^{0}C
3 pIJ702 at 45^{0}C
4 pBROC139 at 45^{0}C

S. thermonitrificans pBROC139 cultures were an intense yellow colour. Photographs were taken of the broths (Figure 7.3). At this stage in the project the sole indication of expression of a product from *S. thermonitrificans* pBROC139 was the production of yellow pigmented broths, in contrast to control cultures. Figure 7.3 illustrates that the expression of a yellow pigment from *S. thermonitrificans* pBROC139 cultures occurs at both 30^{0}C and 45^{0}C in defined medium. Hence the production of the yellow colour is not prevented as a result of the elevated growth temperature. This growth temperature contrasts with that of the host for the DNA insert, *S. clavuligerus*, which has an optimum growth temperature of 26^{0}C and a maximum of 30^{0}C.

7.3.2 The Influence of Temperature on the Apparent Rate of Production of Yellow Pigment in Broths

To investigate the influence of temperature on the rate of production of the yellow colour, the experimental procedure shown in Table 7.1 was carried out. In this case all the inoculum conditions were identical apart from the experimental variable, temperature. Mycelia were pelleted and photographs were taken of the supernatants (Figure 7.4).

It can be seen from Figure 7.4 that the intensity of yellow colouration in culture broths cultivated at 45^{0}C was greater than that for broths cultivated at 30^{0}C. The yellow tint in broths produced by cultivation at 30^{0}C cannot be seen easily on the photograph. The rate of production of this colour was far more rapid in broths at the elevated temperature (48 hours at 45^{0}C, compared to 2 weeks for growth at 30^{0}C for the photographs shown in Figure 7.3). The experiment was repeated using a growth temperature of 50^{0}C for both inoculum and experiment. Once again, even at the elevated temperature, yellow broths were produced (photographs were not taken). This shows that the product may be expressed up to a temperature of at least 50^{0}C. All these experiments were repeated several times to confirm the results.

Hence it has been shown in a crude manner that expression of a yellow product in *S. thermonitrificans* pBROC139 cultures readily occurs in defined medium (7.1.1). It has also been shown that this product is expressed both at the maximum growth temperature for *S. clavuligerus,* 30^{0}C and at temperatures up to 50^{0}C, the practically attainable maximum growth temperature for*S. thermonitrificans* (7.2.2). The apparent rate of production of yellow pigment in broths was increased at elevated temperatures, shown by visual comparison of broths. This rate increase is probably linked to the faster growth rate at the elevated temperatures.

7.4.0 Identification of the Yellow Product

7.4.1 Solvent Extraction using Butanol

The strategy described by Kenig and Reading (1979) was used in the extraction of the yellow pigment with 50 ml supernatants from *S. thermonitrificans* pBROC139, *S. albus* pBROC139 and *S. lividans* pBROC139 culture broths. The plasmid-containing strain,*S. albus* pBROC139, was constructed to prepare DNA for transformation into *S. thermonitrificans* and *S. lividans* pBROC139 was supplied by Beecham (Czaplewski, 1989). The extraction strategy used butanol for solvent extraction, followed by concentration of the non-aqueous phase (Kenig and Reading, 1979). The butanol phase characteristically turns vivid yellow when holomycin-containing broths are used (M. Burnham, personal communication). However, no partitioning of a yellow pigment into butanol occurred for any of the three different broths. Following concentration of the butanol fractions by evaporation, small amounts of a yellow oil remained. The colour of the oil is not necessarily diagnostic, as It is common for any impure oil to be yellow in colour (R A Hill, personal communication; see 7.2.1). The oil from each extraction was applied to a TLC plate together with a pure standard of holomycin and developed in chloroform:methanol, 9:1. A pattern of visible spots appeared, but none corresponded with that of the holomycin standard. This TLC plate was overlaid with agar seeded with a low concentration of *Flavobacterium spp.* (a sensitive organism for the detection and assay of holomycin). The method is described in Section 2.15. Inhibition of growth of *Flavobacterium spp.* was detected with the holomycin standard. However no growth inhibition occurred on the control or pBROC139 tracks from any of the cultures. The overlay was repeated with a range of *Flavobacterium spp.* concentrations, but no sensitivity to any component in tests was detected by the bioassay, (the holomycin standard in each case resulted in growth inhibition of *Flavobacterium*). These results suggested that the yellow colour was produced in very small amounts in all the heterologous hosts and that partitioning into the butanol phase was negligible. Alternatively, it could be concluded that the yellow colour could not be attributed to holomycin.

7.4.2 Solvent Extraction using Ethyl Acetate

A modified extraction procedure was devised in consultation with Dr R.A.Hill from the Department of Chemistry. This involved using ethyl acetate as the organic solvent and extraction of broths at pH7 and pH9. Ethyl acetate was preferred to butanol as a solvent because it has a much higher volatility and is relatively less toxic than butanol.

A 4-day culture of *S. thermonitrificans* pBROC139 grown from a spore inoculum in 2 litres of TSB at 37^{0}C, was clarified by centrifugation and the supernatant used in the extraction procedure. TSB, a complex medium rich in nitrogen sources was the medium of choice at this stage in the investigation for identification of the product. This strategy was invoked to reduce the probability of a nutrient limitation in the medium influencing synthesis of product from pBROC139. It was conceivable that, if the minimal medium 579MM was used, a nutrient limitation (*e.g.* a lack of a specific amino acid) could restrict the formation of the product from *S. thermonitrificans* pBROC139. One third broth volume of ethyl acetate was used to extract 1.1 litres of culture supernatant at pH7.0, the broth pH was altered to pH9.0 by the addition of 1M sodium hydroxide and the extraction procedure repeated. A final extraction was made with one third volume of butanol. Extracts were evaporated to dryness yielding 300 mg of an oily product at pH7.0, and 70 mg of oily product at pH9.0.

The samples were loaded onto mini-TLC plates together with an authentic holomycin standard, and developed in ethyl acetate. None of the extracts from cultures of *S. thermonitrificans* pBROC139 showed components with the same mobility as the holomycin standard at the detection levels employed. The detection methods included visualisation under normal lighting, long wavelength ultraviolet light, and after staining with iodine (which reacts with and stains carbon double bonds). The mini-TLC plates were overlaid with agar containing *Flavobacterium,* but no sensitivity to the organism was detected in test tracks. *Flavobacterium* showed a very sensitive growth inhibition to the presence of the holomycin standard. Regardless of these negative results, each extract was subjected to preparative TLC, and the five major bands were excised and sent for analysis by low field NMR. It was considered possible that any of the extracted components could have been precursors or the degradative products from the product of the plasmid pBROC139. However, no conclusive data were received from low field NMR. This was probably due to the lack of purity of the samples.

7.4.3 Solvent Extraction using Broths Produced at 30^{0}C and 50^{0}C and An Attempt to Develop a Simple Assay for Expression of a Yellow Colour

As a consequence of the lack of visible yellow colour in the culture broth grown in 2 litres of TSB, the experiment was repeated with smaller culture volumes at a range of temperatures in defined medium 579MM (note the change in medium from that used in 7.4.2). The intensity of yellow appeared to be greater and more reproducible in defined medium broths and the easily-observed formation of yellow-pigmented broths using defined media was

Table 7.3 List of Samples Collected from Broths Cultivated at 30^{0}C and 50^{0}C

Culture	Temperature	pH	
pIJ702	30^{0}C	pH7	
pIJ702	30^{0}C	pH9	
pBROC139	30^{0}C	pH7	
pBROC139	30^{0}C	pH9	
pIJ702	50^{0}C	pH7	
pIJ702	50^{0}C	pH9	
pBROC139	50^{0}C	pH7	
pBROC139	50^{0}C	pH9	
pIJ702	30^{0}C)	Butanol fractions	(Butanol fractions for pBROC139 cultures were lost during evaporation)
pIJ702	50^{0}C)		

Holothin

Isolation: Extracted from mycelium with Me_2CO. Also obtained by acid hydrolysis of holomycin
Nature: Basic, -HCl : olive-green crystal.
Melting Point: -HCl: > 300^{0}C
Formula: $C_5H_4ON_2S_2.HCl$
UV max (solvent unspecified): 226, 290 and 381nm.

Figure 7.5a Chemical Characteristics of Holothin (Data from Umezawa, 1967)

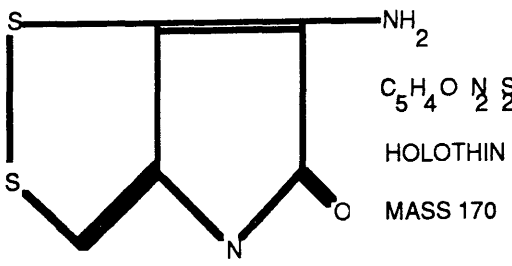


Figure 7.5b Structure of Holothin

considered preferable at this stage. *S. thermonitrificans* minimal medium (579MM) containing 82 mM glucose and 3 mM phosphate was used in all subsequent studies.

Primary seed cultures of *S. thermonitrificans* pBROC139 in TSB were established at 30^{0}C and 50^{0}C. These primary seed stage cultures were inoculated into 500ml of 579MM (containing 82 mM glucose and 3 mM phosphate) after cultivation for 5 days (for 30^{0}C cultures) and 24 h (for 50^{0}C cultures), to a final inoculum concentration of 0.06 ml inoculum per ml medium. Cultures were subsequently grown for 48 (50^{0}C) and 96 hours (30^{0}C), resulting in broths which were a vivid yellow colour in contrast to the brown tinted control cultures. Broths were harvested and clarified by centrifugation at 3,000g in a JA14 rotor for 10 minutes. The method described for ethyl acetate extraction in 7.4.2 was used and extracts concentrated by evaporation, resulting in 10 samples, listed in Table 7.3.

Each extract was loaded onto a mini TLC plate together with a holomycin standard and developed in ethyl acetate. The plates were then overlaid with *Flavobacterium* -containing agar. However, no spots corresponding to components having the same mobility as holomycin were identified in any sample. Neither were there any zones of clearing in the seeded agar which would correspond to a compound to which low concentrations of *Flavobacterium* were sensitive. Holomycin (<<1ng) gave a zone of growth inhibition of*Flavobacterium* around 2.0 cm diameter after overnight incubation.

Each of the 10 samples was dissolved in analytical grade methanol at a concentration which would give spectrophotometric readings in the range of absorbance 0 to 2 over 560-230 nm wavelength. No constructive information could be deduced, because the spectra consisted of broad absorbance peaks over the ultraviolet range of the electromagnetic spectrum. Difference spectra were attempted. This involved identifying a range of wavelengths from which absorptions emanating from yellow compounds would be negligible. The concentrations of pBROC139 samples were then corrected so that the absorption for both control and test samples were the same at these wavelengths. Using this strategy, the control pIJ702 sample was used as a blank in a single beam spectrophotometer, the blank was scanned, the scan stored and subtracted from the test sample to give the difference spectrum (alternatively a dual-beam spectrophotometer could have been used). However all difference spectra showed a part of the spectrum which was negative for large regions. This is probably a result of expression of the *mel* gene in pIJ702 cultures in some conditions, encoding a tyrosinase which, when expressed, results in brown-tinted broths. In plasmid pBROC139 the *mel*

gene is inactivated by the 5.3kb DNA insert. Spectrophotometry of culture broths could not be employed as a direct assay method for the yellow colour, because the broth colour in wild-type cultures used to change during the time course of a fermentation and could not be used as an assay blank. Similarly, pIJ702-containing (control) cultures had broth pigments which varied with time and growth conditions.

It was concluded from this work that the yellow pigment was probably not holomycin. Additionally, the lack of holomycin production in both defined and complex medium, and at temperatures of 30^{0}C, 37^{0}C and 50^{0}C, suggested that the temperature conditions and medium-determined nutrient supply cannot be invoked as wholly responsible for the absence of holomycin production. Holothin, a de-acetylated, and therefore more polar homologue of holomycin was identified as a potential product.

An assay for the yellow pigment was necessary to investigate, quantitatively, the physiology of production. Assay of the yellow pigment was therefore made more difficult than originally anticipated as a result of its unknown nature. A purification strategy was therefore adopted. It was envisaged that the purification of the product would both enable the identification and assay-design for the product in culture broths, which could then be used in quantitative studies of the formation of the heterologous product at elevated temperatures in *Streptomyces*.

7.5 Purification of The Yellow Product

Isolation of the yellow product was achieved by Dr Martin Gilpin and Mr S. Spear at Beecham Pharmaceuticals, Brockham Park. Their work is described in the following paragraphs.

Two litres of *S. thermonitrificans* pBROC139 culture broth grown on 579MM were supplied to Beecham. The broth was extracted with ethyl acetate at pH7, the phases separated and evaporated. Antibacterial activity against *Staphylococcus spp.* was detected in the extracts; this was thought to be due to extraction of thiostrepton into the ethyl acetate phase. Thiostrepton is the antibiotic used for plasmid selection during growth of plasmid-containing strains. No holomycin-like antimicrobial activity was detected.

The aqueous phase was loaded onto a column of Diaion HP20, an adsorption resin, and eluted with 20% (v/v) propanol. Yellow fractions were then collected, pooled and loaded onto a finer-grade column matrix. The pigment was eluted using 20% (v/v) propanol; fractions containing the yellow colour

were evaporated to dryness (yielding 7 mg of product) and submitted for ion-bombardment mass spectrometry. The resulting spectrum could not be interpreted. However this was thought to be a result of the hydrophilicity of the pigment, and the compound was resubmitted for a more suitable form of mass spectrometric analysis (method not given). In addition, it was submitted for nuclear magnetic resonance spectroscopy. Nothing could be interpreted from the NMR and MS results.

Therefore following the isolation of the yellow product at Beecham, further purification was continued in Glasgow. The strategy was based on a putative purification scheme for holothin, the de-acetylated homologue of holomycin. Structure and chemical characteristics of holothin are given in Figure 7.5.

7.5.1 Discontinuous Fed Batch Cultivation for Reproducible, Rapid Production of High-Intensity-Yellow Broths

Culture broths for subsequent purification of pigment were prepared according to the fed-batch regime described in Section 2.16. This regime was found to generate large volumes of intense yellow broths reproducibly, and in a short time at a growth temperature of 45^{0}C. Possible reasons for this may have been:

1.Cultivation in TSB at 45^{0}C using a very high concentration of *S. thermonitrificans* pBROC139 spores in a 500 ml flask with a small medium volume (75 ml) resulted in rapid growth to a high biomass level. It is probable that stationary phase was reached in a short period of time. A 'switch' into yellow production was observed between 20-30 hours under these conditions.

2. Subsequent growth in larger medium volumes (the "feeding" stage) may have provided an environment conducive to a slower growth rate during the period of yellow production due to the decrease in the rate of oxygen transfer to the medium.

3. The use of a defined medium with a relatively low initial phosphate concentration (following the initial growth to high biomass concentrations), may have maintained the phosphate concentration at a lower level during the period of yellow production. There was no evidence that phosphate concentration influenced formation; however pigment production on low-phosphate defined-medium broths was consistently more reproducible than in high-phosphate media (TSB and 579MM containing 10mM phosphate). Inorganic nitrogen concentrations in all defined media were 50 mM. There was no visible production of a yellow product during growth on TSB with a "normal"

Figure 7.6 Scheme for Normal Performance Liquid Chromatography with Amberlite XAD4 Adsorption resin.

Column Type	Pharmacia C 16/40
Column size	16 mm internal diameter 40 cm length
Column Volume	77 ml
Bed Height	38 cm
Stationary phase	Amberlite XAD 4 resin
Mobile phase	20% propanol
Column flow rate	0.5 ml/min

Table 7.4 Results for the Thin Layer Chromatography of Fractions Collected from the Amberlite XAD4 Adsorption Column.

Stationary phase; silica, mobile phase;20% propanol

Yellow fractions	Rf 0.58 (Yellow spot visible) Rf 0.15 (visible only under medium wave UV)
Brown fraction	Rf 0.58 (yellow spot visible) Rf 0.15 (visible only under medium wave UV) A brown smear was seen between the two spots.

Stationary phase; silica, mobile phase; acetone; propanol; water

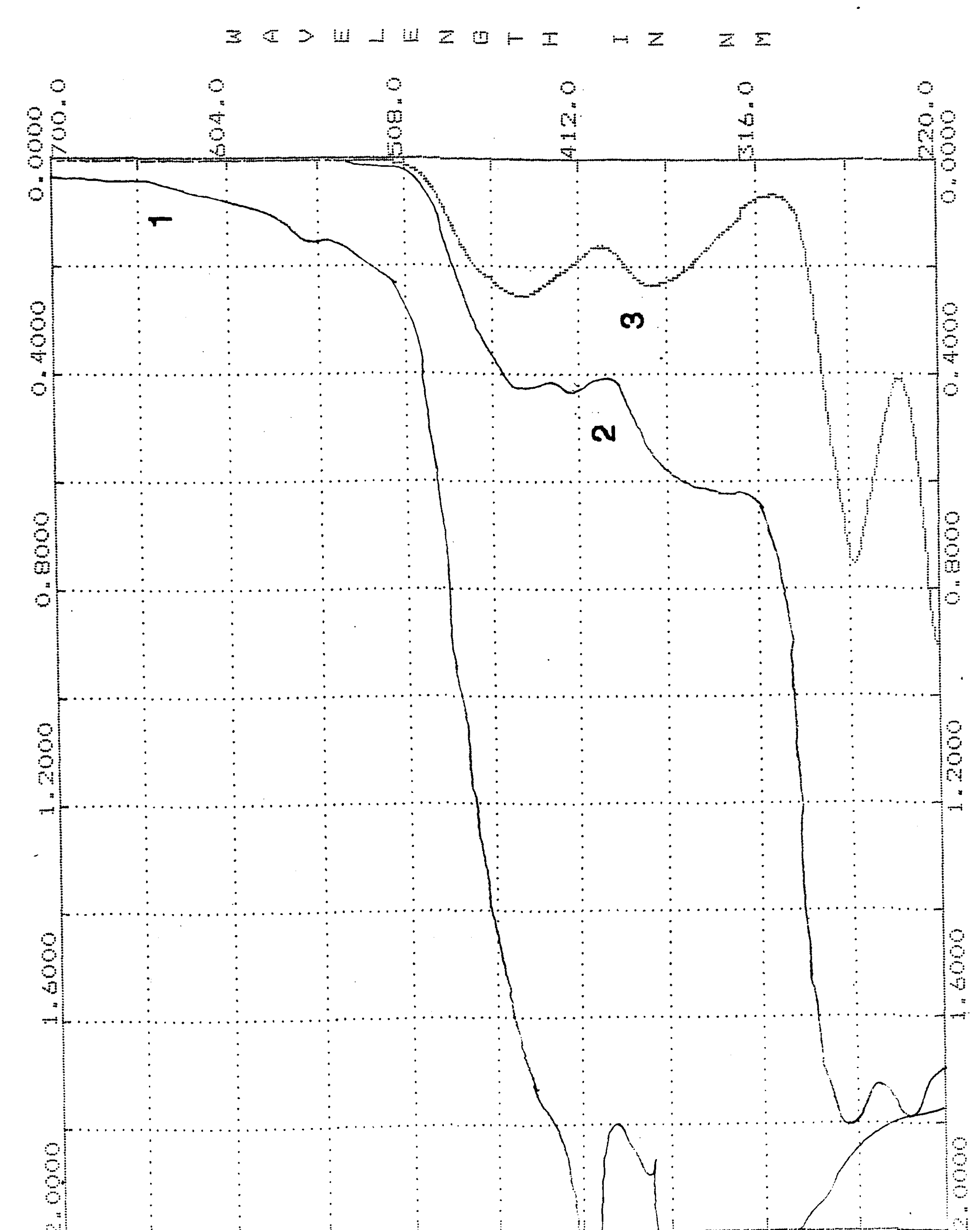
Yellow fractions	Rf 0.78 (yellow spot visible) Rf 0.63 (brown spot visible) Rf 0.09 (visible only under medium wave UV)
Brown fractions	Rf 0.78 (yellow spot visible) Rf 0.63 (brown spot visible) Rf 0.09 (visible only under medium wave UV)

Figure 7.7. Ultraviolet and Visible Scans of the Components Eluted From the Normal Performance Adsorption Column (described in 7.5.4).

Spectrum 1; 1μg of the brown fraction dissolved in 1ml AnalR methanol.

Spectrum 2; 0.5μg of the yellow fraction dissolved in 1ml AnalR methanol.

Spectrum 3; 1μg of the yellow fraction dissolved in 80% AnalR propanol.



A spectroscopic scan of the fractions collected from the column was not carried out, as the fractions were eluted in a highly concentrated state and it was considered that little useful information would be gained by diluting and then scanning each sample, given the paucity of information that was known about the pigment. Instead, the pooled fractions were scanned across both the ultraviolet wavelengths and the visible wavelengths of the spectrum (Section 7.5.4).

S. thermonitrificans pBROC139 spore concentration of 2 x 10^5 spores per ml final concentration and with normal medium/flask volume ratios. However during growth on liquid Emersons medium under 'normal' conditions there was a visible production of yellow pigment (L.Czaplewski, personal communication). Liquid Emerson is a complex medium with a relatively low phosphate concentration compared to TSB.

7.5.2 Column Chromatography

Amberlite XAD 4 resin was chosen as a suitable resin for isolation of the yellow pigment from culture broths. It is a cross-linked polystyrene adsorbent with no ionic functional groups, and is conventionally used in the adsorption of organic solutes from polar solvents. A long column, with a moderate internal diameter was used giving a column height-diameter ration of 1:25 for optimal resolution. This ratio is suitable for exclusion and adsorption chromatography (Pharmacia). The details relating to this chromatographic step are given in Figure 7.6

The column was loaded with 3 litres of culture broth for each column volume of matrix in the preparative mode. Final resolution was unavoidably impaired due to expansion of the matrix on interaction with organic solvents. This reduced the total volume of elution solvent, with only minor additional impairment of resolution. Continuous gradient elution of 10-100% (v/v) propanol, a stepped gradient (50% and 100% v/v propanol) and isocratic elution using 20% (v/v) propanol were tested for column development. Isocratic elution using 20% (v/v) propanol was found to be adequate, together with a fraction size of 1 ml.

A range of pigments was eluted from the column. The first 46 ml of eluate appeared brown. After 49 ml, an intense yellow colour was eluted, whose concentration decreased after the collection of 66ml of eluate. Coloured fractions were pooled into brown fractions and yellow fractions, evaporated to dryness and their mass determined. At this stage, very little information was known about the pigments and a more sensitive technique for identification was not available. The final mass of the collected yellow samples was 33.2 mg, and 125.9mg for the brown fractions. They were analysed by TLC (Section 7.5.3) and by ultraviolet and visible spectroscopy (Section 7.5.4).

7.5.3 Thin Layer Chromatography of Fractions

Two different solvent systems were used for these separations, 20% propanol, and 1;1;1 acetone; propanol; water. The results are tabulated in Table 7.4. Holomycin had negligible solubility in these solvents. The experiment was

carried out on silica TLC plates containing a fluorescent marker, viewed under medium and short wave ultra violet light, and stained with iodine vapour. Iodine is a staining agent which will denature and stain carbon double bonds. All the components which were visible using these techniques are described in Table 7.4.

The data shown in Table 7.4 indicated that the compounds isolated by chromatography did not possess mobilities characteristic of holomycin, and that the isolates were a mixture of compounds. Further purification was therefore required.

7.5.4 Spectrophotometric Investigation of Fractions

The samples collected from the adsorption column were scanned spectrophotometrically from 230 nm to 560 nm using methanol as a solvent (Figure 7.7). Maximum intensity absorbances were recorded for the yellow fractions at 440 nm, 421nm, 342 nm, 271 nm and 232 nm. This did not correspond with the spectrum of holomycin or holothin (shown in Figure 7.2 and Figure 7.5b). The brown fractions scanned under the same conditions showed a broad absorption over the range 500-220 nm. Dilutions to a low absorbance showed the same broad absorption. This suggested that the brown fractions were a mixture of compounds with overlapping absorption spectra.

7.5.5 Bioassay Development

Isolation had reached a stage at which a bioassay could be developed. It was hoped that the purification and identification would provide standards with which to develop a TLC or HPLC assay. Concentration of the yellow extract by passing down an Amberlite XAD 4 column and subsequent evaporation resulted in the precipitation of a yellow powder. This powder was washed several times in HPLC grade methanol and de-ionised water and evaporated to dryness. Samples were then dissolved in warmed L broth with shaking, to the maximum solubility of the compound. This was described as 100% concentration, and estimated to be a solubility of 1 mg/ml for the impure yellow compound in aqueous solvents. The supernatant was filter-sterilized and frozen for further use.

A matrix experiment was established using overnight cultures of *Klebsiella aerogenes*, *Escherichia coli* ML308 and *Bacillus subtilis* EME106. A turbidimetric approach was adopted in preference to an agar diffusion assay as a result of the low solubility of the yellow pigment(s). Dilutions of test

Table 7.5 Development of a Bioassay for the Yellow Product.

Concentration of Y in L Br (as a % of maximum solubility)	Test Organism	Concentration of Test organism	Result
100%	*E. coli*	1×10^{-3}	Turbid Growth
100%	*K. aerogenes*	1×10^{3}	Turbid Growth
100%	*B. subtilis*	1×10^{3}	Clear
50%	*E. coli*	1×10^{3}	Turbid Growth
50%	*K. aerogenes*	1×10^{3}	Turbid Growth
50%	*B. subtilis*	1×10^{3}	Turbid Growth
25%	*E. coli*	1×10^{3}	Turbid Growth
25%	*E. coli*	1×10^{4}	Turbid Growth
25%	*K. aerogenes*	1×10^{3}	Turbid Growth
25%	*K. aerogenes*	1×10^{4}	Turbid Growth
25%	*B. subtilis*	1×10^{4}	Turbid Growth
25%	*B. subtilis*	1×10^{4}	Turbid Growth

Figure 7.8 Scheme for Silica Gel Filtration Chromatography (HPLC mode)

TSK Silica Gel Filtration column G2000 SW	7.8 mm internal diameter x 300 mm length
Guard column	SW XL
Mobile phase	50 mM potassium phosphate buffer pH6.0
Injection volume	20µl (loop injection)
Flow rate	0.5 ml/min

organisms were made in L broth, together with dilutions of the test solution (yellow pigment dissolved in L broth). Tubes were incubated overnight and scored on the basis of presence or absence of growth of the test organism, the results are given in Table 7.5. The experiment was repeated three times to confirm the result. Test organism concentrations at dilutions greater than 1 in 10^4 gave similar results to those described in Table 7.5.

On the basis of the results presented in Table 7.5, *Bacillus subtilis* was identified as a suitable sensitive strain for assay of the yellow product. It was noted that high concentrations of test sample were required to obtain a growth response. Under the same test conditions *Bacillus subtilis* was insensitive to holomycin and in solid media, insensitive to thiostrepton at a concentration of 10 µg/ml. Further work was continued in the purification of the yellow product, which was considered more important at this stage than studying the expression of the product in *S. thermonitrificans* pBROC139.

7.6.0 High Performance Liquid Chromatography and Fast Protein Liquid Chromatography

A Phillips HPLC system was kindly made available by Bioflux, Department of Biochemistry, Glasgow, and a Pharmacia FPLC system was available in the same department.

For HPLC, the column was linked to a multiwavelength detector which could operate in single wavelength or scanning mode (200-400 nm and 400-600 nm). Data handling was effected using an IBM-compatible personal computer using software working in the Microsoft Windows environment. A range of data output was available; three dimensional plots, contour plots, two dimensional plots and spectra. This detection system was found to be ideal for an unknown compound. The FPLC system is well documented as a chromatographic system for the purification of proteins.

7.6.1 Silica Gel Filtration Chromatography (HPLC-mode)

In view of the paucity of information regarding the yellow product a silica gel filtration column was used in the initial stages of HPLC purification. It was known that this column retards the elution of small molecules, presumably due to adsorption, and it was this retardation which was potentially useful in achieving a separation of an unknown compound. (D. Mousedale, personal communication). The operating separation range of this column for proteins and peptides was in the range of between 500 and 60000 Da molecular weight. Small molecules were expected to elute towards the exclusion limit of

Figure 7.9 a The Elution Profile of the Yellow Compound from the TSK Gel Filtration Column described in 7.6.1(Scanned at a Wavelength of 280 nm). (Analytical sample -20µl)

The yellow fraction is marked with an arrow.

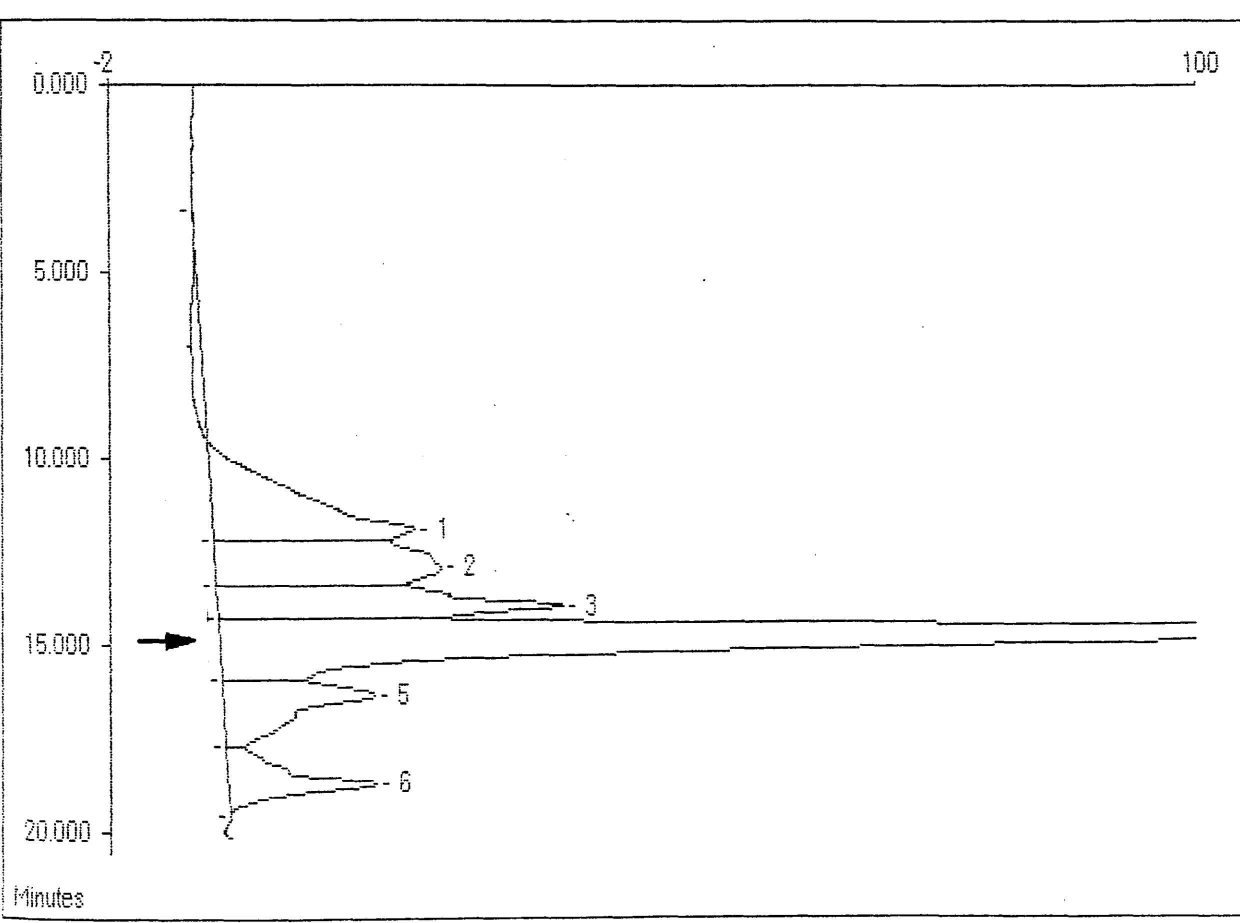
Online Channel 2 Sample : 3 Run Type : Unknown 11:38 Thu Aug 17 1989

Method : 4 Results File : Raw Data : Saved as TSK.003

Mode : Manual Batch Name : TSK

Scale : 1.0000000 Sample Amount : 1.0000000 Sample Name :

Comments :

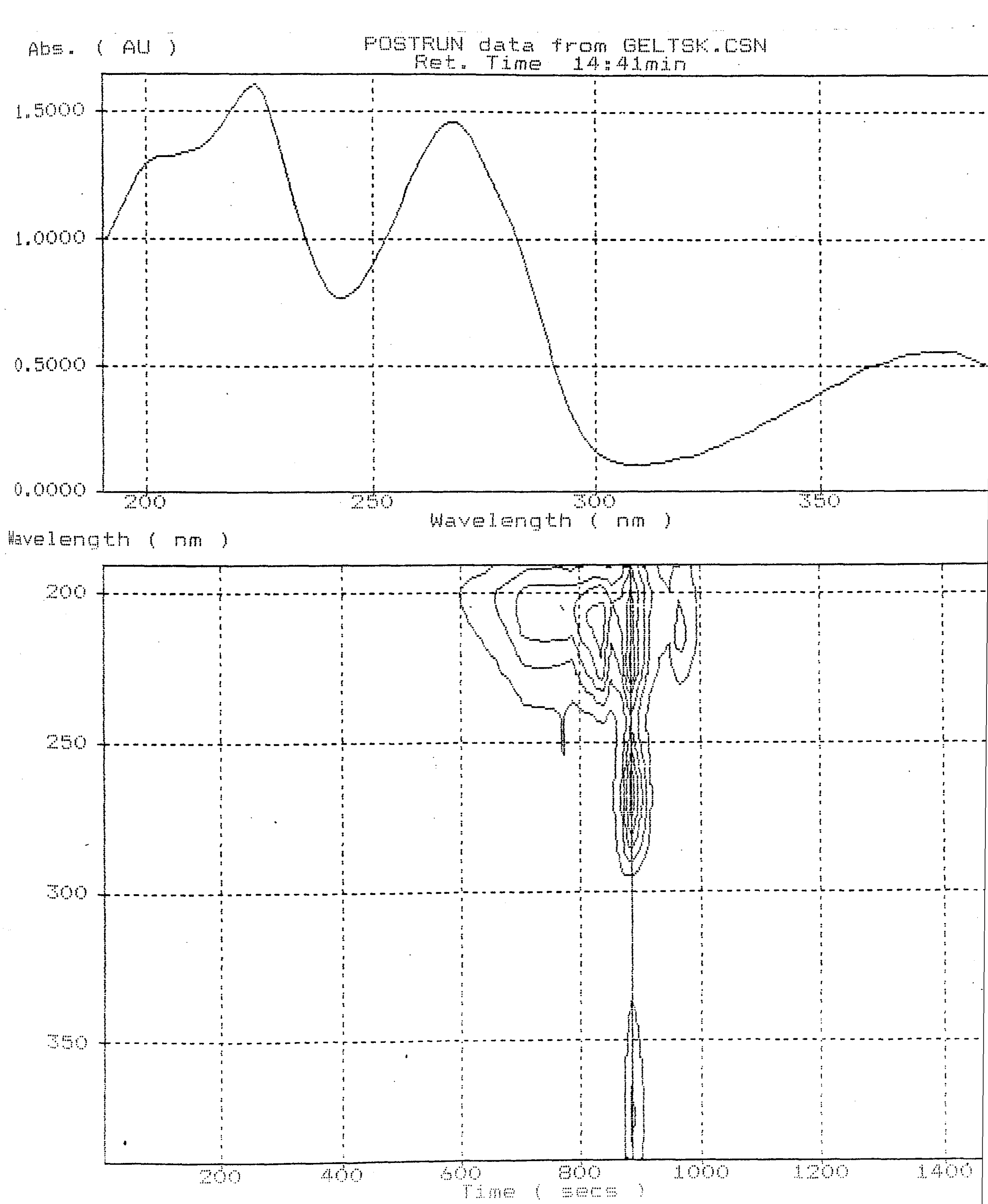


Peak	Type	RT(mins)	Area	Height	Base	Conc
1	BV	11.800	1186.279	18.900	7.631	12.106
2	VV	12.833	1340.658	21.144	7.852	13.684
3	VV	13.833	1269.016	32.616	8.065	12.953
4	VV	14.767	4650.641	91.736	8.284	47.470
5	VV	16.183	801.053	14.201	8.567	8.176
6	VB	18.533	549.433	13.805	9.088	5.608

Figure 7.9b A Scan of the Component with an Elution Time of 14.7 minutes from the TSK Gel Filtration Column described in 7.6.1. (Analytical sample - 20μl)

i) Ultraviolet Scan over the Region 200-400nm

ii) Contour Plot of the Elution Profile of the Column Effluent over the Region 200-400nm.



the column (about 13-15 ml). The scheme for this chromatographic step is given in Figure 7.8.

Six peaks were found on analytical runs scanning in single wavelength mode at 280 nm, a wavelength suitable for the detection particular absorption characteristics of aromatic ring systems (Figure 7.9a). The spectrum of the yellow compound in potassium phosphate buffer was also recorded (Figure 7.9b). The retention time in phosphate buffer pH6.0 was found to be 14.7 min.

Several preparative runs were made, loading a high concentration of the sample in the maximum loading volume (20 μl), and manually collecting 100 μl fractions. In preparative runs ammonium formate pH5.0 was used as a volatile buffer that would not interfere with subsequent analysis. Fractions corresponding to the yellow peak were pooled, washed and evaporated to dryness, yielding 7.3mg of product. The yellow product was submitted for ^{1}H NMR analysis in deuterated water at the Chemistry department, Glasgow University. However no interpretable signal was obtained, due to the presence of contaminants.

7.6.2 Ion Exchange Chromatography

It was noted that ultraviolet spectra of the yellow peak from the gel filtration column in phosphate buffer pH6 had some similarity to the spectrum for holothin in an unspecified solvent, (Figure 7.9b and Figure 7.10). Therefore, the successive strategy was to identify if the yellow compound possessed a free amino group; this would intimate that the compound might be holothin.

FPLC glass ion-exchange columns were chosen for investigation of the ionic interactions of the yellow compound, because binding of the pigment to the column could easily be observed. Ionised compounds are retained on charged columns to different extents depending on their pKa and the pH of the solution. Mono S is a strong cation exchanger based on Pharmacia Mono beads with sulphonic acid groups attached to the matrix. Mono Q is the anion exchange equivalent having quaternary amine groups which remain charged over the pH range 2-12. The scheme used for Cation exchange chromatography is described in Figure 7.11.

The sample was loaded by loop injection in 2 ml ammonium formate at pH5, and binding assessed visually. The yellow pigment was eluted in the first 5ml without binding to the column. These fractions were pooled, the pH adjusted to 2.48 with formic acid and re-applied to the column . No binding of yellow pigment occurred and it was washed from the column. Elution of the column

Figure 7.10. Ultraviolet Absorption Data for Holothin, Holomycin and the Yellow Product from pBROC139 broths.

UV max for holothin in an unspecified solvent (From Umezawa,1967)	= 226, 296, 380 nm
UV max for holomycin in methanol (From Kenig,1979)	= 246, 301, 388 nm
UV max for yellow product in phosphate buffer pH 6	= 226, 270, 381nm

Figure 7.11 Scheme for Cation-exchange and Anion-exchange Chromatography (FPLC mode).

Mono S - Cation Exchange

Column dimensions	5 x 50 mm
Stationary phase	Mono S beads
Mobile phase	Ammonium formate (50 mM) pH 5 and pH 2.48
Column flow rate	1 ml/min
Fraction size	1 ml
Chart recorder speed	1 ml/min

Mono Q - Anion Exchange

Column dimensions	5 mm internal diameter x 50 mm length
Stationary phase	Mono Q beads
Mobile phase	Ammonium Bicarbonate (50 mM) pH 8
Column flow rate	1 ml min^{-1}
Fraction size	1 ml
Chart recorder speed	1 ml min^{-1}

with formic acid pH 2.48 and a low-sensitivity detector setting, resulted in an off-scale absorbance reading for the first 2 ml of column eluate. Hence some contaminating components of the mixture applied to the column had bound, and some purification of the yellow pigment had occurred.

A second approach was taken, using a Mono Q column under conditions in which a free amine group would be de-protonated. The scheme used for anion-exchange chromatography is described in Figure 7.11. A 500 µl sample was loaded in ammonium bicarbonate buffer pH8.0. No binding occurred and the pigment was eluted from the column. The lack of interaction of the compound with Mono S and Q columns therefore suggested that no free amine groups were present in the yellow substance. To confirm these results, a sample from FPLC was applied to a Polypore H cation exchange column (HPLC). This column was used only for confirmation because binding of a charged group would have been almost irreversible in view of the strong cationic nature of the matrix. As expected no binding occurred in formate buffer pH 2.48, further consolidating the information that no free amine group was present in the yellow compound.

7.6.3 Reverse Phase Chromatography

Reverse phase chromatography was the final method chosen for purification. In this form of chromatography the stationary phase is a non-polar hydrophobic surface and the mobile phase is a polar solvent such as acetonitrile or an alcohol. Competition occurs between the stationary and mobile phases for the solute, with the result that solutes are eluted in order of increasing hydrophobicity. Analytical scale separations on the reverse phase column with small amounts of the yellow product in 0.1% trifluoroacetic acid indicated that binding was occurring, and that this step would be effective in purification. A method was developed on an analytical scale (Figure 7.12) which provided good resolution of components. The post chromatographic spectrum is shown in Figure 7.13.

The column was loaded in batch manner in preparative runs (that is, repeated loading through the injection loop so that up to 2 ml of solution could be loaded onto the column before elution). This is an advantage of this type of chromatography, and although resolution may have been impaired, it was preferable to carrying out excessively large numbers of elutions. A particular problem encountered was the low solubility of the yellow compound in a wide range of solvents, which became increasingly noticeable as the material increased in purity.

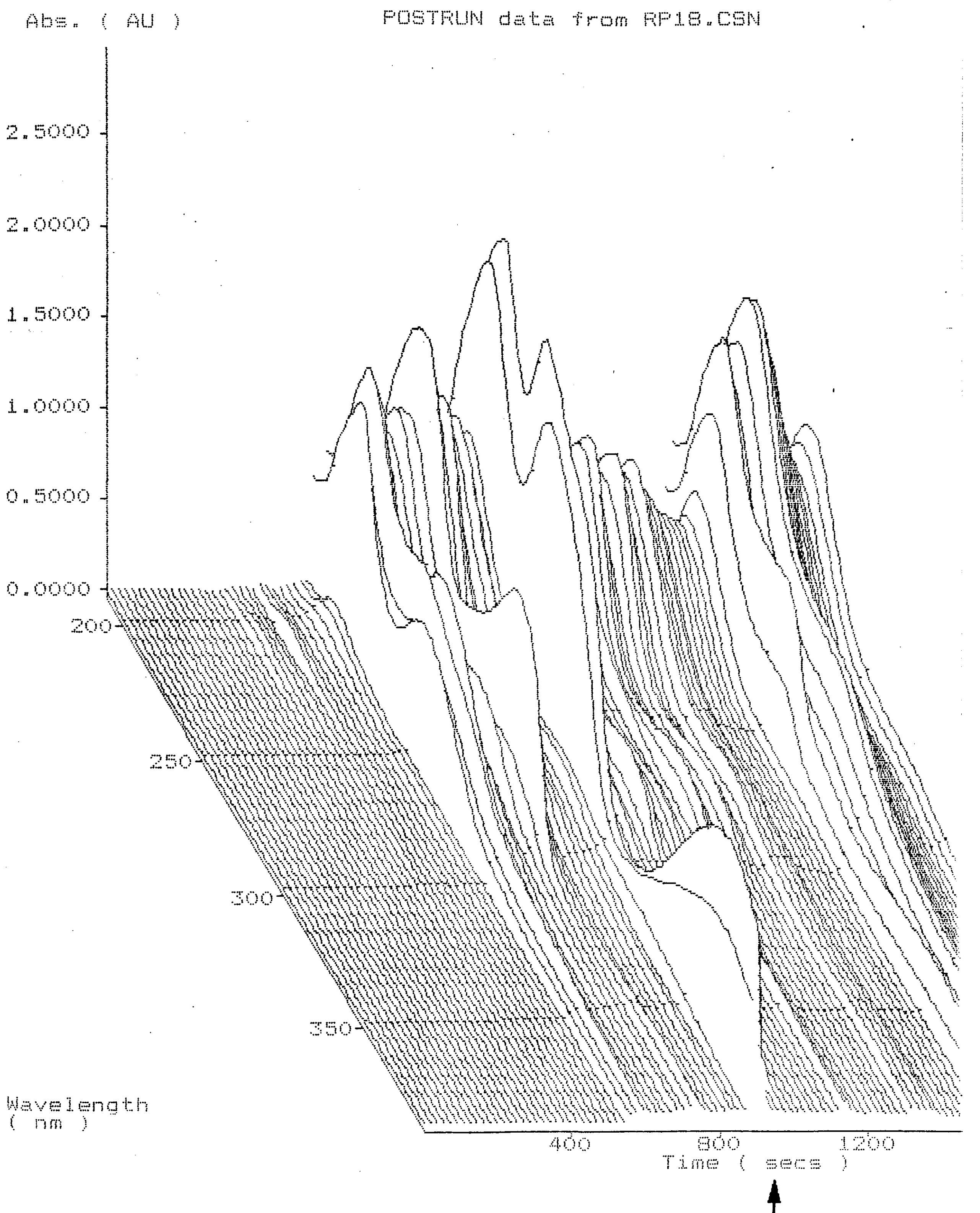
During

Figure 7.12 Scheme for Reverse Phase Chromatography

Spherisorb 5 RP18 column	4.6 mm internal diameter x 220 mm length
Guard column	SW XL
Mobile phase	A = 0.1% trifluoroacetic acid
	B = 0.1% trifluoroacetic acid in acetonitrile
Method	100% A 2 minutes
	Linear gradient 100%-79.3% A in 5 minutes
	Isocratic at 79.3%A, 20.7%B for 15 min
	Linear gradient 79.3%A-0%A in 5 minutes
	Linear gradient 0%-100%A in 2 minutes (reset)
Injection volume	20µl (loop injection)
Flow rate	0.5 ml min^{-1}
Retention time	14.7 min

Figure 7.13. Scan of the Components Eluted from the Reverse Phase Column[182] over the Ultraviolet Wavelength Range, using the Procedure described in Figure7.12 (Analytical Sample - 20µl)

The yellow fraction is marked with an arrow.



preparative runs it was common for the yellow compound to be eluted at a concentration beyond its solubility product, and most of the pigment would crystallize out of solution rapidly. These crystals when re-applied onto the column showed several peaks eluting over the isocratic section of the elution profile on either side of the major peak of yellow colour, separated by 500 μl (1 min in time). The yellow fractions collected from the major peak in preliminary preparative runs were pooled and re-applied to the column for a final preparative run. Three peaks of high intensity yellow colour were eluted from the column. The satellite peaks were visibly yellow in the collected fractions and the three peaks were collected separately. The quantities recovered were;-

Peak I	-	Satellite peak	1.1 mg
Peak 2	-	Main peak	1.4 mg
Peak 3	-	Satellite peak	0.7 mg

Initially the satellite peaks were thought to be precursors or degradative products of the major peak. However it was suggested that they could be a range of chemically- similar amides, an argument which would also explain the co-crystallization which was observed. (Professor C. Brookes, personal communication). All three samples were evaporated and sent for ^{1}H NMR analysis. However, the background level of impurity was still too high for a clear signal to be detected.

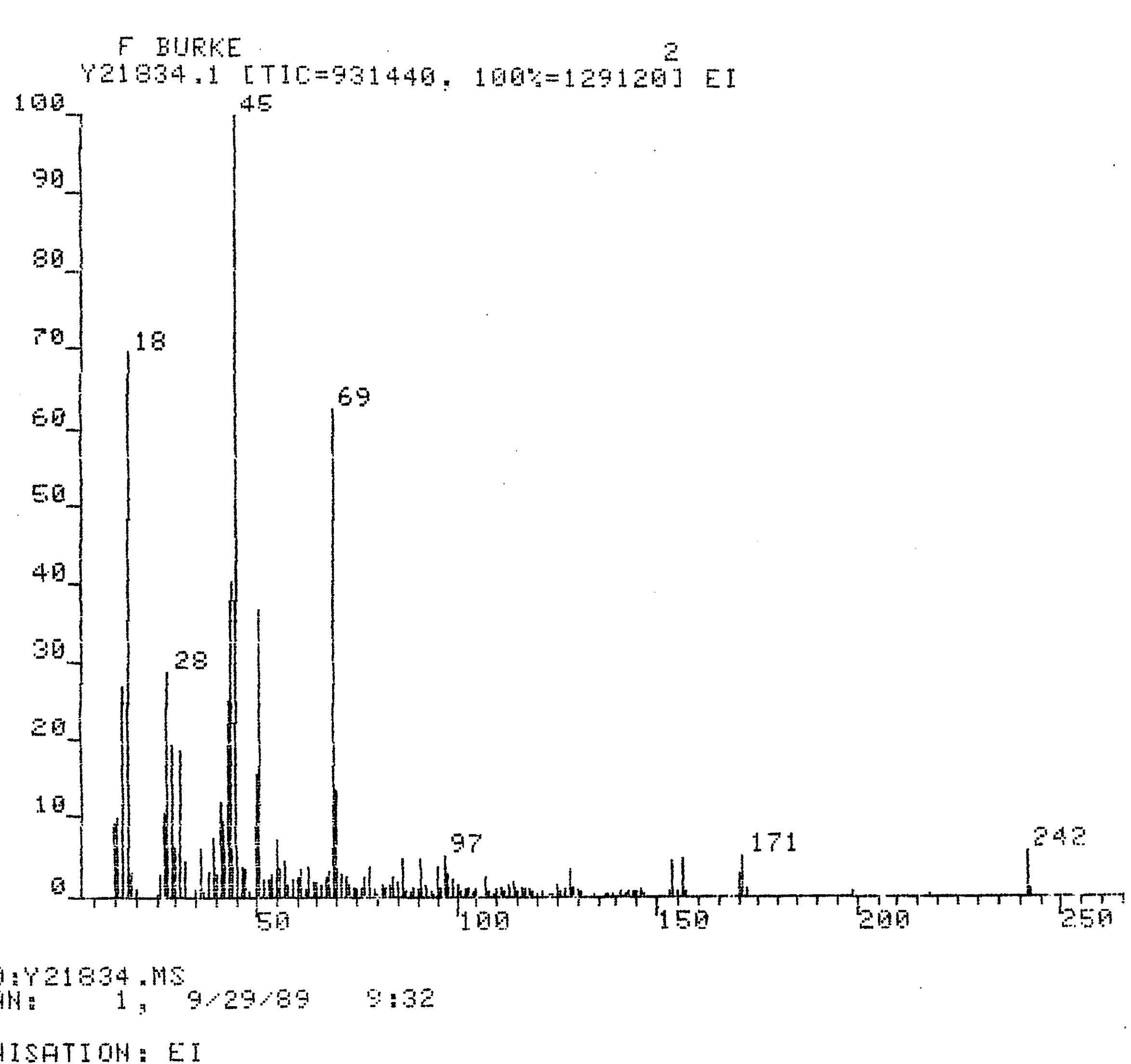
The sample collected from the major peak was submitted for electron impact mass spectrometry. Strong spectra can be obtained from less than 100 ng of sample in mass spectrometry and this technique was more suited to the analysis of the samples at this stage because yields were low and the compound was difficult to purify. Low field mass spectrometry is ideal for obtaining a fragmentation pattern of the compound, providing molecular mass data and structural information, and is complemented by high field mass spectrometry, which can be used to provide accurate ion mass data from which an elemental composition can be estimated.

The major compound in each fraction had a fragmentation pattern with a molecular ion of mass 242 atomic mass units (Figure 7.14). However the elemental composition data did not suggest a compound which could be related to holomycin and neither did it suggest any 'sensible' alternative structure (Figure 7.15).

7.7.0 An Additional Purification

A second major purification was carried out in which the intermediate ion-exchange and gel filtration steps were omitted. The peaks of yellow colour

Figure 7.14. Low Field Electron-Impact Mass Spectrum for the Purified sample Prepared from Reverse Phase Chromatography described in 7.6.3



R
DP0:Y21834.MS
SCAN: 1, 9/29/89 9:32

IONISATION: EI
NO. PEAKS: 131
BASE/NREF INT: 129120./ 129120.
TIC: 931440.
MASS RANGE: 15 - 243
RETN TIME/MISC: 0: 0/ 0/ 0/ 0

MEASURED MASS	% INT. BASE
243	1.0
242	5.7
218	0.3 *
199	0.7
172	0.9
171	5.2
170	2.8
157	0.7
156	4.7
155	0.6
154	4.4
153	0.5
147	0.2
146	0.9
145	0.6
144	0.6
143	0.6
142	0.2
141	0.6
139	0.2
138	0.3
137	0.2

Figure 7.15. Atomic Composition Report from High Field Mass Spectrometry of the Yellow Compound collected from Reverse Phase Chromatography and Described in 7.6.3

PAGE 1

ATOMIC COMPOSITION REPORT

RANGE: 12C0-20.13C0-1.1H0-100.16O0-4.14N0-3.32S0-2
RUN : B3220 SCAN : 1

Peak Mass	% INT	SMPS	12 C	13 C	1 H	16 O	14 N	32 S	MASS MMU	DEVIATION PPM
243.0839	18.5	29	18	0	11	1	0	0	2.91	12.0
			15	0	15	1	0	1	-.46	-1.9
			13	0	13	0	3	1	.88	3.6
			7	0	19	3	2	2	.20	.8
			10	0	17	0	3	2	-2.49	-10.2
			13	1	12	3	1	0	-1.17	-4.8
			11	1	18	1	0	2	.64	2.6
			9	1	16	0	3	2	1.98	8.2
242.0798	100.0	43	14	0	12	3	1	0	-1.88	-7.8
			12	0	18	1	0	2	-.07	-.3
			10	0	16	0	3	2	1.27	5.2
			13	1	11	3	1	0	2.59	10.7
			16	1	9	0	2	0	-.10	-.4
			10	1	15	3	1	1	-.78	-3.2
218.1085	7.8	21	17	0	14	0	0	0	-1.08	-5.0
			12	0	14	2	2	0	2.94	13.5
			9	0	18	2	2	1	-.43	-2.0
			7	1	15	4	3	0	-1.14	-5.2
			13	1	17	0	0	1	.01	.1
			5	1	21	2	2	2	.67	3.1
172.0854	7.3	21	7	0	12	3	2	0	.62	3.6
			10	0	10	0	3	0	-2.06	-12.0
			4	0	16	3	2	1	-2.75	-16.0
			5	0	18	1	1	2	2.43	14.1
			11	1	11	1	0	0	1.07	6.2
			9	1	9	0	3	0	2.41	14.0
			8	1	15	1	0	1	-2.30	-13.4
			3	1	15	3	2	1	1.72	10.0
			6	1	13	0	3	1	-.96	-5.6
171.0792	66.1	43	12	0	11	1	0	0	-1.83	-10.7
			7	0	11	3	2	0	2.20	12.8
			10	0	9	0	3	0	-.48	-2.8
			4	0	15	3	2	1	-1.17	-6.9
			11	1	10	1	0	0	2.64	15.5
			8	1	14	1	0	1	-.73	-4.3
			6	1	12	0	3	1	.61	3.6
			0	1	18	3	2	2	-.07	-.4
			3	1	16	0	3	2	-2.76	-16.1
170.1068	16.2	29	13	0	14	0	0	0	-2.78	-16.3
			8	0	14	2	2	0	1.25	7.2
			5	0	18	2	2	1	-2.12	-12.5
			12	1	13	0	0	0	1.69	[illegible]
			[illegible]	1	15	[illegible]	[illegible]	0	[illegible]	[illegible]
			7	1	17	0	0	1	-1.68	[illegible]
			4	1	17	2	2	1	2.05	[illegible]

eluted from the reverse phase column in repeated preparative separations were sub-divided into early, middle and late fractions. Each set of fractions was then concentrated and reapplied to the column in three separate final preparative separations. Using this strategy five peaks of yellow colour were identified. During this purification the low solubility of all the yellow compounds resulted in low recovery efficiencies, and it was decided to merely subdivide peaks in order to recover the majority of the major peak at a high degree of purity. This was achieved and a yield of product of an estimated 99% or better purity was obtained (as judged by HPLC analysis and detection) (Figure 7.16). In retrospect this estimation was erroneous, possibly due to the co-purification of two products which is described in later sections.

The mass spectrum for this sample of purified product was similar to that obtained for the previous purification. The trace was of greater intensity, due to the larger sample size, and higher temperature for vaporisation (290^{0}C in contrast to 260^{0}C, Figure 7.17). The interpretation of mass spectra is discussed in section 7.8.

This remainder of the sample was sent for NMR analysis by Dr Julia Foster at the University of Leeds. The supposedly 'pure' sample showed too many peaks to be a compound of the estimated mass for a compound related to holomycin. It was suggested that the compound was either far more complicated than originally thought, or that co-purification of an additional compound had occurred (J. Foster, personal communication). No signal could be detected in ^{13}C NMR due to lack of a sufficient quantity of sample. During analysis of the sample at Leeds, it was unfortunately misplaced. Therefore work using this purified sample of yellow product could not be continued. All further work was carried out using crude precipitates crystallised from adsorption column eluates.

7.7.1 Comparison of 3-Dimensional Test Spectra (those of *S. thermonitrificans* pBROC139) with 3-Dimensional Control Spectra (those of *S. thermonitrificans* pIJ702)

A sample of (crude) control extract (run over Amberlite XAD4) from broths of *S. thermonitrificans* pIJ702 was prepared in the same manner and was run using the conditions optimised for samples from broths of *S. thermonitrificans* pBROC139 in both preparative and analytical modes. In the preparative mode the column was loaded with between 1 and 2 ml of extract, and in analytical mode with 20µl of extract. No yellow component from control *S. thermonitrificans* pIJ702 extracts was eluted from the column in either mode using ultraviolet and visible spectroscopy, and a visible yellow component

Figure 7.16 Ultraviolet Scan of the Single Component Eluted from the Reverse Phase Column in 7.7.0 and Collected as a Sample for Further Structural Analysis (Preparative sample).

i) Ultraviolet scan at a wavelength of 302nm (a portion of the plot which was within the scale of the detector).

ii) Contour plot of the elution profile of the column.

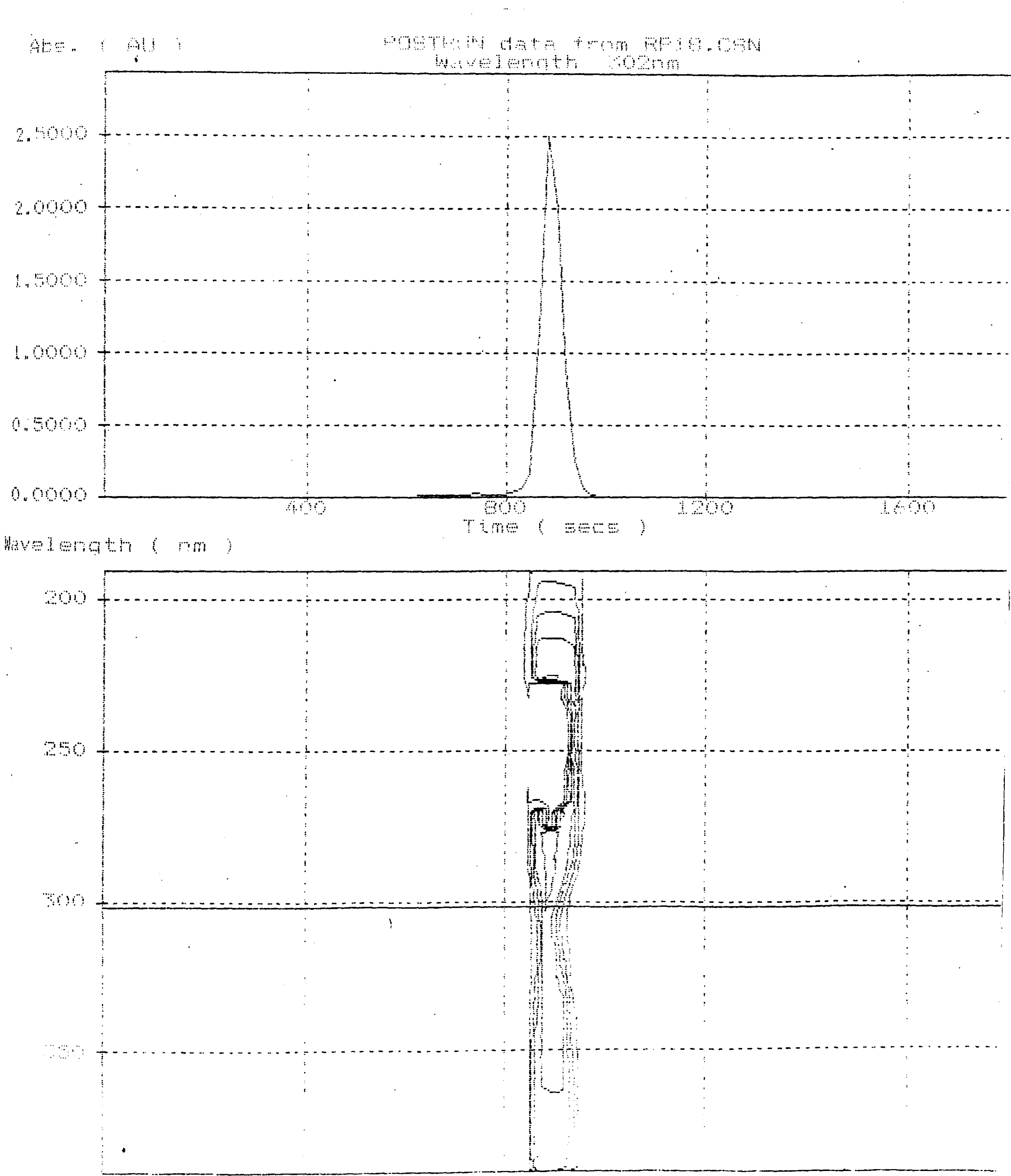
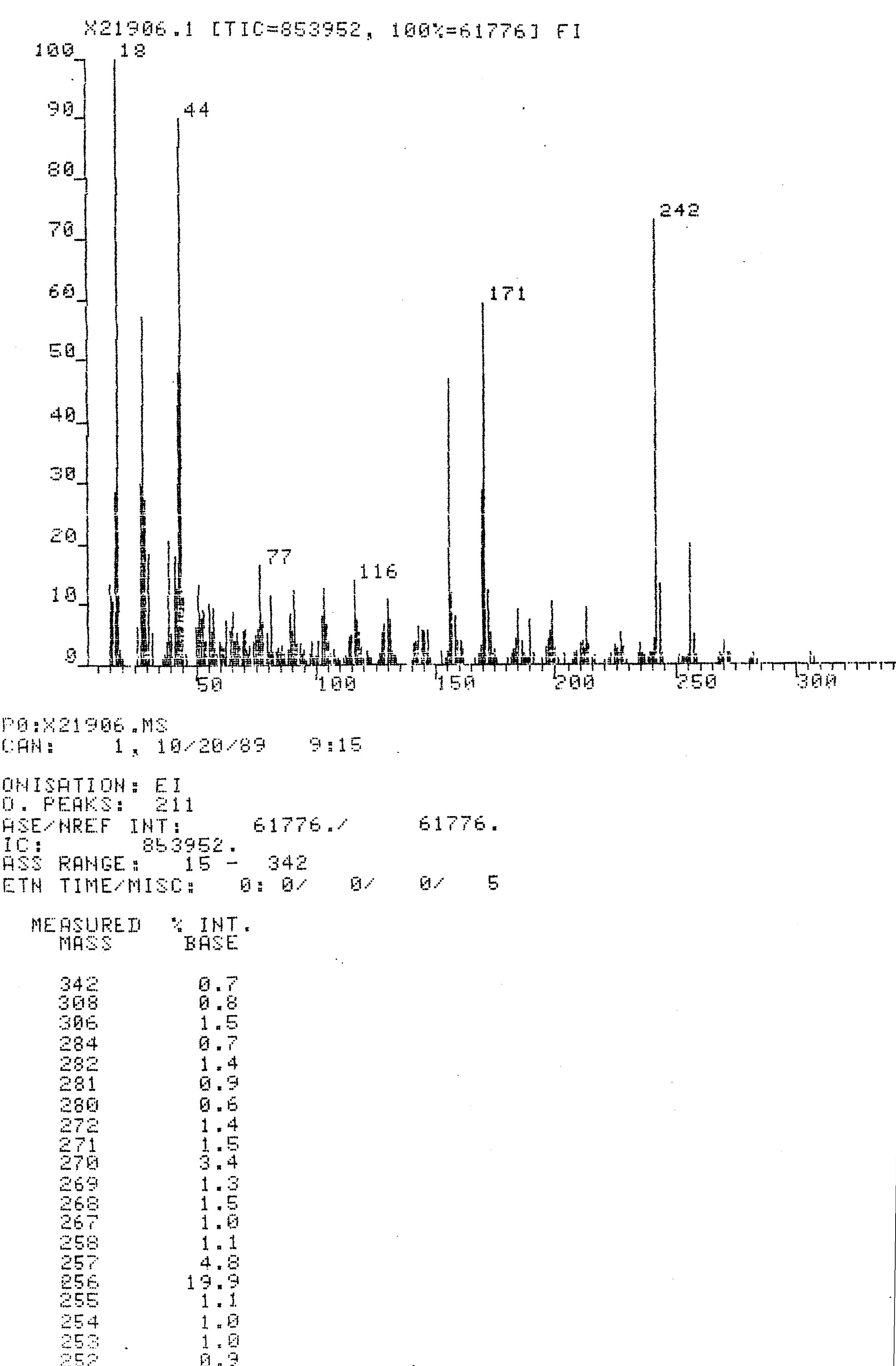


Figure 7.17 Low-field Mass Spectrum for the Yellow Sample Prepared as Described in 7.7.0.



MEASURED MASS	% INT. BASE	
342	0.7	
308	0.8	
306	1.5	
284	0.7	
282	1.4	
281	0.9	
280	0.6	
272	1.4	
271	1.5	
270	3.4	
269	1.3	
268	1.5	
267	1.0	
258	1.1	
257	4.8	
256	19.9	
255	1.1	
254	1.0	
253	1.0	
252	0.9	
251	1.1	*
244	1.8	*

Figure 7.18. Ultraviolet Scan of Components Eluted by Reverse Phase Chromatography described in 7.7.1, The Control extract from *S. thermonitrificans* pIJ702. (Preparative Scale sample - between 1 and 2 ml).

Note the absence of an eluate with an absorbance at 380nm (characteristic for the yellow compound). The expected position for this signal is marked with an arrow, and can be compared with Figure 7.19, run under the same conditions with an analytical scale sample of the test extract from *S. thermonitrificans* pBROC139.

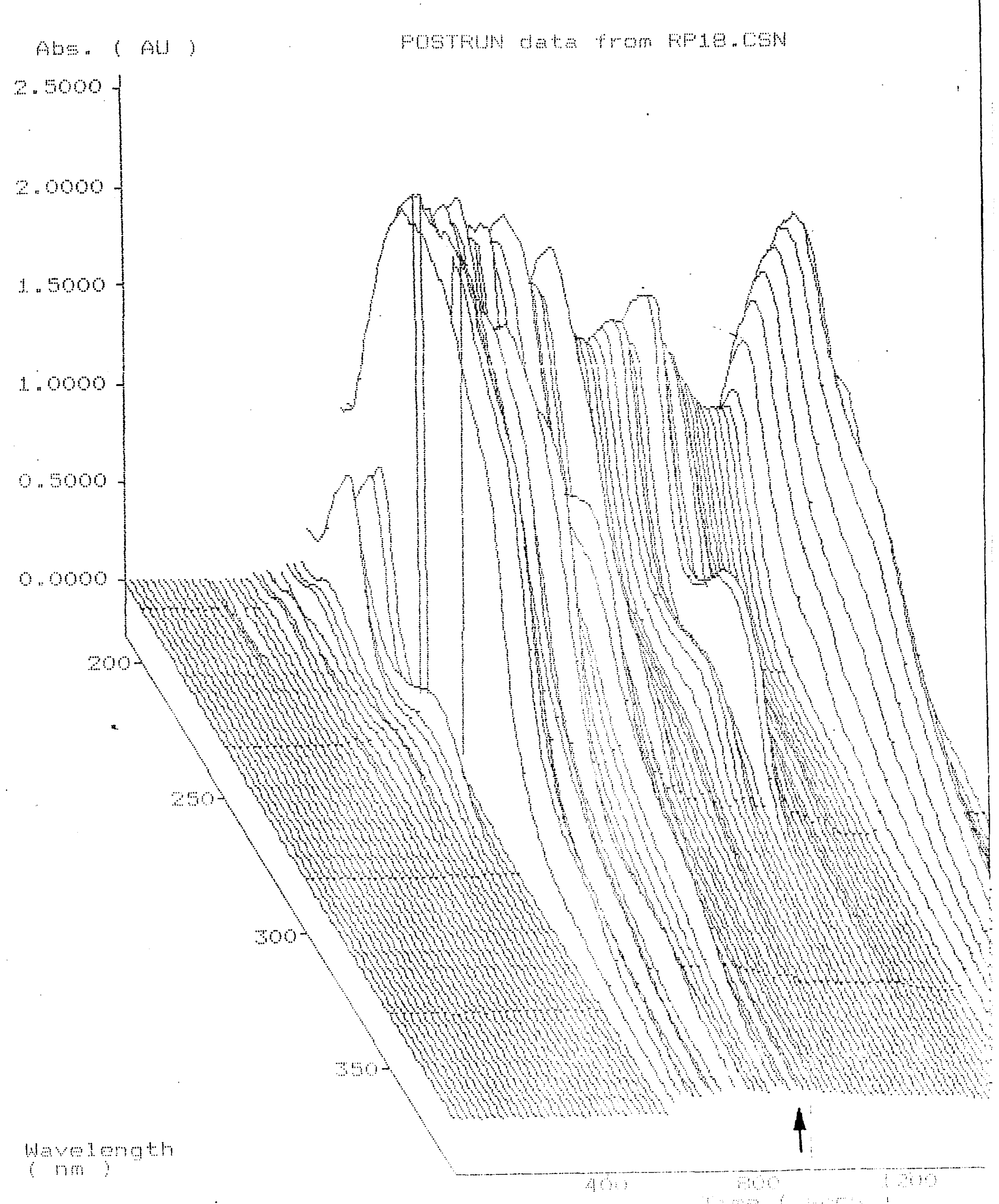
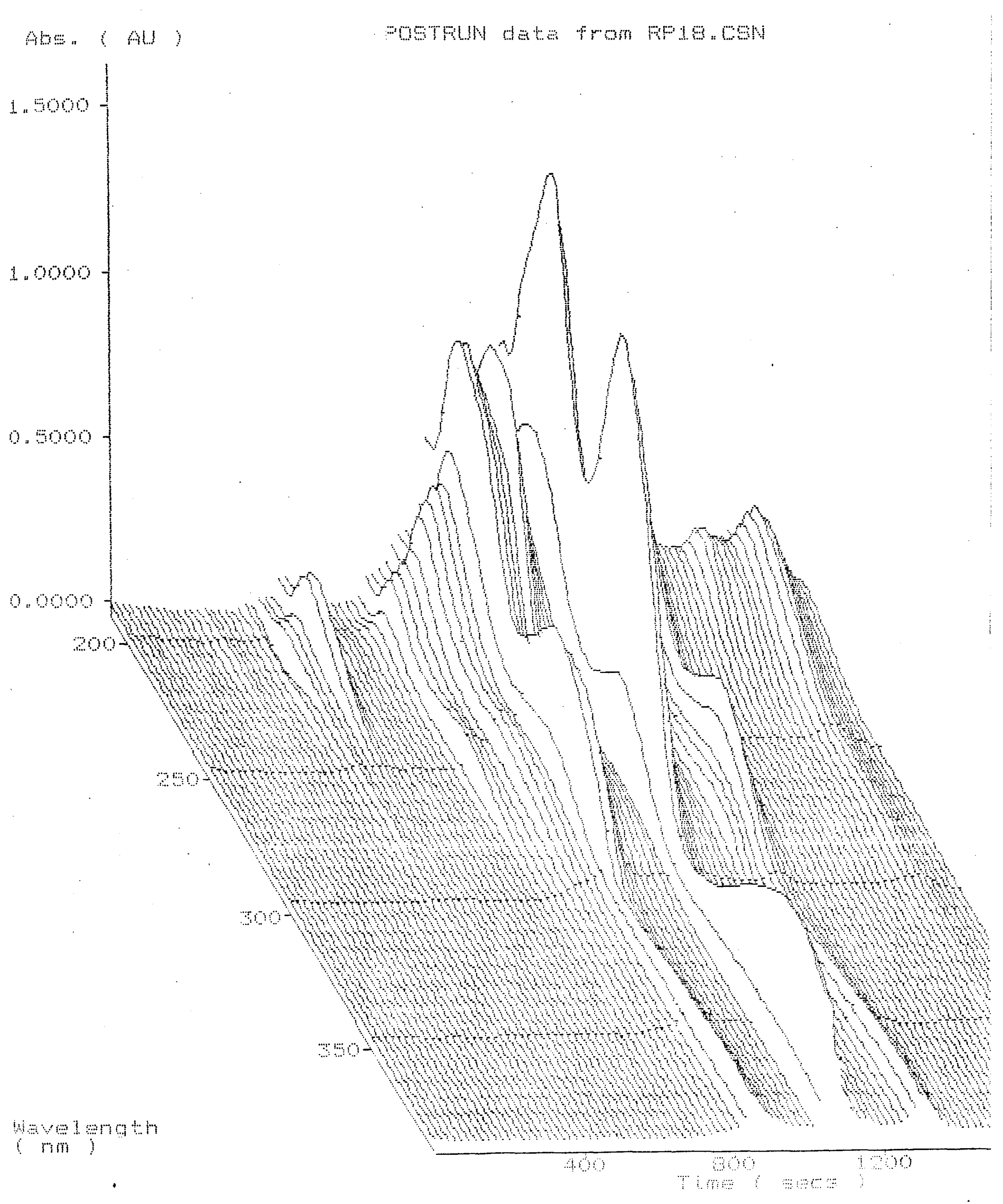


Figure 7.19. Ultraviolet Scan of the Components Eluted by Reverse Phase Chromatography described in 7.7.1. Extract from *S. thermonitrificans* pBROC139 (Analytical Sample, 20 µl).

The yellow fraction is marked with an arrow.



could not be observed in the fractions collected (Figures 7.18 and 7.19). This was an extremely important result and showed that the peak of absorption in the visible section of the spectrum from broth extracts of *S. thermonitrificans* pBROC139 was most probably related to the DNA insert in plasmid pBROC139. The comparison of elution spectra from control (pIJ702) and test cultures (pBROC139) under the same conditions, with equivalent concentrations of extract, indicated that the yellow compound was unlikely to be a host-encoded product (not shown). Neither could the detection of a yellow product have been due to the use of thiostrepton as an antibiotic for plasmid maintenance, or due to the production of a product encoded by the vector pIJ702 in *S. thermonitrificans.*

7.7.2 Options Available for Continuing the Identification

A repeated purification on a larger scale would have been the ideal approach, to obtain more product at a higher degree of purity for further analysis. Resolution could have been improved by the use of preparative scale reverse-phase chromatography columns in contrast to the use of overloaded analytical scale columns in which resolution was undoubtedly impaired.

It was established that a preparative scale RP18 (reverse-phase C18) column was available for use in the laboratories of Smithkline Beecham, and that scale-up using the methods already devised and implemented in Glasgow would be possible. Alternatively, another purification strategy could have been devised, perhaps enabling the removal of the contaminant(s), by binding the impurity(s) to an ion exchange column and collecting the purified sample in the flow-through. Reverse phase chromatography techniques could also have been extended to include ion-suppression chromatography (which may have removed contaminants) and experimenting with the influence of temperature variation on resolution.

7.8.0 Interpretation of Structural Information

7.8.1 Interpretation of Mass Spectra

The mass spectrum for holomycin has ions of mass 214 and 172 atomic mass units (Kenig and Reading, 1979). In comparison, the mass spectrum for the yellow product contained major ions of 270, 256, 242, 213, 185, 171 and 156 units (Figure 7.17). The ion at 242 was suggested to be the molecular ion in view of its abundance (T. Ritchie, personal communication). The mass spectra suggested that the purified product did not contain holomycin. Neither was a potential alternative structure suggested from the elemental composition

report shown in Figure 7.15. However, by manual comparison with the fragmentation pattern of holomycin, a putative compound having a nucleus with the illustrated structure was deduced (Figure 7.20).

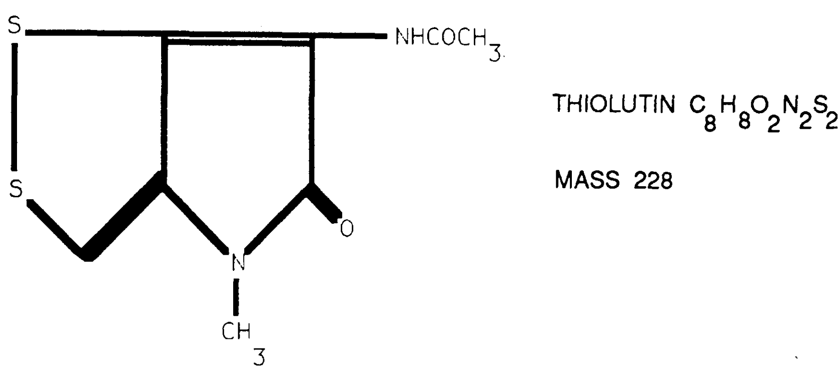


Figure 7.20 Putative Structure for a Compound with a Different Structural Nucleus to Holomycin

It differs from holomycin as the ring N is methylated. A molecular ion of mass 242 could be generated by a molecule in this homologous series with an additional methylene group in the side chain. A putative fragmentation pattern of this compound would give a spectrum similar to the one obtained for the yellow product (Figure 7.21).

The first putative fragmentation of this proposed compound of mass/charge 242 could be the elimination of an ethyl group, leading to an ion with an even number of electrons (213 atomic mass units) and a positive charge on the carbonyl group. Rearrangement of the ethyl group could give ethene and a hydride radical. A second possible fragmentation could divide the N-H and carbonyl groups, removing a propionyl group. This would leave a molecular ion of 185 atomic mass units, and charge stabilization would occur through the lone electron pairs on the nitrogen atom. A third possible fragmentation would divide the side chain from the double ring structure which, if followed with a bimolecular reaction by hydride attack would give rise to an ion of 171 atomic mass units that would be relatively stable. However it is normally considered unlikely that bimolecular reactions give rise to major peaks in mass spectra (M. Stark, personal communication). A peak at 69 could be due to a rearrangement of the smaller fragment. Fission of the C-N bond to demethylate the ring structure followed by a rearrangement could give rise to an ion with a mass value in the range 154-156, although demethylation is considered an unlikely event. The expected resulting structure would be stabilised by charge delocalisation through the N in the ring structure and by the lone electron pairs on the N atom in the side chain.

In the interpretation of mass spectra the natural abundance of isotopes can

Figure 7.21 Fragmentation Pattern for a Putative Compound with Mass 242.

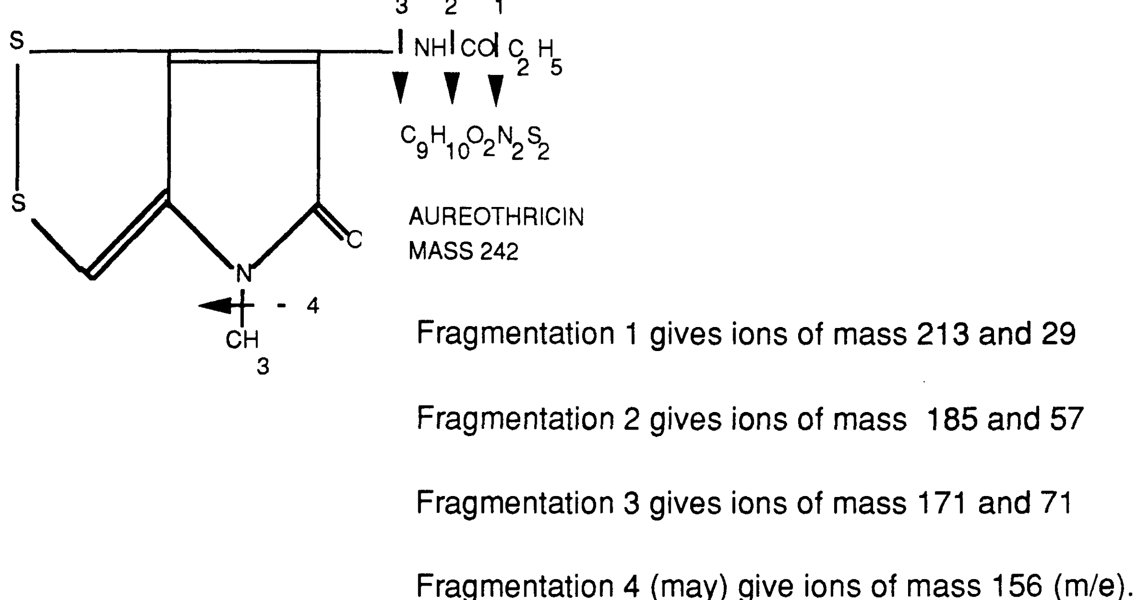
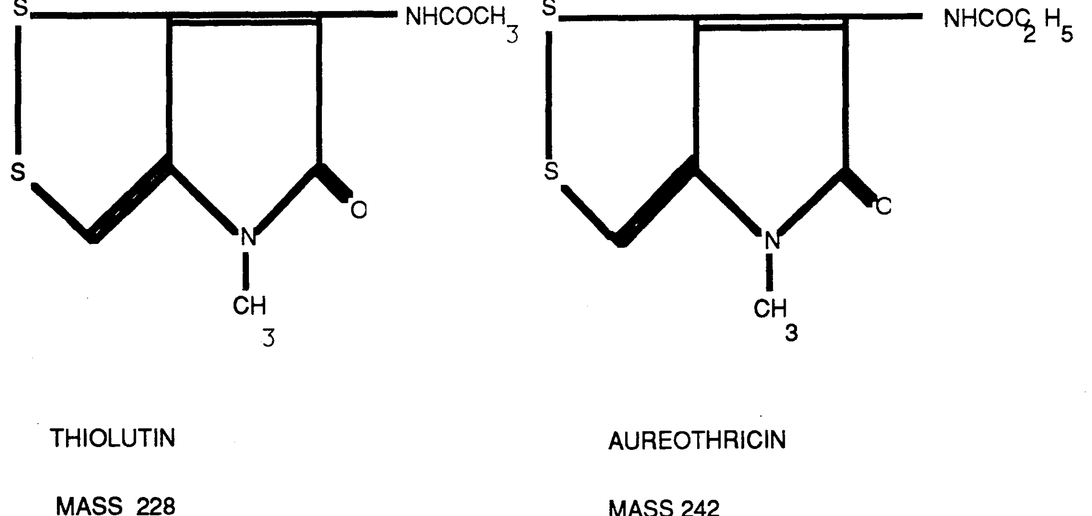


Figure 7.22 Structures and Masses of Aureothricin and Thiolutin.



yield useful information about the type of atoms of which the discrete ions are composed. "Credible" ions may be described as those which have a signal of intensity corresponding to 1% of the major peak, at a mass 1 higher than the major ion. This is due to the natural abundance of ^{13}C, with contributions from the natural abundance of ^{15}N. All the fragment ions discussed had an ion at 1 atomic mass unit higher than the peak of interest. Indicative of structures containing sulphur are satellite peaks at 2 atomic mass units above the major peak. This is due to the natural abundance of ^{34}S which is present at a concentration of 4.2% of total sulphur. Although these diagnostic peaks were present in the mass spectra of the yellow compound, the relative proportions of these (sulphur) diagnostic peaks, 2 mass units higher than the major fragment ions, were variable both within a sample and between samples (Figures 7.14 and 7.17). The proportions of the putative sulphur diagnostic ions do not correspond with theoretically-predicted proportions. As a result, no definite information concerning the presence or absence of sulphur in the compound can be deduced from the fragmentation pattern.

A putative structure having the fragmentation pattern described for a compound of 242 mass units could not be proposed for molecular ions of 256 or 270. However it was thought that these putative molecular ions could be contaminants, due to repeated methylation of a compound of mass 242 and present in the sample at low concentrations.

By inspection of the relevant literature it was found that compounds with the holomycin ring structure in an N methylated form had the names thiolutin (m/e 228) and aureothricin (m/e 242). Structures given in Figure 7.22. No samples of aureothricin were held either by Beecham or Pfizer (the patent holders). However 10 mg of thiolutin were made available for the project by Pfizer.

Both aureothricin and thiolutin belong to the pyrrothine class, and may be synthesised chemically in a similar manner to the N-demethyl pyrrothines such as holothin and holomycin (Ettlinger *et al.*,1959). However, they have not previously been identified as mixtures in culture broths with N-demethylated pyrrothines. They tend to be produced in concert with a homologous series of N-methylated pyrrothines and polyenes (hamycin and neopentaene in particular, which are also yellow crystalline compounds and are soluble in butanol). Several *Streptomyces* are known to produce this range of compounds; *S. albus* strain NRRL 2401 (the strain used for commercial production of thiolutin), *S. pimprina, S. thioluteus, S. farcinicus, S. celluloflavus,* and *Streptomyces sp 2336* (Umezawa, 1963; Tanner *et al.*, 1950).

7.8.2 Further Investigation

A mass spectral comparison of the thiolutin and holomycin standards was made (shown in the Appendix). Mass spectra were compared with the mass spectra already obtained for the purified yellow compound (Figure 7.14. and 7.17). Low field mass spectral analysis of the thiolutin standard showed the expected molecular ion at mass 229 (in contrast to the reported value of 228 a.m.u. for thiolutin), and a contaminating ion of mass 242 was observed in the same sample. Similarly the molecular ion for the holomycin standard was at a mass value of 213 in contrast to 214, the value reported by Kenig *et al.*. This could have been the result of a slight deviation in calibration. By comparison of fragment abundance compared to the internal standard (for thiolutin), it is proposed that the thiolutin sample is contaminated with aureothricin at a level of between 5 to 10% according to mass/charge ratio data. This was confirmed by TLC; two yellow spots were evident in thin layer chromatograms overloaded with samples of thiolutin during which the solvent front was allowed to run beyond the edge of the plate. Thin layer chromatograms of the thiolutin standard were compared with the yellow products crystallised from adsorption-column extracts of *S. thermonitrificans* pBROC139 and *S. albus* pBROC139. Results are tabulated in Table 7.6.

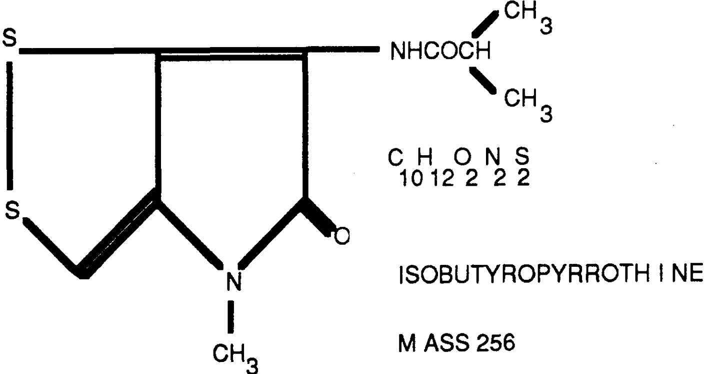
TLCs were also developed in acetonitrile and mixtures of chloroform 9; methanol 1, giving single spots for the thiolutin and holomycin standard and single yellow spots for the impure yellow extract (data not shown, described below). In each case the crude yellow samples from both *S. thermonitrificans* and *S. albus* contained yellow spots running in a more hydrophilic manner than the standard together with five or six contaminating components (impurities were identified using a range of stains and visualisation methods). The samples of crude yellow extract consistently behaved in a more hydrophilic manner than holomycin, aureothricin and thiolutin. Samples obtained from *S. albus* and *S. thermonitrificans,* were indistinguishable on these chromatograms.

Low field mass spectra from holomycin and (aureothricin-contaminated) thiolutin authentic standards, together with spectra of the partially-purified yellow compound were sent to the Microbial Chemistry Department, SmithKline Beecham. The spectra were compared and analysed by computer for subsequent matching to a database of mass spectral information. The yellow sample obtained from *S. thermonitrificans* pBROC139 was identified as potentially containing isobutyropyrrothine, an N-methylated pyrrolidinone nucleus with a branched side chain, with the structure shown in Figure 7.23. The fragment ion at 242 mass could not be attributed to fragmentation of this

Table 7.6 Data from Thin Layer Chromatographic Comparison of Thiolutin standard with yellow products from *S. thermonitrificans* pBROC139 and *S. albus* pBROC139.

Mobile phase;	Butanol. Solvent front in excess of plate length.
Plate length:	80 mm
Thiolutin:	spots at 46 mm and 50 mm
Yellow compound: (*S. thermonitrificans* pBROC139)	spot at 13 mm
Yellow compound; (*S. albus* pBROC139)	spot at 13 mm

Figure 7.23 Structure of Isobutyropyrrothine



compound but could be potentially identified as aureothricin (M. Gilpin, personal communication).

This information was in agreement with the predicted fragmentation for a compound of 242 mass units, with added provision for the ions of higher mass isobutyropyrrothine and aureothricin. Repeated purification and analysis, based on the work described in this chapter, would be required to confirm this.

7.9 Discussion and Future Work

This chapter has described preliminary studies of the expression of a yellow product, originally thought to be holomycin, from *S. thermonitrificans* pBROC139. Information which suggests the product was not holomycin has been shown. A purification strategy for the yellow pigment has been carried out, leading to the isolation of a compound which might be a mixture of two yellow components. The attempted structural elucidation of the product has also been carried out and is described in this chapter.

Several factors may have had important effects on potential expression of the pathway encoded by the insert in pBROC139. Firstly, the physiology of *S. thermonitrificans* could have potentially prevented complete expression as a result of the growth temperature. A product was formed from *S. thermonitrificans* pBROC139 cultures at the normal growth temperatures for *S. thermonitrificans* (shown by yellow broths). Experiments in which the temperature was varied from 30^{0}C to 50^{0}C, and in which the products were analysed by solvent extraction and thin layer chromatography suggested the same yellow product was formed in all cases (sections 7.4.1 and 7.4.3). Hence the elevated growth temperature of *S. thermonitrificans* was not detrimental to formation of the yellow product from *S. thermonitrificans* pBROC139. Secondly, in view of the small size and uncharacterised nature of the DNA insert in the vector pIJ702, it was thought that the insert may encode an incomplete pathway for holomycin, or alternatively, a yellow product unrelated to holomycin. The purification and analysis described in this chapter has shown that the plasmid pBROC139 does not enable the formation of holomycin in heterologous hosts. It has been suggested, however, that the related pyrrothine antibiotics, aureothricin and isobutyropyrrothine have been detected in extracts from *S. thermonitrificans* pBROC139 broths. These antibiotics possess an N-methylated internal ring structure in contrast to des-N-methyl pyrrothine antibiotics (the holomycin class).

The ultimate aim of this project was to mass-balance an antibiotic fermentation, using defined media. Hence it was thought necessary to investigate the influence of nutrient conditions on the production of yellow

product, although this investigation was necessarily simplistic due to the lack of an assay at that stage in the project. Fermentations of *S. thermonitrificans* pBROC139 in TSB complex medium were shown to lack holomycin formation (7.4.2). In 579MM production of yellow broths, using mycelium pre-grown in complex medium, occurred reproducibly at an early stage in growth, in contrast to the expression in a complex medium, TSB. Growth in defined media automatically stresses *Streptomyces* by the initiation of the stringent response (Ochi, K. 1986; Strauch *et al.,* 1991) due largely to the lack of amino acids in the medium. The relatively rapid production of a yellow colour in defined media from mycelium grown in complex medium may suggest that production occurs in response to an environmental stress such as induction of this response.

Investigation of the biosynthetic pathway would be a logical extension of this purification of the product. Energetically the formation of holomycin from cysteine is favoured over that from a precursor of the ß lactam pathway, acetyl cysteinyl valine (Czaplewski, 1989). If incorporation of radiolabel from ^{1}H-labelled cysteine into the yellow product occurs, this would strongly suggest a biosynthetic path derived from cysteine. Further studies of the yellow product could then be attempted. Using cysteine tritiated at C1, C2 and C3 positions, products with different labelling arrangements would result and the exact positions of these carbons could be identified using ^{1}H NMR. Doubly-labelling precursors and investigation into the positions of the labelled moeities in the products would provide information with which a biosynthetic pathway, supported by good evidence, could be postulated. The biosynthetic pathway derived by cysteine condensation is preferred to the modification of aceto cysteinyl valine because a yellow pigment is expressed from pBROC139 in the non ß -lactam-producing heterologous hosts *S. lividans, S. thermonitrificans* and *S. albus*. This would suggest that precursors for ß-lactam and cephem biosynthesis are not required for formation of this antibiotic. It would also suggest that the precursors are readily available metabolites in *Streptomyces.* A postulated pathway would be a very simple condensation pathway requiring three to four genes, adequately encoded on a 5.3 kb segment of DNA (described in Czaplewski, 1989, for holomycin).

Further study using the strain *S. thermonitrificans* pBROC139 would be to test the effect of temperature on expression in a materially-balanced system of cultivation. This would involve quantitative mass balanced fermentation, in the same manner as described in Chapter 5, with the added component of antibiotic assay. Growth efficiencies in terms of yield of mycelium per mole of

glucose utilised would be calculated. Samples of mycelia would be required for copy number determinations, and a correlation of the production titre and rate of production of the yellow pigment with plasmid copy number and dry weight would be made. From these results a specific production rate for the yellow product could be obtained. Fermentations at 45^0C and 37^0C would yield data for comparison, and the feasibility of producing antibiotics at elevated temperature could then be discussed on the basis of the quantitative productivity results.

In conclusion, some of the products from culture broths of *S. thermonitrificans* pBROC139 have been partially purified and identified as possibly isobutyropyrrothine and aureothricin. Continued purification and analysis would be required to confirm or disprove this view.

Final Discussion and Conclusions

8.1 The Glasgow Project

The purpose of this project was to test the feasibility of expressing antibiotic biosynthetic pathways cloned from mesophiles in a selected thermophilic strain. This required the selection of a suitable strain (work of L. Harvey), the development of techniques for plasmid transformation of the chosen strain (work of L. Czaplewski) and finally, the development and implementation of fermentation techniques (work described in this thesis).

A defined medium, defined inocula and growth conditions suitable for homogeneous filamentous growth in a 7 litre volume Bioengineering fermenter have been developed. In addition, growth of *S. thermonitrificans* at a range of dilution rates in chemostat culture have yielded data which suggest that the growth energetics of this strain are similar to those of common mesophilic prokaryotic bacteria, and that maximal molar growth yields are high, even on defined medium. Hence, *S. thermonitrificans* appears to be attractive as a host organism for the expression of antibiotic biosynthetic pathways at elevated temperature.

A recombinant strain, *S. thermonitrificans* pBROC139 was constructed (Czaplewski, 1989), and the pathway expressed at temperatures up to 50^{0}C (this work). This temperature is 20^{0}C in excess of the maximum growth temperature of *S.clavuligerus,* from which the DNA insert in pBROC139 came. It has been shown (albeit in a very primitive manner) that the formation of yellow coloured broths is considerably more rapid at elevated temperature, as would be expected. By HPLC and 3 dimensional spectral analysis of separated components, the yellow product has been shown to be insert specific. Thus the long term aim of the project has been successful.

It would have been more satisfying if either an accurate assay for the yellow product had been developed, or a recombinant strain *S. thermonitrificans* pPFZ163 constructed, to give a strain which could form oxytetracycline. However, alternative strategies for enabling the successful conclusion of these aims have been presented in the respective chapters. It was not envisaged at the outset that the purification of the yellow compound would have been as complex as it has proved to be.

The investigation into the growth characteristics and energetics of *S.*

thermonitrificans have been worthy of investigation from an academic viewpoint, both as a study of the growth characteristics of a streptomycete, and of a moderate thermophile. There is scanty information in the literature concerning the growth energetics of *Streptomyces*, partly because the growth of *Streptomyces* cannot normally be divorced from the production of secondary metabolites. The wild-type *S. thermonitrificans* is therefore an ideal strain in which to investigate streptomycete growth in isolation from antibiotic production.

8.2 Future Potential of *S. thermonitrificans*

S. thermonitrificans has been shown to be potentially useful for the production of antibiotics at elevated temperature. On a large scale, the increased rate of growth and product formation would be expected to improve the productivity of a process. In addition, dramatic reductions in the the total energy cost for cooling the fermentation, as a result of operation at an elevated temperature is an important economic consideration. The physiology of *S. thermonitrificans* would be suitable for efficient growth at elevated temperature; however, an accurate assessment of the feasibiltity of antibiotic production at elevated temperature would require the use of energy balances, and a cost estimation of a potential process.

S. thermonitrificans may additionally be useful as a source of thermostable enzymes. Preliminary investigation has shown that *S. thermonitrificans* possesses lipase activity in solid culture, and the study of this activity is currently underway (R. Kok, personal communication).

As the successful cloning of streptomycete secondary metabolite pathways increases, *S. thermonitrificans* may be worthy of further study as a heterologous host for investigating the regulation of novel, cloned pathways. Strains of *S. thermonitrificans* expressing a heterologous product in a fully-defined system, may be used to identify targets for optimising product formation in the infancy of a commercial project. The controlled growth conditions, availability of defined medium and lack of other antimicrobial product or polysaccharide, provides a very simple system for investigation of the physiologyical regulation of product formation. The information so gained could be used in the fermentation development of the host for optimum productivity.

8.3 General Comments Concerning the Study of *Streptomyces*

Streptomyces are industrially-important micro-organisms, which possess a

complex growth performance. They have different phases of growth and antibiotic production, and undergo changes in mycelial morphology in submerged culture. Although the productivity of an antibiotic fermentation can be optimised and regulated by physiological conditions, identification of molecular mechanisms influencing the regulation of the switch into antibiotic production is still elusive. Even with precise molecular techniques, clear answers to questions relating to physiological switches in *Streptomyces* are not obtainable (see Strauch and Bibb, 1991). Additionally, analysis of the literature relating to the physiological regulation of secondary metabolism shows that conflicting reports often exist. This has been attributed to differences in cultivation conditions, and underlies one of the major hurdles required to study any aspect of *Streptomyces i.e.* consistent, comparable growth conditions (Trilli, 1990). The mycelial growth habit of *Streptomyces*, relying on hyphal extension leading to biomass accretion, results in a mixed population of cells which have had different previous growth histories. When this is combined with the slower growth rates and the (commonly) non-exponential biomass accretion characteristic of *Streptomyces,* the precise study of a single event occurring in batch culture is difficult. For example, if an antibiotic is produced by 5% of cells at a particular stage in growth in a normal batch culture, due to the differences in the age of hyphae, or due to pelletted morphology, then a specific change in any component of the total population is difficult to investigate and quantify.

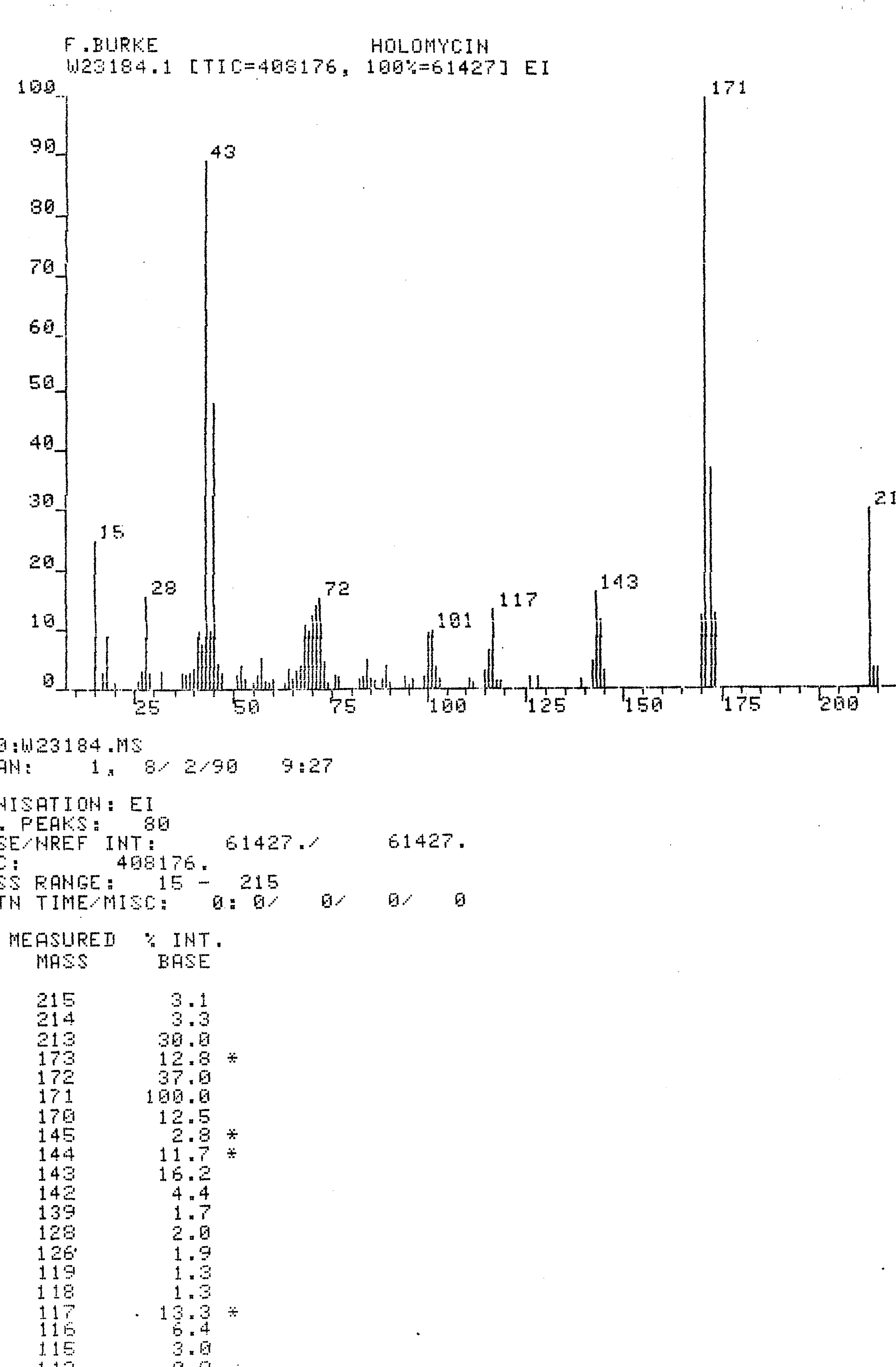
Continuous culture is acknowledged as a preferred culture system to batch culture for the physiological study of a microorganism in submerged culture. Most streptomycete fermentations for the production of secondary metabolites are influenced by some form of nutrient limitation during stationary phase. The relationship between growth rate and nutrient limitation in batch culture prevent investigation into their independent influences on the initiation of secondary metabolite production, but continuous culture allows these influences to be investigated independently. A limit is reached in the physiological study of an organism, which requires molecular techniques to continue the investigation. This limit has been reached with *S.coelicolor* - the physiological studies have specified that both phosphate and nitrogen medium concentrations are important in regulating the switch into antibiotic production, but molecular techniques would be required to elucidate the control mechanisms (Hobbs *et al.,* 1990). In spite of this limit, the use of biomass prepared in defined, reproducible growth conditions, is one hurdle which ensures that some of the differences between results are not due to differences in growth conditions. Therefore investigations combining physiological and molecular approaches would appear to be the ideal.

The limitation of continuous culture for the physiological study of an organism, defined by its non-invasive capacity, could possibly be alleviated by the use of *in vivo* NMR, although the technique is not widely available and dedicated equipment is required. The use of *in vivo* NMR has already been established for a number of years in the study of reaction rates, transport and intracellular changes in the concentration of molecules by the use of natural abundance ^{13}C, ^{15}N and ^{31}P resonance spectroscopy (Gadien, 1982). In *Saccharomyces cerevisiae* , *in vivo* ^{31}P NMR has been used to determine the concentration of sugar phosphates and of ADP and ATP in flux-control studies (Bailey *et al.,* 1990). Methods for studying the intracellular environment of mammalian cells using *in vivo* ^{31}P and ^{13}C natural abundance NMR have been developed (Fernandez *et al.,* 1990). Hence, it is likely that developing the technique for the study of streptomycete developmental and antibiotic production processes would not be insurmountable. As phosphorylated intermediates are implicated as key regulatory molecules in the formation and production of a wide range of antibiotics, the use of ^{31}P in-vivo NMR for studying the regulation of the initiation of secondary metabolism in *Streptomyces* is an attractive idea.

8.4 Final Conclusion

This project (the combined work described in Czaplewski, 1989 and this thesis) has developed a streptomycete thermophilic host/vector system which is simultaneously amenable to precise physiological and molecular investigation. Using this system a heterologous product has been expressed at $50^{0}C$ with apparent rate advantages over expression at $30^{0}C$. Hence the production of antibiotics at elevated temperature is feasible, and is worthy of further investigation.

Figure A.1 Electron-Impact Mass Spectrum of the Holomycin Standard Used in this Study.

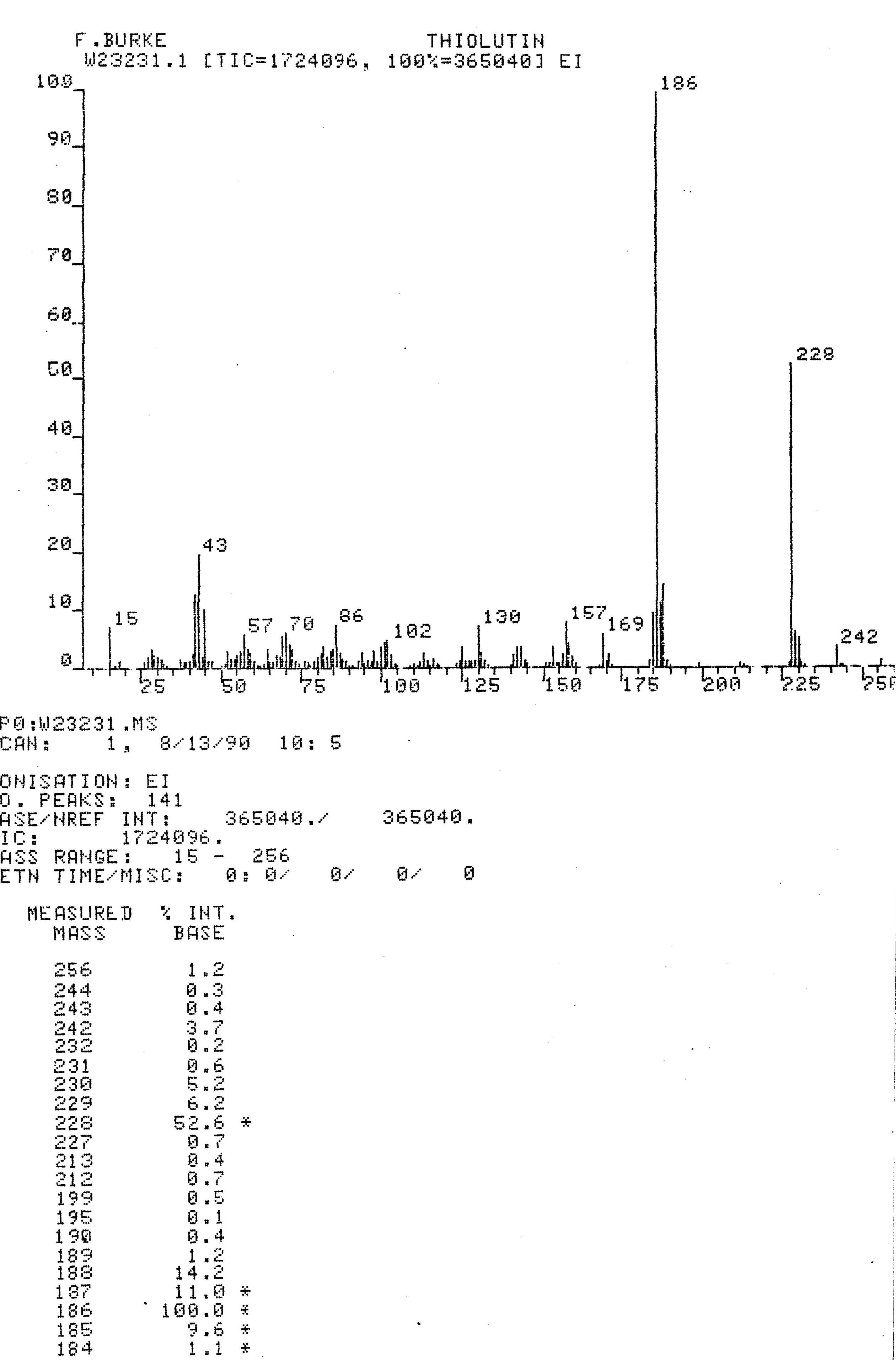


R
DP0:W23184.MS
SCAN: 1, 8/ 2/90 9:27

IONISATION: EI
NO. PEAKS: 80
BASE/NREF INT: 61427./ 61427.
TIC: 408176.
MASS RANGE: 15 - 215
RETN TIME/MISC: 0: 0/ 0/ 0/ 0

MEASURED MASS	% INT. BASE	
215	3.1	
214	3.3	
213	30.0	
173	12.8	*
172	37.0	
171	100.0	
170	12.5	
145	2.8	*
144	11.7	*
143	16.2	
142	4.4	
139	1.7	
128	2.0	
126	1.9	
119	1.3	
118	1.3	
117	13.3	*
116	6.4	
115	3.0	
112	0.9	
111	1.3	

Figure A.2 Electron-Impact Mass Specturm For the Thiolutin Standard discussed in 7.8.2



MEASURED MASS	% INT. BASE	
256	1.2	
244	0.3	
243	0.4	
242	3.7	
232	0.2	
231	0.6	
230	5.2	
229	6.2	
228	52.6	*
227	0.7	
213	0.4	
212	0.7	
199	0.5	
195	0.1	
190	0.4	
189	1.2	
188	14.2	
187	11.0	*
186	100.0	*
185	9.6	*
184	1.1	*
173	0.1	

Adams, H. L. and Thomas, C. R. (1988). The use of image analysis for the morphological measurements on filamentous microorganisms. Biotechnology and Bioengineering **32:** 707-712.

Agrawal, P. and Lim, H. (1984). Advances in Biochemical Engineering and Biotechnology **30:** 61.

Aharonowitz, Y. (1983). Metabolic regulation of antibiotic biosynthesis: effect of carbon, nitrogen and specific and specific amino acid metabolism. In "Bioactive Microbial Products 2: Development and Production". (Nisbet, L.J., and Winstanley, D.J. eds), Society for General Microbiology, Academic Press.

Aharonowitz, Y. and Demain, A. L. (1977). Influence of inorganic phosphate and organic buffers on cephalosporin production by *Streptomyces clavuligerus.* Archives of Microbiology **115:** 169-73.

Aharonowitz, Y. and Demain, A. L. (1978). Carbon catabolite regulation of cephalosporin production in *Streptomyces clavuligerus.* Antimicrobial Agents and Chemotherapy **14:** 159.

Aharonowitz, Y. and Demain, A.L. (1979). Nitrogen nutrition and regulation of cephalosporin production in *Streptomyces clavuligerus.* Canadian Journal of Microbiology **25:** 61-67.

Aiba, S., Imanaka, T. and Koizumi, J. (1983). Tranformation of *Bacillus stearothermophilus* with plasmid DNA and its application to molecular cloning. In "Biochemical Engineering III", Annals of the New York Academy of Sciences, **413** pp57-70.

Allan, E. J. and Prosser, J. I. (1985). A kinetic study of the colony growth of*Streptomyces coelicolor* A3(2) and J802 on solid medium. Journal of General Microbiology **131:** 2521-2532.

Andrews, G. (1989). Estimating cell and product yields. Biotechnology and Bioengineering **33:** 256-265.

Atkinson, B. and Mavituna, B. (1983). Biochemical Engineering and Biotechnology Handbook. Stockton Press, New York.

Atkinson, D. E. (1969). Regulation of enzyme function. Annual Reviews in Microbiology **23:** 47-68.

Bader, F. G. (1986). Physiology and Fermentation Development. In "The Bacteria: a treatise on structure and function". Volume 9, Antibiotic-Producing Streptomyces. pp 281-321. (eds Queener S. W. and Day L. E.) Academic Press. New York

Bailey, J. E. and Ollis, D. (1986). Biochemical Engineering Fundamentals. Second edition, McGraw Hill.

Bailey, J. E., Birnbaum, S., Galazzo, J. L., Khosla, C. and Shanks, J. (1990). Strategies and challenges in metabolic engineering. In "Biochemical Engineering V", Annals of the New York Academy of Sciences p1-15.

Banks, G. T. (1977). Aeration of mould and streptomycete culture fluids. In Topics in Enzyme and Fermentation Biotechnology 1, edited by A Wiseman, pp. 71-110. Ellis Horwood, Chichester.

Barabas, G., Penyige, A., Szabo, J. and Ensign, J. C. (1988). ADP-ribosylation posttranslationally modifies proteins in *Streptomyces griseus*. In "Biology of Actinomycetes 88", edited by Y Okami, Japan Scientific Societies Press.

Bayer, T., Herold, T., Holzhauer, K., Zhou, W. and Schugerl, K. (1990). Comparison of cephalosporin C production in stirred-tank and airlift tower loop reactors. Annals of the New York Academy of Sciences **6:** 589.

Behal, V. (1987). The tetracycline fermentation and its regulation. Critical Reviews in Biotechnology **5:** 275-318.

Behal, V., Gregrova-Prusakova, J. and Hostalek, Z. (1982). Effect of inorganic phosphate and benzylthiocyanate on the activity of anhydrotetracycline oxygenase in *Streptomyces aurefaciens*. Folia MIcrobiologia **27:** 153.

Behal, V., Hostalek, Z. and Vanek, Z. (1979). Anhydrotetracycline oxygenase activity and biosynthesis of tetracyclines in *Streptomyces aurefaciens*. Biotechnology Letters **1:** 177.

Berdy, J. (1980). C.R.C. Handbook of Antibiotic Compounds. Published by CRC Press, Florida.

Betenbaugh, M., Beaty, C. and Dhurjati, P. (1989). Effects of plasmid amplification and recombinant gene expression on the growth kinetics of recombinants *E coli.* Biotechnology and Bioengineering **33:** 1425-1436.

Bhate, D. S., Hulyalkar, R. K. and Nenon, S. K. (1960). Isolation of iso-butyropyrrothine along with thiolutin and aureothricin from a *Streptomyces sp. Experentia* **16:** 504-505.

Bhate, D. S., Lavate W. V. and Acharya, S. P. (1964). Hindustan Antibiotics Bulletin **6:** 153-156.

Bibb, M. J., Ward, J. M. and Hopwood D. A, (1978). Transformation of plasmid DNA into *Streptomyces* at high efficiency. Nature **237:** 389-400.

Bibb, M.J., Freeman, R. F. and Hopwood, D. A. (1977). Physical and genetical characterisation of a second sex factor, SCP2 for *S coelicolor* A3(2). Molecular and General Genetics **154:** 155-166.

Binnie, C., Warren, M. and Butler, M. J, (1989). Cloning and heterologous expression in *Streptomyces lividans* of *Streptomyces rimosus* genes involved in oxytetracycline biosynthesis. Journal of Bacteriology **171:** 887-895.

Birch, A., Hausler, A. and Hutter, R. (1990). Genome rearrangement and genetic instability in *Streptomyces* spp. Journal of Bacteriology **172:** 4138-4142.

Brana, A. F., Manzanal, M. B. and Hardisson, C. (1982). Mode of cell wall growth of *Streptomyces antibioticus*. FEMS Microbiology Letters **13:** 231-235.

Brana, A., Wolfe, S. and Demain, A. L. (1985). Ammonium repression of cephalosporin production by *Streptomyces clavuligerus.* Canadian Journal of MIcrobiology **31:** 736-743.

Braun, S. and Vecht-Lifshitz S. (1991). Mycelial morphology and metabolite prodution. Trends in Biotechnology **9;** 63-68.

Bremer, H. and Dennis, P. P. (1987). Modulation of chemical composition and other parameters of the cell by growth rate. In "*Escherichia coli* and *Salmonella typhimurium,* Cellular and Molecular Biology" (ed. F.C. Neidhardt) Volume 2. American Society for Microbiology, Washington D.C. pp 1527-1542.

Brock, T. D. (1986). Introduction: an overviw of the thermophiles, in "Thermophiles, General, Molecular, and Applied Microbiology" (Brock, T. D. Ed.). 1-16. Wiley, New York.

Bu'lock, J. D. (1961). Intermediary metabolism and antibiotic synthesis. Applied Microbiology **3:** 293-342.

Bu'lock, J. D. (1975). Secondary metabolism in fungi and its relationships to growth and development. In "The Filamentous Fungi. 1. Industrial Mycology, pp35. Edited by Smith, J E and Berry D R, Edward Arnold, London.

Bu'lock, J. D. (1988). Secondary metabolites in the context of biotechnology. Plenary lecture at the Second International Symposium on Overprodution of MIcrobial Products, Ceske Budejovice, Czechoslovakia.

Bu'lock, J. D., Detroy, R. W., Hostalek, Z. and Munim-al-Shakarchi, A. (1974). Regulation of secondary biosynthesis in *Gibberella fujikuroi.* Transaction of the British Mycological Society **62:** 377-389.

Buckland, B. C., Omstead, D. R. and Santamarnia, V. (1985). Novel betalactams. In 'Comprehensive Biotechnology V'. Edited by M Moo-Young pp49-68. Pergamon, Oxford.

Bull, A. T., Huck, T. A, and Bushell, M. E. (1990). Optimization strategies in microbial process development and operation. In "Microbial Growth Dynamics", Special Publications of the Society for General MIcrobiology 28 145-169. Ed. R.K. Poole, M. J. Bazin and C.W. Keevil.

Bushell, M. E. (1988). Growth, product formation and fermentation technology. In Actinomycetes in Biotechnology, ed. M. Goodfellow, S. T. Williams and M. Modarski, pp185-215. Academic Press, London.

Bushell, M. E. (1989). The process physiology of secondary metabolite production. In "Microbial products: New approaches." Symposium of the Society for General Microbiology **44:** pp. 95-121.

Butler, M. J., Friend, E. J., Hunter, I. S., Kaczmarek, F. S., Sugden, D. A. and Warren, M. (1989). Molecular cloning of resistance genes and architecture of a linked gene cluster involved in biosynthesis of oxytetracycline by *Streptomyces rimosus.* Molecular and General Genetics **215:** 231-238.

Buttner, M. J. (1989). RNA polymerase heterogeneity in *Streptomyces coelicolor* A3(2). Molecular Microbiology **3:** 1653-1659.

Byrne, G., and Ward, O. P. (1989). Effect of nutrition on pellet formation by *Rhizopus arrhizus.* Biotechnology and Bioengineering **33:** 912-914.

Cashel, M. and Rudd, K.E. (1987) The stringent response. In "*Escherichia coli* and *Salmonella typhimurium*: Cellular and Molecular Biology". Neidhart, F.C. (Ed-in-chief). Washington, D.C.: American Society for MIcrobiology, pp. 1410-1438.

Caslavska, J., Stastna, J., Ludvik, J. and Blumauerova, M. (1978). Ultrastructure of some species of *Streptomyces* during submerged cultivation. Zentralblatt fur Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene 181-191.

Chaney, A., L. and Marback, E. P. (1962). Modified reagents for determination of urea and ammonia. Clinical Chemistry **8:**130.

Chater, K. F. (1984). Morphological and physiological differentiation in *Streptomyces.* In "Microbial Development" pp89-115 Edited by R. Losick and L Shapiro. Cold Spring Harbor Laboratories.

Chater, K. F. (1989). Multilevel regulation of *Streptomyces* differentiation. Trends in Genetics **5:** 372-376.

Chater, K. F. and Hopwood, D. A. (1989). Diversity of Bacterial Genetics. In "Genetics of Bacterial Diversity"pp24-46. Hopwood and Chater, eds. Academic Press.

Chesbro, W. (1988). The domains of slow bacterial growth. Can. J. Microbiol 34:427-435.

Colombo, A. L., Crespi-Perellino, N. and Micalizio, S. (1982). Relationships between growth, cyclic AMP and tylosin production in two mutants of *Streptomyces fradiae.* Biotechnology Letters **4:** 747.

Cooney, C. L., Wang, H. Y. and Wang, D. I. C. (1977). Computer aided materials balancing for the prediction of fermentation parameters. Biotechnology and Bioengineering **19:** 55-67.

Cortes, J., Llras, P., Castro, J. M. and Martin, J. F (1986). Glucose regulation of cephamcin biosynthesis in *Streptomyces lactamdurans* is exerted on the formation of α-aminoadipyl-cysteinyl valine and deacetoxycephalosporin C synthase. Journal of General Microbiology **132:** 1805.

Czaplewski, L. G. (1989). Molecular Genetics of *S. thermonitrificans* ISP5579. PhD Thesis, University of Glasgow.

Dalili, M., and Chau, P. (1988). Production of Actinomycin D with Immobilized *Streptomyces parvullus* under Nitrogen and Carbon Starvation Conditions. Biotechnology Letters **10:** 331-336.

Davies, J. (1990). What are antibiotics? Archaic functions for modern activities. Molecular Microbiology (1990) **4:** 1227-1232.

Demain, A. L. (1989). Carbon source regulation of idiolite biosyntheis in actinomycetes. In "Regulation of Secondary Metabolism" p127-135. Edited by S. Shapiro, published by C.R.C. Press.

Desai, A. J. and Dhala, S. A. (1967). *Streptomyces thermonitrificans* , a thermophilic streptomcete. *Antonie van Leeuwenhoek* **33:** 137-144.

Donadio, S., Tuan, J., Staver, M., Weber, J., Paulus, T., Maine, G., Leung, J., Dewitt, J., Vara, J., Wang, Y., Hutchinson, R. and Katz, L. (1989). Genetic Studies on Erythromycin Biosynthesis in *Saccharopolyspora erythraea.* In "Genetics and Molecular biology of Industrial Microorganisms", pp53-61. Edited by Hershberger C., Queener S., Hegermann G. Based on the Proceedings of the Fourth ASM Conference on the Genetics and Molecular Biology of Industrial Microorganisms, Bloomington 1988.

Doull, J. and Vining, L. (1990). Nutritional control of actinorhodin production by *Streptomyces colicolor* A3(2): suppressive effects of nitrogen and phosphate. Applied MIcrobiology and Biotechnology 12:449-454.

Dyke, S., Floyd, A., Sainsbury, M., and Theobald, R. (1978). Organic Spectroscopy; An Introduction. Published by Longman Press.

Dykstra, K., Li, X. and Wang, H. (1988). Computer modelling of antibiotic fermentation with on-line product removal. Biotechnology and Bioengineering **32:** 356-362.

Edwards, C. and Ball, A. S. (1987). Respiratory chain composition and activity in some thermotolerant streptomycetes. FEMS Microbiology Letters **40:** 61-66.

Effenberger, W., Muller, P. J. and Bocker, H. (1983). Uncoupling of respiration in turimycin fermentations. Zentralblatt fur Bakteriologie, Microbiologie und Hygiene **23:** 557.

Ensign, J. C. (1976). Properties and germination of *Streptomyces* spores and a suggestion for functions of antibiotics. In "Microbiology 1976" pp531, edited by Schlessinger D. American Society for Microbiology, Washington D C.

Ensign, J. C. (1978). Formation properties and germination of actinomycete spores. Annual Review of Microbiology **32:** 185-219.

Ensign, J. C. (1988). Physiological regulation of sporulation of *Streptomyces griseus.* In "Biology of Actinomycetes 88", edited by Y Okami, Japan Scientific Societies Press.

Ensign, J. C., Grund, A. P., and Coleman, R. H. (1980) Differentiation of actinomycetes: do antibiotics play a role?. Biology of Actinomycetes and Related Organisms **15:**72.

Ensign, J. C., McBride, M. J., Stoxen, L. J., Bertinuson, A., Pomplun, M. and Ho, A. (1986). The life cycle of *Streptomces:* germination and properties of spores and regulation of sporulation. In "Biological Biochemical and Biomedical Aspects of Actinomycetes", pp225. Edited by Szabo G., Biro S. and Goodfellow M. Akademiai Kaido, Budapest.

Epp, J.K., Burgett, S.G. and Schoner, B.E. (1987). Cloning and nucleotide sequence of a carbomycin resistance gene from *Streptomyces thermotolerans.* Gene **53:** 73-83.

Epp, J.K., Huber, M. and Turner, J.Schoner, B.E. (1989). Cloning of carbomycin biosynthetic and resistance genes and their expression in heterologous hosts. In "Genetics and Molecular Biology of Industrial Microorgansisms' p35-40. Edited by C Hershberger, S Queener and G Hegerman. Proceeding of th fourth ASM Conference of the same title.

Erickson, L. E., Minkewich, I. G. and Eroshin, V. K. (1979). Utilisation of mass-energy balance regularities in the analysis of continuous culture data. Biotechnology and Bioengineering **21:** 575-591.

Escalante, L., Lopez, H., Nateos, R., Lara, F. and Sanchez, S. (1982). Transient repression of erythromycin formation in *Streptomyces erythreus.* Journal of General Microbiology **128:** 2011.

Esener, A. A., Roels, J. A. and Kossen, N. W. F. (1983). Biotechnology and Bioengineering **25:** 2093-2098.

Esener, A. A., Veerman, T., Roels, J. A., and Kossen, N. W. F. (1982). Modeling of bacterial growth; formulation and evaluation of a structural model. Biotechnology and Bioengineering **24:** 1749-1764.

Ettlinger von L., Gaumann, E., Hutter, R., Keller-Sckierlein, W., Kradolfer, F., Neipp, L., Prelog, B. and Zahner, H. (1959). Stoffwechselprodukte von Actinomyceten: Holomycin. *Helvetica Chimica Acta* **42:** 563-569.

Evans, C.G.T., Herbert, D. and Tempest, D.W. (1970).The continuous culture of microorganisms. 2 Construction of a chemostat. In "Methods in MIcrobiology (J. R. Norris and D. W. Ribbons, eds.) pp277-323. Academic Press.

Farrand, S. G., Jones, C. W., Linton, J. D. and Stephenson, R. J. (1983). The effect of temperature and pH on the growth efficiency of the thermoacidophilic bacterium *Bacillus acidocaldarius* in continuous culture. Archives of Microbiology **135:** 276-283.

Fawcett J. K and Scott J. E. (1960). A rapid and precise method for the determination of urea. J. Clin. Pathol. **13:**156.

Fernandez, E., Mancuso, A., Murphy, M., Blanch, H. and Clark, D. (1990). Nuclear magnetic resonance methods for observing the intracellular environment of mammalian cells. In "Biochemical Engineering V", Annals of the New York Academy of Sciences pp458-475.

Fiske, C.H., Subbarow, Y. (1925). The colorimetric determination of phosphorous. J. Biol. Chem. **66**: 375.

Flores, M. E. and Sanchez, S. (1985). Nitrogen regulation of erythromycin formation in *Streptomyces erythraeus.* FEMS Microbiology Letters **26:** 191-194.

Frankena, J., Verseveld, H. W., van and Stouthamer, A. H. (1985). A continuous culture study of the bioenergetic aspects of growth and production of exocellular protease in *Bacillus licheniformis.* Applied MIcrobiol. Biotechnol. **22**: 169-176.

Freifelder, D. (1982). Physical Biochemistry. Applications to Biochemistry and Molecular Biology. Published by W H Freeman.

Fryday, A. and Bushell, M. E. (1983). The application of materials balancing to the characterization of sequential secondary metabolite formation in *Streptomyces cattleya* NRRL8057. Journal of General Microbiology **129:** 1733-1741.

Gadian, D. G. (1982). Nuclear Magnetic Resonance and Its Applications to Living Systems. Oxford University Press, London.

Gaeumann, E., Prelog, V. and Vischer, E. (1961). Holothin and derivatives thereof. US Patent 3014922.

Gardner, A. and Cadman, T. (1990). Product deactivation in recombinant *Streptomyces*. Biotechnology and Bioengineering **36:** 243-251.

Gil, J. A., Naharro, G., Villanueva, J. R., and Martin, J. F. (1985). Characterization and regulation of p-aminobenzoic acid synthase from *Streptomyces griseus*. Journal of General Microbiology **131:** 1279.

Grafe, U. (1989). Autoregulatory secondary metabolites from actinomycetes in *Streptomyces*. In "Regulation of Secondary Metabolism" p75-126. Edited by S. Shapiro, published by C.R.C. Press.

Grafe, U. (1990). Lipid composition of slowly sedimenting chemostat mutants of *Streptomyces lividans.* Poster at the International Symposium on Genetics and Product Formation in *Streptomyces*, Erfurt, GDR.

Grafe, U., Bocker, H. and Thrum, H. (1977). Regulative influence of *o*-aminobenzoic acid on the biosynthesis of nourseothricin in cultures of *Streptomyces noursei* JA 3890b. Zeitschrift fur Allgemeine MIkrobiologie **17:** 201-209.

Gray, D., Gooday, G. and Prosser, J. I. (1990). Apical hyphal extension in *Streptomyces coelicolor* A3(2). Journal of General Microbiology **136:** 1077-1084.

Gray, P. P. and Bhuwapathanapun, S. (1980). Production of the macrolide antibiotic tylosin in batch and chemostat cultures. Biotechnology and Bioengineering **22:** 1785-1804.

Gray, P. P. and Vu-Trong, K. (1987). Production of the macrolide tylosin in cyclic fed-batch culture. Biotechnology and Bioengineering **39:** 33-40.

Grund, A. D. and Ensign, J. C. (1985). Properties of the germination inhibitor of *Streptomyces viridochromogenes* spores. Journal of General Microbiology **131:** 833.

Hamana, K. and Matsuzaki, S. (1987). Distribution of polyamines in actinomycetes. FEMS MIcrobiology Letters **41:** 211-215.

Hanel, F., Grafe, U., Roth, M., Bormann, E.J., and Krebs, D. (1984). Influence of inorganic phosphate on the lipid synthesis of a phosphate deregulated mutant of *Streptomyces noursei.* Journal of Basic MIcrobiology **25**: 325.

Hara, O. and Beppu, T. (1982). Mutants blocked in streptomycin prduction in *Streptomyces griseus*- the role of A-factor. Journal of Antibiotics 35: 1208-1215.

Hara, O., Horinouchi, S., Uozumi, T. and Beppu, T. (1983). Genetic analysis of of A-factor synthesis in *Streptomyces coelicolor* A3(2) and *Streptomyces griseus.* Journal of General Microbiology **129:** 2939-2944.

Hartley, B. S, and Payton, M. (1982). Industrial prospects for thermophiles and thermophilic enzymes. Biochemical Society Symposium **48:** 133-146.

Heijnen, J. J., Roels, J. A. and Stouthamer, A. H. (1979). Application of balancing methods in modeling the penicillin fermentation. Biotechnology and Bioengineering **21:** 2175-2201.

Heinen, H. and Lauwers, A. M. (1983). Changes in Enzyme stability and fatty acid conposition of *Streptomyces sp.* a facultative thermophilic actinomycete. Archives of Microbiology **134**: 247-250.

Herbert, D. (1976). Stoichiometric aspects of microbial growth. In "Continuous Culture 6; Applications and New Fields". pp1-39 Edited by A. Dean and D. Ellwood. Chichester, Ellis Horwood.

Hill, A. V. (1928). The diffusion of oxygen and lactic acid through tissues. Proceedings of the Royal Society B **104:**39-46.

Hobbs, G., Frazer, C., Gardner, D. C. J., Petty, J., Oliver, S. G. and Cullum, J. (1988). Growth of *Streptomyces coelicolor* in continuous culture. Abstracts of the 112th Meeting of the Society for General Microbiology, Reading, P63.

Hobbs, G., Frazer, C. M., Gardner, D., Flett, F. and Oliver, S. G. (1989). Dispersed growth of *Streptomyces* in liquid culture. Applied Microbiology and Biotechnology **31:** 272-277.

Hobbs, G., Frazer, C. M., Gardner, D., Flett, F. and Oliver, S. G. (1990). Pigmented antibiotic production by *Streptomyces coelicolor* A3(2): kinetics and the influence of nutrients. Journal of General Microbiology **136:** 2291-2296.

Hodgson, D. A. (1982). Glucose repression of carbon source uptake and metabolism in *Streptomyces coelicolor* A3(2). Journal of General Microbiology **128:** 2417.

Hodgson, D. A. (1989). Increasing production of secondary metabolites. SERC Bulletin **4:** No3 p18-19.

Hodgson, D. A., Hood, D. and Swoboda, U. (1990). The interaction of primary and secondary metabolism: proline metabolism and undecylprodigiosin anabolism in *Streptomyces coelicolor* A3(2). Journal of Cellular Biochemistry, **14A:** pp87.

Hopwood, D. A. (1957). Genetic recombination in *Streptomyces coelicolor.* Journal of General MIcrobiology **16**:ii-ii.

Hopwood, D. A. (1988). Towards an understanding of gene switching in Streptomyces, the basis of sporulation and antibiotic prduction. Proceedings of the Royal Society: **B235:** 121-138.

Hopwood, D. A., Bibb, M. J., Chater, K. F., Kieser, T., Bruton, C. J., Kieser, H. M., Lydiate, D. J., Smith, C. P., Ward, J. M. & Schrempf, H. (1985a). Genetic manipulation of *Streptomyces*: a laboratory manual. The John Innes Foundation.

Hopwood, D. A., Wright, H. M., Bibb, M. J. and Cohen, S. N. (1977). Protoplast fusion and genetic recombination in *Streptomyces*. Nature **268:** 171-174.

Hopwood, D. A. and Chater, K. F. (1986). *Streptomyces* Genetics. In "The Bacteria" **9** . Edited by Queener S. W. and Day L. E. Academic Press.

Hopwood, D.A. and Kieser, T. (1990). The *Streptomyces* Genome. In "The Bacterial Chromosome". Drlica, K. and Riley M (ed.). ASM, Washington D.C. pp 147-162.

Horinouchi, S. and Beppu, T. (1988). Regulation of secondary metabolism in *Streptomyces.* In "Biology of Actinomycetes 88", p71-76. Edited by Y. Okami, Japan Scientific Societies Press.

Hostalek, Z, Tobek, I., Bobyk, M. A. and Kulaev, I.S. (1976). Folia Microbiologica **21**:131-136.

Hunter, I. S. (1985). Gene cloning in *Streptomyces.* In: DNA Cloning, Volume II pp19. I.R.L Press, Oxford.

Hunter, I. S. and Baumberg, S. (1989). Molecular genetics of antibiotic formation. In "Microbial products: New approaches." The Society for General Microbiology Symposium **44** pp. 121-162 (eds. Baumberg S, Hunter I S and Rhodes P M). Cambridge University Press.

I. D. Hamilton (1976). PhD Thesis, University of Glasgow.

I. S. Hunter (1977). PhD Thesis, University of Glasgow.

James, P. D. A. (1990). Ph. D. Thesis. University of Liverpool.

James, P. D. A. and Edwards, C. (1988). The effects of cultural conditions on growth and secondary metabolism in *Streptomyces thermoviolaceus.* FEMS Microbiology Letters **52:** 1-6.

Janglova, Z., Suchy, J., and Vanek,. Z. (1969). Intracellular adenosine-5'-triphosphate concentration in *Streptomyces aurefaciens.* Folia Microbiologia **14:** 208.

Jones, C. W. (1977). Aerobic respiratory systems in bacteria. In Symposium of the Society for General MIcrobiology **27:** 23-59.

Jones, C. W. and Poole, R.K.(1985). The analysis of cytochromes. Methods in Microbiology **18:** 285-327.

Jones, C. W. (1988). Membrane -associated energy tranduction in bacteria. In "Bacterial Energy Transduction". Ed. C. Anthony. Academic Press New York, pp 1-82.

Jones, G. H. (1985). Regulation of phenoxazinone synthase expression in *Streptomyces antibioticus.* Journal of Bacteriology **163:** 1215.

Jones, P., Moore, D. and Trinci, A.P.J. (1988). Effects of junlon and hostacerin on the electrokinetic properties of spores of *Aspergillus niger*, *Phanerochaete chrysosporium* and *Geotrichum candidum.* Journal of General Microbiology **134**:235-240.

Joshi, S. and Yamazaki, H. (1987). Repeated batch antibiotic fermentation by *Streptomyces* immobilized on cotton cloth. Biotechnology Techniques **1:** 45-48.

Kannan, L. V. and Rehacek, Z. (1970). Formation of poly-B-hydroxybutyrate by actinomycetes. Indian Journal of Biochemistry **7:** 126-129.

Katz, E. (1980). Kynurenine formamidase isozymes in *Streptomyces parvulus.* In "Biochemical and Medical Aspects of Tryptophan Metabolism" pp159. Edited by Hayasishi O. and Ishimusra Y. Elsevier.

Katz, E., Thompson, C. J. and Hopwood, D. A. (1983). Cloning and expression of the tyrosinase gene from *Streptomyces antibioticus* in *Streptomyces lividans.* Journal of General Microbiology **129:** 2703.

Kendrick, D. and Ensign, J. C. (1983). Sporulation of *Streptomyces griseus* in submerged culture. Journal of Bacteriology **155:** 357-366.

Kenig, M. and Reading, C. (1979). Holomycin and and antibiotic (MM19290) related to tunicamycin, metabolites of *Streptomyces clavuligerus.* Jounal of Antibiotics **32:** 449-554.

Khosla, C. and Bailey, J. E. (1988a). Heterologous expression of a bacterial haemoglobin improves the growth properties of recombinant *Escherichia coli.* Nature **331:** 633-635

Khosla, C. and Bailey, J. E. (1988b). The *Vitreoscilla* hemoglobin gene: molecular cloning, nucleotide sequence and genetic expression in *Escherichia coli.* Molecular and General Genetics **214:** 158-161.

Khosla, C., Curtis, J., DeModena, J., Rinas, U., and Bailey, J. E. (1990). Expression of Intracellular hemoglobin improves protein synthesis in oxygen-limited *Escherichia coli.* Biotechnology **8:** 849-853.

Kirby, R. (1978). An unstable genetic element affecting the production of the antibiotic holomycin by *Streptomyces clavuligerus.* FEMS Microbiological Letters **3:** 283-286.

Kirst, H. A. *et al.,* (1986). Preparation and evaluation of 3,4-ester derivatives: of 16-membered macrolide antibiotics related to tylosin. Journal of Antibiotics **39** 1724-1735.

Kominek, L. A., (1972). Biosynthesis of novobiocin by *Streptomyces niveus.* Antimicrobial agents and Chemotherapy **1:** 123.

Kondo, S., Yasui, K., Natsume, M., Katayama, M., and Marumo, S. (1988). Isolation, physico-chemical properties and biological activity of pamamycin-607, an aerial mycelium-inducing substance from *Streptomyces alboniger.* Journal of Antibiotics **41:** 1196-1204.

Konstantinov, C., and Yoshida, T. (1989). Physiological state control of fermentation processes. Biotechnology and Bioengineering **33:** 1145-1156.

Kretschmer, S., Riesenberg, D. and Bergter, F. (1981). Comparative analysis of mycelial growth in actinomycetes. Zentralblatt fur Bakteriologie, Microbiologie und Hygiene. 1 Abteilung, Supplement **11:** 131-135.

Kristjansson, J. K. (1989). Thermophilic organisms as sources of thermostable enzymes. Trends in Biotechnology **7** :349-353

Kulaev, I. S. (1985). Some Aspects of environmental regulation of microbial phosphorous metabolism. In "Environmental Aspects of Microbial Physiology" p1-23 pub. Elsevier.

Larsson, G. and Enfors, S. (1990). The pH-auxostat as a tool for studying microbial dynamics in continuous fermentation. Biotechnology and Bioengineering **36:** 224-232.

Lee, S. B. and Bailey, J. (1984). Genetically structured models for *lac* promoter-operator function in the chromosome and in multicopy plasmids: *lac* operator function. Biotechnology and Bioengineering **26:** 1372-1382.

Lee, S. B. and Bailey, J. (1984). Genetically structured models for *lac* promoter-operator function in the chromosome and in multicopy plasmids: *lac* promoter function. Biotechnology and Bioengineering **26** :1383-1389.

Leonardo, E. D. and Sedivy, J. M. (1990). A new vector for cloning large eukaryotic DNA segments in *Escherichia coli.* Biotechnology **8:** 841-843.

Liao, J. (1989). Fermentation data analysis and state estimation in the presence of incomplete mass balance. Biotechnology and Bioengineering **33** :613-622.

Llefke, E., Kaiser, D. and Onken, U. (1990). Growth and product formation of actinomycetes cultivated at increased total pressure and oxygen partial pressure. Applied Microbiology and Biotechnology **12**:674-679.

Lilley, G. and Bull, A. T. (1974). The production of ß-1,3 glucanase by a thermophilic species of *Streptomyces.* Journal of General Microbiology **83:** 123-133.

Lilley, G., Clark, A. and Lawrence, G. (1981). Control of the production of Cephamycin C and Thienamycin by *Streptomyces cattleya* NRRL 8057. J. Chem. Tech. Biotechnol. **31**:127-134.

Linton, J. D. (1990). The relationship between metabolite production and the growth efficiency of the producing organism. FEMS Microbiology Reviews **75:** 1-18.

Linton, J. D., Gouldney, D. and Woodard, S. (1988). Efficiency and stability of exopolysaccharide prodution from different carbon sources by *Erwinia herbicola.* Journal of General Microbiology **134:** 1913-1921.

Llras, P., Asturias, J. and Martin, J. (1990). Phosphate control sequences involved in transcriptional regulation of antibiotic biosynthesis. Trends in Biotechnology **8:** 184-189.

Locci, R. and Sharples, G. P. (1983). Morphology pp199. In "Biology of the Actinomycetes". (eds. Goodfellow, M., Modarski, M. and Williams, S. T.) Academic Press. London.

Lohr, D., Buschulte, T., and Gilles, E. (1989). Continuous cultivation of *Streptomyces tendae* in different media. Applied Microbiology and Biotechnology **32:** 274-279.

Luedeking, R. (1967). In "Biochemical and Biological Engineering **1**" p208. N. Blakebrough (ed.) Academeic Press, New York .

Lydiate, D. J., Malpartida, F. and Hopwood, D. A. (1985). Plasmid SCP2*: its functional analysis and development into useful cloning vectors. Gene **35:** 223-235.

Madry, N. and Pape,. H. (1982). Formation of secondary metabolism enzymes in the tylosin producer *Streptomyces* T59-235. Archives of Microbiology **131:** 170.

Madry, N., Sprinkmeyer, R. and Pape, H. (1979). Regulation of tylosin synthesis in *Streptomyces*: effects of glucose analogs and inorganic phosphate. European Journal of Applied Microbioloogy and Biotechnology **7:** 365.

Magnola, S., Leenutaphong, D., DeModena, J., Curtis, J., Bailey, J. E., Galazzo, J. L. and Hughes, D. E. (In Press). Actinorhodin production by *Streptomyces coelicolor* and growth of *Streptomyces lividans* are improved by the expression of a bacterial hemoglobin.

Majumdar, M. K. and Majumdar, S. K. (1979). Isolation and characterization of three phosphoamido-neomycins and their conversion into neomycin by *Streptomyces fradiae.* Biochemical Journal **120:** 271.

Makins, E. and Holt, G. (1982). LIposome-mediated transformation of streptomycetes by chromosomal DNA. Nature **293:** 671.

Marshall, K. C. and Alexander, M. (1960). Growth of fungi and actinomycetes Journal of Bacteriology **80**:412.

Martin, J. E., Liras, R. and Demain, A. L. (1978). ATP and adenylate energy charge during phosphate-mediated control of antibiotic synthesis. Biochemical and Biophysical Research Communications **83:** 822.

Martin, J. E., and Liras, R. (1989). Organiszation and Expression of genes involved in the biosynthesis of antibiotics and other secondary metabolites. In "Annual Reviews in Microbiology 43:173-206.

Martin, J. F. (1989). Molecular mechanisms for the control by phosphate of the biosynthesis of antibiotics and other secondary metabolites. In "Regulation of Secondary Metabolism" p1213-237. Edited by S Shapiro, published by CRC Press.

Masuma, R., Tanaka, Y. and Omura, S. (1983). Ammonium ion-depressed fermentation of tylosin by the use of a natural zeolite and its significance in the study of the biosynthetic regulation of the antibiotic. Journal of Fermentation Technology **56:** 345-353.

Mathumathi, R., Kumaravel, S. and Dharmalingam, K. (1990). Deamplification and deletion of amplified DNA in *Streptomyces lividans* and *S. fradiae.* Applied Microbiology and Biotechnology **33:**291-295.

Mazodier, P., Petter, R. and Thompson, C. (1989). Intergeneric conjugation between *Escherichia coli* and *Streptomyces* species. Journal of Bacteriology **171:** 3583-3585.

McCarthy, A. J. (1987). Lignocellulose-degrading actinomycetes. FEMS Microbiology Reviews **46** 145-163.

McKay, A., Quilter, J. and Jones, C. W. (1982). Energy conservation in the extreme thermophile *Thermus thermophilus* HB8. Archives of Microbiology **131:** 43-50.

Mendez, C., Barbes, C., Manzanal, M. B. and Hardisson, C. (1985). A simple method to study colony development in *Streptomyces.* FEMS Microbiology Letters **28:** 99-102.
Mendez, C., Brana, A. E., Manzanal, M. B. and Hardissson, C. (1985b). Role of substrate mycelium in colony development in *Streptomyces.* Canadian Journal of Microbiology **31:** 446-450.

MIller, A. L. and Walker, J. B. (1969). Enzymatic phosphorylation of streptomycin by extracts of streptomycin-producing strains of *Streptomyces.* Journal of Bacteriology **99:** 401.

MIller, J. R. and Ingolia, T. D. (1989b). Cloning and characterization of ß-Lactam biosynthetic genes. Molecular Microbiology **3:** 689-695.

Miller, J.R. and Ingolia, T. D. (1989a). Cloning ß-Lactam Genes for *Streptomyces* spp. and Fungi. In "Genetics and Molecular biology of Industrial Microorganisms", Edited by Hershberger C., Queener S., Hegermann G. Based on the Proceedings of the Fourth ASM Conference on the Genetics and Molecular Biology of Industrial Microorganisms, Bloomington 1988.

Minkevich, S.G., Krynitskaya, A.Yu., and Eroshin, V.K. (1989). Bistat - a novel method of continuous cultivation. Biotechnology and Bioengineering **33:** 1157-1161.

Monod, J. (1942) Recherches sur la Croissance des Cultures Bacterriennes. Hermann, Paris.

Monod, J. (1950). La technique de culture continue. Theorie et applications. Annales de L'Institut Pasteur **79:** 390-409.

Neijssel, O. and Tempest, D. W. (1975). The regulation of carbohydrate metabolism in *Klebsiella aerogenes* NCTC 418 organisms, growing in chemostat culture. Archives of Microbiology **106:** 251-258.

Neijssel, O. and Tempest, D. W. (1986). Bioenergetic aspects of aerobic growth of *Klebsiella aerogenes* NCTC 418 in carbon-limited and carbon-sufficient chemostat culture. Archives of MIcrobiology **133:** 300-302.

Nienow, A.W. (1990). Agitators for mycelial fermentations. Trends in Biotechnology **8:** 224-233.

Nisbet, L. J. and Porter, N. (1989). The impact of pharmacology and molecular biology on the exploitation of microbial products. In "Microbial Products: New Appraches", Symposium Volume 44 of the Society for General Microbiology.

Novick, A. and Szilard, L. (1950). Description of the chemostat. Science **112:** 715-716.

Novotna, J., Kadir O., Bulumauerova, M., Behal, V., Hostalek, Z. (1984). Mutants of *Streptomyces aureofaciens* resistant to inhibition of chlortetracycline biosynthesis by phosphate. Folia Microbiology **29:** 399.

Ochi, K. (1986). Occurrence of the stringent response in *Streptomyces* sp. and its signifigance for the initiation of morphological and physiological differentiation. Journal of General Microbiology **132:** 2621-2631.

Ochi, K. (1987a). Changes in nucleotide pools during sporulation of *Streptomyces griseus* in submerged culture. Journal of General Microbiology **133:** 2787-2795.

Ochi, K. (1987b). Metabolic initiation of differentiation and secondary metabolism by *Streptomyces griseus*: significance of the stringent resposne (ppGpp) and GTP content in relation to A-factor. Journal of Bacteriology **169:** 3608-3616.

Okungbawa, J. (1991). PhD Thesis, University of Glasgow.

Okungbawa, J., Holms, W. H. and Hunter, I. S. (1989). Increased gene dosage of phosphoenolpyruvate carboxylase improves conversion of glucose to biomass and decreases acetate excretion in Escherichia coli ML308. Offered Paper at 113rd Meeting of the Society for General Microbiology.

Onadipe, A. and Bushell, M. (1987). The use of multivariate analysis for the design of selective isolation conditions for mutants of *Streptomyces cattleya* with improved antibiottic titre. Journal of Chemical Technology and Biotechnology **39:** 237-249.

Owens, J. D. and Legan, J. D. (1987). Determination of the Monod substrate saturation constant for microbial growth. FEMS Microbiology Reviews **46:** 419-432.

Pall, M. L. (1977) cAMP and the plasma membrane potential in *Neurospora crassa*. Journal of Biological Chemistry **252**: 7146-7150.

Pall, M. L., Trevillyan, J. M. and Hinman, N. (1981). Deficient cyclic AMP control in mutants of two genes of *Neurospora crassa.* Molecular and Cell Biology **1:** 1-18.

Papoutsakis, E. (1984). Equations and calculations for fermentations of butyric acid bacteria. Biotechnology and Bioengineering **26:** 174-187.

Peretti, S. and Bailey, J. (1987). Simulations of host-plasmid interactions in *Escherichia coli*: copy number, promoter strength and ribosome binding site strength effects on metabolic activity and plasmid gene expression. Biotechnology and Bioengineering **29:** 316-328.

Pirt, S. J. (1965). The maintenance energy of bacteria in growing cultures. Proceedings of the Royal Society B **163**:224.

Pirt, S. J. (1966). A theory of the mode of growth of fungi in the form of pellets in submerged culture. Proceedings of the Royal Society B **166:** 369-373.

Pirt, S. J. (1974). Principles of Microbe and Cell Cultivation. Blackwell, Oxford.

Pirt, S. J. (1990). The dynamics of microbial growth - a personal view. In "MIcrobial Growth Dynamics" pp1-17. Special Publications of the Society for General Microbiology Volume 28. Edited by R. K. Poole, M. J. Bazin and C. W. Keevil.

Pirt, S. J. and Callow, D. S. (1960). Studies of the growth of *Penicillium chrysogenum* in continuous flow culture with reference to penicillin production. Journal of Applied Bacteriology **23:** 87.

Pond, J. L. and Langworthy, T. A. (1987). Effect of growth temperature on the long-chain diols and fatty acids of *Thermomicrobium roseum*. Journal of Bacteriology **169:** 1328-1330.

Rafii, F.,Crawford, D., Bleakley, B. H. and Wang, Z. (1988). Assessing the risks of releasing recombinant *Streptomyces* in soil. Microbiological Sciences **5:** 358-362.

Rebello, A., Gil, J., Liras, P., Asturias, J. A. and Martin, J. F. (1989). Cloning and characterisation of a phosphate-regulated promoter involved in phosphate control of candicidin biosynthesis. Gene **79:** 47-58.

Rhodes, P. M. (1984). The production of oxytetracycline in chemostat culture. Biotechnology and Bioengineering **26:** 382-385.

Rhodes, P. M., Winskill, N., Friend, E. J. and Warren, M. (1981). Biochemical and genetic characterisation of *Streptomyces rimosus* mutants impaired in oxytetracycline biosynthesis. Journal of General Microbiology **124:** 329-338.

Richards, H. and Ward, J. (1990). Studying plasmids in *Streptomyces.* SERC Bulletin **4** No6 p14-15.

Richardson, M., Kuhstoss, S., Huber, M., Ford, L., Godfrey, O., Turner, J. and Nagaraja Rao. (1989). Cloning of genes involved in spiramycin biosynthesis from *Streptomyces ambofaciens.* In "Genetics and Molecular Biology of Industrial Microorgansisms' p40-44. Edited by C. Hershberger, S. Queener and G. Hegerman. Proceeding of th fourth ASM Conference of the same title.

Riesenberg, D. and Bergter, F. (1979). Dependance of macromolecular composition and morphology of *Streptomyces hygroscopicus* on specific growth rate. Zentralblatt fur Bakteriologie, Microbiologie und Hygiene **19:** 415-430.

Riesenberg, D., Bergter, G. and Kari, C. (1984). Effect of serine hydroxamate and methyl a-D-glucopyranoside treatment on nucleoside polyphosphate pools, RNA and protein accumulation in *Streptomyces hygroscopicus.* Journal of General Microbiology **130:** 2549-2558.

Roels, J. (1980). Application of macroscopic principles to microbial metabolism. Biotechnology and Bioengineering **22:** 2457-2514.

Rols, J.L., Condoret, J.S., Fonade, C., and Goma, G. (1990). Mechanism of enhanced oxygen transfer in fermentation using emulsified oxygen-vectors. Biotechnology and Bioengineering **35:** 427-435.

Russell J (1986). Heat production by ruminal bacteria in continuous culture and its relationship to maintenance energy. Journal of Bacteriology **168** 694-701.

Russell N J and Fukaunaga N (1990). A comparison of thermal adaptation of membrane lipids in psychrophilic and thermophilic bacteria. FEMS Microbiology Reviews **75** 171-182.

Rye, A., Drozk, J. W., Jones, C. W. and LInton, J. D. (1988). Growth efficiency of *Xanthomonas campestris* in continuous culture. Journal of General Microbiology **134** 1055-1061.

Salas, J. A., Guijarro, J. A. and Hardisson, C. (1983). High calcium content in *Streptomyces* spores and its release as an early event during spore germination. Journal of Bacteriology **155:** 1316.

Sanchez, S. and Quinto, C. M. (1975). D-Glucose isomerase: constitutive and catabolite repression-resistant mutants of *Streptomyces phaeochromogenes.* Applied Microbiology **30:** 750.

Sankaran, L. and Pogell, B. M. (1975). Biosynthesis of puromycin in *Streptomyces alboniger*: regulation and properties of O -demethylpuromycin O-methyltransferase. Antimicrobial Agents and Chemotherapy **8:** 721.

Schatz, A., Bugie, E. and Waksman, S. A. (1944). Streptomycin, a substance exhibiting antibiotic activity against gram-positive and gram-negative bacteria. Proceedings of the Society for Experimental Biology and Medicine **55:** 66.

Schugerl, K., Wittler, R. and Lorenz, T. (1983). The use of molds in pellet form. Trends in Biotechnology **1:** 120-122.

Scott, W. A., Mishra, N. C. and Tatum, E. L. (1973). Biochemical genetics of morphogenesis in Neurospora. Brookhaven Sumposia in Biology **25:** 1-18.

Seno, E. T. and Chater, K. F. (1983). Glycerol catabolic enzymes and their regulation in wild type and mutant strains of *Streptomyces coelicolor* A3(2). Journal of General Microbiology **129:** 1403-1413.

Shapiro, S. (1989). Nitrogen assimilation in actinomycetes and the influence of nitrogen nutrition on actinomycete secondary metabolism. In "Regulation of Secondary Metabolism" p135. Edited by S. Shapiro, published by CRC Press.

Shapiro, S. and Vining, L. C. (1983). Nitrogen metabolism and chloramphenicol production by *Streptomyces venezuelae.* Canadian Journal of Microbiology **29:** 1706-1714.

Shipston, N. and Bunch, A.W. (1989). The physiology of L-methionine catabolism to the secondary metabolite ethylene by *Escherichia coli.* Journal of General Microbiology **139**: 1489-1497.

Shreve, B. and Turner, J. R. (1985) Method of producing antimicrobial agents and bioconverting microorganism therefor. United States Patent 4522919

Sikyta, B., Slezak, J. and Herold, H. (1961). Growth of *Streptomyces aurefaciens* in continuous culture. Applied Microbiology **9:** 233-238.

Sinclair, C. G. (1987). MIcrobial process kinetics. In "Basic Biotechnology" p75-127. Ed J D Bu'lock and B Kristiansen. Academic Press.

Solomon, B. O. and Erickson, B. S. (1981). Biomass yields and maintenance requirements for growth on carbohydrates. Process Biochemistry February 44-49.

Sonnleitner, S. and Fiechter, A. (1983). Advantages of using thermophiles in biotechnological processes, expectations and realities. Trends in Biotechnology **1:** 74-80.

Steel, R. and Maxon, W. D. (1966). Studies with a multiple-rod mixing impeller. Biotechnology and Bioengineering **8:** 109-115.

Stouthamer, A. H. (1977). Energetic aspects of the growth of micro-organisms. Symposia of the Society for General Microbiology **27:** 285-315.

Stouthamer, A. H. , Bulthius, B. A. and van Verseveld, H. W. (1990). Energetics of growth at low growth rates and its relevance for the maintenance concept. In "MIcrobial Growth Dynamics" pp85-103. Special Publications of the Society for General Microbiology Volume 28. Edited by R K Poole, M J Bazin and C W Keevil.

Stouthamer, A. H. and van Verseveld, H. W. (1987). Microbial energetics should be considered in manipulating metabolism for biotechnological purposes. Trends in Biotechnology **5:** 149-155.

Stowell, J. D., and Bateson, J. D. (1983). Economic aspects of industrial fermentation. In "Bioactive Microbial Products II- Development and Production". (Nisbet L. J. and Winstanley D. J. eds). Academic Press.

Strauch, E., Takano, E. Baylis, H. A. and Bibb, M. J. (1991). The stringent response in *Streptomyces coelicolor* A3(2). Molecular Microbiology **5:** 289-298.

Streekstra, H., Teixeira de Mattos, M., Neijssel, O. and Tempest, D. (1987). Overflow metabolism during anaerobic growth of *Klebsiella aerogenes* NCTC 418 on glycerol and dihydroxyacetone in chemostat culture. Archives of Microbiology **147:** 268-275.

Stuttard, C. (1979). Transduction of auxotrophic markers in a chloramphenicol producitng strain of *Streptomyces*. Journal of General Microbiology **110:** 479-482.

Suarez, J. E. and Chater, K. F. (1980). Polyethylene glycol-assisted transfection of streptomycete protoplasts. Journal of Bacteriology **142:** 8-14.

Szeszak, F., Vitalis, S., Toth, F., Valu, G., Fachet, J., and Szabo, G. (1990). Detection and determination of factor C - a regulatory protein - in *Streptomyces* strains by antiserum and monoclonal antibody. Archives in Microbiology **154**: 82-84.

Tempest, D. W. and Neijssel, O. M. (1984). The status of Y_{ATP} and maintenance energy as biologically interpretable phenomena. Annual Reviews of Microbiology **38:** 459-86.

Terry, J. and Springham, D. G. (1981). The effect of inorganic phosphate on cyclic adenosine 3',5' -monophosphate in streptomycin-producing cultures of *Streptomyces griseus*. Canadian Journal of Microbiology **27:** 1044.

Tough, A. (1990). PhD Thesis, University of Aberdeen.

Trilli, A. (1990). Kinetics of secondary metabolite production. In "MIcrobial Growth Dynamics" pp103-127. Special Publications of the Society for General Microbiology Volume 28. Edited by R K Poole, M J Bazin and C W Keevil.

Trilli, A., Crossley, M. V. and Kontakou, M. (1987). Relation between growth rate and erythromycin production in *Streptomyces erythraeus.* Biotechnology Letters **11:** 765-770.

Trinci, A. P. J. (1969). A kinetic study of the growth of *Aspergillus nidulans* and other fungi. Journal of General Microbiology **57:** 11-24.

Trinci, A. P. J., Robson, G.D., Wieb, M. G., Cuncliffe, B. and Naylor, T. W. (1990). Growth and morphology of *Fusarium graminearum* and other fungi in batch and continuous culture. In "MIcrobial Growth Dynamics" pp17-39. Special Publications of the Society for General Microbiology Volume 28. Edited by R K Poole, M J Bazin and C W Keevil.

Trinci, A.P. J. (1973). The hyphal growth unit of wild type and spreading colonial mutants of neurospora crassa. Archiv fur Mikrobiologie **91**:127-136.

Trinci, A.P.J. (1983). Effect of junlon on morphology of *Aspergillus niger* and its use in making turbidity measurements of fungal growth. Transactions of the British Mycological Society **87:**20-24.

Tsai, R. and Lee, Y. (1989). A criterion for selecting fermentation stoichiometry methods. Biotechnology and Bioengineering **33:** 1347-1349.

Tsai, S. and Lee, Y. (1988). Application of metabolic pathway stoichiometry to statistical analysis of bioreactor measurement data. Biotechnology and Bioengineering **32:** 713-715.

Umezawa, H. (1967). Index of Antibiotics from Actinomycetes. Universiy of Tokyo Press, Tokyo.

Vanek, Z., Novak, J, and Jechova, V. (1988). Primary and secondary metabolism. In "Biology of Actinomycetes 88", edited by Y. Okami, Japan Scientific Societies Press.

Vining, L. C. (1990). Functions of secondary metabolites. Annual Review of Microbiology **44:** 395-427.

Vining, L. C. and Doull, J. L. (1988). Catabolite repression of secondary metabolism in actinomycetes. In "Biology of Actinomycetes 88", edited by Y. Okami, Japan Scientific Societies Press.

Virolle, M. (1986). Cited in Hopwood D. A. *et al.,* Regulation of gene expression in antibiotic-producting *Streptomyces.* Symposium of the Society for General Microbiology 39. Edited by I. R. Booth and C. F. Higgins.

Vivian, A. (1971). Genetic control of fertility in *Streptomyces coelicolor* A3(2);Plasmid involvement in the interconversion of UF and IF strains. Journal of General Microbiology **69:** 353-364.

Vu-Trong, K., Bhuwapathanapun, S. and Gray, P. (1980). Metabolic regulation in tylosin-producing *Streptomyces fradiae:* regulatory role of adenylate nucleotide pool and enzymes involved in biosynthesis of tylonolide precursors. Antimicrobial Agents and Chemotherapy **17:** 519.

Wallace, R. J. and Holms, W. H. (1986). Maintenance coefficients and rates of turnover of cell material in E*scherichia coli* ML308 at different growth temperatures. FEMS Microbiology Letters **37:** 317-320.

Wang, D. I. C. and Fewkes, R. C. J. (1977). EFfect of operating and geometric parameters on the behavour of non-Newtonian mycelial antibiotic fermentations. Developments in Industrial Microbiology 18: 39-57.

Wanner, U. and Egli, T. (1990). Dynamics of microbial growth and cell composition in batch culture. FEMS Microbiology Reviews **75:** 19-44.

Weast, *et al.,* (1972). C.R.C. Handbook of Chemistry and Physics. 53rd edition Cleveland, Ohio.

Weigel, B. J., Burgett, S. G., Chen, V. J., Skatrud, P. L., Frolik, C. A., Queener, S. W. and Ingolia, T. D. (1988). Cloning and expression in *Escherichia coli* of Isopenicillin N synthetase genes from *Streptomyces lipmanii* and *Aspergillus nidulans.* Journal of Bacteriology **170:** 3817-3826.

Wellington, E. M. H., Cresswell, N. and Saunders, V. A. (1990). Growth and survival of streptomycete inoculants and extent of plasmid transfer in sterile and non sterile soil. Applied and Environmental Microbiology **56:** 1413-1419.

Werner, W., Rey, H. and Wielinger, H. (1970). Z. analyt. Chem. **252**:224.

Westlake, D. W. S., Sal, F., McGrath, R. and Vining, L. C. (1968). Influence of nitrogen source on formation of chloramophenicol in cultures of *Streptomyces sp* 3022a Canadian Journal of Microbiology **14:** 587-593.

Westpheling, J., Ranes, M. and Losick, R. (1985). RNA polymerase heterogeneity in *Streptomyces coelicolor.* Nature **313:** 22-27.

White, P. J., Young, J., Hunter, I. S., NImmo, H., and Coggins, J. R.(1990). Purification of 3-dehydroquinase from Streptomyces coelicolor A3(2). Biochemical Journal **265:** 735-738.

Wiegel, J. (1990). Temperature spans for growth: hypothesis and discussion. FEMS Microbiology Reviews **75:** 155-170.

Willets, N and Wilkins, B. (1984). Processing of plasmid DNA during bacterial conjugation. MIcrobiological Reviews **48:** 24-41.

Williams, P. J le B. and McDonald, I. J. (1966). Permeability of a micrococcal cell to maltose and ssome related sugars. Canadian Journal of Microbiology **12:** 1213.

Williams, S. T., Goodfellow, M., Wellington, E. M. H., Sneath, P. H. and Sackin, M. J. (1983). Numerical classification of *Streptomyces* and related genera. Journal of General Microbiology **129:** 1743-1813.

Woese, C. (1987) Bacterial Evolution. Microbiological Reviews **51**:221-271.

Young, M. C., Kempe, L. L. and Bader, F. G. (1985). Effects of phosphate, glucose and ammonium on cell growth and lincomycin production by *Streptomyces lincolnensis* in chemically defined media. Biotechnology and Bioengineering **27:** 327-333.