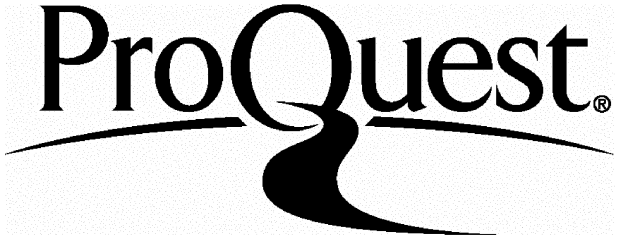
Enhanced production of a recombinant, thermostable α-amylase in *Streptomyces lividans*: Effects of plasmid construction and culture conditions.

A thesis submitted to the University of London for the Degree of Doctor of Philosophy by

Susan C. Robinson

Department of Biochemical Engineering
University College London
Torrington Place
London WC1E 7JE

September 1998



Abstract

Members of the genus *Streptomyces* are well known for their ability to produce a wide range of antibiotics. In addition to this valuable attribute, many of these bacteria also secrete a variety of proteins. This has led to their being considered as alternative host organisms for the production of heterologous gene products. This work examines the potential of *Streptomyces lividans* as a host for the secretion of recombinant proteins using the production of a thermostable α-amylase as a model system.

Two new streptomycete expression vectors were constructed. Incorporation of the *sti* region from pIJ101, which carries a site for second strand initiation of plasmid replication, was found to increase levels of α-amylase production by over 1000-fold. This was likely due to improvements in plasmid copy number and form. The study confirmed that use of promoter arrays to drive recombinant gene expression can be an effective way to achieve high level protein production. Over one and a half times more α-amylase was produced when P_{mel} was placed in tandem with P_{amy} in front of the α-amylase gene.

As expected, medium composition also played an important role in determining the behaviour of the system. The most favourable condition for high level enzyme production was found to be minimal medium lacking rapidly metabolisable sugars. Glucose was found to repress production of the α-amylase in the *S. lividans* system, likely through the action of the Reg1 protein. A statistical experimental approach allowed the identification of other influential medium variables, with succinate, calcium and phosphate levels proving key. Unexpectedly, above optimal growth temperatures were found to significantly boost levels of α-amylase production from the *S. lividans* host. Possible reasons for this phenomenon are explored and discussed.

In summary, this work highlights the strong potential of the *Streptomyces* system. It demonstrates that *S. lividans* can provide a viable, and competitive, alternative to *E. coli* as a host for the production of heterologous proteins.

' The outcome of any serious research can only be to make two questions grow where one question grew before.' Thorstein Veblen, 1857-1929.

Acknowledgements

Firstly, I would like to thank all of the people who, over the years, have encouraged me to think freely and have confidence in my own beliefs, opinions and abilities.

Special acknowledgement goes to Dr John Ward, whose patient teaching of Molecular Biology made this work possible. I feel truly fortunate to have had such a capable and knowledgeable supervisor to guide me over the course of this work. I am also grateful to Dr Andy Ison for all of the advice and encouragement he has given me during my time at University College London.

I would like to thank each of my parents for their love and support. I am indebted to them, both for instilling in me a curiosity about the world around me, and for allowing me every opportunity to explore it.

Finally, I would like to thank Sarah and John for putting up with my ramblings and reminding me that there are more important things to life than work.

Contents

Figures

Chapter 1: Introduction

Chapter 3: Initial Media Experiments

Chapter 4: Construction & Evaluation of New α-Amylase Expression Vectors

Chapter 5: Performance of *S. lividans* pQR620 Under Fermentation Conditions

Chapter 6: The Effect of Incubation Temperature on α-Amylase Production

Chapter 7: The Effect of Medium Composition on α-Amylase Production

Chapter 8: Discussion

Tables

Chapter 1: Introduction

Chapter 2: Materials & Methods

Chapter 4: Construction & Evaluation of New α-Amylase Expression Vectors

Chapter 5: Performance of *S. lividans* pQR620 Under Fermentation Conditions

Chapter 6: The Effect of Incubation Temperature on α-Amylase Production

Chapter 7: The Effect of Medium Composition on α-Amylase Production

Chapter 8: Discussion

Chapter 1
Introduction

1.1: The Genus *Streptomyces*

The genus *Streptomyces* consists of a large, wide ranging group of Gram positive, spore forming, soil bacteria. They are characterised by a complex cycle of morphological differentiation which closely resembles that of filamentous fungi (Chater, 1998; Schauer *et al.*, 1988). Their developmental cycle stretches over a period of many days during which time spatial and temporal activation of genes leads to formation of substrate mycelium, formation of aerial mycelium and finally sporulation. The length of the cycle depends on the substrate upon which they are growing, but typically lasts from 4 to 10 days.

Due to their saprophytic lifestyle the group are natural and highly active secretors of a large range of enzymes. These include proteases, nucleases, lipases and a variety of enzymes which hydrolyse different types of polysaccharides (Virolle & Bibb, 1988). Several streptomycete produced enzymes are in commercial production. The most important of these is glucose isomerase which is used for bulk production of high fructose corn syrups. Other industrially important enzymes include amylase, cellulase, xylanase and chitinase for degradation of biomass, proteases for detergents and tanning of leather, lytic enzymes for food preservation and wine and beer clarification and pectic enzymes for fruit juice processing (reviewed by Piret & Demain, 1988). Many other enzymes are showing promise for a large range of applications. Examples include pronase, l-asparaginase and urate oxidase for medical use, bacteriolytic enzymes as immunostimulants, restriction endonucleases for molecular biology and acylases and lipases for resolving racemic mixtures and yielding chiral compounds.

Undoubtedly, the most important characteristic possessed by members of the genus is their ability to produce antibiotics. Over 60% of known naturally occurring antibiotics come from *Streptomyces* species (Demain, 1988). As a result, intense commercial interest has been present in the genus for several decades. Large scale production of clinically and agriculturally important secondary metabolites, such as streptomycin,

actinomycin, erythromycin and oxytetracycline, is an important sector for the pharmaceutical and agrochemical industries.

1.1.1: The streptomycete genome.

In comparison with other prokaryotes, *Streptomyces* possess relatively large genomes; typically ranging from 5 x 10^3 to 7 x 10^3 Kb (Birch *et al.*, 1990). This is approximately 1.5 to 2 times the size of the *E. coli* genome (Piret & Demain, 1988). Frequently extra chromosomal DNA is carried. Such genetic elements can be present in a wide range of sizes and copy numbers (Piret & Demain, 1988).

The most notable characteristic of the streptomycete genome is its extremely high guanine (G) and cytosine (C) content, which stands at around 73% (Enquist & Bradley, 1971; Benigni *et al.*, 1975; Williams *et al.*, 1983). Use of G or C in the first and second codon positions is prominent, i.e. 66% and 53% respectively in a recent study of 34 sequenced *Streptomyces* genes (Molnar, 1994). There is an extremely strong bias towards the use of these two nucleotides in the third position. Wright and Bibb (1992) found that between 76 and 98% of the nucleotides in this position were G or C, depending on the gene being studied. Beyond this almost 'compulsory' G or C at the third position, the preference of C over G at these positions is suggested wherever the code allows choice (Molnar, 1994).

Although it is apparent from previous expression studies that streptomycetes do possess tRNA species capable of reading all 61 possible amino acid codons, it is also apparent that some codons are infrequently used and several are virtually forbidden, e.g. TTA and CTA (Bibb *et al.*, 1985b). This biased codon usage is likely to reflect quantitative differences in the pools of major tRNA species. A large body of research has been conducted to try and gain an understanding of the role of the TTA codon within this group of organisms. This codon is present in very few native genes and its occurrence is non-random with respect to the class of genes concerned. It is mostly contained in genes encoding proteins needed only during morphological and physiological differentiation (Chater, 1998; Leskiw *et al.*, 1991). This codon is thought to play a regulatory role in gene expression in *S. lividans* (White & Bibb, 1997). It is solely recognised by the $tRNA_{leu}$-like product of the *bld*A gene which becomes available only

at the later stages of growth (Leskiw *et al.*, 1993). Genes containing TTA codons are expressed exquisitely at these late stages even if driven by vegetatively expressed promoters (White & Bibb, 1997; Ueda *et al.*, 1993a).

Another interesting aspect of *Streptomyces* species is the phenomenon of genetic instability. This manifests itself as extraordinarily high mutation rates affecting certain species specific traits (Piret & Demain, 1988). Unstable phenotypes are irreversibly lost at spontaneous frequencies as high as 0.1% of plated spores. Most of these instabilities are the result of extensive chromosomal deletions which are frequently accompanied by intense DNA amplifications, site specific exclusions and integrations that take place in the absence of any obvious selection pressure (Piret & Demain, 1988). Amplification and deletion events in *Streptomyces* species are two to three orders of magnitude larger than similar events in *E. coli* and *B. subtilis* (Birch *et al.*, 1990). The exact reasons why the cells can tolerate such gross loss of genetic information are not known. It seems that the regions which are prone to deletion events tend to be the large segments of the streptomycete genome which are free of essential genes.

1.1.2: Streptomycete gene expression.

Research conducted in the late 1970's and early 1980's suggested that the investigation of the *Streptomyces* genetic system would provide an opportunity to learn about new aspects of bacterial gene expression. The available evidence indicated that the transcription control mechanisms present were likely to include many interesting, and perhaps novel, features. The very complex developmental cycle undertaken by these organisms tended to suggest that regulation and co-ordination of gene expression would need to at least match this level of complexity. The promoter regions of other prokaryotes were known to be relatively rich in A and T (Rosenberg & Court, 1979). Whether this held true in *Streptomyces* was also of great interest. Therefore these factors, coupled with growing interest in these organisms as expression hosts, led to the study of gene expression in *Streptomyces* becoming an active research area.

Over the last 15 years a great deal of progress has been made in gaining an understanding of the mechanisms in operation. This work has confirmed that a tremendous level of complexity is present. Current understanding of streptomycete

genetic signals and regulation of gene expression is reviewed in the Sections which follow. Despite intensive research, our knowledge of the systems in operation is still relatively basic. However, this is a fast moving field with new pieces of understanding falling into place month by month. Over the next decade a clear picture should begin to emerge as to how these organisms control expression of their genes both temporally and spatially and in response to varying environmental and nutritional conditions.

1.1.2.1: Promoters.

Examination of the 150 or so *Streptomyces* promoters identified indicates that they display extreme heterogeneity (Strohl, 1992; Bourn & Babb, 1995). This is in direct contrast with most of the large number of promoter regions identified and studied in *E. coli* and *B. subtilis*, where the majority correspond closely to the *E. coli* promoter consensus sequence (-35 TTGACA and -10 TATAAT).

Initial research has placed *Streptomyces* promoters into two groupings. The predominant class of sequences that initiate transcription do not function when transplanted into *E. coli*, e.g. the *tsr* and *aph* promoters (Binnie *et al.*, 1997; Bibb *et al.*, 1985b). DNA fragments encoding such promoter activity lack any segments resembling the promoter regions of *E. coli* and *B. subtilis*. In the majority of these cases the specific nucleotides recognised by RNA polymerase are unknown. The second group of transcription and initiation signals can function as promoters in both *S. lividans* and *E. coli*, for example the *dag*A p4 promoter (Buttner *et al.*, 1987; Jaurin & Cohen, 1985). This class of promoters are AT rich and have been shown to contain all the elements characteristic of *E. coli* promoters, i.e. -35 and -10 regions and spacing of 16 to 18 bp between regions (Bourn & Babb, 1995). It is common for genes to possess both types of transcription signal in their promoter regions, e.g. the *dag* promoters (Buttner *et al.*, 1987).

Studies have uncovered extremely complicated promoter regions. Many contain multiple promoters (as many as 8), tandem promoters and overlapping promoters on opposite strands (Strohl, 1992; Bourn & Babb, 1995; Binnie *et al.*, 1997). Multiple promoters provide an obvious means of achieving differential gene expression, likely through the production of alternative sigma factors (Section 1.1.3.1).

In the *E. coli* and *Bacillus* systems another well supported and widely held view is that initial binding of the RNA polymerase to a promoter sequence primarily involves recognition of the -35 region (Dombroski *et al.*, 1992). Again *Streptomyces* seem to provide an 'exception to the rule'. For the *tsr* p2 promoter it is possible to destroy the sequence up to -22 nucleotides upstream of the start codon without affecting transcription (Janssen & Bibb, 1990). The p1 and p2 promoters of *Micromonospora echinospora*, which are functional in *S. lividans*, only need 5 and 17 bp of native upstream sequence respectively for proper initiation (Baum *et al.*, 1989). The exact mechanism which allows these promoters to function is not known.

In the *E. coli* and *Bacillus* systems it is not only the promoter sequence itself which is critical for effective transcription, other factors such as spacing and upstream sequences are important too. An average distance of 23 nucleotides separates the transcription start site from the coding region in a typical *E. coli* gene (Rosenberg & Court, 1979). The streptomycete system again seems to accept a much wider variation. A comparison of 48 genes found a huge range of distances between the transcription start sites and the coding regions of between 9 and 345 nucleotides (Strohl, 1992). Most sites were around 100 nucleotides away. In genes with long 5' untranslated sequences significant secondary structures were found (Strohl, 1992; Jaurin & Cohen, 1985). These were hypothesised to contain regulatory sites such as anti-terminators.

1.1.2.2: Transcription terminators.

The mechanism by which transcription is terminated in *E. coli* has been widely documented. In most cases, downstream of the coding region of a gene there is a palindromic GC rich region which is followed by an AT rich region (Dale, 1989). As a consequence, a stable hairpin loop structure is formed in the corresponding mRNA. This is followed by four or more U residues where the RNA polymerase dissociates. If such a structure is present no other help is needed for termination. In situations where no such structure is present termination is dependent on the participation of the Rho protein (Dale, 1989).

Termination in *Streptomyces* seems to be analogous to Rho-independent termination in *E. coli* (Binnie *et al.*, 1997; Molnar, 1994). Streptomycete terminators also possess

long imperfect inverted repeat sequences which are able to form hairpin loop structures. They do not, however, possess the stretch of T residues seen in *E. coli* (Binnie *et al.*, 1997; Molnar, 1994; Bibb *et al.*, 1985b).

1.1.2.3: Translational signals.

The Shine-Dalgarno sequences of streptomycete genes tend to lie much farther away from the initiation codon than seen in either *E. coli* or *Bacillus* species (Binnie *et al.*, 1997; Piret & Demain, 1988). This is not the only difference seen when compared to these other systems. McLaughlin *et al.* (1981) proposed that the ribosome binding sites of Gram positive bacterial mRNAs are typically able to form strong complexes with the 3' end of 16s rRNA. In fact, in *B. subtilis* rather extensive complementarity between the 3' end of the 16s rRNA and the Shine-Dalgarno region is required for translational initiation (Moran *et al.*, 1982). In contrast, in *E. coli* a rather poor Shine-Dalgarno region is tolerated (Davis *et al.*, 1990). The sequence of the 3' end of *S. lividans* 16s rRNA has been determined and is identical to that of *B. subtilis* (Bibb *et al.*, 1985b). However, in direct contrast with the hypothesis put forward by McLaughlin *et al.* (1981), streptomycete ribosomes do not require a high degree of complementarity in order to initiate translation. This is underlined by the ability of *Streptomyces* to express *E. coli* genes, such as *amp*C, which have relatively poor Shine-Dalgarno sequences, and by the fact that a wide range of ribosome binding sites have been found in *Streptomyces* (Strohl, 1992).

Interestingly, a significant number of streptomycete genes are apparently translated from leaderless transcripts, where the start point of transcription coincides with the first base of the translation initiation codon (Binnie *et al.*, 1997). Such genes include *aph* and *erm*E (Chang & Chang, 1988; Schmitt-John & Engels, 1992).

1.1.3: Regulation of streptomycete gene expression.

In line with their complex life cycles, members of the genus *Streptomyces* have been found to possess extraordinarily intricate systems for the regulation of gene expression. This complexity far outstrips that previously observed during research in *E. coli* and *B. subtilis* (Saier, 1995; Titgemeyer, 1995, Delic *et al.*, 1992). Much effort has been concentrated into understanding the genetic switches responsible for the onset of

secondary metabolism and morphological differentiation. This work has identified a network of interconnected mechanisms for controlling gene expression. The evidence suggests regulatory links exist between catabolite repression, the sensing of environmental stimuli, cellular differentiation and control of secondary metabolism. Some of the control mechanisms at work in the streptomycete system are now beginning to be elucidated. It is likely that a greater number are yet to be discovered and investigated. Current understanding of several systems, thought to play central roles, is reviewed below.

The vast majority of research into the regulation of gene expression has been conducted in *S. coelicolor* A3(2). Although some exceptions will be seen, current evidence indicates that the majority of control systems observed to function in this species will also operate in other members of the genus. The intensive research currently being undertaken to understand this particular organism will, therefore, be expected to provide valuable insights into control of *Streptomyces* gene expression.

1.1.3.1: Role of RNA polymerase sigma (σ) factors.

The core RNA polymerase of bacteria is able to polymerise ribonucleoside triphosphates into RNA. However, recognition of promoter sequences requires the presence of the sigma (σ) subunit (Buttner, 1989). This subunit confers the ability to specifically bind to a promoter and initiate transcription. Cells which possess the ability to produce different σ factors, with differing promoter specificities, have a powerful mechanism for controlling the expression of discrete gene sets. Such systems have been identified and studied in both *E. coli* and *Bacillus* species where they have been shown to act as control mechanisms for such diverse processes as chemotaxis and sporulation (Buttner, 1989).

To date, several different σ factors have been found within streptomycete species (Table 1.1). A number are homologues of σ subunits previously identified in other bacteria (Kang *et al.*, 1997). However, although similar in structure, it seems that the functions of these peptides have not been strictly maintained within the different species (Chater, 1998). It is generally accepted that a significant number of σ factors have yet to be identified and studied. Those to which specific functions have been

assigned have been found to be involved in the control of key cellular processes (Table 1.1).

The wide variation observed within streptomycete promoter sequences, alongside the complexity of the morphological and metabolic systems seen within the genus, would tend to suggest that alternative σ factors may play a far more central role in the regulation and co-ordination of gene expression than seen in other bacterial species.

Table 1.1: RNA polymerase σ factors identified in *Streptomyces* species.

Sigma Factor (gene)	Function (known/postulated)	Reference
σ^{WhiG} (*whiG*)	Initiation of sporulation Transport	Chater *et al.*, 1989 Tan *et al.*, 1998
σ^{E} (*sigE*)	Regulation of extracytoplasmic functions	Bourn & Babb, 1995
σ^{F} (*sigF*)	Spore wall thickening / maturation	Potuckova *et al.*, 1995
σ^{HrdA} (*hrdA*)	Unknown function	Buttner , 1989
σ^{HrdB} (*hrdB*)	Transcription housekeeping genes Recognition of '*E. coli* like' promoters. Essential for growth	Buttner, 1989. Labes *et al.*, 1997
σ^{HrdC} (*hrdC*)	Control of morphology	Buttner , 1989
σ^{HrdD} (*hrdD*)	Production of pigmented antibiotics Stationary phase gene expression	Fujii *et al.*, 1996 Kang *et al.*, 1997
σ^{52}	Stationary phase gene expression	Kang *et al.*, 1997
σ^{31}	Stationary phase gene expression	Kang *et al.*, 1997

1.1.3.2: Regulation of responses to the nutritional environment.

A number of different types of regulatory phenomena, such as catabolite repression, inducer exclusion and inducer expulsion, have been described and characterised in enterics and low GC Gram positive bacteria. Although studies have illustrated that

streptomycetes can use a wide variety of sugars for growth, knowledge concerning specific pathways of carbohydrate metabolism and their regulation is still extremely limited. The phosphoenolpyruvate sugar phosphotransferase system (PTS) has long been known to be central to sugar transport and catabolic regulation in many bacteria (Davis *et al.*, 1990). Until recently it was unclear whether a similar system was in operation within the streptomycetes. Many groups reported evidence that the genus lacked a PTS system (Angell *et al.*, 1992; Garcia-Dominguez *et al.*, 1989). However, in 1995 Titgemeyer *et al.* reported the existence of PEP-dependent PTS systems in *S. lividans*, *S. coelicolor* and *S. griseofuscus*. The presence of this system means that, as in other bacterial regulation systems, one or more of the PTS proteins may interact directly with other enzymes, or phosphorylate target proteins, to control metabolic activity (Titgemeyer *et al.*, 1995). Characterisation of the PTS proteins so far identified, indicates that several of the components in the streptomycete system are dissimilar to their analogues in the enteric PTS system (Titgemeyer *et al.*, 1995). This may suggest that the sugar transport systems of actinomycetes may play a wider role in regulation than seen in other bacterial systems, e.g. control of differentiation and antibiotic production. Classical ATP-dependent protein kinase activities are also detected within *Streptomyces* cells (Vanek *et al.*, 1995). This system provides an additional mechanism for the regulation of gene expression in response to levels of intracellular metabolites.

1.1.3.3: Two-component regulatory systems.

Another type of regulatory system, which is widespread amongst eubacteria, is the two-component regulatory system. Such systems provide a mechanism to allow cells to sense and respond to a wide variety of environmental stimuli (Vanek *et al.*, 1995). A membrane bound sensor protein autophosphorylates in response to a particular stimuli. This protein then phosphorylates a response regulator protein, changing its conformation. This response regulator typically interacts with a target promoter to modulate gene expression. At least 5 such systems have been identified within the genus *Streptomyces* (Chang *et al.*, 1996). These systems have been reviewed by Beppu (1995) and Chater and Bibb (1996).

1.1.3.4: Diffusible factors.

A number of diffusible self-regulatory factors (γ-butyrolactones) are produced and secreted by *Streptomyces* species (Chater, 1998). These molecules operate in an analogous way to eukaryotic hormones. Following production they disperse in the cells' local environment, acting as triggers for differentiation for other streptomycete cells in the area (Beppu, 1995; Schneider *et al.*, 1996). The best understood of these factors is A-factor. This molecule is produced by *S. griseus* and is required for sporulation and antibiotic production within the species (Schneider *et al.*, 1996). A-factor is thought to diffuse across the cell membrane and then complex with a specific cytoplasmic receptor protein. The action of binding this receptor protein acts to derepress a key gene, triggering the onset of secondary metabolism and aerial mycelium formation in *S. griseus* cells (Onaka *et al.*, 1995). Although it is thought that this family of extracellular signal molecules are vitally important within the streptomycetes, the functions of many of the molecules identified have yet to be established. Further research will determine how large a role these molecules actually play in regulating cellular behaviour.

1.1.3.5: Role of repressor and inducer proteins.

DNA binding proteins play central roles in regulation within bacterial cells. One of the largest, most diverse, classes of DNA binding proteins are transcription factors that act to regulate gene expression (Pabo & Sauer, 1992). As with other systems, such inducer and repressor proteins are very important players in the regulation of streptomycete gene transcription. Transcription of the agarase gene, *dagA*, of *S. coelicolor* A3(2) is thought to be regulated by the presence of a specific repressor protein whose activity can be inhibited by partly hydrolysed agar (Sevin-Gonzalez *et al.*, 1994). Delic *et al.* (1992) found evidence that a repressor protein acts to regulate expression of the chitinase gene, *chi*63, of *S. plicatus*. They postulate that the protein is inactivated in the presence of chitin. A repressor protein is also thought to play a role in regulating expression from the actinorhodin biosynthetic gene cluster in *S. coelicolor* A3(2) (Caballero *et al.*, 1991). Martin *et al.* (1989b) postulate that inducer or repressor proteins could be crucial for the control of transcription from phosphate regulated genes. A great number of additional regulatory proteins, similar to those detailed

above, are sure to be described as more is learnt about the many mechanisms which are acting to control gene expression within these organisms.

1.1.4: Protein secretion by *Streptomyces.*

1.1.4.1: Signal peptides.

As widely documented in the secretion systems of other prokaryotic and eukaryotic organisms, most extracellular streptomycete proteins are synthesised as intracellular precursors carrying a signal sequence at their amino termini (Izard & Kendall, 1994; Freudl, 1992). This signal peptide acts to channel the polypeptide into the cell's secretory pathway and is removed during translocation (Gilbert *et al.*, 1995). No obvious consensus sequence can be seen in the *Streptomyces* signal sequences which have so far been studied (Lammertyn *et al.*, 1998). This mirrors the situation in the *Bacillus* and *E. coli* systems where a consensus sequence is also lacking (Pugsley, 1993).

In line with those seen in these species, *Streptomyces* signal peptides display the set of biochemical characteristics typical of these sequences, as defined by Von Heijne and Abrahmsen (1989). All possess a positively charged N terminus of variable length which is rich in Arg or Lys residues (Gilbert *et al.*, 1995). In *Streptomyces* the positive charge tends to be provided by Arg residues. Next follows a hydrophobic core with residues which have a marked preference to form helices. This region is usually 12 to 20 amino acids long in *Streptomyces* (Gilbert *et al.*, 1995). Lastly comes the C terminal region. This region frequently contains a helix breaking proline residue followed by a stretch of 5 to 10 amino acids which adopt a ß-turn configuration. The C terminal region usually ends with the sequence Ala-X-Ala just before the cleavage site of the signal peptides (Lammertyn & Anne, 1998). *Streptomyces* signal sequences conform very strongly to this '-1, -3' rule.

Overall the signal peptides of Gram positive bacteria tend to be considerably longer than those of Gram negatives, i.e. 30 AAs compared to 24 AAs, with an average length of 35 AAs, for streptomycete sequences (Gilbert *et al.*, 1995; Lammertyn & Anne, 1998). The N region also tends to be more highly charged than in Gram negative proteins (Freudl, 1992; Lammertyn & Anne, 1998).

In addition to the three regions discussed above a forth region has been identified which is present in exceptionally long signal peptides such as many found in *Streptomyces* (Park *et al.*, 1991; Lammertyn & Anne, 1998). This region has been designated the 'Arg cluster', and is located between the N terminal region and the hydrophobic region. Deletion of this cluster from several signal peptides, e.g. of the *Streptomyces* KSM-9 cellulase, results in secretion being almost completely abolished.

Over the last few years a number of studies have looked into the effects of different signal peptide mutations on the secretion of a variety of different proteins from streptomycete cells (reviewed by Lammertyn & Anne, 1998). These studies have shown that the alteration of amino acid residues can have a wide range of contradictory effects on levels of protein production and transcription. Fass and Engels (1996) observed that the total charge, and the position of positive charges, were important in determining levels of tendamistat secretion from *S. lividans*. However, they found that changes in the signal sequence had no effect on the levels of tendamistat mRNA produced by the cells. In contrast, Hale and Schottel (1996) found changes in the N terminal charge of the *S. scabies* esterase signal peptide produced significant reductions in both levels of protein secretion and transcription. The current evidence suggests that the important features of streptomycete signal sequences may vary depending on the particular signal peptide and protein being studied.

1.1.4.2: The secretion mechanism.

Of all the aspects of protein production in *Streptomyces*, probably the least is known about the actual secretion mechanisms which exist to move proteins out of the cells. In Gram negative bacteria the major export system is called the general secretory pathway (GSP) or Sec-dependent pathway (reviewed by Pugsley, 1993). It requires a minimum of six Sec proteins for targeting and translocation of secretory proteins across the plasma membrane. Proteins secreted using this pathway are characterised by the presence of a signal peptide. This sequence is believed to retard the folding of the preprotein and assist in directing the preprotein to the cytoplasmic membrane through interaction with Sec proteins. The signal peptide is cleaved by a signal peptidase during or shortly after the translocation process.

It seems almost certain that an analogous secretory pathway operates for the secretion of proteins from *Streptomyces* cells. Genes encoding homologues of the components that make up the Gram negative pathway have been recently identified (Table 1.2). It is therefore likely that the general mechanism of protein secretion described in *E. coli* is in operation within streptomycetes, despite the clear differences between cell wall structure. The ability of streptomycete cells to use signal peptides from *E. coli* proteins provides strong evidence in support of this theory (Morosoli *et al.*, 1997). Structural differences between *E. coli* Sec proteins and their streptomycete homologues may indicate that differences in the specificity and function of each protein are present.

Some proteins in *E. coli,* which lack signal sequences, are known to be secreted by Sec-independent mechanisms which involve use of dedicated transporters (Gilbert *et al.*, 1995). The contradictory nature of many of the results seen in studies looking into the effects of alterations in signal peptide structure would be consistent with the presence of an alternative pathway, or pathways, for secretion in *Streptomyces*. Considering the complexity of the regulatory and metabolic systems present in the genus it would be surprising if additional pathways for protein secretion were not eventually described.

Table 1.2: Homologues of *E. coli* Sec proteins identified in *Streptomyces* species.

Protein Homologue	**Function in *E. coli* Secretory Pathway (known/postulated)**	**Reference**
Sec A	ATPase	Blanco *et al.*, 1996
Sec E	Membrane protein involved in translocation	Miyake *et al.*, 1994
Sec Y	Membrane protein involved in translocation	Ostiguy *et al.*, 1996
Sec D	Membrane protein involved in release of secreted protein	Loriaux, 1995
Sec F	Membrane protein	Loriaux, 1995
Type 1 signal peptidase	Cleavage of signal peptide	Lammertyn *et al.*, 1998
GroES	Molecular chaperone	Marco *et al.*, 1992

1.2: History of Recombinant Protein Production from Streptomycetes

1.2.1: Reasons for using streptomycetes as host systems.

The *Streptomyces* system offers a range of potential benefits when compared to other proven bacterial hosts for recombinant protein expression, such as *E. coli* and *B. subtilis*. The vast majority of streptomycetes lack pathogenicity (Piret & Demain, 1988). This can be an important advantage when choosing an organism for the production of proteins for clinical use. It can also simplify, somewhat, the precautions which are required for growth on a large scale. Another benefit for the system is the extremely large body of understanding, mainly unpublished, which exists on the large scale fermentation and downstream processing of these organisms, thanks to their roles as prolific antibiotic producers. Such intimate knowledge about a particular organism can be invaluable when attempting to optimise production of a protein on a commercial scale. Although knowledge of the genetic system of *Streptomyces* currently lags behind that of *E. coli*, a wide range of techniques for genetic manipulation of the genus have been established (Hopwood *et al.*, 1985; Baltz, 1998). Unlike other bacterial hosts, these organisms also offer the possibility of prolonged expression through their active secondary metabolism.

The main benefit for use of *Streptomyces* as host cells is undoubtedly their prolific ability to express and fully secrete a range of proteins. Successful secretion of proteins with a size ranging from 8kDa (α-amylase inhibitor Haim II) to 130kDa (β-galactosidase) has been documented (Nagaso *et al.*, 1988; Eckhardt *et al.*, 1987). This method of production has several positive consequences. Full secretion of a protein to the culture supernatant typically presents a far simpler purification challenge than is seen for the traditional *E. coli* host system. No cell disruption stage is required to free the protein product from an intracellular location. As cell breakage is not required, contamination of the product with intracellular proteins is avoided. This means that a system where the product is fully secreted can often produce a high specific activity product. The downside of this strategy is that the protein of interest tends to be present in a large volume. Degradation of the protein at air/liquid interfaces within the fermenter can also be a problem, especially in extended fermentations. High level recombinant protein expression in *E. coli* often leads to the production of inclusion

bodies consisting of inactive, misfolded protein. Although the streptomycete system can not carry out the complex post-translational modifications required for activity of some eukaryotic proteins, it is capable of secreting many proteins in a native, active form, with the correct tertiary structure (Bender *et al.*, 1990a).

1.2.2: *Streptomyces* host strains.

Due to the number of diverse species and strains of *Streptomyces* there are a very wide range of possible host strains available (Goodfellow *et al.*, 1987). However, most of these are relatively unknown and uncharacterised and many have undesirable characteristics such as high extracellular protease levels. Consequentially most of the work conducted in *Streptomyces* has been done in host strains where a proven track record of successful recombinant protein production exists. Currently the strain most extensively used for the expression of cloned genes is *Streptomyces lividans* 66 (strain 1326) and its genetically marked TK derivatives (Gilbert *et al.*, 1995).

This strain has several advantageous characteristics which favour its use as a host. The genome is well characterised. It has a reduced restriction modification barrier and secretes relatively small amounts of proteases (Engels & Koller, 1992). The strain grows and sporulates well on solid media. It also grows well in liquid media (without sporulation) and is then easily converted to protoplasts which regenerate readily and can be transformed very effectively by a wide variety of plasmids (Hopwood *et al.*, 1985).

Almost all studies involving expression of cloned genes in *Streptomyces* have used *S. lividans* 66 or a derivative as their production host. Few reports of comparisons between *S. lividans* 66 and other *Streptomyces* strains exist. However these reports do provide fairly strong evidence that use of other strains should be investigated. Hoshiko *et al.* (1987) observed a 20-fold increase in amylase activity when the alpha-amylase gene was cloned using a multicopy vector in the original strain (*S. hygroscopicus* AA69-4) compared with when *S. lividans* 1326 was used as the host. Proteolytic degradation did not seem to be the cause of the lower yields and it was suggested that the original host may have a more efficient mechanism for the secretion of this enzyme. Large variability in the production capacity of different strains for the same recombinant protein has been reported, even when the strains have been transformed

with the same genetic construct (Inokoshi *et al.*, 1993). These differences may be caused by as yet uncharacterised factors and point to the fact that various host strains should be considered when optimising the production of a recombinant protein from this genus.

1.2.3: *Streptomyces* cloning vector systems.

The vast majority of *Streptomyces* cloning vectors are based on the replication determinant from plasmid pIJ101 (Fig. 1.1). This is a naturally occurring plasmid which was isolated by Kieser *et al.* in 1982 from *S. lividans* ISP5434. A wide variety of constructs have been developed from this plasmid, most notably plasmid pIJ702 (Fig. 1.2)(Katz *et al.*, 1983). Derivatives of this vector, which carries the *tsr* gene that confers resistance to the antibiotic thiostrepton and the tyrosinase gene (*mel*) of the melanin biosynthesis pathway, have been used extensively for cloning both prokaryotic and eukaryotic proteins in *S. lividans* (Gilbert *et al.*, 1995). More recently *Streptomyces* / *E. coli* shuttle vectors have been developed from these plasmids, both simplifying and speeding up genetic manipulation (Dyson & Evans, 1996). pIJ702 derived vectors have copy numbers which range between 40 and 500 (Brasch & Cohen, 1995; Kieser *et al.*, 1982). The maximal copy number has been shown to fluctuate throughout growth, with numbers being maximal after exponential growth (Wrigley-Jones *et al.*, 1993).

This family of plasmids have been shown to replicate using asymmetric rolling circle replication (Kataoka *et al.*, 1994; Suzuki *et al.*, 1997a). Such a mechanism requires a plus (+) origin, a minus (-) origin and a replication protein (Rep). It is thought to involve the following sequence of events (Novick, 1989; Gruss & Ehrlich, 1989; te Riele *et al.*, 1986a). Firstly Rep recognises the + origin and nicks the DNA initiating replication. This triggers displacement of the original plus strand and polymerisation of a new plus strand by 3' extension. Strand displacement continues with bound Rep protein remaining at the + origin. Eventually the minus strand origin, which is carried on the plus strand, is exposed. This sequence forms a hairpin which is used to initiate lagging strand synthesis, primed by host cell factors. These factors convert the displaced plus strand from ss to dsDNA. The Rep protein ligates the ends of the displaced plus strand to form a complete circle. The nicks which initiate and terminate a round of plus strand synthesis occur at the same site. Many streptomycete plasmids,

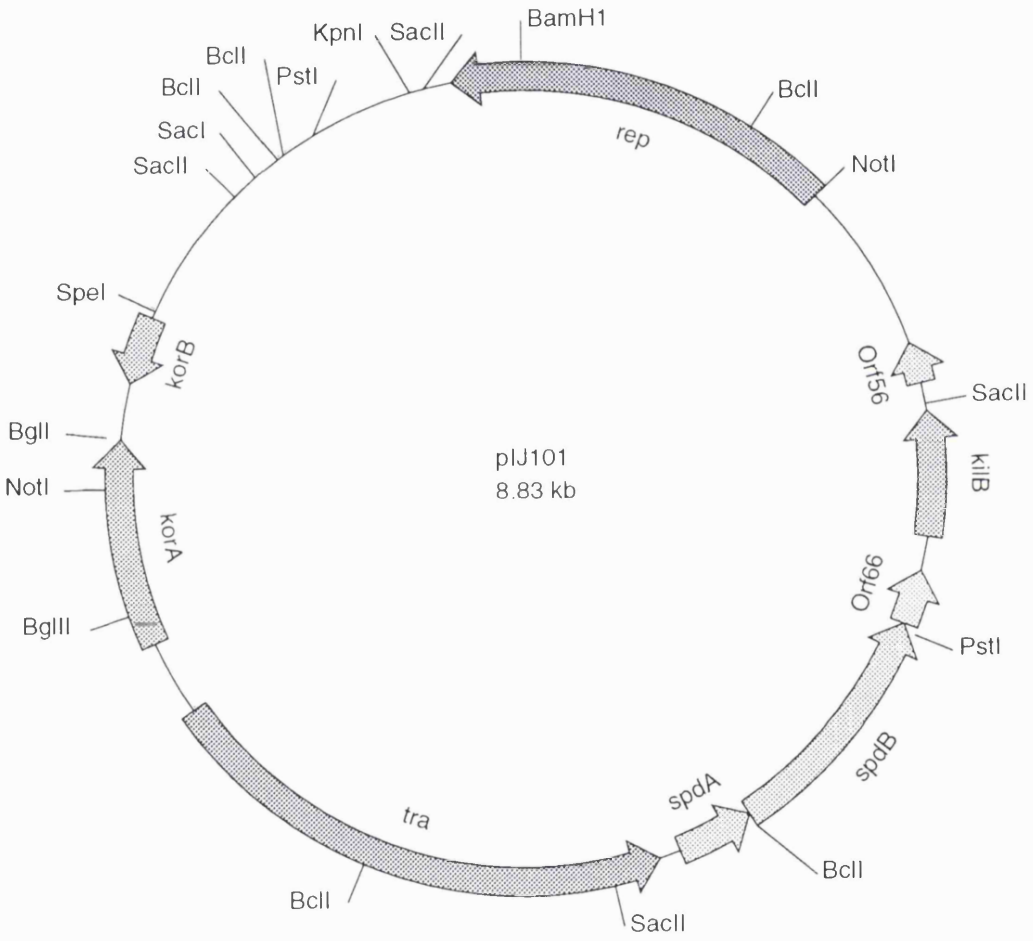


Figure 1.1: Plasmid pIJ101 (Kieser *et al.*, 1982).

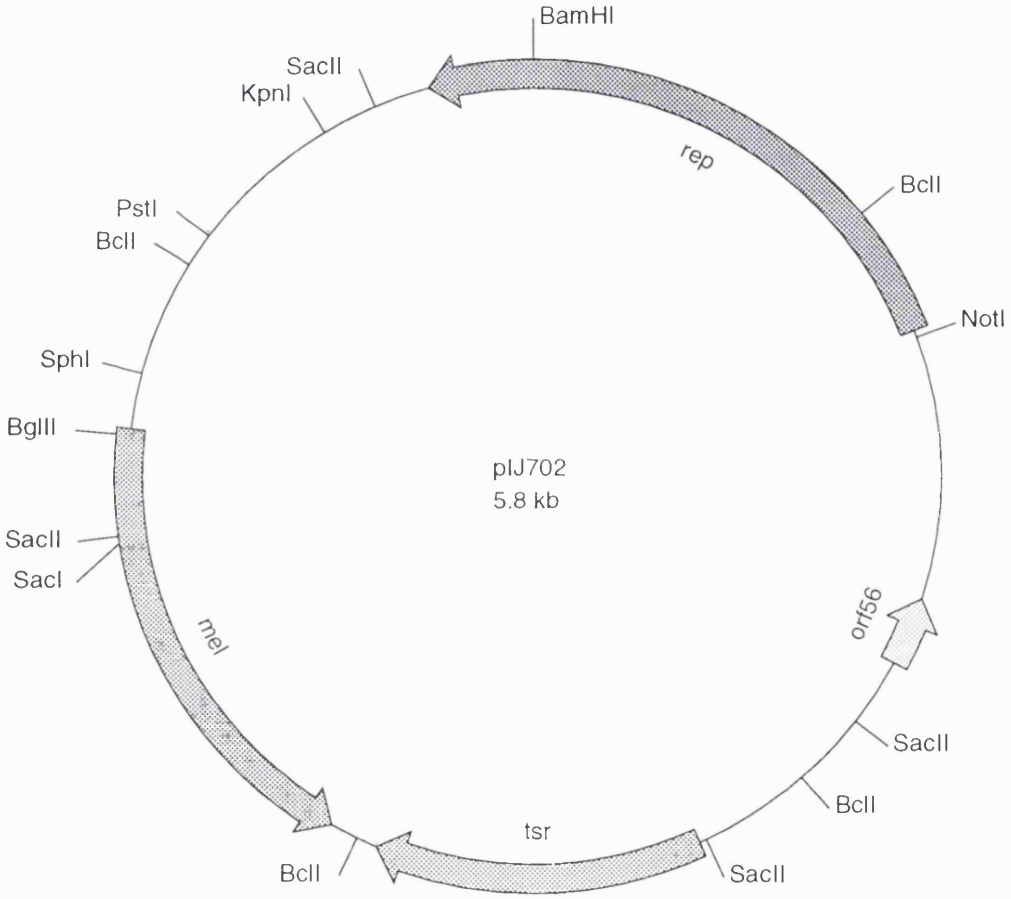


Figure 1.2: Plasmid pIJ702 (Katz *et al.*, 1983).

including pIJ702, lack the minus replication origin which is present on their parent plasmid pIJ101 (Zaman *et al.*, 1993a). Such plasmids are capable of replication but the process tends to be inefficient, with conversion of the lagging strand relying on sequences which can act as weak minus origins (Kataoka *et al.*, 1994).

1.2.4: Overview of recombinant protein expression by streptomycetes.

1.2.4.1: Recombinant proteins successfully produced by *Streptomyces*.

A large number of recombinant proteins have been expressed successfully by streptomycetes since the early 1980s (reviewed by Binnie *et al.*, 1997 & Gilbert *et al.*, 1995). A great number have been homologous proteins, which originated in other *Streptomyces* species. Examples of these proteins are listed in Table 1.3. Generally expression and secretion of such proteins has been driven by genetic signals and signal sequences native to the particular gene.

Many heterologous genes have also been expressed by streptomycete host cells. Examples are listed in Table 1.4. These genes have originated in a very wide range of different organisms, both prokaryotic and eukaryotic. Secretion of the protein of interest was not achieved for many heterologous proteins expressed in the early 1980's. However, this early research did show the potential of the system for protein expression. In 1984, using *S. lividans* as a host, Gray *et al.* expressed bovine growth hormone (Gray *et al.*, 1984). This was the first eukaryotic protein to be produced in *Streptomyces*. Although the protein remained intracellular, it was present in an active mature form at levels of up to three times that achievable using the *E. coli* systems of the time. Fusion of heterologous genes with signal peptides from streptomycete proteins naturally secreted at very high levels, e.g. the tendamistat and *Streptomyces* subtilisin inhibitor (SSI) signal peptides, has proved to be a successful strategy for the secretion of gene products (Bender *et al.*, 1990b; Taguchi *et al.*, 1992). In 1993, Fornwald *et al.* reported successful secretory production of the human T cell receptor CD_4 and its derivatives. Using an *S. lividans* production system they secreted correctly folded, disulphide bonded proteins at high levels (50mg/l and above). These represent much higher yields than those achieved in equivalent *E. coli* and animal cell culture.

Table 1.3: Examples of homologous proteins expressed in *S. lividans*.

Protein	Gene	Origin	Reference
phosphoenolpyruvate carboxylase	*ppc*	*S. coelicolor*	Bramwell *et al.*, 1993
esterase	*est*	*S. scabies*	Hale *et al.*, 1992
tendamistat	*ai*	*S. tendae*	Schmitt-John & Engels, 1992
α-amylase	*amy*	*S. griseus*	Garcia-Gonzalez *et al.*, 1991
subtilisin inhibitor (SSI)	*ssi*	*S. albogriseolus*	Obata *et al.*, 1989
α-amylase	*aml*	*S. venezuelae*	Virolle *et al.*, 1988
α-amylase	*aml*	*S. limosus*	Virolle & Bibb, 1988
α-amylase	*amy*	*S. hygroscopicus*	Hoshiko *et al.*, 1987
β-lactamase	*bla*	*S. albus G*	Dehottay *et al.*, 1986
cholesterol oxidase	*cho*	*Streptomyces* SA-COO	Murooka *et al.*, 1986
agarase	*dag*A	*S. coelicolor*	Kendall & Cullum, 1984
tyrosinase	*mel*C	*S. antibioticus*	Katz *et al.*, 1983

Table 1.4: Examples of heterologous proteins expressed in *S. lividans*.

Protein	Origin	Promoter	Signal Peptide	Reference
TNF	mouse	VSI	*aml*	Lammertyn *et al.*, 1998
TNF	mouse	VSI	VSI	Lammertyn *et al.*, 1997
transglutaminase	*Streptoverticillium*	*mel*	TGase	Washizu *et al.*, 1994
sCD_4 receptor	human	STI-II	STI-II	Fornwald *et al.*, 1993
mAb FV domain	mouse	SSI	SSI	Ueda *et al.*, 1993b
aphidaecin	honeybee	SSI	SSI	Taguchi *et al.*, 1992
interferon-α	mouse	*aml*	*aml*	Anne *et al.*, 1991
β-lactamase	*E. coli*	*dac*	*dac*	Piron-Fraipont *et al.*, 1990
hirudin	leech	tendam./*mel*	tendam	Bender *et al.*, 1990b
phosphotriesterase	*Flavobacterium*	*opd/mel*	*opd*	Steiert *et al.*, 1989
peptide pheromone	*Enterococcus*	SSI/*mel*	SSI	Taguchi *et al.*, 1989b
proinsulin	monkey	tendam./*mel*	tendam	Koller *et al.*, 1989
interferon α-2	human	*aph*	none	Pulido *et al.*, 1986
interleukin-2	human	*E. coli* consensus	none	Munoz *et al.*, 1985
growth hormone	bovine	*aph*	none	Gray *et al.*, 1984

1.2.4.2: Genetic signals used for recombinant protein production.

1.2.4.2.1: Promoters.

In order for the products of cloned genes to be synthesised in large amounts, it is crucial that transcription of the gene is optimised as far as is possible. High production yields have been obtained for many streptomycete genes cloned in *S. lividans* under the control of their own promoters (Kendall & Cullum, 1984; Katz *et al.*, 1983). Most of these studies have not considered how efficient a particular promoter was in driving transcription. In many cases gene dosage effects are credited with raising the levels of the products concerned (Gilbert *et al.*, 1995). There are very few reports describing optimisation of the expression of homologous cloned genes by modification of the promoter (Schmitt-John & Engels, 1992; Vigal *et al.*, 1991b). In several cases levels of expression have been raised almost by chance. One example of this is the finding that if additional promoters are located close and in tandem with the promoter region of the gene which is being expressed, significantly increased transcription can occur. In 1989 Taguchi *et al.* observed that, in addition to transcription from the promoter of the gene they were trying to express, read-through from the *mel* promoter in pIJ702 was increasing transcription (Taguchi *et al.*, 1989b). Such effects have been repeated several times (Bender *et al.*, 1990b; Koller & Riess, 1989).

Both Gram positive and Gram negative promoters have been used to express heterologous proteins in *S. lividans* (Lammertyn *et al.*, 1998; Munoz *et al.*, 1985). However, most groups have achieved expression by fusing structural genes with streptomycete promoters known to control the expression of genes encoding proteins which are naturally produced in great abundance, e.g. the *S. lividans* ß-galactosidase gene (Lichenstein *et al.*, 1988), the *S. fradiae* aminoglycoside phosphotransferase (*aph*) gene (Pulido *et al.*, 1986), the *S. albogriseolus* subtilisin inhibitor (SSI) gene (Ueda *et al.*, 1993b), the *S. erythraeus* erythromycin resistance (*erm*E) gene (Chang & Chang, 1988) and the *S. tendae* tendamistat gene (Koller *et al.*, 1989). In some cases this approach has worked very well, in others it has not. Only a very few studies have actually compared the efficiency of various promoters to direct the expression of a particular heterologous gene (Chang & Chang, 1988; Brawner *et al.*, 1990).

From these studies, no one particular promoter seems guarantee high levels of expression. Different genes are expressed to different levels under the control of the same promoter (Bender *et al.*, 1990a; Bender *et al.*, 1990b; Koller *et al.*, 1989). It is clear that promoters from genes encoding proteins naturally formed in large amounts do not necessarily express heterologous proteins at high levels (Brawner *et al.*, 1990; Pulido *et al.*, 1986). In some of these cases low production yields may be caused by low stability of the mRNA obtained from the transcription of fusions of streptomycete promoters and foreign genes (Gilbert *et al.*, 1995).

1.2.4.2.2: Transcription terminators.

The presence of a terminator downstream of a cloned gene may help to raise overexpression by increasing the stability of mRNA produced. Evidence that this does occur has come from several studies. In 1987, Pulido and Jimenez reported that the addition of an *aph* transcriptional terminator downstream of the human interferon - α2 gene cloned into *S. lividans* increased the stability of the specific mRNA and led to a 4-fold increase in protein production (Pulido & Jimenez, 1987). However, other studies have found differently. Taguchi *et al.* (1993) observed a 3.5-fold increase in the production of SSI when they deleted the first of two putative terminators present downstream of the SSI gene.

1.2.4.3: Future prospects for improving levels of cloned gene transcription.

What is clear from the literature is that a great deal more research must be done in all aspects of this area before the routine optimisation of transcription of both homologous and foreign genes will be possible. Where data is available the rate of transcription of a gene is directly proportional to the rate of secretion of the product (Piret & Demain, 1988; Vigal *et al.*, 1991a). This strongly suggests that the bottleneck of secretory expression is at the level of transcription. In order to alleviate this bottleneck vastly more efficient expression cassettes must be designed. Further comparisons of the performance of certain promoters in expressing various genes and mutagenesis studies of promoter regions will undoubtedly be the first steps towards this goal. Obtaining a greater understanding of the regulatory sequences used to control transcription within the streptomycete system will also be of importance. As more understanding of sigma

factor and other regulatory cascades is gathered, it can be envisioned that particular promoters will be used to gain expression at a particular time or under a certain condition. Martin *et al.* (1989b) cloned a small gene which coded for a small, 15 kDa protein called Saf. Streptomycete cells transformed with this gene are found to overproduce many enzymes. Expression of *saf* in cells has been observed to increase the production of several secretory proteins in *S. lividans* by 1.5 to 4-fold (Molnar, 1994). Utilisation of extra and intracellular regulatory signals such as Saf and A-factor (Section 1.1.3.4) may provide another route for increasing gene expression.

1.2.4.4: Secretion of recombinant proteins.

The streptomycete system is proving to be particularly useful for the secretory production of a wide range of foreign proteins (reviewed by Molnar, 1994, Gilbert *et al.*, 1995 & Binnie *et al.*, 1997). In contrast to *E. coli* or *B. subtilis,* the secretion machinery does not seem severely biased against eukaryotic proteins, which tend to be secreted in amounts similar to prokaryotic proteins expressed in the same system (Molnar, 1994).

As previously stated (Section 1.2.4.1), many of the heterologous proteins which have been produced from *Streptomyces* have been produced as fusion proteins. Many reports have indicated that fusion of the gene of interest with a signal sequence of a protein normally secreted in large amounts by *Streptomyces* does not guarantee efficient secretion and accurate processing (Bender *et al.*, 1990a; Morosoli *et al.*, 1997 & Sathyamoorthy & Speedie, 1995). For reasons which are still not understood, some signal peptides are more efficient than others at directing the secretion of fusion proteins (Chang & Chang, 1988; Page *et al.*, 1996). A large body of research is currently underway aimed at gaining a greater understanding of the important characteristics for secretion and processing of proteins within the streptomycete system. Current evidence suggests that the modulation of signal peptides may be a valuable tool for increasing the potential of the streptomycete host system. Several signal peptide mutations have been identified which have favoured the kinetics of protein secretion (Vigal *et al.*, 1991b; Lammertyn & Anne, 1998). Strong evidence also exists that interactions between signal peptides and mature protein sequences are important in determining the efficiency of secretion (Lichenstein *et al.*, 1988; Pugsley, 1993).

Obtaining a detailed understanding of such effects is likely to be key in allowing the development of generic systems which will allow the routine high level expression and secretion of homologous and heterologous proteins by *Streptomyces*.

1.2.4.5: Barriers for high level recombinant protein production.

Experience over the last decade has suggested that GC bias does not prevent expression of genes from less biased organisms. However, there is good evidence that in some cases it can have a strong influence on expression. Ueda *et al.* modified various codons in the *Streptomyces* subtilisin inhibitor (SSI) gene (Ueda *et al.*, 1993a). The replacement of two frequent Leu codons by rare TTA Leu codons resulted in a 40% decrease in SSI production by *S. lividans*. Leskiw *et al.* (1991) found that introduction of a TTA codon in a cloned heterologous gene dramatically reduced its expression in *S. lividans*. Such evidence strongly suggests that rare codons in cloned genes might have to be replaced by codons more commonly used in *Streptomyces* to obtain efficient protein production.

S. lividans was chosen as a good host strain partly because of its relatively low levels of extra and intracellular proteases (Section 1.2.2). Despite this, levels of proteases which can cause significant degradation of a potentially valuable secreted recombinant product are sometimes present. Fornwald *et al.* (1993) reported that proteolysis limited the accumulation of soluble derivatives of human T cell receptor CD_4. In initial experiments they found a decrease in proteins present after prolonged growth. A serine protease was thought to be responsible. Several studies have been undertaken to further define the protease complement of *S. lividans* (Aphale & Strohl, 1993; Aretz *et al.*, 1989). A variety of protease types have been observed to be produced under a range of differing environmental conditions. Bascaran *et al.* (1990) found that extracellular protease production by *S. clavuligerous* tended to be triggered under sub-optimal conditions and in the presence of amino acids. Ammonium was found to interfere with protease formation whenever it was present in the medium. Nutritional effects are also likely to govern the production of proteases by *S. lividans*. There are obviously a very wide spectrum of variables involved in the proteolytic degradation of recombinant products. It is likely to be some time before it is possible to accurately tailor a medium to give the most favourable protease complement for a certain recombinant protein.

Until we have a greater understanding of the mechanisms involved, finding the best medium conditions for expression will be partly trial and error. Ultimately, a combination of nutrient optimisation with protease deficient strains may be required to increase the stability of protease sensitive products.

Currently the most significant barrier to the more widespread use of *Streptomyces* strains as hosts for recombinant protein production is our lack of knowledge of the intricacies of the system. At the present time there are few generalisations as to what will consistently work to allow optimal production of a chosen protein. When building an expression system it is necessary to try a range of different promoters, signal peptides and medium compositions to gain high level expression and secretion. It is difficult to predict whether a particular combination of factors will, or will not, combine to produce an effective system. A greater understanding of the mechanisms in operation must be obtained so that high level production from a cloned gene can become less a matter of chance and more of a matter of design.

1.3: Aim

The central aim of this work was to gain further understanding as to what effects the production of a plasmid-borne gene product in *S. lividans* through optimising the level of secretory expression of a model homologous protein. It was hoped both positive and negative influences would be identified through this research. The effect of inclusion of the pIJ101 minus origin (*sti*) on levels of recombinant protein expression was investigated alongside the effect of variation in medium and cultural conditions. Although a large part of this work was done in shake flasks, a significant number of large scale fermentations were conducted so that the effect of scale up on the expression system could be quantified and analysed.

Many authors have stated their belief that the streptomycete system can become a viable alternative to the *E. coli* system for the routine expression of cloned gene products. Despite this, very few studies have directly compared the two systems for expression of an identical protein. An even lower number have conducted a comparison based on the levels of expression and recovery which could be achieved from the host systems on a larger, more industrially relevant, pilot plant scale. Another aim of this research was to conduct such a comparison.

1.3.1: *S. thermoviolaceus* α-amylase as a model protein.

The α-amylase from *Streptomyces thermoviolaceus* CUB74 was chosen as the model protein for this work (Bahri & Ward, 1990b). This is one of the smallest of the streptomycete α-amylases so far identified, at 49.2 kDa (460 amino acids) (Bahri & Ward, 1993). It is a thermostable protein with an optimum temperature of approximately 50°C. A system for the periplasmic expression of this protein in *E. coli* is well developed and characterised (French, 1993; Pierce, 1996). Large scale fermentation and recovery operations have been optimised for this host system (Pierce, 1996). Vectors have already been developed for the expression of this protein by *S. lividans* (Bahri, 1990). However, only very low levels of protein expression and secretion have been obtained (Section 3.2)(French, 1993).

The α-amylase is a highly suitable model protein. In addition to being easy and rapid to assay in solution, its production by cells growing on solid, starch containing, media is also simple to detect through the presence of visible zones of starch hydrolysis. As a thermostable protein it is possible to assay for this α-amylase at 50°C, therefore reducing the chance of interference from amylases naturally produced by host organisms. Good levels of expression of several other homologous α-amylase genes have been achieved when cloned into *S. lividans* or *S. coelicolor*, i.e. *S. venezuelae aml* (Virolle *et al.*, 1988), *S. limosus aml* (Virolle & Bibb, 1988), *S. griseus amy* (Martin *et al.*, 1989a), *S. hygroscopicus amy* (Hoshiko *et al.*, 1987), *Streptomyces* sp. WL6 *amy* (Chen *et al.*, 1995). Several of these gene sequences possess a very high level of similarity with the *S. thermoviolaceus amy* gene (Bahri & Ward, 1993; Mellouli *et al.*, 1998)

The *S. thermoviolaceus* α-amylase belongs to the endoglucanase group of starch degradation enzymes. The protein acts to specifically cleave the 1,4-α linkages of starch. The major products of the action of this α-amylase are maltotetraose, maltotriose and maltose; with maltose accumulating over extended periods while maltotetraose disappears (Bahri & Ward, 1990b). The mechanisms involved in the transcription of the *amy* gene were investigated by Bahri and Ward (1993). The α-amylase open reading frame was found to be 1380bp long with a GC content of 60%. It

was isolated on a 1.7kb *Bam*HI-*Sph*I genomic DNA fragment. Putative -10 and -35 regions of the promoter driving *amy* expression (P_{amy}) were identified (TACGGT and TTGACC respectively). These regions are identical to the -10 and -35 promoter regions found in the α-amylase genes of *S. limosus*, *S. venezuelae* and *S. griseus*. Although the promoter displays some consensus with the *E. coli* consensus sequence, studies have indicated that it is not functional in *E. coli* (Bahri & Ward, 1993). A perfect inverted repeat of 11bp is found downstream of the *amy* gene. This sequence acts as a transcription terminator. The protein possesses a signal sequence of 21 amino acids at its N terminus (Gilbert *et al.*, 1995). This is significantly shorter than would be expected for a streptomycete protein (Section 1.1.4.1) and is the shortest of the streptomycete α-amylase leader sequences so far identified. Despite its length the signal sequence displays all of the other characteristics associated with streptomycete signal peptides (Section 1.1.4.1). Bahri (1990) concluded that *S. lividans* was able to process the α-amylase protein correctly to the mature form found in the parent strain.

A series of direct and inverted repeats are found in the upstream region of the α-amylase gene (Bahri & Ward, 1993). Operator like structures have often been described as having such features (Virolle & Gagnat, 1994). Virolle and Gagnat (1994) identified several repeats involving the sequence CTTGCAG in the *S. limosus aml* promoter region. An inverted repeat including this sequence is also found in the *S. thermoviolaceus amy* gene sequence. In both genes the inverted repeats are located just downstream from the transcription start point.

When Virolle and Gagnat (1994) cloned the promoter region of the *S. limosus aml* gene on a high copy number plasmid and transformed a strain already carrying a vector for expression of *aml* they found an increase in α-amylase expression. They attributed this result to the binding and titration of negative regulatory proteins by the putative operator sequences. Upstream regions (-344 to -86) were found to play a role in determining levels of gene transcription (possibly due to interactions with other DNA binding proteins). However, the primary operator was proposed to be the inverted repeat sequence contained in the -2 to +29 region. They postulate that repression might involve repressor mediated formation of a DNA loop that blocks transcription initiation. The high level of homology found to exist between the *S. limosus aml* gene and the *S.*

thermoviolaceus amy gene (69.5%), along with the presence of the inverted repeat sequence in the *S. thermoviolaceus* gene, strongly suggest that regulation of transcription of both of these genes may share common mechanisms. The results of Virolle and Gagnat (1994) therefore indicate that repressor proteins may be implicated in controlling levels of expression of the *S. thermoviolaceus* α-amylase.

In *S. thermoviolaceus* production of the α-amylase can be induced and repressed by maltotriose and manitol respectively (Bahri, 1990). Under maximum induction the cells were found to produce 9 units of α-amylase per ml of supernatant after 18 hours incubation. In contrast, no evidence of induction of *amy* by inducers which acted in the wild type system could be found when the gene was cloned into pIJ702 in *S. lividans* (Bahri, 1990). Action of inducers can not, however, be ruled out by this evidence as their structures or levels and patterns of production may differ between the two organisms.

Chapter 2
Materials & Methods

2.1: Reagent and Antibiotic Sources

The majority of chemicals used in this study were obtained from BDH (Poole, Dorset) or Sigma (Poole, Dorset). They were of AnalaR grade or of the highest grade available. Casamino acids, yeast extract, Bacto-tryptone and Bacto-peptone were obtained from Difco (Detroit, USA). Malt extract broth, tryptone soya broth, nutrient broth and nutrient agar were purchased from Oxoid (Unipath Ltd., Basingstoke). Bacteriological agar was obtained from Gibco (Paisley, Scotland). Ampicillin and kanamycin were from Sigma (Poole, Dorset). Thiostrepton was a generous gift of S. J. Lucania, the Squibb Institute for Medical Research, NJ, USA.

2.2: Bacterial Strains

Streptomyces lividans 66, strain TK24, was used as the host strain for the streptomycete plasmids used in the study. *E. coli* JM107 was used as the host for the α-amylase expression vector pQR126. It was also used as the host for the streptomycete plasmids with shuttle vector capability during genetic manipulation procedures, to simplify and speed up the work. The genotypes of these organisms are listed in Table 2.1.

Table 2.1: Bacterial strains used in this study (described using standard phenotype nomenclature).

Bacterial Strain	Genotype	Reference
S. lividans 66 (TK24)	Str-6, SLP2-, SLP3-	Hopwood *et al.*, 1983
E. coli JM107	*end*A1 *gyr*A96 *thi*-1 *hsd*R17 *sup*E44 *rel*A1 Δ(*lac-pro*AB) (*F' tra*D36, *pro*AB, *lacI*^{q} ZΔM15)	Yanish-Perron *et al.*, 1985

The *Streptomyces* strains used in this study were maintained as spore suspensions in 20% v/v glycerol at -20°C. *E. coli* strains were maintained as cell suspensions under the

same conditions. Stock cultures of each strain were maintained at -70°C in 20% v/v glycerol.

2.3: Plasmids Used and Constructed During this Study

A list of the plasmids used and constructed during the course of this study are shown in Table 2.2.

Table 2.2: Plasmids used and constructed in this study. Abbreviations used: Ap^r - ampicillin resistance, Kn^r - kanamycin resistance, Cm^r - chloramphenicol resistance, Tc^r - tetracycline resistance, Ts^r - thiostrepton resistance, *amy* - *S. thermoviolaceus* α-amylase gene, *sti*$^+$ - site for second-strand initiation of plasmid replication in *Streptomyces*, *mel*$^+$ - melanin production via tyrosinase.

Plasmid	Genotype & Characteristics	Hosts Allowing Plasmid Replication		Promoter(s) driving *amy* expression in *S. lividans*	Reference
		S. lividans	*E. coli*		
pQR300	Ap^r *lacZα amy*		✓	-	Bahri (1990)
pQR126	Kn^r *lacZα amy*		✓	-	Ward (unpublished)
pIJ303	Ts^r *sti*$^+$	✓		-	Kieser *et al.* (1982)
pIJ702	Ts^r *mel*$^+$	✓		-	Katz *et al.* (1983)
pQR311	Ts^r *mel*$^+$ *amy*	✓		P_{amy}	Bahri (1990)
pQR318	Ts^r Cm^r Tc^r *amy*	✓	✓	P_{pBR325}	Bahri (1990)
pQR443a	Ts^r Ap^r *mel*$^+$ *sti*$^+$	✓	✓	-	Zaman (1991)
pQR620	Ts^r Ap^r *sti*$^+$ *amy*	✓	✓	P_{amy} P_{mel}	this study
pQR621	Ts^r Ap^r *sti*$^+$ *amy*	✓	✓	P_{amy}	this study

2.4: *S. lividans* Culture Conditions

2.4.1: Liquid media.

2.4.1.1: Minimal medium plus succinate (MMS).

This medium was based on the minimal liquid medium (NMMP) of Hopwood *et al.* (1985). Succinate (succinic acid, disodium salt) was used as a supplementary carbon source. The PEG 6000 contained in the original formulation was not included in this recipe as it was considered likely to interfere with any chromatographic downstream processing operations which might be carried out on the broth.

MMS g/l:

Succinate	5
$(NH_4)_2SO_4$	2
Casamino acids	5
$MgSO_4.7H_2O$	0.6
NaH_2PO_4	2.17
K_2HPO_4	3.6
Minor elements solution (see below)	1ml
Distilled water	to 1000ml.

Minor elements solution g/l:

$ZnSO_4.7H_2O$	1
$FeSO_4.7H_2O$	1
$MnCl_2.4H_2O$	1
$CaCl_2$.anhydrous	1

2.4.1.2: Tryptone soya broth (TSB).

Tryptone soya broth was made up in distilled water as directed by the manufacturers (30g/l).

2.4.1.3: Yeast extract / malt extract medium (YEME) (Hopwood *et al.*, 1985).

YEME g/l:

Yeast extract	3
Bacto-peptone	5
Malt extract	3
Glucose	10
Sucrose	340

After autoclaving, $MgCl_2.6H_2O$ (2.5M) was added at a concentration of 2ml/l.

When growing *S. lividans* cells for preparing protoplasts, sucrose was omitted from the medium and 25ml/l of 20% glycine was added.

2.4.1.4: Modified yeast extract / malt extract medium (MYEME) (Erpicum *et al.*, 1990).

This medium is a modification of that described in Section 2.4.1.3.

MYEME g/l:

Yeast extract	0.3
Bacto-tryptone	0.5
Malt extract	0.3
Glucose	1
Sucrose	34
NH_4Cl	50mM
BES buffer	100mM

The pH was adjusted to 7.4 with 4N NaOH prior to sterilisation.

2.4.1.5: *Streptomyces* minimal medium (SMM)(Madden *et al.*, 1996).

SMM g/l:

NaH_2PO_4	3
K_2HPO_4	1.5
$MgSO_4.7H_2O$	0.6
$FeSO_4.7H_2O$	0.01
$(NH_4)_2SO_4$	2
Carbon source	5

After sterilisation, the medium was supplemented with (per litre):

10ml 10% (w/v) $CaCl_2$
2ml trace elements solution (see below)
1ml vitamin solution (see below)

Trace elements solution (per litre): 40 mg $ZnCl_2$, 200mg $FeCl_3.6H_2O$, 10mg $CuCl_2.2H_2O$, 10mg $MnCl_2.4H_2O$, 10mg $Na_2B_4O_7.10H_2O$, 10mg $(NH_4)_6Mo_7O_{24}.4H_2O$.

Vitamin solution (per litre): 1g riboflavin, 1g nicotinamide, 100mg *p*-aminobenzoic acid, 500mg pyridoxine.HCl, 500mg thiamine.HCl, 200mg biotin.

2.4.1.6: Malt extract peptone medium + phosphate (MEPP) (Wrigley-Jones, 1990).

MEPP g/l:

Glycerol	20
Malt extract broth	10
Bacto-peptone	10
K_2HPO_4	1.74

Prior to autoclaving, the medium was made up to 1 litre with distilled water and the pH was adjusted to 7.

2.4.1.7: *Streptomyces* defined medium (SDM): (E. Daae, personal communication).

This medium was formulated to allow examination of growth of *S. lividans* in a fully defined medium.

SDM g/l:

NaH_2PO_4	2.17
K_2HPO_4	3.6
$MgSO_4.7H_2O$	0.6
$FeSO_4.7H_2O$	0.01
Carbon source	28mM
Nitrogen source	24mM

After sterilisation, the medium was supplemented with (per litre):

1ml 10% (w/v) $CaCl_2$
2ml trace elements solution (Section 2.4.1.5)
1ml vitamin solution (Section 2.4.1.5)

In this study glucose was used as a carbon source. Aspartate was used as a nitrogen source (Chapter 7).

2.4.2: Solid media.

2.4.2.1: ½ Strength tryptone soya broth (½ TSB) agar.

½ TSB agar g/l:

Tryptone soya broth	15
Bacteriological agar	20
Distilled water	to 1l

This agar was routinely used for growth and sporulation of *S. lividans*. 1% (w/v) insoluble potato starch was routinely added to allow the visualisation of zones of starch hydrolysis due to α-amylase activity.

2.4.2.2: R2 agar (Hopwood *et al.*, 1985).

This agar was made up in two parts:

R2/A g/l:

K_2SO_4	0.5
$MgCl_2.6H_2O$	20.2
$CaCl_2.2H_2O$	5.9
Glucose	20
Proline.	6
Casamino acids	0.2
Trace elements solution (Section 2.4.1.5) .	4ml
Bacteriological agar	44

This was dispensed into 500ml bottles, in 200ml aliquots, and autoclaved.

R2/B g/l:

TES Buffer (adjusted to pH 7.4 with NaOH) .	11.5
Yeast extract	10
Sucrose	203

This was also dispensed into 200ml amounts prior to autoclaving.

Following sterilisation the bottles were allowed to cool slightly. An aliquot of R2/B was poured into an aliquot of R2/A. 2ml of 1% (w/v) KH_2PO_4 was then added to each 400ml of R2. The agar was swirled to mix and the plates were poured.

2.4.3: Sterilisation conditions.

All media were sterilised by autoclaving at 121°C for 20 minutes.

2.4.4: Antibiotic addition.

Thiostrepton was routinely added to liquid and solid media used for the growth of *S. lividans* strains carrying plasmids with the thiostrepton resistance gene (*tsr*). A stock solution of thiostrepton (50mg/ml) was made by diluting the antibiotic in dimethylsulphoxide (DMSO). This was stored at -20°C. The antibiotic was added to a

final concentration of 5μg/ml in liquid media and 50μg/ml in solid media. No thiostrepton was added to 20l fermenter cultures.

2.4.5: Preparation and transformation of *Streptomyces* protoplasts.

Preparation of *S. lividans* protoplasts was carried out as described by Hopwood *et al.* (1985). *S. lividans* TK24 was grown in 25ml YEME (Section 2.4.1.3) in 500ml flasks for 36 to 40 hours with shaking. The mycelia were harvested at 4000rpm for 10 minutes, washed with 15ml 10.3% (w/v) sucrose and were then respun. The pellet was re-suspended in 4ml of protoplast (P) buffer (see below) containing 1mg/ml lysozyme and was incubated at 30°C for 1 hour with occasional swirling. Following this incubation, an additional 5ml of P buffer was added and the mixture was filtered through non-absorbent cotton wool. The protoplasts were sedimented gently by spinning at 3000rpm for 5 minutes. They were then re-suspended in 1ml of P buffer and dispensed into 50μl aliquots. These were stored at -70°C until required.

The procedure of Thompson *et al.* (1982) was used for the transformation of protoplasts. An aliquot of protoplasts was thawed out quickly in warm water. Up to 5μl of plasmid DNA was added to the tube. 200μl of transformation (T) buffer (see below) was then added and the liquid was mixed by pipetting up and down gently several times. Aliquots of this mixture were then plated out onto dried R2 agar plates (100μl was usually spread). If more than one transformation was being carried out, each was done individually.

After the plates had been spread they were incubated at 30°C for 15 to 24 hours. They were then overlaid with soft agar containing thiostrepton at 50μg/ml. Following several more days incubation at 30°C, resistant colonies were identified and subcultured onto ½TSB plates.

P buffer:

The following basal solution was made up, dispensed into 80ml aliquots and autoclaved:

Sucrose	103
K_2SO_4	0.25
$MgCl_2.6H_2O$	2.02
Trace element solution (Section 2.4.1.5)	2ml
Distilled water	to 800ml.

Before use, the following solutions were added into each aliquot:

KH_2PO_4 (0.5% w/v)	1ml
$CaCl_2.2H_2O$ (3.68% w/v)	10ml
TES buffer (5.73% adjusted to pH 7.2)	10ml

T buffer:

The following sterile solutions were mixed:

Sucrose (10.3% w/v)	25ml
Distilled water.	75ml
Trace element solution (Section 2.4.1.5)	0.2ml
K_2SO_4 (2.5% w/v)	1ml

9.3ml of this solution was removed to a fresh sterile tube and the following solutions were added:

$CaCl_2$ (5M)	0.2ml
Tris-maleic acid buffer*	0.5ml

1.5ml of this solution was then added into 0.5g of PEG 1000 which had been previously autoclaved. The resulting solution was mixed well prior to use.

(* A 1M solution of Tris was made up. This was adjusted to pH 8 with maleic acid.)

2.4.6: Preparation of *S. lividans* spores.

Half TSB agar plates were spread with a loop of *S. lividans* spores from a stock culture. The plates were incubated at 30°C until sporulation occurred. Approximately 3ml of sterile 20% (v/v) glycerol was added to the surface of each plate. The spores were

scraped from the surface of the colonies with a stiff loop. The solution was then removed from the plate into a sterile container where it was mixed with other spores of the same strain. When all of the spores had been harvested, the spore preparation was dispensed into 1ml aliquots. These were frozen at -20°C until required for use. A sufficient volume of spores from each strain was prepared to allow their use for the duration of the study.

2.4.7: Shake flask culture conditions.

Unless otherwise stated, all shake flask cultures were conducted in 2l flasks containing 500ml of medium. The flasks had 4 tall baffles (15cm) to ensure good aeration and mixing. A stainless steel spring (1.3cm diameter coil, 19sw gauge wire, Alliance Spring Co., London) was coiled at the bottom of each flask to help break up *S. lividans* cell pellets (Hopwood *et al.*, 1985). Following inoculation (Section 2.4.9.1), flasks were placed in an orbital incubator (New Brunswick Scientific Co. Ltd., NJ, USA) at 300rpm.

This incubator was initially located in a 30°C warm room, with the lid left open, for the duration of the experiments described in Chapters 3, 4 and 5. Although the temperature regulation of this warm room was generally good the temperature was occasionally found to vary by a few degrees, particularly during periods of warm weather. Following the discovery that supernatant α-amylase levels were significantly affected by incubation temperature (Chapter 6), the incubator was moved out of the warm room to another location in the hope that here the temperature regulation would be more reliable. Despite this action, the incubator temperature still tended to fluctuate from 30°C during warm weather. Occasions where such temperature fluctuations occurred are noted in the text.

Shake flask experiments were conducted to examine the effect of incubation temperature on α-amylase production from the *S. lividans* α-amylase expression systems and on tyrosinase production from *S. lividans* pIJ702 (Sections 6.3 & 6.5.1). These flasks were incubated in the same orbital incubator at 34°C. The incubator was found to maintain this higher temperature without the occurrence of significant fluctuations.

2.4.8: Growth of *S. lividans* in 20l fermenters.

S. lividans pQR620 was grown in 20l LH fermenters (LH, Reading) with a 13l working volume. 11.5l of medium was made up and sterilised *in situ* in each fermenter. Three shake flask cultures were then used as the inoculum for one fermentation (Section 2.4.9.2), bringing the volume in each vessel up to the working volume, i.e. 11.5% v/v inoculum. Dissolved oxygen tension (DOT) and pH were measured using Ingold electrodes (Ingold, Switzerland). The pH was maintained at 7 by automatic addition of 4N sodium hydroxide and 4N ortho-phosphoric acid unless otherwise stated. Automatic heating and cooling were used to maintain the temperature within the vessels. Antifoam (Polypropylene glycol) was added automatically when a probe sensed a build up of foam in the fermenter headspace. Each fermentation was aerated at a flow rate of 10 l/min (0.8vvm). Three Rushton turbine impellers were used to mix the broth. Each fermentation was stirred at 500rpm. Four baffles were also present in the vessels to ensure good mixing. A real time data acquisition system (RT-DAS) allowed fermentation conditions to be monitored and recorded. Mass spectrometry was used to monitor exhaust gas composition and to allow the calculation of oxygen uptake (OUR) and carbon dioxide evolution (CER) rates.

2.4.9: Inoculum preparation.

2.4.9.1: Shake flask cultures.

Shake flask cultures were each inoculated with 2ml of spore preparation per 500ml of broth. Aliquots of frozen spores in 20% glycerol (Section 2.4.6) were removed from the -20°C freezer and thawed out just prior to their use as inocula. Where necessary thiostrepton was added into the broth at the same time as the inoculum (Section 2.4.4).

2.4.9.2: Fermentations.

Each 20l fermentation was given an inoculum of three flasks each containing 500ml of broth. Flasks contained MMS medium and thiostrepton unless otherwise stated. Two different strategies for preparing these flasks were used over the course of this study (Sections 2.4.9.2.1 & 2.4.9.2.2). The strategy used for each fermentation is stated in the text. Wherever possible two fermentations were run in parallel. This meant that two

sets of inoculum flasks were usually prepared in parallel. Two extra flasks were prepared than would be needed in case of contamination, i.e. 8 flasks in total (6 for use as inocula and 2 spare). When the fermenters were ready for inoculation the flasks were examined and any which looked suspect/turbid were discarded. If all of the flasks looked uncontaminated 6 were chosen at random. These flasks were sampled to allow their characteristics (DCW, α-amylase levels, protein levels) to be determined and were then immediately used as inocula. A sample from each flask was streaked out onto an agar plate to check that no contamination was present (Section 2.6.1).

2.4.9.2.1: One-stage inoculation strategy.

5µl of frozen spore stock was spread onto a series of large (14cm) ½ TSB, 1% starch agar plates (Section 2.4.2.1). These were incubated at 30°C for 10 days to allow sporulation to occur. The spores from four of the plates were then harvested with a stiff loop into sterile distilled water (10ml/plate). The spores were pooled in a sterile container and an aliquot of the suspension (5ml per flask) was used to inoculate 8 x 2l flasks containing MMS medium. The flasks were then placed at 300rpm in a 30°C orbital incubator for 50 hours.

2.4.9.2.2: Two-stage inoculation strategy.

An initial seed flask containing MMS medium was inoculated with an aliquot of *S. lividans* pQR620 spores (Section 2.4.9.1). This was placed in a 30°C orbital incubator at 300rpm for 40 to 50 hours. Eight main inoculum flasks were then inoculated with 25ml of this seed flask. They were incubated, as previously described, for around 70 hours.

2.5: *E. coli* Culture Conditions

2.5.1: Growth of *E. coli* on liquid and solid media.

E. coli strains were grown either in nutrient broth (number 2) or on nutrient agar at 37°C. Each was prepared according to the manufacturers instructions. Nutrient agar containing 1% insoluble starch (w/v) was used to allow detection of zones of starch hydrolysis due to α-amylase diffusion from *E. coli* pQR126 cells. The antibiotics

ampicillin and kanamycin were added when required to ensure plasmid maintenance. Ampicillin was added to a final concentration of 500μg/ml in solid media and 50μg/ml in liquid media. Kanamycin was added to a final concentration of 25μg/ml in solid media and 2.5μg/ml in liquid media.

2.5.2: Preparation and transformation of *E. coli* competent cells.

E. coli JM107 was grown in 100ml of nutrient broth for between 2.5 and 3 hours (OD_{600} of ~ 0.7). When the correct cell density was reached the broth was decanted into sterile, ice cold centrifuge bottles. The cells were then harvested by spinning in a benchtop centrifuge at 4000rpm for 5 minutes. The supernatant was discarded and the cell pellets were re-suspended in 20ml of ice cold 75mM $CaCl_2$, 15% glycerol. The cells were spun down again and the resulting pellets were re-suspended in a total volume of 5ml of ice cold 75mM $CaCl_2$, 15% glycerol. The competent cell suspension was dispensed into 0.25ml aliquots. Cells were used for transformation immediately or were stored at -70°C until required.

The following procedure was used for the transformation of *E. coli*. An aliquot of competent cells was taken from the freezer and thawed out on ice. 1 to 2μl of plasmid DNA was then added into each aliquot of cells. This volume was increased when cells were being transformed with a ligation reaction. The tube was mixed gently and the cells were left on ice for 45 minutes. When this time had elapsed, the cells were incubated in a 37°C water bath for 10 minutes. Following this heat shock step, the cells were added into 5ml of nutrient broth and were shaken for 1 to 2 hours at 37°C. Aliquots of this culture (100 or 200μl) were then plated onto appropriate nutrient agar plates for incubation at 37°C.

2.6: Analytical Procedures

2.6.1: *S. lividans* sample preparation.

25ml samples of broth were obtained at each sample point for both shake flask and fermenter grown *S. lividans* cultures. 4.5ml of each sample was spun down in a microfuge (10000rpm for 10 minutes). The supernatant was removed away from the cell pellets into fresh tubes. α-Amylase assays were conducted immediately on the

recovered supernatant. The remaining supernatant was stored at -20°C to allow assay of protein and/or protease levels at a later time. Recovered cell pellets were also stored at -20°C so that internal α-amylase levels could be monitored. The remainder of each sample of broth was used for dry cell weight measurement. A loop full of each sample was plated out onto tryptone soya broth or nutrient agar plates to allow contamination to be detected if present.

2.6.2: Determination of *S. lividans* dry cell weights.

Growth of *S. lividans* cultures was monitored by dry cell weight (DCW) measurement. 5ml samples of broth were filtered through pre-dried and weighed AP25 pre-filters (Millipore, Watford) under vacuum. Whole broth samples were shaken well as each aliquot was removed, to ensure that the pellets were evenly suspended. Each sample was done in duplicate or triplicate, depending on the number of samples being processed. Filters were washed with 5ml of sterile distilled water. Each was then dried to constant weight at 80°C using a HP53 halogen moisture analyser (Mettler Toledo, Leicester). This allowed the filters to be dried more rapidly than was possible in a heated oven. Once filters had been dried each was weighed, so that dry cell weight values could be calculated.

2.6.3: Assay of α-amylase activity.

α-Amylase activity was measured by monitoring the rate of decrease of a coloured starch/iodine complex using a modified version of the assay of Blanchin-Roland and Masson (1989). An appropriate dilution was made of the sample to be assayed in 0.5ml of 15mM sodium phosphate buffer (pH5.8). This was incubated at the assay temperature of 50°C for 3 minutes to allow the solution to reach the correct temperature before the assay was commenced. The assay was started by adding 0.5ml of 0.5% (w/v) soluble starch in 15mM sodium phosphate buffer (pH5.8) into the enzyme solution. This solution had previously been heated to boiling point and filtered, whilst hot, through Whatman number 1 filter paper. It was kept at 50°C during each set of assays and was freshly made up daily. Following addition of the starch solution, each assay tube was vortexed and immediately returned to the 50°C water bath. 50μl aliquots were removed at various time points over a 20 minute period. These were immediately

diluted with 1ml of iodine solution (freshly prepared by adding 200µl 2.2%I_2/4.4%KI (w/v) into 100ml of 2% (w/v) KI solution). The rate of decrease in absorbance over time was then measured at 600nm. One unit of enzyme activity corresponds to the hydrolysis of 143µg/minute of soluble starch at 50°C.

Some experiments were conducted during the course of the study where starch was contained in the *S. lividans* culture medium being evaluated. When α-amylase assays were conducted on supernatant samples of such media, care was taken that levels of starch present in the supernatant did not affect the α-amylase assay. The supernatant was diluted in sodium phosphate buffer in the same way as would be done in each assay. 50µl of this dilution was then added into 1ml of iodine stop solution. If a dark colour developed the assay would not be a reliable indicator of actual α-amylase activity and it was not conducted. This was not found to be a problem in the cultures discussed in this work.

2.6.4: Assay of protein levels.

Supernatant protein levels were determined by the method of Bradford (1976) using BioRad protein assay reagent (BioRad, Munich, Germany). Assays were conducted as described by the manufacturers. Bovine serum albumin was used to produce a standard curve for each set of assays.

2.6.5: Assay of supernatant protease activity.

Supernatant protease activity was detected using azocasein as a substrate for proteolysis. 0.5ml of supernatant was mixed with 0.5ml of buffer solution containing 0.4% azocasein, 10mM $CaCl_2$ and 100mM Tris.HCl (pH 7.5). This was incubated in a 30°C water bath for 10 minutes. Once this incubation period had been completed 50µl of 15% (w/v) trichloroacetic acid solution was added and the tube was vortexed. The assay mixture was then left at room temperature for 10 to 15 minutes. This allowed undigested azocasein to precipitate. Finally the tube was spun in a microfuge for 10 minutes at 10000rpm and the optical density of the supernatant was read at 420nm. One unit of protease activity was defined as a change in OD_{420} of 1 optical density unit/ml in an hour.

2.6.6: Measurement of sample pH.

The pH of *S. lividans* broth samples was measured using pH indicator strips.

2.6.7: Assay of intracellular tyrosinase activity in *S. lividans* pIJ702 cells.

S. lividans pIJ702 was grown in MMS medium at 30 and 34°C (Section 2.4.7). 25ml samples were taken from the flasks at regular intervals. The samples were spun down in a benchtop centrifuge at 4000rpm for 10 minutes. Each pellet was re-suspended in a known volume of 0.1M sodium phosphate buffer (pH6). The cells were disrupted by sonication (8μ amplitude for 10 x 10 second cycles) and the cell debris were separated from the cell extract by centrifugation in a microfuge (10 minutes at 10000rpm).

Tyrosinase activity was measured in the supernatant using the dopachrome method of Lerch and Ettlinger (1972) with L-dihydroxyphenylalanine (L-DOPA) as substrate. 20mM L-DOPA was dissolved in 0.1M sodium phosphate buffer (pH6). 1ml of this solution was placed in a cuvette. The reaction was started by adding 100μl of cell extract into the solution. The formation of dopachrome at 30°C was then followed using a DU 7500 spectrometer (Beckman, High Wycombe). Absorbance readings were taken at an optical density of 475nm over 3 minutes. The molar extinction coefficient for dopachrome at 475nm is 3600 (Katz *et al.*, 1983). One unit of tyrosinase activity is defined as the amount of enzyme that catalyses the oxidation of 1μmol of L-DOPA per minute (Katz *et al.*, 1983).

2.6.8: Preparation of *S. lividans* cell extract.

Mycelia from 4.5ml of broth were washed with 1ml of sterile distilled water and spun down in a microfuge (5 minutes at 10000rpm). The pellet was then re-suspended in a known volume of 15mM sodium phosphate buffer. The cells were disrupted by sonication (8μ amplitude for 10 x 10 second cycles) and the cell debris were separated from the cell extract by centrifugation (10 minutes at 10000rpm). Levels of α-amylase activity in the cell extract were then assayed using the procedure described in Section 2.6.3.

2.6.9: Preparation of *E. coli* periplasmic extract.

E. coli pQR126 was grown overnight at 37°C in 100ml of nutrient broth. The broth was decanted into sterile centrifuge bottles and was spun down in a benchtop centrifuge (4000rpm for 5 minutes). The recovered cell pellets were re-suspended in 20ml of 20% (w/v) sucrose, 1mM EDTA solution containing 500μg/ml lysozyme. This was left at room temperature with occasional shaking for 10 to 15 minutes. 20ml of sterile distilled water was added into the lysis mixture and the solution was left to stand for a further 10 to 15 minutes. The mixture was then spun down (4000rpm for 10 minutes) to recover the cell periplasmic fraction from the cell debris.

2.6.10: SDS-polyacrylamide gel electrophoresis.

Supernatant protein profiles were observed using SDS-polyacrylamide gel electrophoresis (SDS-page). Gels were run as described by Maniatis *et al.* (1982) using 10% resolving gels and 5% stacking gels. 30% (w/v) acrylamide, 1.034% bis-acrylamide (ratio 29:1) was obtained from Severn Biotech Ltd. (Kidderminster, Worcs.). Tris-glycine buffer was used for electrophoresis (5 x buffer contained 15.1g/l Tris.base, 94g/l glycine, 5g/l SDS). Mid-range protein molecular weight markers (Promega, Madison, WI, USA) were run on each gel as standards.

The low levels of protein present in *S. lividans* supernatant meant that it was necessary to concentrate the protein in supernatant samples prior to loading onto SDS-page gels. This was done using trichloroacetic acid (TCA) precipitation from a volume of supernatant that contained between 50 and 70μg of protein. When the protein profiles of supernatants from the same experiment were being compared, a set volume of supernatant from each sample was used, so that levels of protein were directly comparable. 5% of a 10% TCA solution was added into each sample. Each tube was mixed, left on ice for 10 minutes and pelleted in a microfuge (5 minutes at 10000rpm). The supernatant was removed from the pellets and replaced with 500μl of ice cold acetone. This was vortexed well and left on ice for a further 10 minutes. Pellets were recovered again, as described previously. The supernatant was discarded and each pellet was dried in a 60°C oven. The protein preparations were then dissolved in 10μl of 100mM Tris (pH8.8) and 10μl of 2 x SDS loading buffer (2 x Buffer; 100mM

Tris.HCl (pH6.8), 4% SDS, 0.2% bromophenol blue, 20% glycerol, 200mM dithiothreitol (added just prior to use)). The protein samples and protein standards were boiled for 3 minutes before being loaded onto SDS-page gels.

2.6.11: Experiments to determine the relationship between size of zones of starch hydrolysis and α-amylase activity on solid media.

2.6.11.1: Zones produced by liquid α-amylase preparations.

Konker and Jaeger (1987) showed how a sensitive plate assay for bacterial lipases was developed. A similar strategy was followed here for α-amylase. An *E. coli* JM107 pQR126 periplasmic extract (Section 2.6.9) and a *S. lividans* TK24 pQR318 supernatant preparation containing α-amylase were produced. Each was assayed in triplicate for α-amylase activity and was spun down and filtered (0.2 μm). These last steps were of importance as they ensured that no remaining organisms were present which would grow on the agar plates and invalidate the experiment.

The enzyme preparations were diluted with sterile distilled water to a range of concentrations from 0.5 units of α-amylase per ml to 0.15×10^{-3} units of α-amylase per ml. A preliminary experiment had suggested that a linear relationship between zone size and α-amylase activity only existed around this range of concentration. At higher concentrations something other than α-amylase controlled zone size. The most likely cause was that the diffusion of the enzyme through the agar was limiting above a certain concentration.

A series of large (14 cm) petri dishes were filled with $^1/_2$ TSB, 1% starch agar to a depth of 5mm. Several wells were made in each plate using a sterile core borer (5mm diameter). Wells were spaced at 4cm intervals. Thirty μl of each dilution was then added into each well. The plates were left on the bench overnight (12 h) to allow the liquid to soak into the agar without evaporating. They were then placed in a 50°C incubator for 30 hours. Each dilution from each species was carried out in duplicate.

Following incubation the plates were flooded with an iodine solution so that the zones of hydrolysis could be seen and measured more easily. The radius of each zone was then measured from the edge of the well to the edge of the cleared area. Some zones

had a distinctly skewed shape. In such cases the thickest and thinnest points of the zone were measured and the average was used as the radius. The zones tended to extend more towards the edges of each plate, indicating that this was probably a diffusion effect.

2.6.11.2: Zones produced by growing colonies.

$^1/_2$ TSB, 1% starch agar plates were produced as described previously (Section 2.4.2.1). These were then stab inoculated with *S. lividans* pQR620. *E. coli* JM107 pQR126 cells were stab inoculated in a similar manner into nutrient agar plates containing 1% starch (Section 2.5.1). *S. lividans* TK24 and *E. coli* JM107 were also inoculated onto plates as controls. *S. lividans* cultures were incubated at 30°C and *E. coli* cultures were incubated at 37°C. Growing both of the cultures at one temperature was discounted because it was felt that the plasmid copy numbers inside the cells would be affected so much by temperature that the results would be invalid.

Zone diameters produced by the resulting colonies were measured twice a day for 4 days and then again at 7 days. An iodine solution was not used here to help show up zones of hydrolysis because it might have had an adverse effect on cell growth and production. Zone edges were therefore detected by eye. No visible zones were produced by *E. coli* JM107 control colonies. Small zones (1-2mm) could be seen around some, but not all, of the *S. lividans* colonies after 170 hours incubation. Measured zone sizes for each strain and time point were averaged and plotted to show the development of zones of starch hydrolysis over time (Section 3.6).

2.6.12: Plackett-Burman analysis of the effects of medium components on *S. lividans* pQR620.

A Plackett-Burman experimental design was used to identify which components of MMS medium had significant effects on of the performance of different aspects of the *S. lividans* pQR620 system. This is called a two-level fractional factorial design as the effects of the different variables are assessed at both a high level and a low level. Use of these designs is reviewed by Stowe and Mayer (1966) and Greasham (1993).

Two separate analyses were carried out using a Plackett-Burman matrix for the study of seven variables with 8 experiments (Table 2.3). The experiments were all conducted in

2l shake flasks each containing 500ml of liquid medium. The levels at which each variable would be studied were selected carefully. This was to ensure that a large enough differential was achieved in each case to prompt the display of any effect, without causing such a large effect that responses due to the alteration of other variables would be masked. See Sections 2.6.12.2 & 2.6.12.3 for the levels used in each of the analyses. Once these levels were set, the exact composition of the medium contained in each flask was defined by the matrix. Each flask/experiment was given a number from 1 to 8. The medium components chosen to act as variables were weighed or measured out and were added into each flask at the correct level. In both studies variable G was assigned to be a dummy variable. Although this variable also appears at defined levels in each flask according to the Plackett-Burman matrix, in reality nothing at all was added into the flasks. This dummy variable is an important feature in such experiments. It allows levels of experimental error to be measured and therefore allows real effects to be detected despite any underlying experimental error which may be present.

As can be seen from the matrix (Table 2.3), each variable appeared 4 times at a high level and 4 times at a low level in each study. Each time variable A was tested at its high level, variable B was tested twice at its high and low levels. Likewise, each time variable A was tested at its low level, variable B was again tested twice at a high level and twice at a low level. This is another crucial feature of the Plackett-Burman designs as it means that the matrix design cancels out the effect of variable B when calculating the effect of variable A. As the same design holds true for all of the other variables, each variable is independently evaluated. This prevents any estimate of the interactions between variables, a characteristic of the design which is acceptable in situations where the interactions between variables are not apparent (Greasham, 1993).

When the different medium components had been measured out, 500ml of a basal medium was added to each flask (Sections 2.6.12.2 & 2.6.12.3). Each flask was swirled to dissolve any remaining solids and the 8 flasks were autoclaved together. Thiostrepton and DMSO were used as variables in the studies which were conducted. These medium components were added into flasks just prior to inoculation.

Table 2.3: Plackett-Burman matrix for the analysis of 7 variables with 8 experiments. +, high level of variable; -, low level of variable.

Experimental Trial (Flask)	Assigned Variables A	B	C	D	E	F	Dummy Variable G
1	+	+	+	-	+	-	-
2	-	+	+	+	-	+	-
3	-	-	+	+	+	-	+
4	+	-	-	+	+	+	-
5	-	+	-	-	+	+	+
6	+	-	+	-	-	+	+
7	+	+	-	+	-	-	+
8	-	-	-	-	-	-	-

When the different medium components had been measured out, 500ml of a basal medium was added to each flask (Sections 2.6.12.2 & 2.6.12.3). Each flask was swirled to dissolve any remaining solids and the 8 flasks were autoclaved together. Thiostrepton and DMSO were used as variables in the studies which were conducted. These medium components were added into flasks just prior to inoculation.

The 8 flasks which made up each study were all inoculated with 25ml from a single seed flask. The seed flasks used contained 500ml of MMS medium and thiostrepton. Each was inoculated with a 1ml aliquot of frozen *S. lividans* pQR620 spore stock and was incubated at 30°C and 300rpm. The seed flask for Plackett-Burman study 1 was grown for 24 hours prior to being used as inoculum. The seed flask used in study 2 was incubated for twice this time prior to use. Following inoculation, the main flasks were incubated at 30°C and 300rpm in an orbital incubator. Regular samples were taken to allow biomass, α-amylase, supernatant protein and protease levels to be measured. At the sample points the flasks were shuffled around in the incubator to try to minimise any effects due to position, e.g. possible temperature fluctuations.

2.6.12.1: Determination of the effects of changing levels of a variable on the system.

Once each study had been completed, and the data from each experiment within that study gathered, it was possible to calculate the effect of each variable on a certain

response of system performance, e.g. peak biomass level or rate of supernatant α-amylase production. It also allowed the level of significance of any effects seen to be defined. This was done using the following calculations:

Initially, the effect (E) of a variable on a response of the system was calculated using:

$$E_x = \frac{\text{total of 4 responses at high level (+)}}{4} - \frac{\text{total of 4 responses at low level (-)}}{4} \quad (1)$$

This calculation was also made for the dummy variable (i.e. for E_G in these studies). If no interactions between variables had occurred, and all levels were reproduced perfectly with no error in the measurement of responses, the experimental design ensures that the effect shown by the dummy variable(s) will be 0. Therefore, cases where E_G was found to deviate from 0 indicated that either experimental errors had been made during measurement of the responses or that interactions between variables had occurred. Cases where large deviations in E_G values from 0 were observed are therefore discussed as they may be indicative of underlying interactions of variables (Section 7.5.11.2).

The variance of each effect (V_{eff}) can be estimated by averaging the square of the effect of the dummy variable(s) (E_d):

$$V_{eff} = \frac{\sum (E_d)^2}{n} \quad (2)$$

where n = number of dummy variables/degrees of freedom for error.

Each of the two studies conducted as part of this work only contained a single dummy variable (variable G). This meant that the variance for each effect was simply equal to the square of the effect of the dummy variable:

$$V_{eff} = \frac{E_G{}^2}{1}$$

The standard error (SE) of an effect was then calculated by taking the square root of the variance:

$$SE_{eff} = \sqrt{V_{eff}} \quad (3)$$

The significance level of each effect was then determined using the Student t test (two-tailed). The t value for each effect was calculated as follows:

$$t_x = \frac{E_x}{SE_{eff}} \qquad (4)$$

where t_x is the effect of variable x.

Obtaining a positive t value for a variable indicated that increasing its level had a positive effect on the response of the system. Conversely, obtaining a negative t value indicated that the effect on the response was negative. Once values of t had been calculated for the effect of each variable on a response of the system, standard tables of the Student t distribution were consulted. This allowed the probability of finding the observed effect by chance to be evaluated. Observed effects were only accepted as a direct result of modifying the level of a variable if they were found to have a confidence level of 80% or greater.

An example of the calculations described above, where the effect of changing the levels of variables on maximum specific growth rate (μ_{max}) for Plackett-Burman study 1 were analysed, is included in Appendix 2.

If a classical approach had been used to conduct these experiments and examine the effects of each variable at two different concentrations, by altering a single medium component at a time, a total of 2^7 or 128 experiments would have been required. Use of this approach therefore provided a rapid but powerful method of identifying important medium components for different system responses.

2.6.12.2: Plackett-Burman study 1.

The variables chosen for analysis in the first Plackett-Burman study, and their high and low levels, are detailed in Table 2.4.

The basal medium added into each flask consisted of 3.6g/l $K_2HPO_4.3H_2O$, 2.17g/l $NaH_2PO_4.1H_2O$. This was made up as a single batch in distilled water so that the basal medium added into each flask would be identical. It was intended that this basal medium would act to reduce the likelihood of any severe pH changes by buffering the

system. Such changes could have altered the outcome of the experiment by causing unpredictable variations in the flasks.

Table 2.4: Chosen variables and their levels used in Plackett-Burman study 1.

	Variable	High Level (+) per l	Low Level (-) per l
A	Casamino acids	15g	5g
B	Succinate	15g	5g
C	Minor elements (Section 2.4.1.1)	8ml	2ml
D	$(NH_4)_2SO_4$	8g	2g
E	$MgSO_4$	1.8g	0.6g
F	Thiostrepton (in DMSO)	5mg	--
G	Dummy	--	--

2.6.12.3: Plackett-Burman study 2.

The variables chosen for analysis in the second Plackett-Burman study, and their high and low levels, are detailed in Table 2.5.

Table 2.5: Chosen variables and their levels used in Plackett-Burman study 2.

	Variable	High Level (+) per l	Low Level (-) per l
A	$(NH_4)_2SO_4$	2g	--
B	K_2HPO_4/NaH_2PO_4	3 x buffer	1 x buffer
C	Thiostrepton /DMSO	Thiostrepton & DMSO	DMSO only
D	$FeSO_4$ (1g/l)	6ml	--
E	$CaCl_2$ (1g/l)	6ml	--
F	Protease inhibitors	+	--
G	Dummy	--	--

Two separate batches of basal medium were made up for this study. One batch contained K_2HPO_4 and NaH_2PO_4 at the level usually used in MMS medium (i.e. 1 x buffer ; 3.6g/l $K_2HPO_4.3H_2O$ (16mM), 2.17g/l $NaH_2PO_4.1H_2O$ (16mM)). The other contained 3 times these concentrations (i.e. each at 48mM). Casamino acids (5g/l), succinate (5g/l), minor elements solution (2ml/l)(Section 2.4.1.1) and $MgSO_4$ (0.6g/l) were also added into each batch. These were then aliquotted into the appropriate flasks as defined by the buffer concentration level required. Just prior to inoculation, thiostrepton dissolved in DMSO was added into flasks which needed a high level of this variable. 50µl of stock solution was added to each giving a final concentration of thiostrepton of 5µg/ml. The other four flasks each had 50µl of DMSO added into them giving the low level of variable C. Complete, mini EDTA-free protease inhibitor cocktail tablets were obtained from Boehringer-Mannheim (Lewes, UK). These were chosen for use in these experiments because it was expected their components would not drastically interfere with *S. lividans* cell growth if added into the broth. 12 tablets were dissolved in 6ml of sterile distilled water. The solution was then filtered through a 0.2µm filter (Millipore, Watford) to ensure sterility. Prior to inoculation, 0.5ml of this solution was added into the flasks earmarked to contain a high level of protease inhibitor. An additional 1ml of the solution was added to these flasks at 45 hours.

2.7: Molecular Biology Techniques

2.7.1: Preparation of plasmid DNA from *E. coli.*

Plasmid DNA was prepared from *E. coli* cultures using Wizard plus miniprep and midiprep kits (Promega, Madison, WI, USA) as described in the manufacturers' instructions.

2.7.2: Preparation of plasmid DNA from *S. lividans.*

Plasmid DNA was isolated from *Streptomyces* cells using alkaline lysis as described by Hopwood *et al.* (1985).

2.7.3: Preparation of total DNA from *S. lividans.*

Total DNA was prepared from *S. lividans* cultures using the method of Fisher (described in Hopwood *et al.*, 1985). 50mg of mycelia were re-suspended in 500µl of

lysis solution containing 2mg/ml lysozyme and 50μg/ml RNase in 0.3M sucrose, 25mM Tris (pH8), 25mM EDTA. This was incubated at 37°C until the cells became translucent. 250μl of 2% SDS was added and the solution was vortexed for approximately 1 minute. The solution was then extracted several times with neutral phenol-chloroform (250μl aliquots) until very little white interface was seen when the solvent was added. Phases were separated during these extractions with 2 minute spins in a microfuge. The resulting aqueous layer was removed to a clean tube and 0.1 volume of 3M sodium acetate (pH4.8) was added. After a brief mix 1 volume of isopropanol was added to the tube. The tube was incubated at room temperature for 5 minutes and then spun at top speed in a microfuge for 5 minutes. The supernatant was removed from the tube and the total DNA pellet was re-suspended in 500μl of Tris/EDTA (TE) buffer (10mM Tris.HCl pH7.5, 1mM EDTA). The resulting DNA solutions were stored at -20°C.

2.7.4: Restriction digests.

Restriction digests were performed using the method of Maniatis *et al.* (1982). All reactions were carried out at 37°C using a universal 10 x restriction buffer (50mM $MgCl_2$, 500mM Tris.HCl pH7.5). Restriction enzymes were obtained from New England Biolabs (Beverly, MA, USA). Reactions were stopped by the addition of an aliquot of loading buffer (0.1M EDTA, 40% (w/v) sucrose, 0.15 mg/ml bromophenol blue).

2.7.5: Agarose gel electrophoresis.

DNA fragments were separated by gel electrophoresis in 1% agarose gels as described by Maniatis *et al.* (1982). The running buffer used was Tris-borate-EDTA buffer (TBE: 90mM Tris.base, 90mM boric acid, 1mM EDTA, 0.05μg/ml ethidium bromide). Gels were visualised and photographed under ultra-violet light.

2.7.6: Extraction of DNA bands from agarose gels.

DNA bands were extracted from agarose gels using a Geneclean kit (Bio101 Inc., Vista, CA, USA). The procedure was conducted as described in the manufacturers' instructions.

2.7.7: Ligation of DNA.

Ligation reactions were carried out as described by Maniatis *et al.* (1982) except that reactions were carried out overnight at 4°C. Reactions were carried out in 10 x ligation buffer (0.66M Tris.HCl pH7.5, 0.1M $MgCl_2$, 0.1M dithiothreitol, 1mM ATP) using T4 DNA ligase (New England Biolabs, Beverly, MA, USA).

2.7.8: Detection of single-stranded plasmid DNA.

Total DNA samples prepared from cells of *S. lividans* pIJ303, pQR620, pQR621, pIJ702, pQR318 and pQR311 were loaded onto a 1% agarose gel. The gel was electrophoresed (Section 2.7.5) until the loading buffer front was approximately halfway down the length of the gel. Single-stranded DNA was expected to run ahead of the other plasmid forms which were present, therefore care was taken not to loose any from the end of the gel. DNA bands were then visualised under ultra-violet light and photographed. The pattern of bands was also traced onto an acetate sheet so that their exact positions could be directly compared with the position of any ssDNA detected.

Once the visible band positions had been recorded the gel was washed in 0.2N HCl for 10 minutes and then neutralised in 1M Tris (pH7.4), 1.5M NaCl for 30 minutes. No denaturation step was included in this procedure so that only ssDNA which had been present naturally in the cells would be detected. DNA bands were then transferred onto a nitrocellulose membrane using 20 x SSC as the transfer buffer (1 x SSC buffer contained 8.77g/l NaCl, 4.41g/l sodium citrate). Southern blotting was carried out overnight as described in Maniatis *et al.* (1982). The membrane was then briefly dried between two pieces of filter paper and the DNA was attached to the membrane using UV-crosslinking.

The membrane was incubated in pre-hybridisation buffer (10 x SSC, 150μg/ml denatured herring sperm, 0.2% bovine serum albumin, 20% formamide, 0.5% SDS) for 5 hours at 55°C. Denatured, [^{32}P]dCTP-labelled pIJ702 was then added into the tube. The membrane was then incubated overnight at 55°C to allow this probe to hybridise to any ssDNA that was present. When this incubation period was complete the membrane was washed sequentially in 2 x SSC, 0.1% SDS; 0.5% 2 x SSC, 0.1% SDS and 0.1 x SSC, 0.1% SDS. Each wash step lasted 15 minutes. The membrane was then placed

between 2 pieces of cling film and exposed to X-ray film overnight. The autoradiograph was developed and the image was photographed to the same scale as the original gel. The autoradiograph was compared to the acetate indicating the position of visible DNA bands on the agarose gel. This allowed the distance from the wells of both visible DNA bands and ssDNA bands to be established.

Chapter 3
Initial Media Experiments

3.1: Aims

The major aim of the work detailed in this chapter was to identify a suitable liquid medium for use throughout the study. Levels of α-amylase production from *S. lividans* pQR311 and pQR318 were examined as part of the media evaluation. Although only very low levels of α-amylase were obtained in liquid media, large zones of starch hydrolysis were produced by the recombinant *Streptomyces* colonies on agar plates. This suggested that higher levels of α-amylase were produced by these strains on solid media than in liquid media. Experiments were also conducted to investigate this possible inconsistency.

3.2: Background

At the start of this project two high copy number vectors were available for expression and secretion of the α-amylase in *Streptomyces* (Bahri, 1990). In the first of these constructs, pQR311, gene expression is directed from the native *amy* promoter. The second plasmid, pQR318, does not contain the natural promoter. Instead transcription of the α-amylase gene is believed to be controlled by read through from a pBR325 promoter. Both of these constructs are based on the streptomycete plasmid pIJ702 (Fig. 1.2). French (1993) looked at production of α-amylase from these plasmids in a variety of media under shake flask and bioreactor conditions. Tryptone soya broth (TSB) was found to give the best profile for enzyme production. *S. lividans* pQR318 produced up to 3.5 U/ml α-amylase under these conditions. *S. lividans* pQR311 produced lower levels of enzyme (around 2 U/ml supernatant). The pattern of α-amylase production over time was similar from each plasmid.

Over the last 10 years a system for the periplasmic expression of the α-amylase in *E. coli* has been developed and optimised (French, 1993; Pierce, 1996). Two high copy number plasmids are available for the expression of the enzyme; pQR126 (French, 1993) and pQR187 (J.M. Ward, unpublished). Total α-amylase production from the system can reach 40-50 U/ml culture (equivalent to 100 to 130 mg/ml α-amylase).

However, the enzyme is found in three distinct cellular locations: cell associated, periplasmic and extracellular. This means that the recoverable level of enzyme is effectively far lower than the actual level of α-amylase within the cells as a considerable amount is lost during protein purification. What proportion of the α-amylase is located in a particular position in the cells depends both on the strain of *E. coli* and the plasmid used for expression (Pierce, 1996). Between 20 and 40% tends to be located periplasmically; 10 to 30% can be cell associated. The majority of the enzyme, 40 to 70%, is lost into the extracellular medium. This release of product is usually caused by disruption of the outer cell membrane giving rise to periplasmic leakage.

A comparison of this data shows that α-amylase production from the *S. lividans* expression system is 15 to 20-fold lower than that from the *E. coli* system. Therefore, based on the data shown previously, *E. coli* would definitely be the host system of choice for production of the enzyme. Levels of α-amylase production would have to be raised significantly before the balance would swing towards the *Streptomyces* system being the more viable option.

3.3: Preliminary Media Evaluations

Preliminary shake flask experiments were undertaken to give an indication of which medium should be used as a base for the study and to reconfirm the levels of α-amylase production from the *S. lividans* expression vectors. It was suggested that *Streptomyces* minimal medium (SMM) might be a suitable basal medium for product expression (Section 2.4.1.5) (E. Madden, personal communication). Supernatant acidification, due to organic acid production during rapid growth, is a recognised problem in streptomycete cultures (Ahmed *et al.*, 1984; Dekleva & Strohl, 1987). Madden *et al.* (1996) consistently found that the presence of ammonium ions completely suppressed organic acid excretion by the cells. The suggested medium contained 2g/l $(NH_4)_2SO_4$ (15mM) specifically for this reason. Succinate, Casamino acids and starch (each at 5g/l) were used as additional supplements. These had been observed to give good growth of non-recombinant *S. lividans* and were considered to be promising starting points for the study. Modified yeast extract/malt extract (MYEME) medium was also

tested (Section 2.4.1.4). This is a complex medium which had been found to give high levels of recombinant β-lactamase production in *S. lividans* (Erpicum *et al.*, 1990). The plasmid used in the study by Erpicum *et al.* (1990) was based on pIJ702 and therefore would be expected to behave in a very similar way to pQR318 and pQR311. This parallel, along with the similarity of the products (both β-lactamase and α-amylase are secreted degradative enzymes), suggested that MYEME might also be suitable for α-amylase production. In addition, TSB was used to allow comparison of the results with those of French (1993). Each flask was inoculated with an aliquot of the appropriate frozen spore stock and was incubated at 30°C. No detectable α-amylase was produced by *S. lividans* pQR311 in any of the media tested. *S. lividans* pQR318 produced only low levels of extracellular α-amylase (Fig. 3.2).

3.3.1: Tryptone soya broth (TSB).

The level and patterns of growth seen in TSB were very similar to those seen by French (1993), i.e. a peak biomass of around 4g DCW/l (Fig. 3.1). However, comparison of α-amylase production shows a very different picture. In this time course experiment only 0.27 units of α-amylase were produced per ml of supernatant. This represents a 10-fold reduction in the level of enzyme compared to that obtained in the previous study. The productivity of the cells was around 60 U α-amylase/g DCW (Fig. 3.3). Medium pH started off at 7 and rose steadily over time to around pH 8 (Fig. 3.5).

Several possible explanations exist for the large variation in performance seen. The inoculum used in French's experiment was very different from that used here, i.e. vegetative versus spore inoculum (French, 1993). Several authors have described how system performance can be very strongly influenced by the inoculum used. Stoichev *et al.* (1981) found that the type and age of inoculum governed the pattern of glucose isomerase production seen from *Streptomyces* strains 1339 and 765. A 60 hour vegetative inoculum gave optimal results. Elibol *et al.* (1995) obtained a very different picture when they looked at actinorhodin production by *S. coelicolor* A3(2). They found that a spore inoculum was preferable and observed lower peak growth and pigment production when a vegetative inoculum was used. A small cell inoculum of *S. venezuelae* gave the highest titres of chloramphenicol production in a study by Chatterjee *et al.* (1983). The advantage seen increased as the cultures aged. When a

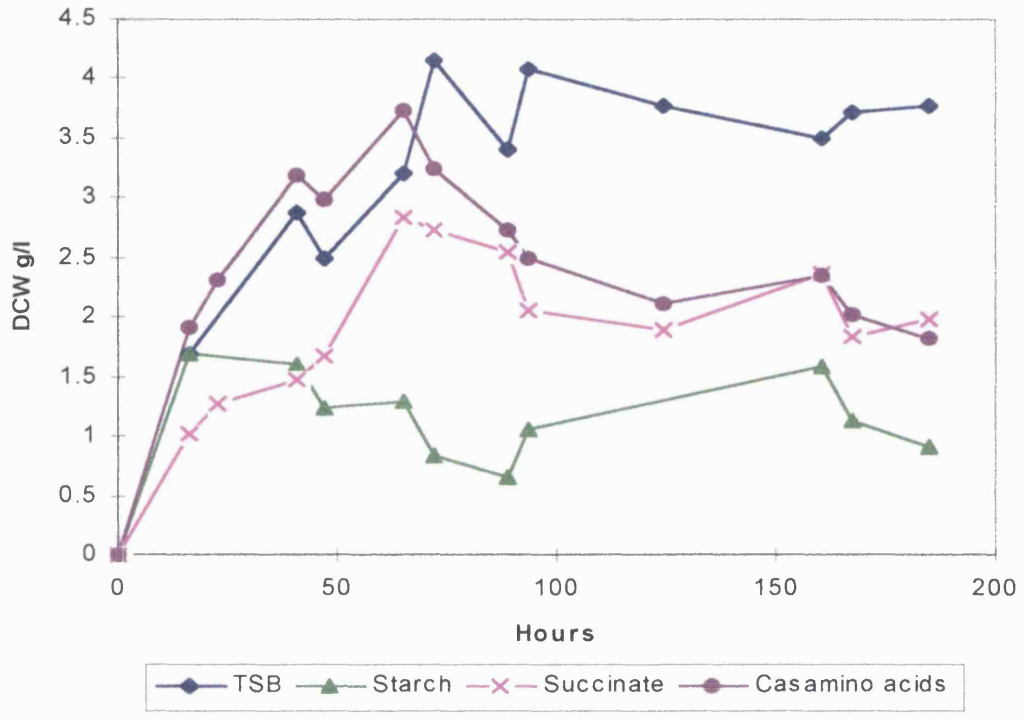


Figure 3.1: Growth of *S. lividans* pQR318 in TSB and SMM supplemented with starch, succinate and Casamino acids. No dry cell weight measurements were available from the culture grown in MYEME because of the nature of the medium.

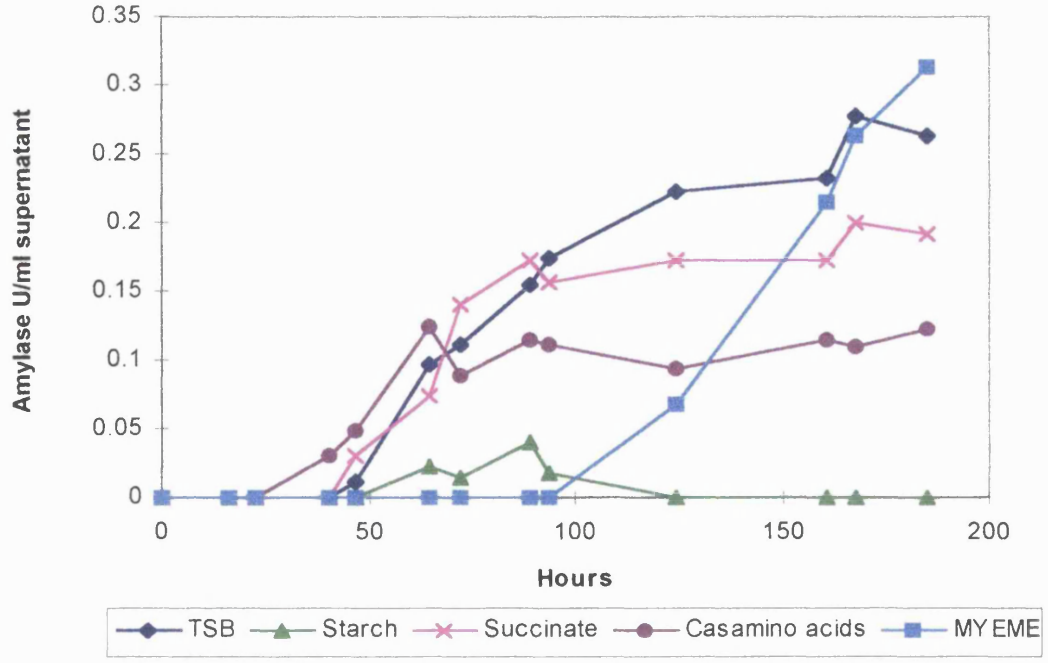


Figure 3.2: α-Amylase production by *S. lividans* pQR318 grown in MYEME, TSB and SMM supplemented with starch, succinate and Casamino acids.

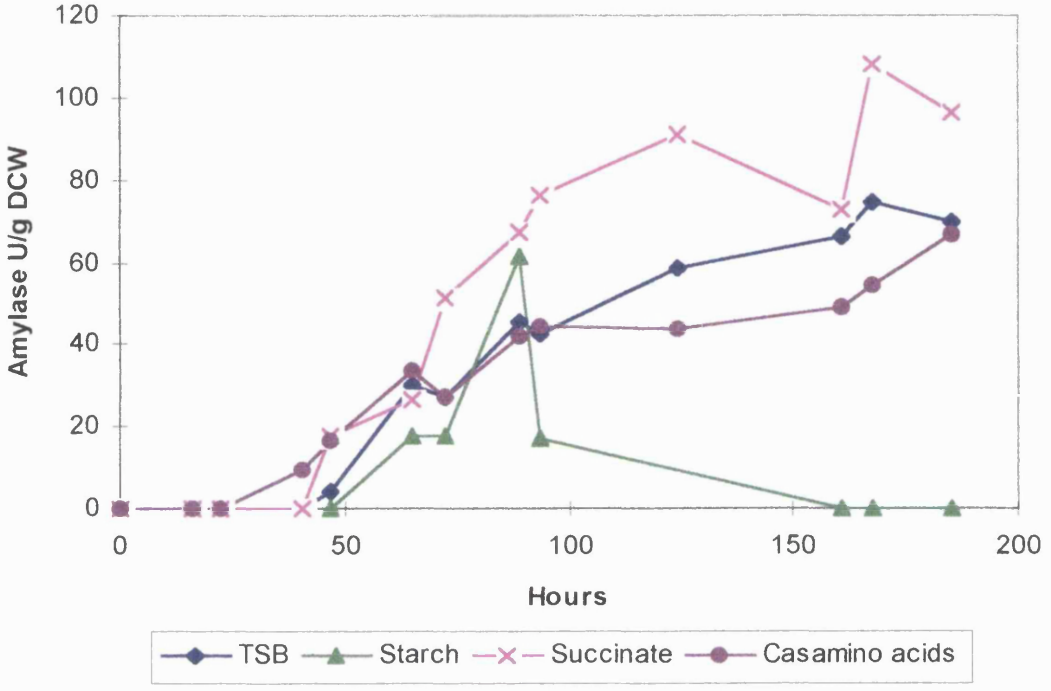


Figure 3.3: α-Amylase productivity levels for *S. lividans* pQR318 grown in TSB and SMM supplemented with starch, succinate and Casamino acids.

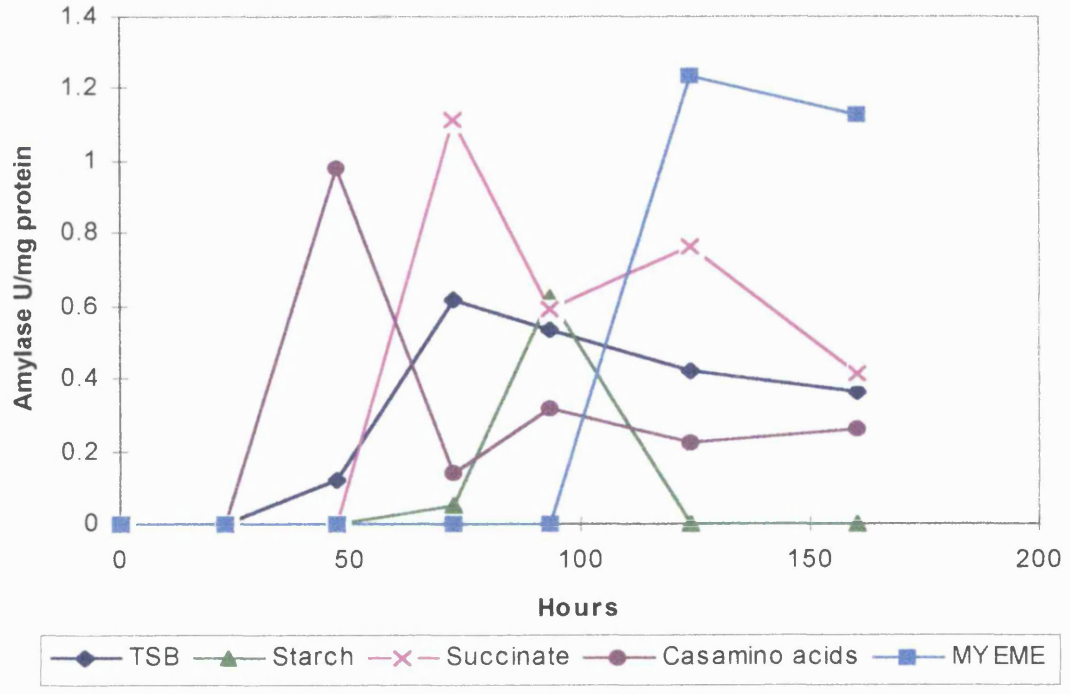


Figure 3.4: α-Amylase specific activity levels for *S. lividans* pQR318 grown in MYEME, TSB and SMM supplemented with starch, succinate and Casamino acids.

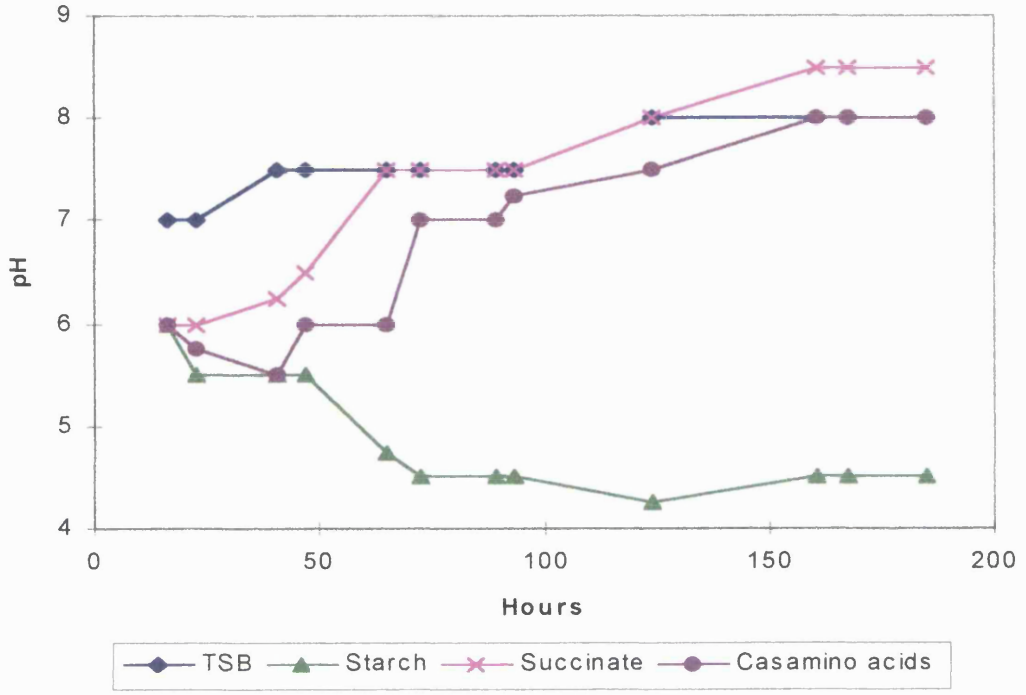


Figure 3.5: Supernatant pH fluctuations for *S. lividans* pQR318 grown in TSB and SMM supplemented with starch, succinate and Casamino acids.

large inoculum was given, the antibiotic was produced earlier but the rate of synthesis was lower. These studies are indicative of the large effect that choice of inoculum can have. It seems entirely plausible that the marked difference in α-amylase levels observed could result from variations between inocula.

Another possible reason for the differences seen would be loss of plasmid from the cells or reduced plasmid copy number. The spore stocks used to inoculate these flasks were produced at the beginning of the project, shortly before this experiment was conducted. Spores were harvested from ½ TSB, 1% starch agar plates. These plates contained thiostrepton to ensure plasmid maintenance. Each flask also contained the antibiotic to maintain selection throughout the time course. Although occasional instances of instability have been noted, the majority of available data indicates that plasmids based on the pIJ702 replicon are stable in the absence of selective pressure. Payne *et al.* (1990) found that production from a parathion hydrolase gene cloned into pIJ702 only varied slightly over 20 cell generations. This indicates strongly that the plasmid displays good segregational stability. Wrigley-Jones *et al.* (1993) observed the streptomycete plasmid pIJ303 to be 'remarkably stable under normal growth conditions' in *S. lividans*. In shake flask studies, plasmid copy numbers above 500 were present at the end of rapid growth under optimal conditions. Relatively high copy numbers (50 to 350) were maintained well into stationary phase. Over the course of growth similar patterns of fluctuation in copy number are likely to be displayed by pIJ702 based vectors as they contain the same minimal replicon.

Structural plasmid instability has been observed among this family of plasmids (Pigac *et al.*, 1988; Zaman *et al.*, 1993b). Such rearrangements could also have a detrimental affect on α-amylase production. Cells grown from the spore stock were found to contain a plasmid of the correct size for pQR311. Deletion or recombination of the plasmid with loss of the α-amylase gene was therefore improbable. The evidence suggests that plasmid instability or rearrangement is unlikely to be the cause of the differences in production levels seen in the two experiments. Variation in inoculum type is therefore the most probable explanation for the observed decrease in α-amylase production.

3.3.2: Modified yeast extract/malt extract medium (MYEME).

MYEME medium contains a high concentration of sucrose (34%)(Section 2.4.1.4). The medium was extremely viscous and difficult to handle. Accurate measurement of dry cell weight was impossible. Very little growth was observed in the flask until around 100 hours incubation. At this point biomass was seen to accumulate quickly. A steep rise in α-amylase production occurred concurrently with this increase in biomass (Fig. 3.2). The peak specific activity seen compared well with the other flasks (Fig. 3.4). Supernatant pH remained neutral throughout the experiment.

Erpicum *et al.* (1990) also obtained slow growth in MYEME, with one of their cultures taking around 150 hours to reach peak biomass. As this is a rich, complex medium, where no nutrient limitation should initially be present, oxygen limitation is the most likely cause of the slow growth. The very high viscosity of the medium would lead to reduced oxygen transfer to the liquid and to less efficient mixing. Both of these factors could lead to reduced growth rates. However, Erpicum *et al.* (1990) still found this to be significantly better for β-lactamase production than the same medium without sucrose. They concluded that this was because a high water activity had a positive affect on the system, either by increasing product stability or by influencing synthesis or secretion of the β-lactamase. The rapid production of α-amylase from the cells seen during the final stages of the time course looked promising. It was not possible to continue the experiment for a longer period of time to see if the high production rate was maintained. An additional experiment would be needed, perhaps with smaller volumes of medium in the shake flasks to maximise oxygenation, to evaluate the true potential of this medium for α-amylase production from *S. lividans* and to draw any conclusion about the effect of water activity on the system.

3.3.3: Supplemented *Streptomyces* minimal medium (SMM).

3.3.3.1: SMM + succinate or Casamino acids.

The SMM flasks supplemented with succinate and Casamino acids followed very similar patterns. In each case growth peaked around 3g DCW/l. Very little lag phase was seen in the flasks. Supplementation with succinate was more favourable for α-amylase production than supplementation with Casamino acids. It produced a higher

titre of enzyme per ml of supernatant and gave a higher specific activity (Figs. 3.2 & 3.4). In fact, the cells grown in SMM + succinate produced the highest level of α-amylase per unit biomass when compared to the other media tested in the time course (Fig. 3.3). The pH of the succinate flask rose steadily from inoculation and was above pH 8.0 at the end of the experiment (Fig. 3.5). In the flask supplemented with Casamino acids an initial pH drop was seen. However, after 40 hours the trend was reversed and the pH started to climb. The final pH of this flask was 8.0.

3.3.3.2: SMM + starch.

The final flask in this time course was SMM supplemented with starch. The flask initially started to grow well but stopped at around 1g DCW/l (Fig. 3.1). A small amount of α-amylase production was seen but quickly fell off (Fig. 3.2). This is reflected in the specific activity and productivity data (Figs. 3.4 & 3.3). In stark contrast to the other two flasks, the pH of this flask fell to 4.5 and remained at this level for the duration of the time course. In this experiment the acidification of SMM supplemented with starch had been unexpected as the medium formulation chosen had been previously found to suppress organic acid production completely (Madden *et al.*, 1996). It was decided to carry out another experiment, to confirm the findings, before drawing conclusions about the reasons for the patterns of growth and enzyme excretion seen.

3.4: Medium Acidification

3.4.1: Acidification in glucose and starch containing media.

This experiment was conducted to confirm that the supernatant acidification seen in the previous experiment was reproducible and, if so, to investigate the reasons for the effect. *S. lividans* pQR318 spores were inoculated into SMM supplemented with glucose, starch and glutamate (each at 2.5 g/l w/v). Flasks supplemented with glucose + starch and starch + glutamate (each at 1.5 g/l w/v) were also used to see if this affected the patterns seen. Glutamate was chosen as one of the supplements because it feeds into central metabolism at the tricarboxylic acid (TCA) cycle as α-ketoglutarate. The flasks were incubated at 30 °C as in the previous experiment.

The results showed that the acidification was a reproducible effect (Fig. 3.10). Under these conditions the performance of the cells fell into two groups: flasks containing

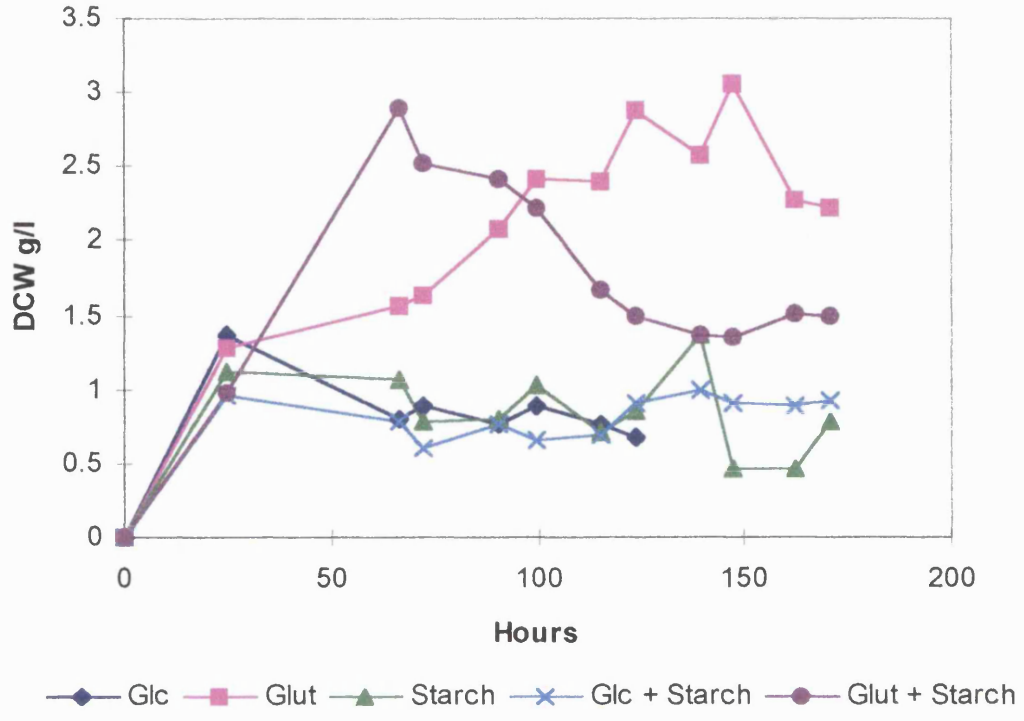


Figure 3.6: Growth of *S. lividans* pQR318 in SMM supplemented with glucose, glutamate, starch, glucose + starch and glutamate + starch.

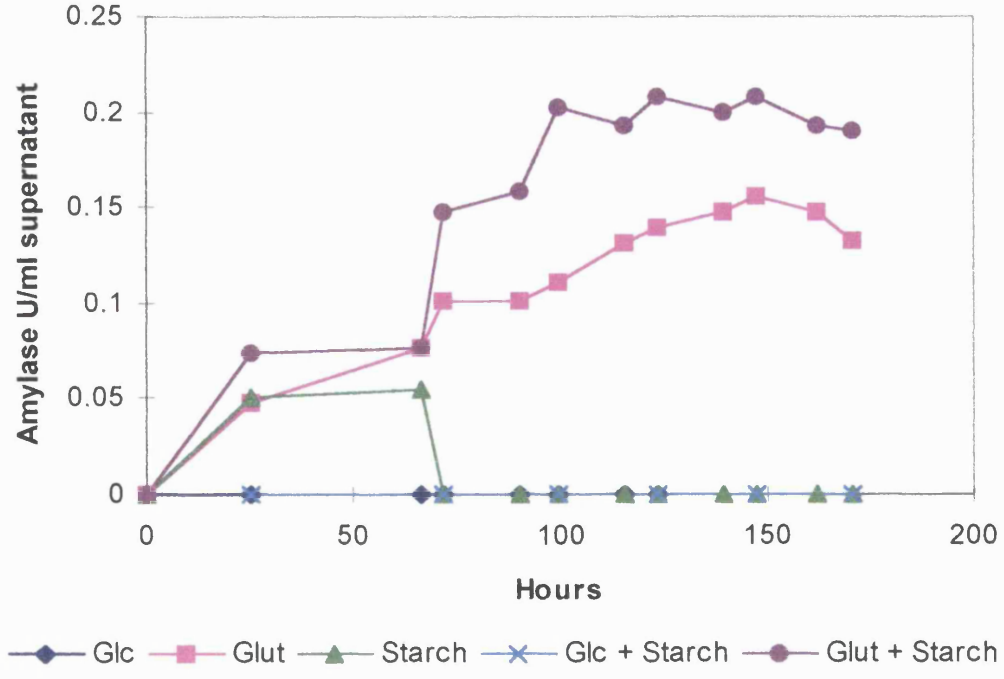


Figure 3.7: α-Amylase production by *S. lividans* pQR318 in SMM supplemented with glucose, glutamate, starch, glucose + starch and glutamate + starch.

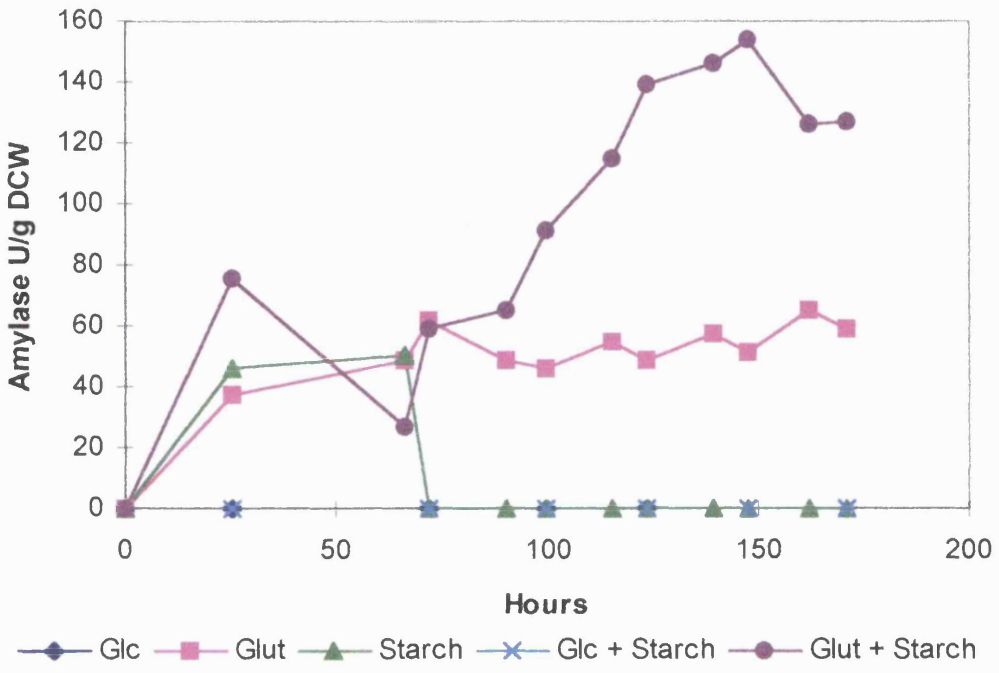


Figure 3.8: α-Amylase productivity levels for *S. lividans* pQR318 grown in SMM supplemented with glucose, glutamate, starch, glucose + starch and glutamate + starch.

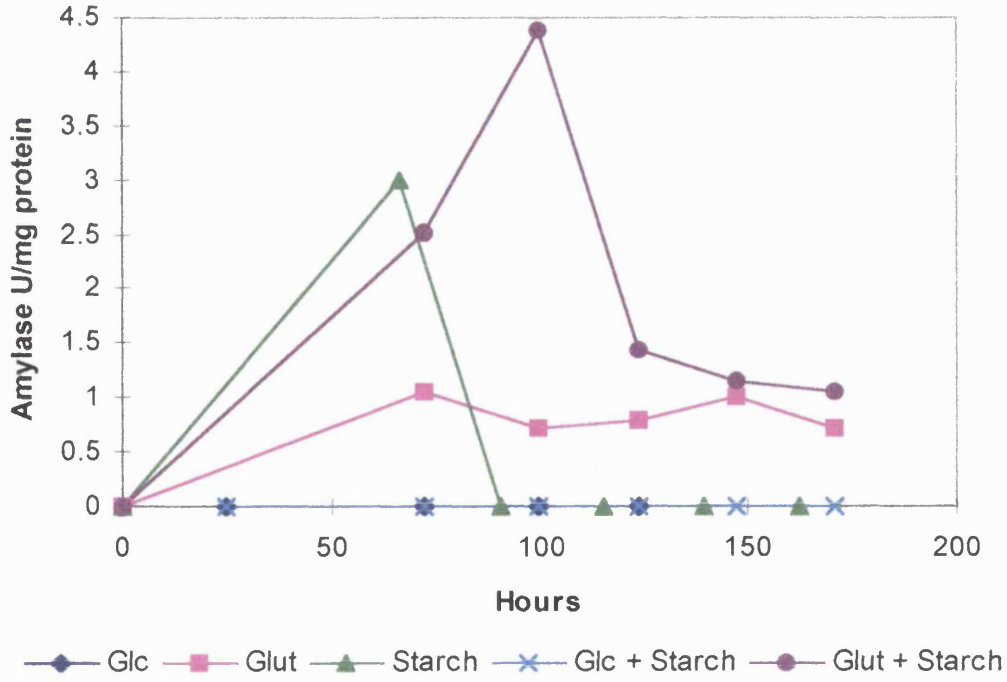


Figure 3.9: α-Amylase specific activity levels for *S. lividans* pQR318 grown in SMM supplemented with glucose, glutamate, starch, glucose + starch and glutamate + starch.

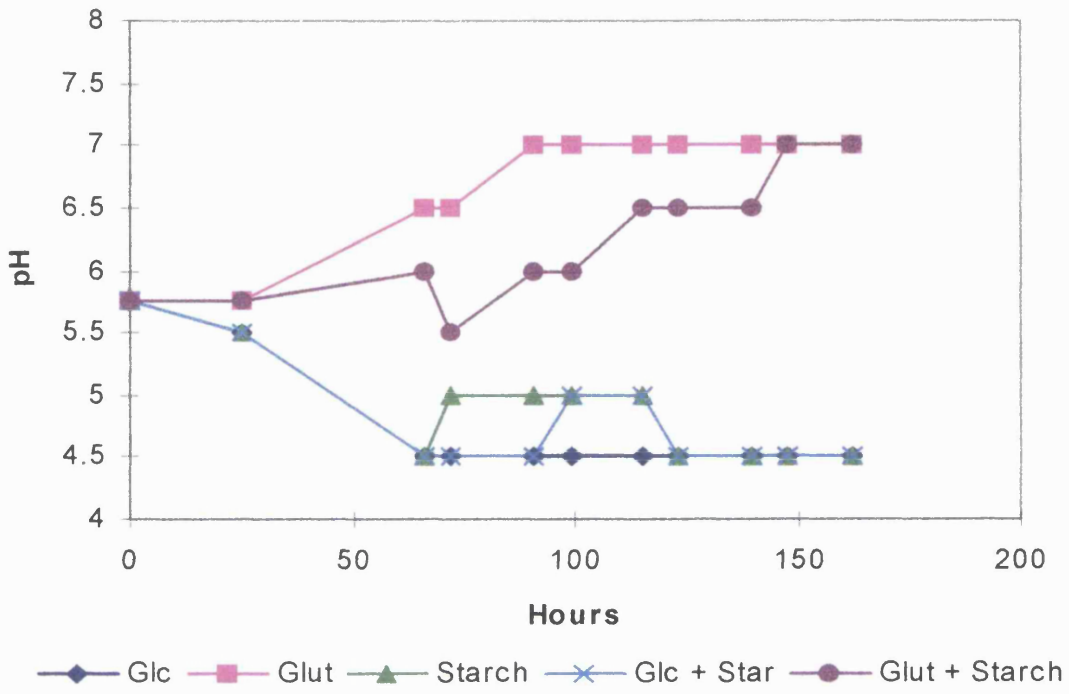


Figure 3.10: Supernatant pH fluctuations for *S. lividans* pQR318 grown in SMM supplemented with glucose, glutamate, starch, glucose + starch and glutamate + starch.

glutamate and flasks without it. The flasks containing glucose, starch and glucose + starch reached a biomass of around 1g DCW/l and then plateaued (Fig. 3.6). Only the flask containing starch alone showed any sign of α-amylase production (Fig. 3.7). However, this too was curtailed and no activity was present in the culture supernatant after 70 hours.

The flasks where glutamate was present showed more normal patterns of growth (Fig. 3.6). Supplementing the medium with glutamate + starch led to fast growth and a rapid entry into death phase. The flask containing only glutamate grew more steadily; taking about twice the time to reach its peak biomass. α-Amylase production levels from these flasks were comparable to those seen in the first experiment (Fig. 3.7). The flask supplemented with glutamate + starch gave the highest peak α-amylase titre and the highest productivity and specific activity (Figs. 3.8 & 3.9).

3.4.2: Possible mechanisms of acidification.

The pH profiles seen in these flasks may help to indicate why the various patterns of growth and α-amylase production were obtained. Many studies have described how supernatant pH fluctuations, along with a knowledge of medium composition, can be interpreted to allow certain conclusions to be drawn about substrate use and flow through central metabolism. *Streptomyces* species will accept quite a broad range of pH. However, they rarely grow in lower than pH 5.0 or above pH 9 (Lee & Lee, 1994). The optimum pH is around 7. Inhibition of growth and metabolism, as seen in the SMM + glucose, starch and glucose + starch flasks, has been commonly associated with supernatant pH values below 5.0 (Ahmed *et al.*, 1984; Dekleva & Strohl, 1987).

Organic acid excretion is usually found when cells are grown in a medium containing a rapidly metabolised carbon source such as glucose. Two main reasons for medium acidification have been put forward. Firstly, organic acid byproducts are believed to be excreted under aerobic conditions due to imbalances in the flux of metabolites through glycolysis and the TCA cycle (Goel *et al.*, 1995; Surowitz & Pfister, 1985). Secondly, some authors also postulate that the uptake of ammonium ions from the medium is a major cause of pH drop (DelaCruz *et al.*, 1992; Ahmed *et al.*, 1984).

Although most agree in principle that the presence of organic acids constitutes an important problem, the exact reason for their production is widely disputed. Surowitz and Pfister (1985) found that pyruvate excretion accompanied growth of *S. alboniger* in glucose-containing media. Under these circumstances they noted increased activity of the glycolytic enzymes, leading to pyruvate synthesis (phosphofructokinase and pyruvate kinase). However, this occurred in the absence of any increased activity of pyruvate catabolising enzymes (pyruvate dehydrogenase and citrate synthetase). This mismatch of activities was thought to explain acidification. In contrast, others have suggested that the accumulation of organic acids results from limitation of the TCA cycle capacity due to repression of TCA cycle enzymes during rapid growth (Goel *et al.*, 1995; Dekleva & Strohl, 1987). The common message in these studies is that the regulation of enzyme activities may play a central role in the phenomenon. Another relevant question is whether it is the acidification itself that causes inhibition of cell growth and metabolism or whether the acidification is a symptom of a physiological state which is unfavourable for the cells. DelaCruz *et al.* (1992) found that adding organic acid into the cultures in varying amounts caused no adverse effects. This raises the possibility that alternative, unidentified products are causing the observed inhibition of cell growth.

3.4.3: Influence of nitrogen source on acidification.

It is clear that acidification is heavily influenced by nitrogen source, although exactly how these effects are mediated is not clear (Ahmed *et al.*, 1984; Madden *et al.*, 1996). Adding TCA cycle precursors to try to address the metabolic imbalances assumed to be present has varying effects. When the constraint is at the level of TCA enzymic capacity this has been unsuccessful (Goel *et al.*, 1995). DelaCruz *et al.* (1992) found that incorporating a readily utilisable carbohydrate into a peptide-based complex medium resulted in enhanced growth and expression. Cultures without glucose supplements, or after glucose depletion, became more basic with time, suggesting that different types and levels of byproducts were produced in response to different initial glucose concentrations. They put forward two hypotheses to explain their results. When excess glucose is present with a complex nitrogen source they postulate that acidification occurs, not due to the production of organic acids by the cells, but because

deaminated acid products are being created from the peptides and amino acids present in the nitrogen source (Fig. 3.11A). When no glucose is available, but a complex nitrogen source is present, they suggest that the carbon skeletons from the nitrogen source are used for biosynthesis, resulting in the production of ammonium byproducts. This results in basification of the medium (Fig. 3.11B). The findings of Erpicum *et al.* (1990) are used as evidence to support this theory. They saw increased productivity from recombinant *S. lividans* when ammonium was added to MYEME medium to reduce the likelihood of amino acid catabolism. When ammonium was not included in their glucose supplemented complex medium, large pH fluctuations were observed and foreign protein production was reduced.

Madden *et al.* (1996) agree that the presence of ammonium plays a crucial role in preventing acidification. As previously mentioned, they found it completely abolished acid production. However, their findings contradict the hypothesis of DelaCruz *et al.* (1992) as to the source of acid production: they favour the overflow of α-ketoglutarate and pyruvate due to imbalances in central metabolism and found no evidence of acids from amino acid deamination. No acids were excreted when Madden *et al.* (1996) grew *S. lividans* in SMM containing only amino acids in both the presence and absence of ammonium. Use of radiolabelled substrates indicated that the pyruvate and α-ketoglutarate produced by cells grown in a medium containing glucose and amino acids (without ammonium) contained carbon from each of the substrates. Preliminary results indicated that this was also true of complex medium constituents such as malt extract and peptones.

3.4.4: Effect of individual medium components on supernatant pH fluctuations.

In these experiments acidification was only seen in the presence of the rapidly metabolised carbon sources, glucose and starch. This trend agreed well with the majority of other studies. In each case growth was subject to inhibition. This could have been due to low pH environments being unfavourable to growth or because the internal state of the cells was unsuited to further metabolism. Some evidence of reassimilation of organic acids exists in the flasks containing starch towards the middle of the time course. In each case a slight pH rise was seen in conjunction with a small increase in biomass levels. However, this effect was transitory and the pH soon

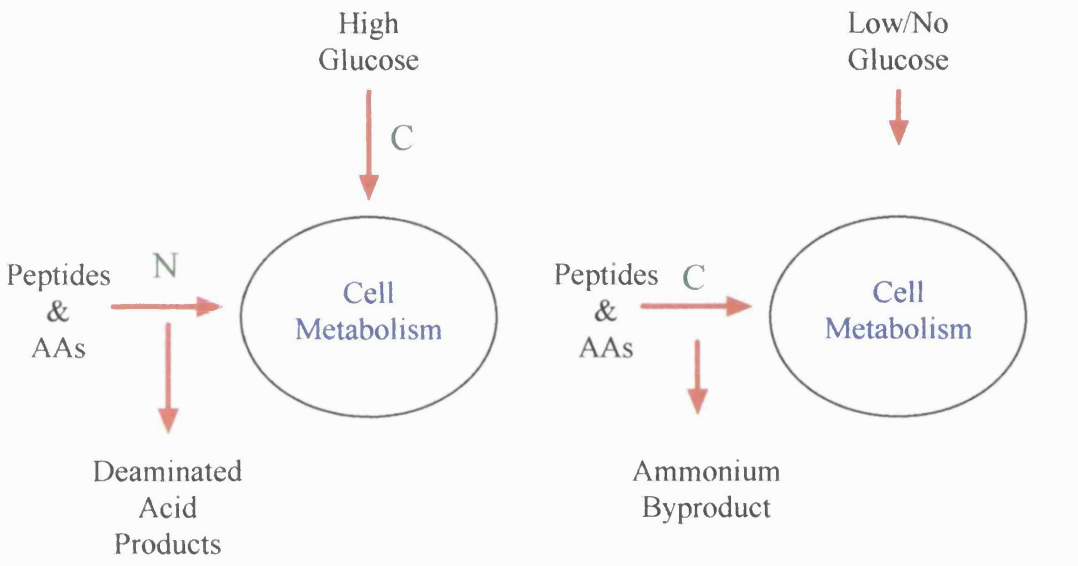


Figure 3.11: Hypotheses to account for byproduct accumulation under conditions of excess glucose (A) and insufficient/no glucose (B) conditions in complex media (from DelaCruz *et al.*, 1992).

dropped again. Ammonium must have been used as a nitrogen source by the cultures. This may also have contributed to the acidification of the medium as an accumulation of protons would be expected to occur when it was removed from the supernatant (Shapiro, 1989).

Growth on SMM + glutamate was more steady, as would be predicted in the absence of a rapidly utilised carbon source. The pH also showed a paced increase up to 7 where it levelled off. This must reflect the pattern seen when glutamate is used as a carbon source for growth. A similar pattern was seen from the SMM + Casamino acids culture in the first time course, although a slight acidification at the start of growth was observed. Differences between these two flasks must represent the effect of having a more varied mixture of amino acids to use as substrates.

No significant acidification was seen when starch was used in conjunction with the amino acid glutamate. As acidification had been seen in the flask with starch alone this difference must have been due to the presence of glutamate. It is possible that, as this amino acid feeds into the TCA cycle, it is acting to counteract overactive glycolysis. Alternatively, it could be acting as an inducer of TCA enzymes or a repressor of glycolytic enzymes; effectively giving more balanced metabolism. The pH remained in the same range over the first 100 hours of the experiment with no net rise. This was an unusual pattern as all of the other flasks in the study quickly went either up or down in a definitive manner. It would be explained if the glutamate was being used as a carbon source, as well as a nitrogen source, by the cells alongside the starch. It would seem feasible that any acidification, associated with the use of starch as a carbon source would therefore be balanced by a net production of ammonium ions as glutamate was assimilated. When the starch was exhausted, a typical pattern of pH rise would occur. This would lead to the scheme outlined in Figure 3.12 as an alternative to that of DelaCruz *et al.* (1992) (Fig. 3.12). Evidence for such a pattern would be supported by the radiolabelling studies of Madden *et al.* (1996) which suggested that carbon from both glucose and amino acids was found in pyruvate and α-ketoglutarate secreted by such cultures.

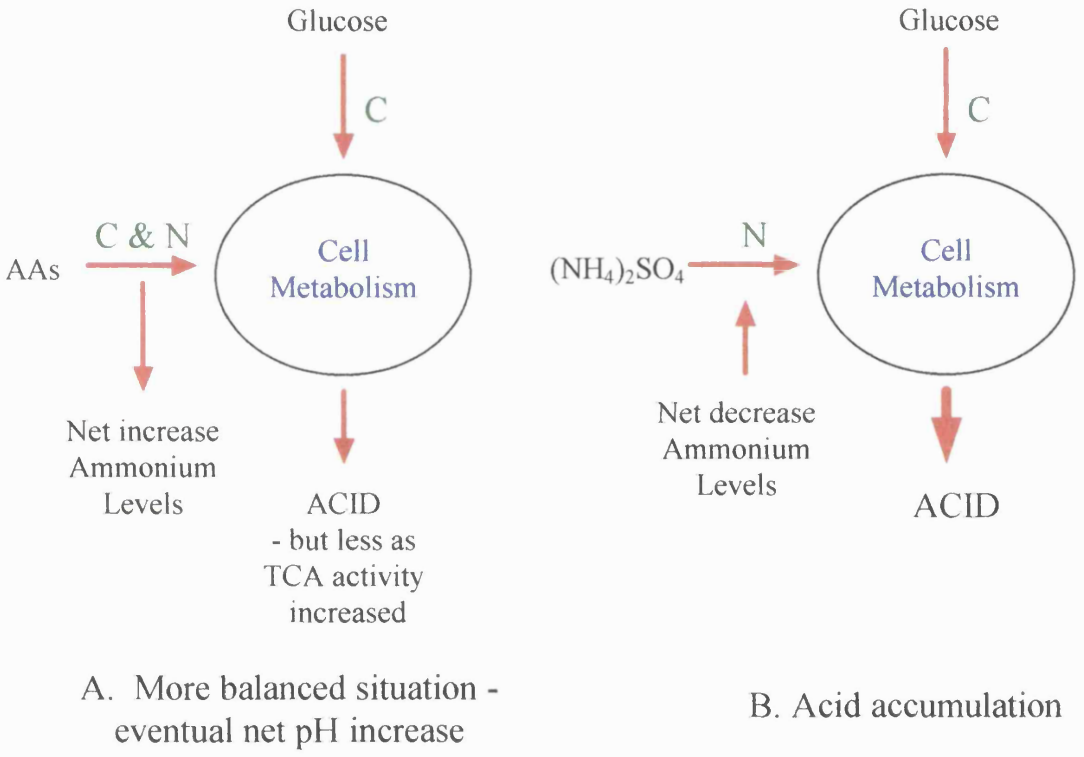


Figure 3.12: Hypotheses suggested by the results of this study to account for supernatant pH fluctuations seen under conditions where both amino acids and $(NH_4)_2SO_4$ are present (A) and only $(NH_4)_2SO_4$ is present (B) in supplemented SMM.

3.4.5: Amino acids may be the preferred nitrogen source of *S. lividans* TK24.

Very little is known about nitrogen metabolism in *Streptomyces*. Where research has been done into nitrogen uptake and assimilation, large variations in mechanism and regulation are found to occur from species to species (Fisher, 1988; Shapiro, 1989). This lack of solid information makes it difficult to draw conclusions about the effects of nitrogenous medium components on the system.

Ammonium ions are generally considered to be the preferred nitrogen source for almost all micro-organisms (Miller & Churchill, 1986). However, evidence is gathering that this does not hold true for *Streptomyces*. Several studies have indicated that the presence of ammonium can be inhibitory for the production of secondary metabolites (Aharonowitz & Demain, 1979; Cimburkova *et al.*, 1988). Recent evidence has suggested that ammonium is not necessarily the preferred nitrogen source in many streptomycetes. Zhang *et al.* (1996) found that *S. griseofuscus* NRRL 5324 used glutamate over ammonium as a nitrogen source for rapid initial cell growth. Such findings may indicate that amino acid medium constituents would be used in preference to ammonium in *S. lividans*. A comparison of the results from the starch + glutamate flask and the starch flask support this hypothesis. As the patterns of growth and acidification changed from the point of inoculation when glutamate was added, it is fair to assume that the changes were due to the immediate use of the amino acid in preference to $(NH_4)_2SO_4$. If the ammonium was used to the exclusion of the glutamate, the patterns in the two flasks would have been expected to agree until the ammonium was exhausted.

3.4.6: Oxygenation levels may influence acidification.

The results obtained in the time course experiments directly contradict those of Madden *et al.* (1996). Ammonium had no obvious effect in suppressing acid production. This result was surprising as the medium composition was directly comparable, and the concentration of $(NH_4)_2SO_4$ used was identical in each case (15mM). Ahmed *et al.* (1984) found plasmids to have no influence on acidification. The same is likely to hold true in this system, as the plasmids were not directing the production of large amounts of product. One major difference between the two studies is likely to have been the

levels of oxygenation within the flasks. Different sized flasks containing differing proportions of liquid were used. Madden *et al.* (1996) used 2l flasks containing 500ml of liquid (25% full), whereas 500ml flasks containing 200ml of medium (40% full) were used in this study. Both sets of flasks contained springs for pellet disruption and were incubated in a 28°C orbital incubator at 200rpm. This variation means that the cells in this study were growing in significantly lower oxygen concentrations.

Chatterjee *et al.* (1983) examined the effects of aeration on chloramphenicol production in *S. venezuelae*. They discovered increasing culture acidification as oxygenation was reduced. This suggests that major changes in central metabolism occur when oxygen is limited. As oxygen acts as the terminal electron acceptor in oxidative phosphorylation this is not too surprising. It may mean that uptake and use of medium components are altered in response to oxygen levels in a way that is most favourable to the cells. Vecht-Lifshitz *et al.* (1990) found that the maximal size of pellets was governed mainly by oxygen supply in *S. tendae*. A trend was seen toward the formation of many small pellets when oxygen concentrations were low and this effect could be seen even at the early stages of a fermentation. Therefore, another possible explanation would be that a smaller pellet size altered the pattern of nutrient use in some way, possibly by altering the transport of medium components into the cells. Further experimentation would be needed to explain these results fully and gain a greater understanding of the mechanisms involved in acidification.

3.4.7: Glucose may repress α-amylase production.

The fact that no enzyme production was seen in any of the flasks containing glucose may indicate that glucose repression was also acting to inhibit α-amylase expression. Some α-amylase was produced in the flask containing starch alone. The pattern of α-amylase activity seen provides evidence for glucose repression. At the very beginning of the time course some enzyme was produced. The α-amylase would then have immediately started to break the starch down into maltose. This would have been further metabolised into glucose in the cells. If glucose repression was operating this would have then fed back and inhibited the production of further α-amylase. The acidification seen in the flask meant that no conclusions could be drawn. The small amount of enzyme that was present in the supernatant disappeared rapidly. Low pH

may have caused the breakdown of this enzyme, along with any additional enzyme made by the cells. Alternatively, the conditions within the flask may have been favourable for protease production. Higher levels of α-amylase were obtained in the flask containing both starch and glutamate. The pattern of α-amylase production seen here is also suggestive of glucose repression as the productivity of the cells remained around the same level up until the cells entered stationary phase, at which point the starch would have been used up. Then productivity increased rapidly as if inhibition had been relieved.

Additional experiments would be required to give a definitive answer as to why no enzyme is produced in the presence of glucose. A shake flask experiment where glucose and glutamate are both used as supplements might be more revealing as the glutamate would be expected to counteract the production of organic acids and the concurrent inhibition of growth and metabolism associated with acidification. A more direct way of testing the system would be to use a bioreactor where the pH could be maintained at a suitable level despite acidification.

Further experimental work was conducted to investigate the observed glucose effect. These experiments are described and discussed in Chapter 7.

3.5: Minimal Medium plus Succinate (MMS)

The final medium tested in the preliminary experiments was a variation of minimal liquid medium (Hopwood *et al.*, 1985) supplemented with succinate (called MMS)(Section 2.4.1.1). The basal composition of this medium includes Casamino acids and is buffered to around pH 7.0. The findings of the previous experiment suggest this composition would tend to prevent medium acidification. It was decided to supplement the medium with succinate as an additional carbon source. This was chosen as it had given high α-amylase productivities as a supplement to SMM in the first time course (Fig. 3.3). These cultures were grown in 2l baffled flasks with springs in a volume of 500ml to prevent any possibility of oxygen limitation.

This medium was the only one tested where α-amylase production from *S. lividans* pQR311 was observed (Fig. 3.14). However, this was only a very small level of

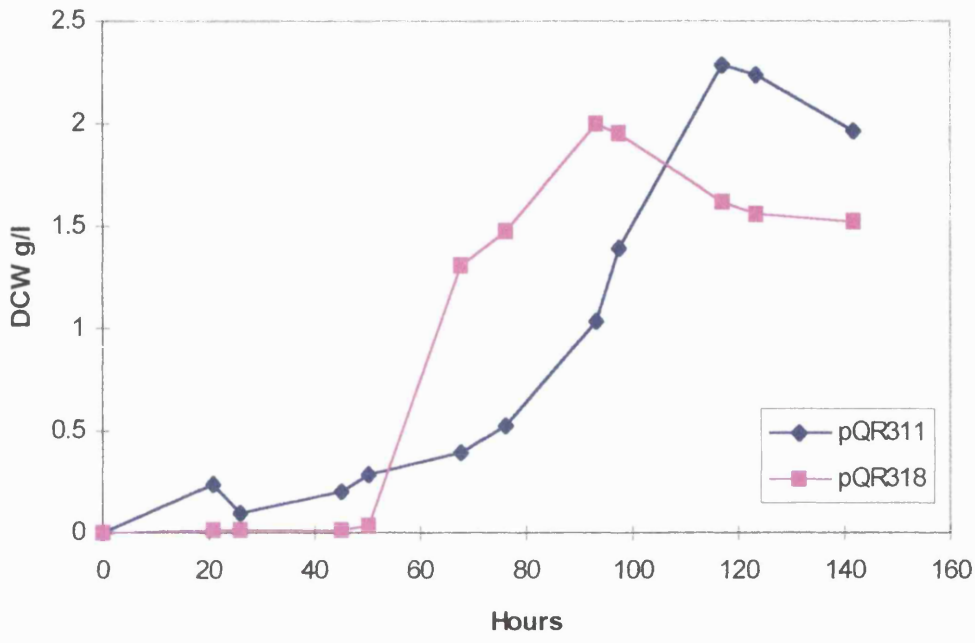


Figure 3.13: Growth of *S. lividans* pQR318 and *S. lividans* pQR311 in MMS.

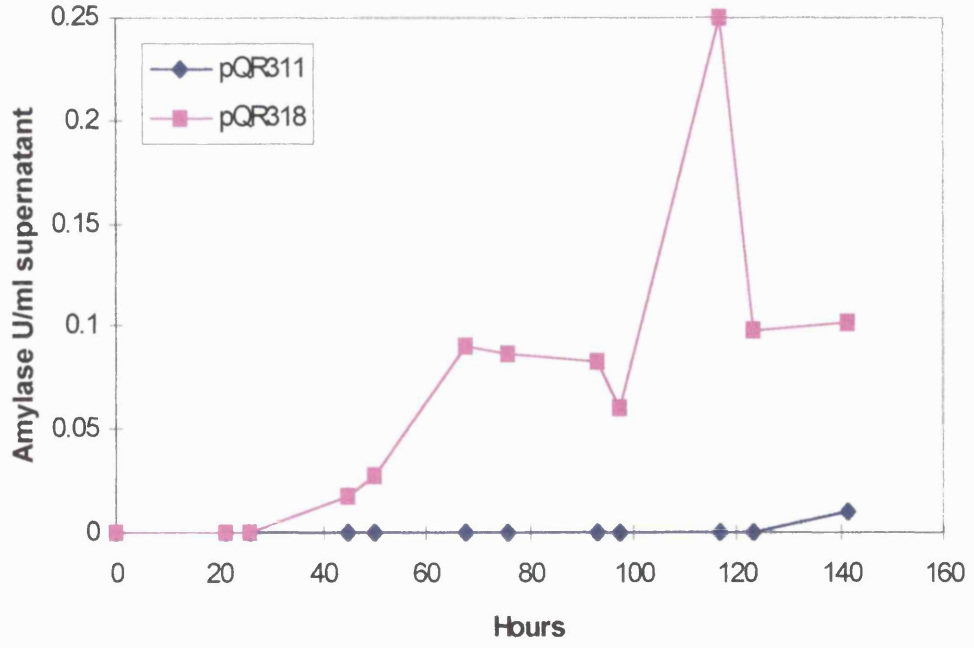


Figure 3.14: α-Amylase production by *S. lividans* pQR318 and *S. lividans* pQR311 grown in MMS.

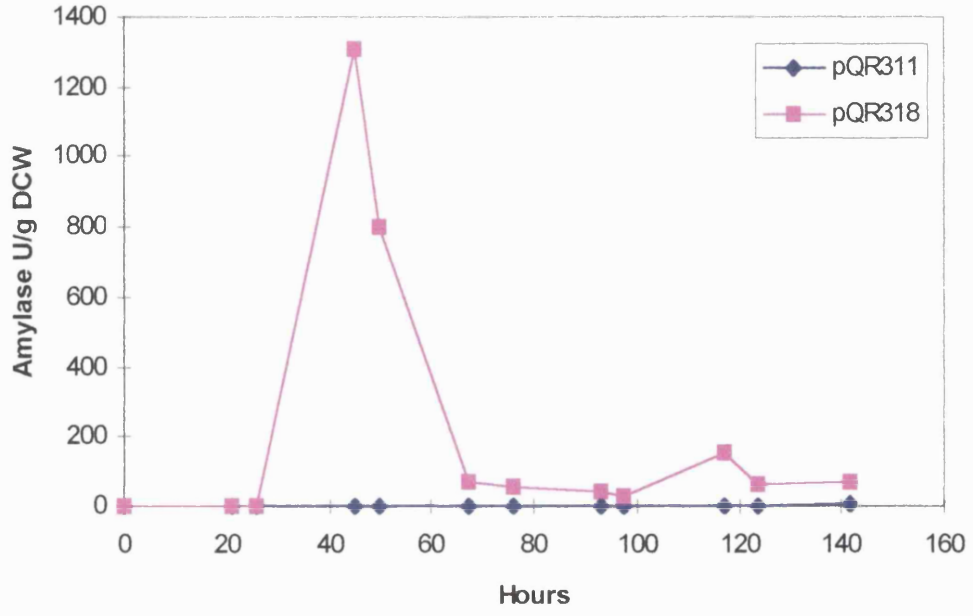


Figure 3.15: α-Amylase productivity levels for *S. lividans* pQR318 and *S. lividans* pQR311 grown in MMS.

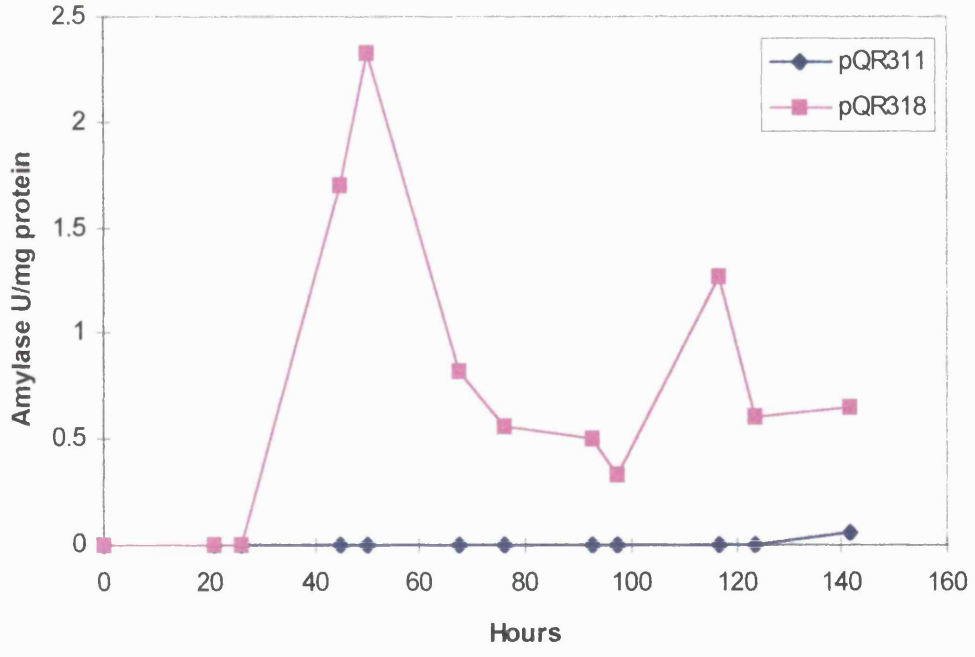


Figure 3.16: α-Amylase specific activity levels for *S. lividans* pQR318 and *S. lividans* pQR311 grown in MMS.

activity towards the end of the experiment. Both of the cultures grew to around 2g DCW/l (Fig. 3.13). A long lag time was seen in each of the flasks. The lag of the *S. lividans* pQR311 was significantly longer than that of *S. lividans* pQR318. This difference is likely to be due to variations in the spore inocula between the two strains. Although fairly little biomass was present in the *S. lividans* pQR318 culture over the first 60 hours, what was there was productive with each gram of cells making over 1000 U of α-amylase at one point (Fig. 3.15).

The peak levels of α-amylase produced by *S. lividans* pQR318 during this experiment were similar to those seen in the other two experiments (Fig. 3.14 versus Fig. 3.2 & Fig. 3.7). As expected no significant acidification was seen in this medium. The supernatant became gradually more alkaline as the experiment progressed.

3.6: α-Amylase Production on Solid Media

As discussed previously (Section 3.2) French (1993) found the *S. lividans* constructs to produce approximately 15 to 20-fold lower levels of enzyme than the *E. coli* constructs. The results described above indicated that this differential was even greater. However, when the relevant cells were plated out on starch containing agar plates the zones of starch hydrolysis seen were fairly similar. *E. coli* strains produced zones about twice the size of those seen from the *Streptomyces* strains. Although only 40-70% of the *E. coli* produced α-amylase would be expected to be extracellular (Pierce, 1996) this would still represent a far larger amount of α-amylase than that thought to be produced by the *Streptomyces*. This would lead to the expectation that the zones of starch hydrolysis produced by colonies of *E. coli* pQR126 and pQR187 would be far larger than those produced by *S. lividans* pQR318 and pQR311.

This inconsistency between observed and expected results raised the possibility that lower levels of α-amylase were produced by the *Streptomyces* strains in liquid than were produced on solid media. It was important to look at this possibility. If such behaviour was being displayed by the system it would suggest that trying to use genetic means to further raise the levels of α-amylase produced in *Streptomyces* would still only result in low α-amylase levels in liquid media. Attempting to understand the difference in levels under the different conditions and trying to overcome any problem

would therefore become more important aims. Two experiments were conducted to try to unravel the inconsistency.

3.6.1: The linear relationship between zone size and log α-amylase activity.

The size of radii of zones of starch hydrolysis, produced by known levels of α-amylase, were determined as described in Section 2.6.11.1. A plot of zone radius versus log α-amylase concentration showed that a linear relationship exists between these parameters at this range of concentrations (Fig. 3.17). Measurement of very small and very large zones was difficult to carry out accurately. This was reflected by larger deviations from the predicted relationship below zone radii of 4mm and above zone sizes of 10mm. *E. coli* α-amylase and *S. lividans* α-amylase produced zones of the same size at the same concentration. This indicates that the enzyme obtained from both sources acts in the same way in this system and that other factors such as proteases or differences in protein processing are unlikely to be playing a role. These results show that it only requires a very small amount of α-amylase to produce a large zone of hydrolysis, e.g. 0.003 units of amylase produced a zone of 10mm radius under these conditions. This data, and the data from the preliminary experiment indicated that zone size became limited by something other than enzyme concentration at between 0.8 and 0.5 α-amylase U/ml. Zone diameter remained around 60mm for all concentrations between 0.8 and 15 α-amylase U/ml after 100 hours incubation.

3.6.2: Production of zones of starch hydrolysis over time by *E. coli* pQR126 and *S. lividans* pQR318 colonies.

A second experiment was undertaken to look at the size of zones produced over time by *E. coli* pQR126 and *S. lividans* pQR318 colonies (Section 2.6.11.2). The results from this experiment are shown in Figure 3.18. The zone from the *E. coli* colony grew quickly and kept growing over the course of the experiment, reaching a diameter of around 70mm after 200 hours. No zone was visible from the *S. lividans* pQR318 colony for the first 20 hours. Then it grew steadily with time, reaching a peak of around 30mm after 200 hours. These zone sizes were fairly representative of those seen when the two strains had been plated out previously.

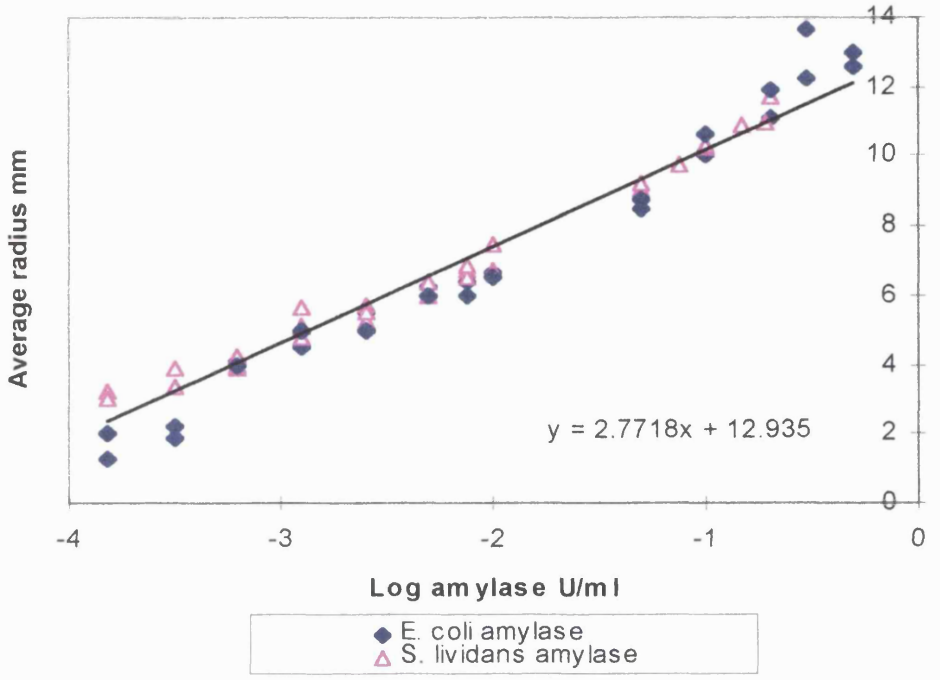


Figure 3.17: Graph showing the relationship between size of starch hydrolysis zones and log α-amylase activity/ml on starch containing agar plates.

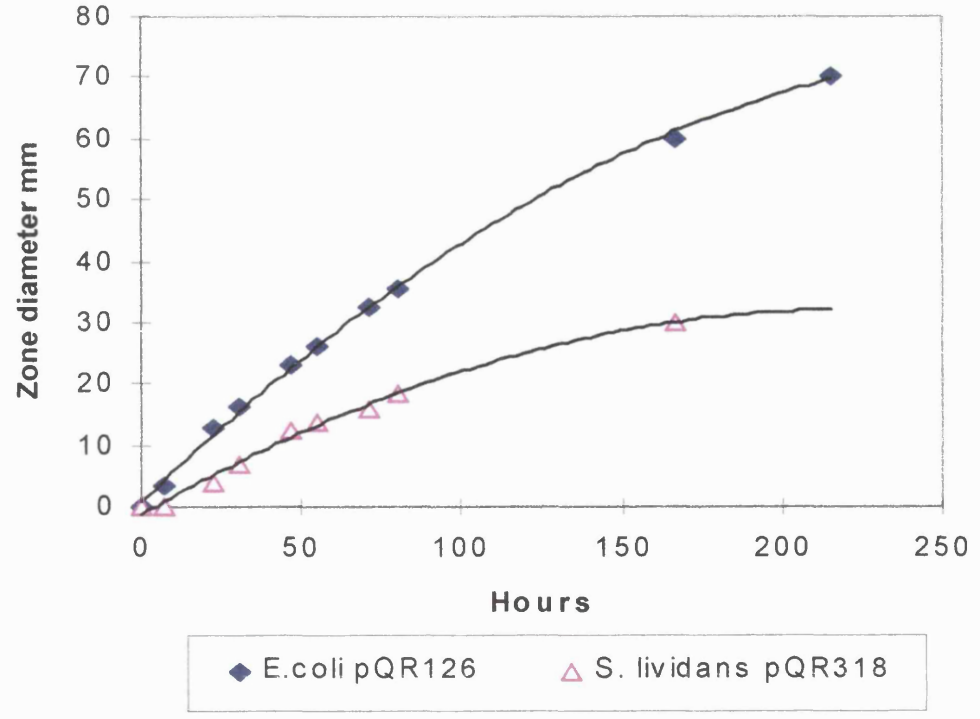


Figure 3.18: Production of zones of starch hydrolysis over time from colonies of *E. coli* pQR126 (37°C) and *S. lividans* pQR318 (30°C).

3.6.3: Starch hydrolysis zone sizes on solid media correlated with observed levels of α-amylase production in liquid media.

The large number of parameters affecting the system make it difficult directly to compare the two hosts. The time over which the enzyme was allowed to diffuse affected the final zone size. The first experiment therefore gave a false picture as the α-amylase was present in the well at the start of the experiment and was not produced over time as would be the case from a colony. The first experiment was conducted at the optimum temperature for the α-amylase. The zones produced at 37°C and 30°C would therefore be expected to be correspondingly smaller for the same level of activity.

It was felt that the best way to compare levels of activity produced was to read off the size of the zones at 42 hours from the time course experiment and then calculate how much α-amylase activity this represented, using the relationship described by the first experiment. This method assumed that the effects of temperature and rate of α-amylase production were negligible. The *E. coli* pQR126 colony had produced a zone of 12mm radius after 42 hours. The *S. lividans* colony had produced a zone of 4.5mm radius over the same period. These correspond to α-amylase levels of 0.46 U/ml and 9.1×10^{-4} U/ml respectively.

Although this is only a rough comparison it indicates that the relative levels of α-amylase production seen in liquid culture are repeated on solid media. The above comparison suggests that over 500 times more α-amylase had been produced by the *E. coli* pQR126 colony after 42 hours than had been produced by the *S. lividans* pQR318 colony. This differential is probably an overestimate, as the temperature effects and growth rates of the cells will tend to exaggerate slightly the performance of the *E. coli* at 42 hours. The small amounts of activity required to produce large zones, and the fact that maximum zone sizes are probably often limited by factors other than the level of α-amylase present, lead to the conclusion that the observations seen in liquid and on solid media do concur. Higher levels of production or excretion by *S. lividans* pQR318 on solid media can therefore be discounted.

3.7: Key Findings

3.7.1: Choice of a liquid medium for use throughout the study.

The main aim of the work described in this chapter was to decide on a basal medium to be used throughout the course of the study. The preliminary experiments suggested that rapidly utilised carbon sources were not suitable for good growth and α-amylase production, as medium acidification tended to occur. Madden *et al.* (1996) found that 6 to 18% of the carbon provided to *S. lividans* cultures was excreted as organic acid. This represents a significant waste of substrate which would be better used if it were to be channelled into growth and product. Some signs were also present that glucose repression of α-amylase production might be occurring.

Both of these problems could theoretically be overcome by using fed-batch culture (Payne *et al.*, 1990; DelaCruz *et al.*, 1992; Goel *et al.*, 1995; Zhang *et al.*, 1996). The literature suggests that this would have the potential to be a very good way of maximising α-amylase production. The point of this study, however, was to investigate the factors affecting recombinant protein production in *S. lividans*, not to optimise protein production. It was envisaged that the majority of the work would be carried out in shake flask culture rather than on a large scale. This in itself meant that using fed-batch was not a viable option. Use of media based on rapidly metabolised carbon sources such as glucose and starch was therefore discounted, because acidification was likely to cause a problem.

TSB gave very good growth. However, the cell productivity was poor and very high levels of background protein were produced by the cells, so the specific activity was low. These findings suggested this medium was not particularly suitable for α-amylase production. MYEME medium looked promising but the high viscosity of the medium, and the extremely long lag period prior to growth and enzyme production, meant that it would not be a viable option for the study. Easy and accurate measurement of dry cell weight would also have been difficult.

MMS medium was eventually singled out as the basal medium of choice. Levels of α-amylase produced in this medium were relatively high and α-amylase specific activities were good. This medium was buffered and did not support acidification. It also

allowed α-amylase production from the early stages of growth right through until late stationary phase. Exponential growth was fairly rapid and significant cell lysis was not observed after peak biomass had been obtained. The fact that this was the only medium where any production was seen from *S. lividans* pQR311 added to the case.

3.7.2: Type, age and size of inoculum.

As previously discussed (Section 3.3.1), the type and size of inoculum used was likely to account for the differences in the levels of α-amylase seen between these experiments and those conducted by French (1993). These findings underlined the importance of inocula upon the performance of a system. At this stage of the study it was felt that attempting to define an 'optimum' inoculum was not an attainable goal. Choosing to use one particular type of inoculum was discounted, as what might be good under some conditions would probably give a poor performance under others. However, it would only be possible to make valid comparisons between experiments when the inoculum used was of similar age, type and size. It was decided to keep the inocula used throughout a group of experiments as consistent as possible. A comparison of different groups of experiments would then allow conclusions to be drawn about which type of inoculum performed best under which circumstances.

3.7.3: Requirement for new α-amylase expression vectors.

The expression vectors pQR318 and pQR311 gave low levels of α-amylase production from *S. lividans* both in liquid and on solid media. These results confirmed that significantly higher levels of enzyme production would need to be obtained from the system before *S. lividans* could be considered as a viable alternative host organism to *E. coli*. Construction of a series of new expression vectors was the next logical step towards the goal of attaining significantly higher levels of α-amylase.

3.8: Summary

Minimal medium with succinate (MMS) was chosen as the most suitable medium to use throughout the study. Media containing rapidly utilised carbon sources, such as glucose and starch, were not suitable. Medium acidification tended to occur when they were present and growth and α-amylase production were repressed. The type of nitrogen

source supplied affected supernatant pH fluctuations. The findings suggested amino acids were used in preference to ammonium. Presence of ammonium ions in the medium did not prevent acidification. Only low levels of α-amylase production were obtained from *S. lividans* pQR318 and pQR311. No variation was found between levels of enzyme production from the strains on solid or in liquid media.

Chapter 4

Construction and Evaluation of New α-Amylase Expression Vectors

4.1: Aims

The low levels of α-amylase production given by vectors pQR311 and pQR318 made it hard to determine whether changing a culture parameter had had a true effect on the expression system, or whether effects were due to natural variation. The main aims of the work described in this chapter were to investigate the effect of alterations in vector construction on expression level and, through this work, to produce new plasmids capable of directing significantly higher levels of α-amylase production in *S. lividans*. It was hoped raised production could be achieved by creating plasmids with increased efficiencies of plasmid replication and higher levels of transcription from the α-amylase gene. Following plasmid construction, a shake flask study was conducted to evaluate the performance of the new expression vectors.

4.2: Background

Although the complex regulatory systems allowing temporal and spatial regulation of gene expression within *Streptomyces* are not yet fully understood, expression vectors have been constructed and many different proteins have been produced successfully at high levels (reviewed by Gilbert *et al.*, 1995). A wide variety of regulatory signals were used in these constructs yet no defined guidelines have emerged for the construction of vectors that will reliably express large amounts of protein.

Extra-chromosomal vectors need to have stable modes of replication and inheritance, as well as possessing efficient transcription and translation signals. The vast majority of multicopy *Streptomyces* plasmids, e.g. pIJ702 derived vectors, are based on the minimal replicon of plasmid pIJ101. Significantly, these plasmids lack the primary site for second-strand initiation (*ssi* or minus-strand replication origin) which is necessary for efficient rolling circle plasmid replication (Section 1.2.3)(del Solar *et al.*, 1987; Gruss & Ehrlich, 1989; Zaman *et al.*, 1993a). Absence of this signal leads to a reduction in copy number and causes the build up of a pool of single-stranded (ss) DNA within the cells (Deng *et al.*, 1988 ; Kieser *et al.*, 1988). This is thought to contribute to plasmid

structural instability (Pigac *et al.*, 1988; Zaman *et al.*, 1993b). Both of the streptomycete α-amylase expression vectors, pQR311 and pQR318, lack the minus-strand replication origin.

4.3: New α-Amylase Expression Vectors

4.3.1: Strategies for obtaining increased expression of the α-amylase gene.

When planning the construction of new α-amylase expression vectors it was decided to use two different strategies to try to achieve increased levels of gene expression. The first strategy involved attempting to increase plasmid stability and copy number by incorporating the minus-strand origin on the new constructs. It was hoped such a change would lead to higher α-amylase expression levels over the course of a fermentation.

Zaman (1991) constructed a series of pIJ702 based vectors which contained the site for second-strand initiation (*ssi*) on a 0.53 kb *Spe*I-*Sac*II fragment from pIJ101 (called the *sti* region). It was decided to use the plasmid pQR443a as a base for the new constructs (Fig. 4.1). In addition to carrying the minus-strand origin this plasmid is a *Streptomyces-E. coli* shuttle vector. This was a useful characteristic as it allowed *E. coli* to be used as a host during plasmid construction and selection, thereby speeding up and simplifying the process.

The second strategy used to try and raise α-amylase production levels was to place the natural *amy* promoter in a tandem arrangement with another streptomycete promoter. Several authors had observed increased levels of gene expression when the *mel* promoter, of the tyrosinase (*mel*) genes on pIJ702 (Katz *et al.*, 1983; Leu *et al.*, 1989), was placed upstream of other streptomycete promoters (Koller & Riess, 1989; Koller *et al.*, 1989; Bender *et al.*, 1990b; Taguchi *et al.*, 1992). Steiert *et al.* (1989) found that the *mel* promoter in combination with the *opd* promoter region, contributed to increased gene expression over either promoter alone. It was hoped such an effect could be exploited to raise levels of α-amylase gene expression and secretion.

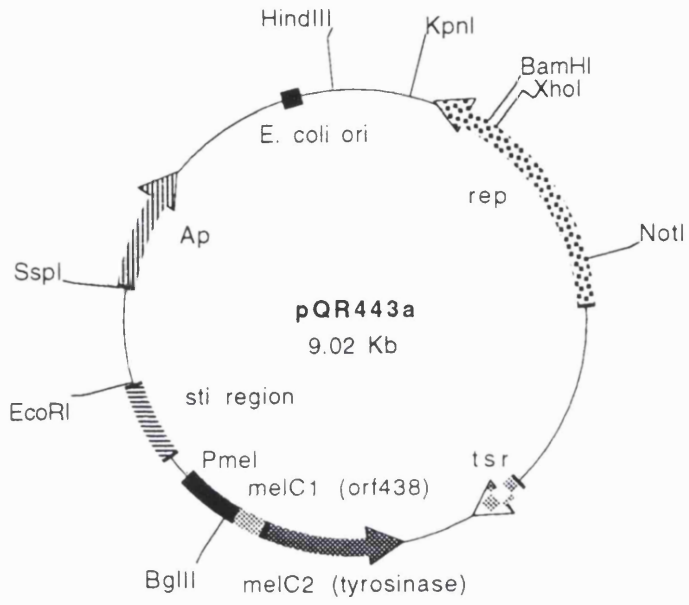


Figure 4.1: Plasmid pQR443a (Zaman, 1991). This plasmid was used as the base for the new α-amylase expression vectors pQR620 and pQR621. It contains the *sti* region from plJ101 and is a *Streptomyces-E. coli* shuttle vector.

4.3.2: Construction of plasmids pQR620 and pQR621.

A 3.1 kb *Bam*HI-*Bgl*II fragment was isolated from the *E. coli* α-amylase expression plasmid, pQR300. This fragment contained the entire *S. thermoviolaceus* α-amylase gene and its promoter region. The fragment was ligated into the *Bgl*II site of pQR443a (Fig. 4.1). This site is within an open reading frame sequence (*orf*438) that lies between the *mel* promoter and the *mel* structural genes. This produced two new plasmids, pQR620 and pQR621. In pQR620 the *amy* fragment is located so that the *amy* promoter and the *mel* promoter are arranged in tandem upstream of the *amy* gene (Fig. 4.2). Plasmid pQR621 contains the *amy* fragment inserted in the opposite orientation (Fig. 4.2). In this plasmid α-amylase expression is driven by the *amy* promoter alone.

Both of these new constructs successfully transformed *S. lividans* protoplasts to thiostrepton resistance. Large spore preparations were prepared from each new strain (Section 2.4.6). These were aliquotted, frozen and used as a source of inocula for the entirety of the study.

4.4: Evaluation of the New Plasmids in *S. lividans* TK24

4.4.1: Shake flask comparisons between new and old constructs.

Shake flask experiments were conducted to compare the patterns of growth and α-amylase production from the new constructs, pQR620 and pQR621, with those from the old constructs, pQR311 and pQR318. *S. lividans* pIJ702 was also included as a control. This combination of flasks allowed analysis of the effects of the *mel* promoter and the *ssi* sequence on the system.

This experiment was conducted in 2l baffled shake flasks containing 500ml of MMS medium (Sections 2.4.1.1 & 2.4.7). Each flask was inoculated with an aliquot of frozen spores and was incubated at 30°C.

4.4.1.1: *S. lividans* TK24 pQR620 and pQR621 produced high levels of α-amylase.

After a couple of days incubation it was clear that the cells containing the new constructs were producing significantly higher levels of secreted α-amylase than those

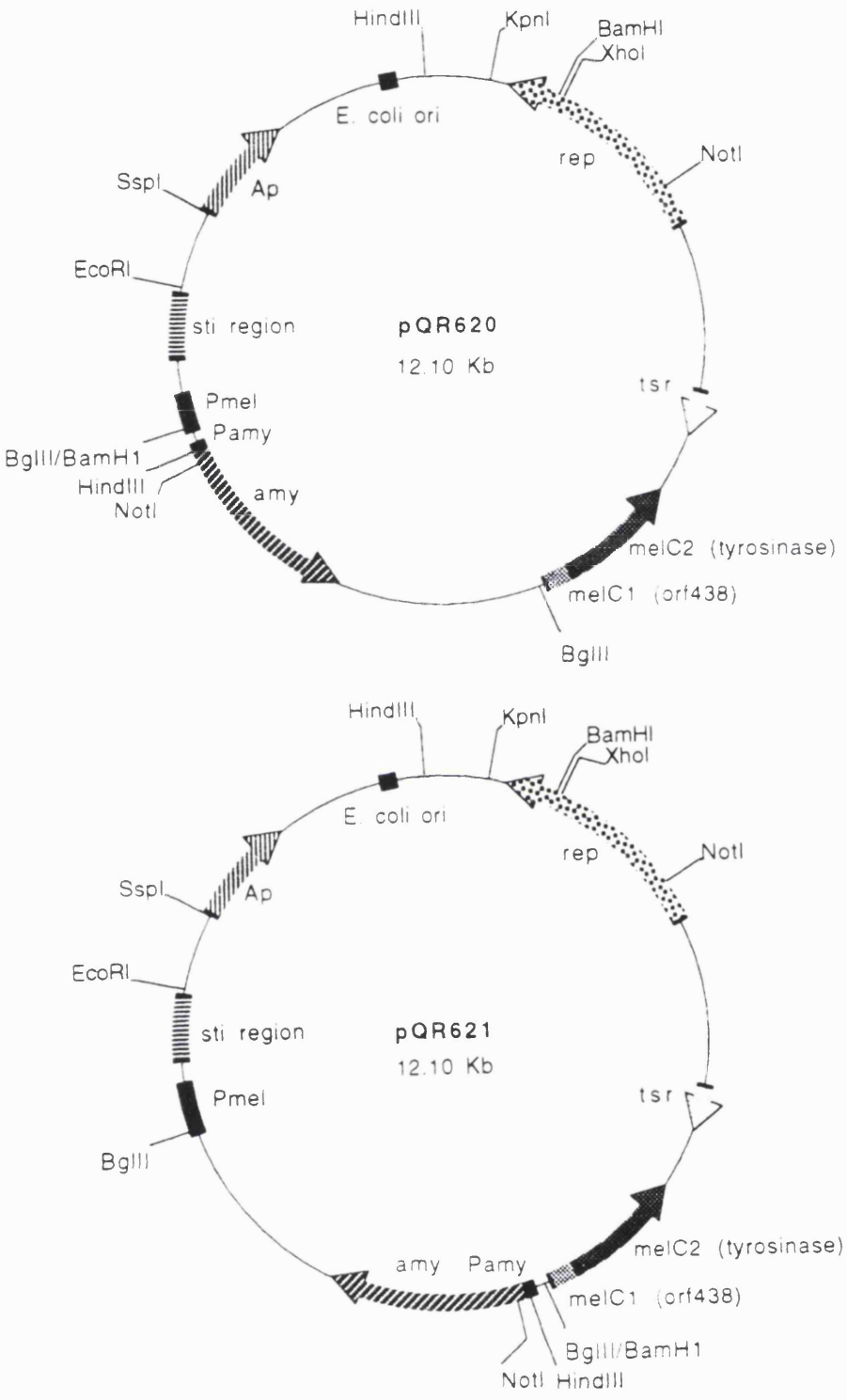


Figure 4.2: The newly constructed α-amylase expression vectors pQR620 and pQR621. A 3.1 kb *Bgl*II-*Bam*H1 fragment carrying the α-amylase gene (*amy*) and promoter was isolated from pQR300 (Bahri, 1990). This fragment was inserted into the *Bgl*II site on pQR443a (Fig. 4.1) in both orientations to give plasmids pQR620 and pQR621. In pQR620 the tyrosinase promoter (P_{mel}) and the amylase promoter (P_{amy}) are positioned in tandem upstream of the *amy* gene. In pQR621 only the P_{amy} is located upstream of the *amy* gene.

containing the old constructs. *S. lividans* pQR620 and pQR621 steadily produced enzyme over the length of the time course (Fig. 4.4). *S. lividans* pQR620 produced the greatest amount of enzyme, peaking at 13.4 α-amylase U/ml and then levelling off at around 9.0 U/ml. *S. lividans* pQR318 secreted 0.8 α-amylase U/ml supernatant. The cells carrying plasmid pQR311 only made 0.085 α-amylase U/ml supernatant. As expected, no detectable α-amylase was produced by *S. lividans* pIJ702. The levels and patterns of growth seen in each flask were very similar (Fig. 4.3). This was reflected in the maximum specific growth rates of the cultures (μ_{max})(Table 4.1). All fell within 0.007 h^{-1} of each other, with the exception of *S. lividans* pQR311 which was slightly slower growing at a μ_{max} of 0.041 h^{-1}. Again, the differences seen probably reflect variations in the spore inocula given to the flasks (Section 3.3.1). Slightly higher peak supernatant protein levels were seen from the cells containing pQR311 and pQR318 than were seen from the other cultures (Fig. 4.5). *S. lividans* pQR620 and pQR621 cells secreted significant levels of protein into the supernatant at an earlier stage in the time course than the other strains. This difference can be attributed to the shorter lag in growth exhibited by these strains (Fig. 4.3). The productivity and specific activity data confirmed the trends seen for supernatant α-amylase levels (Figs. 4.6 & 4.7). *S. lividans* pQR620 performed better than the other cultures in each case.

Densitometry scanning had previously indicated that 1 unit of α-amylase was equivalent to 2.6μg of protein (French, 1993). This allowed the peak levels of α-amylase protein/l to be calculated for each culture (Table 4.1). During this experiment, cell extracts were assayed for α-amylase activity alongside the culture supernatants. This was considered prudent as it was conceivable that at higher levels of enzyme production a bottleneck would form at secretion in the *S. lividans* cells. However, no detectable levels of α-amylase were found in any of the cell extracts tested.

4.4.2: Analysis of single-stranded (ss) DNA levels in the cells.

Shake flask cultures of *S. lividans* pQR620, pQR621, pQR311 and pQR318 were grown in MMS as described in Section 4.4.1. Cultures of *S. lividans* pIJ702 and pIJ303 were also grown to act as positive and negative controls respectively. The flasks were incubated at 30°C for 44 hours and were then harvested. Each supernatant was assayed

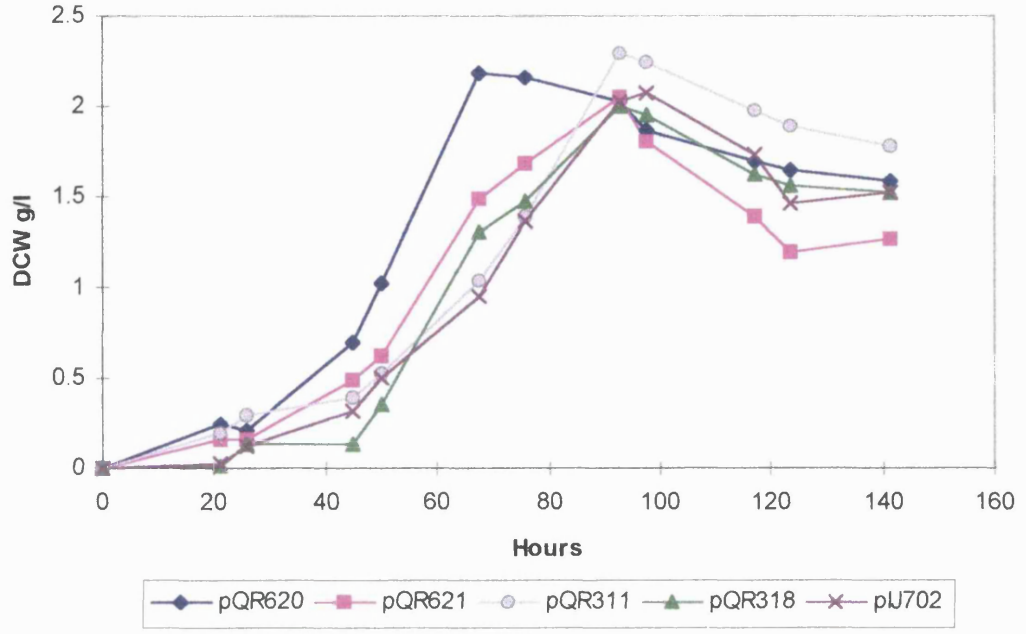


Figure 4.3: Growth curves for the time course experiment comparing *S. lividans* pQR620 (*amy*$^+$, *sti*$^+$, P_{mel}, P_{amy}), *S. lividans* pQR621 (*amy*$^+$, *sti*$^+$, P_{amy}), *S. lividans* pQR311 (*amy*$^+$, *sti*$^-$, P_{amy}) and *S. lividans* pQR318 (*amy*$^+$, *sti*$^-$, P_{pIJ325}). *S. lividans* pIJ702 (*amy*$^-$, *sti*$^-$) was included in the experiment as a control. The cultures were grown in MMS at 30°C. The average standard error of the mean (SEM) was 0.026 g DCW/l.

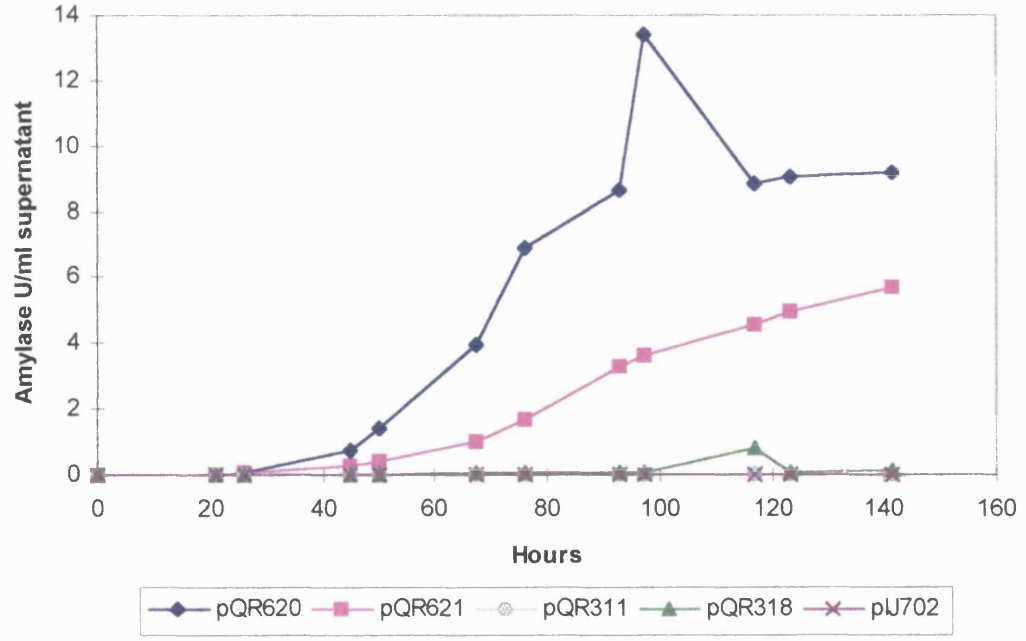


Figure 4.4: α-Amylase production in the time course experiment comparing *S. lividans* pQR620 (*amy*$^+$, *sti*$^+$, P_{mel}, P_{amy}), *S. lividans* pQR621 (*amy*$^+$, *sti*$^+$, P_{amy}), *S. lividans* pQR311 (*amy*$^+$, *sti*$^-$, P_{amy}) and *S. lividans* pQR318 (*amy*$^+$, *sti*$^-$, P_{pBR325}). *S. lividans* pIJ702 (*amy*$^-$, *sti*$^-$) was included in the experiment as a control. The cultures were grown in MMS at 30°C. The average standard error of the mean (SEM) was 0.105 U/ml.

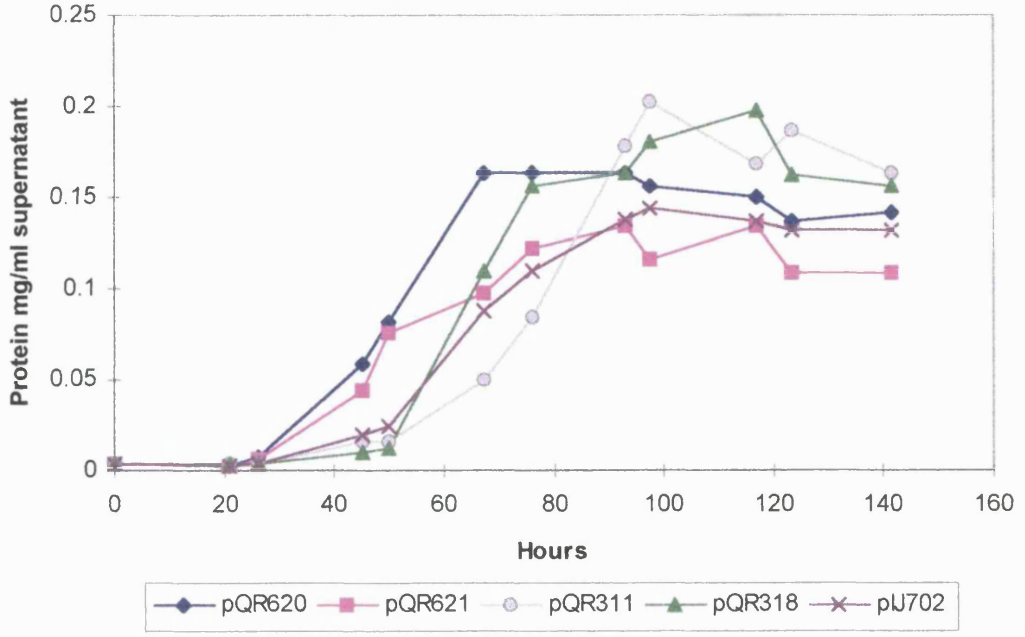


Figure 4.5: Supernatant protein levels in the time course experiment comparing *S. lividans* pQR620 (*amy*$^+$, *sti*$^+$, P_{mel}, P_{amy}), *S. lividans* pQR621 (*amy*$^+$, *sti*$^+$, P_{amy}), *S. lividans* pQR311 (*amy*$^+$, *sti*$^-$, P_{amy}) and *S. lividans* pQR318 (*amy*$^+$, *sti*$^-$, P_{pBR325}). *S. lividans* pIJ702 (*amy*$^-$, *sti*$^-$) was included in the experiment as a control. The cultures were grown in MMS at 30°C. The average standard error of the mean (SEM) was 0.003 mg/ml.

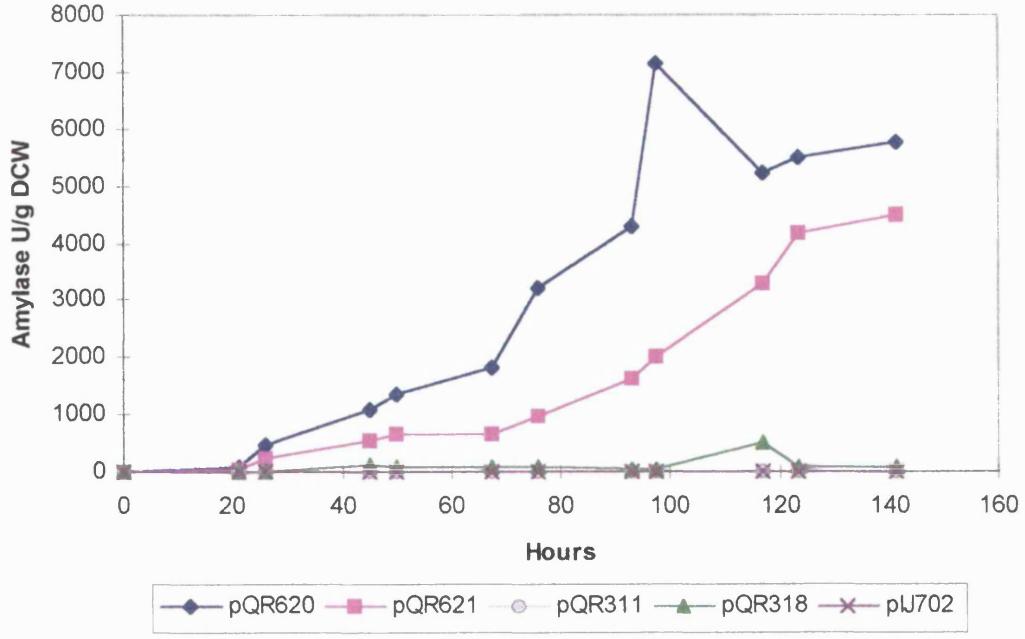


Figure 4.6: α-Amylase productivity levels in the time course experiment comparing *S. lividans* pQR620 (*amy*$^+$, *sti*$^+$, P_{mel}, P_{amy}), *S. lividans* pQR621 (*amy*$^+$, *sti*$^+$, P_{amy}), *S. lividans* pQR311 (*amy*$^+$, *sti*$^-$, P_{amy}) and *S. lividans* pQR318 (*amy*$^+$, *sti*$^-$, P_{pBR325}). *S. lividans* pIJ702 (*amy*$^-$, *sti*$^-$) was included in the experiment as a control. The cultures were grown in MMS at 30°C.

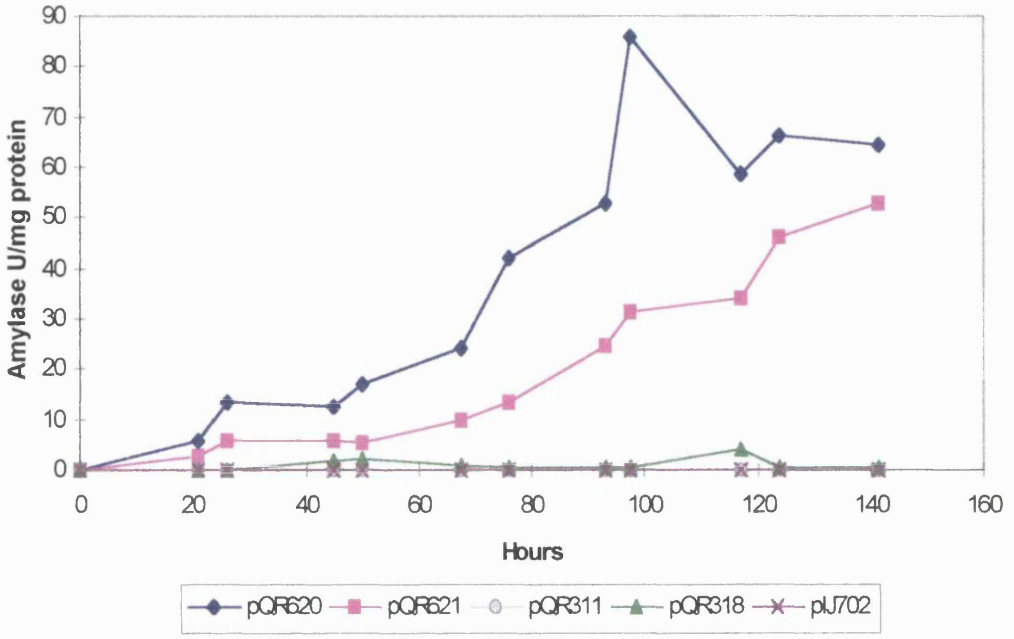


Figure 4.7: α-Amylase specific activity levels in the time course experiment comparing *S. lividans* pQR620 (*amy*$^+$, *sti*$^+$, P_{mel}, P_{amy}), *S. lividans* pQR621 (*amy*$^+$, *sti*$^+$, P_{amy}), *S. lividans* pQR311 (*amy*$^+$, *sti*$^-$, P_{amy}) and *S. lividans* pQR318 (*amy*$^+$, *sti*$^-$, P_{pIJ325}). *S. lividans* pIJ702 (*amy*$^-$, *sti*$^-$) was included in the experiment as a control. The cultures were grown in MMS at 30°C.

Table 4.1: Peak levels of different culture characteristics in the time course experiment comparing *S. lividans* pQR620 (*amy*$^+$, *sti*$^+$, P_{mel}, P_{amy}), *S. lividans* pQR621 (*amy*$^+$, *sti*$^+$, P_{amy}), *S. lividans* pQR311 (*amy*$^+$, *sti*$^-$, P_{amy}) and *S. lividans* pQR318 (*amy*$^+$, *sti*$^-$, P_{pBR325}). *S. lividans* pIJ702 (*amy*$^-$, *sti*$^-$) was included in the experiment as a control. The cultures were grown in MMS at 30°C. Levels of α-amylase protein (mg/l) were calculated using the specific activity value determined by French (1993)(1 unit α-amylase = 2.6μg of protein).

Plasmid	μ_{max} h^{-1}	**Peak DCW** g/l	**Peak α-Amylase** U/ml	**Peak Protein** mg/ml	**Peak Productivity** U/g DCW	**Peak Specific Activity** U/mg	**α-Amylase** mg/l
pQR620	0.057	2.18	13.40	0.164	7166	85.90	34.84
pQR621	0.054	2.05	5.71	0.134	4496	52.87	14.85
pQR318	0.058	2.00	0.80	0.198	494	4.04	2.08
pQR311	0.041	2.29	8.50×10^{-3}	0.202	4	0.05	0.21
pIJ702	0.051	2.07	---	0.144	---	---	---

for α-amylase activity. No α-amylase production was detected from the cells containing the plasmids pQR311, pIJ303 or pIJ702. The supernatants from the *S. lividans* pQR620, pQR621 and pQR318 cultures were found to contain 0.508, 0.228 and 0.010 α-amylase U/ml respectively. These levels correlated well to those seen in the previous experiment at the equivalent point in the time course (Fig. 4.4). This indicated that the plasmid levels and forms present in the cells were representative of the normal situation. Total DNA was prepared from 0.1g of cells taken from each culture (Section 2.7.3).

4.4.2.1: Plasmid topoisomer profiles.

Total DNA from each of the preparations was run on an agarose gel (Section 2.7.8). Bands were visualised and photographed (Fig. 4.8).

The plasmid topoisomer profiles observed were noteworthy. The DNA preparations from cells containing pIJ303, pQR620 and pQR621, which all possess the minus-strand origin, showed a distinctly different pattern from those of pIJ702, pQR311 and pQR318, where no *ssi* is present. The first group of plasmids were present almost exclusively as open circles. These molecules co-migrated with the chromosomal DNA present in the preparations. The other preparations contained a very high proportion of supercoiled plasmid DNA along with open circular forms and chromosomal DNA. Faint bands which may represent multimeric plasmid forms could also be seen in some of the lanes, e.g. the pQR621 and pQR311 lanes. Very similar profiles were seen in *S. lividans* TK24 pIJ303 and pIJ702 cell lysates by Wrigley-Jones (1990). In this study plasmid topoisomer profiles were followed throughout growth. pIJ303 was found to exist mainly as open circles (80 to 100%) in the early stages of growth. Levels of supercoiled plasmid were seen to increase gradually during the course of experiments (up to around 50% of plasmid molecules at the end of stationary phase). In contrast, pIJ702 cultures mostly yielded supercoiled plasmid forms (up to 100% of plasmids present). Levels of open circular plasmid increased slightly as the cultures aged.

4.4.2.2: No ssDNA was present in *sti*$^+$ cells.

After the gel had been photographed the DNA was blotted directly onto a nitrocellulose membrane, without a prior denaturation step (Section 2.7.8). The membrane was then

probed with [α-^{32}P]dCTP-labelled pIJ702. As no denaturation step was included in the procedure, only DNA which was present in the cells in single-stranded form was detected by the probe.

The results observed in this experiment were exactly as expected. No ssDNA was detected in the total DNA preparations containing the plasmids pIJ303, pQR620 and pQR621, each of which contained the site for second-strand initiation (Fig. 4.9 - lanes b, c and d). Large amounts of ssDNA were detected in the DNA preparations containing pIJ702, pQR311 and pQR318 (Fig. 4.9 - lanes e, f and g). The hybridising bands had migrated approximately 2.75 cm from the wells. On the stained agarose gel, these were found to correspond to the faint shadowy bands seen under the highly visible lower bands in lanes e, f and g (Fig. 4.8).

4.5: Impact of Inclusion of the *sti* Region on the New Constructs

4.5.1: Replication efficiency of pQR620 and pQR621.

The results of the ssDNA detection experiment clearly indicated that the plasmids containing the minus-strand origin were capable of more efficient replication than those lacking the minus origin. Many authors have described the build up of pools of ssDNA in cells containing plasmids which replicate using a rolling circle mechanism (Deng *et al.*, 1988; Kieser *et al.*, 1988; Pigac *et al.*, 1988; te Riele *et al.*, 1986a). Single-stranded plasmid molecules are intermediates in rolling circle replication (Section 1.2.3). They build up in the cytoplasm of cells containing plasmids which lack a site for second-strand initiation as, without the *ssi*, no efficient signal exists on the molecules to prompt host cell factors to convert the ssDNA to dsDNA (del Solar *et al.*, 1987; Gruss & Ehrlich, 1989; Novick, 1989). Such plasmids, e.g. pIJ702, pQR311 and pQR318, rely on other, less efficient, random initiation mechanisms to complete the replication cycle and produce dsDNA (te Riele *et al.*, 1986b; Kataoka *et al.*, 1994).

Zaman *et al.* (1993a) found that presence of the *sti* region was sufficient for the efficient initiation of second-strand synthesis to form double-stranded plasmid derivatives. The results of this study strongly support this finding. No ssDNA was detected in the DNA preparations from pIJ303, pQR620 and pQR621. This suggests

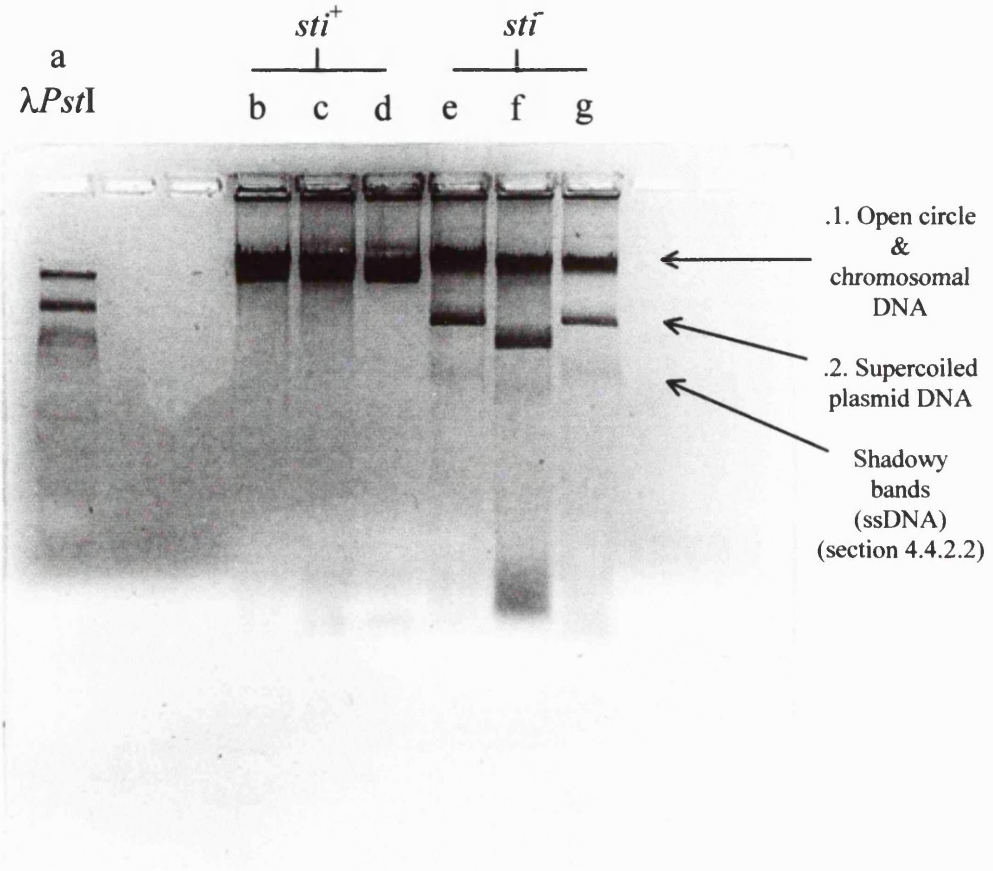


Figure 4.8: Agarose gel (1%) showing the total DNA preparations used in the ssDNA detection experiment. Samples were loaded as follows: lane **(a)** λ *Pst*I markers; lane **(b)** *S. lividans* pIJ303 (*sti*$^+$), lane **(c)** *S. lividans* pQR620 (*sti*$^+$), lane **(d)** *S. lividans* pQR621 (*sti*$^+$), lane **(e)** *S. lividans* pIJ702 (*stī*), lane **(f)** *S. lividans* pQR318 (*stī*), lane **(g)** *S. lividans* pQR311 (*stī*). Different plasmid topoisomers were visible in the DNA preparations: **(1)** open circular plasmid forms and chromosomal DNA, **(2)** supercoiled plasmid forms.

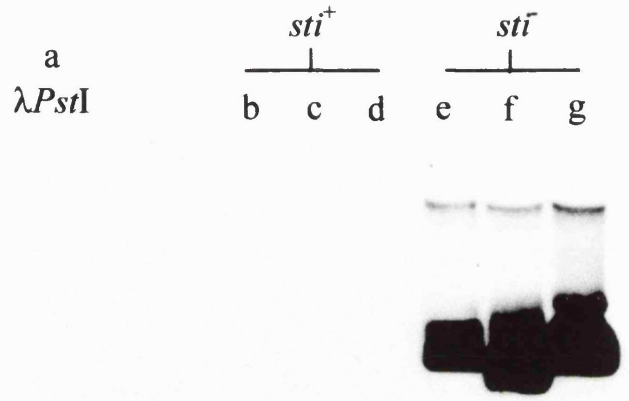


Figure 4.9: Results of Southern hybridisation for the detection of ssDNA in the total DNA preparations. The total DNA preparations were run on a 1% agarose gel (see Fig. 4.8). The DNA was then blotted directly onto a nitrocellulose membrane without a prior denaturation step (section 2.7.9). This was then probed with [α-^{32}P]dCTP-labelled pIJ702. Only plasmid DNA present in the cells as ssDNA should be detected by this procedure. Samples were loaded as follows: lane **(a)** λ *Pst*I markers; lane **(b)** *S. lividans* pIJ303 (*sti*$^+$), lane **(c)** *S. lividans* pQR620 (*sti*$^+$), lane **(d)** *S. lividans* pQR621 (*sti*$^+$), lane **(e)** *S. lividans* pIJ702 (*stī*), lane **(f)** *S. lividans* pQR318 (*stī*), lane **(g)** *S. lividans* pQR311 (*stī*).

that the ssDNA molecules were converted into dsDNA as soon as the *ssi* was exposed and able to interact with host cell factors (Section 1.2.3).

The plasmid topoisomer profiles seen in the DNA extracts can also be explained by differences in replication. Open circular plasmids are more likely to be present when active replication is occurring as the plasmids are nicked to initiate the process (Gros *et al.*, 1987; Ilyina & Koonin, 1992; Suzuki *et al.*, 1997b). Plasmids would tend to exist in supercoiled form in the absence of active replication. The profiles observed therefore provide further evidence that choosing to incorporate the minus-strand origin on the new α-amylase expression vectors produced constructs capable of very efficient replication.

4.5.1.1: Increased plasmid copy number.

The absence of an *ssi* on plasmids which replicate via rolling circle replication has been shown to have several further implications. In some hosts, e.g. *Bacillus subtilis*, loss of the minus-strand origin has no effect on plasmid copy number. However, in *Streptomyces* the copy number of *sti*$^-$ plasmids is found to be lower than equivalent *sti*$^+$ plasmids (Gruss & Ehrlich, 1989). In 1982, Kieser *et al.* noted that derivatives of pIJ101 had copy numbers of between 40 and 300 per cell. Deng *et al.* (1988) found that *sti*$^-$ plasmids had copy numbers at the lower end of this range (50 to 100). Addition of the *sti* region in the natural orientation with regard to the basic replicon led to an increase in copy number. Wrigley-Jones *et al.* (1992 & 1993) monitored the copy numbers of pIJ303 and pIJ702 (*sti*$^+$ and *sti*$^-$ respectively). pIJ303 was found to exist at over 400 copies per chromosome between the initial rapid growth phase and the stationary phase. In contrast, pIJ702 only reached a maximum of 200 copies per chromosome. These results suggest that the new α-amylase expression vectors pQR620 and pQR621, which are both *sti*$^+$, will achieve maximum copy numbers 2 to 3-fold greater than the *sti*$^-$ constructs pQR311 and pQR318. No direct measurements of plasmid copy number were made from the cultures, because accurately assessing levels in *S. lividans* has been shown to be difficult. Plasmid levels can vary greatly depending on when samples are taken during growth (Wrigley-Jones, 1990). The increased α-amylase production seen from the new *sti*$^+$ plasmids provided evidence that increases in copy number had taken place (Section 4.4.1.1).

4.5.1.2: Improved plasmid structural and segregational stability.

Plasmid structural and segregational stability are also likely to have been increased by the incorporation of the *ssi* on the new expression vectors. te Riele *et al.* (1986b) found that around a third of the copies of the *ssi*⁻ staphylococcal plasmid pC194 were present as ssDNA. Plasmids based on the pIJ101 minimal replicon belong to the same family of vectors and can therefore be assumed to accumulate similar levels of ssDNA when no *ssi* is present. High levels of ssDNA were certainly present in cells containing the plasmids pQR311 and pQR318 (Fig. 4.9). Many studies have found that large pools of ssDNA play a role in plasmid structural instability, probably by stimulating intermolecular recombination (Suzuki *et al.*, 1997a; Zaman *et al.*, 1993b; Gruss & Ehrlich, 1989; Pigac *et al.*, 1988; Lee *et al.*, 1986; del Solar *et al.*, 1987). The plasmids pQR443a, pQR311 and pQR318 had been previously observed to exhibit good structural stability (Bahri, 1990; Zaman, 1991). The contribution of the *sti* region to improving this aspect of the α-amylase expression vectors was probably therefore of only marginal benefit. Other factors such as the location of the *ssi* with respect to the *rep* gene and the type of *E. coli* plasmid used to produce shuttle vectors have also been shown to affect structural stability (Zaman *et al.*, 1993b; Lee *et al.*, 1986). Incorporation of the *sti* region should not therefore be considered as the total solution to this problem.

Segregational plasmid instability is thought to be governed by two main factors. Firstly, the probability of generation of plasmid free cells from plasmid harbouring cells is important. Secondly, if the growth rate of plasmid free cells is higher than plasmid containing cells, plasmid free cells will eventually dominate the population. Lee *et al.* (1992) looked at the segregational stability of the streptomycete plasmid pDML6. They found the growth rates of plasmid containing and plasmid free cells to be the same. This meant that the probability of generation of plasmid free cells was the most important factor. Segregational instability may be of importance in the α-amylase expression system. As analysis of plasmid distribution throughout the mycelia is extremely difficult, gauging the significance of any such effect is almost impossible. If present, segregational plasmid loss would have the greatest impact on productivity as the system was scaled up, because use of thiostrepton selection would be unlikely on a

fermenter scale. Incorporation of the *sti* region onto the new constructs should reduce the risk of significant segregational instability under fermentation conditions by raising plasmid copy numbers and reducing the chance for plasmid loss upon cell division.

4.5.2: Inclusion of the *sti* region boosted α-amylase production by over 1000-fold.

The major difference between the plasmids pQR621 and pQR311 is that pQR621 contains the site for second-strand initiation. In each case α-amylase expression is driven by an identical promoter arrangement. A comparison of α-amylase production from *S. lividans* pQR311 and *S. lividans* pQR621 will therefore give a direct indication of the effect of the *sti* region on the system. In the shake flask comparison experiment *S. lividans* pQR621 produced 1043-fold more α-amylase per gram of cells than *S. lividans* pQR311 (Table 4.2). This is a highly significant increase in production.

Table 4.2: Comparison of the productivity values from *S. lividans* pQR620 (amy^+, sti^+, P_{mel}, P_{amy}), *S. lividans* pQR621 (amy^+, sti^+, P_{amy}), *S. lividans* pQR311 (amy^+, sti^-, P_{amy}) and *S. lividans* pQR318 (amy^+, sti^-, P_{pBR325}). The cultures were grown in MMS at 30°C.

Plasmid	Presence of *sti* region	Promoter(s)	Peak Productivity U/g DCW	Relative Peak Productivity - compared too	
				pQR311	pQR621
pQR620	sti^+	P_{mel}, P_{amy}	7165.78	1662.6	1.6
pQR621	sti^+	P_{amy}	4496.06	1043.2	1.0
pQR318	sti^-	P_{pBR325}	493.83	114.6	0.1
pQR311	sti^-	P_{amy}	4.31	1.0	9.6×10^{-4}

The increased copy number of pQR621 relative to pQR311 is likely to have helped raise levels of production. However, the copy number of the sti^+ plasmid was only predicted to be 2 to 3-fold greater than that of the sti^- plasmid. This differential is unlikely entirely to explain the very large increase in α-amylase production observed. Plasmid structural and segregational instability were not thought to be major factors in the low level production seen from pQR311. Improvements in these characteristics would

therefore be predicted to have little effect on the performance of the system. It could also be the case that the single-stranded pool of plasmid in, for example, pQR311, may not be a substrate for the *Streptomyces* transcription apparatus, whereas all of the double-stranded molecules in the pQR620 and pQR621 containing cells will be able to be transcribed. Such an effect could account for the observed differences in α-amylase levels between the *sti*$^+$ and *sti*$^-$ plasmids. Another possible explanation for the increase in production was that expression from the α-amylase gene was enhanced when the plasmids were present as open circles. This conformation may favour the initiation of transcription from the α-amylase gene. DNA secondary structures and/or operator regions may be less exposed, and unable to interact with cellular factors needed for high level expression, when the DNA is supercoiled. The role of such sequences in α-amylase expression may be very important (Section 1.3.1). Therefore, this explanation for the large rise in levels is plausible.

4.6: Effect of Promoter Arrangement on Expression

4.6.1: Tandem arrangement of P_{mel} and P_{amy} led to a 1.6-fold increase in α-amylase production.

The only difference between the plasmids pQR620 and pQR621 is the orientation of insertion of the DNA fragment containing the α-amylase gene. This means that in pQR620 expression from the gene should be directed by both the *amy* and *mel* promoters. In pQR621 expression is due to the *amy* promoter (P_{amy}) only. Comparison of the strains containing these constructs showed that presence of the *mel* promoter (P_{mel}) led to a 1.6-fold increase in production (Table 4.2). Both of these constructs contained the *sti* region. Therefore, the rise in α-amylase levels obtained from pQR620, compared with pQR621, must have been due to the effect of the dual promoter in front of the *amy* gene in pQR620 versus the single amylase promoter in pQR621. This increase was less spectacular than that generated by the inclusion of the *sti* region. However, it still represented a significant and worthwhile rise in α-amylase production.

4.7: Concluding Remarks

4.7.1: Effect of promoter spacing and surrounding DNA sequence.

The *mel* promoter is generally considered to give strong expression (Schmitt-John & Engels, 1992). This factor, along with its presence on the commonly used plasmid pIJ702, has meant that many studies have used the promoter in combination with a variety of other promoters and genes. A wide range of outcomes have been obtained in these studies. Schmitt-John and Engels (1992) looked at the effect of different promoter arrangements on production of tendamistat from pIJ702-based vectors. The tendamistat promoter alone gave only slightly lower levels of production than the tendamistat promoter in tandem with P_{mel}. No increase in expression was seen when the P_{mel} was used with the promoters from the neomycin resistance gene (*aph*p1 and *aph*p2) when compared to expression from the *aph* promoters alone. Likewise, no additional expression was seen when the P_{mel} promoter was placed in tandem with a synthetic consensus *Streptomyces/E. coli*-like promoter. In fact in these two constructs northern blotting revealed that the promoter was inactive. The highest levels of expression were mediated by the *mel* promoter in tandem with the *erm*E-up promoter. In this case very strong signals were detected from both promoters. The *mel* promoter by itself directed very good expression and seemed to give the strongest signal of all the promoters examined. Taguchi *et al.* (1992) found that the presence of P_{mel} helped to increase expression of a *Streptomyces* subtilisin inhibitor (SSI)/pheromone peptide fusion protein above the level seen when the SSI promoters were present alone. In this construct the gene was inserted into the *Sph*I site of the *mel* gene on pIJ702. Koller *et al.* (1989) saw a 3 to 5-fold decrease in production of tendamistat when the *mel* promoter was removed from upstream of the tendamistat promoter.

The very wide variations seen in these results strongly suggest that the sequences around the promoter play an important part in its function. The spacing of the promoter in relation to other promoters and the gene of interest must also be crucial. Regulatory proteins and DNA secondary structure are also likely to play a role (Section 1.3.1).

4.7.2: Sequences capable of acting as sites for second-strand initiation may be carried on many inserts.

Another explanation for these wide variations is suggested by the very large rise in expression seen due to increased efficiency of plasmid replication (Section 4.5). It is possible that some of the promoter or gene inserts used have carried sequence or regions of secondary structure which could act as weak (or strong) priming sites to initiate the conversion of ssDNA to dsDNA in plasmid replication. Suzuki *et al.* (1997a) found three sequences on the streptomycete plasmid pSN22 which could act as minus-strand origins. Two of the *ssi*s were present on pSN22 in the wrong orientation for activity. When inserted in the correct orientation with regard to the *rep* gene each of the sequences efficiently directed the conversion of ssDNA to dsDNA during replication. del Solar *et al.* (1987) found 6 sequences which could act as minus-strand origins on the staphylococcal rolling circle plasmid pLS1. Three of these sequences were on the correct strand for activity. These studies indicate that sequences which could improve replication are fairly common. The results of this study suggest that even a small increase in replication efficiency could lead to greatly increased levels of expression from a plasmid, such as pIJ702, which possesses no minus-strand origin. As the vast majority of *Streptomyces* research is carried out using vectors based on pIJ702, it is possible that potentiation of plasmid replication by such random sequences could be common.

4.7.3: Understanding plasmid copy number control.

The control of copy number in rolling circle plasmids is complex and not well understood. Larson and Hershberger (1986) looked at control of copy number in the streptomycete plasmid SCP2. They made a series of deletions to try and inactivate negative control of copy number. Plasmids with three different copy numbers were isolated. The normal plasmid content was 1 to 2 copies per cell. Cells containing plasmids with moderate copy number (approximately 10 to 20 plasmids/cell) were also found. When the plasmid carried the *rep* gene alone an ultrahigh copy number resulted. This was around 1000 times the normal SCP2 copy number. These findings suggested several regions of the plasmid played a role in regulating copy number.

Runaway replication and ultrahigh copy numbers have not been observed from *sti*$^+$ plasmids based on the pIJ101 minimal replicon. Instead the maximum copy numbers reported have been in the region of 300 to 500 (Kieser *et al.*, 1982; Wrigley-Jones *et al.*, 1993). This may indicate that these plasmids also carry regions which negatively control copy number. In this study, increasing plasmid copy number by ensuring the efficiency of plasmid replication has proved to be an effective way of raising levels of α-amylase production. Therefore, obtaining additional copy number increases might raise levels even further. Attempting to define and remove any regions exerting negative copy number control in pQR620 could therefore be considered as one possible avenue to follow if even better α-amylase expression vectors are to be constructed. This would only give viable plasmids if such negative control regions lie outside of the *rep* gene. It is also possible that increasing plasmid levels further would actually be detrimental for α-amylase production as the demands made on the cells might be too great to allow good growth and product formation.

4.7.4: Secretion of α-amylase.

This shake flask study indicated that the cells could support the production of 35 milligrams of α-amylase per litre of broth over 4 days without any bottleneck occurring in secretion, as demonstrated by there being no detectable α-amylase in the cells during this time. No detrimental effects on growth rates or levels were observed during α-amylase production. These findings could have been predicted from the observations of other studies which have suggested that homologous proteins tend to be efficiently secreted by *S. lividans*. Obata *et al.* (1989) cloned the gene for *Streptomyces* subtilisin inhibitor (SSI) from *S. albogriseolus* S-3253 into pIJ702, forming plasmid pJS1. Around 100 milligrams of SSI was produced per litre of culture medium by *S. lividans* 66 pJS1 over a period of 7 days. Koller and Riess (1989) cloned the gene for the α-amylase inhibitor of *S. tendae* (tendamistat) into pIJ350. This plasmid directed the production and secretion of between 600 and 700 mg/l of tendamistat from *S. lividans* TK24 over a 5 day period. Kendall and Cullum (1984) conducted a similar study with the agarase gene from *S. coelicolor* A3(2). They obtained secretion of 138 mg/l broth from *S. lividans* TK64 after a week of incubation. These results suggest that the secretory machinery of *S. lividans* can cope with a very high level of protein. They may

indicate that levels of α-amylase production could be increased several fold more without problems in secretion being encountered.

4.7.5: Achieving further increases in α-amylase levels.

The results discussed in Section 4.7.1, along with the findings of this study, indicate that the use of promoter arrays is potentially a good way of raising levels of expression from cloned genes in *Streptomyces*. However, it is obvious that a great deal more knowledge must be gained before it will be possible to predict which promoter combinations will work, and how the components should be positioned, to optimise expression. The very large increases in α-amylase production obtained from the constructs containing the *sti* region suggest that ensuring the efficiency of plasmid replication is potentially of more importance initially than the promoter driving expression.

The replication of the α-amylase expression vectors would be hard to optimise further until more is understood about the mechanisms of plasmid replication and copy number control. This means that additional increases in α-amylase expression from the plasmids could only realistically be made by further improving levels of transcription or translation from the α-amylase gene. As using the *amy* and *mel* promoters in tandem gave a significant rise in α-amylase expression, adding in additional promoters might lead to even higher levels of production. Alongside P_{mel} several other *Streptomyces* promoters are recognised as giving 'strong' expression. These include the *erm*E, *aph*, *Streptomyces* subtilisin inhibitor and *S. ghanaensis* phage I19 14II promoters (Bibb *et al.*, 1985a; Janssen *et al.*, 1985; Taguchi *et al.*, 1989a; Labes *et al.*, 1997). Inserting one, or more, of these promoters upstream of the α-amylase gene of pQR620 might give further increases in expression. Transcription of the α-amylase gene in the construct pQR318 is driven by a piece of pBR325 sequence present in the plasmid (P_{pBR325}). Bahri (1990) identified the probable promoter sequence as -10 (AAAGCT) -35 (TTAACT). In *S. lividans* this sequence gave α-amylase production over 100-fold greater than the native α-amylase promoter. It would therefore also be of interest to try this promoter sequence in tandem with the *mel* promoter as it too might be an effective combination. Insertion of the *sti* region into pQR318 would allow this particular genetic combination to be studied.

4.8: Summary

Two new α-amylase expression vectors were constructed. Each contains the *sti* region from pIJ101 which encodes the site for second-strand initiation. This site is necessary for efficient plasmid replication. In plasmid pQR620 expression of the α-amylase gene is driven by the *mel* and *amy* promoters located in tandem. In pQR621 expression is driven by the *amy* promoter alone. No single-stranded DNA was detected in *S. lividans* cells carrying the new plasmids. Single-stranded DNA was detected in cells carrying the plasmids pQR311 and pQR318; which do not contain the *sti* region. This indicated that the new plasmids, pQR620 and pQR621, replicated more efficiently than pQR311 and pQR318. Inclusion of the *sti* region on the expression vectors increased α-amylase production by over 1000-fold. Presence of the *mel* promoter in tandem with the *amy* promoter led to a further 1.6-fold increase in production. *S. lividans* pQR620 produced 13.4 α-amylase U/ml supernatant. This is equivalent to approximately 35 mg/l α-amylase. No intracellular α-amylase was detected suggesting that the *S. lividans* secretory machinery can handle this level of protein.

Chapter 5

Performance of *S. lividans* pQR620 Under Fermentation Conditions

5.1: Aims

The work described in this chapter had two main aims. It was hoped that moving from shake flasks to 20l fermenters would quickly identify any major problems caused by scale-up and would allow the effects of increased shear forces and pH control to be evaluated. Previous results had suggested that inocula characteristics had a large effect on the performance of cultures. Two different inocula preparation strategies were therefore used for these fermentations to try to define further the characteristics of a good inoculum.

5.2: Background

A series of 4 fermentations were carried out in MMS to evaluate growth and α-amylase production patterns from *S. lividans* pQR620 under more controlled conditions. The fermentations were conducted as described in Section 2.4.8. Twenty litre vessels were chosen for this work as they provided a large enough working volume to allow regular samples to be taken over a prolonged period and were big enough to show any problems associated with scale-up. Fermentation parameters were maintained as described in Section 2.4.8 unless otherwise stated.

5.2.1: Inoculation strategies.

A decision was made to use two different inoculation strategies for these fermentations (Sections 2.4.9.2.1 & 2.4.9.2.2). It was hoped a comparison of fermentation performance would then indicate which strategy had been most favourable for high levels of α-amylase production. The characteristics of the inocula used in fermentations A, B, C and D are shown in Table 5.1.

5.2.1.1: One-stage inoculation strategy.

For the first two fermentations (A and B) the inocula were produced using a one-stage inoculation strategy (Section 2.4.9.2.1).

5.2.1.2: Two-stage inoculation strategy.

The inocula for fermentations C and D were produced using a two-stage inoculation strategy (Section 2.4.9.2.2).

Table 5.1: Characteristics of the inocula used in fermentations A, B, C and D. Flasks contained *S. lividans* pQR620. They were sampled immediately prior to use as inocula (see Section 5.2.1 for further details).

Fermentation	Inoculum type given to flask	Age of inoculum at transfer to fermenter	Inoculum characteristics at point of transfer to fermenter (total levels in 1.5l volume)		
			DCW g	α-Amylase U	Supernatant Protein mg
A	spores from plate	50 h	0.784	985	18
B		50 h	0.828	1545	40
C	25 ml from seed flask	70 h	2.052	8270	133
D		70 h	2.405	6155	111

5.3: Fermentations A and B (given one-stage inocula)

5.3.1: Fermentation conditions.

These fermentations were run in parallel. In fermentation A the pH was automatically maintained at pH 7. No pH control was used in fermentation B and the pH was allowed to follow its natural path. Aside from this difference it was intended that the fermentations would be run in an identical manner. Over the first 15 hours a problem occurred with the air supply to the fermenters. This affected fermentation B to a far greater extent than fermentation A. Although the dissolved oxygen tension (DOT) in fermentation A dropped, it did not fall below 45% saturation. Oxygenation levels in fermentation B fell to around 5% saturation several times over a 5 hour period (9 to 14h)(Fig. 5.1).

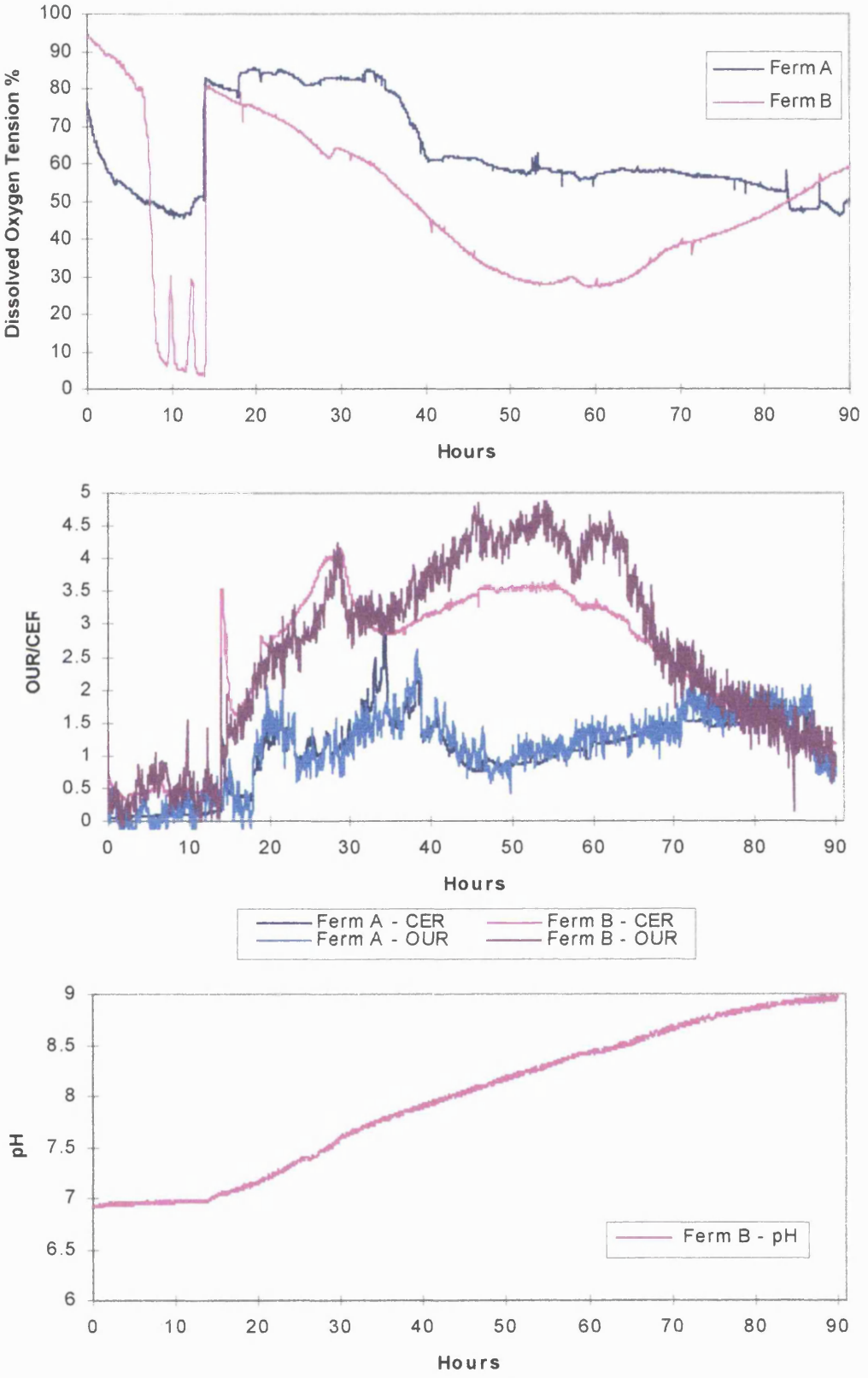


Figure 5.1: Logged data for fermentations A and B. Dissolved oxygen concentrations, oxygen uptake rates and carbon dioxide evolution rates are shown for both fermentations. The pH of fermentation A was controlled to 7 so no profile is included here.

5.3.2: Performance of fermentations A and B.

Both cultures experienced an initial lag period of around 20 hours. Fermentation B grew more quickly than fermentation A (μ_{max} of 0.067 versus 0.046 h^{-1}) and reached a higher peak biomass level (2.05g DCW/l versus 1.73g DCW/l)(Fig. 5.2). Stationary phase was reached around 10 hours earlier in fermentation B than in fermentation A (60 versus 70 hours). The α-amylase production profiles mirrored each other despite the variations in growth between the two cultures (Fig. 5.3). α-Amylase was secreted steadily into the media during exponential growth. The rate of production increased during early stationary phase and then levelled off. No significant drops in supernatant α-amylase were seen at the end of either fermentation. This indicates that significant degradation of α-amylase, due to proteases or high alkalinity levels, was not occurring. The supernatant protein production and specific activity levels were also very similar for the two fermentations (Figs. 5.4 & 5.6).

Some differences in productivity were seen between the cultures early on in growth with quite high levels of α-amylase in fermentation B over the first two samples (Fig. 5.5). This was likely to be due to the high α-amylase levels, which were present in the inoculum of fermentation B, giving artificially high values. Despite these early differences, productivity peaked in both cultures between 80 and 90 hours at around 5800 α-amylase U/g DCW. The oxygen uptake rate (OUR) and carbon dioxide evolution rate (CER) profiles for the two cultures showed that more active metabolism was occurring in fermentation B than in fermentation A (Fig. 5.1). This probably represents the differences in growth rates and biomass levels achieved in the two cultures. However, it may be indicative of other, more significant, differences in metabolism between the cells in each fermenter. The respiratory quotient (RQ) data obtained from these fermentations was too noisy to interpret, probably due to the wide range of carbon sources available to the cells in MMS medium.

The pH in fermentation B behaved as predicted from the shake flask experiments conducted using MMS (Section 3.5). No pH drops were observed, instead the pH climbed steadily throughout the growth and stationary phases.

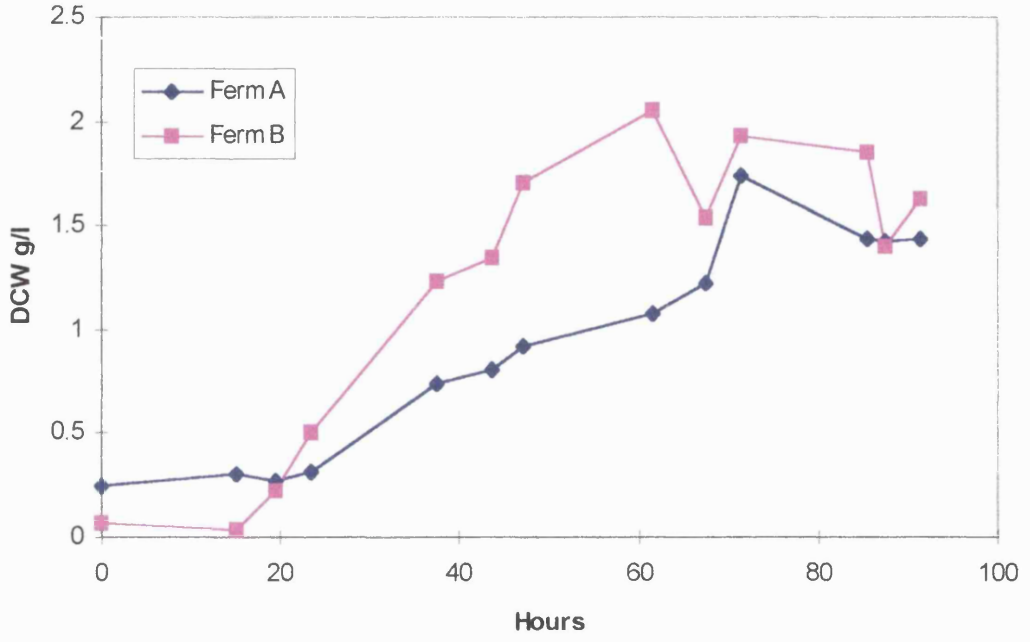


Figure 5.2: Growth curves for *S. lividans* pQR620 fermentations inoculated with single-stage inocula. The cells were grown in MMS medium at 28°C. The pH in fermentation A was maintained at 7, whilst that of fermentation B was allowed to follow its natural course. The average standard error of the mean was 0.068 g DCW/l.

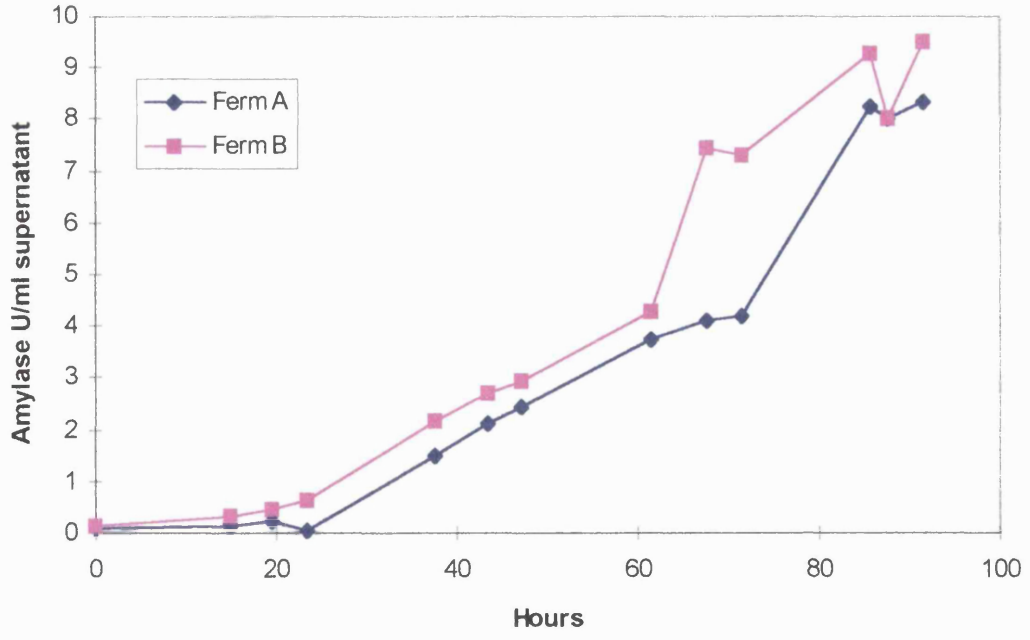


Figure 5.3: α-Amylase production profiles for *S. lividans* pQR620 fermentations inoculated with single-stage inocula. The cells were grown in MMS medium at 28°C. The pH in fermentation A was maintained at 7, whilst that of fermentation B was allowed to follow its natural course. The average standard error of the mean was 0.095 α-amylase U/ml supernatant.

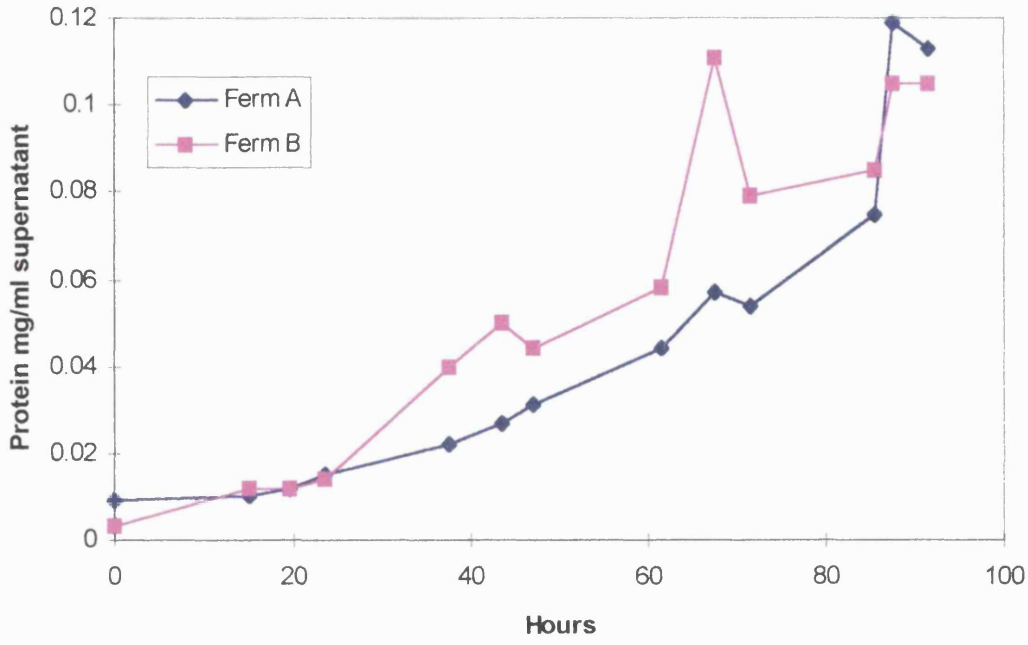


Figure 5.4: Supernatant protein levels for the *S. lividans* pQR620 fermentations inoculated with single-stage inocula. The cells were grown in MMS medium at 28°C. The pH in fermentation A was maintained at 7, whilst that of fermentation B was allowed to follow its natural course. The average standard error of the mean was 0.002 mg/ml supernatant.

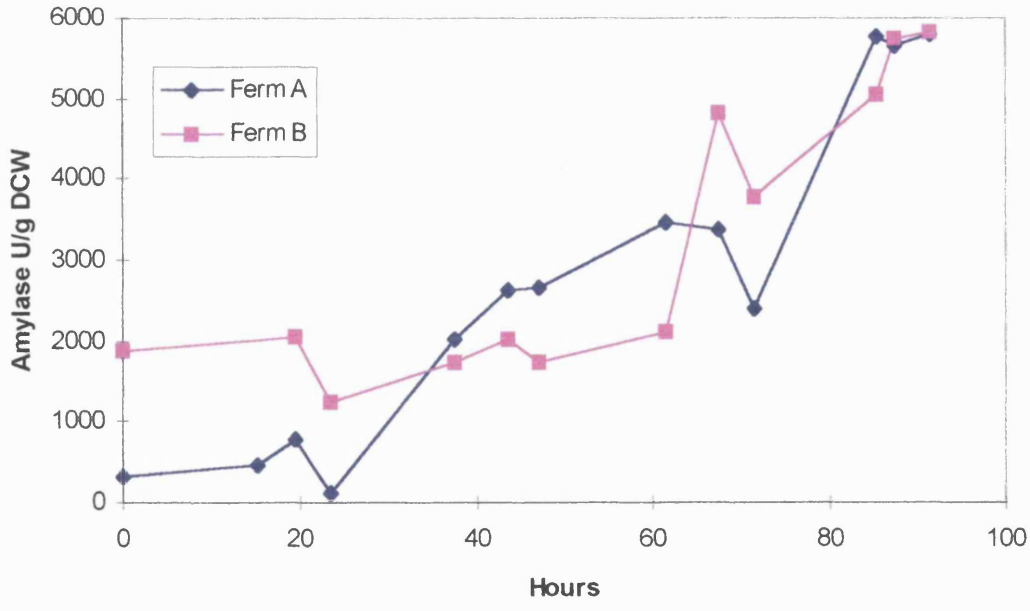


Figure 5.5: α-Amylase productivity levels for the *S. lividans* pQR620 fermentations inoculated with single-stage inocula. The cells were grown in MMS medium at 28°C. The pH in fermentation A was maintained at 7, whilst that of fermentation B was allowed to follow its natural course.

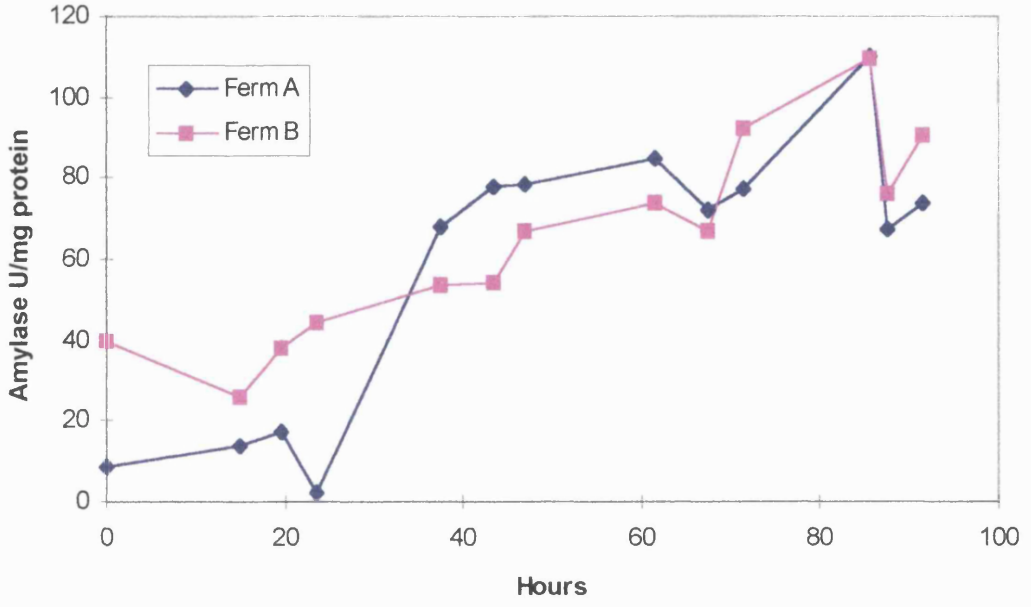


Figure 5.6: α-Amylase specific activity levels for the *S. lividans* pQR620 fermentations inoculated with single-stage inocula. The cells were grown in MMS medium at 28°C. The pH in fermentation A was maintained at 7, whilst that of fermentation B was allowed to follow its natural course.

5.3.3: Effect of fluctuations in oxygen level and pH.

It was difficult to see what, if any, effects low oxygen saturation and lack of pH control had on the system. α-Amylase and protein production levels were remarkably consistent between the two fermentations considering the differences in conditions. In fact, the cell productivities peaked within 30 α-amylase U/g DCW (5807 U/g DCW versus 5834 U/g DCW respectively). The peak specific activities obtained were also effectively identical (110 α-amylase U/mg protein and 109 α-amylase U/mg protein respectively). These similarities were present throughout the course of the fermentations.

The growth patterns seen were the opposite of those expected. The pH of fermentation B deviated fairly rapidly from the 'optimum' value for *Streptomyces* of pH 7.0 (Lee & Lee, 1994)(pH 7.5 by 30h and pH 8.0 by 40h)(Fig. 5.1). Some inhibition of growth would have been predicted at these higher pH values, but this did not occur. The transient low oxygen levels also appeared to have little effect on this culture. The lag periods seen in the two fermentations were of the same length. It is conceivable that the brief period of low DOT in fermentation B influenced the culture somehow and actually prompted faster growth and increased metabolism.

5.3.3.1: Effect of transient low DOT levels.

Wrigley-Jones (1990) studied the effect of low DOT on cell growth and plasmid copy number in *S. lividans* TK24 pIJ303. In this study low oxygen concentrations were imposed after a brief period of normal growth. Initially levels of 5% DOT were maintained. Under these conditions the cells were found to keep on growing. In fact, the logged fermentation data obtained was very similar to that seen under normal (high DOT) conditions. This suggested the critical DOT level for *S. lividans* was below 5% oxygen saturation. Next DOT was reduced to < 2% saturation. Despite these highly oxygen limited conditions, no significant reduction in peak biomass concentration was seen. Growth rate was reduced. Significant cell lysis did not occur. The plasmid pIJ303 remained stable although copy numbers were reduced by around 50% from those found under normal conditions. These results were taken as evidence that fermentative growth may occur under such conditions. The only deviation seen from

the 'normal' culture pattern under these conditions was that no characteristic rise in medium pH was seen towards the end of the experiment. Lee and Lee (1994) found similar results in continuous fermentation studies with *S. lividans* pIJ702.

Although no experiments were conducted, Wrigley-Jones (1990) postulated that, if normal culture conditions were resumed after a period of DOT stress, the plasmid copy number would be restored to normal levels. The results from fermentations A and B indicate that this prediction was correct. The α-amylase productivities obtained suggest that plasmid copy numbers must have been roughly comparable in the two cultures. Fermentation B exhibited the characteristic pH rise, suggesting the oxygenation status of the cells quickly returned to normal. No firm evidence was found to suggest that either lack of pH control, or transient low oxygen concentrations, had any significant effect on α-amylase production from *S. lividans* pQR620 under these conditions.

5.3.4: Effect of inocula variation.

The differences that were observed can easily be explained by the variations between the inocula given to each fermentation. The inoculum given to fermentation B had a slightly higher level of biomass than that given to fermentation A (Table 5.1). More cells were present initially in fermentation B giving this culture the potential to grow more rapidly. The cells used to inoculate fermentation B also seemed to be more metabolically active than those given to fermentation A. They had secreted twice as much protein into the supernatant and had produced one third more α-amylase at the point of inoculation.

5.4: Fermentations C & D (given two-stage inocula)

5.4.1: Fermentation conditions.

The inocula used for these fermentations were produced using the two-stage inoculation strategy described in Section 2.4.9.2.2, i.e. the main inoculum flasks were inoculated from a seed flask. In each of these fermentations the pH was automatically maintained at 7 for the duration of the experiment. All other fermentation parameters were maintained as for fermentations A and B (Section 2.4.8). Oxygen saturation was observed to remain above 75% in each vessel, so no possibility of oxygen limitation existed in either culture.

5.4.2: Performance of fermentations C and D.

No distinct lag period was seen in either fermentation (Fig. 5.7). Fermentation D had a very high μ_{max} of 0.145 h^{-1}. In contrast, the μ_{max} value for fermentation C was lower at 0.079 h^{-1}. Fermentation D reached stationary phase extremely rapidly (around 25 h growth). It took fermentation C an additional 10 to 15 hours to reach the same stage. Very little drop in dry cell weight was seen over the prolonged stationary phases of the cultures. Although fermentation D grew more rapidly, it reached its peak supernatant α-amylase level after that of fermentation C and secreted less α-amylase during the stationary phase (Fig. 5.8). A drop in supernatant α-amylase level was seen in both cultures after 70 hours. It is possible that this drop represented protease degradation of the enzyme. However, a corresponding drop in overall supernatant protein levels was not seen at this point. This may indicate that an α-amylase inhibitor was being produced by the cells in late stationary phase. *Streptomyces* species are known to produce such enzyme inhibitors, e.g. tendamistat from *S. tendae* (Koller & Riess, 1989) and *haim*II from *S. griseosporeus* (Nagaso *et al.*, 1988), therefore this is a possible explanation for the drop in activity.

The very rapid growth seen in fermentation D was accompanied by high levels of background protein production (Fig. 5.9). Such behaviour was not observed in fermentation C. This meant that overall supernatant protein levels remained significantly lower in fermentation C than in fermentation D. These profiles had a big impact on the specific activity levels in each culture (Fig. 5.11). Fermentation C peaked at 419 α-amylase U/mg protein. This compared to a peak level of 230 α-amylase U/mg protein in fermentation D. The productivity of the cells followed similar patterns in exponential and early stationary phase but deviated in mid-stationary phase when α-amylase production from fermentation D levelled off for a time (Fig. 5.10). Peak productivity in fermentation C was 6017 α-amylase U/g DCW. In fermentation D the cells reached 4479 α-amylase U/g DCW. Similar trends were observed in the OUR/CER profiles from each culture but fermentation D was significantly more metabolically active than fermentation C over stationary phase (Fig. 5.12). As the biomass levels present in each case were relatively close, this must represent a difference in some aspect of metabolism between the cultures.

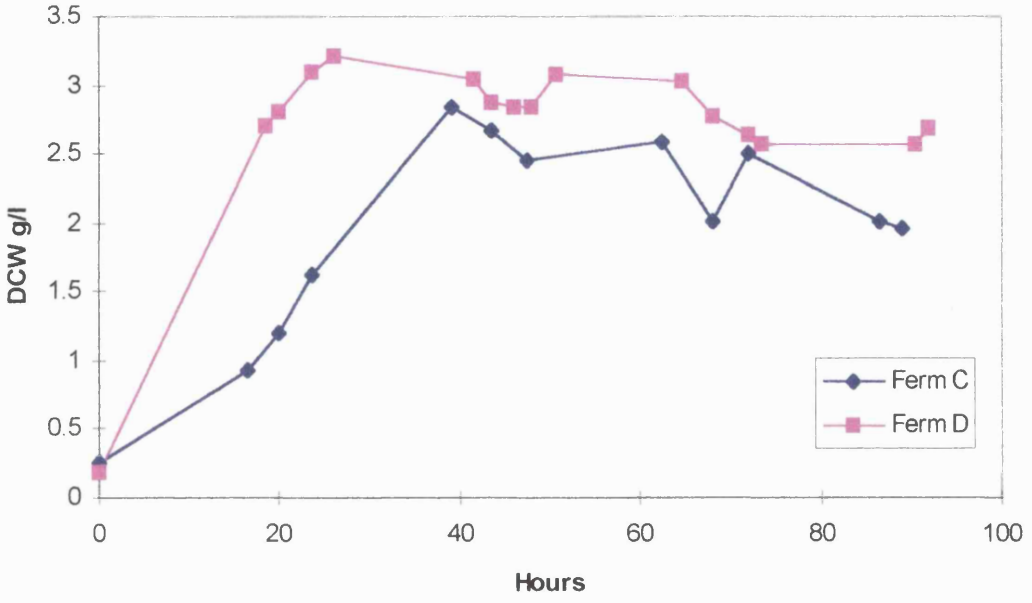


Figure 5.7: Growth curves for the *S. lividans* pQR620 fermentations inoculated with two-stage inocula. The cells were grown in MMS medium at 28°C. The pH of both fermentations was maintained at 7. Average standard error of the mean was 0.129 g DCW/l.

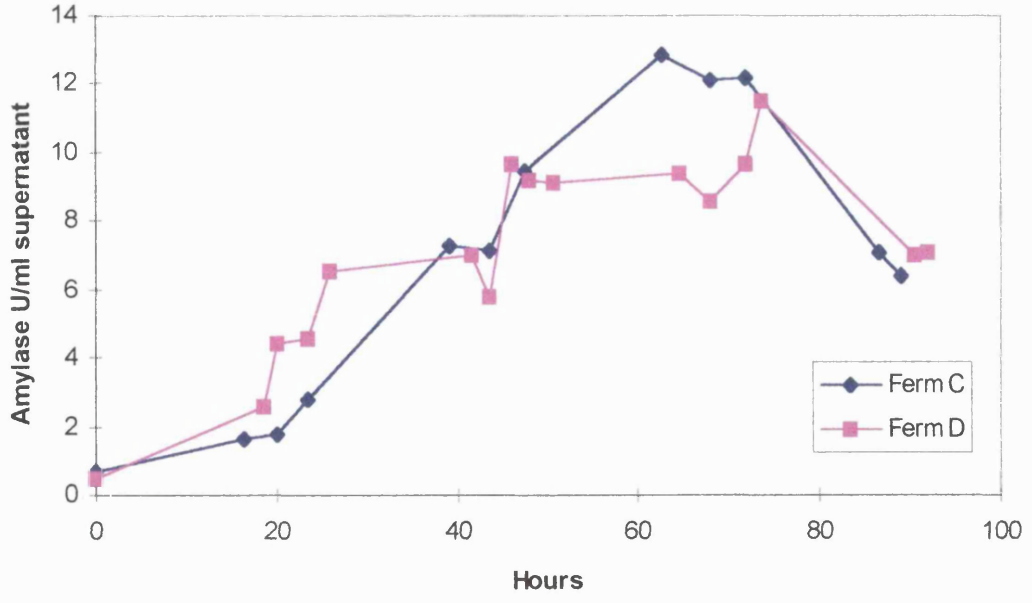


Figure 5.8: α-Amylase production profiles for the *S. lividans* pQR620 fermentations inoculated with two-stage inocula. The cells were grown in MMS medium at 28°C. The pH of both fermentations was maintained at 7. Average standard error of the mean was 0.314 α-amylase U/ml.

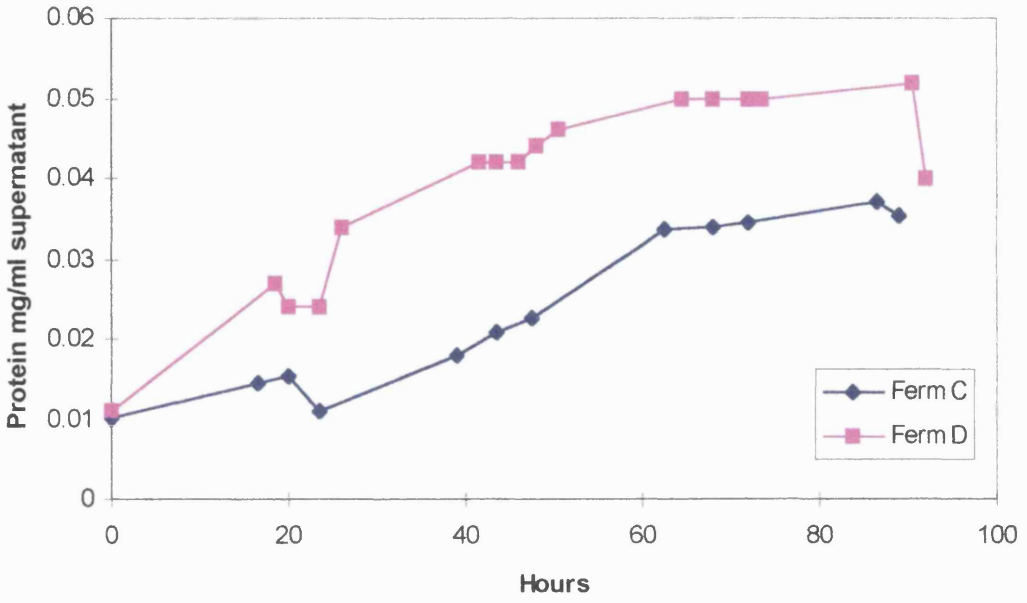


Figure 5.9: Supernatant protein levels for the *S. lividans* pQR620 fermentations inoculated with two-stage inocula. The cells were grown in MMS medium at 28°C. The pH of both fermentations was maintained at 7. Average standard error of the mean was 0.001 mg/ml.

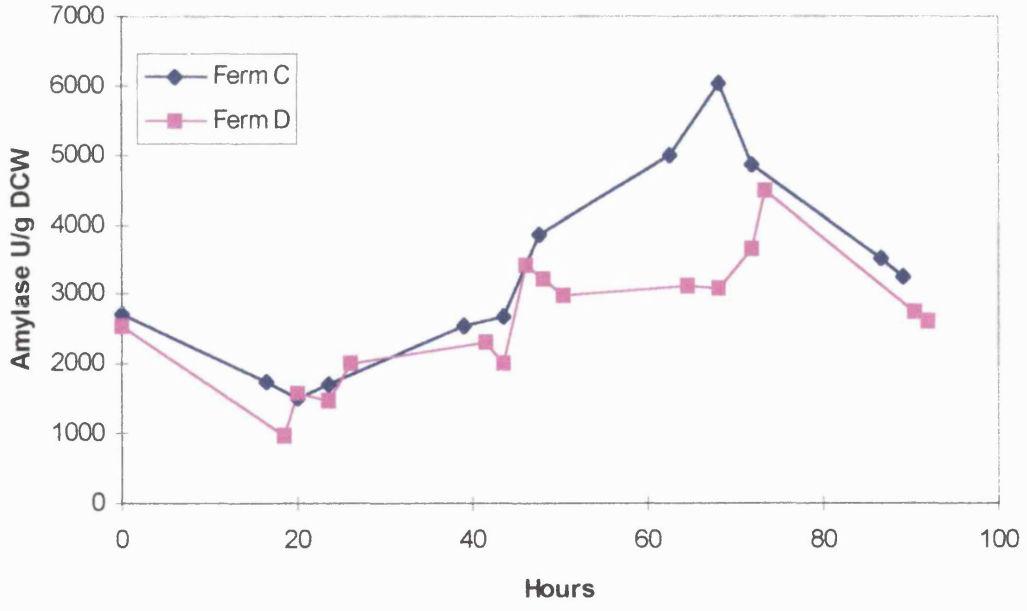


Figure 5.10: α-Amylase productivity profiles for the *S. lividans* pQR620 fermentations inoculated with two-stage inocula. The cells were grown in MMS medium at 28°C. The pH of both fermentations was maintained at 7.

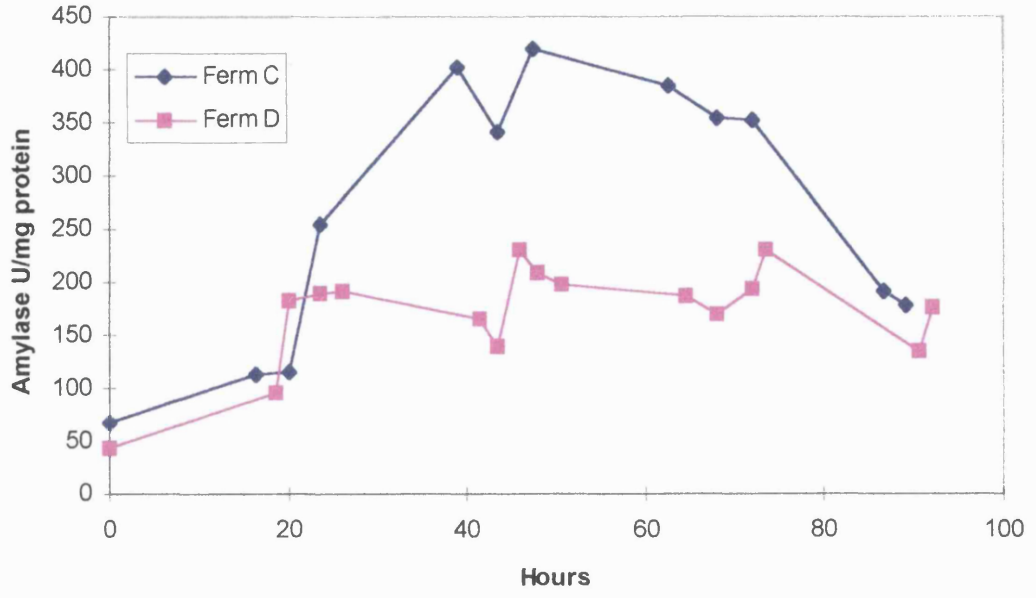


Figure 5.11: α-Amylase specific activity profiles for the *S. lividans* pQR620 fermentations inoculated with two-stage inocula. The cells were grown in MMS medium at 28°C. The pH of both fermentations was maintained at 7.

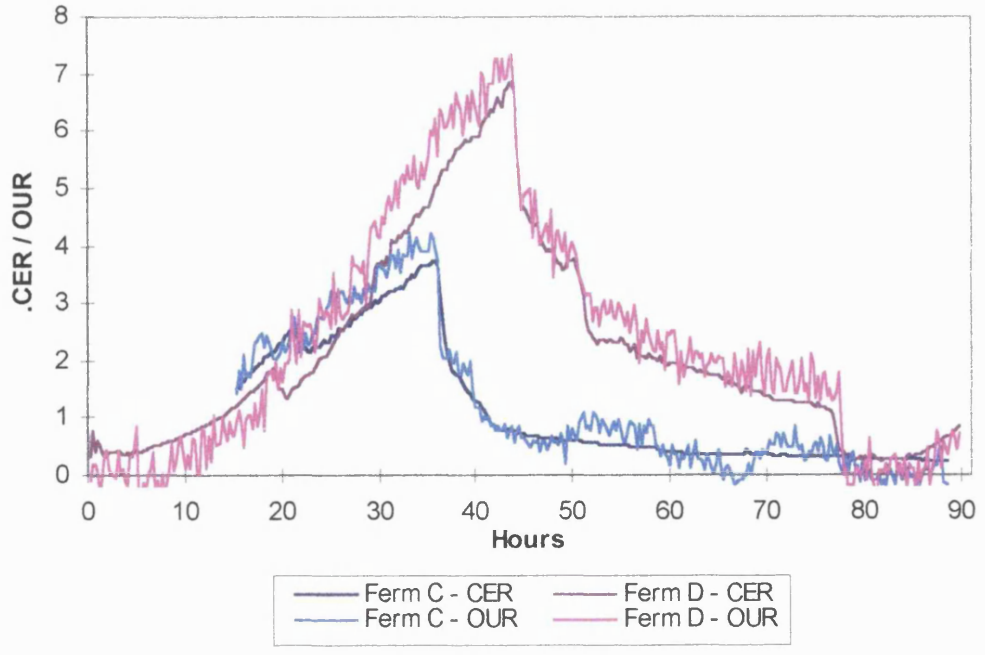


Figure 5.12: Oxygen uptake rate and carbon dioxide evolution rate profiles from fermentations C and D.

5.4.3: Effect of inocula variation.

These fermentations were carried out under identical conditions and would therefore be expected to be comparable. However, crucial differences between the behaviour of the two cultures were seen. This, again, points to the inoculum having a critical effect on the outcome of each fermentation. The inoculum given to fermentation D had a higher level of total biomass (i.e. 118% of that given to fermentation C). However, it had produced less α-amylase and protein than the inoculum used for fermentation C (Table 5.1). The fermentation started from this inoculum showed similar behaviour.

5.5: Overall Comparison of Fermentations A, B, C and D

5.5.1: Comparison of the two inocula production strategies.

The only theoretical difference between fermentation A and fermentations C and D was the strategy used to produce the inocula. The younger, single stage, inoculum used for fermentation A gave a much longer lag period and slower growth, with a lower peak biomass (around 1 to 0.5 g DCW less)(Figs. 5.2 & 5.7). These differences were reflected in the OUR and CER data from the 3 fermentations as the traces from C and D showed a much sharper change in metabolism on the transition into stationary phase (Figs. 5.1 & 5.12). The fermentations inoculated with single stage inoculum flasks, i.e. A and B, produced far higher levels of supernatant protein than fermentations C and D, i.e. 0.119 and 0.111mg/ml versus 0.037 and 0.052mg/ml respectively (Figs. 5.4 & 5.9).

These results suggested that the second strategy should be the preferred choice for producing inocula for fermentations, i.e. seed flask ⇨ main inoculum flasks ⇨ fermenter. With this strategy, lag periods were very short and good levels of growth were achieved, whilst production of contaminating background protein was minimised.

5.5.2: Comparison of α-Amylase productivity levels.

Although differences were seen between growth and protein production patterns for the four fermentations, the α-amylase productivities observed were remarkably similar in each case (Fig. 5.13). Peak levels for the fermentations A, B and C fell within 210 α-amylase U/g DCW of each other (at 5807, 5834 and 6017 U/g DCW respectively). The

peak level for fermentation D was lower at 4479 U/g DCW. This seemed to be mainly due to the failure of the culture to produce much α-amylase in mid-stationary phase.

The closeness in values of productivity over exponential growth was noteworthy. The growth rates over this phase of the cultures covered a very wide range, so it was surprising to see such a closeness in productivities. This trend may suggest that something was limiting α-amylase production in the cells to a certain level. Such a pattern may be explained by limitation of a host cell factor required for α-amylase production.

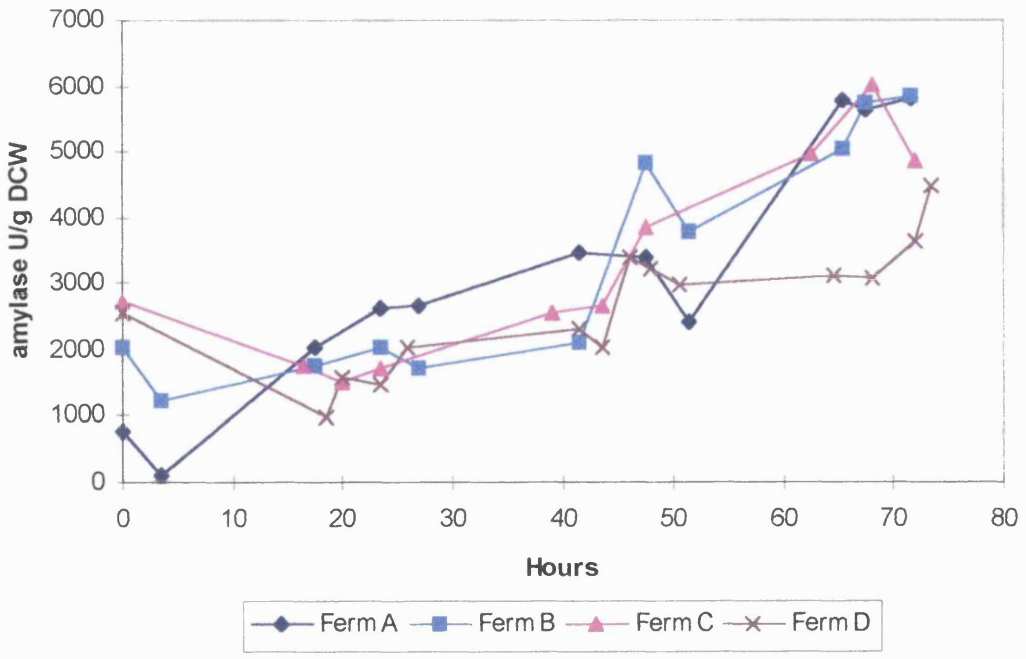


Figure 5.13: Graph showing the productivity profiles for fermentations A, B, C and D. Cultures A and B were given a single-stage inoculum. Cultures C and D were given a two-stage inoculum. The pH in fermentation B was not controlled. Each of the other cultures were maintained at pH 7. The profiles have been adjusted so that 0 hours is at the start of the period of exponential growth in each case.

5.6: Key Findings

5.6.1: Production of 'optimal' inocula.

It was obvious from the results of the four fermentations that the metabolic state, type, age and level of the inoculum have a great influence on the performance of the system (Sections 5.3.4 and 5.4.3). Even if the optimum inoculum could be defined, the evidence suggests it would be almost impossible to reproduce consistently. For example, the three flasks that went to make up the inoculum for fermentation D should have been identical at the point of use. No differences between the cultures were visible to the naked eye. The flasks used were identical, with matching baffles and springs. Each contained 500ml of the same batch of MMS medium and were autoclaved together. The flasks were each inoculated with 25mls from a single seed flask. Some variation could have entered at this point. However, great care was taken to ensure the seed flask was well mixed as each inoculum was withdrawn, to minimise any such problem. The flasks were incubated in the same incubator for the same period of time.

Despite these precautions the 3 flasks contained very different levels of growth, α-amylase and protein at the point when they were used as inocula (Table 5.2). Two of the flasks had performed in a similar way. However, flask 3 had much higher biomass and α-amylase levels and lower total protein levels. Differences in pellet sizes were also observed. Flasks 1 and 2 had small pellets (almost mycelial), whereas flask 3 had big pellets (0.5 to 1mm diameter). As all other conditions were identical it seems these variations might have been purely due to the orientation and positioning of each flask within the orbital incubator. Despite efforts to reduce the risk of variation, such differences in inoculum flasks were obtained throughout this study.

Using the available facilities, it was difficult to see how these variations could be eliminated. Producing a large volume of vegetative inoculum in a fermenter and then freezing it in aliquots was considered. However, the problems involved in producing and storing such large volumes of culture, and the risks of introducing contamination, were considered too great to make such a strategy viable. It was decided the best option was to continue producing inoculum flasks as described above and to be aware of potential variation.

Table 5.2: Characteristics of the three flasks which made up the inoculum given to fermentation D. Although each flask had been produced in a seemingly identical manner large differences existed between the three flasks (Section 5.6.1).

Inoculum Flask	DCW g/l	α-Amylase U/ml	Supernatant Protein mg/ml	Productivity U/g DCW	Specific Activity U/mg protein
1	1.27	2.4	0.074	1889	32
2	1.36	2.25	0.088	1654	25
3	2.18	7.66	0.060	3514	128

5.6.2: α-Amylase production was both growth and non-growth associated.

α-Amylase production did not seem to be entirely constitutive in these fermentations. This is illustrated well with a plot of product versus cell concentration. Such plots help to present a clear picture of whether production is growth associated, non-growth associated or constitutive (Wallace *et al.*, 1992). An idealised plot is shown in Figure 5.14. If cell growth and production are concurrent, the plot will slope upwards from left to right (line .1.). If production is suppressed during cell growth a horizontal line will be seen (line .2.). Non-growth associated production is represented by a vertical line or a line sloping upwards from right to left (lines .3. and .4.). Figure 5.15 shows such a plot for the four fermentations.

Growth associated production of α-amylase was seen in all four cultures. This was followed in cultures A, B and D with a period of growth without α-amylase production (represented by horizontal lines). In each case this was observed just prior to peak biomass and entry into stationary phase. This effect was only transitory and each culture soon began to secrete α-amylase to the medium again. No such reduction in α-amylase production was seen from fermentation C, although production did ease off just after the cells in this culture reached stationary phase. Strong non-growth associated α-amylase production was then observed from each of the cultures. Patterns began to fluctuate towards the ends of each experiment.

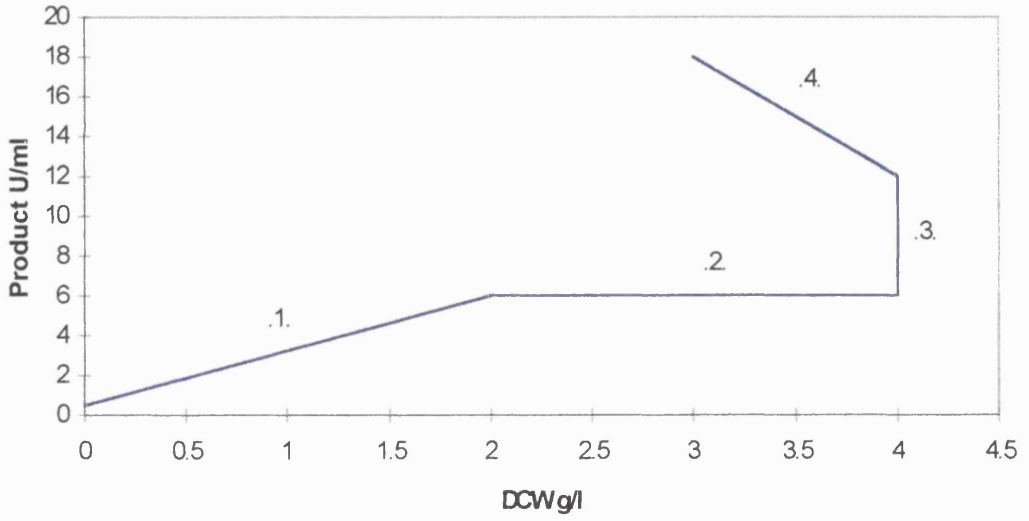


Figure 5.14: Idealised plot of product versus cell concentration. Line .1. - growth associated product production. Line .2. - production suppressed during growth (horizontal line). Lines .3. and .4. - non-growth associated production.

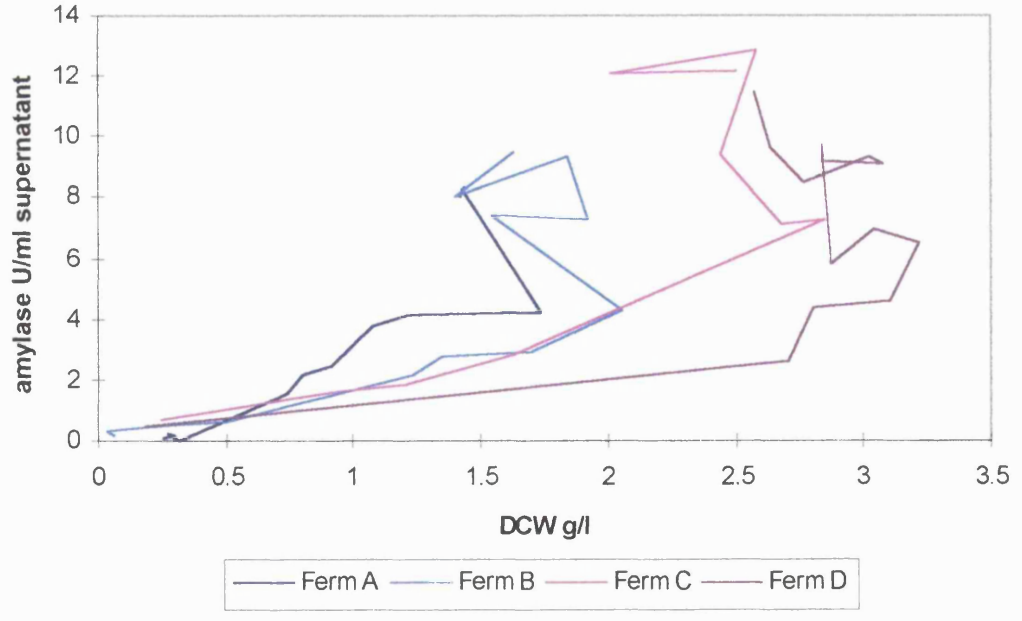


Figure 5.15: Plot of supernatant α-amylase levels versus cell concentrations for the *S. lividans* pQR620 fermentations A, B, C and D.

5.6.2.1: RNA polymerase sigma factor profiles.

Temporal and spatial gene expression in *Streptomyces* is believed to be controlled by cascades of RNA polymerase sigma (σ) factors, each of which recognises a different class of promoter (Buttner, 1989; Strohl, 1992; Lonetto *et al.*, 1994)(Section 1.1.3.1). These results showed that σ factors capable of interacting with the *mel* and/or *amy* promoters were present for the majority of the cells' life cycle. Changes in gene expression will be present as the cells move from exponential growth into stationary phase. Alterations in the σ factor profiles are likely to play a crucial role in this transition (Buttner *et al.*, 1988; Buttner, 1989). The periods seen in fermentations A, B and D when growth continued but α-amylase production stopped may have coincided with such a transitionary period. Fluctuations in the levels of different σ factors have been observed in other prokaryotic systems, e.g. *Bacillus subtilis* and *E. coli* (Arnosti *et al.*, 1986; Straus *et al.*, 1987). Changes in half life, reversible modifications, proteolysis and interactions with activating factors are thought to alter patterns of gene expression by determining the activities of different σ factor fractions in response to various stimuli (Tilly *et al.*, 1986; Arnosti *et al.*, 1986; Straus *et al.*, 1987; Potuckova *et al.*, 1995). This complexity allows rapid changes in patterns of σ factor use within the cells. Such a mechanism means that a short period of non-production is feasible due to changes in σ factor levels or activity. Further experiments would be required to confirm these findings and determine the pattern of transcription from the *mel* and *amy* promoters over the course of a fermentation.

5.6.3: Stability of α-amylase in the culture supernatants.

Degradation of the α-amylase by proteases or high alkalinity did not present a particular problem under the conditions tested, although some reduction in activity levels were observed at the very end of fermentations C and D. In these cases overall levels of protein did not drop concurrently with the α-amylase, suggesting that the cause might be specific to the product. Again, further work would be required to determine the reason for this reduction in activity. The performance of fermentation B also suggested that a brief period of oxygen limitation had little effect. The results from these fermentations suggested that the *S. lividans* pQR620 α-amylase production system was quite robust.

5.6.4: Comparison of shake flask and fermenter cultures.

5.6.4.1: α-Amylase and background protein levels.

The performance seen from *S. lividans* pQR620 on a shake flask scale compared well with those seen from the fermentations inoculated using single-stage inocula, i.e. fermentations A and B. Among these cultures the growth rates and peak biomass values were fairly consistent (0.057, 0.046 and 0.067 h^{-1} and 2.18, 1.73 and 2.05g DCW/l respectively). Each also showed an initial lag period of around 20 hours prior to exponential growth. The growth patterns seen in the shake flask cultures therefore differed from those seen in fermentations C and D in the same ways that fermentations A and B had been observed to differ (Section 5.5).

The peak α-amylase level observed in the flask was higher than that seen in any of the fermentations. However, growth and α-amylase production were monitored for a longer period in the shake flask culture than in the fermentations. It is possible that, if they had been monitored, the α-amylase level in fermentations A and B would have continued to rise. These similarities between the shake flask and fermentations A and B may have been due to similarities between the inoculum given to each culture.

Productivity level seemed to be the most comparable characteristic of those calculated for the fermentations. The productivity data from the shake flask was generally lower than that from the fermentations (Fig. 5.16), although, it did follow the same general fluctuations in pattern. The difference in levels may have been partly due to its low initial α-amylase level when compared to the other cultures. Each of the fermentations' inocula contained a significant number of α-amylase units. This would have tended artificially to raise productivity levels early in the cultures before much growth had occurred. However, as the productivity levels remained low over growth, this is unlikely to have contributed significantly to the difference. The peak productivity in the shake flask was actually higher than those from the fermentations.

Another fairly clear cut difference was that the shake flask culture produced far higher levels of contaminating background protein than the fermentations. Protein levels in fermentations A and B, which had grown in a comparable manner to the shake flask,

peaked at 0.119 and 0.111 mg/ml supernatant respectively. The shake flask culture of *S. lividans* pQR620 had a peak supernatant protein level of 0.164 mg/ml, around a third higher than the levels in fermentations A and B, and over two thirds higher than the levels in fermentations C and D. These differences obviously affected the α-amylase specific activity values in each culture and indicated that, if purity of the α-amylase was the main consideration, producing the enzyme in a fermenter would give a significant advantage.

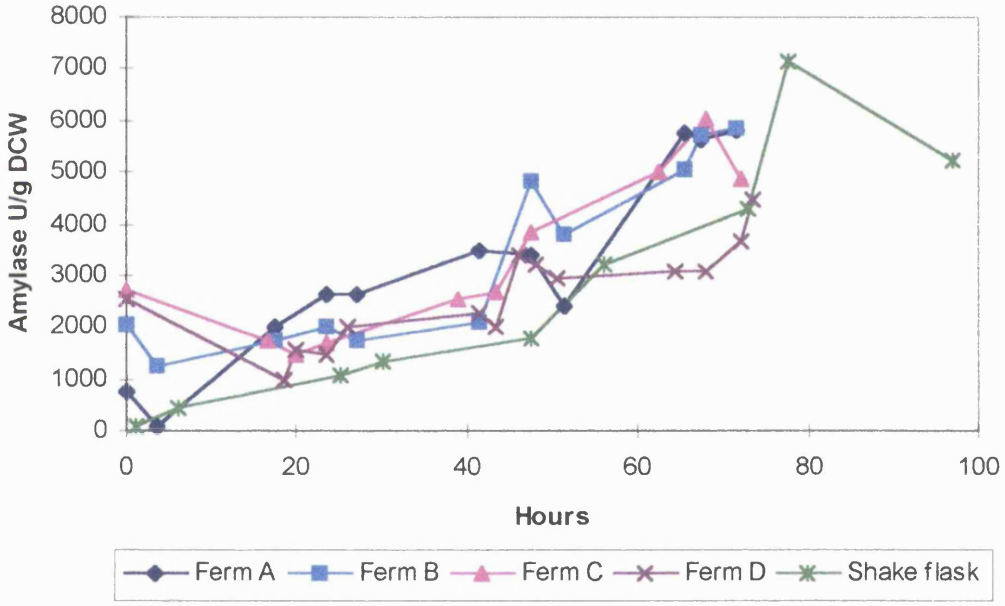


Figure 5.16: Graph showing the productivity profiles of *S. lividans* pQR620 in fermentations A, B, C and D and in shake flask culture. The profiles have been adjusted so that 0 hours is at the start of the period of exponential growth in each case.

5.6.4.2: Effect of shear levels and pellet sizes.

Several possible explanations exist for the variations in protein levels and productivities between the shake flask and fermenter grown cultures. The cells grown in the fermenters would have been subjected to higher levels of shear stress than those grown in the shake flasks (Braun & Vecht-Lifshitz, 1991). Although these forces are difficult to quantify and evaluate, they could have been the major reason for the differences seen. The baffles and springs in the shake flask would have helped to break up the pellets in the medium to give a smaller average size than would otherwise have been present (Vecht-Lifshitz *et al.*, 1990). This is taken to a greater extreme in fermenters. Many species will only form dispersed growth when high shear conditions are present. Pelleting organisms such as *S. hygroscopicus* and *S. lividans* are found to form very small pellets under such conditions (Whitaker, 1992; Kennedy *et al.*, 1994). The expected trends in pellet size were confirmed visually for the shake flask and fermentation cultures discussed here. The pellets in the fermentations tended to be much smaller than those in the shake flasks. Image analysis studies would be required to quantify such differences.

Pellet size differences would be expected to have a major effect on the α-amylase expression system. Distances of diffusion from the medium into the centre of smaller pellets would be low. The cells in the centre of small pellets would therefore be in an entirely different environment from those at the centre of large pellets (Bushell, 1988; Braun & Vecht-Lifshitz, 1991). They would be in a far better situation with regard to supply of nutrients and oxygen. A large proportion of the total cell population will be inside pellets in these *S. lividans* cultures. If diffusion limited the nutrient supply to these cells their metabolism could be greatly affected and α-amylase production could become less favourable. In a similar way, production of contaminating proteins from these cells might be enhanced. Nutrient limitation could trigger the secretion of degradative enzymes or secondary metabolites as the cells attempted to improve conditions in their local environment (Bushell, 1989; Wilson & Bushell, 1995). The existence of such a situation in larger pellets might explain the patterns seen from the fermenter and shake flask grown cells. As inoculum size and type is also known to affect pellet size (Whitaker, 1992; Vecht-Lifshitz *et al.*, 1990; Braun & Vecht-Lifshitz,

1991), such effects may also explain the differences in growth, α-amylase and protein production seen from different inocula. Further work would be needed to confirm this hypothesis.

5.6.4.3: Effects of oxygenation levels and antibiotic concentration.

Variations in oxygenation levels are present between shake flask and fermenter conditions (Kennedy *et al.*, 1994; Clark *et al.*, 1995). Exactly what effects these differing levels had on the cultures was difficult to say. However, the evidence from fermentations A and B, where the oxygen supply was disrupted for a period (Section 5.3.3), suggests that varying oxygenation levels were not likely to be the cause of the differences seen.

Another, less obvious, difference between the cultures was the presence of the antibiotic thiostrepton (Section 2.4.4). This was added into the flask at a concentration of 5μg/ml. Only residual thiostrepton from the inoculum flasks was present in the fermenters, so the levels were significantly different from those in the flask. It was possible that the thiostrepton, or the DMSO it was dissolved in, led to increased general protein production to the detriment of α-amylase production. No mention of such an effect occurs in the *Streptomyces* literature. However, it is possible that this difference helped to contribute to the variations seen (see Section 8.2 for further discussion).

5.6.4.4: Differences in plasmid copy number.

Wrigley-Jones *et al.* (1993) found that the plasmid copy number of the *sti*$^+$ plasmid pIJ303 in *S. lividans* under fermentation conditions was only 50 to 75% of that seen in shaken culture. They postulated that this was due to the increased growth rate of the cells within the fermenter environment. As the plasmid levels were still high they felt that gene expression from the plasmid was unlikely to be adversely affected. The performance of fermentation D may suggest that gene expression was reduced because of lower copy number. This culture grew about twice as quickly as fermentation C which was given a similar inoculum (Fig. 5.7). It produced less α-amylase than the other fermentations, which had been slower growing (Section 5.5).

In these experiments the productivities of the cells grown in the fermenters were greater than those from the cells grown in the shake flask over the majority of the life cycle. This pattern would have been expected to be reversed if a lower copy number was affecting expression in the fermentations. As the growth rates in fermentations A, B and C and the shake flask were relatively close it is possible a decrease in copy number was not seen in these fermentations when compared to shaken culture. These results may suggest that more steady growth within the fermenter would be optimal for α-amylase production by allowing plasmid copy number to remain high.

If the plasmid copy number was indeed reduced in all the fermentations, as Wrigley-Jones *et al.* (1993) observed with *S. lividans* pIJ303, then the reduction in copy number did not significantly reduce the α-amylase levels seen in the supernatants. If enzyme production was lowered because of a fall in copy number the reduction must have been more than compensated for by a different favourable condition present in the fermenters. More in depth investigations into the copy number of pQR620 under various conditions would need to be conducted before any conclusion could be drawn about which of the above situations applied.

5.6.4.5: Effect of growth temperature.

Another possible reason for the variations seen between the shake flask and fermenter grown cultures was the growth temperature. The shake flask study had been carried out at 30°C and the fermentations at 28°C. When these experiments were conducted such a temperature difference was expected to be of very little consequence to expression of α-amylase (Section 6.2.1). Subsequently, it was found that higher temperatures were more favourable for α-amylase production (Chapter 6). The *S. lividans* pQR620 flask discussed in this chapter therefore probably contained slightly higher levels of α-amylase than would have been expected from a flask grown at 28°C (Section 6.4.1). This variation in temperature would not be expected significantly to change growth levels or patterns. These findings suggested that the differential in α-amylase productivity levels between the shake flask and fermenter cultures compared here would be slightly wider if the shake flask had been incubated at 28°C. The points discussed above are therefore still valid in the light of these findings.

5.7: Summary

Four fermentations were carried out in MMS medium to evaluate the performance of *S. lividans* pQR620 under more controlled conditions. Each of the cultures was grown at 29°C. The α-amylase productivities seen in the fermentations were closely comparable although differences had been observed in growth and protein production patterns. Significantly higher levels of contaminating protein were produced by fermentations A and B than were produced by fermentations C and D. Faster growth and higher peak biomass levels were obtained in cultures C and D. The evidence indicated that these outcomes were a function of the type of inoculum used and suggested that inocula should be prepared using a two-stage strategy to optimise growth and α-amylase specific activity. Transient low DOT levels and pH control had no obvious effects on the performances of the cultures. Significant degradation of α-amylase was not observed under the conditions used. α-Amylase production was both growth and non-growth associated suggesting RNA polymerase σ factors capable of recognising the *mel* and/or *amy* promoters were present in the cells for the majority of the culture time. The *S. lividans* pQR620 expression system performed better on a large-scale than it had done in shake flasks. Both α-amylase productivities and specific activities were higher in fermenter grown cultures than in comparable shake flask cultures.

Chapter 6

The Effect of Incubation Temperature on α-Amylase Production

6.1: Aims

The work detailed in this chapter was conducted to define and investigate the effect of variations in incubation temperature on α-amylase production from the *S. lividans* α-amylase expression systems.

6.2: Background

During the course of the work documented in Chapters 4 and 5 it was difficult to reproduce the same levels of α-amylase from experiment to experiment. On some occasions very large increases in α-amylase yields were observed (up to 50 α-amylase U/ml supernatant). Many of the experiments had been conducted over the course of a particularly hot summer. Analysis of the results indicated that the highest levels of α-amylase had been achieved during periods of relatively high temperature. This raised the possibility that increased temperatures of incubation stimulated α-amylase production from *S. lividans* pQR620 and pQR621.

The orbital incubator used for these experiments was located in a controlled temperature room which was maintained at 30°C. Slight temperature fluctuations were experienced in this environment when the door was opened at times when the external temperature was higher than that of the room. Very little information on the effect of temperature on *Streptomyces* production systems is available in the primary literature. However, the evidence that was available suggested that transient changes in temperature of a few degrees above or below 30°C would have little effect on the cells.

6.2.1: *S. lividans* grows optimally at 29°C.

The majority of the members of the genus *Streptomyces* are mesophiles. Generally *Streptomyces* fall into the lower temperature range for such bacteria with optimum growth in the range of 25 to 30°C (Bader, 1986). Most fermentations are run at between 27 and 34°C to achieve maximum productivity. The optimum growth temperature of

wild type *S. lividans* is 29°C (Kluepfel *et al.*, 1986; Lee & Lee, 1994). The cells can sustain growth at temperatures as high as 46°C and significant levels of growth are seen at 40°C, suggesting that *S. lividans* is a thermotolerant organism. The vast majority of studies using *S. lividans* are conducted at either 30°C (i.e. Hopwood *et al.*, 1985; Vigal *et al.*, 1991b; Katz *et al.*, 1983; Taguchi *et al.*, 1989b; Stein *et al.*, 1989; Parro & Mellado, 1994; Payne *et al.*, 1990) or 28°C (*i.e.* Eckhardt *et al.*, 1987; Bender *et al.*, 1990a; Fornwald *et al.*, 1987; Dehottay *et al.*, 1986; Wrigley-Jones *et al.*, 1992; Erpicum *et al.*, 1990). Occasionally, other temperatures are used, *e.g.* Kendall and Cullum (1984) grew cultures at 32°C; Kendall and Cohen (1987) used 34°C. Choice of incubation temperature seems to be arbitrary and is rarely justified. Only a small number of published studies have sought to optimise expression of recombinant or natural products by streptomycetes through alterations in growth temperature.

6.2.2: Growth temperature can affect recombinant protein production.

Most available evidence would tend to suggest α-amylase production would be better at low or optimal temperatures. Wrigley-Jones *et al.* (1993) found that temperature affected the plasmid copy number of pIJ303. At 28°C copy numbers remained relatively high for the duration of the fermentation. In contrast, when *S. lividans* pIJ303 was grown at 37°C plasmid levels peaked approximately 200 copies per chromosome **lower** than the maximum level seen at 28°C. Also, at the higher temperature plasmid levels declined rapidly in stationary phase to an undetectable level. These low copy numbers would be expected to have a significant negative effect on α-amylase production levels from the *S. lividans* expression system at temperatures above 30°C.

Lee and Lee (1994) looked at the effects of temperature on growth, plasmid stability and β-lactamase production from *S. lividans*. The vector used in their study was based on pIJ702. Approximately 92% of cells harboured plasmids. This level remained constant over a temperature range of 24 to 36°C. The plasmid copy number was not measured. Peak biomass levels obtained were also constant over this range of temperature. However, levels of β-lactamase production were best at the sub-optimal temperature of 27°C. Haas-Lauterbach *et al.* (1993) found that production of tendamistat from a streptomycete plasmid in *S. lividans* TK24 was better at 19°C than at 28°C. Levels were reduced even further when the cells were grown at 37°C.

Production of several natural products has also been observed to be reduced at higher temperatures. Stoichev *et al.* (1981) found that glucose isomerase production by *Streptomyces* species 1339 fell by over a third when the growth temperature was raised from 30 to 36°C. Production of papain and trypsin inhibitors by *Streptomyces* species 22 was maximal at 18°C, although growth was only slightly affected by temperatures in the 18 to 37°C range (Kourteva *et al.*, 1995).

Reports of maximal product production at higher than optimum temperatures are scarce in the *Streptomyces* literature, although they do occur. Reusser (1985) found that *Streptomyces espinosus* produced up to 100-fold more lincomycin at temperatures above 28°C. Highest levels of production occurred at 45°C. Kluepfel *et al.* (1986) looked at the production of cellulases and xylanase by *S. lividans* 1326. Maximal levels of cellulase were produced between 34 and 37°C. Xylanase production was best at 40°C. These levels were 2 to 5-fold greater than those obtained at 31°C. Flickinger *et al.* (1990) found that toyocamycin production by *Streptomyces chrestomyceticus* peaked at between 34 and 36°C. Each of these reports refer to natural products. No reference could be found in the literature to increased production from a plasmid borne gene in *Streptomyces* at temperatures above those considered as optimal, i.e. 28 to 30°C.

6.3: Shake Flask Study to Evaluate the Effect of Raised Incubation Temperature on α-Amylase Production from the *S. lividans* Expression Systems

A preliminary shake flask study was conducted to investigate the effect of temperature on α-amylase production from *S. lividans* pQR620, pQR621, pQR311 and pQR318, and to see whether increases in incubation temperature could have caused the variations seen from experiment to experiment.

This experiment was conducted in 2l baffled flasks containing 500ml of MMS medium. Each flask was inoculated with an aliquot of the appropriate spore stock and was then placed in an orbital incubator at 300rpm. One set of four flasks was incubated at 30°C. An identical set of flasks was incubated at 34°C. Samples were taken at regular time points over the course of the experiment. When the flasks were returned to the incubator following sampling, a conscious effort was made to place each flask in a

different position on the shaking platform from that which each had been in over the previous period of incubation. This was to help to eliminate any variations due to the positioning of flasks within the incubator.

Figures 6.1, 6.2, 6.3 and 6.4 show the results of this experiment. Each set of figures compares the performance of one of the *S. lividans* strains at 30 and 34°C.

6.3.1: Significantly increased α-amylase productivity was obtained in shake flask culture at 34°C.

The results of this experiment showed that incubation temperature did have a very dramatic effect on the levels of α-amylase produced. The productivity of *S. lividans* pQR620 at 34°C was almost 3-fold greater than its productivity at 30°C (Fig. 6.1e). A jump in productivity at the higher temperature was also seen from *S. lividans* pQR621, with 1.4-fold more α-amylase produced per gram of cells at 34°C than at 30°C (Fig. 6.2e). As only very low levels of α-amylase activity were produced by *S. lividans* pQR311 and pQR318, interpreting the influence of temperature on enzyme production was more difficult (Figs. 6.3b & 6.4b). *S. lividans* pQR311 produced higher levels of α-amylase at 34°C than at 30°C, with a 1.3-fold increase in productivity at 34°C (Fig. 6.3e). In contrast, lower levels of α-amylase productivity were seen for *S. lividans* pQR318 at 34°C than at 30°C (Fig. 6.4e). When considering these results, it must be noted that the peak measured level of α-amylase for the 30°C culture is an outlying point which could be due to error.

The patterns and levels of growth seen in the flasks were similar at both temperatures (Figs. 6.1a, 6.2a, 6.3a & 6.4a). Only *S. lividans* pQR620 displayed any real variation with a shorter lag period and faster growth at 34°C than at 30°C (Fig. 6.1a). The differences in growth pattern that were seen in the flasks could again be explained by inoculum variations (Section 5.6.1). Other studies have observed similar trends, with levels of streptomycete growth remaining approximately constant over a wide range of temperature (Lee & Lee, 1994; Reusser, 1985; Kourteva *et al.*, 1995). Again these results provide evidence that high levels of α-amylase production (up to 100mg/l) do not seem to retard *S. lividans* cell growth (Section 4.7.4).

Figures 6.1a-e: Effect of incubation temperature on *S. lividans* pQR620.

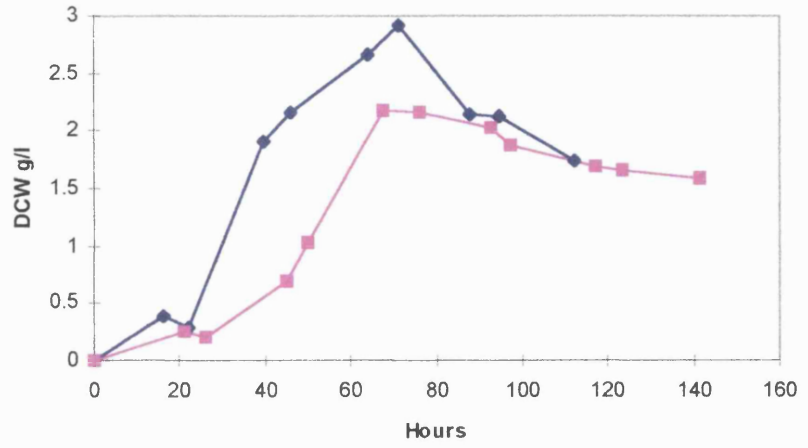


Figure 6.1a: Growth curves for *S. lividans* pQR620 grown at 30 and 34°C (average SEM 0.054 g DCW/l).

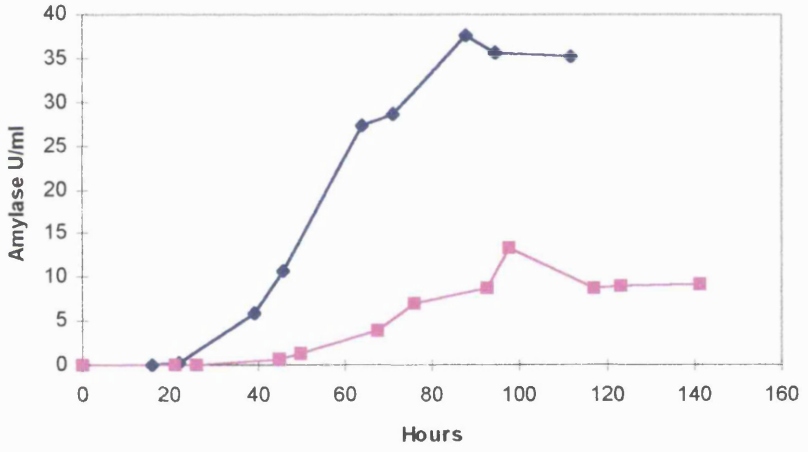


Figure 6.1b: Supernatant α-amylase levels for *S. lividans* pQR620 grown at 30 and 34°C (average SEM 0.470 U/ml).

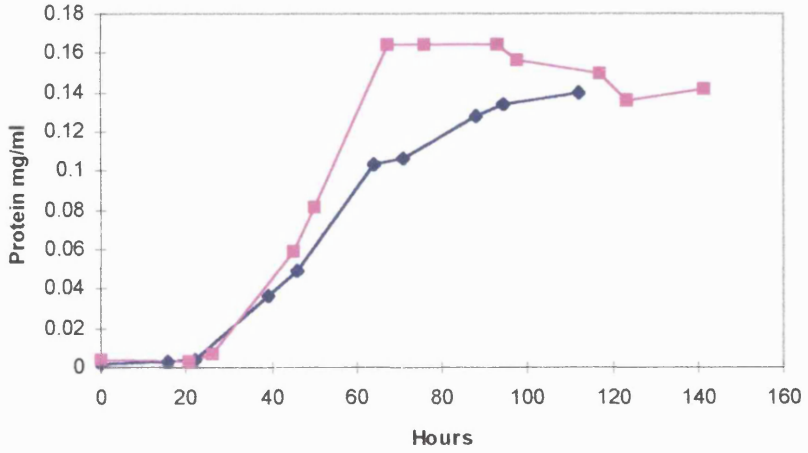


Figure 6.1c: Supernatant protein levels for *S. lividans* pQR620 grown at 30 and 34°C (average SEM 0.002 mg/ml).

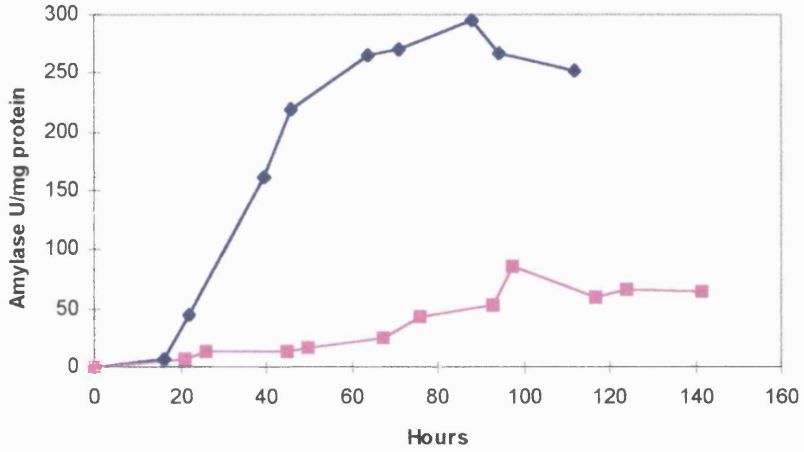


Figure 6.1d: α-Amylase specific activity levels for *S. lividans* pQR620 grown at 30 and 34°C.

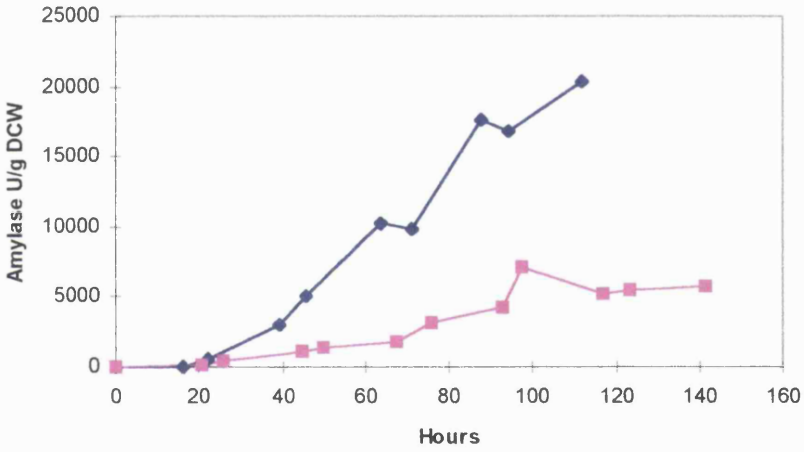


Figure 6.1e: α-Amylase productivity for *S. lividans* pQR620 grown at 30 and 34°C.

Figures 6.2a-e: Effect of incubation temperature on *S. lividans* pQR621.

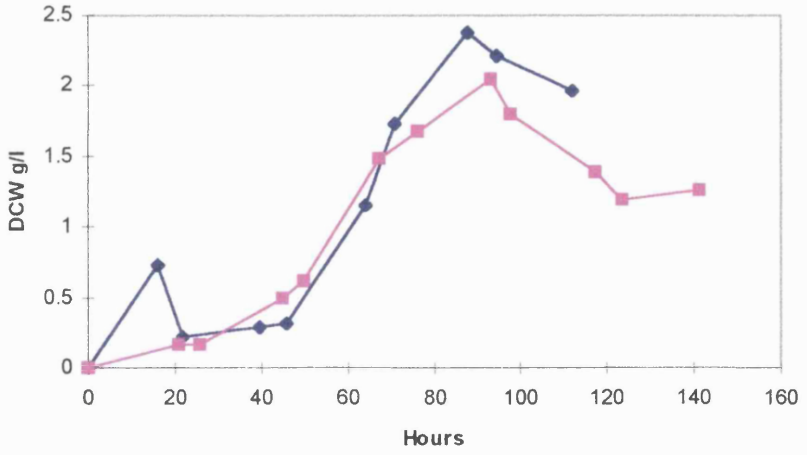


Figure 6.2a: Growth curves for *S. lividans* pQR621 grown at 30 and 34°C (average SEM 0.058 g DCW/l).

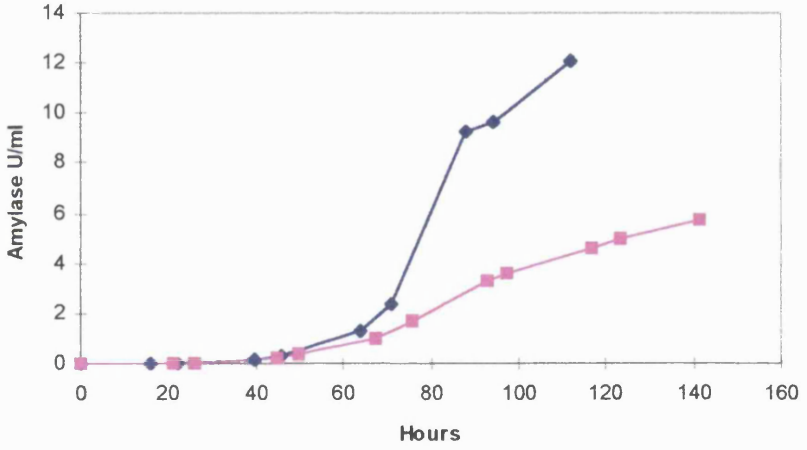


Figure 6.2b: Supernatant α-amylase levels for *S. lividans* pQR621 grown at 30 and 34°C (average SEM 0.072 U/ml).

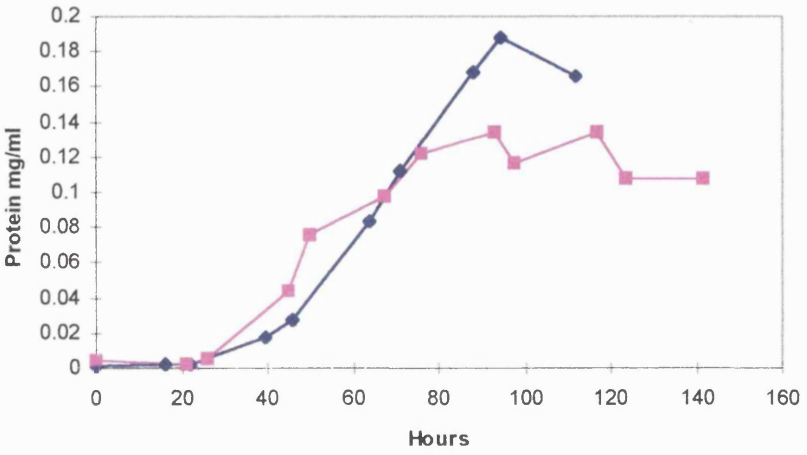


Figure 6.2c: Supernatant protein levels for *S. lividans* pQR621 grown at 30 and 34°C (average SEM 0.002 mg/ml).

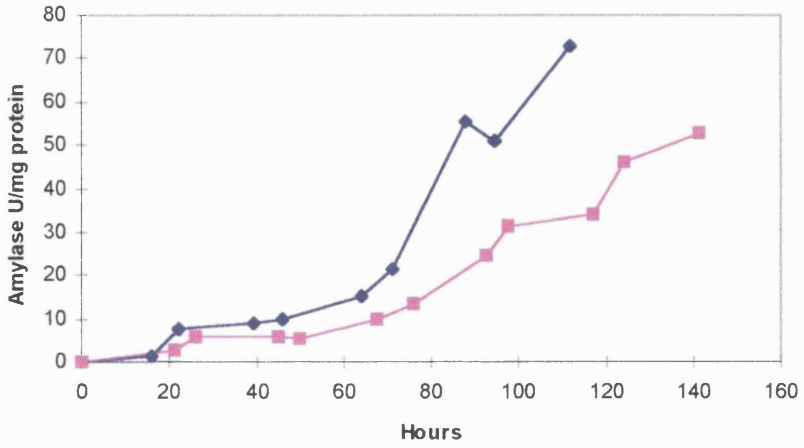


Figure 6.2d: α-Amylase specific activity levels for *S. lividans* pQR621 grown at 30 and 34°C.

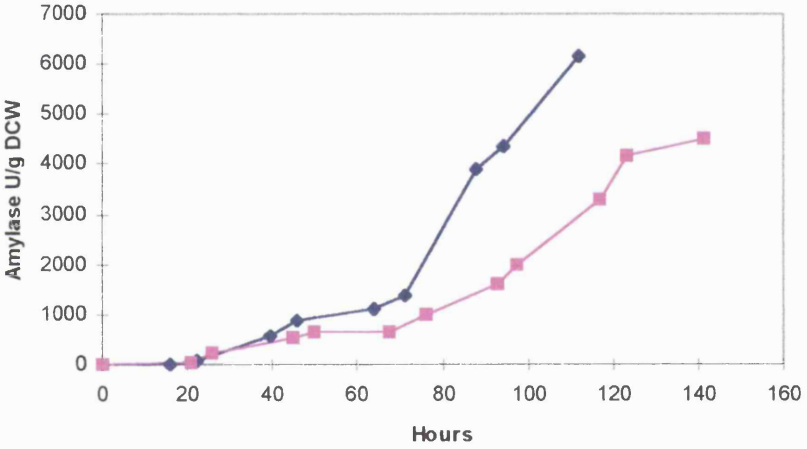


Figure 6.2e: α-Amylase productivity for *S. lividans* pQR621 grown at 30 and 34°C.

Figures 6.3a-e: Effect of incubation temperature on *S. lividans* pQR311.

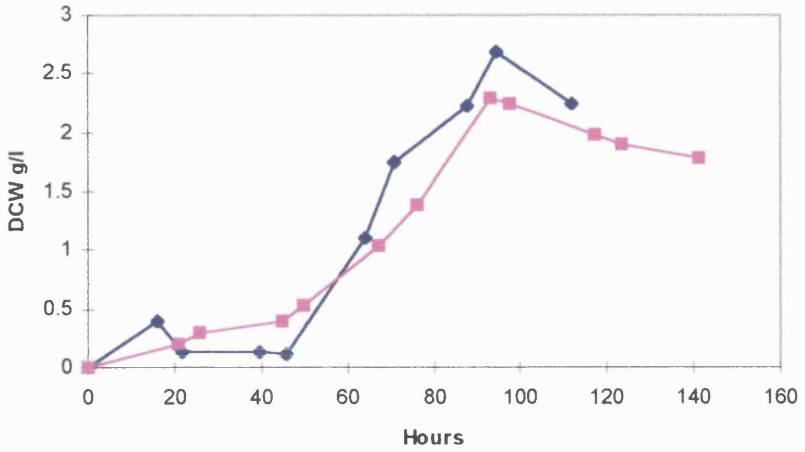


Figure 6.3a: Growth curves for *S. lividans* pQR311 grown at 30 and 34°C (average SEM 0.055 g DCW/l).

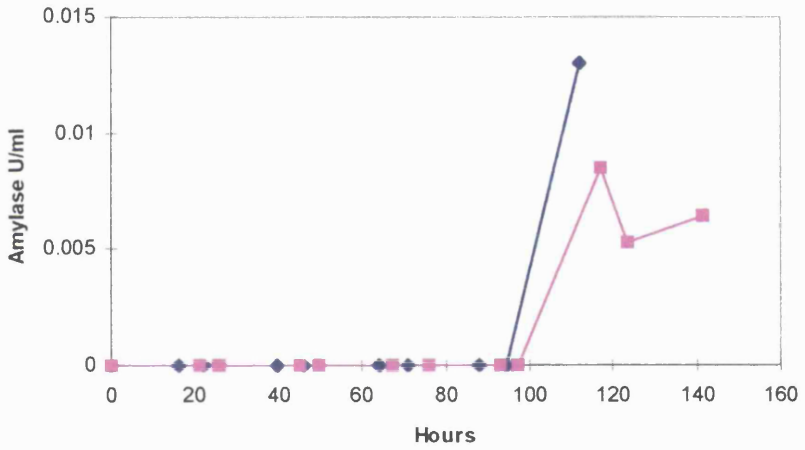


Figure 6.3b: Supernatant α-amylase levels for *S. lividans* pQR311 grown at 30 and 34°C (average SEM 0.001 U/ml).

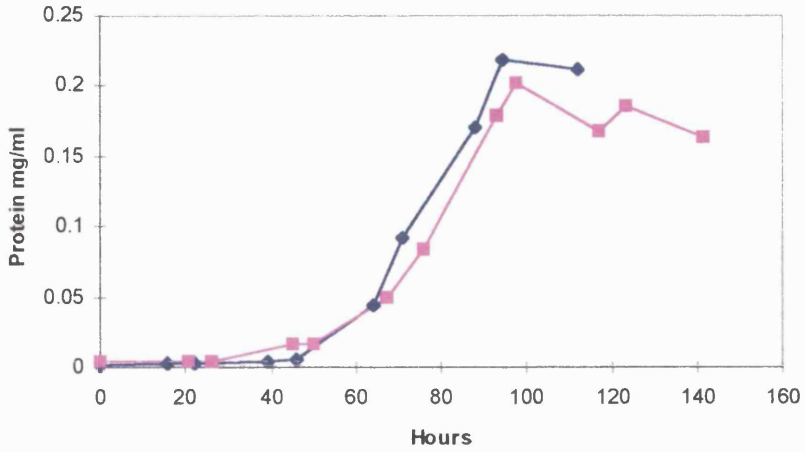


Figure 6.3c: Supernatant protein levels for *S. lividans* pQR311 grown at 30 and 34°C (average SEM 0.001 mg/ml).

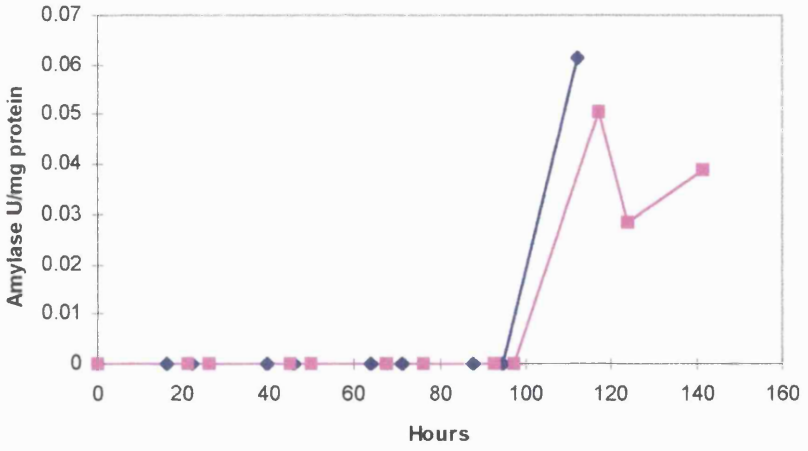


Figure 6.3d: α-Amylase specific activity levels for *S. lividans* pQR311 grown at 30 and 34°C.

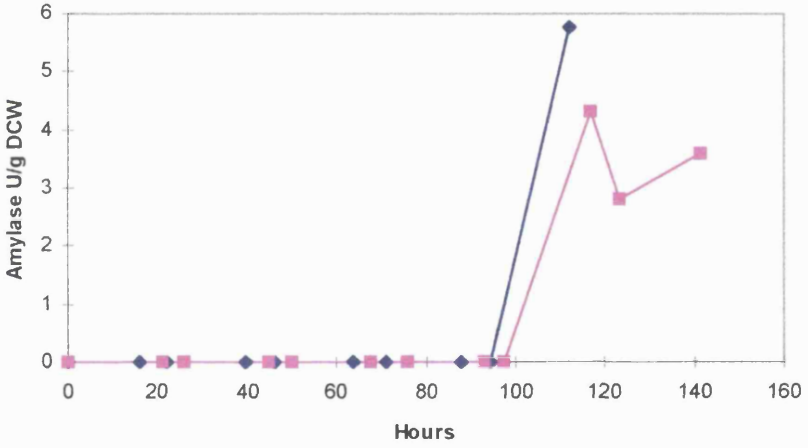


Figure 6.3e: α-Amylase productivity for *S. lividans* pQR311 grown at 30 and 34°C.

Figures 6.4a-e: Effect of incubation temperature on *S. lividans* pQR318.

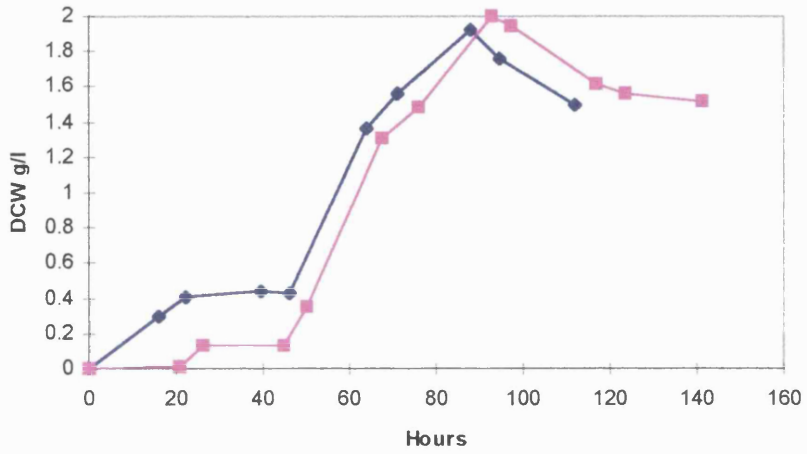


Figure 6.4a: Growth curves for *S. lividans* pQR318 grown at 30 and 34°C (average SEM 0.038 g DCW/l).

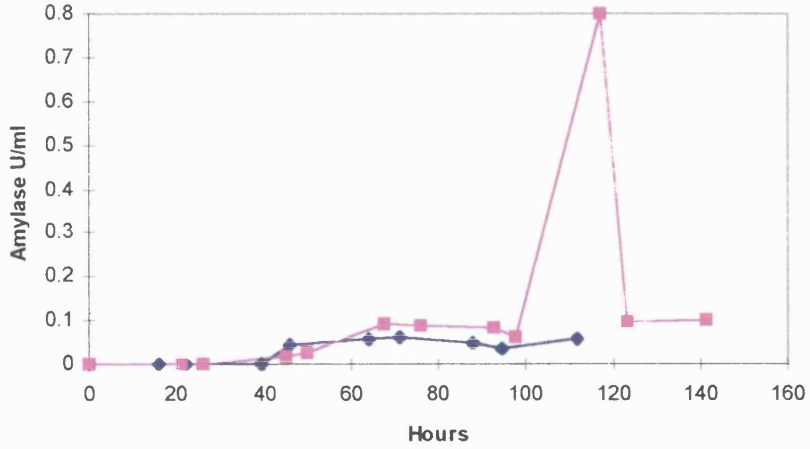


Figure 6.4b: Supernatant α-amylase levels for *S. lividans* pQR318 grown at 30 and 34°C (average SEM 0.004 U/ml).

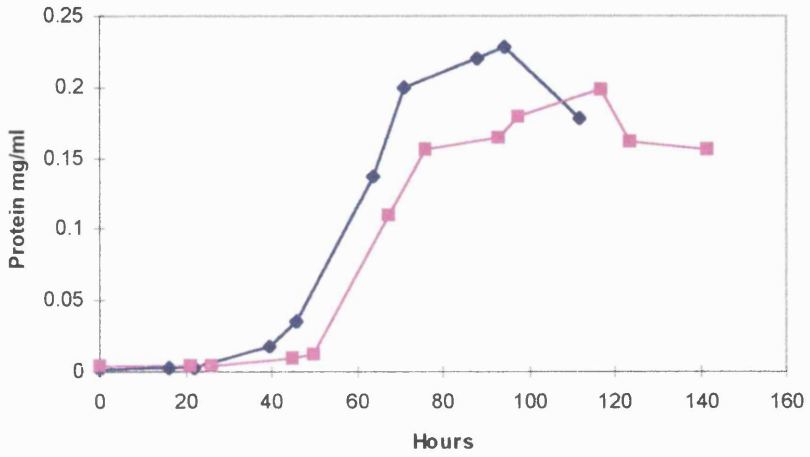


Figure 6.4c: Supernatant protein levels for *S. lividans* pQR318 grown at 30 and 34°C (average SEM 0.003 mg/ml).

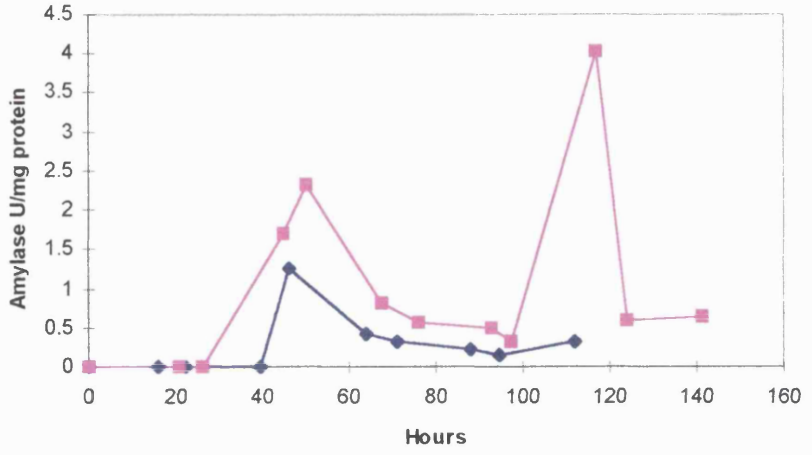


Figure 6.4d: α-Amylase specific activity levels for *S. lividans* pQR318 grown at 30 and 34°C.

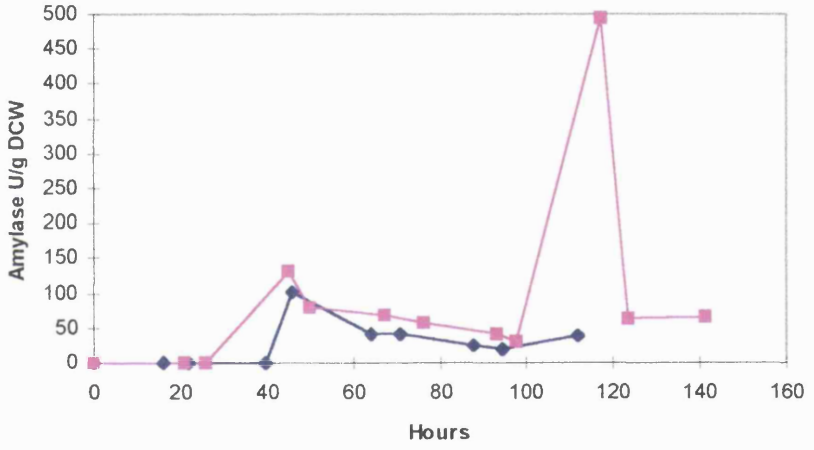


Figure 6.4e: α-Amylase productivity for *S. lividans* pQR318 grown at 30 and 34°C.

6.3.2: Production of background protein at 34°C.

The specific activity values obtained from the *S. lividans* pQR620 cultures suggest that the high levels of α-amylase expression found at 34°C have a negative affect on the production or secretion of other proteins (Fig. 6.1d). At 34°C, the peak specific activity value obtained was 294 α-amylase units per mg of supernatant protein. At 30°C this value was 86. This represented a 3.4-fold increase in specific activity at the higher incubation temperature, although only a 2.8-fold increase in α-amylase productivity was observed. Each of the supernatant protein measurements included the α-amylase fraction, so the difference in relative levels at 34°C was likely to have been even greater. Assuming that 1 unit of α-amylase equals 2.6μg of protein (French, 1993), in the *S. lividans* pQR620 culture grown at 34°C, the results show that approximately two thirds of the supernatant protein was α-amylase (*i.e.* 100μg α-amylase/ml in a total level of 140μg of protein/ml)(Fig. 6.1c). The *S. lividans* pQR620 culture grown at 30°C produced around 35μg of α-amylase per ml of supernatant in a total background protein of 164μg protein per ml (*i.e.* approximately one fifth of the total protein was α-amylase).

Several possible mechanisms could allow very high levels of α-amylase production to affect the levels of other protein secreted by the cells. The cells transcription and translation machinery could be sequestered by such high levels of α-amylase gene expression. The precursor pools within the cells could be altered in a way which was detrimental to the production of other secreted proteins or the cells' export machinery could be monopolised by the α-amylase. Other factors such as morphology and medium composition are likely to influence background protein levels, so further work would be needed to confirm that high levels of α-amylase expression directly affect production of other secreted proteins.

The increase in specific activity seen at 34°C, when compared to 30°C, in *S. lividans* pQR621 exactly corresponded to the increase in α-amylase productivity values seen at the higher temperature (both values were 1.4-fold higher at 34°C)(Figs. 6.2d & 6.2e). This suggested that no large detrimental effect on background protein secretion was seen due to α-amylase production at this level and incubation temperature. The protein

production profiles for *S. lividans* pQR311 and pQR318 were very close at both temperatures (Figs. 6.3c & 6.4c). As high levels of α-amylase were not produced by either of these strains, these profiles were likely to be more representative of the behaviour of wild-type *S. lividans* in shake flask culture at 30 and 34°C.

6.3.3: Roles of the *mel* promoter and *sti* region in the temperature effect.

This experiment showed that temperature did have a highly significant effect on α-amylase expression. The results may help to narrow down the possible reasons for the increase in α-amylase production with temperature. No increase in α-amylase production was seen at the higher incubation temperature with *S. lividans* pQR318. In fact, levels of productivity obtained at 34°C were lower than those obtained at 30°C (Fig. 6.4e). This suggested that the particular property, or properties, of the system responsible for the temperature enhancement are not present in the *S. lividans* pQR318 system. Both *S. lividans* pQR311 and pQR621 displayed increased α-amylase productivities when incubated at the higher temperature, suggesting that the element, or elements, responsible for the increases was present in these expression systems. The only element common to pQR311 and pQR621, but not present on pQR318, is the native upstream region of the α-amylase gene. The explanation for the temperature effect might therefore be found in this region. These results strongly suggest that incorporation of the *sti* region, and the ensuing improvements of the plasmids' replication characteristics, do not play a role in the system's response to temperature. Both pQR621 (sti^+) and pQR311 (sti^-) respond in a similar way.

As described in Section 6.3.1, *S. lividans* pQR620 produced 3-fold more α-amylase at 34°C than at 30°C. *S. lividans* pQR621 was found only to produce 1.4-fold more α-amylase at the higher temperature. The larger magnitude of the response in pQR620 might indicate that the *mel* promoter region also plays a role. It is possible that temperature has an effect on the α-amylase genes upstream region which then feeds back in some way to enhance levels of transcription from the *mel* promoter region. Alternatively, expression from the *mel* promoter could remain constant at both temperatures, with expression only being affected and increased from the α-amylase promoter when there is a temperature rise.

Alternatively, the rise in expression at higher temperatures could be due to another aspect of α-amylase production, secretion or stability. The fact that *S. lividans* pQR318 produced lower levels at 34°C than at 30°C may only be a characteristic of that particular plasmid. As the experiment with pQR318 was conducted only once, and because the measured α-amylase levels were so low, and were therefore more likely to be significantly affected by slight errors, it may be unwise to conclude that the explanation for the response is associated with the plasmids. Temperature has a very large influence on the behaviour of bacterial cells and enzymes (Cossins & Bowler, 1987). Several different factors could be acting together to raise α-amylase levels. Since no concrete explanation could be reached based on these findings, no possible mechanisms for the temperature effect could be ruled out at this stage of the study.

6.3.4: Temperature fluctuation in shake flask cultures.

The variations in α-amylase level obtained during shake flask experiments could definitely be explained by relatively small fluctuations in incubation temperatures. This finding meant that obtaining reliable data, unaffected by temperature variations between flasks, would be almost impossible to obtain from shake flask studies on the *S. lividans* α-amylase expression system. Heat is produced by growing cells through respiration. In shake flask cultures heat transfer to the surrounding air tends to be poor. This means that shake flasks frequently run 2 to 3°C higher than the shaker room temperature during periods of peak oxygen uptake (Bader, 1986). It is virtually inevitable that the cells contained in different flasks will have differing respiration rates and therefore differing levels of heat generation. There is also an uneven distribution of temperature depending on the location of the flask on the shaker. Those nearest the centre and near the drive frequently run 1°C higher than more remote flasks (Bader, 1986). Such effects could drastically affect the α-amylase production levels between flasks and, if comparing different conditions (*e.g.* medium components), could result in false assumptions about the affect of a change on α-amylase production.

The findings of this shake flask experiment therefore indicated that, to establish a true picture of α-amylase production from the *S. lividans* expression systems, experiments would always need to be conducted in fermenters where temperatures could be maintained more satisfactorily. However, even under such conditions it would still be

necessary to bear the effect of temperature in mind. Gradients may exist in fermenters, especially in any stagnant zones that may be present (Braun & Vecht-Lifshitz, 1991; Votruba & Vanek, 1989). Ensuring adequate mixing during growth of the cells in fermenters was therefore of paramount importance if reliable results were to be obtained. Although the evidence suggested that shake flask conditions were not ideal for the investigation of α-amylase production, it was impossible to conduct every experiment on a fermenter scale. Therefore, in subsequent shake flask experiments, it was necessary to take into account possible variations in α-amylase levels due to temperature differences.

6.4: Evaluation of the Effect of Temperature on α-Amylase Production from *S. lividans* pQR620 Under Fermentation Conditions

A series of fermentations were conducted to define the effect of temperature under more closely controlled conditions because the temperatures of incubation for the previous shake flask experiment were likely to have been subject to variations. These experiments used *S. lividans* pQR620.

Fermentations C and D (described in Chapter 5) were used as examples of 28°C cultures. Four additional fermentations were carried out at 24°C, 32°C, 34°C and 36°C. All of the fermentations were inoculated using the two-stage inoculum strategy described in Section 5.2.1.2. Again each of the inocula were sampled prior to use so their characteristics could be defined and compared (Table 6.1). MMS medium was used for each culture. The pH was maintained at 7 in each case and the vessels were stirred at 500 rpm. DOT levels remained above 60% saturation over the course of each fermentation.

6.4.1: Higher levels of α-amylase production were again obtained at raised incubation temperatures.

These fermentations confirmed the finding that temperatures above the *S. lividans* optimal growth temperature were preferable for high α-amylase productivities (Fig. 6.9). The lowest levels of α-amylase were obtained in the supernatants of the fermentations grown at 24 and 28°C (Fig. 6.6). Approximately 5-fold more α-amylase

Table 6.1: Characteristics of the inocula used in the fermentation experiments examining the effect of incubation temperature on *S. lividans* pQR620. Flasks were sampled immediately prior to use as inocula (see Section 5.2.1.2 for further details).

Temperature of Fermentation	Age of Inoculum h	Characteristics of inoculum at point of transfer to fermenter (total levels in 1.5l volume)		
		DCW g	α-Amylase U	Supernatant Protein mg
24°C	75	2.227	11163	174
28°C (C)	70	2.052	8270	133
28°C (D)	70	2.405	6155	111
32°C	58	1.624	4320	41
34°C	74	3.298	12485	82
36°C	75	2.461	9720	259

was produced when the cells were incubated at 34 or 36°C (Fig. 6.9). Levels of α-amylase production at 32°C were between these two extremes.

No decisive trend could be seen in the patterns and levels of growth of the fermentations (Fig. 6.5). Differences between inoculum characteristics had been present which might, again, have been responsible for the variations seen (Table 6.1). The fact that no clear temperature emerged as best for fast growth and high biomass levels provides further evidence that *S. lividans* can grow well over a wide range of temperature (Section 6.3.1).

The supernatant protein levels observed did seem to present a clear picture upon first inspection, with much higher levels being present at 32, 34 and 36°C when compared with levels at 28 and 24°C (Fig. 6.7). However, most of the protein detected at 34 and 36°C would have been α-amylase as each culture produced around 0.1mg of α-amylase per ml of supernatant. This meant that very little background protein was present in either of these cultures; a finding which may provide additional evidence that high level α-amylase production affects the amount of other protein produced by the cells (Section 6.3.2). The protein levels secreted by the 32°C culture were therefore distinct from the

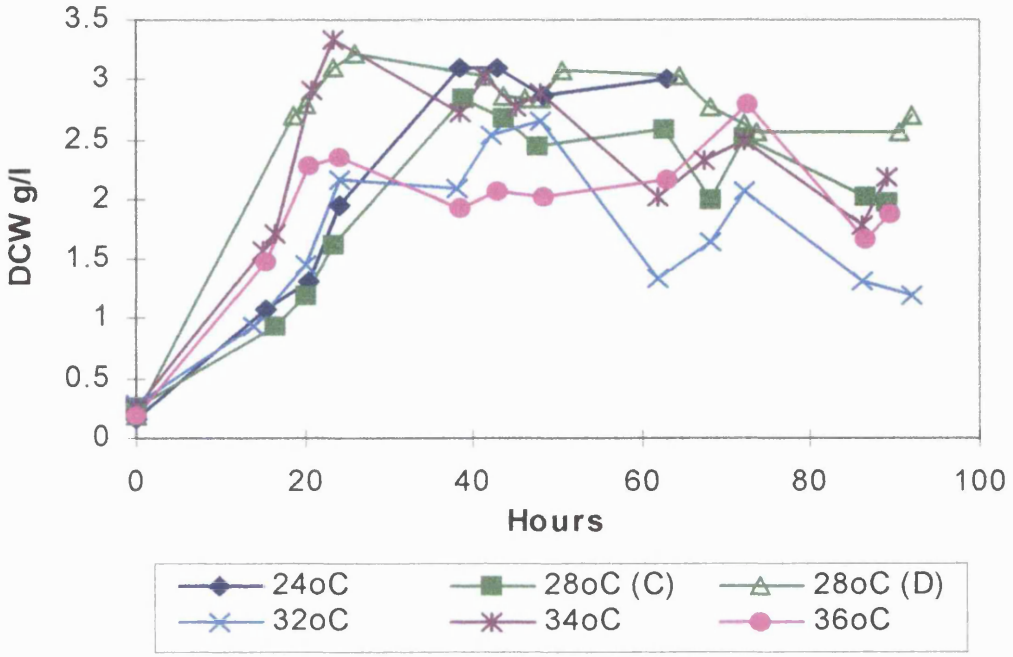


Figure 6.5: Growth patterns in the fermentation experiments examining the effect of incubation temperature on the performance of the *S. lividans* pQR620 system (average SEM 0.119 g DCW/l).

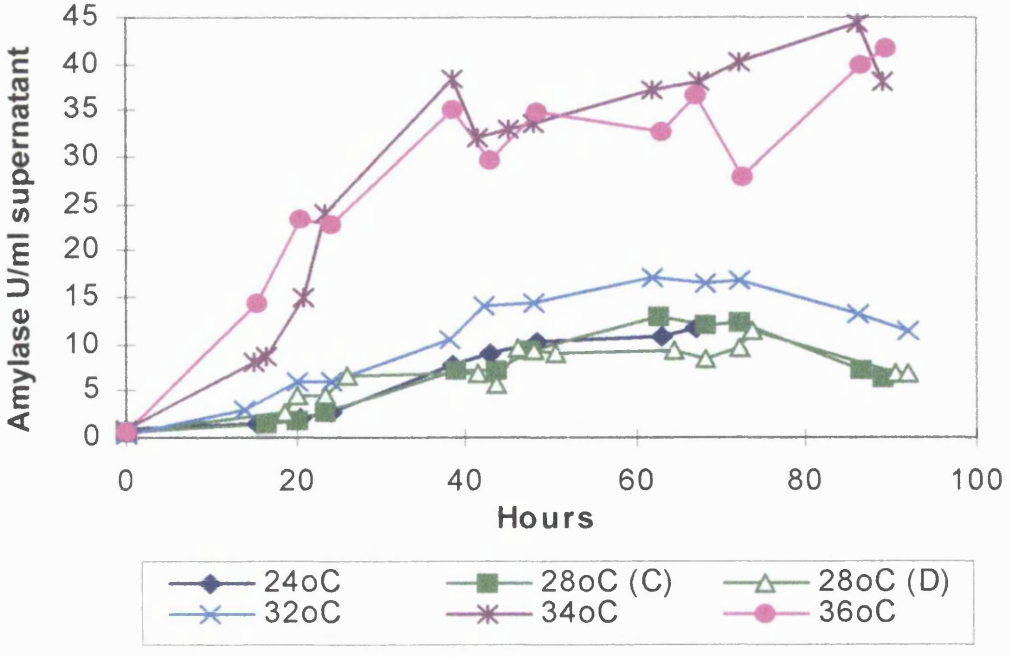


Figure 6.6: Supernatant α-amylase levels in the fermentation experiments examining the effect of incubation temperature on the performance of the *S. lividans* pQR620 system (average SEM 0.573 U/ml).

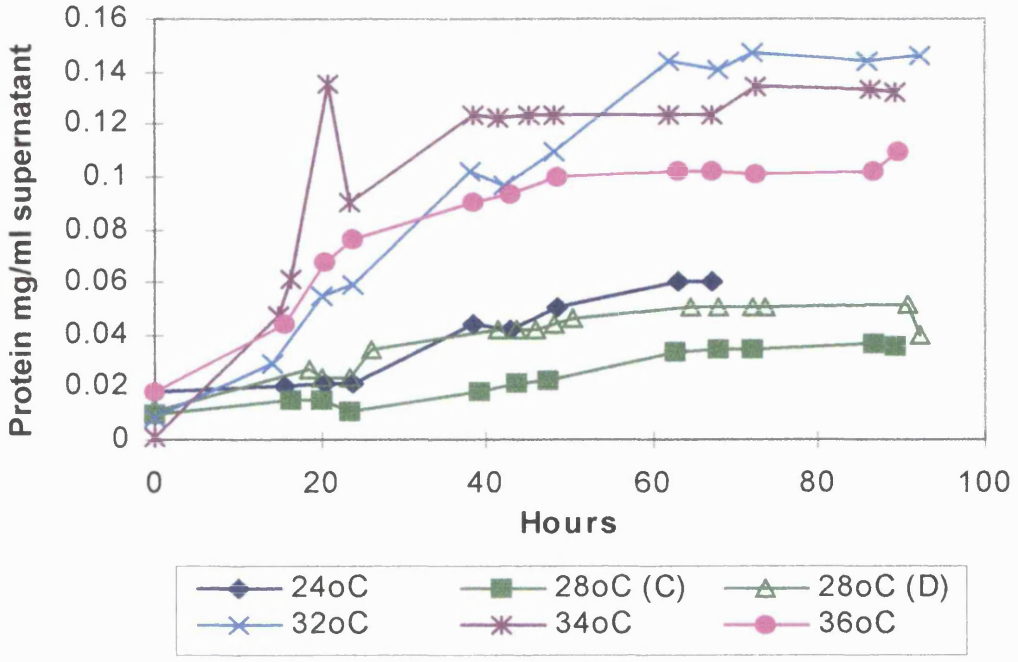


Figure 6.7: Supernatant protein levels in the fermentation experiments examining the effect of incubation temperature on the performance of the *S. lividans* pQR620 system (average SEM 0.002 mg/ml).

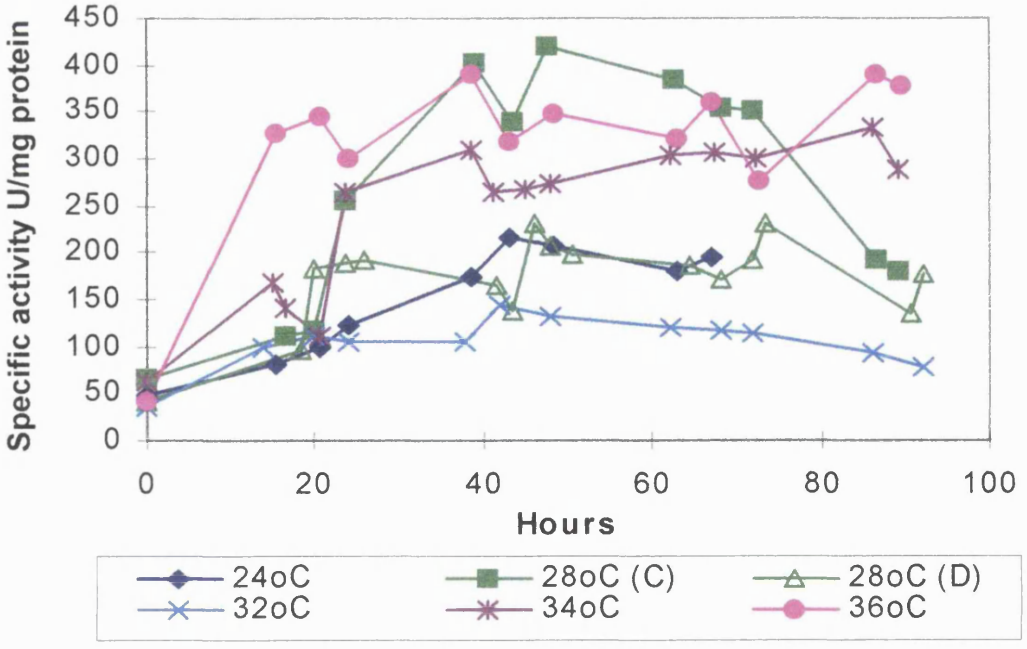


Figure 6.8: α-Amylase specific activity levels in the fermentation experiments examining the effect of incubation temperature on the performance of the *S. lividans* pQR620 system.

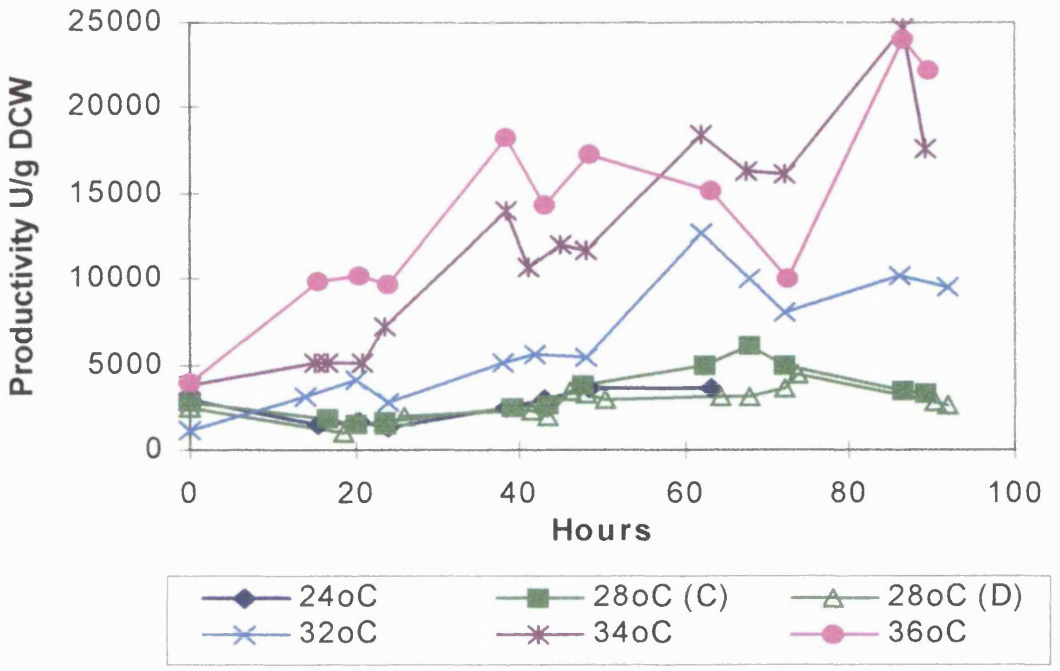


Figure 6.9: α-Amylase productivity levels in the fermentation experiments examining the effect of incubation temperature on the performance of the *S. lividans* pQR620 system.

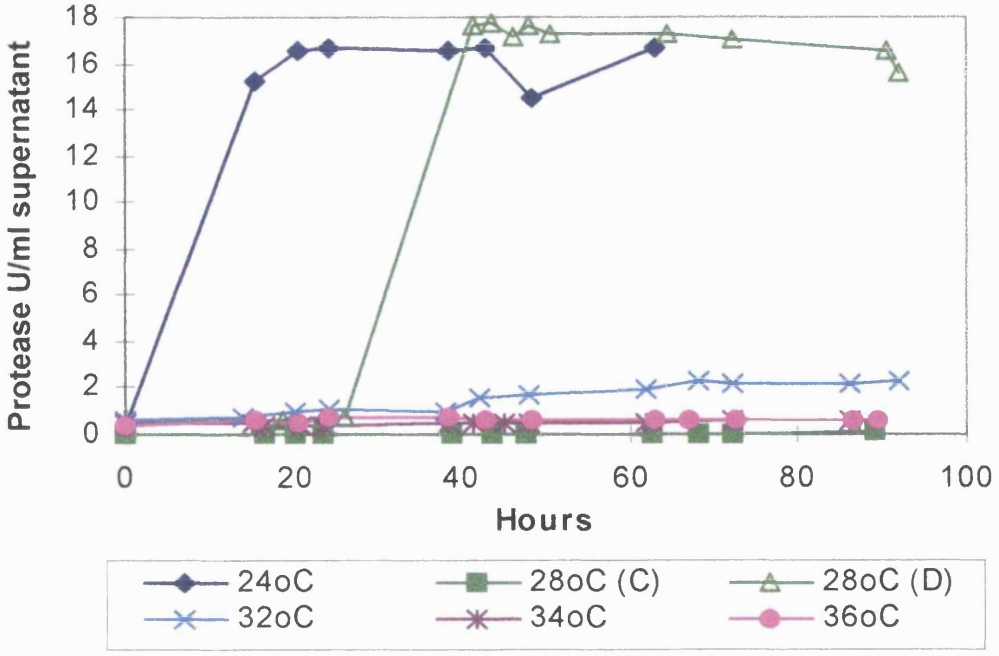


Figure 6.10: Protease levels in the fermentation experiments examining the effect of incubation temperature on the performance of the *S. lividans* pQR620 system.

other cultures. Only about 0.04mg of the 0.15mg of protein that was present in the supernatant was α-amylase. The inoculum given to the 32°C fermentation was significantly younger than those given to the other fermentations (Table 6.1). This factor may have been responsible for the high levels of general protein secretion.

6.4.2: Presence of supernatant proteases.

The supernatants of these fermentations were assayed for general protease activity. No strong evidence of drops in α-amylase levels or general protein production levels were found in these experiments. However, it was conceivable that high levels of protease activity had been present in the 24 and 28°C cultures from the start of each fermentation, which would have kept levels of α-amylase, and other protein, low from the start of the experiments. Such a difference could be a simple explanation for the patterns seen.

Very low levels of protease activity were detected in all but two of the cultures (Fig. 6.10). Protease activity was detected early on in the growth of the 24°C culture, and from 25 hours onwards in one of the 28°C fermentations (fermentation D). When proteases were produced, levels increased over a brief period to a certain level and then remained fairly constant for the rest of the experiment. The very quick rise in levels may suggest that production of proteolytic activity was triggered by the presence, or absence, of a particular factor, e.g. exhaustion of a medium component (see Section 7.5.11.3 for further discussion). Temperature differences might play a role in protease production but no real evidence exists for this in these results. One of the 28°C fermentations (D) made protease and the other, fermentation C, did not. This variation between the two 28°C fermentations also provides evidence that proteolytic degradation of α-amylase activity in these cultures was not acting to keep levels of the product low.

6.4.3: Optimum incubation temperature for α-amylase production.

A graph of peak α-amylase productivity versus temperature indicates that the optimum incubation temperature for high level α-amylase production from *S. lividans* pQR620 is likely to be somewhere between 34 and 36°C (Fig. 6.11). Further fermentations would need to be undertaken to define the optimum temperature more precisely.

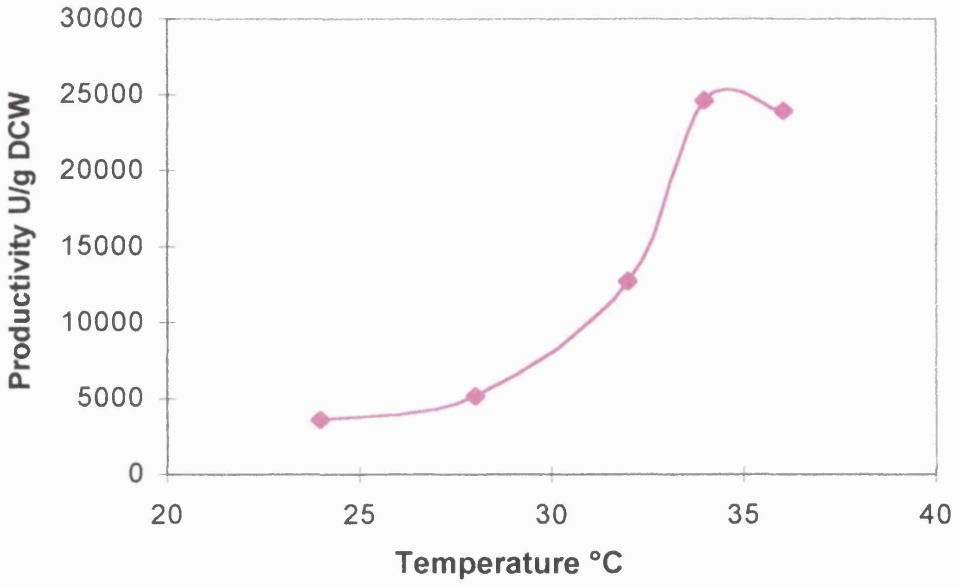


Figure 6.11: Plot of peak α-amylase productivity versus incubation temperature. This plot indicates that the optimum incubation temperature for high level α-amylase production from *S. lividans* pQR620 is likely to lie between 34 and 36°C.

6.4.4: Possible mechanisms for the effect of temperature on α-amylase production.

Changes in temperature of the order of 1% can cause the rates of biological reactions to change by nearly 10% (Cossins & Bowler, 1987). Temperature can therefore affect the fermentation process in many complex and unknown ways. Bader (1986) gave an overview of some possible temperature effects. He concluded that it was difficult to provide a complete picture of temperature effect 'as this alone could easily be a topic for a book'. Possible reasons for the effect of temperature on the *S. lividans* α-amylase expression system are discussed at length in Chapter 8. A summary of these possible explanations can be found in Figure 6.12. Suggestions as to how the phenomenon could be further explored are discussed in Section 8.4.9.

6.5: Experiments to Further Investigate the Temperature Effect

The time constraints placed on this study meant that little could be done fully to explore the many of possible explanations which could lie behind the dramatic increase seen in α-amylase levels at higher incubation temperatures. It was only possible to define the effect seen from the expression systems. Despite the limited time available, two experiments were undertaken.

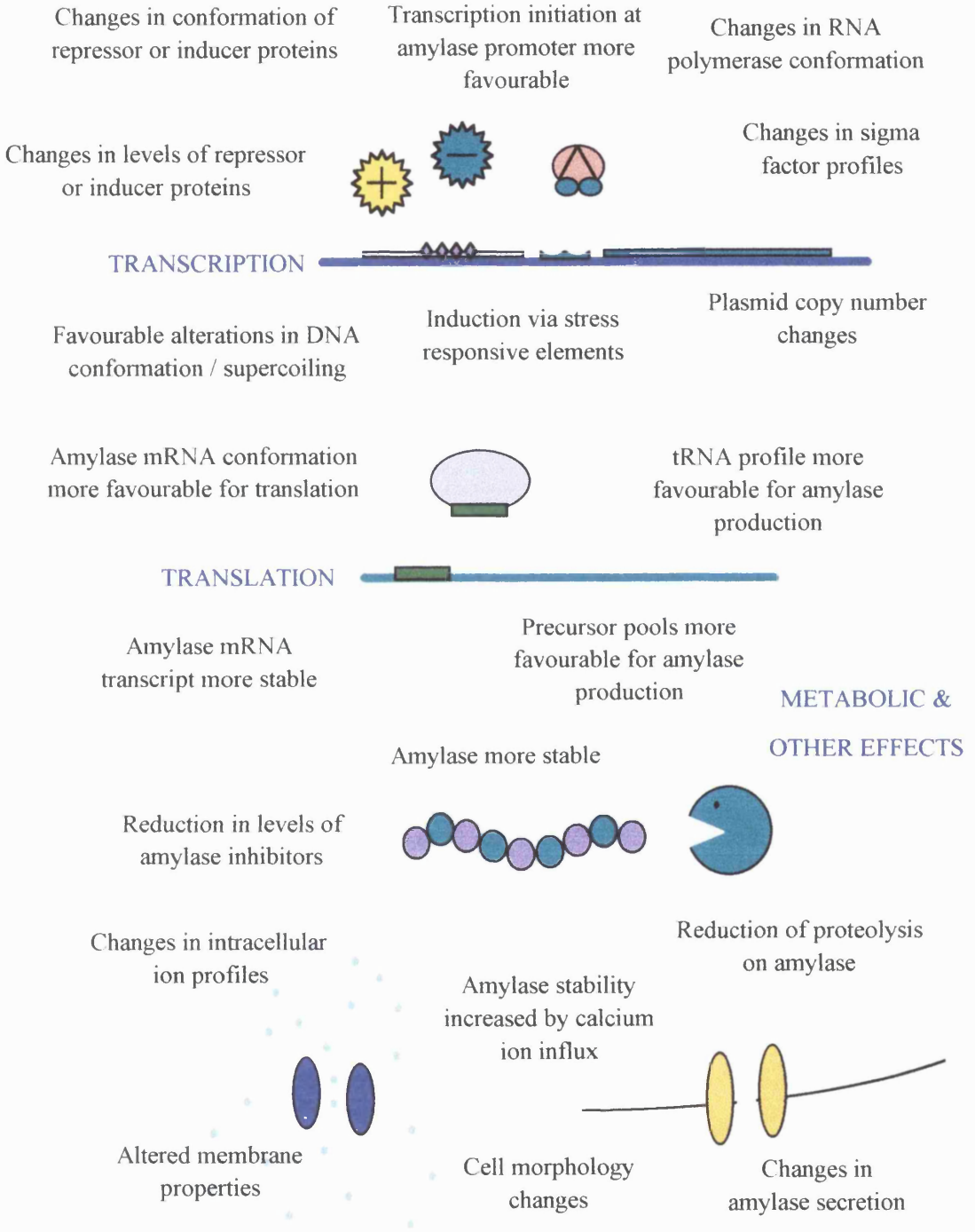


Figure 6.12: Summary of effects which could be responsible for increased levels of α-amylase production from *S. lividans* pQR620, pQR621 and pQR311 at temperatures above those optimal for growth.

6.5.1: Tyrosinase production from *S. lividans* pIJ702 at 30 and 34°C.

Several pieces of indirect evidence suggested that the *mel* promoter region was not involved in mediating the temperature response (Section 6.3.3). An experiment was conducted to try to confirm that it was not involved. *S. lividans* pIJ702 was grown in MMS medium at 30 and 34°C. This plasmid carries the region coding for expression of tyrosinase with transcription being driven by the *mel* promoter region (Fig. 1.2).

At 30°C intracellular tyrosinase productivity peaked at 26000 U/g DCW. At 34°C a peak level of 12500 U/g DCW was detected. Gardner and Cadman (1990) found that tyrosinase was inactivated by heat, therefore a direct comparison of these figures would not give a true reflection of total levels made. Approximate deactivation constants were calculated from the equations stated in their research (Appendix 1). This allowed the estimated true activity to be calculated. If heat inactivation had not been acting in the system, the 30°C culture was predicted to have made a total of 97000 tyrosinase U/g DCW. At 34°C 83000 tyrosinase U/g DCW would have been expected. As the predicted levels of enzyme production were not boosted at the higher temperature these results provided further evidence that the *mel* promoter region was not directly involved in the increase in α-amylase expression.

6.5.2: Possible links to the cellular stress response.

6.5.2.1: Patterns of heat shock protein production.

Many of the effects discussed as possible explanations for the behaviour of the cultures at raised temperatures could be associated with a stress response by the cells, e.g. synthesis of new σ factors, changes in protein or protease levels (Section 8.4). The non-transient pattern of α-amylase production seen in the fermentation experiments did not tend to support the idea that the α-amylase was being produced as, or due to, the action of a heat shock protein (Fig. 6.6). The vast majority of proteins produced following a heat shock are only produced transiently. Tilly *et al.* (1986) found that heat shock protein synthesis by *E. coli* at 42°C peaked between 5 and 8 minutes after heat shock. Up to 50-fold increases in protein production were seen during this period. Levels then quickly declined to new steady state levels which were typically around twice the levels at 30°C. Similar patterns are seen in yeast although raised expression tends to last for

20 to 40 minutes in this organism (Panaretou, 1993). The continued raised α-amylase production rates over the entire length of the *S. lividans* cultures would therefore be at odds with the explanation that the effect was a heat shock associated response.

6.5.2.2: Possible role of stress responsive elements.

A large amount of work has looked at the response of yeast (*Saccharomyces cerevisiae*) to increased temperatures (reviewed by Piper, 1997). In this organism, particular sequences in the upstream and promoter regions of genes have been shown to control activation of gene transcription upon heat shock. Heat shock elements (HSEs) and general stress responsive elements (STREs) are the best understood of these sequences (Piper, 1995; Piper, 1997). STRE sequences consist of the consensus sequence AGGGG or CCCCT. These sequences are recognised and bound by transactivators which trigger high level gene expression. Induction via this mechanism can be activated by diverse types of sublethal stress. Two examples of CCCCT sequences are found in the α-amylase sequence at -92 and +45, raising the possibility that an analogous mechanism of heat induction operates in *S. lividans* (Fig. 6.13). No research has been conducted into whether, or not, STRE or HSE sequences are active in organisms other than yeasts. The universal nature of many other responses to heat makes it a possibility.

6.5.2.3: Effect of ethanol shock on *S. lividans* pQR620 cells.

Many of the major changes induced in yeast by stressful levels of ethanol are identical to those caused by heat stress (Piper, 1995). In yeast sublethal ethanol stress induces practically identical changes to protein synthesis as heat shock. Heat and ethanol can both exert an effect via stress responsive elements (STREs)(Piper, 1995).

It was decided to examine the response of *S. lividans* pQR620 cells to ethanol shock to see if this could, by itself, prompt the effect seen at higher temperatures. It was hoped that this experiment would provide evidence that the response was not associated with heat shock of the cells. As this had not been previously undertaken, finding the correct ethanol level to give a response, without adversely affecting the cells, was likely to be difficult. In yeast the level of ethanol must be above a certain threshold level to exert an effect (Piper, 1997). A level of 4% v/v is typically used. The *Streptomyces* cells

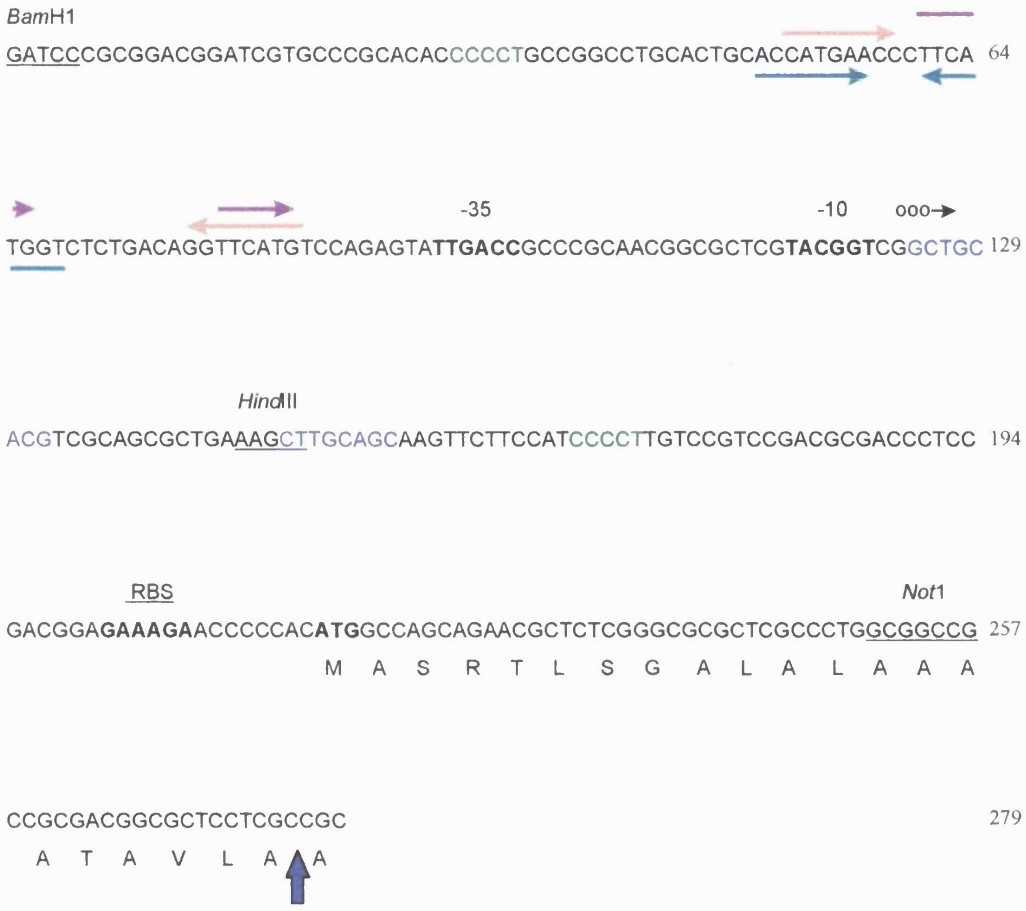


Figure 6.13: Upstream sequence of the *S. thermoviolaceus* α-amylase gene showing putative -10 and -35 regions, transcription start point, ribosome binding site and possible signal peptidase cleavage site. Inverted and direct repeat sequences postulated to play a role in the regulation of α-amylase expression are shown with arrows. The inverted repeat sequence shared with *S. limosus* is shown in blue. Bases shown in green are sequences possibly involved in regulation of transcription when the cells are under stress.

were likely to be far less tolerant of the alcohol than yeast. To maximise the chances of obtaining the correct level it was decided to add ethanol to two shake flasks. In one flask ethanol was added at 1% v/v. In the other it was added at 3% v/v. Both additions were made towards the end of exponential growth, as would be done in a yeast ethanol shock experiment. A control flask where no ethanol was added was included as a control. Each flask contained MMS medium and was given an identical inoculum of *S. lividans* pQR620 spores. Intracellular α-amylase levels were measured alongside supernatant α-amylase and protein measurements.

This experiment again coincided with a period of warm weather. Over the first 24 hours of incubation the temperature of the orbital incubator containing the flasks maintained a temperature of around 32°C, 2 degrees above the desired temperature. Following this period the temperature dropped back down to 30°C and remained at that level until the end of the experiment.

6.5.2.3.1: Effect of increased temperature over the first day of incubation.

The first notable result from this experiment was that the control flask, where no ethanol was added, achieved a higher level of α-amylase production than would have been expected from a culture incubated at 30°C. It displayed behaviour more like that of a flask grown at 34 or 36°C (Fig. 6.15). This suggests that a high temperature over the first day of incubation somehow conditioned the cells to produce high levels of α-amylase although the incubation was quickly returned back to 30°C (see Section 6.6.1 for discussion). Growth, α-amylase and productivity profiles were highly comparable for the control and 1% ethanol flask prior to ethanol addition (Figs. 6.14, 6.15 & 6.16). The 3% ethanol flask exhibited a longer lag phase than the other flasks and had not reached the same level of growth as the 1% flask when the ethanol addition was made at 65 hours (Fig. 6.14). Addition of ethanol halted rapid growth of the *S. lividans* cells but did not seem to increase significantly the rate of cell death observed in stationary phase (Fig. 6.14).

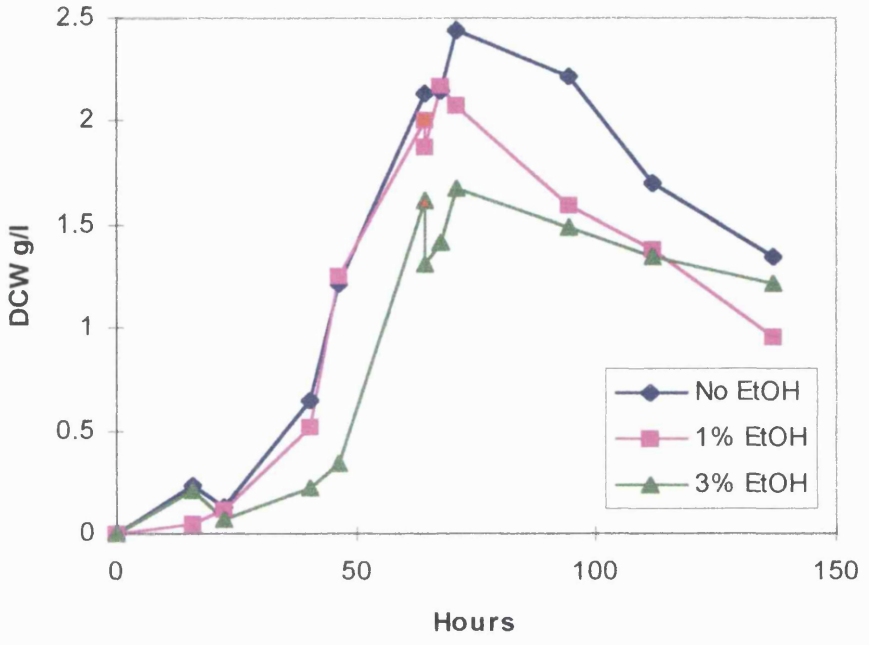


Figure 6.14: Growth curves for the shake flask study examining the effect of ethanol shock on *S. lividans* pQR620 (average SEM 0.048 g DCW/l). Ethanol was added at 1% v/v or 3% v/v at 65 hours (points with red centres). No ethanol was added to the control flask.

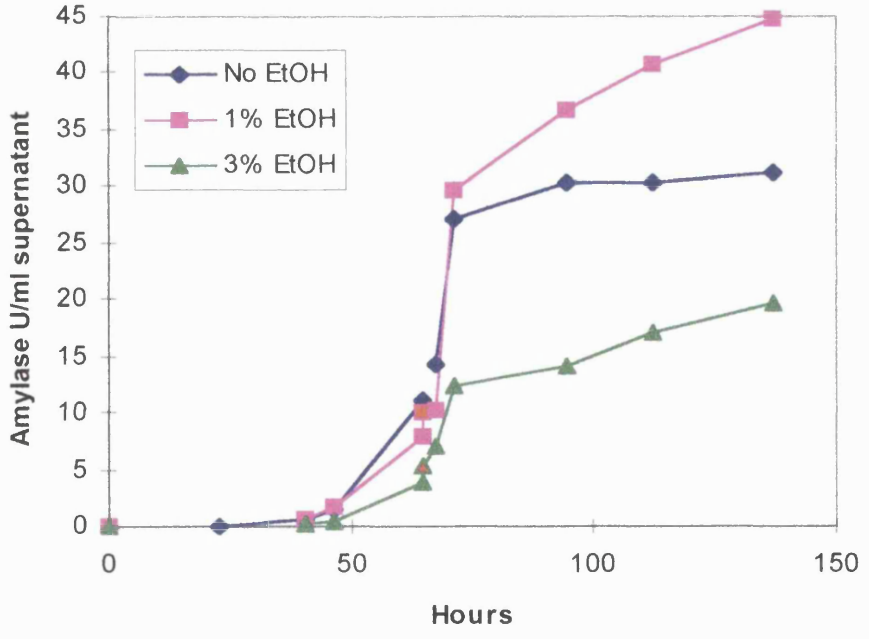


Figure 6.15: Supernatant α-amylase levels for the shake flask study examining the effect of ethanol shock on *S. lividans* pQR620 (average SEM 0.444 U/ml). Ethanol was added at 1% v/v or 3% v/v at 65 hours (points with red centres). No ethanol was added to the control flask.

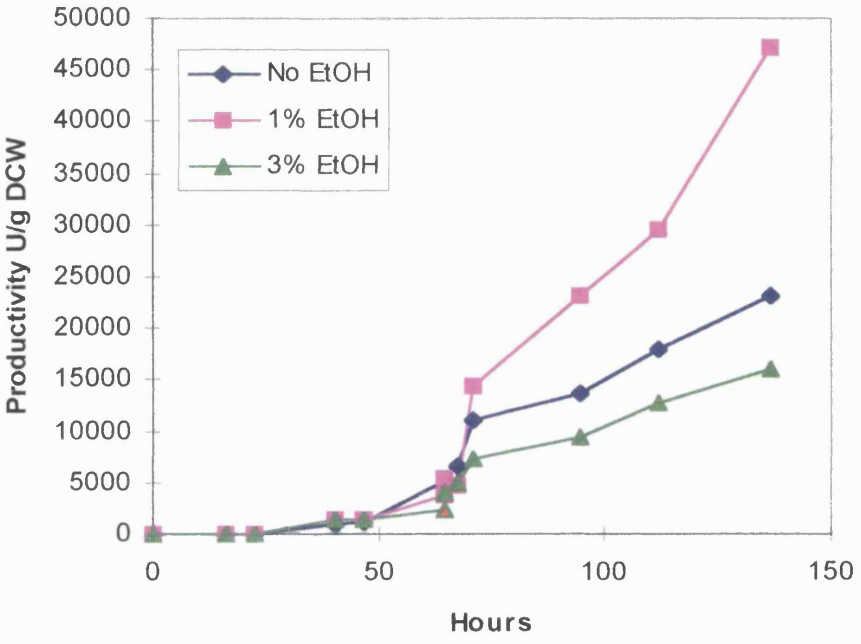


Figure 6.16: α-Amylase productivity levels for the shake flask study examining the effect of ethanol shock on *S. lividans* pQR620. Ethanol was added at 1% v/v or 3% v/v at 65 hours (points with red centres). No ethanol was added to the control flask.

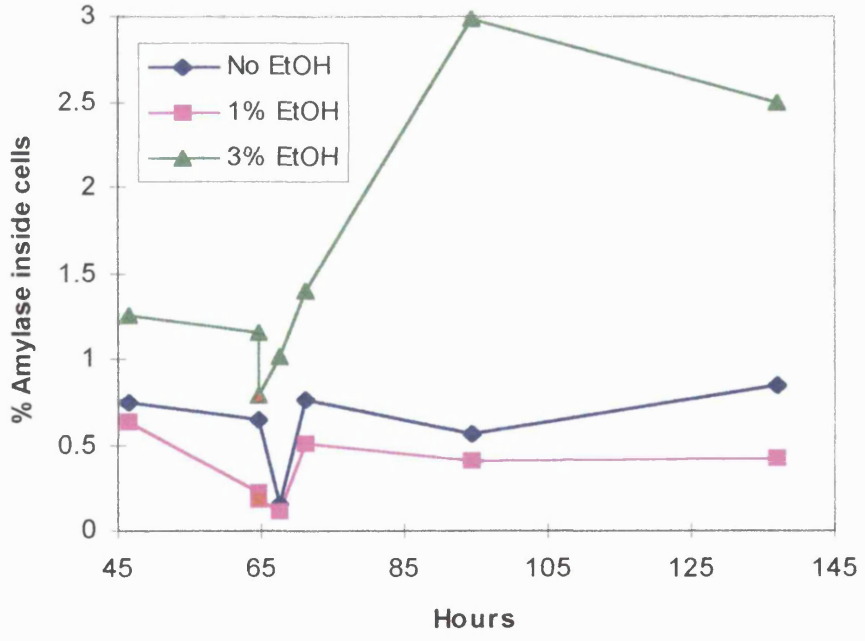


Figure 6.17: Plot showing the percentage of total α-amylase located intracellularly for the shake flask study examining the effect of ethanol shock on *S. lividans* pQR620. Ethanol was added at 1% v/v or 3% v/v at 65 hours (points with red centres). No ethanol was added to the control flask.

6.5.2.3.2: Addition of 1% ethanol increased the α-amylase productivity of the *S. lividans* pQR620 cells.

The cells incubated with 1% ethanol produced α-amylase more quickly than those in the control flask and maintained their faster rate of production over stationary phase (Figs. 6.15 & 6.16). The cells reached a productivity of around 48000 α-amylase U/g DCW, approximately twice the levels seen in the 34 and 36°C fermentations (Figs. 6.16 & 6.9). Intracellular α-amylase levels were comparable between the control flask and the 1% ethanol flask (Fig. 6.17). In each case α-amylase retained in the cell remained at below 1% of total α-amylase throughout the experiment. Levels of α-amylase in the 3% ethanol flask, relative to the other flasks, were low, although the cells continued to produce α-amylase throughout stationary phase (Fig. 6.16). This probably indicates that this level of ethanol was having a significant effect on the functioning of the cells. The intracellular α-amylase profile of the cells in this flask tended to confirm this as up to 3% of the total α-amylase produced was being retained inside the cells (Fig. 6.17).

Alcohols can partition into the lipid bilayer of cell membranes. This is postulated to have several possible effects (Piper, 1997). It may affect the signalling of a stress response or affect the sensing system responsive to stress. It can also permeabilise membranes (Panaretou & Piper, 1992). Alteration of membrane properties could also lead to changes in the secretion system. The 3% ethanol flask provides evidence that this level of alcohol can effect the secretion machinery of *Streptomyces* cells, as around 3-fold more α-amylase was found to be intracellularly located in this culture than under any other conditions.

6.5.2.4: A direct link between heat shock and increased α-amylase production could not be discounted.

It had been hoped that this experiment would provide evidence that the temperature effect was not linked to a stress response in the cells. However, addition of 1% ethanol significantly boosted the α-amylase productivity of the cells to higher levels than had been observed previously. As this experiment was only conducted once further experiments would be required to confirm that the findings were reproducible. Some thermostable enzymes have been shown to have enhanced activity at relatively low

concentrations (15 to 20%) of organic solvents such as acetone (Veronese *et al.*, 1984). The results could therefore have been due to the potentiation of the α-amylase activity by the ethanol and not due to increased production. A protein gel was run to allow a visual comparison of the α-amylase bands from the cultures to see if more enzyme was present in the 1% ethanol flask than in the control flask. The TCA precipitation carried out to prepare the protein samples resulted in losses of protein from the samples. This meant that a direct comparison could not be made. Further experiments would therefore be needed to examine the effect of ethanol on the α-amylase.

6.6: Concluding Remarks

6.6.1: Potential for greater optimisation of growth temperature profile.

The fact that increased levels of α-amylase productivity seemed to be triggered throughout a five day experiment when the cells were only incubated at a high temperature over the initial day of the experiment was interesting (Section 6.5.2.3.1). This suggests that the cells could be being conditioned in the early stages of growth to be more productive. It also suggests that a further increase in levels of α-amylase could be obtained from the cells by determining a regime in which temperature shifts are used to optimise the system. Ohno *et al.* (1993) found a similar effect in antibiotic production from *B. subtilis* NB22. They determined that maintaining cultures at a temperature lower than that optimal for growth during exponential phase was crucial for high level production of the antibiotic, even though it was not produced until stationary phase. They optimised a regime of temperature shifts by lowering the temperature of shake flasks for one day only over a five day incubation. A similar experiment could lead to the discovery of a better protocol for growth of *S. lividans* pQR620 and to increases in α-amylase yield from the cultures.

6.6.2: Role of temperature in the regulation of streptomycete expression.

Kluepfel *et al.* (1986) found that production of the CM-cellulase and xylanase proteins from *S. lividans* was optimal at 34 to 40°C. Each of these enzymes was found to be thermostable with an optimum temperature for enzyme activity of between 55 and 60°C. *Streptomyces chrestomyceticus* was found to produce toyocamycin optimally at 34 to 36°C (Flickinger *et al.*, 1990). Reusser (1985) saw full induction of lincomycin

from *Streptomyces espinosus* at 45°C. None of these authors attempted to give an explanation for their results or to postulate what mechanisms were involved in the increased levels of production at temperatures above those optimal for growth. Although these proteins were all natural products, in contrast to the plasmid borne α-amylase, the production profiles bear many similarities. It is possible that these products belong to a group of proteins which are preferentially produced by *Streptomyces* at higher temperatures. It would be interesting to see if the regulation of production of these gene products was controlled through a common mechanism.

If the mechanism of the temperature effect on α-amylase was elucidated and found not to be specific to the α-amylase, it might be possible to use the mechanism to trigger high level production of other plasmid borne gene products from *S. lividans*. Such plasmids may be a potent way of producing high levels of recombinant protein.

6.7: Summary

A shake flask study was conducted to examine the effect of higher than normal incubation temperature on growth and α-amylase production from *S. lividans* pQR620, pQR621, pQR311 and pQR318. Cultures grown at 30°C were compared with cultures grown at 34°C. Incubation temperature was found to have a dramatic effect on α-amylase productivity from *S. lividans* pQR620, pQR621 and pQR311 cells. The higher incubation temperature was preferable for high level α-amylase production. Growth levels and patterns were not noticeably affected by the increased temperature. Fermentation studies using *S. lividans* pQR620 confirmed these findings with around 5-fold more α-amylase produced per gram of cells at 34 and 36°C than was produced at 28°C. This corresponded to a peak level of approximately 100 milligrams per litre of α-amylase protein. The results of these studies suggested that the optimum temperature for α-amylase production fell somewhere in between 34 and 36°C. A myriad of possible reasons exist which could explain how increased incubation temperature could act to increase levels of production. The evidence suggests that neither the *sti* region or the *mel* promoter region are responsible for the effect. Addition of 1% ethanol to the cells at 30°C also boosted α-amylase production levels. This finding meant that a direct link between high level α-amylase production and the heat shock response could not be ruled out.

Chapter 7

The Effect of Medium Composition on α-Amylase Production

7.1: Aims

The work described in this chapter was conducted to evaluate the response of *S. lividans* pQR620 to changes in medium composition. A statistical experimental approach was used to identify which components of MMS medium had the greatest influence on the performance of the system.

7.2: Background

Many studies have shown that to attain high level production of a recombinant protein, identifying a suitable culture medium for bacterial growth is often as important as optimising the genetic instructions for expression of the protein. This may be especially relevant in streptomycete expression systems where it is likely medium composition would need to be fine-tuned to prolong the period of time over which a protein is produced. This could involve manipulating the medium to encourage a quick entry into secondary metabolism or altering nutrient levels to prolong primary metabolism.

A detailed understanding of the metabolism of several bacteria, such as *E. coli*, has been built up over the last few decades. The understanding of actinomycete metabolism is sparse by comparison. This has meant that media formulation is largely being done empirically. When variations in levels of a medium component have had an effect on levels of a product, it is often difficult to explain exactly why. Despite this lack of detailed understanding many studies have been published into the effects of variations in medium constituents on antibiotic production. By comparison, very few studies have investigated the effects of media composition on recombinant protein production by *Streptomyces*.

The work detailed in this chapter was conducted to answer some of the questions raised in the preliminary shake flask media studies (Chapter 3). It was also conducted to identify the key medium components influencing production of α-amylase in MMS medium. Although it was likely that many of the effects seen could not be completely

explained, any additional information gathered about the response of the expression system to changes in medium composition would be valuable.

7.3: The Effect of Glucose on the *S. lividans* pQR620 System

Shake flask studies with *S. lividans* TK24 pQR318 had indicated that α-amylase production was poor in glucose containing media (Section 3.4.7). The evidence suggested that glucose repression of α-amylase production was the likely explanation. However, severe acidification had been seen in the relevant shake flask cultures. This raised the possibility that the enzyme was being produced but that it was rapidly degraded in the prevailing environment.

In order to investigate further the cause of the poor production, a 20l fermentation was conducted using the *S. lividans* pQR620 expression system. It was hoped that the increased levels of α-amylase expression obtained from the newly constructed vector would make it easier to distinguish true medium component driven effects from those caused by other variations, for example inoculum size or age. The fermentation was carried out at 28°C in *Streptomyces* defined medium (SDM) supplemented with glucose and aspartate (Section 2.4.1.7). It was conducted as described in Section 2.4.8. The pH of the medium was automatically controlled to 7. This allowed the effects of glucose to be considered separately from those of acidification. The inoculum flasks were prepared using the two-stage inoculum strategy (Section 2.4.9.2.2).

7.3.1: Glucose represses production of α-amylase by *S. lividans* pQR620.

The results of this fermentation showed a classic pattern of glucose repression. This is especially well illustrated by the growth curve and productivity profiles of the fermentation (Figs. 7.1 & 7.5). Biomass levels increased steadily to a peak of around 3g DCW/l at 70 h and then levelled off, indicating entry into stationary phase. HPLC analysis indicated that this change had occurred at the time of glucose exhaustion. The amount of α-amylase produced per gram of dry cell weight remained at a basal level during exponential growth. As the cells entered stationary phase (70 h) repression of α-amylase production was lifted and the enzyme was produced rapidly. Supernatant α-amylase peaked at 5.6 U/ml at 94 h.

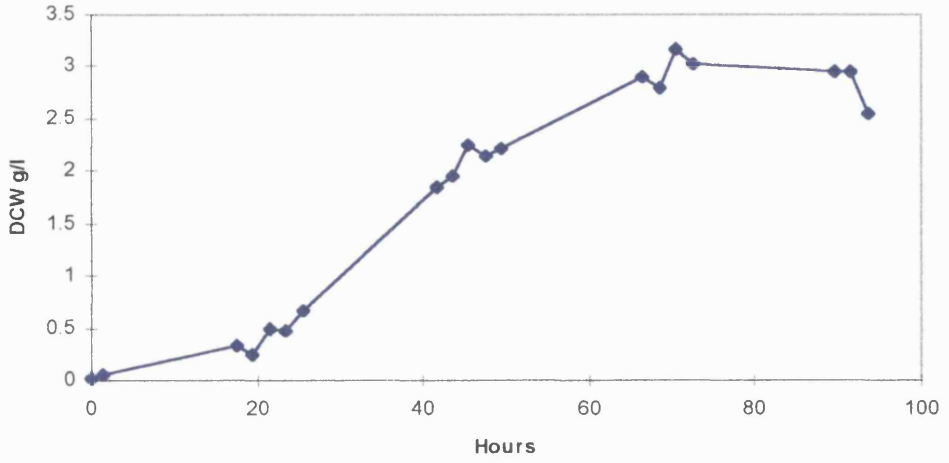


Figure 7.1: Growth curve for *S. lividans* pQR620 grown in SDM medium supplemented with glucose and aspartate. Average SEM 0.087 g DCW/l.

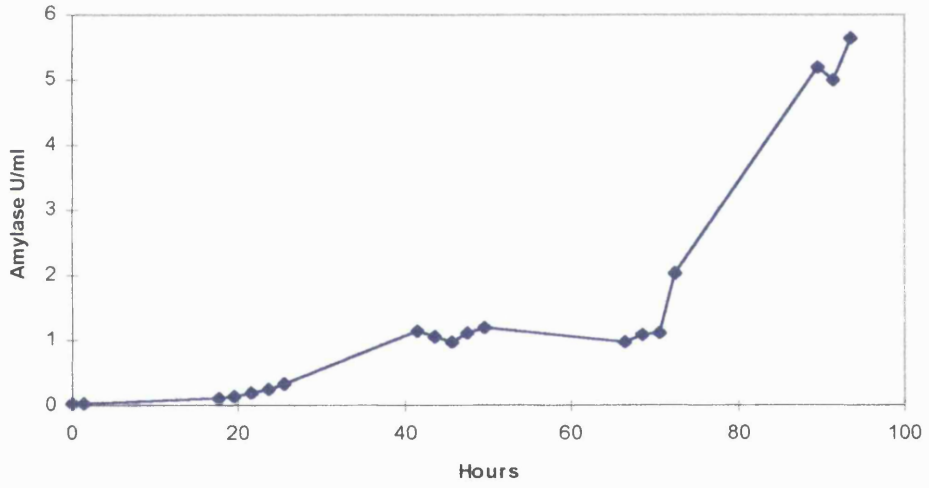


Figure 7.2: α-Amylase production by *S. lividans* pQR620 grown in SDM medium supplemented with glucose and aspartate. Average SEM 0.072 units/ml.

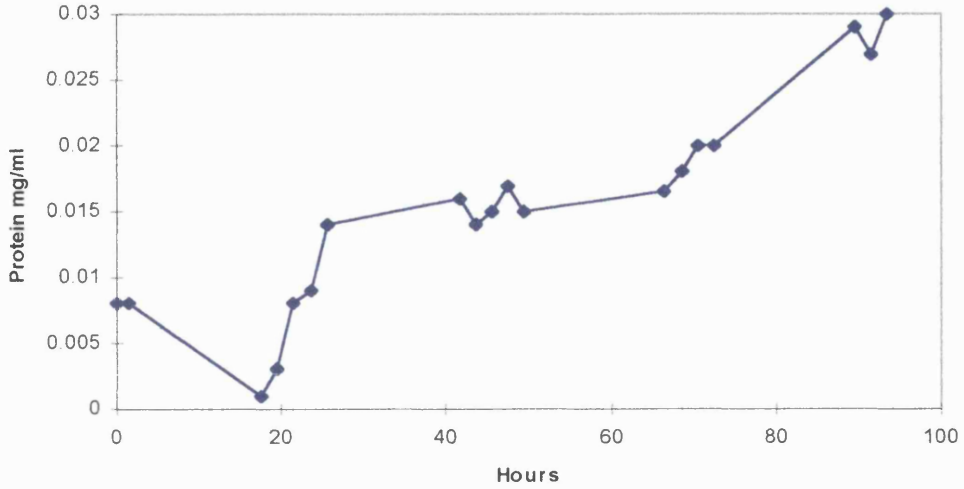


Figure 7.3: Supernatant protein levels for *S. lividans* pQR620 grown in SDM medium supplemented with glucose and aspartate. Average SEM 0.002 mg/ml.

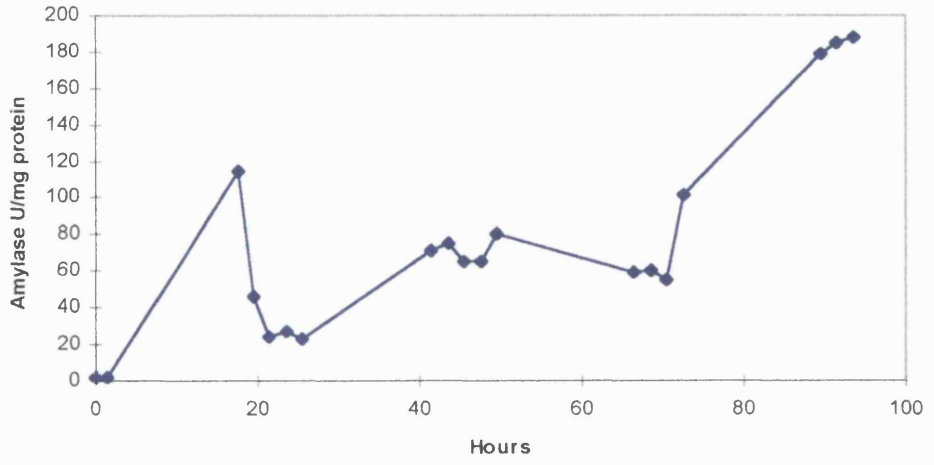


Figure 7.4: Specific activity profile for *S. lividans* pQR620 grown in SDM medium supplemented with glucose and aspartate.

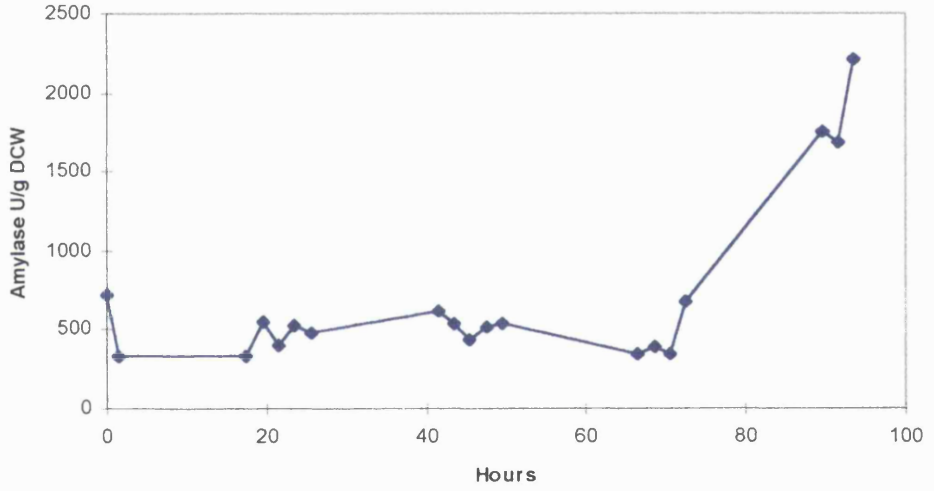


Figure 7.5: Productivity profile for *S. lividans* pQR620 grown in SDM medium supplemented with glucose and aspartate.

Supernatant protein carried over from the inoculum flasks was initially metabolised by the *S. lividans* pQR620 cells (Fig. 7.3). This may indicate that it was used as a nitrogen source in preference to the aspartate. Aspartate had previously been observed to give good levels of growth in this defined medium (E. Bull-Daae, personal communication). However, the sluggish growth and initial use of supernatant protein may indicate that it is far from ideal for the *S. lividans* cells. As cell growth took off supernatant protein levels also increased. This trend continued during stationary phase with supernatant protein levels peaking at 0.03mg/ml. The maximum α-amylase specific activity reached was 188 U/mg of protein (Fig. 7.4).

These results effectively ruled out breakdown of the α-amylase due to low pH as an explanation for the results obtained in the preliminary shake flask studies. Another potential cause of the pattern of α-amylase accumulation seen could be proteolytic degradation. Glucose may have promoted the production of a protease or proteases that act to rapidly breakdown α-amylase. The lack of variation seen in the basal level of α-amylase produced per gram of biomass provides evidence against this possibility (Fig. 7.5).

7.3.2: The Reg1 protein is likely to control expression of the α-amylase.

Over the last decade α-amylases from several streptomycete species have been cloned and expressed in *S. lividans* and *S. coelicolor* (Section 1.3.1). In the majority of wild type systems studied, expression of the enzyme is negatively regulated by glucose, e.g. *S. venezuelae aml* (Virolle *et al.*, 1988), *S. hygroscopicus amy* (Hoshiko *et al.*, 1987), *S. kanamyceticus amy* (Flores *et al.*, 1993) and *S. griseus amy* (Vigal *et al.*, 1991a). In each case this pattern of regulation was maintained upon expression of the genes in a new host strain (*S. lividans* or *S. coelicolor*). However, the α-amylases of *S. thermoviolaceus* and *S. limosus* were not subject to glucose repression in their wild type strain (Bahri & Ward, 1990a; Long *et al.*, 1987). Virolle and Bibb (1988) reported that, upon cloning into *S. lividans* or *S. coelicolor*, the *S. limosus aml* gene became subject to glucose repression. This was an interesting observation as it indicated that the gene 'adopted' the regulatory characteristics of its new host. The results of this fermentation indicate that the *S. thermoviolaceus amy* gene also displays this behaviour when present in a *S. lividans* host cell.

Glucose catabolite repression of protein production has been described many times in the streptomycete system, e.g. actinomycin synthesis in *S. antibioticus* (Vining & Doull, 1988), agarase production in *S. coelicolor* (Servin-Gonzalez *et al.*, 1994), xylanase production in *Streptomyces* sp. CH-M-1035 (Flores *et al.*, 1996). Although the phenomenon has been widely observed, little is understood about the mechanism of glucose repression in the streptomycete system. The interesting regulatory pattern of the *S. limosus aml* gene attracted the attention of researchers as a suitable model system for study. Regulation of *aml* expression by glucose was found to occur at the level of transcription (Virolle & Bibb, 1988). A series of direct and inverted repeats are found in the promoter regions of the known streptomycete α-amylase genes (Yin *et al.*, 1997). A high degree of conservation exists in the sequences and positioning of the repeats with respect to the -10 and -35 regions of the genes, suggesting that the regulatory mechanisms involved in control of transcription will largely be common. The results of this study confirm that the *S. thermoviolaceus amy* gene responds to the presence of glucose in a *S. lividans* host as would be expected if this assumption were true.

Virolle and Gagnat (1994) originally postulated that the repeat sequences governed catabolite repression of gene expression by acting as operator sequences for positive and negative regulatory proteins (Section 1.3.1). However, recent work by Nguyen *et al.* (1997) has provided evidence that glucose repression of the α-amylase genes in *S. lividans* is controlled by the product of the *reg1* gene. Reg1 is a helix-turn-helix protein belonging to the same family of regulatory proteins as the LacI and GalR proteins of *E. coli*. Catabolite repression of *aml* production was found to be completely abolished in a mutant strain of *S. lividans* where the *reg1* gene had been removed (Nguyen *et al.*, 1997). The LacI/GalR regulatory protein family recognise operator sites of the GN_4CGN_4C type, where the central CG pair are essential for protein binding. Putative sites of this type have been identified in the α-amylase genes of *S. limosus* and *S. lividans* (*amy* and *amlB*) (Nguyen *et al.*, 1997). Inspection of the *S. thermoviolaceus amy* gene shows that this gene also possesses a putative binding site for the Reg1 protein (+1 to +24)(Fig. 6.13). Interestingly, the location of the sequence is closely comparable to that observed in the *S. limosus aml* gene (-116). This similarity provides further evidence that the mechanism of regulation of the *S. thermoviolaceus amy* gene

is analogous to that of the *S. limosus aml* gene. It can therefore be concluded that transcription of the *amy* gene on pQR620 is likely to be under the control of Reg1.

Despite these advances in understanding many questions remain as to the precise mechanism of catabolite repression in *Streptomyces*. Repression of *aml* was found to be dependent on the presence of functional glucose kinase in *S. coelicolor* (Virolle & Bibb, 1988). Despite the very high copy numbers of pQR620 thought to reside inside *S. lividans* cells in the *amy* expression system constructed in this work (Section 4.5.1.1), no titration effect of the putative repressor protein was observed. These observations suggest that although Reg1 may act to directly control transcription of the α-amylase gene, other additional layers of regulation exist which play a role in governing the cells' response to their environment. It would be interesting to determine exactly why glucose repression of α-amylase production was not observed in *S. thermoviolaceus and S. limosus*. Are alternative mechanisms of catabolite repression functioning in these organisms? A large amount of further investigation would be required to elucidate fully these mechanisms.

7.4: Performance of the *S. lividans* pQR620 System in the Complex Medium MEPP

Only low levels of biomass had been achieved in the media used for growth of the *S. lividans* pQR620 to this point in the study. Levels typically reached 2.5g DCW/l in MMS medium. This represents a very low level of growth considering that significantly higher biomass yields can be obtained from streptomycetes in complex media. DelaCruz *et al.* (1992) obtained over 8g DCW/l of recombinant *S. lividans* using LB broth supplemented with tryptone and yeast extract.

For financial reasons the majority of industrial *Streptomyces* fermentations are conducted in complex media (Greasham, 1993). Use of a medium such as MMS would be unlikely to be cost effective for the commercial production of a recombinant protein such as α-amylase. It would be more financially viable if the recombinant product being expressed was a high value pharmaceutical.

It was decided to grow *S. lividans* pQR620 in a more complex medium, which was known to give a high biomass yield, to see how the system responded to such an

environment. Wrigley-Jones (1990) had observed *S. lividans* biomass yields of up to 15g DCW/l in 5l fermentations using malt extract peptone medium supplemented with phosphate (MEPP)(Section 2.4.1.6). This medium contained no rapidly utilisable sugars which might cause catabolite repression of α-amylase expression. It was therefore considered a suitable choice for this investigation. The experiment was conducted in a 20l fermenter, as described in Section 2.4.8. Medium pH was automatically maintained at 7. Inoculum flasks were prepared using the two-stage inoculum strategy (Section 2.4.9.2.2).

7.4.1: α-Amylase productivity in MEPP medium.

As expected, the *S. lividans* pQR620 cells grew rapidly in this medium with a peak biomass level of over 9g DCW/l (Fig. 7.6). This was more than three times the level routinely obtained in MMS medium. The supernatant α-amylase profile initially followed a similar pattern to that of biomass, with rapid increases in levels seen over exponential growth (Fig. 7.7). However, α-amylase production continued well into the stationary phase of the culture. Over 17 units of α-amylase per ml of supernatant were obtained. This was 5 units more α-amylase per ml of culture than had been observed in equivalent cultures grown in MMS medium (Fig. 5.8). Extracellular proteases or α-amylase inhibitors may have been produced towards the end of stationary phase as levels of the enzyme declined after 70 h.

Although higher overall levels of supernatant α-amylase were achieved in this fermentation, the high level of biomass present meant that this actually represented a very low level of productivity from the *S. lividans* pQR620 cells (Fig. 7.10). The cells grown in MEPP medium had a peak productivity of 2174 units of α-amylase per gram DCW. This compared poorly with the level routinely achieved in MMS medium of 6000 units of α-amylase per gram DCW (Fig. 5.13).

High basal levels of protein were seen in MEPP medium (Fig. 7.8). This was expected due to the complex nature of the medium. In addition to this significant basal level, the cells secreted very large amounts of protein over the course of the fermentation. Levels peaked at 0.3 mg/ml of supernatant, approximately 6 times the levels found in equivalent MMS medium cultures (Fig. 5.9). As a consequence of this behaviour the α-

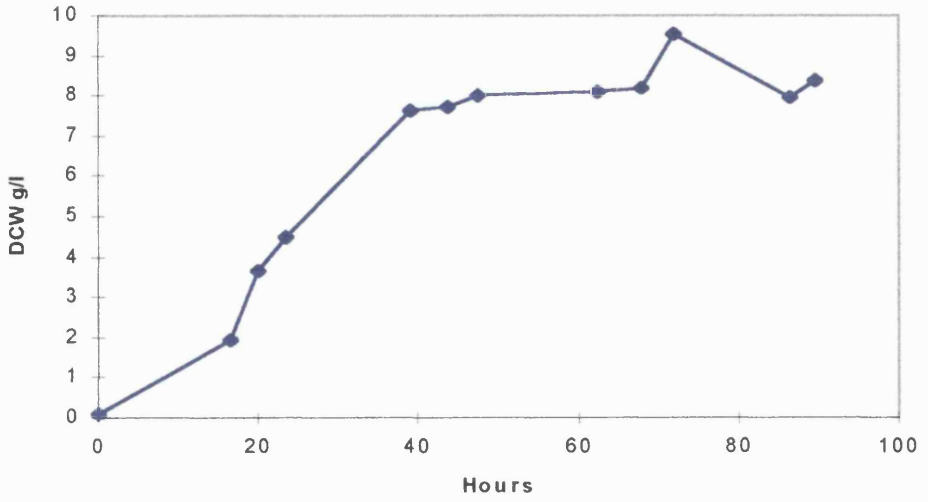


Figure 7.6: Growth curve for *S. lividans* pQR620 grown in MEPP medium. Average SEM 0.162g DCW/l.

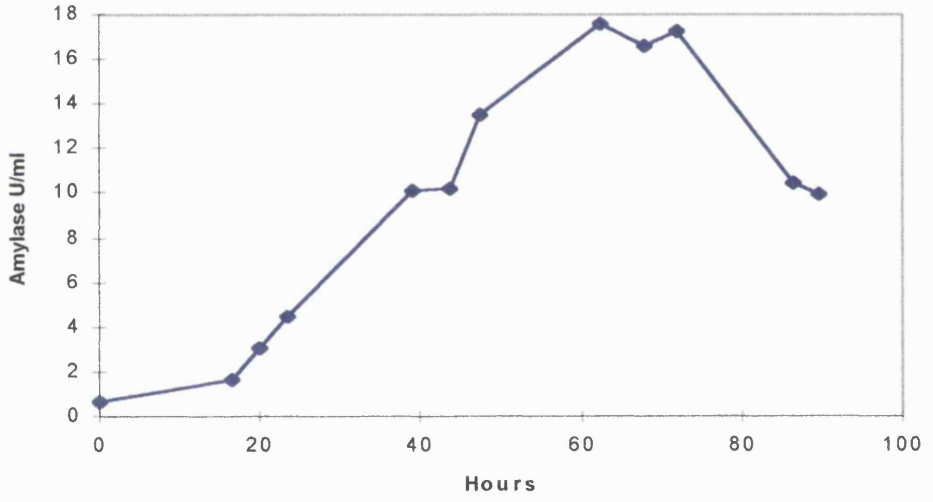


Figure 7.7: α-Amylase production by *S. lividans* pQR620 grown in MEPP medium. Average SEM 0.426 U/ml.

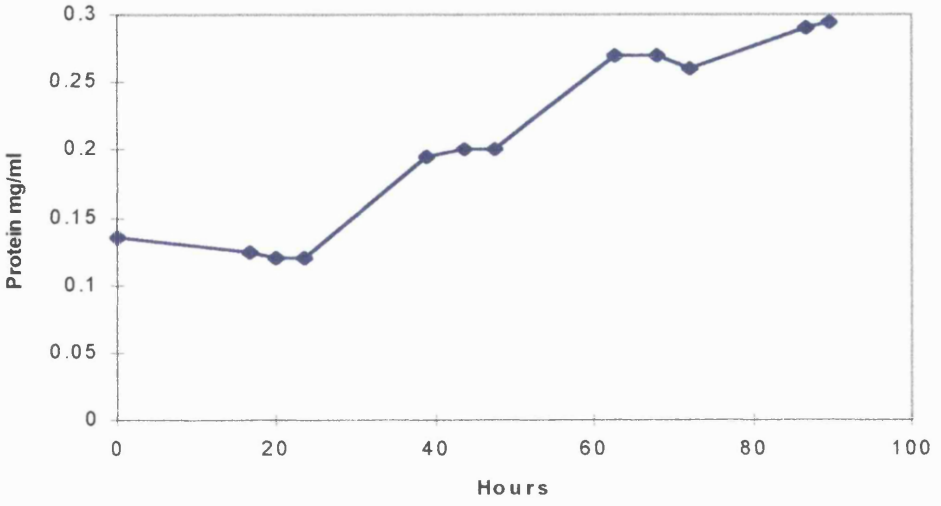


Figure 7.8: Supernatant protein levels for *S. lividans* pQR620 grown in MEPP medium.

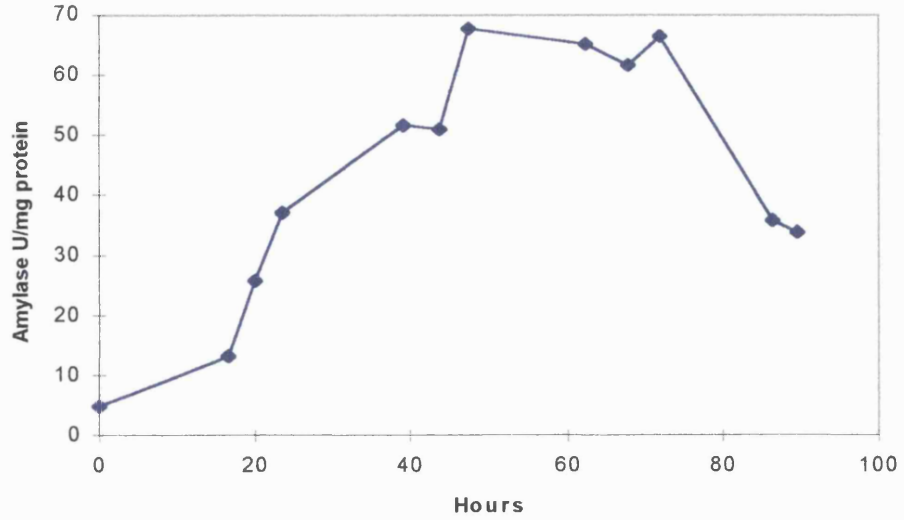


Figure 7.9: Specific activity profile for *S. lividans* pQR620 grown in MEPP medium.

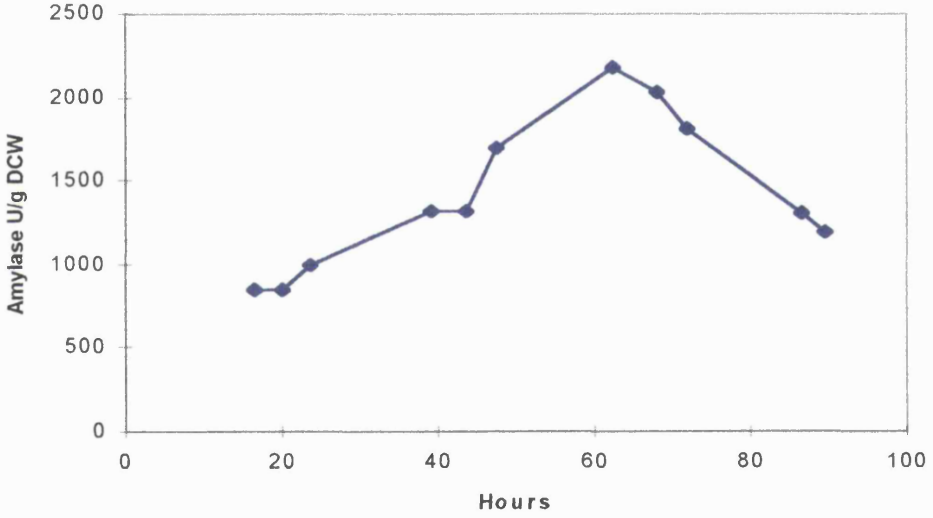


Figure 7.10: Productivity profile for *S. lividans* pQR620 grown in MEPP medium.

amylase specific activity values achieved were very low (Fig. 7.9). Specific activity peaked at 68 U α-amylase per mg protein, 4 to 5 times lower than that seen in MMS fermentations (Fig. 5.11).

Comparison of the overall level of α-amylase achieved in MEPP medium and MMS medium indicates that higher total levels were produced in MEPP (2.2×10^5 units versus 1.6×10^5). At first glance this might indicate that MEPP would be the medium of choice if commercial production of α-amylase was the final goal. However, the very high levels of protein also obtained in MEPP medium would complicate any protein purification operations probably negating any advantage gained through having a higher overall α-amylase production level.

7.4.2: Minimal medium provides favourable conditions for α-amylase production.

The level of α-amylase productivity seen from the *S. lividans* pQR620 cells illustrates that the conditions favourable for high level growth are not necessarily favourable for high level recombinant protein production. Virolle and Bibb (1988) observed that levels of induced and uninduced α-amylase production were consistently higher when host strains were grown in minimal medium lacking sugars when compared with levels when the cells were grown in richer sugar containing media. The patterns of α-amylase production seen throughout this study mirrored this finding.

The α-amylase productivity of the cells in MEPP medium was significantly lower than that of the cells in MMS medium (Figs. 7.10 & 4.6). However, it was significantly higher than that seen in glucose and aspartate containing medium during exponential growth (Fig. 7.5). This tends to suggest that although production of α-amylase may be repressed in MEPP medium it is not as tightly controlled as when under conditions of glucose repression. The high level general protein secretion seen in MEPP when compared to that in glucose and aspartate medium (Figs. 7.8 & 7.3) indicates that the regulation of α-amylase production in MEPP is likely to be specific to this enzyme alone.

These observations may indicate that catabolite repression of α-amylase expression is only completely relieved in minimal media. Alternatively it may indicate that low nutrient environments, where the cells are experiencing stress, are favourable for α-

amylase expression. It would make sense for a cell experiencing a poor nutrient supply to try to improve the situation, and liberate additional resources, by secreting hydrolytic enzymes. These findings may provide additional evidence that the switch on of high level α-amylase expression is linked to the cells' stress response system (Section 6.5.2).

7.4.3: Effects of variation in amino acid, phosphate and minor element levels.

Several studies have found that the amino acid composition of a medium is important in governing protein production patterns from streptomycete cells. Chatterjee *et al.* (1983) found that production of chloramphenicol by *S. venezuelae* varied depending on the amino acids used for growth. Sanchez and Brana (1996) found that mixtures of amino acids and peptides could act as factors for the activation of secondary metabolism in *S. clavuligerus*. Strauch *et al.* (1991) observed that starvation for many amino acids triggered more pronounced changes in intracellular nucleoside triphosphate concentrations than starvation for a single amino acid. It is possible that differences in the amino acid profiles of MEPP and MMS medium were responsible for the variation in α-amylase production seen. Although production of tyrosinase from the *mel* promoter in pIJ702 is constitutive in *S. lividans*, it is inducible by L-methionine and L-leucine in *S. antibioticus* (Hintermann *et al.*, 1985). The additional α-amylase production seen in MMS medium could have been due to a previously unrecognised amino acid effect, which causes induction or de-repression of expression from the *mel* promoter on pQR620.

The patterns of α-amylase and general protein production observed in MEPP and MMS medium could also be explained by differences in the phosphate or minor element concentrations in the different media. Lounes *et al.* (1995) observed that excess phosphate ions stimulated total protein production in *S. ambofaciens* whilst simultaneously preventing the expression of two proteins. Enhancement of general protein secretion at high phosphate levels was reported again by these authors (Lounes *et al.*, 1996). Their studies indicate that phosphate concentration has a series of complex effects both on primary and secondary metabolism. Variations in levels of minor elements such as copper, zinc and manganese have also been shown to have varying effects on streptomycete metabolism and protein expression (Chatterjee *et al.*, 1983; Ahmed *et al.*, 1984; Bader, 1986; Weinberg, 1989). The very divergent

compositions of MEPP medium and MMS medium mean that significant differences in levels of minor elements almost certainly existed between the two systems. It is possible that the α-amylase and protein profiles observed in this study largely reflected the levels of such medium components.

Parro and Mellado (1994) found that, in rich media, levels of extracellular agarase produced by *S. lividans* were limited by high rates of intracellular degradation of the agarase precursor. Negative regulation of the secretion machinery was also postulated to play a role under certain conditions. Such effects could be further possible reasons for the reduced levels of α-amylase observed in MEPP medium.

7.5: Identification of Influential MMS Medium Constituents Using a Statistical Experimental Approach

A large number of variables exist that will exert an effect on the performance of the *S. lividans* pQR620 α-amylase production system. Results obtained, and observations made, during the course of this study suggested that variables such as inoculum size and age, incubation temperature, speed of shaking or stirring and levels of aeration had, or were likely to have, an influence on the performance of the system. The results presented above indicate that levels of individual medium components are likely to be equally, if not more, influential than these factors.

Classical experimental methodology often altered a single variable at a time to analyse the effect of that variable on the performance of a particular system. When a large number of variables exist in a system such an approach is impractical and time consuming. These considerations have led to the use of statistical experimental designs (reviewed by Greasham, 1993). Such designs allow efficient, rapid analysis of a large number of variables in a manageable number of experiments. Recently, these approaches have been used to develop a defined medium for vancomycin production (McIntyre *et al.*, 1996), to optimise a culture medium for recombinant interferon-gamma production by Chinese hamster ovary cells (Castro *et al.*, 1992), to develop a defined medium for physostigmine production by *S. griseofuscus* (Zhang *et al.*, 1996) and to analyse the effect of varying medium components on antibiotic production by *S.*

aureofaciens (Ternel *et al.*, 1997). They have also been used in industrial media development programmes, although little of this work is in the public domain.

7.5.1: The Plackett-Burman design.

One method, which has been use to great effect in media optimisation studies, is the Plackett-Burman design (Plackett & Burman, 1946; Stowe & Mayer, 1966). This is a two-level factorial design which allows the analysis of N-1 variables with N experiments (in increments of 4). In each set of experiments one, or more, variables are left 'unassigned'. This allows the estimation of experimental error and the calculation of levels of significance for any effects which are detected (Section 2.6.12.1). Each variable is tested at a high level and a low level. The design of each set of experiments allows the independent evaluation of the effect of each variable on parameters of the system such as growth rate, peak biomass or peak levels of enzyme production (see Section 2.6.12 for further explanation). Although these experimental designs do not allow the optimal level of a variable to be defined, they do allow the identification of the most important and influential variables within a system. Further research can therefore be targeted towards optimising, or understanding, the factors which have the greatest effects.

As a large number of different components were present in MMS medium use of a Plackett-Burman experimental design was chosen as a powerful way to identify factors which affected the performance of the *S. lividans* pQR620 α-amylase expression system. This method was particularly valid in the light of the finding that temperature had a significant effect on levels of α-amylase production. During previous experiments it had been impossible to remove any possible effect seen because of variation in the temperature at different points in the orbital incubator (Section 6.3.4). Use of this experimental approach meant that any such effect would be accounted for within the calculated error and would not affect the overall results.

7.5.2: Possible limiting nutrients in MMS medium.

In order to allow an easier interpretation of the results of these experiments the likely limiting factor(s) in MMS medium were identified (Table 7.1). These calculations were based on the typical elemental composition of bacteria given by Greasham (1993) and

Table 7.1: Comparison of the levels of each element provided by MMS with the level of each element required for the production of 3 grams of *S. lividans* DCW (based on typical bacterial composition given by Greasham (1993)). The amount of each element provided in MMS by Casamino acids was calculated from a detailed composition provided by Difco (Detroit, USA).

Element	Grams of Element Required for 3 g DCW	Grams of Element Provided by MMS	Possible Limiting Factor(s)
Carbon *	3.60	2.14	✓
Nitrogen	0.3750	0.7865	-
Phosphorous	0.0750	0.9760	-
Sulphur	0.0180	0.5762	-
Potassium	0.0690	1.2330	-
Magnesium	0.0090	0.0090	?
Sodium	0.0240	2.1030	-
Calcium	0.0180	0.0004	✓
Iron	0.0003	0.0002	✓
Copper	0.0006	-	✓
Manganese	0.0003	0.0003	?

* This calculation is based on 60% of the carbon metabolised being used up in cell maintenance (Bader, 1986).
? Possibly limiting under certain growth conditions.

Table 7.2: Table showing the percentage of each element contributed by the individual components of MMS Medium.

	Percentage of Total Provided by Medium Component										
Element	C	N	P	S	K	Mg	Na	Ca	Fe	Cu	Mn
Medium Component											
Succinate	42						41				
Casamino acids	58	46		2			42				
$(NH_4)_2SO_4$		54		84							
$MgSO_4$				13		100					
K_2HPO_4			50		100						
NaH_2PO_4			50				17				
Minor Elements				<1				100	100		100

the peak level of growth seen routinely in the medium in previous experimental work, i.e. 3g DCW/l. It was assumed that 60% of the carbon metabolised was used for maintenance and was not incorporated into the cells (Bader, 1986). This analysis indicated that the limiting component of MMS medium was likely to be carbon. Calcium, iron and copper were also possible limiting factors. However, it is likely that these, and other minor elements, were also added in the water used to produce the medium or as trace contaminants in other medium components. No allowance was made in these calculations for the use of medium components as substrates for α-amylase production.

7.5.3: Choice of variables for study.

In the first Plackett-Burman experiment it was decided to use Casamino acids, succinate, minor elements, ammonium sulphate, magnesium sulphate and thiostrepton as variables for study. The experiment was conducted as described in Section 2.6.12. Details of the high and low levels used for each component are given in Section 2.6.12.2. The results of the analysis are shown in Table 7.3. The growth curves, α-amylase, protein and protease production patterns, specific activity and productivity profiles for the 8 shake flasks which made up this experiment are shown in Figures 7.11 to 7.16. A great deal of variation was observed between the 8 individual flasks for each of these measured parameters indicating that the different combinations of medium components were influencing the behaviour of the *S. lividans* pQR620 cells in a variety of ways.

A second analysis was conducted to follow up some of the effects seen in the first study and to consider the response of the system to some additional variables. In this second experiment it was chosen to vary the levels of ammonium sulphate, phosphate, thiostrepton, $FeSO_4$ and $CaCl_2$. A sterile protease inhibitor cocktail was also used as a variable, to investigate the effects of protease activity on the system. Details of the high and low levels used for each component are given in Section 2.6.12.3. The results of this second analysis are shown in Table 7.4. The growth curves, α-amylase, protein and protease production patterns, specific activity and productivity profiles for the 8 shake flasks which made up this experiment are shown in Figures 7.17 to 7.22. Again the

Table 7.3: Results of Plackett-Burman analysis 1. The effect of each variable on a parameter of the system, and its significance level (%), is shown. Positive effects are shown as black; negative effects are shown as *blue*.

Variable	Peak dry cell weight (g/l)		μ_{max} (h^{-1})		Peak α-amylase (U/ml)	
	E_x	Signif. %	E_x	Signif. %	E_x	Signif. %
Casamino acids	0.3175	--	0.00233	--	*-2.7925*	--
succinate	0.3825	--	0.00728	**90**	5.2875	**80**
minor elements	0.5925	**80**	0.00508	**80**	3.8775	**80**
$(NH_4)_2SO_4$	*-0.1475*	--	0.00233	--	*-0.1325*	--
$MgSO_4$	0.3475	--	*-0.00338*	***80***	0.0875	--
thiostrepton	0.3425	--	0.00103	--	3.6675	**80**
dummy	*-0.1325*		0.00097		1.1375	
V_{eff}	0.0176		9.41×10^{-7}		1.2939	
SE_{eff}	0.1325		0.00097		1.1375	

Variable	Peak rate α-amylase prodn. (U/h)		Peak productivity (U/g DCW)		Peak supernatant protein (mg/ml)	
	E_x	Signif. %	E_x	Signif. %	E_x	Signif. %
Casamino acids	*-0.0880*	--	*-1448*	--	26.5	**98**
succinate	0.1516	--	1868	--	37.5	**99**
minor elements	0.0839	--	1051	--	*-13*	***95***
$(NH_4)_2SO_4$	0.0475	--	485	--	*-0.5*	--
$MgSO_4$	*-0.0165*	--	37	--	*-31*	***98***
thiostrepton	0.1049	--	545	--	83	**99**
dummy	0.0500		875		*-0.5*	
V_{eff}	0.0025		765625		0.25	
SE_{eff}	0.0500		875		0.50	

Variable	Peak specific activity (U/mg)		Peak protease (U/ml)		Initial protease (U/g DCW)	
	E_x	Signif. %	E_x	Signif. %	E_x	Signif. %
Casamino acids	*-20.958*	***80***	8.165	**95**	4770	--
succinate	53.258	**90**	8.995	**95**	32048	**95**
minor elements	51.763	**90**	0.540	--	*-2043*	--
$(NH_4)_2SO_4$	*-3.023*	--	*-0.540*	--	14370	**90**
$MgSO_4$	40.478	**90**	*-0.560*	--	*-17196*	***90***
thiostrepton	*-27.088*	***80***	7.985	**95**	*-3496*	--
dummy	6.133		0.490		1839	
V_{eff}	37.613		0.240		3381921	
SE_{eff}	6.133		0.490		1839	

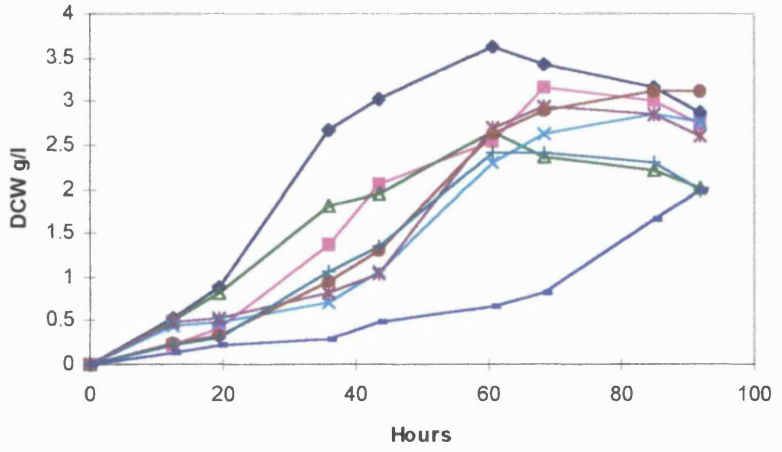


Figure 7.11: Growth curves for the 8 experiments in Plackett-Burman analysis 1.

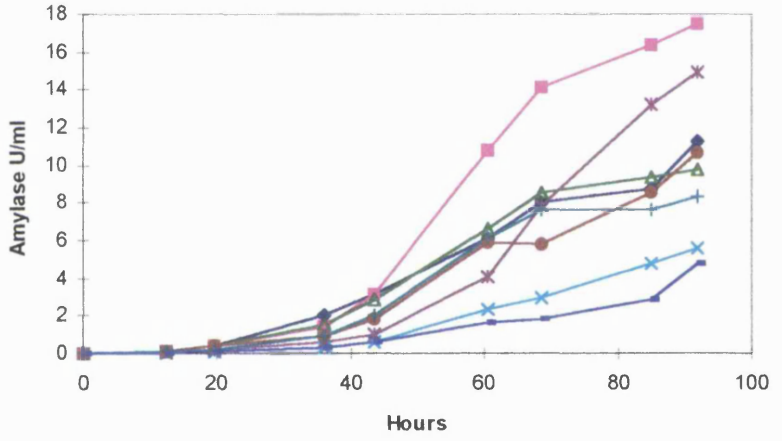


Figure 7.12: Supernatant α-amylase levels for the 8 experiments in Plackett-Burman analysis 1.

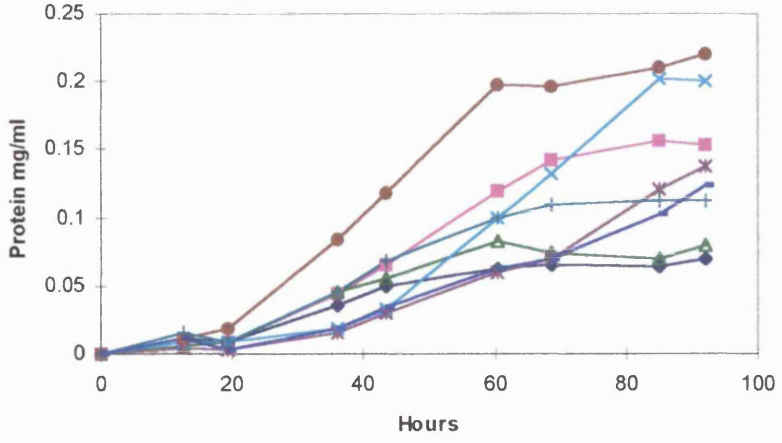


Figure 7.13: Supernatant protein levels for the 8 experiments in Plackett-Burman analysis 1.

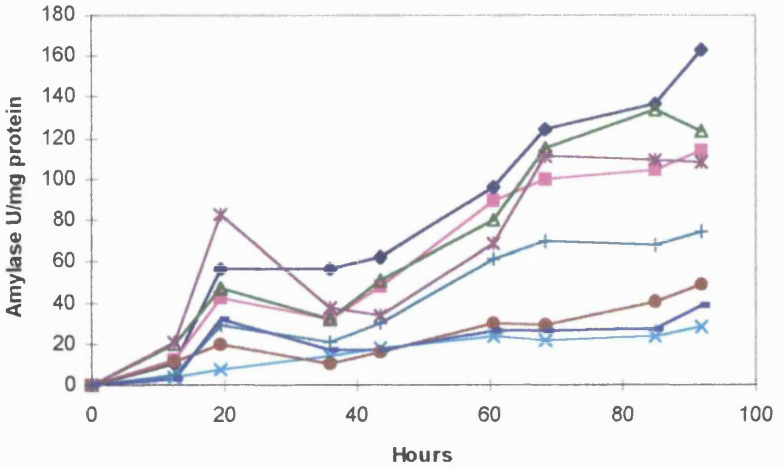


Figure 7.14: Specific activity profiles for the 8 experiments in Plackett-Burman analysis 1.

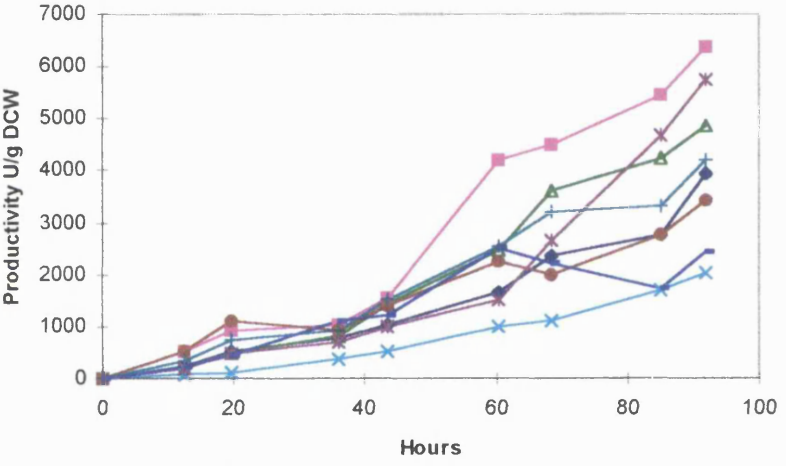


Figure 7.15: Productivity profiles for the 8 experiments in Plackett-Burman analysis 1.

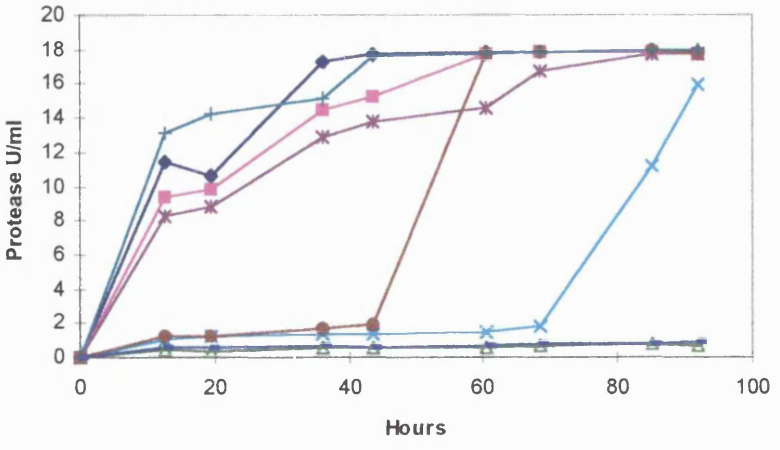


Figure 7.16: Protease production profiles for the 8 experiments in Plackett-Burman analysis 1.

Table 7.4: Results of Plackett-Burman analysis 2. The effect of each variable on a parameter of the system, and its significance level (%), is shown. Positive effects are shown as black; negative effects are shown as *blue*.

Variable	Peak dry cell weight (g/l)		μ_{max} (h^{-1})		Peak α-amylase (U/ml)	
	E_x	Signif. %	E_x	Signif. %	E_x	Signif. %
$(NH_4)_2SO_4$	0.350	**98**	0.0039	--	1.5175	**80**
phosphate	*-0.035*	***80***	0.0053	--	5.3375	**95**
thiostrepton	0.310	**95**	*-0.0068*	--	0.4875	--
$FeSO_4$	*-0.095*	***90***	0.0020	--	*-2.0075*	***90***
$CaCl_2$	0.365	**98**	0.0114	**80**	1.0075	**80**
protease inhibitor	*-0.390*	***98***	0.0040	--	*-0.2875*	--
dummy	*-0.010*		0.0034		*-0.2875*	
V_{eff}	1×10^{-4}		1.16×10^{-5}		0.0826	
SE_{eff}	0.010		0.0034		0.2875	

Variable	Peak rate α-amylase prodn. (U/h)		Peak productivity (U/g DCW)		Peak supernatant protein (mg/ml)	
	E_x	Signif. %	E_x	Signif. %	E_x	Signif. %
$(NH_4)_2SO_4$	0.0598	--	14	--	*-27*	***80***
phosphate	0.1566	**80**	3572	**80**	*-14*	--
thiostrepton	*-0.0123*	--	*-992*	--	*-1*	--
$FeSO_4$	*-0.0777*	--	*-692*	--	*-64*	***95***
$CaCl_2$	0.0578	--	17	--	*-68*	***95***
protease inhibitor	*-0.0438*	--	1241	--	73	**95**
dummy	*-0.0483*		725		5	
V_{eff}	0.0023		525625		25	
SE_{eff}	0.0483		725		5	

Variable	Peak specific activity (U/mg)		Peak protease (U/ml)		Initial protease (U/g DCW)	
	E_x	Signif. %	E_x	Signif. %	E_x	Signif. %
$(NH_4)_2SO_4$	28.5	**95**	*-0.02475*	***90***	381	--
phosphate	60.0	**98**	16.1675	**99**	12762	**80**
thiostrepton	9.5	**90**	0.0075	--	*-1834*	--
$FeSO_4$	*-1.5*	--	0.0475	--	678	--
$CaCl_2$	71.0	**99**	0.1425	**80**	*-1902*	--
protease inhibitor	*-61.0*	***98***	*-0.2825*	***90***	*-1389*	--
dummy	1.0		*-0.0275*		2036	
V_{eff}	1.0		7.56×10^{-4}		4145296	
SE_{eff}	1.0		0.0275		2036	

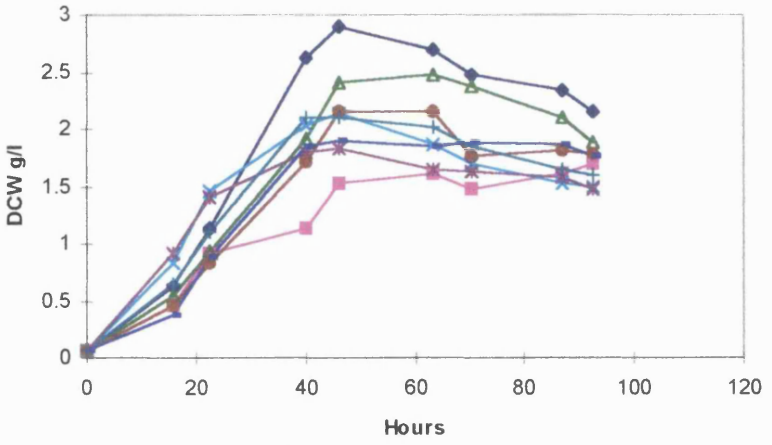


Figure 7.17: Growth curves for the 8 experiments in Plackett-Burman analysis 2.

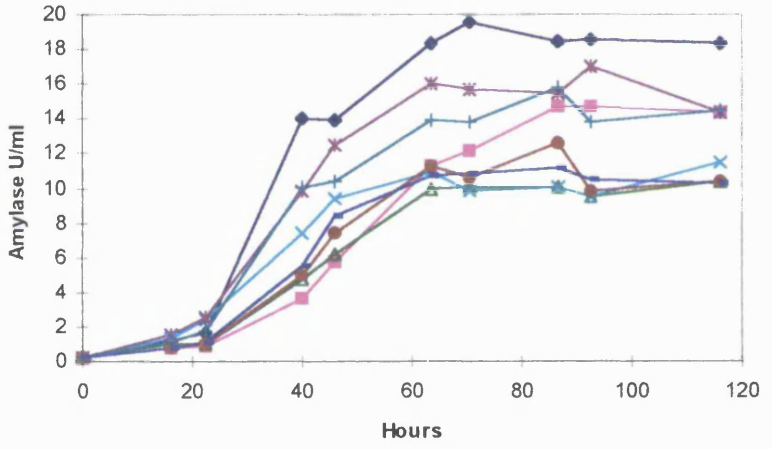


Figure 7.18: Supernatant α-amylase levels for the 8 experiments in Plackett-Burman analysis 2.

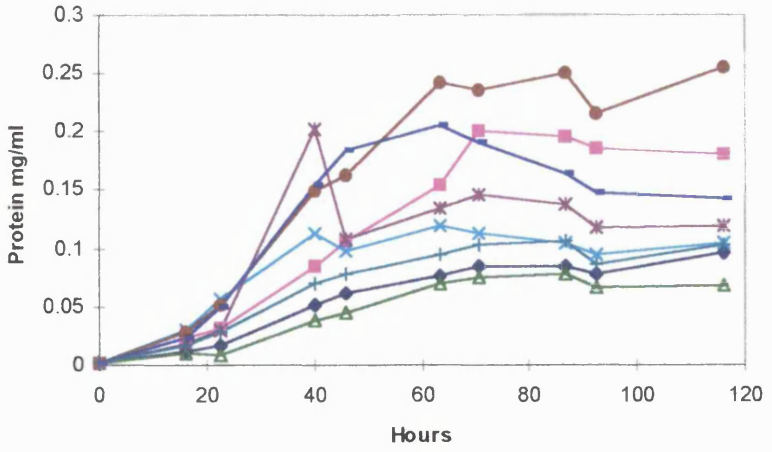
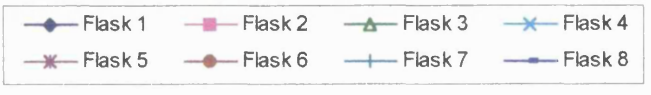


Figure 7.19: Supernatant protein levels for the 8 experiments in Plackett-Burman analysis 2.



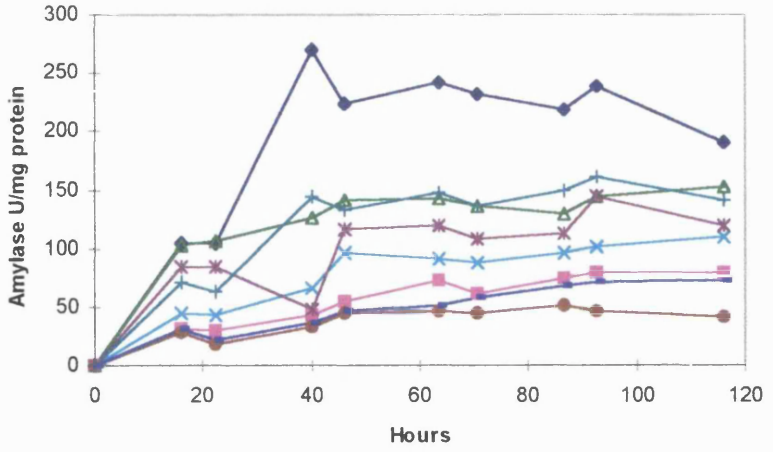


Figure 7.20: Specific activity profiles for the 8 experiments in Plackett-Burman analysis 2.

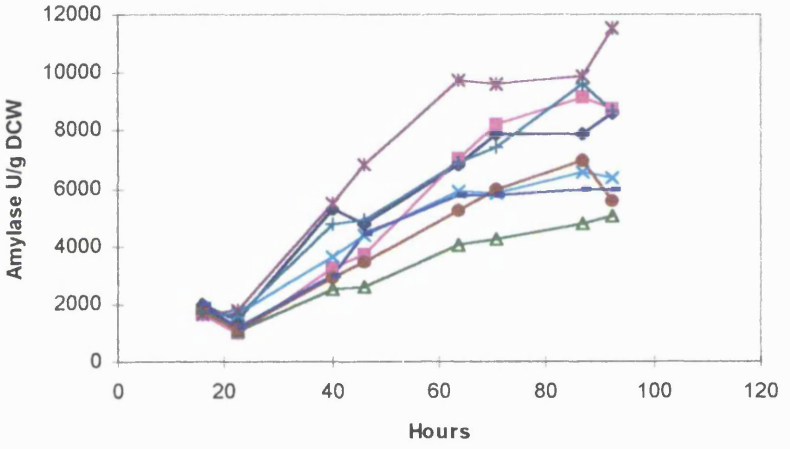


Figure 7.21: Productivity profiles for the 8 experiments in Plackett-Burman analysis 2.

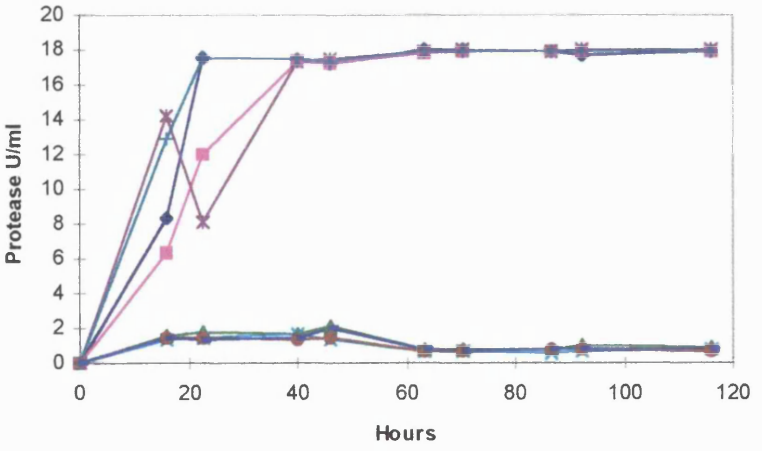


Figure 7.22: Protease production profiles for the 8 experiments in Plackett-Burman analysis 2.

variation between the shake flask profiles indicated that the different medium combinations in the study were acting to influence the behaviour of the cells.

The results of these analyses will be discussed together as this allows a more complete picture of the possible effects prompted by the variations in medium component levels to be given.

7.5.4: Effect of variation in Casamino acids level.

Analysis of MMS medium suggested that carbon might be limiting growth of the *S. lividans* pQR620. Although 58% of the carbon in MMS is provided in the form of Casamino acids (Table 7.2), increasing the level of this component did not lead to increased biomass production (Table 7.3). Increases in background protein and protease levels were, however, observed. Raising the level of Casamino acids had no significant effect on α-amylase production. A significant drop in the peak α-amylase specific activity was measured, presumably in response to the changes in background protein level.

These results suggest that Casamino acids are not particularly favourable medium constituents for α-amylase production by *S. lividans* pQR620, as they tend to prompt the cells to produce contaminating protein rather than α-amylase. They do provide further confirmation that the amino acid composition of a medium plays an important role in governing the protein production pattern of streptomycete cells (Section 7.4.3). It would be interesting to look at the response of the system if the Casamino acids were removed from the media formulation, or were present at a much lower level than that tested here, i.e. well below 5 g/l. It would seem likely that they provide important factors for α-amylase production. However, the complex mixture of different amino acids provided to the cells in this experiment almost certainly caused many other effects on cell metabolism, both positive and negative. Testing individual amino acid effects on the system would provide more useful information than the grouping of them used here.

7.5.5: Effect of variation in succinate level.

Increasing the level of succinate was found to have a wide range of positive effects on *S. lividans* pQR620. Peak α-amylase, peak specific activity, peak supernatant protein and initial and peak protease levels were all significantly greater at the high succinate level (Table 7.3). Maximum specific growth rate was positively affected by the change in succinate concentration, but no significant increase in peak biomass was detected. These findings, alongside those for Casamino acids, do not suggest that raising the level of carbon in the medium relieved any growth limitation. They point to one of the other possible factors identified being the true limiting factor for growth (Table 7.1). High levels of initial and peak protease production were seen when high levels of succinate were present. This could suggest that cell growth rate was being limited by the level of easily obtainable and metabolizable forms of nitrogen in the medium surrounding the cells.

Flores *et al.* (1996) found that succinate strongly repressed the production of β-xylosidase and β-xylanase from *Streptomyces* spp. CH-M-1035. No such effect was seen in the *S. lividans* pQR620 α-amylase expression system. The situation seen in these experiments seemed to more closely mirror the trends seen in work by Chatterjee *et al.* (1983). They found succinate to be a good carbon source for growth and chloramphenicol production by *S. venezuelae*. They postulated that it was particularly suitable as it provided a slowly metabolised carbon source which influenced endogenous metabolic precursor pools in a way which was favourable for production of the antibiotic. The beneficial effects of succinate on α-amylase production levels in the *S. lividans* system were also likely to have been due to favourable effects of the substrate on precursor pools.

Peak α-amylase specific activity was significantly increased in high succinate conditions although levels of background protein were also positively affected. The succinate may have boosted α-amylase production by a greater amount than it boosted general protein production. Alternatively, the high levels of protease produced may have been acting preferentially on the background proteins, therefore reducing the levels of contaminating protein present (see Section 7.5.10 for further discussion). It is possible a combination of these mechanisms was in operation.

7.5.6: Effect of variations in levels of minor elements ($ZnSO_4$, $FeSO_4$, $MnCl_2$, $CaCl_2$) and $MgSO_4$.

In the first Plackett-Burman analysis minor elements solution and $MgSO_4$ were used as separate variables. Increased levels of minor elements boosted peak dry cell weight, maximum specific growth rate, peak α-amylase level and peak specific activity whilst at the same time reducing the peak level of contaminating supernatant protein (Table 7.3). This was a favourable result from the point of view of α-amylase production as it meant that higher levels of less contaminated enzyme were being produced more quickly by the cells. Raising levels of $MgSO_4$ acted to reduce maximum specific growth rate and peak supernatant protein. This led to an increase in peak specific activity. Presence of the higher level of $MgSO_4$ at the start of the culture was found to suppress early protease production.

Calcium, iron and copper were identified as possible limiting factors in the growth of *S. lividans* (Table 7.1). As raising the level of minor elements increased the peak dry cell weights obtained it would seem that calcium, iron, or both elements, are the true limiting factors in MMS medium. In order to investigate further which components were causing which effects $FeSO_4$ and $CaCl_2$ were used as individual variables in the second Plackett-Burman study. Elevated $FeSO_4$ levels were found to have a negative effect on peak dry cell weight, α-amylase and supernatant protein (Table 7.4). Raised levels of $CaCl_2$ increased peak dry cell weight, maximum specific growth rate, peak α-amylase, specific activity and protease level whilst reducing peak supernatant protein (Table 7.4). The results of this analysis therefore indicate that calcium is the major limiting factor in MMS medium.

The trends seen in Plackett-Burman study 1 when levels of minor elements were raised, were consistent with the results seen in the second study due to increases in the level of $CaCl_2$. This raised the possibility that the overriding effects of high levels of minor elements could have been caused by the $CaCl_2$ component of the solution, with $ZnSO_4$, $FeSO_4$ and $MnCl_2$ having little effect on the measured responses. Evidence for this comes from a study on chloramphenicol production by Chatterjee *et al.* (1983). They found that omitting iron and manganese had no significant affect on *S. venezuelae.*

Weinberg (1989) concluded than manganese is generally not important for actinomycetes. Greater protease activity was measured in the presence of high level $CaCl_2$, but not where there were high levels of minor elements or high $FeSO_4$. By elimination, this indicates that zinc or manganese ions may have acted to reduce levels of protease production. Increasing levels of $FeSO_4$ produced negative effects on the system. This suggests that the high level of $FeSO_4$ tested (0.014mM) could have been toxic to the cells.

The positive effects of $CaCl_2$ on α-amylase levels could have been predicted as several studies have reported a positive link between calcium ions and α-amylase activity and stability. Nadia *et al.* (1994) also observed increases in α-amylase and protease production by *S. enissus* and *S. violarus* at elevated Ca^{2+} concentrations. Vukelic *et al.* (1992) found calcium ions protected *S. rimosus* α-amylase from inactivation by p-chloromercuribenzoate. They found chloride ions exerted no effect. Bahri and Ward (1990b) found the *S. thermoviolaceus* α-amylase to have a surprisingly extended stability in the presence of Ca^{2+}. The presence of calcium ions substantially increased the stability of the enzyme to thermal inactivation. In Plackett-Burman study 2 no significant increase in the rate of α-amylase production was seen in the test flasks. However, the peak α-amylase level was raised. This provides evidence that such a mechanism was in operation in these cultures.

Varying the levels of calcium, magnesium, zinc and iron would have been expected to exert complex effects on the *S. lividans* pQR620 cells themselves. Many metabolic enzymes require metal ions as cofactors. High levels of these elements can cause a wide range of inhibitory and stimulatory effects within the cells (Bader, 1986; Weinberg, 1989; Chatterjee *et al.*, 1983). These ions can also form insoluble salts and remove other nutrients from solution. It is certain that many of the effects caused by the changes in levels made would not have been detected in the limited number of responses that were monitored for the studies. Such effects could be acting in concert with any stabilising/activity enhancing effects of calcium ions to influence internal precursor pools and conditions in a manner which affected α-amylase production either positively or negatively.

7.5.7: Effect of variation in ammonium sulphate level.

Raising the level of $(NH_4)_2SO_4$ was found only to significantly affect levels of initial protease production (Table 7.3). This was unexpected considering the wide range of documented effects of ammonium ions on streptomycete cells. This result provides further evidence for the hypothesis that amino acids are used in preference to ammonium as nitrogen sources by the *S. lividans* pQR620 cells (Section 3.4.5).

As this was a surprising initial result, the importance of ammonium sulphate to the α-amylase expression system was investigated further in Plackett-Burman study 2. In this experiment the low level chosen was 0 g/l. This allowed the effect of the presence of $(NH_4)_2SO_4$ to be evaluated, rather than the effect of different levels of the component. The presence of ammonium sulphate was found to be necessary for good growth (Table 7.4). It was also found to have a positive effect on peak α-amylase and peak specific activity. In contrast to the observed effect seen in the first study, the presence of ammonium sulphate produced a significant negative effect on levels of protease and supernatant protein.

The dependence of the majority of significant effects on the absolute presence or absence of $(NH_4)_2SO_4$ again suggests that ammonium ions are playing a role in the regulation of metabolism rather than being used as a source of nitrogen. Many authors have illustrated that the presence of NH_4^+ ions can lead to the suppression of actinomycete secondary metabolism (DelaCruz *et al.*, 1992; Greasham, 1993; Lounes *et al.*, 1995; Tang & Hutchinson, 1995). Erpicum *et al.* (1990) found that supplementation of their cultures with 50mM ammonium enhanced recombinant protein production. These results may indicate that ammonium acts to suppress the switch from primary to secondary metabolism by streptomycete cells, allowing primary metabolic activity to be extended. This could allow enhanced production of proteins whose precursor molecules are provided mainly by primary metabolic pathways. Efforts have been made to relate the action of NH_4^+ ions in nitrogen catabolite repression to mechanisms of nitrogen assimilation. However, no clear link has so far been found (Doull & Vining, 1990). The results of this study suggest that actual assimilation of the nitrogen might not be necessary for repression to occur. Other mechanisms might exist to sense levels of nitrogen in the cells' surrounding

environment and to allow appropriate alterations in metabolic activity to be made. Taken together, the results of the two Plackett-Burman studies suggest that α-amylase production by the *S. lividans* cells occurs more favourably in the presence of ammonium sulphate. Raising the level of the component above 15mM would have little further effect on the system and would represent a waste of substrate.

7.5.8: Effect of presence of thiostrepton and dimethylsulphoxide (DMSO).

Thiostrepton was used as a variable in the first Plackett-Burman study. For this set of experimental flasks the high level was presence of thiostrepton and its solvent, DMSO. 'Low level' flasks contained no antibiotic or DMSO. As both the thiostrepton and the DMSO had effectively been used together as variables in the first study, the two components of the antibiotic solution were separated for the second Plackett-Burman study. Here only high level flasks contained thiostrepton. All of the flasks had DMSO added. This combination of experiments permitted the results of the first and second studies to be compared, effectively allowing the effects of thiostrepton and DMSO to be considered separately.

Addition of thiostrepton into recombinant streptomycete cultures is generally considered to be unnecessary for plasmid maintenance and protein production (Section 3.3.1). As the antibiotic is not thought to significantly increase the copy number of pIJ101-based plasmids, it would therefore not be expected to produce major effects on the cells. Contrary to expectations, the presence of these components affected the *S. lividans* pQR620 cells in several ways when compared with cells which had not been exposed to the antibiotic or solvent. Presence of thiostrepton and DMSO significantly boosted the production of supernatant protein, α-amylase and protease (Table 7.3). It led to a reduction in the peak specific activity obtained. When tested as a separate variable in study two, thiostrepton was found to significantly increase peak specific activity and dry cell weight (Table 7.4).

A comparison of these results suggested that DMSO had a number of effects upon the *S. lividans* pQR620 system. In fact, the results indicated that the solvent actually played more of a role in determining the behaviour of the system than the thiostrepton itself. Thiostrepton, when used as a single variable, was found to cause a significant increase

in dry cell weight levels. However, presence of thiostrepton and DMSO in cultures, was not found to affect growth. This would tend to indicate that DMSO has a negative effect on levels of biomass.

DMSO has been shown to cause sublethal stress in *E. coli* (LaRossa *et al.*, 1994). Such an effect could cause a reduction in cell growth. The observed effect of thiostrepton on biomass was unexpected. Thiostrepton exerts its antibiotic effect by complexing with 23s ribosomal RNA, thereby blocking protein synthesis (Cundliffe, 1989; Thompson & Cundliffe, 1991; Smith *et al.*, 1995). *Streptomyces* cells are protected from the antibiotic by a methylase which acts on the ribosomal protein and prevents the complexing of the thiostrepton molecule. In the *S. lividans* pQR620 system this methylase is coded for by the *tsr* gene on the plasmid (Fig. 4.2). The presence of thiostrepton has been shown to inhibit the activity of the methylase (Bechthold & Floss, 1994). It has been postulated that the methylase plays an additional role within the cells, possibly by acting as a regulator of intermediary metabolism (Thompson & Cundliffe, 1981; Thompson *et al.*, 1982; Cundliffe, 1989). The presence of thiostrepton in the *S. lividans* pQR620 cells within the Plackett-Burman studies may conceivably have boosted cell growth by inhibiting some regulatory or other function of the RNA methylase.

Thiostrepton was also found significantly to increase the peak α-amylase specific activity of cultures. However, no significant increases or decreases were observed in the levels of α-amylase or supernatant protein respectively. It is possible that this response was due to the combined effect of a slight increase in peak α-amylase level coupled with a slight decrease in supernatant protein. Although such trends can be seen within the data neither of these effects was picked up as significant at the 80% level. Thiostrepton might therefore have acted to increase plasmid stability or copy number within the *S. lividans* cells. Alternatively, it might have interfered with general protein production within the cells. As thiostrepton acts by complexing with ribosomal proteins (see above) it is possible that, even in a situation where the 23s protein is protected by methylation, the antibiotic molecules may reside in close proximity to the active ribosomes. This could hinder the production of some proteins.

Further comparison of the two Plackett-Burman studies suggests that DMSO acts to increase peak α-amylase, peak supernatant protein and peak protease levels, whilst decreasing the peak specific activity obtained. These observations could be explained in several ways. DMSO has been shown to be an effective permeabilizing agent for plant, insect, and gram negative cells. Wahl *et al.* (1995) found that both intracellular and extracellular concentrations of a recombinant monoclonal antibody increased sharply after the addition of 2.8% DMSO to a plant cell expression system. They suggested that the rise in extracellular production was due to an increased secretion due to permeabilization of the membrane. Alterations in patterns of substrate uptake were considered a likely cause of the rise in intracellular production. Schmidt *et al.* (1989) saw similar behaviour in a tobacco cell expression system. In this case the effects were seen at much lower level of DMSO. A 100% increase in productivity was seen in an insect cell/Baculovirus expression system by Radford *et al.* (1992) upon addition of 1% DMSO. No possible explanation was put forward for this effect. Ames *et al.* (1984) found that DMSO also acted to permeabilize Gram negative cell walls.

The increased peak α-amylase, protein and protease levels in the *S. lividans* pQR620 cultures in the presence of DMSO could have been due to increased secretion/leakage of proteins from the cells. This could have led to an overall decrease in specific activity. Alternatively, permeabilization of the cell walls could have allowed an influx of substrates such as Ca^{2+} ions. As previously described this could have caused a wide range of effects within the cells (Section 7.5.6). DMSO itself has been shown to act as a stabilising agent for proteins in aqueous solution (Wahl *et al.*, 1995). This attribute of the solvent might have been responsible for the patterns observed. Another possible explanation is that the DMSO triggered a stress response within the cells. This could have led to changes in protein and protease levels and, as discussed in Section 8.2 and 8.4.9, might be linked to increases in α-amylase production.

7.5.9: Effect of variation in phosphate level.

Raising the level of phosphate in the medium was found significantly to affect many of the responses being monitored for this study. Peak α-amylase activity, productivity, specific activity and the rate of α-amylase production all rose, as did the measured peak

protease and initial protease levels (Table 7.4). Peak dry cell weight was found to decrease at the higher level of phosphate.

Numerous studies have shown the importance of phosphate levels to the behaviour of streptomycete cells (Bader, 1986; Martin, 1989; Martin *et al.*, 1989b; Doull & Vining, 1990; Lounes *et al.*, 1995). Many of the effects described are thought to be directly linked to the regulatory roles of phosphate within the cell. Increased levels were found to be very favourable for the production of α-amylase by *S. lividans* pQR620. Differential expression of various genes is observed as a function of phosphate concentration. Many primary metabolic enzymes appear to be stimulated by the presence of the element (Martin, 1989). However, stimulation of secondary metabolic pathways, at the expense of primary, has also been reported (Lounes *et al.*, 1996). Although it is generally accepted that phosphate levels play a controlling role in streptomycete metabolism the mechanisms by which effects are mediated are still far from clear. A close link with levels, and forms, of carbon and nitrogen is postulated (Lounes *et al.*, 1996).

The increases in production of α-amylase at the high level would tend to indicate that phosphate acts to boost primary metabolic pathways in the *S. lividans* pQR620 system. It is also possible that the higher level of the element acted soley on expression of the α-amylase gene itself or on one or more of its precursor production pathways. DNA levels within the cell can also be increased at high phosphate concentrations (Martin, 1989). The observed effects could therefore also have been due to an increase in plasmid copy number or speed of plasmid replication.

The significant decrease in dry cell weight observed at the high phosphate concentration was not expected and is hard to explain. It is possible that the cells reduced the amount of storage material being carried in response to the conditions of plentiful phosphate. Phosphate salts typically carry relatively high levels of metal ions, such as Zn^{2+} and Fe^{2+}, as contaminants (Weinberg, 1989). As raising the level of $FeSO_4$ was found negatively to affect peak dry cell weight (Section 7.5.6) it is possible that this response to the high level of phosphate was caused by a contaminating metal ion.

7.5.10: Addition of a protease inhibitor cocktail.

The addition of a protease inhibitor cocktail into cultures at several time points caused an interesting pattern of effects. As expected the inhibitors acted to reduce significantly the level of peak protease observed (Table 7.4). Levels of peak supernatant protein were found to rise, presumably because of a reduction in general proteolysis. The level of α-amylase production in these cultures was not significantly affected by the addition of the protease inhibitors. This would tend to indicate that the α-amylase is not susceptible to degradation by the proteases routinely secreted by the streptomycete cells in this system. As would be predicted if this were a correct assumption, the peak α-amylase specific activity was significantly reduced by the introduction of the inhibitor cocktail. The dry cell weights obtained in the study were reduced by the addition of protease inhibitors. This was not an unexpected effect, as even though the cocktail chosen for this work was EDTA and PMSF free, one or more of its components may have acted upon the cells in a negative way.

7.5.11: Other factors which may affect the system's behaviour.

7.5.11.1: Maximum specific growth rate.

Considerable evidence exists that specific growth rate plays a role in controlling the switch between primary and secondary metabolism (Doull & Vining, 1990). Secondary metabolism is generally thought to be associated with low growth rates (Demain, 1989). High growth rates can repress key enzymes of antibiotic synthesis (Yoon & Choi, 1997). It is postulated that interacting mechanisms exist which sense changes in growth rate due to carbon, nitrogen and phosphate depletion and allow patterns of metabolism to be altered according to the prevailing conditions (Doull & Vining, 1990; Vining & Doull, 1988). In the two Plackett-Burman studies succinate, minor elements (Table 7.3) and $CaCl_2$ (Table 7.4) were found significantly to increase the maximum specific growth rates of the cells. The peak α-amylase levels for each of these variables were also found to rise significantly. This raises the possibility that high specific growth rates somehow play a controlling role in high level α-amylase production. Alternatively, this finding may be co-incidental or the high growth rates may be indicative of an active metabolism or pattern of metabolism which was favourable for

α-amylase expression and secretion. $MgSO_4$ was found to significantly reduce the maximum specific growth rate in study 1 (Table 7.3). However, no decrease in peak α-amylase was seen. This would provide evidence that no link exists between growth rate and α-amylase production and that the above effects were co-incidental. Further research would be needed to confirm or rule out a link.

7.5.11.2: Interactions between medium components.

The effect of the dummy variable on each monitored response is a measure of the inherent error in each set of calculations (Section 2.6.12.1). The error will partly be made up of the experimental error in each set of measurements, but also includes the affects of any interactions which have occurred between the different variables being tested. This means that a large dummy effect (relative to other measured effects) may indicate responses where interactions of the different components being tested have played a major role. In particular this was seen for the peak rate of α-amylase production and peak productivity responses in both Plackett-Burman studies (Table 7.3 & 7.4). This may indicate that these parameters of the system are largely controlled by interactions of two or more of the variables being considered in each study. In contrast the dummy variable had very little effect on peak supernatant protein in the studies. This would therefore tend to indicate that increasing or decreasing levels of each component had a specific and particular effect on the system. This type of analysis can only provide a pointer as to where interactions may be having a large effect, it cannot indicate which variables are interacting. Any such effects should become clearer and easier to interpret as more is learnt about the complex mechanisms controlling streptomycete metabolism.

7.5.11.3: Production of proteases.

The graphs showing the measured protease levels over the two studies (Figs. 7.16 & 7.22) are interesting as they show that protease production is rather an all or nothing affair. This is illustrated well by flasks 4 and 6 in the first Plackett-Burman study (Fig. 7.16) where basal levels of protease production were seen prior to rapid and sudden protease production. Similar patterns had previously been seen in 20l MMS fermentations (Section 6.4.2). These results provide further evidence that protease

production was triggered by a particular event in these cultures. As the production did not occur at the point where dry cell weight levelled off, it is possible that the trigger was the depletion of a nutrient or nutrients below a threshold level. Many of the flasks produced proteases immediately after the point of inoculation (Fig. 7.22). In these cases, it is possible the trigger was the presence of some nutrient at a threshold level. Again the reasons for these patterns should become clearer as more is understood about the effect of the medium environment upon protease production.

7.5.11.4: *S. lividans* pQR620 growth may be limited by high level α-amylase production.

Even in the flasks where certain components were found to significantly increase the level of peak dry cell weight obtained, the highest biomass level measured was 3.5g DCW/l (Fig. 7.11). This represents a relatively small increase in biomass from the level normally obtained in MMS cultures, i.e. approximately 2.5g DCW/l (Fig. 4.3). These findings may suggest that a non-nutrient factor is acting to limit levels of growth in the *S. lividans* pQR620 MMS system. Oxygen was unlikely to have been the limiting factor as over 4 grams dry cell weight per litre had previously been obtained in shake flasks in rich TSB medium (Figure 3.1). As the *S. lividans* pQR620 cells were producing high levels of α-amylase it is possible that the limitation was imposed by the demands being made on the cells by enzyme production.

7.5.12: The medium compositions tested prompted large differences in culture performance.

The graphs charting each of the studies show the wide range of effects caused by the relatively minor range of medium alterations being investigated (Figs. 7.11 to 7.22). Figure 7.23 shows a SDS-page gel of supernatant samples from each of the flasks in Plackett-Burman study 1. This again illustrates the power of the variations made on the system, with wide variations in protein production profiles seen between the flasks.

A very wide range of peak productivities were obtained over the two studies. These values ranged from 1500 to 12000 α-amylase units per gram dry cell weight. The highest peak productivity value was over double that previously achieved in unmodified MMS medium at this growth temperature. This would suggest that additional large

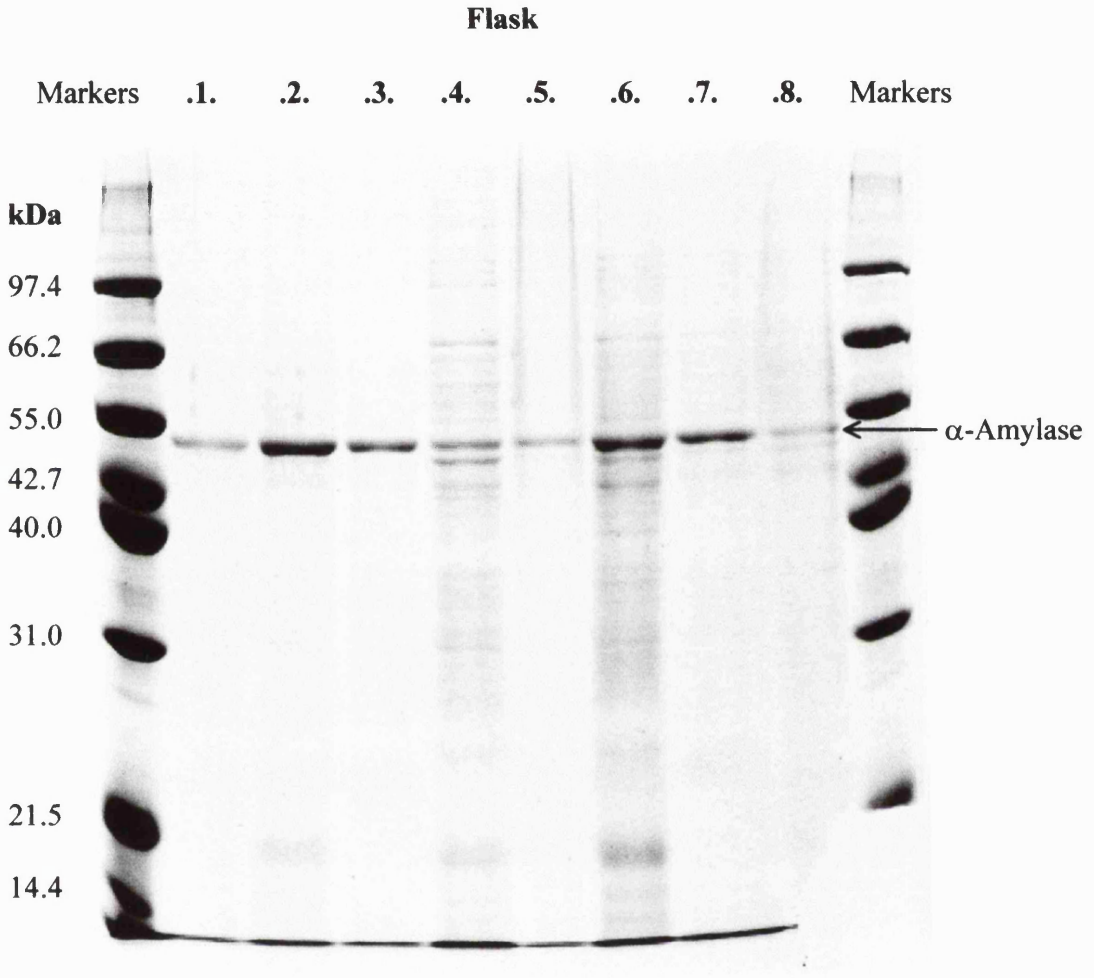


Figure 7.23: SDS-page gel showing supernatant samples from Plackett-Burman study 1 (87 h growth) flasks 1 to 8. The protein from 0.5 ml of each supernatant sample was loaded onto the gel.

increases in α-amylase yields could be obtained from the *S. lividans* pQR620 system if further medium optimisation was to be undertaken and again highlights the power of medium composition in determining the outcome of a fermentation. If MMS medium were to be further optimised this could be achieved using other statistical experimental design approaches such as response surface designs (Greasham, 1993). This would follow on from this study and determine the optimal levels of each of the major variables identified using the Plackett-Burman designs.

7.6: Summary

The work detailed in this chapter confirmed that medium composition plays an important role in the behaviour of the *S. lividans* pQR620 α-amylase expression system. Glucose was found to repress production of the recombinant enzyme. The streptomycete cells grew well in the complex medium MEPP. However, although high levels of biomass were obtained, the α-amylase productivity of the cells was very low and large amounts of supernatant protein were produced. This confirmed that conditions favourable for good growth were not necessarily favourable for high level recombinant protein production. Generally the cells seemed to perform best in minimal media lacking rapidly metabolisable sugars. Two experiments were conducted using a Plackett-Burman statistical experimental design, allowing the identification of medium components which were important for the performance of particular aspects of the system. A wide range of effects were seen when the levels of different MMS medium components were altered, further illustrating the importance of medium composition to the behaviour of the *S. lividans* pQR620 α-amylase expression system.

Chapter 8
Discussion

8.1: Genetic Construction of *Streptomyces* Expression Systems

The main aim of the work described in this thesis was to gain further understanding of the factors which influence expression of recombinant proteins in the streptomycete system. The results indicate that current understanding of the construction of high level expression vectors is still relatively poor and provide strong evidence that the system is far from being exploited to the full.

Very few of the streptomycete expression vectors in common use carry a site for second strand initiation (*ssi*) of plasmid replication (Section 4.2). Inclusion of this site on the *Streptomyces amy* constructs led to a more than 1000-fold increase in levels of α-amylase production (Section 4.5.2). This single alteration to the plasmids had a greater affect on levels of expression of the recombinant protein than any other change made over the course of this work. The observations described in this thesis suggest that incorporation of a *ssi* into expression constructs, such as pIJ702, could provide a significant increase in levels of recombinant protein production from many systems.

Gaining an understanding of the mechanism by which the *ssi* gave increased protein production would be likely to open up further possibilities for optimising gene expression. The available evidence suggested several likely reasons for the patterns of production seen. The number of *amy* gene sequences in a form available for transcription may have been raised on the ssi^+ plasmids, through increased copy number or faster conversion of ssDNA to dsDNA (Section 4.5). If this is found to contribute to the effect, further research into the control of copy number in streptomycete plasmids might allow the construction of ultrahigh and run-away replication vectors which give increased product yields.

Presence of high levels of open circle plasmid were found to correlate to high levels of α-amylase production (Section 4.4.2.1). Such plasmid structures might provide easier access for cellular factors and allow more efficient expression. The additional levels of α-amylase produced at high temperatures may provide additional evidence of the

importance of plasmid conformation (Section 8.4.3). Incorporation of carefully designed DNA sequence around a gene of interest, which would make important regions of the expression cassette more 'accessible' to the transcription machinery, could be considered if it were shown that the overall structure of the plasmid acted to limit expression levels significantly.

This study, in common with others, shows that using promoter arrays to drive expression of recombinant proteins can be an affective method for achieving high level protein production (Section 4.7.1). Although many promoters of streptomycete origin have now been described (Bourn & Babb, 1995; Strohl, 1992) only a very small number have been used in expression studies. It is therefore probable that combinations of promoters which would drive expression of a recombinant protein to a far greater extent than P_{amy} and P_{mel} will be identified. A greater understanding of the promoters which are available, and how they can work in conjunction with each other, is likely to be an integral part of future work on streptomycete expression vector construction.

Little is presently known about the mechanisms acting to control levels and patterns of expression in *Streptomyces* host systems. Currently the construction of particularly good vectors tends to require luck along with experience. Successful combinations of expression signals are often reapplied as they are discovered. In the future, a more logical, 'jigsaw like' approach is likely to prevail, with people fitting together certain attributes to build expression vectors which fit their exact needs. Production of α-amylase from *S. lividans* pQR620 was not constant over time. Instead, rates of production fluctuated, and sometimes stopped, over certain periods of cell growth (Section 5.6.2.1). This pattern may have corresponded to alterations in types and/or levels of RNA polymerase sigma factors which recognised P_{mel} and P_{amy}. It is possible that as knowledge of the regulation of this system increases it will be possible to pick a certain promoter, or promoters, which will give gene expression only when desired, e.g. at peak biomass. This type of control would be useful in instances where the recombinant protein being produced was toxic to the cells or had negative effects on some aspect of cell growth or metabolism. Exerting this level of control in other host systems, such as *E. coli,* is often difficult. Therefore, if a thorough understanding of the

Streptomycete system can be achieved, the complexity of the life cycle and regulatory system could actually be an advantage of the system rather than a hindrance.

One or more regulatory proteins are postulated to be acting to regulate expression of the α-amylase protein in the *S. lividans* pQR620 system, e.g. Reg1 is likely to control glucose repression of *amy* (Section 7.3.2). The Plackett-Burman medium studies and temperature work provided further evidence that a number of regulatory systems were in operation (Sections 7.4.3, 7.5.7 & 7.5.9). The statistical analyses also indicated that precursor supply could strongly affect the production patterns obtained (Sections 7.5.5 & 7.5.9). These findings suggest that the expression system could be improved by using metabolic engineering approaches, such as those described by Chater (1990), to relieve repression and optimise precursor supply. Again, such an approach would be likely to be generally applicable to other proteins being expressed in the streptomycete system.

Despite the large body of research being conducted into the organisation and operation of actinomycete gene expression, our understanding of the system is still extremely poor relative to that of other bacterial systems such as *E. coli*. Although the intricacy of the *Streptomyces* system is currently acting as a barrier for routine high level recombinant protein production, it is likely that, in the long term, the complexity of the system will allow the production of elegant expression systems. The vector construction work described in this thesis has highlighted the need for further understanding of the topic. It has also illustrated how important some of the as yet unexploited genetic signals are for overall levels of protein production from *Streptomyces* expression vectors.

8.2: Influence of Medium Composition on Levels of Recombinant Protein Production

The results of research into the effect of medium composition on α-amylase expression levels reinforce the importance of this aspect of a production system on recombinant protein yield. They also act to highlight the general lack of detailed understanding of streptomycete metabolism and its control mechanisms. A large body of research has been conducted over the last few years to try to build a more complete picture of the systems which are operating in these organisms and how the systems interact. In the large part the findings reported in this research are contradictory and have allowed few

solid conclusions to be drawn. When compared with other recent studies, the work described in this thesis is similarly inconclusive.

Over the course of this study the *S. lividans* cells had nitrogen provided to them in a number of forms, e.g. as amino acids, in the form of $(NH_4)_2SO_4$ and as part of more complex medium components. The type of nitrogen source provided was shown to influence strongly the pattern of acidification observed in rich media (Section 3.4.4). However, a number of contradictory hypotheses have emerged to explain how the effects are mediated (Section 3.4.3). Evidence that ammonium ions were not used in preference to other forms of nitrogen was uncovered (Sections 7.3.1 & 7.5.7), although their presence had a positive influence upon several parameters of the α-amylase expression system. Further work will be required to clarify the nitrogen utilisation patterns of *Streptomyces* species and define the mechanisms involved in uptake and assimilation of the element.

α-Amylase production from the *S. lividans* expression vectors was found to be higher in defined medium than in media containing more complex or rapidly metabolisable carbon sources (Sections 3.7.1 and 7.4.2). Presence of glucose was found to cause repression of α-amylase expression (Section 7.3.1). However, even if this repression could be overcome, it is likely that yields of the enzyme would be poor in media containing high levels of a rapidly metabolisable carbon source, due to medium acidification with its associated inhibition of growth (Section 3.4). One possible way of overcoming the problem of acidification would be to use fed-batch culture. This has been shown to be a successful approach for improving production of natural products and recombinant proteins in the streptomycete system (DelaCruz *et al.*, 1992; Ates *et al.*, 1997; Zhang *et al.*, 1996). However, it would be unlikely to completely relieve glucose repression of α-amylase expression from *S. lividans* pQR620. If a recombinant protein being expressed in the *S. lividans* system was not sensitive to catabolite repression, fed-batch culture would probably provide a strong approach for optimising production.

Although some studies have found high levels of recombinant protein production from *S. lividans* in complex media (Erpicum *et al.*, 1990; Flickinger *et al.*, 1990; Reichl *et al.*, 1992), this work, alongside several other studies (Section 7.4.2; Yoon & Choi,

1997; Zhang *et al.*, 1996), indicated that minimal medium may be the best option for obtaining peak levels of the desired product whilst minimising levels of contaminating background protein. This is an important consideration because the purity of a recombinant protein will affect the complexity and cost of any recovery operations needed for protein purification. The additional cost of a defined medium versus a more complex formulation could well be offset by savings gained in purification costs (see Section 8.5 for further discussion). The good performance of the *S. lividans* system in defined medium may therefore make it highly suitable for expression of high value pharmaceutical proteins, where obtaining high levels of purity are an issue. As more is learnt about the systems of acidification, catabolite repression and background protein expression in *S. lividans* it should be possible to optimise production of lower value products in complex media, therefore allowing routine use of the system for production of lower value products.

Use of Plackett-Burman analysis to identify important components in MMS medium proved to be a useful approach which showed the potential for increases in levels of α-amylase production from *S. lividans* pQR620 through medium optimisation. This type of methodology is undoubtedly useful in a commercial setting where increasing product yield is the main aim for the majority of research. However, the work described in this thesis has also highlighted its use as a tool for understanding the effects of different medium components on an expression system.

The experimental results obtained in the Plackett-Burman analysis of the *S. lividans* pQR620 system indicated that the level of calcium ions was highly influential in determining the final outcome of a culture (Section 7.5.6). Raising the level of $CaCl_2$ in MMS medium consistently produced an increase in peak α-amylase level. Several possible explanations for this effect were discussed (Section 7.5.6). One possible explanation for the effect of temperature in raising α-amylase expression was that an influx of calcium ions into the cells acted to inhibit degradation or stimulate production of the enzyme (see Section 8.4.7 for discussion). The observed effects of calcium in the Plackett-Burman analyses would provide supporting evidence for Ca^{2+} ions playing a role in the temperature effect.

Analysis of the effect of protease inhibitor cocktail addition indicated that proteases capable of degrading the α-amylase protein were not present or active in the culture supernatant of *S. lividans* pQR620 under the conditions tested (Section 7.5.10). This undoubtedly would have helped to enhance the specific activity of the α-amylase in routine MMS culture conditions. This is an important finding from the point of view of assessing the potential of *S. lividans* as a host organism for recombinant protein production on the basis of the results described in this thesis. Only by conducting further expression studies with other proteins would it be possible to assess if proteolytic degradation would be a problem under the conditions found to be optimal for α-amylase expression from *S. lividans* pQR620. It is likely that, to a great extent, this would depend on the particular gene product in question. Taking a long term view, strain development approaches could be used to solve such a problem by producing low protease strains of *S. lividans*. This would be analogous to similar work done to minimise the effect of proteolytic degradation in *E. coli* host strains.

Throughout the study shake flask cultures were found to produce much higher levels of contaminating background protein than fermenter grown cultures (Section 5.6.4.1). One possible explanation for this finding was the presence of the antibiotic thiostrepton or its solvent, DMSO (Section 5.6.4.3). The results of the Plackett-Burman analyses showed that although thiostrepton itself had some influence upon the cultures, DMSO acted on the system in a number of ways (Section 7.5.8). These included the action of increasing peak levels of supernatant protein. In Plackett-Burman analysis 1, DMSO was not added to flasks 1, 3, 7 and 8; it was added into the other four flasks (Section 2.6.12). The protein levels in the flasks where DMSO was not added more closely matched the level typically seen in fermenter grown cultures (Figs. 7.13, 5.4 & 5.9). Where DMSO was added the protein levels rose to a level more typical of shake flask grown cultures (Figs. 7.13 & 4.5). Taken together these findings provide evidence that the higher levels of protein observed in shake flask culture might be caused by the addition of DMSO alongside thiostrepton. If further research confirmed this hypothesis, there might be a case for using other antibiotic selection systems, where use of DMSO would not be required, in streptomycete vectors.

Throughout this study different patterns of growth and metabolism were routinely seen from shake flask cultures which had been seeded with identical inocula. This is well illustrated in Table 5.1 which shows the characteristics of two sets of inocula flasks. The results of the fermentations, unsurprisingly, indicated that the differences in inocula affected the outcome of each fermentation (Section 5.3.4). Some of the differences observed between inoculum flasks were likely to be due to the effect of slight differences between incubation temperatures of cultures (Section 6.3.4). It is also likely that other factors were playing a role. Recent research indicates that compounds which serve as 'hormonal regulators', e.g. A-factor and other similar molecules, are present in a wide variety of *Streptomyces* species (Onaka *et al.*, 1995). Cascades of these molecules are thought to act as chemical messengers which regulate and control morphogenesis and secondary metabolism within streptomycete cultures (Onaka *et al.*, 1995; Vujaklija *et al.*, 1991; Horinouchi *et al.*, 1983). It is likely that the conditions in which spore inocula are produced and initially germinated will affect the patterns and time of production of such messengers (Karandikar *et al.*, 1996). This suggests that patterns of growth and metabolism in large scale cultures may be largely determined by inoculum preparation stages. Obtaining a greater understanding of the regulatory mechanisms involved, in order to optimise production of inocula, could lead to significant increases in productivity over and above those obtained through traditional medium optimisation strategies.

8.3: Growth of Recombinant *Streptomyces* in Fermenters

Under the conditions studied in this thesis, α-amylase productivity from *S. lividans* cells in fermenter grown cultures was higher than that measured in shake flask culture (Section 5.6.4.1). The scale up factor involved between the 500ml flasks and the 20l fermenters was relatively minor when compared to a typical industrial scale fermentation. It was, however, large enough to gain some perspective on the possible problems, and advantages, which would be encountered upon moving the expression system to even larger vessels.

Acidification proved to be as much of a problem in the streptomycete α-amylase expression system as it had been in other streptomycete antibiotic and heterologous

protein production systems (Section 3.4; Payne *et al.*, 1990; DelaCruz *et al.*, 1992; Dekleva & Strohl, 1987; Ahmed *et al.*, 1984). The pH value of the culture supernatant was obviously an important factor in determining the outcome of each culture. In this respect, the controlled fermenter environment provided more suitable conditions for the *S. lividans* cells than did shake flask culture (Section 7.3.1). The use of the fermenter allowed testing of medium formulations which would have been otherwise impossible in shake flasks, as acidification would have acted to inhibit growth (Sections 7.3 & 7.4). In contrast to falling pH, pH increase up to 8 seemed to have little obvious effect on the *S. lividans* pQR620 cells (Sections 5.3.3 & 3.3.3.1). Despite the lack of any observed effect on the parameters monitored in this work, severely alkaline pH would be expected to influence the cells' behaviour in a variety of ways. No attempt was made to define the optimum pH for the α-amylase expression system. Such work could further enhance the level of protein production. If a different heterologous protein had been expressed from the system, alkaline pH may have had more of an influence on the peak levels of product seen. The thermostable nature of the α-amylase is likely to mean it has increased stability to a variety of harsh conditions, including pH extremes (Imanaka, 1994). Again, to assess the real relevance of the observations described to the streptomycete host system, a range of other proteins would need to be expressed to see if they were more sensitive to such supernatant variations.

Close control of growth temperature was key for good performance of the α-amylase expression system (Sections 6.3.1 & 6.4.1). Again, this was not realistically possible in the shake flask system (Section 6.3.4), whereas it could be achieved in fermenter grown cultures. An 'optimum' temperature for α-amylase production was identified as part of this work (Section 6.4.3). However, evidence was produced which suggested that even greater levels of enzyme production could be achieved by identifying the best overall temperature profile for high level recombinant protein production (Section 6.6.1). Potentially, the fermentation could be split into two sections, i.e. one for *S. lividans* growth (30°C) and one for α-amylase expression (34-36°C). Optimisation of the culture temperature profile would potentially be a potent way of increasing the α-amylase yield from the *Streptomyces* cells without further alterations to the genetic system.

Visual assessment of shake flask cultures alongside fermentation broth strongly indicated that pellet size tended to be significantly smaller in the fermenter environment (Section 5.6.1). Although not really explored in this study, this could have a number of important effects on the behaviour of the *S. lividans* cells as diffusion distances from the outside to the inside of the pellets would lead to the creation of nutrient gradients (Section 5.6.4.2). Examining the relationship between pellet size and performance of the expression system would be an interesting avenue for further exploration. This could be done by altering stirrer speed and fermenter impeller geometry to create a range of different shear stress environments. Such a study would also allow host cells behaviour to be contrasted with that of wild type *S. lividans*, possibly giving an indication of whether high level protein secretion altered the robustness of the recombinant cells to mechanical stresses.

8.4: Possible Mechanisms for the Effect of Temperature on α-Amylase Production

As discussed in Section 6.2.2 very few studies have examined the effect of temperature on streptomycete systems and no example of increased expression of a plasmid borne gene at raised temperature has been described for the genus. This means much of the evidence for possible mechanisms behind the effect observed in this study must be taken from other systems. A large body of evidence suggests that many responses to raised temperature are conserved throughout nature (Chaloupka, 1985; Panaretou, 1993). The seemingly universal nature of these responses means that drawing evidence from other systems as to the mechanism of temperature influence on α-amylase production is valid.

8.4.1: Effects on transcription.

Regulation of protein synthesis in prokaryotes takes place predominantly at the level of transcription (Votruba *et al.*, 1991). In order for transcription initiation to occur, RNA polymerase must interact with the promoter region of a gene to form an open complex (Ueshima *et al.*, 1989). The early stages of this process seem to involve the recognition of the -35 and -10 regions followed by the wrapping of DNA around the back of the RNA polymerase where favourable DNA-protein interactions may occur (Perez-Martin

& Espinosa, 1994). This stage in α-amylase expression could be affected by temperature in numerous ways.

The heat shock response of *E. coli* is mediated by the production of the RNA polymerase sigma (σ) factor, σ^{32}, which recognises heat shock gene promoter regions and leads to their transcription (Ueshima *et al.*, 1989; Straus *et al.*, 1987). During normal growth σ^{32} has a very short half life and exists only at low levels (Tilly *et al.*, 1986). When the cells are subjected to a rapid rise in temperature, levels of σ^{32} increase. This is largely because the stability of the protein is dramatically increased (Straus *et al.*, 1987). Under these conditions, levels of the major σ factor, σ^{70}, also rise (Arnosti *et al.*, 1986). In contrast, heat shock in *B. subtilis* leads to a decrease in levels of the major σ factor, σ^{43} (Arnosti *et al.*, 1986).

Sigma factors are thought to play an important role in temporal and spatial gene expression in *Streptomyces* (Servin-Gonzalez *et al.*, 1994; Westpheling *et al.*, 1985; Buttner, 1989)(Section 1.1.3.1). This makes it extremely likely that synthesis of new σ factors or varying levels of other σ factors would be involved in the response to increased temperature. Such changes in σ factor profiles could lead to significant increases in transcription from the α-amylase gene. Temperature could also affect the synthesis of RNA polymerase subunits (Chaloupka, 1985; Votruba *et al.*, 1991) or alter the half lives of σ factors (Straus *et al.*, 1987) in ways which lead to increased transcription of the α-amylase coding region. The conformation of RNA polymerases can also be altered by temperature (Chaloupka, 1985). This could occur in a manner which made their interaction with the α-amylase promoter region more favourable. The synthesis of auxiliary transcription factors which helped to increase the rate of transcription initiation could also be triggered by raised temperatures.

8.4.2: Effects on repressor and inducer proteins.

Repressor and/or inducer proteins are likely to play an active role in the regulation of α-amylase transcription from the expression vectors (Sections 1.3.1 & 7.3.2). Variations in temperature would be expected to exert some effect on the activity of these proteins. Firstly, as with RNA polymerase subunits, higher temperatures could lead to conformational changes in the proteins. Inducer conformation could be changed in a

way which meant binding could occur more easily or that binding could be tighter (Bader, 1986; Chaloupka, 1985). Either of these effects could lead to increases in α-amylase expression. Converse effects on a repressor protein would also lead to increased α-amylase transcription.

Evidence from the *Bacillus* and *E. coli* systems shows that at superoptimal temperatures the portion of proteins degraded during one cell cycle increases tremendously (Pine, 1973; Strnadova *et al.*, 1986). This is thought to be due to a combination of alterations in protein stability and stimulation of levels of intracellular proteases at higher temperatures (Strnadova *et al.*, 1986; Chaloupka, 1985). As the evidence suggests presence of repressor proteins may well be involved in regulation of α-amylase transcription, inactivation of a repressor protein, by proteases, at higher temperatures would definitely be a conceivable explanation for the high levels of α-amylase production seen at 34 and 36°C. An even simpler explanation would be that expression of repressor proteins was itself repressed by high temperatures. The synthesis of heat shock proteins has been observed in *Streptomyces* (Votruba & Vanek, 1989). It is also possible that one of these heat shock proteins acts as an inducer for α-amylase transcription or interacts with a repressor protein to relieve repression.

8.4.3: Changes in DNA conformation.

Changes in DNA conformation have also been seen with varying temperature (Chaloupka, 1985; Votruba *et al.*, 1991). Such changes can exert large effects on levels of transcription from a particular gene by altering interactions between the DNA, RNA polymerases and other binding proteins, such as repressors or inducers (Chaloupka, 1985; Perez-Martin & Espinosa, 1994). Ueshima *et al.* (1989) found that the effect of supercoiling was markedly different among a range of test promoters. Their results confirmed that some promoters are activated when they are located in supercoiled DNA. One such promoter was the P2 promoter of the *rpoH* gene. This gene codes for σ^{32}, the σ factor which co-ordinates the heat shock response in *E. coli*, which may imply that temperature induced changes in DNA conformation help to switch on the response. The authors postulated that supercoiling activated the promoter by facilitating local DNA unwinding at the promoter region thereby increasing the rate of open complex formation. It has been suggested that the *dagA* promoters of *S.*

coelicolor A3(2) may be located in a chromosomal domain that is subject to regulation by DNA looping or supercoiling (Servin-Gonzalez *et al.*, 1994). More localised DNA bending can also affect the interaction of RNA polymerase subunits with DNA (Perez-Martin & Espinosa, 1994).

Streptomycetes may make use of changes in DNA topology, due to changes in environmental conditions, as an alternative method for transcription regulation. Slight alterations in the DNA conformation of the plasmids could block repressor binding or allow more efficient interactions and faster open complex formation at the α-amylase promoter region. Alternatively, more local effects around the *amy* gene could alter transcription. The α-amylase gene was cloned from the thermophilic streptomycete *S. thermoviolaceus* which grows over a temperature range of 25 to 58°C, with an optimal temperature of 50°C (James & Edwards, 1989). In the natural environment of the wild type organism at 50°C the topology of the α-amylase sequence is likely to be different from that of the *amy* coding region in *S. lividans* at 30°C, purely because of the large difference in temperature. It is therefore possible that at the lower temperature the conformation of the α-amylase promoter and upstream region is simply not favourable for efficient interactions with the transcription machinery. The gene was never designed to be read at low temperature. Incubating the *S. lividans* strains at 34 and 36°C could have altered the DNA conformation enough to allow the interactions to occur.

8.4.4: Effects on plasmid copy number.

Studies on a range of prokaryotic systems have tended to suggest that at above optimal incubation temperatures plasmid copy numbers tend to be reduced (Son *et al.*, 1987; Muth *et al.*, 1989). The reasons for this trend are not well understood. The work by Wrigley-Jones *et al.* (1993) on the effect of raised incubation temperature on *S. lividans* pIJ303 strongly suggested that plasmid copy number profiles in *S. lividans* pQR620, pQR621 and pQR311 would also be less favourable at higher temperatures (Section 6.2.2). The results from this study provide convincing evidence that an increase in plasmid copy number, and copies of the *amy* gene, were not the reason for increased α-amylase production at higher incubation temperatures. However this explanation cannot be discounted, as no copy number profiles were taken during the experiments.

The presence of a section of DNA from the thermophilic streptomycete might somehow improve the copy number of the plasmids at raised temperature leading to a gene dosage effect on α-amylase production.

8.4.5: Effects on translation.

It is possible that temperature also regulates enzyme synthesis at the level of mRNA translation (Votruba *et al.*, 1991). The steady state concentration of functionally active mRNA is important for overall levels of translation (McCarthy, 1991). Temperature can significantly alter the rate of mRNA breakdown (Chaloupka, 1985). Yamamori and Yura (1980) found that the mRNA transcripts of some heat shock proteins were stabilised by an upward temperature shift. Votruba *et al.* (1991) suggested that synthesis of an extracellular protease from *Bacillus megaterium* could be controlled by alterations in mRNA stability at different temperatures.

The mRNA transcript from the α-amylase gene may have become more stable at higher incubation temperatures allowing higher levels of translation to occur. As with the coding sequence's DNA topology, the origin of the gene from the thermophile *S. thermoviolaceus* may mean that the mRNA transcripts take up a more stable structure at higher temperatures (Section 8.4.3). The number of ribosomes present in cells appears to be fairly constant over a large temperature range, although numbers do decrease somewhat when incubation temperatures are significantly above optimal levels (Chaloupka, 1985). If a large fraction of the mRNA present in the cells became destabilised when temperature was increased, but the α-amylase mRNA transcripts remained or became stable, more of the translation machinery could be dedicated to building the α-amylase protein. This could lead to higher levels of α-amylase production. Increased levels of translation have been shown to feedback and raise the stability of mRNA transcripts (McCarthy, 1991). It is postulated that this effect is due to ribosomes protecting the mRNA from ribonuclease attack. Such an effect could act to amplify levels of α-amylase translation achieved from the system.

Messenger RNA structure can be altered by varying temperature (Chaloupka, 1985). This situation, and its possible effects, would be closely analogous to that discussed for changes in DNA topology (Section 8.4.3). The topology of mRNA is known to affect

levels of translation (Anne *et al.*, 1995; Gold, 1988). Inducible *erm* genes are transcribed to yield mRNA that can adopt alternative conformations, therefore determining its availability for translation, i.e. a translational attenuation mechanism (Cundliffe, 1989). It is therefore feasible that a rise in temperature induced a change in the structure of the α-amylase mRNA transcript which was more favourable for translation initiation or which increased the rate of protein translation.

8.4.6: Effects on central metabolism and precursor pools.

A wide range of changes occur in intracellular metabolite pools when temperature is raised or lowered. As the production and activity of intracellular enzymes will be affected by temperature in many different ways the effects of such changes are impossible to define fully (Votruba *et al.*, 1991). Many may be very subtle and unpredictable (Bader, 1986). This complexity is illustrated by the conflicting nature of many results reported in the literature.

It is generally agreed that the levels of internal precursor pools are significantly altered by changes in growth temperature. One reason for this is the much increased level of protein turnover which goes alongside temperature increase and acts to alter levels of amino acids within the cells (Chaloupka & Strnadova, 1982). Changes in tRNA synthetase levels are also seen at raised temperatures; some are increased, others decreased (Chaloupka, 1985). This inevitably leads to alterations in levels of charged tRNA within the cells, a situation which can profoundly affect translation patterns (Gold, 1988).

Another major reason for changes in precursor levels at increased temperature is thought to be linked to changes in cell membrane permeability. There is strong evidence that, as incubation temperature rises, cell cytoplasmic membranes exhibit an increasing permeability to protons. This tends to act to dissipate proton-motive force (Farrand *et al.*, 1983). This means cells must respire more rapidly to survive. In most organisms maintenance energy therefore increases with increasing temperature, leading to decreases in biomass and product yields (Chaloupka, 1985; Votruba & Vanek, 1989). Fluxes through central metabolic pathways therefore seem to be altered to meet the cells immediate needs. In micro-organisms capable of gaining energy by the glycolytic

pathway, as well as by aerobic oxidation, the former system often operates preferentially at higher temperatures (Chaloupka, 1985). Alongside these 'more global' changes, alterations in the activities of individual enzymes can lead to radical shifts in precursor fluxes through competing pathways (Votruba & Vanek, 1989). The dominance of primary metabolic pathways at raised temperatures can mean that synthesis of products derived from these pathways can be more favourable under such conditions (Votruba & Vanek, 1989). However, antibiotic production rates frequently rise with increasing temperature, so the situation is not straightforward (Bader, 1986). It has been suggested that some streptomycete heat shock proteins might participate in turning on or turning off genes associated with secondary metabolism (Votruba & Vanek, 1989).

Cell morphology and differentiation might also be affected by variations in intracellular pools as levels of metabolic regulators could be dramatically altered (Reichl *et al.*, 1992; Bader, 1986; Chaloupka, 1985). As previously discussed, morphological changes could exert a dramatic effect on α-amylase production (Section 5.6.4.2).

The effects discussed above could obviously have increased levels of α-amylase production by the *S. lividans* cells in a whole myriad of ways. Precursor pools could have become more favourable as temperatures of incubation rose. The α-amylase productivities seen in the four 28°C fermentations were very close despite wide variations in other culture parameters, possibly suggesting that production of the enzyme was limited by some factor (Section 5.5.2). Changes in precursor pools could act to relieve such a limitation. Better charged tRNA profiles may have led to increased translation efficiencies. Intracellular protease levels might have been reduced giving increased α-amylase protein stability or increased α-amylase mRNA stability. It could also be envisaged that morphological changes were responsible for the pattern of α-amylase production seen.

8.4.7: Effects on membrane structure and function.

Temperature has been observed to affect the secretion of certain enzymes (Jeenes *et al.*, 1991; Chaloupka, 1985; Yatvin, 1987). Aono (1988) found that a penicillinase from a *Bacillus* species cloned in *E. coli* was secreted more efficiently at higher temperatures.

Such examples of increased secretion at high temperatures are few and far between. In general it seems that protein secretion is favoured at temperatures below those optimal for growth (Jeenes *et al.*, 1991). Changes in the secretion of specific proteins with temperature are postulated to be due to alterations in membrane architecture and constitution (Bader, 1986; Yatvin, 1987; Chaloupka, 1985). In addition to altering the lipid composition of the membrane, temperature may effect the interactions which occur between membrane proteins (Chaloupka, 1985). This could lead to changes in the functioning of the secretory machinery of the *Streptomyces* cells.

Differences in secretion of α-amylase may have some effect on the levels of α-amylase seen in the culture supernatants. However, it would be difficult to believe that increased efficiencies of excretion of the protein could lead to the large increases in α-amylase productivity seen from the cells. Less than 1% of total α-amylase was found to be located intracellularly in the shake flask temperature experiments, suggesting that no bottleneck in secretion was present. Increasing the efficiency of secretion would therefore not be expected to have a noticeable effect on productivity. Such a situation might, however, occur if intracellular proteases were acting to degrade the majority of α-amylase protein before it could be secreted. Reducing the pool of α-amylase waiting to be secreted from the cell, thereby cutting the residency time of each protein within the cell, could act to increase overall peak levels of product in the supernatant.

It is well documented that increases in temperature act to raise the permeability of cell membranes (Votruba & Vanek, 1989; Panaretou, 1993; Panaretou & Piper, 1992). This can lead to an influx of ions from the supernatant and changes in the intracellular environment. Effects of ionic strength on *in vitro* enzyme activities have long been recognised (Votruba & Vanek, 1989). Ionic strength can also affect DNA topology and levels of transcription from certain promoters (Ueshima *et al.*, 1989; Nakanishi *et al.*, 1975). Protease activities could also be repressed or stimulated by such environmental alterations (Veronese *et al.*, 1984; James *et al.*, 1991). Therefore a change in the membrane permeability of the *S. lividans* cells could have a multitude of effects. The net effect of which this be an enhancement in levels of α-amylase produced by the cells.

In particular, studies have examined the possible consequences of an influx of calcium ions into the cells due to altered membrane permeability. Experiments on HeLa, *Drosophila* and yeast cells suggest that changes in levels may mediate the activation of certain second messengers leading to the activation of gene transcription events (Panaretou, 1993). The evidence suggested that such influxes of ions were not directly responsible for the activation of the heat shock response. However, increases in incubation temperature can indirectly lead to a strong stimulation of levels of activity of Ca^{2+} activated enzymes and pathways by allowing the ions into the cells (Piper, 1997).

Calcium ions have been observed to substantially increase the stability of the *S. thermoviolaceus* α-amylase to thermal denaturation (Bahri & Ward, 1990b). This could be acting in the *S. lividans* expression systems to reduce the level of α-amylase inactivated over the length of the experiments thereby increasing the final peak levels achieved. However, Bahri and Ward (1990b) found that at 37°C the enzyme was 100% stable whether or not calcium ions were present. This evidence would tend to suggest that the overall influence of any stabilising effect of Ca^{2+} ions entering the cells would be marginal and would be unlikely to explain the substantial increases in peak α-amylase levels observed. An alternative mechanism, whereby increased intracellular Ca^{2+} levels could raise α-amylase levels, would be that calcium protected the α-amylase from proteolytic attack. Protein bound ligands, such as metal ions, often drastically reduce the rate of proteolysis of a given protein (Veronese *et al.*, 1984). The observed effects of increasing $CaCl_2$ level in the Plackett-Burman analyses would provide strong evidence that calcium could be playing such a role (Section 7.5.6).

8.4.8: Direct effects on the α-amylase protein.

Other direct effects on the α-amylase protein could be envisaged to be responsible for the increased production at higher temperatures. Bahri and Ward (1990b) observed a 12% loss of α-amylase activity when the enzyme was incubated at 4°C for an hour. This trend was reproducible whether or not Ca^{2+} ions were present. Since the enzyme was 100% stable under the same conditions at 37°C these results indicate that low temperature alone can have an effect on α-amylase stability. Incubating the enzyme at 28 or 30°C would not be expected to have quite the same effect as incubating the

enzyme at 4°C, but some losses in α-amylase levels could occur over the course of a long experiment. However, the results of the 24°C and 28°C fermentations tend to suggest that if such an effect is present its affect is negligible, as approximately the same levels of α-amylase accumulated in each culture (Fig. 6.9). Further experiments would need to be done to assess the stability of the α-amylase between 20 and 40°C before any conclusions could be drawn about the importance of stability at different temperatures.

As previously discussed some *Streptomyces* species produce α-amylase inhibitor proteins (Section 5.4.2). If such molecules were produced by *S. lividans* at optimal and low growth temperatures, but not at above optimal growth temperatures, the pattern of α-amylase accumulation seen in these experiments might be obtained. Alternatively, an α-amylase inhibitor could be produced over the whole range of growth temperatures but might be denatured, or rendered more susceptible to proteolytic attack, at higher temperatures. Again further experimentation would be needed to investigate such an effect.

8.4.9: Future work to investigate the temperature effect.

The very wide range of effects which could play a role in raising α-amylase production from the cells at higher temperatures suggest that a great deal of work would be required to unravel the reason for the phenomenon. Several different effects could be acting together to prompt the response seen from the cells.

Experiments to analyse levels of transcription from the *amy* promoter region could indicate whether regulation at the level of transcription was involved in changing levels of α-amylase production with temperature. This could be done using S1 nuclease analysis and Northern blotting. Such experiments would also indicate whether a different start site was used. If levels of transcription were found to differ significantly between two temperatures further studies could be done to pinpoint the mechanism of the effect. If levels of transcription were not found to rise at increased incubation temperatures experiments could be conducted to see of the effect was mediated at the translational level. Amounts of α-amylase mRNA transcript could be monitored at the

different temperatures using Northern blotting. Pulse chase experiments could be conducted following the addition of rifampicin to see if the half life of the mRNA species was increased at higher temperatures (McCarthy, 1991).

Mutagenesis of possible upstream operator regions or the introduction of extra copies of these regions on another plasmid could indicate whether repressor or inducer proteins were involved. Agents which alter DNA topology could be introduced to examine whether changes in DNA conformation were playing a role. One such agent is dimethylsulphoxide (DMSO)(Nakanishi *et al.*, 1975). The observed effects of this solvent on the cells in the Plackett-Burman experiments may provide evidence that such effects are important (Section 8.2). Attempts could be made to isolate the sigma factor(s) responsible for transcription from the *amy* promoter region at higher temperatures. Measuring the plasmid copy number of pQR620 at raised temperature, to see if an increase in α-amylase gene dosage could be the explanation, would be a good starting point for these experiments.

A wide range of experimental methods could be used to assess the effects of some of the other possible factors involved in the system. Incubating α-amylase containing supernatant at different temperatures in the 20 to 40°C range, over an extended time period in the presence of protease inhibitors, might indicate whether the α-amylase was significantly more stable at 36°C than at 28 to 30°C. Adding purified α-amylase into wild type *S. lividans* supernatants grown at 30 and 34°C might allow the detection of α-amylase inhibitor or protease production by the cells at lower temperatures. Levels of intracellular proteolysis of the α-amylase could be examined by looking for breakdown products of the enzyme within cells using western blotting and antibodies against the protein. This could also be done by incubating the α-amylase with cell extracts from cultures grown at 30 and 34°C to see if inactivation occurred. Experiments could also be done to see if Ca^{2+} ions protected the enzyme from proteolytic attack. Membrane stretching agents such as chlorpromazine (Piper, 1997) could be used on 30°C grown cells to see if effects of ion influx or changes in secretion were involved. Alterations in cell morphology could be investigated using image analysis studies.

8.5: Comparison of the *E. coli* and *Streptomyces lividans* Host Systems for Production of the Thermostable α-Amylase

The choice of host organism is a critical factor in determining the complexity and ease of the processing operations required to purify a recombinant protein. Alongside overall yield of protein, these considerations are important when choosing an expression system. Previous work on expression of the *S. thermoviolaceus* α-amylase from *E. coli* and *S. lividans* suggested that *E. coli* would be the host system of choice for the production of this protein (French, 1993; Pierce, 1996)(Section 3.2). These studies indicated that levels of α-amylase production from *S. lividans* would have to be raised significantly before it would be worth considering the streptomycete system as an alternative to *E. coli* periplasmic expression. Over the course of this study, the construction of new *S. lividans* expression vectors, alongside media learnings, have allowed the level of α-amylase to be significantly raised. This alteration in overall productivity of the *S. lividans* system may act to make the streptomycete system a viable option. This section therefore compares and contrasts the two α-amylase expression systems to identify whether the increase in enzyme yields achieved would push the balance towards the *S. lividans* system being that of choice. Figure 8.1 shows a comparison of the processing operations which would most likely be used to purify the α-amylase from each host system. Table 8.1 compares critical parameters of the process streams for each host system prior to the initial cell harvesting and final purification stages of downstream processing.

8.5.1: Host and vector characteristics.

At the current time, *E. coli* is undoubtedly the best understood host system for high level expression of recombinant proteins. The range of expression vectors available for this bacterium far outstrips those available for production of cloned genes in other host organisms. These plasmids are widely available. They are easy and fast to construct and have a history of giving successful results with a range of proteins. Production of a streptomycete expression system is still not straightforward despite the advances in understanding which have been made over the last decade. The use of *E. coli/Streptomyces* shuttle vectors allow the construction of vectors to be speeded up considerably. However, the genetic manipulation and clone selection procedures for

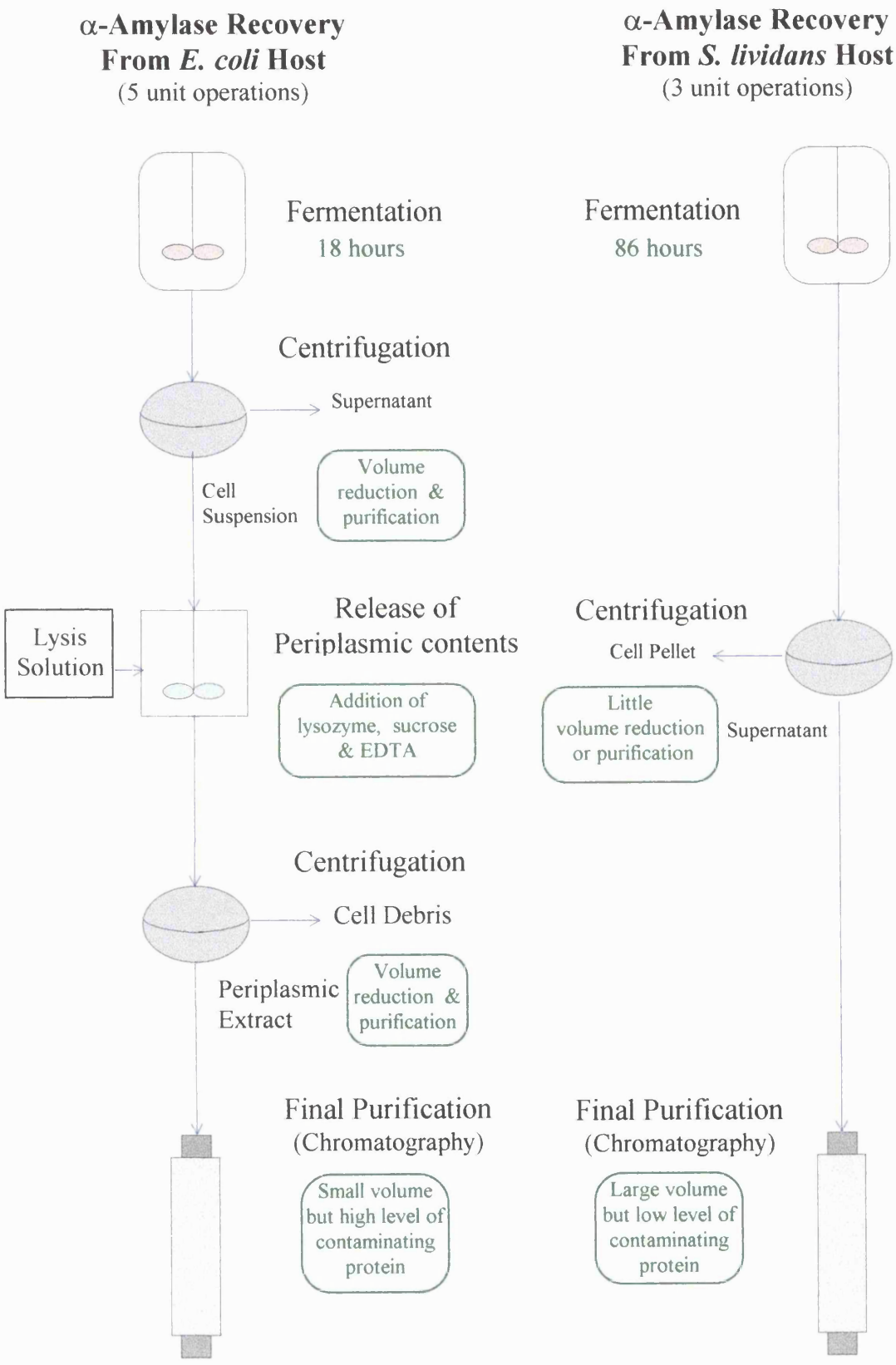


Figure 8.1: Comparison of the processes used to recover the recombinant α-amylase from the periplasm of *E. coli* cells and the supernatant of *S. lividans* cell cultures.

Table 8.1: Comparison of the characteristics of fermentation broths and process feed streams (before final purification/chromatography stage) for *E. coli* pQR126 and *S. lividans* pQR620. Data shown is for a typical *E. coli* pQR126 fermentation (Pierce, 1996) and for the 34°C *S. lividans* pQR620 fermentation described in Chapter 6 (Figs. 6.5 to 6.9).

Parameter of System	***E. coli* pQR126** (for 1 litre broth)	***S. lividans* pQR620** (for 1 litre broth)
Fermentation time (h)	18	86
Fermentations per week (7 days)	9.33	1.95
Broth Characteristics		
Final Biomass (g DCW)	4.05	1.80
Total α-amylase (U)	16780	44390
Location of α-amylase (U) - Extracellular - Periplasmic - Cell associated	 4120 (24.5%) 5120 (30.5%) 7540 (45.0%)	 44390 (100%) - -
Total α-amylase productivity (U/g DCW)	4143	24661
Available α-amylase* (U)	5120 (30.5%)	44390 (100%)
Total protein (mg)	9600	406
α-Amylase specific activity (U/mg)	1.75	109
Characteristics of Feed stream to Final Purification Stage		
Number of unit operations used	5	3
Feed stream volume (ml)	160	970
% of initial broth volume	16 %	97 %
Total α-Amylase (U)	3523	43945
% Recoverable α-amylase* lost in chain of unit operations	31 %	1 %
Total Protein (mg)	142	134
α-Amylase specific activity (U/mg)	24.8	331
Fold purification from initial broth	14	3
Estimated Final Yield [#] / l broth	2818	35156
Estimated yield per week[❖]	26292	68554

[#] Assuming 80% recovery of α-amylase from feed stream in final purification stage.
[❖] Based on the processing of 9330ml *E. coli* broth & 1950ml *S. lividans* broth (i.e. assuming fermentation time to be the limiting factor for production).

streptomycete plasmids are still being developed and optimised. Such factors mean that use of *S. lividans* vectors, such as pQR620, for commercial production of recombinant proteins is still rare.

The *E. coli* vectors include many with genetic elements which allow expression under certain conditions, e.g. high temperature run away replication vectors, or in the presence of certain molecules, e.g. IPTG. Such close control of expression can be invaluable when expressing proteins which may be toxic to the cells or have dramatic effects on central metabolism. Although understanding of streptomycete genetic control is advancing rapidly it is unlikely that similar vector systems will be available for the system for some time to come. This factor may discourage the use of *S. lividans* as a host for expression of proteins which are considered likely to have negative effects on the cells. Such considerations may be influential, and could act to dissuade researchers from trying out the host system.

As outlined above, the *E. coli* host system currently provides many advantages over the streptomycete host system, with regard to ease of genetic manipulation and the level of control available over gene expression. This is likely to change with time. Researchers may be more willing to try out the system as the range of vectors available for *S. lividans* grows, and the number of proteins successfully expressed from the system increases. If it is found to deliver comparable yields of proteins to the more familiar *E. coli* system it could eventually be seen as a viable alternative. To a large extent the success of the streptomycete system, and its future use, will depend on it giving advantages over traditional host systems in fermentation and downstream processing. Advantages here will drive use of the system for commercial protein production.

8.5.2: Fermentation.

The fermentation processes used for the two α-amylase expression systems are very different both in execution and final broth characteristics. *E. coli* grows extremely rapidly relative to *S. lividans*. This fact is reflected in the fermentation times for the systems. The *E. coli* pQR126 system has reached peak α-amylase levels, and is ready for harvest, after 18 hours of growth. In contrast, the *S. lividans* pQR620 system takes 86 hours to reach this point. This has obvious consequences for a production process,

as around 5 *E. coli* fermentations could be conducted in the time taken for a single *S. lividans* fermentation to reach the point of harvest (Table 8.1). On a simplified level, this means that the yield of α-amylase that could be obtained from the *S. lividans* fermentation would need to be over 5-fold higher to justify its use.

Table 8.1 shows the characteristics of 1 litre of *E. coli* and *S. lividans* fermentation broth prior to further processing (cell harvest/separation). A comparison of the total levels of α-amylase in each volume of broth indicate that the *S. lividans* broth contains 2.6 times more than that contained in the *E. coli* broth. However, only the α-amylase located in the periplasm of the *E. coli* cells is available for recovery. Any enzyme located outside of the cells (24.5% of total α-amylase), or in the *E. coli* cytoplasm (45% of total α-amylase) will not be recovered in the downstream processing operations. This contrasts greatly with the *S. lividans* system where all of the α-amylase produced by the cells can theoretically be recovered. This effectively lowers the yield of the *E. coli* fermentation to less than an eighth of that of the streptomycete expression system, i.e. α-amylase available for recovery is 5000 U for 1l of *E. coli* pQR126 broth and 44000 U for 1l of *S. lividans* pQR620 broth. Therefore, on the basis of final α-amylase available for recovery, it would be more economical to conduct a single *S. lividans* pQR620 fermentation than it would be to conduct 5 *E. coli* pQR126 fermentations.

Although the total level of product available for recovery is obviously key, other factors associated with the fermentation process could also play a role in determining which type of fermentation was most suitable for production of a recombinant protein. Large scale fermentation of *Streptomyces* is not as straightforward as large scale fermentation of *E. coli*. The slow growing nature of *S. lividans* means that there is a proportionally greater chance of broth contamination than with the fast growing *E. coli* cells, where it is unlikely contamination would have time to become established. This is magnified by the lengthy inoculum production chain needed for the *S. lividans* cultures. Fermentation reliability will therefore be expected to be greater for the *E. coli* system. A great deal more is invested into a single *S. lividans* pQR620 fermentation than in an *E. coli* pQR126 fermentation, so losses through contamination would need to be taken into consideration when deciding whether use of the Streptomycete system would be viable.

The cost of growth media and any other additives required for high level expression must also be taken into account when choosing an expression system. *E. coli* pQR126 is typically grown in a complex medium such as Terrific broth (Pierce, 1996; French, 1993). The media investigation work described in this thesis strongly suggests that use of a defined medium, such as MMS, is a prerequisite for high level expression of α-amylase by *S. lividans* (Section 8.2). Use of a defined medium will inevitably cost more per fermentation than use of a complex medium. The type of product being produced by an expression system would typically dictate whether such an investment is worthwhile. In a commercial situation, the cost of MMS medium might be decisive in determining that the *S. lividans* host system was not cost effective for production of α-amylase. If the protein being expressed was a high value pharmaceutical this would be less likely to be an issue. In such a situation use of a defined medium could actually be even more advantageous, as it would reduce the level of contaminating protein which needed to be removed from the product in later purification operations and allow a greater understanding of the separation problem to be tackled. Antibiotic addition is usually a necessity in *E. coli* host systems to ensure high level gene expression and plasmid maintenance. This again adds cost to the production process and acts to introduce an additional contaminant to the system which must eventually be removed. The results of this and other studies strongly indicate that the addition of thiostrepton is not necessary to obtain good performance from an *S. lividans* expression system. This can be seen as an advantage of the streptomycete system.

Degradation of the α-amylase by extracellular proteases did not present a problem in the *S. lividans* pQR620 system (Section 8.2). This could, however, have been a significant problem if another protein had been chosen for expression. The digestion of proteins by streptomycete proteases could be a barrier to the successful production of many proteins in *S. lividans*. As a greater number of recombinant genes are expressed in the streptomycete system it will be easier to assess the significance of this factor. If a large proportion of the proteins produced are found to be substantially degraded during growth it would dissuade people from choosing to use *S. lividans* as a host. Periplasmic expression of a protein within *E. coli* can help to reduce levels of proteolysis by limiting the number of host cell proteases to which a product is exposed.

It does not, however, remove the threat of degradation. Many proteins may be sensitive to the *E. coli* periplasmic proteases but not the *S. lividans* extracellular proteases, and vice versa. The sensitivity of a particular protein may, again, be a strong reason for the selection of one of the host systems over the other.

8.5.3: Initial downstream processing operations.

The *S. lividans* α-amylase expression system provides obvious advantages over the *E. coli* system from a downstream processing standpoint. Three unit operations are required to prepare the *E. coli* broth for the final purification steps, versus a single operation for the *S. lividans* broth (Fig. 8.1). Generally, the more steps involved in a process the greater the losses of product through wastage. This generalisation holds true for these systems. Over 30% of the α-amylase which was available for recovery from the *E. coli* broth had been lost from the process feed stream prior to the chromatography unit operations (i.e. mainly lost as enzyme held up and removed within cell debris). In contrast, only a very low percentage of the recoverable α-amylase was lost from the *S. lividans* feed stream (i.e. supernatant lost along with the cell pellet in centrifugation). Fewer unit operations also tends to mean that the capital cost and operating costs of a processing operation are lower, with fewer pieces of equipment required and fewer operators needed to manage the process.

Although the *S. lividans* expression system allows the number of unit operations needed for protein recovery to be minimised, a larger volume must be handled. In the processing operation detailed here the volume is effectively the same as that of the initial fermentation broth. Retention of the α-amylase within the *E. coli* cells allows the processing volume to be significantly reduced at the cell harvest stage. This addresses some of the impact of a larger number of processing steps by reducing operating costs and allowing smaller pieces of equipment to be utilised.

Centrifugation was used for cell harvest for both of the systems in this comparison. On a larger scale, disc stack centrifugation would undoubtedly be the method of choice for *E. coli* cell separation, as it would provide an efficient and rapid harvest. The characteristics of the *S. lividans* broth allow several different unit operations to be considered. The *S. lividans* pQR620 cell pellets tended to be large, ranging up to 1mm

in size. Due to this large size, several unit operations could be used to give efficient recovery of the cells. These include filtration techniques such as microfiltration or depth filtration and steps such as basket centrifugation. Settling could also be considered as a cheap and reliable alternative. Such low shear operations would fulfil the need to maintain the integrity of the cells, therefore minimising contamination of the supernatant with intracellular proteins, whilst at the same time giving efficient separation.

The need for periplasmic lysis is a severe disadvantage for the *E. coli* α-amylase expression system. Although retention of the α-amylase within the periplasm allows a reduction of process volume, the steps involved have implications for the overall process. Lysozyme (20mg/gDCW), sucrose (15% w/v) and EDTA (1mM) are added to the cell suspension to bring about lysis of the outer cell membranes and release the contents of the periplasm (Pierce *et al.*, 1997). Use of these components effectively adds to the overall level of contaminating molecules which have to be separated from the α-amylase later on in the process. The addition of sucrose leads to an increase in viscosity of the periplasmic extract which will act to reduce the efficiency of the centrifugation step which follows cell lysis, leading to increased wastage of α-amylase. Use of these components would also constitute a substantial ongoing cost on a large scale.

8.5.4: Protein loading of the two process streams.

The level of protein contained in each feed stream just prior to the final purification stages of the recovery process will be key to the final overall quality of the α-amylase obtained from each of the two systems. The total protein loading of the process stream was consistently far lower for the *S. lividans* system than for the *E. coli* system. The level of total protein contained in the *E. coli* broth was significantly higher than that in the *S. lividans* broth (Table 8.1). However, the strategy of retaining the α-amylase in the periplasm of the cells allowed the removal of the vast majority of the protein contamination at the two centrifugation unit operations (Fig. 8.1). This meant that the specific activity of the α-amylase in the *E. coli* feed stream was 14 times that of the enzyme's specific activity in the fermentation broth. A more modest 3-fold purification was seen in the *S. lividans* feed stream as the cells were removed away from the α-

amylase containing supernatant. Despite the high level of purification achieved in the centrifugation unit operations for the *E. coli* feed stream, the specific activity of the α-amylase produced by the *S. lividans* system was over 13 times that of the α-amylase produced by the *E. coli* system just prior to final purification steps. This means that the streptomycete system provides a much simpler purification challenge for chromatography unit operations. Significantly less column fouling will occur as there is a lower overall level of background protein present.

8.5.5: Final purification steps.

Ion exchange chromatography can be used to recover the α-amylase from the two process streams (Pierce, 1996). However, the conditions for optimal recovery of the α-amylase from each of the feed streams will be different due to the differing constitutions of the contaminating protein which is present. Further research is needed to determine the optimum conditions for recovery of the α-amylase from each system as little work has been done on this part of the process at the current time. In order to allow a comparison of the overall recovery process it has been assumed that conditions can be identified which allow an 80% recovery of the α-amylase from each chromatography feed stream.

8.5.6: Estimated process yields for the *E. coli* and *S. lividans* α-amylase host systems.

Table 8.1 shows the estimated α-amylase yields per litre of broth and per week for each of the two α-amylase expression systems. Over 12-fold more α-amylase can be recovered from a litre of *S. lividans* pQR620 broth than can be recovered from a litre of *E. coli* pQR126 broth. However, five times as many *E. coli* fermentations could be conducted per unit time. Therefore, 2.6-fold more α-amylase could be recovered per week from the *S. lividans* system than could be recovered from the *E. coli* system.

8.5.7: Future potential of the streptomycete expression system.

This comparison indicates that the *S. lividans* expression system can compete with that of the more widely used *E. coli* system for expression of a recombinant protein. On the basis of the above discussion *S. lividans* would now be considered as the host organism

of choice for the production of the α-amylase. Disadvantages which currently exist in the earlier stages of system development, i.e. complexity of expression vector construction, were more than overcome by the advantages conferred by the system throughout the downstream processing operations. Future developments, such as the ability to recover a larger proportion of the α-amylase from the *E. coli* broth, would necessitate a reassessment of the choice of host system for α-amylase production.

The work described in this study has reaffirmed how much is yet to be learnt about the metabolism and genetic system of *S. lividans*. It has also underlined the potential of the streptomycetes as host organisms for high level recombinant protein production. Only over the next decade, as a greater understanding of these organisms is gained and our ability to manipulate them increases, will the true potential of the genus *Streptomyces* as producers of natural and recombinant products be revealed.

Appendix I

Calculation of the Specific Deactivation Rate Constant (Kd) of Tyrosinase at 30 and 34°C

These calculations were based on those of Gardner and Cadman (1990). The deactivation rate of an enzyme has a dependence on temperature given by;

$$K_d = \left(\frac{KT}{h}\right) \exp^{\left(\frac{\Delta S}{R}\right)} \exp^{-\left(\frac{E_A}{RT}\right)} \qquad (1)$$

Where K= Boltzmann's constant (1.380 x 10^{-20} kJ/K)

h = Plank's constant (2.385 x 10^{-27} kJh)

R = Gas constant (8.314 kJ/kgmolK)

T = Temperature in Kelvin (i.e. °C + 273.15)

E_A = Activation energy of the inactivation reaction (kJ/kgmol)

Δ_S = Entropy change of the inactivation reaction (kJ/kgmol)

E_A and Δ_S change with the medium used. Gardner and Cadman (1990) used a complex and a minimal medium in their calculations. As the values for MMS medium were not known the average values from the study of Gardner and Cadman (1990) were used as they should give an approximation of the true value. Therefore, it was assumed that Δ_S = 95 kJ/kgmol and that E_A/R = 10700 K.

Substituting these values into equation (1) for 30°C (303.15 K):

$$K_d = \left(\frac{(1.380 \times 10^{-20} \times 303.15)}{2.385 \times 10^{-27}}\right) \exp^{\left(\frac{95}{8.314}\right)} \exp^{\left(-\frac{10700}{303.15}\right)}$$

$$K_d = (1.758 \times 10^{9}) \exp^{(11.43)} \exp^{(-35.296)}$$

$$K_d = (1.758 \times 10^{9})(9.204 \times 10^{4})(4.689 \times 10^{-16})$$

K_d30°C = 0.076

The K_d for 34°C was calculated in the same way using T = 307.15 K.

K_d34°C = 0.120

The estimated true productivity and specific productivity were then calculated for each period of the time course using the following equations:

$$R_{est} = \left(\frac{(E_i - E_{i-1})}{t_i - t_{i-1}} \right) + K_d \left(\frac{E_i + E_{i-1}}{2} \right) \quad (2)$$

Where R_{est} = estimated true productivity

E_i = tyrosinase activity at time t_i

E_{i-1} = the tyrosinase activity at the previous sample time t_{i-1}

$$q_{est} = \frac{R_{est}}{X} \quad (3)$$

Where q_{est} = specific productivity

X = dry cell weight

These figures were combined to give the estimated total accumulation of tyrosinase units for each culture.

Appendix II

Sample Calculation from Plackett-Burman Study 1: Determination of Variables which Significantly Affected Maximum Specific Growth Rate.

Following the completion of the 8 experiments which made up study 1, the data obtained was analysed. This allowed the maximum specific growth rate (μ_{max}) to be calculated for each flask. The values for each experiment were as follows:

Flask	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
μ_{max}	0.0297	0.0341	0.0234	0.0207	0.0243	0.0278	0.0313	0.0184

Equation 1, described in Section 2.6.12.1, was then used to calculate the effect of each variable (E_x) on μ_{max}. For example the effect of variable A (E_A) on μ_{max} was calculated as follows:

$$E_A = \frac{\mu max\ flask\ 1 + \mu max\ flask\ 4 + \mu max\ flask\ 6 + \mu max\ flask\ 7}{4} - \frac{\mu max\ flask\ 2 + \mu max\ flask\ 3 + \mu max\ flask\ 5 + \mu max\ flask\ 8}{4}$$

Variable	**a** Σ of μ_{max} values from high level (+) flasks	**b** Σ of μ_{max} values from low level (-) flasks	**Effect variable on μ_{max} (E_x)** $E_x = \frac{(a-b)}{4}$
A Casamino acids	0.1095	0.1002	0.00233
B succinate	0.1194	0.0903	0.00728
C minor elements	0.1150	0.0947	0.00508
D $(NH_4)_2SO_4$	0.1095	0.1002	0.00233
E $MgSO_4$	0.0981	0.1116	-0.00338
F thiostrepton	0.1069	0.1028	0.00103
G dummy	0.1068	0.1029	0.00097

The variance of each effect (V_{eff}) was then calculated from the effect of the dummy variable (E_G). As only a single dummy variable had been used in this experiment this value equalled the square of E_G.

$$V_{eff} = (0.00097)^2 = 9.41 \times 10^{-7}$$

The standard error of each effect (SE_{eff}) equalled the square root of V_{eff}.

$$SE_{eff} = \sqrt{9.41x10^{-7}} = 0.00097$$

Once this value had been determined the t value for each effect was calculated (see below). A table of the Student t distribution was consulted to allow the significance level of each variable's effect to be determined. An effect was only considered to be significant if its confidence level was found to be 80% or above, indicating that the effect was highly unlikely to have been due to chance.

Variable	**Effect variable on μ_{max}** **(E_x)**	**Student t value** $t_x = \frac{E_x}{SE_{eff}}$	**Level of Significance %** (from tables of Student t distribution)
A Casamino acids	0.00233	2.389	70
B succinate	0.00728	7.467	**90**
C minor elements	0.00508	5.210	**80**
D $(NH_4)_2SO_4$	0.00233	2.389	70
E $MgSO_4$	*-0.00338*	*-3.467*	***80***
F thiostrepton	0.00103	1.056	50

Black = positive effect, *Blue* = negative effect

This analysis therefore showed succinate and minor elements both significantly affected μ_{max} in Plackett-Burman study 1. In each case increasing levels of the variable led to increased μ_{max}. Conversely, increasing the level of $MgSO_4$ was found to have a significant negative effect on μ_{max}. The effects of Casamino acids, $(NH_4)_2SO_4$ and thiostrepton were not high enough to be considered significant.

References

Aharonowiz, Y., Demain, A.L. (1979). Nitrogen nutrition and regulation of cephalosporin production in *Streptomyces clavuligerous*. Can. J. Micro. 25, 61-67.

Ahmed, Z.U., Shapiro, S., Vining, L.C. (1984). Excretion of α-keto acids by strains of *Streptomyces venezuelae*. Can. J. Micro. 30, 1014-1021.

Ames, G.F., Prody, C., Kustu, S. (1984). Simple, rapid and quantitative release of periplasmic proteins by chloroform. J. Bact. 160(3), 1181-1183.

Angell, S., Schwarz, E., Bibb, M.J. (1992). The glucose kinase gene of *Streptomyces coelicolor* A3(2): its nucleotide sequence, transcriptional analysis and role in glucose repression. Mol. Micro. 6(19), 2833-2844.

Anne, J., Van Mellaert, L., Cornette, F., Brau, B., Eyssen, H. (1991). Secretion of murine interferon-α by *Streptomyces* using the α-amylase signal peptide. Abstr. P1-001, Eighth International Symposium on Actinomycete Biology, ISBA, Madison, WI, USA.

Anne, J., Van Mellaert, L., Lammertyn, E., Joris, B., Sablon, E., Van Broekhoven, A., Eyssen, H. (1995). Factors influencing secretion of heterologous proteins in *Streptomyces lividans* using mouse tumour necrosis factor α as a model protein. Biotechnologiya, 7-8, Proceedings of the Ninth Symposium on the Actinomycetes, 55-60.

Aono, R. (1988). Cultivation conditions for extracellular production of penicillinase by *Escherichia coli* carrying pEAP31 on a semi-large scale. Appl. Micro. Biotech. 28, 414-418.

Aphale, J.S., Strohl, W.R. (1993). Purification and properties of an extracellular aminopeptidase from *Streptomyces lividans* 1326. J. Gen. Micro. 139, 417-424.

Aretz, W., Koller, K.P., Reiss, G. (1989). Proteolytic enzymes from recombinant *Streptomyces lividans* TK24. FEMS Micro. Letters 65, 31-36.

Arnosti, D.N., Singer, V.L., Chamberlin, M.J. (1986). Characterisation of heat shock in *Bacillus subtilis*. J. Bact. 168(3), 1243-1249.

Ates, S., Elibol, M., Mavituna, F. (1997). Production of actinorhodin by *Streptomyces coelicolor* in batch and fed-batch cultures. Process Biochem. 32(4), 273-278.

Bader, F.G. (1986). Physiology and Fermentation Development, 281-321: The Bacteria, Vol. 9, Antibiotic Producing Streptomyces (Queener, S.W & Day, L.E., eds.), Academic Press, New York.

Bahri, S.M. (1990). Cloning and expression of a thermostable α-amylase from *Streptomyces thermoviolaceus* CUB74. Ph.D. Thesis, University College London.

Bahri, S.M., Ward, J.M. (1990a). Regulation of a thermostable α-amylase of *Streptomyces thermoviolaceus* CUB74: maltotriose is the smallest inducer. Biochimie 72, 893-895.

Bahri, S.M., Ward, J.M. (1990b). Cloning and expression of an α-amylase gene from *Streptomyces thermoviolaceus* CUB74 in *Escherichia coli* JM107 and *Streptomyces lividans* TK24. J. Gen. Micro. 136, 811-818.

Bahri, S.M., Ward, J.M. (1993). Sequence of the *Streptomyces thermoviolaceus* CUB74 α-amylase encoding gene and its transcription analysis in *Streptomyces lividans*. Gene 127, 133-137.

Baltz, R.H. (1998). Genetic manipulation of antibiotic producing *Streptomyces*. Trends Micro. 6 (2), 76-82.

Bascaran, V., Hardisson, C., Brana, A.F. (1990). Regulation of extracellular protease production in *Streptomyces clavuligerus*. Appl. Micro. Biotech. 34, 208-213.

Baum, E.Z., Buttner, M.J., Lin, L., Rothstein, D.M. (1989). Transcription from the P1 promoters of *Micromonospora echinospora* in the absence of native upstream DNA sequences. J. Bact. 171(12), 6503-6510.

Bechthold, A., Floss, H.G. (1994). Overexpression of the thiostrepton-resistance gene from *Streptomyces azureus* in *Escherichia coli* and characterisation of recognition sites of the 23s rRNA A1067 2'-methyltransferase in the guanosine triphosphatase centre of 23s ribosomal RNA. Eur. J. Biochem. 224, 431-437.

Bender, E., Koller, K.P., Engels, J.W. (1990a). Secretory synthesis of human IL-2 by *Streptomyces lividans*. Gene, 86, 227-232.

Bender, E., Vogel, R., Koller, K.P., Engels, J. (1990b). Synthesis and secretion of hirudin by *Streptomyces lividans*. Appl. Micro. Biotech. 34, 203-207.

Benigni, R., Antonov, R.P., Carere, A. (1975). Estimation of the genome size by renaturation studies in *Streptomyces*. Appl. Micro. 30, 324-326.

Beppu, T. (1995). Signal transduction and secondary metabolism: prospects for controlling productivity. TIBTECH 13, 264-269.

Bibb, M.J., Janssen, G.R., Ward, J.M. (1985a). Cloning and analysis of the promoter region of the erythromycin resistance gene (*ermE*) of *Streptomyces erythraeus*. Gene 38, 215-226.

Bibb, M.J., Bibb, M.J., Ward, J.M., Cohen, S.N. (1985b). Nucleotide sequences encoding and promoting expression of three antibiotic resistance genes indigenous to *Streptomyces*. Mol. Gen. Genet. 199, 26-36.

Binnie, C., Cossar, J.D., Stewart, D.I.H. (1997). Heterologous biopharmaceutical protein expression in *Streptomyces*. TIBTECH 15, 315-320.

Birch, A., Hausler, A., Hutter, R. (1990). Genome rearrangement and genetic instability in *Streptomyces* species. J. Bact., 172(8), 4138-4142.

Blanchin-Roland, S., Masson, J.M. (1989). Protein secretion controlled by a synthetic gene in *Escherichia coli*. Protein Eng. 2, 473-480.

Blanco, J., Coque, J., Martin, J. (1996). Characterisation of the *secA* gene of *Streptomyces lividans* encoding a protein translocase which complements an *Escherichia coli* mutant defective in the ATPase activity of SecA. Gene 176, 61-65.

Bourn, W.R., Babb, B. (1995). Computer assisted identification and classification of streptomycete promoters. Nucleic Acids Res. 23(18), 3696-3703.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Bramwell, H., Nimmo, H.G., Hunter, I.S., Coggins, J.R. (1993). Phosphoenolpyruvate carboxylase from *Streptomyces coelicolor* A3(2): purification of the enzyme, cloning of the *ppc* gene and over expression of the protein in a streptomycete. Biochem. J. 293, 131-136.

Brasch, M.A., Cohen, S.N. (1995). Sequences essential for replication of plasmid pIJ101 in *Streptomyces lividans*. Plasmid 33, 191-197.

Braun, S., Vecht-Lifshitz, S.E. (1991). Mycelial morphology and metabolite production. TIBTECH 9, 63-68.

Brawner, M.J., Fornwald, J., Rosenberg, M., Poste, G., Westpheling, J. (1990). Heterologous gene expression in Streptomyces. In Proceedings Vol. I , Sixth International Symposium on Genetics of Industrial Microorganisms. Strasbourg, France.

Bushell, M.E. (1988). Growth, product formation and fermentation technology. Chapter 5, 185-217. In *Actinomyces* in Biotechnology (Goodfellow, M., Williams, S.T., Mordarski, M. eds.). Academic Press.

Bushell, M.E. (1989). The process physiology of secondary metabolite production. In Microbial Products: New Approaches, 44th Symposium of the Society of General Microbiology.

Buttner, M.J., Fearnley, I.M., Bibb, M.J. (1987). The agarase gene (*dagA*) of *Streptomyces coelicolor* A3(2): nucleotide sequence and transcriptional analysis. Mol. Gen. Genet. 209, 101-109.

Buttner, M.J., Smith, A.M., Bibb, M.J. (1988). At least three different RNA polymerase holoenzymes direct transcription of the agarase gene (*dagA*) of *Streptomyces coelicolor* A3(2). Cell 52, 599-607.

Buttner, M.J. (1989). RNA polymerase heterogeneity in *Streptomyces coelicolor* A3(2). Mol. Micro. 3(11), 1653-1659.

Caballero, J.L., Malpartida, F., Hopwood, D.A. (1991). Transcriptional organisation and regulation of an antibiotic export complex in the producing *Streptomyces* culture. Mol. Gen. Genet. 228, 372-380.

Castro, P.M.L., Hayter, P.M., Ison, A.P., Bull, A.T. (1992). Application of a statistical design to the optimisation of culture medium for recombinant interferon-gamma production by chinese hamster ovary cells. Appl. Micro. Biotech. 38, 84-90.

Chaloupka, J., Strnadova, M. (1982). Kinetics of protein turnover in growing cells of *Bacillus megaterium*. J. Gen. Micro. 128, 1003-1008.

Chaloupka, J. (1985). Temperature as a factor regulating the synthesis of microbial enzymes. Micro. Sciences 2(3), 86-90.

Chang, H.M., Chen, M.Y., Shieh, Y.T., Bibb, M.J., Chen, C.W. (1996). The cutRs signal transduction system of *Streptomyces lividans* represses the biosynthesis of the polyketide antibiotic actinorhodin. Mol. Micro. 21(5), 1075-1085.

Chang, S.Y., Chang, S. (1988). Secretion of heterologous proteins in *Streptomyces lividans*. In Biology of Actinomycetes '88 (Okami, Y., Beppu, T., Ogawara, H. eds.). Japan Scientific Societies, Tokyo. 103-107.

Chater, K.F., Bruton, C.J., Plaskitt, K.A., Buttner, M.J., Mendez, C., Helmann, J. (1989). The developmental fate of *S. coelicolor* hyphae depends crucially on a gene product homologous with the motility sigma factor of *B. subtilis*. Cell 59, 133-143.

Chater, K.F. (1990). The improving prospects for yield increase by genetic engineering in antibiotic producing *Streptomyces*. Biotech. 8, 115-121.

Chater, K.F., Bibb, M.J. (1996). Regulation of Bacterial Antibiotic Production. In Products of Secondary Metabolism (Kleinkauf, H., von Dohren, H. eds.). Biotechnology, Vol. 6. VCH, Weinheim, Germany.

Chater, K.F. (1998). Taking a genetic scalpel to the *Streptomyces* colony. Microbiology 144, 1465-1478.

Chatterjee, S., Vining, L.C., Westlake, D.W.S. (1983). Nutritional requirements for chloramphenicol biosynthesis in *Streptomyces venezuelae*. Can. J. Micro. 29, 247-253.

Chen, I., Marcos, A.T., da Costa, S.O.P., Martin, J.F., Padilla, G. (1995). Cloning and characterisation of an α-amylase gene from *Streptomyces* species WL6. Biochem. Mol. Biol. 35(5), 1059-1067.

Cimburkova, E., Zima, J., Novak, J., Vanek, Z. (1988). Nitrogen regulation of avermectin biosynthesis in *Streptomyces avermitilis* in a chemically defined medium. J. Basic. Micro. 28(8), 491-499.

Clark, G.J., Langley, D., Bushell, M.J. (1995). Oxygen limitation can induce microbial secondary metabolite formation: investigations with miniature electrodes in shaker and bioreactor culture. Microbiology 141, 663-669.

Cossins, A., Bowler, A. (1987). Temperature Biology of Animals, Chapman Hall, London, 155-201.

Cundliffe, E. (1989). How antibiotic-producing organisms avoid suicide. Ann. Rev. Micro. 43, 207-233.

Dale, J.W. (ed.)(1989). Chapter 5. Molecular Genetics of Bacteria. John Wiley and Sons, 78-81.

Davis, B., Dulbecco, R., Eisen, H., Ginsberg, H. (1990). Microbiology - 4th Edition. J.B. Lippincott Co., Philadelphia.

Dehottay, P., Dusart, J., Duez, C., Lenzini, M.V., Martial, J.A., Frere, J.M., Ghuysen, J.M., Kieser, T. (1986). Cloning and amplified expression in *Streptomyces lividans* of a gene encoding extracellular β-lactamase from *Streptomyces albus* G. Gene 42, 31-36.

Dekleva, M.L., Strohl, W.R. (1987). Glucose-stimulated acidogenesis by *Streptomyces peucetius*. Can. J. Micro. 33, 1129-1132.

del Solar, G.H., Puyet, A., Espinosa, M. (1987). Initiation signals for the conversion of single stranded to double stranded DNA forms in the streptococcal plasmid pLS1. Nuc. Acid. Res. 15(14), 5561-5580.

DelaCruz, N., Payne, G.F., Smith, J.M., Coppella, S.J. (1992). Bioprocess development to improve foreign protein production from recombinant *Streptomyces*. Biotech. Progress 8, 307-315.

Delic, I., Robbins, P., Westpheling, J. (1992). Direct repeat sequences are implicated in the regulation of two *Streptomyces* chitinase promoters that are subject to carbon catabolite control. Proc. Natl. Acad. Sci. USA 89, 1885-1889.

Demain, A.L. (1988). Actinomycetes: what have you done for us lately? In Biology of Actinomycetes '88 (Okami, Y., Beppu, T., Ogawara, H. eds.) Japan Scientific Societies, Tokyo. 19-25.

Demain, A.L. (1989). Carbon source regulation of idiolite biosynthesis in actinomycetes. In Regulation of Secondary Metabolism in Actinomycetes (Shapiro, S. ed.), CRC Press, Boca Raton. 127-134.

Deng, Z., Kieser, T., Hopwood, D.A. (1988). 'Strong incompatability' between derivatives of the *Streptomyces* multi-copy plasmid pIJ101. Mol. Gen. Genet. 214, 286-294.

Dombroski, A., Walter, W., Record, M.T., Siegele, D., Gross, C. (1992). Polypeptides containing highly conserved regions of transcription initiation factor σ^{70} exhibit specificity of binding to promoter DNA. Cell, 70, 501-512.

Doull, J.L., Vining, L.C. (1990). Physiology of antibiotic production in Actinomycetes and some underlying control mechanisms. Biotech. Adv. 8, 141-158.

Dyson, P.J., Evans, M. (1996). pUCS75, a stable high-copy-number *Streptomyces-Escherichia coli* shuttle vector which facilitates subcloning from pUC plasmid and M13 bacteriophage vectors. Gene 171, 71-73.

Eckhardt, T., Strickler, J., Gorniak, L., Burnett, W.V., Fare, L.R. (1987). Characterisation of the promoter, signal sequence and amino terminus of a secreted β-galactosidase from *Streptomyces lividans*. J. Bact. 169(9), 4249-4256.

Elibol, M., Ulgen, K., Kamaruddin, K., Mavituna, F. (1995). Effect of inoculum type on actinorhodin production by *Streptomyces coelicolor* A3(2). Biotech. Letters 17(6), 579-582.

Engels, J.W., Koller, K.P. (1992). Gene expression and secretion of eukaryotic foreign proteins in *Streptomyces*. In Transgenesis (Murray, J.A. ed.). John Wiley and Sons.

Enquist, L.W., Bradley, S.G. (1971). Characterisation of deoxyribonucleic acid from *Streptomyces venezuelae* spores. Dev. Ind. Micro.12, 225-236.

Erpicum, T., Granier, B., Delcour, M., Lenzini, V.M., Nguyen-Disteche, M., Dusart, J., Frere, J.M. (1990). Enzyme production by genetically engineered *Streptomyces* strains: influence of culture conditions. Biotech. Bioeng. 35, 719-726.

Farrand, S.G., Jones, C.W., Linton, J.D., Stephenson, R.J. (1983). The effect of temperature and pH on the growth efficiency of the thermoacidophilic bacterium *Bacillus acidocaldarius* in continuous culture. Arch. Micro. 135, 276-283.

Fass, S.H., Engels, J.W. (1996). Influence of specific signal peptide mutations on the expression and secretion of the α-amylase inhibitor tendamistat in *Streptomyces lividans*. J. Biol. Chem. 271, 15244-15252.

Fisher, S.H. (1988). Nitrogen assimilation in *Streptomyces*. In Biology of Actinomycetes '88 (Okami, Y., Beppu, T., Ogawara, H. eds.). Japan Scientific Societies, Tokyo. 47-51.

Flickinger, M.C., Greenstein, M., Bremmon, C., Conlin, J. (1990). Strain selection, medium development and scale-up of toyocamycin production by *Streptomyces chrestomyceticus*. Bioprocess. Eng. 5, 143-153.

Flores, M.E., Ponce, E., Rubio, M., Huitron, C. (1993). Glucose and glycerol repression of α-amylase in *Streptomyces kanamyceticus* and isolation of deregulated mutants. Biotech. Letters 15(6), 595-600.

Flores, M.E., Perea, M., Rodriguez, O., Malvaez, A., Huitron, C. (1996). Physiological studies on induction and catabolite repression of β-xylosidase and endoxylanase in *Streptomyces* species CH-M-1035. J. Biotech. 49, 179-187.

Fornwald, J.A., Schmidt, F.J., Adams, C.W., Rosenberg, M., Brawner, M.E. (1987). Two promoters, one inducible and one constitutive, control transcription of the *Streptomyces lividans* galactose operon. Proc. Natl. Acad. Sci. USA 84, 2130-2134.

Fornwald, J.A., Donovan, M.J., Gerber, R., Keller, J., Taylor, D.P., Arcuri, E.J., Brawner, M.E. (1993). Soluble forms of the human T cell receptor CD_4 are efficiently expressed by *Streptomyces lividans*. Biotech. 11, 1031-1036.

French, C. (1993). Production and recovery of a recombinant α-amylase enzyme from *Escherichia coli* and *Streptomyces lividans*. Ph.D. Thesis, University College London.

Freudl, R. (1992). Protein secretion in Gram-positive bacteria. J. Biotech. 23, 231-240.

Fujii, T., Gramajo, H.C., Takano, E., Bibb, M.J. (1996). *redD* and *actII*-ORF4; Pathway-specific regulatory genes for antibiotic production in *Streptomyces coelicolor* A3(2), are transcribed in vitro by an RNA polymerase holoenzyme containing σ^{HrdD}. J. Bact. 178(11), 3402-3405.

Garcia-Dominguez, M., Martin, J., Liras, P. (1989). Characterisation of sugar uptake in wild-type *Streptomyces clavuligerus*, which is impaired in glucose uptake, and in a glucose-utilising mutant. J. Bact. 171, 6808-6814.

Gardner, A.R., Cadman, T.W. (1990). Product deactivation in recombinant *Streptomyces*. Biotech. Bioeng. 36, 243-251.

Gilbert, M., Morosoli, R., Shareck, F., Kluepfel, D. (1995). Production and secretion of proteins by *Streptomyces*. Crit. Rev. Biotech. 15(1), 13-39.

Goel, A., Lee, J., Domach, M.M., Ataai, M.M. (1995). Suppressed acid formation by co-feeding of glucose and citrate in *Bacillus* cultures: emergence of pyruvate kinase as a potential metabolic engineering site. Biotech. Progress 11, 380-385.

Gold, L. (1988). Posttranscriptional regulatory mechanisms in *Escherichia coli*. Ann. Rev. Biochem. 57, 199-233.

Goodfellow, M., Lacey, J., Todd, C. (1987). Numerical classification of thermophilic *Streptomyces*. J. Gen. Micro. 133, 3135-3149.

Gray, G., Selzer, G., Buell, G., Shaw, P., Escanez, S., Hofer, S., Voegeli, P., Thompson, C. (1984). Synthesis of bovine growth hormone by *Streptomyces lividans*. Gene 32, 21-30.

Greasham, R.L. (1993). Media for microbial fermentations. In Biotechnology Vol. 3: Bioprocessing (Stephanopoulos, G. ed.), VCH Publishing, Weinheim. Chapter 7, 127-139.

Gros, M.F., te Riele, H., Ehrlich, S.D. (1987). Rolling circle replication of single-stranded DNA plasmid pC194. EMBO J. 6(12), 3863-3869.

Gruss, A., Ehrlich, S.D. (1989). The family of highly interrelated single-stranded deoxyribonucleic acid plasmids. Micro. Rev. 53(2), 231-241.

Haas-Lauterbach, S., Scharf, M., Sprunkel, B., Neeb, M., Koller, K.P., Engels, J.W. (1993). High yield fermentation and purification of tendamistat disulphide analogues secreted by *Streptomyces lividans*. Appl. Micro. Biotech., 38, 719-727.

Hale, V., McGrew, M., Carlson, B., Schottel, J.L. (1992). Heterologous expression and secretion of a *Streptomyces scabies* esterase in *Streptomyces lividans* and *Escherichia coli*. J. Bact. 174, 2431-2439.

Hale, V.A., Schottel, J.L. (1996). Mutational analysis of the *Streptomyces scabies* esterase signal peptide. Appl. Micro. Biotech. 45, 189-198.

Hintermann, G., Zatchej, M., Hutter, R. (1985). Cloning and expression of the genetically unstable tyrosinase structural gene from *Streptomyces glaucescens*. Mol. Gen. Genet. 200, 422-432.

Hopwood, D.A., Kieser, T., Wright, H.M., Bibb, M.J. (1983). Plasmids, recombination and chromosome mapping in *Streptomyces lividans* 66. J. Gen. Micro. 129, 2257-2269.

Hopwood, D.A., Bibb, M.J., Chater, K.F., Kieser, T., Brunton, C.J., Kieser, H.M., Lydiate, D.J., Smith, C.P., Ward, J.M., Schrempf, H. (1985). Genetic Manipulation of *Streptomyces*: A Laboratory Manual. John Innes Foundation, Norwich.

Horinouchi, S., Hara, O., Beppu, T. (1983). Cloning of a pleiotropic gene that positively controls biosynthesis of A-factor, actinorhodin and prodigiosin in *Streptomyces coelicolor* A3(2) and *Streptomyces lividans*. J. Bact. 155(3), 1238-1248.

Hoshiko, S., Makabe, O., Nojivi, C., Katsumata, K., Satoh, E., Nagaoka, K. (1987). Molecular cloning and characterisation of the *Streptomyces hygroscopicus* α-amylase gene. J. Bact. 109, 1029-1036.

Ilyina, T.V., Koonin, E.V. (1992). Conserved sequence motifs in the initiator proteins for rolling circle DNA replication encoded by diverse replicons from eubacteria, eukaryotes and archaebacteria. Nuc. Acid. Res. 20(13), 3279-3285.

Imanaka, T. (1994). Thermostable enzymes produced by recombinant mesophilic and thermophilic bacteria. In Recombinant Microbes for Industrial and Agricultural Applications (Murooka, Y., Imanaka, T. eds.), Marcel Dekker, Chapter 17.

Inokoshi, J., Takeshima, H., Ikeda, H., Omura, S. (1993). Efficient production of aculaecin A acylase in recombinant *Streptomyces* strains. Appl. Micro. Biol. 39, 532-537.

Izard, J.W., Kendall, D.A. (1994). Signal peptides: exquisitely designed transport promoters. Mol. Micro. 13(5), 765-773.

James, P.D.A., Iqbal, M., Edwards, C., Miller, P.G.G. (1991). Extracellular protease activity in antibiotic-producing Streptomyces thermoviolaceus. Cur. Micro. 22, 377-382.

Janssen, G.R., Bibb, M.J., Smith, C.P., Ward, J.M., Kieser, T., Bibb, M.J. (1985). Isolation and analysis of *Streptomyces* promoters. In Microbiology - 1985 (Leive, L. ed.). American Society for Microbiology, Washington D.C..

Janssen, G.R., Bibb, M.J. (1990). Tandem promoters, tsrp1 and tsrp2, direct transcription of the thiostrepton resistance gene (*tsr*) of *Streptomyces azureus*:

transcriptional initiation from tsrp2 occurs after deletion of the -35 region. Mol. Gen. Genet. 221, 339-346.

Jaurin, B., Cohen, S.N. (1985). *Streptomyces* contain *Escherichia coli*-type A + T-rich promoters having novel structural features. Gene 39, 191-201.

Jeenes, D.J., Mackenzie, D.A., Roberts, I.N., Archer, D.B. (1991). Heterologous protein production by filamentous fungi. Biotech. Gen. Eng. Rev. 9, 327-367.

Kang, J.G., Hahn, M.Y., Ishihama, A., Roe, J.H. (1997). Identification of sigma factors for growth phase-related promoter selectivity of RNA polymerases from *Streptomyces coelicolor* A3(2). Nuc. Acid. Res. 25(13), 2566-2573.

Karandikar, A., Sharples, G.P., Hobbs, G. (1996). Influence of medium composition on sporulation by *Streptomyces coelicolor* A3(2) grown on defined solid media. Biotech. Techniques 10(2), 79-82.

Kataoka, M., Kuno, N., Horiguchi, T., Seki, T., Oshida, T. (1994). Replication of the *Streptomyces* plasmid pSN22 through single-stranded intermediates. Mol. Gen. Genet. 242, 130-136.

Katz, E., Thompson, C.J., Hopwood, D.A. (1983). Cloning and expression of the tyrosinase gene from *Streptomyces antibioticus* in *Streptomyces lividans*. J. Gen. Micro. 129, 2703-2714.

Kendall, K., Cullum, J. (1984). Cloning and expression of an extracellular-agarase gene from *Streptomyces coelicolor* A3(2) in *Streptomyces lividans* 66. Gene 29, 315-321.

Kendall, K.J., Cohen, S.N. (1987). Plasmid transfer in *Streptomyces lividans*: identification of a Kil-Kor system associated with the transfer region of pIJ101. J. Bact. 169(9), 4177-4183.

Kennedy, M.J., Reader, S.L., Davies, R.J., Rhoades, D.A., Silby, H.W. (1994). The scale up of mycelial shake flask fermentations: a case study of gamma linolenic acid production by *Mucor hiemalis* IRL 51. J. Ind. Micro. 13, 212-216.

Kieser, T., Hopwood, D.A., Wright, H.M., Thompson, C.J. (1982). pIJ101, a multi-copy, broad host range *Streptomyces* plasmid: functional analysis and development of DNA cloning vectors. Mol. Gen. Genet. 185, 223-238.

Kieser, T., Deng, Z., Hopwood, D.A. (1988). Strong incompatability and co-integrate formation between derivatives of the *Streptomyces* multi-copy plasmid pIJ101. In Biology of Actinomycetes '88 (Okami, Y., Beppu, T., Ogawara, H. eds.) Japan Scientific Societies, Tokyo. 97-102.

Kluepfel, D., Sharek, F., Mondon, F., Morosoli, R. (1986). Characterisation of cellulase and xylanase activities of *Streptomyces lividans*. Appl. Micro. Biotech. 24, 230-234.

Koller, K.P., Riess, G. (1989). Heterologous expression of the α-amylase inhibitor gene cloned from an amplified genomic sequence of *Streptomyces tendae*. J. Bact. 171(9), 4953-4957.

Koller, K.P., Riess, G., Sauber, K., Uhlmann, E., Wallmeier, H. (1989). Recombinant *Streptomyces lividans* secretes a fusion protein of tendamistat and proinsulin. Biotech. 7, 1055-1059.

Konker, G., Jaeger, K.E. (1987). Specific and sensitive plate assay for bacterial lipases. Appl. Env. Micro. 53 (1), 211-213.

Kourteva, Y., Stefanova, L., Konova, O., Peretz, V., Venkov, C. (1995). Biosynthesis of extracellular low-molecular mass papain and trypsin inhibitors by *Streptomyces* species 22: effect of cultural conditions. Appl. Micro. Biotech. 43, 131-136.

Labes, G., Bibb, M., Wohllebenss, W. (1997). Isolation and characterisation of a strong promoter element from the *Streptomyces ghanaensis* phage I19 using the gentamicin resistance gene (*aacC1*) of Tn1696 as reporter. Microbiology 143, 1503-1512.

Lammertyn, E., Anne, J. (1998). Modification of *Streptomyces* signal peptides and their effects on protein production and secretion. FEMS Micro. Letts. 160, 1-10.

Lammertyn, E., Desmyter, S., Schacht, S., van Mellaert, L., Anne, J. (1998). Influence of charge variation in the *Streptomyces venezuelae* α-amylase signal peptide on heterologous protein production by *Streptomyces lividans*. Appl. Micro. Biotech. 49, 424-430.

Lammertyn, E., van Mellaert, L., Schacht, S., Dillen, C., Sablon, E., van Broekhoven, A., Anne, J. (1997). Evolution of a novel subtilisin inhibitor gene and mutant derivatives for the expression and secretion of mouse tumour necrosis factor α by *Streptomyces lividans*. Appl. Env. Micro. 63(5), 1808-1813.

LaRossa, R.A., Majarian, W.R., Van Dyk, T.K. (1994). A highly sensitive method for detecting environmental insults. International Patent No. WO 9413831 (23.06.94)

Larson, J.L., Hershberger, C.L. (1986). The minimal replicon of a streptomycete plasmid produces an ultrahigh level of plasmid DNA. Plasmid 15, 199-209.

Lee, J.H., Jang, J.D., Lee, K.J. (1992). Segregational instability of a recombinant plasmid pDML6 in *Streptomyces lividans*. J. Micro. Biotech. 2(2), 129-134.

Lee, J.H., Lee, K.J. (1994). Effect of growth rate and cultivation environments on cloned gene stability and the cloned gene product formation in *Streptomyces lividans*. J. Biotech. 33, 195-204.

Lee, Y.H.W., Tzecheng, Z.Y., Wang, S.C., Cheng, W.L., Chen, C.W. (1986). Structural stability of heterologous genes cloned in *Streptomyces* plasmid pIJ702. Biochem. Biophys. Res. Com. 140(1), 372-378.

Lerch, K., Ettlinger, L. (1972). Purification and characterisation of a tyrosinase from *Streptomyces glaucescens*. Eur. J. Biochem. 31, 427-437.

Leskiw, B.K., Bibb, M.J., Chater, K.F. (1991). The use of a rare codon specifically during development. Mol. Micro. 5(12), 2861-2867.

Leskiw, B.K., Mah, R., Lawlor, E.J., Chater, K.F. (1993). Accumulation of *bldA*-specified tRNA is temporally regulated in *Streptomyces coelicolor* A3(2). J. Bact. 15(7), 1995-2005.

Leu, W.M., Wu, S.Y., Lin, J.J, Lo, S.J., Lee, Y.H.W. (1989). Analysis of the promoter region of the melanin locus from *Streptomyces antibioticus*. Gene 84, 267-277.

Lichenstein, H., Brawner, M.E., Miles, L.M., Meyers, C.A., Young, P.R., Simon, P.L., Eckhardt, T. (1988). Secretion of interleukin-1β and *Escherichia coli* galactokinase by *Streptomyces lividans*. J. Bact. 170(9), 3924-3929.

Lonetto, M.A., Brown, K.L., Rudd, K.E., Buttner, M.J. (1994). Analysis of the *Streptomyces coelicolor sigE* gene reveals the existence of a subfamily of eubacterial RNA polymerase σ factors involved in the regulation of extracytoplasmic functions. Proc. Natl. Acad. Sci. USA 91, 7573-7577.

Long, C.M., Virolle, M.J., Chang, S.Y., Chang, S., Bibb, M.J. (1987). α-Amylase gene of *Streptomyces limosus*: nucleotide sequence, expression motifs and amino acid sequence homology to mammalian and invertebrate α-amylases. J. Bact. 169(12), 5745-5754.

Loriaux, A. (1995). Submitted to the EMBL bank (*sec*D of S. coelicolor: Q53955. *sec*F of S. coelicolor: Q53956)

Lounes, A., Lebrihi, A., Benslimane, C., Lefebvre, G., Germain, P. (1995). Influence of nutrient excess on intracellular proteins composition in relation to spiramycin production in *Streptomyces ambofaciens*. Biotech. Letters 17(7), 699-704.

Lounes, A., Lebrihi, A., Benslimane, C., Lefebvre, G., Germain, P. (1996). Regulation of spiramycin synthesis in *Streptomyces ambofaciens*: effects of glucose and inorganic phosphate. Appl. Micro. Biotech. 45, 204-211.

Madden, T., Ward, J.M., Ison, A.P. (1996). Organic acid excretion by *Streptomyces lividans* TK24 during growth on defined carbon and nitrogen sources. Microbiology 142, 3181-3185.

Maniatis, T., Fritsch, E.F., Sambrook, J. (eds.)(1982). Molecular Cloning: A Laboratory Manual. Cold spring Harbour Laboratory, New York.

Marco, S., Parro, V., Carrascosa, J., Mellado, R. (1992). *Streptomyces lividans* possesses a GroEL-like chaperonin. FEMS Micro. Lett. 93, 127-132.

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 in Actinomycetes (Shapiro, S. ed.), CRC Press, Boca Raton.

Martin, J.F., Daza, A., Vigal, T., Alegre, T., Garcia, M.D., Liras, P., Gil, J.A. (1989a). Cloning of amylase and alkaline phosphatase genes from *Streptomyces griseus* as secretion vectors. Biochem. Soc. Trans. 17, 342-344.

Martin, J.F., Daza, A., Asturias, J.A., Rebollo, A., Gil., H.A., Liras, P. (1989b). Cloning and characterisation of master genes involved in the control of secondary metabolism, differentiation and extracellular enzymes in *Streptomyces*. Folia Microbiologica 34(5), 363.

McCarthy, J.E.G. (1991). Optimising post-transcriptional steps of gene expression in *Escherichia coli*. Adv. Gene Technol. 2, 145-175.

McIntyre, J.J., Bull, A.T., Bunch, A.W. (1996). Vancomycin production in batch and continuous culture. Biotech. Bioeng. 49, 412-420.

McLaughlin, J.R., Murray, C.L., Rabinowitz, J. (1981). Unique features of the ribosome binding site sequence of the Gram-positive *Staphylococcus aureus* β-lactamase gene. J. Biol. Chem. 256, 11283-11291.

Mellouli, L., Ghorbel, R., Virolle, M.J., Bejar, S. (1998). Alpha-amylase gene of thermophilic *Streptomyces* sp. TO1: nucleotide sequence, transcriptional and amino acid sequence analysis. FEMS Micro. Letts. 160, 17-23.

Miller, T.L., Churchill, B.W. (1986). Substrates for large-scale fermentations. In Manual of Industrial Microbiology and Biotechnology (Demain, A.L., Solomon, N.A. eds.), American Society of Microbiology, Washington D.C., 122-136.

Miyake, K., Onaka, H., Horinouchi, S., Beppu, T. (1994). Organisation and nucleotide sequence of the *sec*E-*nus*G region of *Streptomyces griseus*. Biochem. Biophys. Acta 1217, 97-100.

Molnar, I. (1994). Secretory production of homologous and heterologous proteins by recombinant *Streptomyces*: what has been accomplished. In Recombinant Microbes for Industrial and Agricultural Applications (Murooka, Y., Imanaka, T. eds.), Marcel Dekker.

Moran, C.P., Lang, N., LeGrice, S.F., Lee, G., Stephens, M., Sonenshein, A.L., Pero, J., Losick, R. (1982). Nucleotide sequences that signal the initiation of transcription in *Bacillus subtilis*. Mol. Gen. Genet. 186, 339-346.

Morosoli, R., Shareck, F., Kluepfel, D. (1997). Protein secretion in *Streptomyces*. FEMS Micro. Letters. 146, 167-174.

Munoz, A., Perez-Aranda, A., Barbero, J.L. (1985). Cloning and expression of human interleukin-2 in *Streptomyces lividans* using the *E. coli* consensus promoter. Biochem. Biophys. Res. Commun. 133, 511-519.

Murooka, Y., Ishizaki, T., Nimi, O., Maekawa, N. (1986). Cloning and expression of *Streptomyces* cholesterol oxidase gene in *Streptomyces lividans* with plasmid pIJ702. Appl. Env. Micro. 52, 1382-1387.

Muth, G., Nussbaumer, B., Wohlleben, W., Puhler, A. (1989). A vector system with temperature-sensitive replication for gene disruption and mutational cloning in *Streptomyces*. Mol. Gen. Genet. 219, 341-348.

Nagaso, H., Saito, S., Saito, H., Takahashi, H. (1988). Nucleotide sequence and expression of a *Streptomyces griseosporeus* proteinaceus α-amylase inhibitor (HaimII) gene. J. Bact. 170, 4451-4457.

Nakanishi, S., Adhya, S., Gottesman, M., Pastan, I. (1975). Selective effects of $MgCl_2$ and temperature on the initiation of transcription at lac, gal and λ promoters. J. Bio. Chem. 250, 8202-8208.

Nguyen, J., Francon, F., Virolle, M.J., Guerineau, M. (1997). Amylase and chitinase genes in *Streptomyces lividans* are regulated by *regI*, a pleiotropic regulatory gene. J. Bact. 179(20), 6383-6390.

Novick, R.P. (1989). Staphylococcal plasmids and their replication. Ann. Rev. Micro. 43, 537-65.

Obata, S., Furukubo, S., Kumagoi, I., Takahashi, H., Miura, K.I. (1989). High level expression in *Streptomyces lividans* 66 of a gene encoding *Streptomyces* subtilisin inhibitor from *Streptomyces albogriseolus*. J. Biochem. 105, 372-376.

Ohno, A., Ano, T., Shoda, M. (1993). Effect of temperature change and aeration on the production of the antifungal peptide antibiotic iturin by *Bacillus subtilis* NB22 in liquid cultivation. J. Ferm. Bioeng. 75(6), 463-465.

Onaka, H., Ando, N., Nihira, T., Yamada, Y., Beppu, T., Horinouchi, S. (1995). Cloning and characterisation of the A-factor receptor gene from *Streptomyces griseus*. J. Bact. 177(21), 6083-6092.

Ostiguy, S., Gilbert, M., Shareck, F., Kluepfel, D., Morosoli, R. (1996). Cloning and sequencing of the *sec*Y homologue from *Streptomyces lividans* 1326. Gene 176, 265-267.

Pabo, C.O., Sauer, R.T. (1992). Transcription factors: structural families and principles of DNA recognition. Ann. Rev. Micro. 61, 1053-1095.

Page, N., Kluepfel, D., Shareck, F., Morosoli, R. (1996). Effect of signal peptide alterations and replacement on export of xylanase A in *Streptomyces lividans*. Appl. Env. Micro. 62(1), 109-114.

Panaretou, B. (1993). The plasma membrane in the tolerance of yeast to stress. Ph.D. Thesis, University College London.

Panaretou, B., Piper, P.W. (1992). The plasma membrane of yeast acquires a novel heat-shock protein (hsp30) and displays a decline in proton-pumping ATPase levels in response to both heat shock and the entry to stationary phase. Eur. J. Biochem. 206, 635-640.

Park, J.S., Horinouchi, S., Beppu, T. (1991). Characterisation of the leader peptide of an endotype cellulase produced by an alkaline *Streptomyces* strain. Agric. Biol. Chem. 55, 1745-1750.

Parro, V., Mellado, R.P. (1994). Effect of glucose on agarase overproduction by *Streptomyces*. Gene 145, 49-55.

Payne, G.F., DelaCruz, N., Coppella, S.J. (1990). Improved production of heterologous protein from *Streptomyces lividans*. Appl. Micro. Biotech. 33, 395-400.

Perez-Martin, J., Espinosa, M. (1994). Correlation between DNA bending and transcriptional activation at a plasmid promoter. J. Mol. Bio. 241, 7-17.

Pierce, J. (1996). A study of the recovery and purification of a recombinant protein. Ph.D. Thesis, University College London.

Pierce, J.J., Turner, C., Keshavarz-Moore, E., Dunnill, P. (1997). Factors determining more efficient large-scale release of a periplasmic enzyme from *E. coli* using lysozyme. J. Biotech. 58, 1-11.

Pigac, J., Vijaklija, D., Toman, Z., Gamulin, V., Schrempf, H. (1988). Structural instability of a bifunctional plasmid pZG1 and single-stranded DNA formation in *Streptomyces*. Plasmid 19, 222-230.

Pine, M.J. (1973). Regulation of intracellular proteolysis in *Escherichia coli*. J. Bact. 115(1), 107-116.

Piper, P. (1997). The yeast heat shock response. In Yeast Stress Responses (Hohmann, S., Mager, W.H. eds.), Springer-Mol. Biol. Intelligence Unit, Chapter 3, 75-99.

Piper, P.W. (1995). The heat shock and ethanol stress responses of yeast exhibit extensive similarity and functional overlap. FEMS Micro. Letters 134, 121-127.

Piret, J.M., Demain, A.L. (1988). Actinomycetes in biotechnology: an overview. In Actinomycetes in Biotechnology (Goodfellow, M., Williams, S.T., Mordarski, M. eds.), Academic Press, Chapter 11.

Piron-Fraipont, C., Lenzini, M.V., Dusart, J., Ghuysen, J.M. (1990). Transcriptional analysis of the DD-peptidase / penicillin-binding-protein-encoding *dac* gene of *Streptomyces* R61: use of the promoter and signal sequences in a secretion vector. Mol. Gen. Genet. 223, 114-120.

Plackett, R.L., Burman, J.P. (1946). The design of optimum multifactorial experiments. Biometrika 33, 305-325.

Potuckova, L., Kelemen, G.H., Findlay, K.C., Lonetto, M.A., Buttner, M.J., Kormanec, J. (1995). A new RNA polymerase sigma factor, σF, is required for the late stages of morphological differentiation in *Streptomyces* species. Mol. Micro. 17(1), 37-48.

Pugsley, A.P. (1993). The complete general secretory pathway in Gram-negative bacteria. Micro. Rev. 57(1), 50-108.

Pulido, D., Vara, J.A., Jimenez, A. (1986). Cloning and expression in biologically active form of the gene for human interferon α2 in *Streptomyces lividans*. Gene 45, 167-174.

Pulido, D., Jimenez, A., (1987). Optimisation of gene expression in *Streptomyces lividans* by a transcriptional terminator. Nuc. Acid. Res. 15, 4227-4240.

Radford, K.M., Reid, S., Greenfield, P.F. (1992). Improved production of recombinant proteins by the Baculovirus expression system. In Animal Cell Technology: Basic and Applied Aspects (Murakami, H. ed.), Kluwer Academic Publishers, 419-424.

Reichl, U., King, R., Gilles, E.D. (1992). Effect of temperature and medium composition on mycelial growth of *Streptomyces tendae* in submerged culture. J. Basic Micro. 32(3), 193-200.

Reusser, F. (1985). Temperature dependence of lincomycin production by thermoduric *Streptomyces espinosus* strains. J. Antibiot. 38(6), 806-807.

Rosenberg, M., Court, D. (1979). Regulatory sequences involved in the promotion and termination of RNA transcription. Ann. Rev. Genet. 13, 319-353.

Saier, M.H. (1995). Differential codon usage: a safeguard against inappropriate expression of specialised genes? FEBS Letters 362, 1-4.

Sanchez, L., Brana, A.F. (1996). Cell density influences antibiotic biosynthesis in *Streptomyces clavuligerus*. Microbiology 142, 1209-1220.

Sathyamoorthy, M., Speedie, M.K. (1995). Monitoring transport of a recombinant phosphotriesterase in *Streptomyces lividans* using a pulse-chase system. Appl. Micro. Biotech. 43, 493-497.

Schauer, A., Ranes, M., Santamaria, R., Guijarro, J., Lawlor, E., Mendez, C., Chater, K., Losick, R. (1988). Visualising gene expression in time and space in the filamentous bacterium *Streptomyces coelicolor*. Science 240, 768-772.

Schmidt, A.J., Lee, J.M., An, G. (1989). Media and environmental effects on phenolics production from tobacco cell cultures. Biotech. Bioeng. 33, 1437-1444.

Schmitt-John, T., Engels, J.W. (1992). Promoter constructions for efficient secretion expression in *Streptomyces lividans*. Appl. Micro. Biotech. 36, 493-498.

Schneider, D., Bruton, C.J., Chater, K.F. (1996). Characterisation of *spaA*, a *Streptomyces coelicolor* gene homologous to a gene involved in sensing starvation in *Escherichia coli*. Gene 177, 243-251.

Servin-Gonzalez, L., Jensen, M.R., White, J., Bibb, M. (1994). Transcriptional regulation of the four promoters of the agarase gene (*dagA*) of *Streptomyces coelicolor* A3(2). Microbiology 140, 2555-2565.

Shapiro, S. (1989). Nitrogen assimilation in actinomycetes and the influence of nitrogen nutrition on actinomycete secondary metabolism. In Regulation of Secondary Metabolism in Actinomycetes (Shapiro, S. ed.), CRC Press, Boca Raton. 135-212.

Smith, T.M., Jiang, Y.F., Shipley, P., Floss, H.G. (1995). The thiostrepton-resistance encoding gene in *Streptomyces laurentii* is located within a cluster of ribosomal protein operons. Gene 164, 137-142.

Son, K.H., Jang, J.H., Kim, J.H. (1987). Effect of temperature on plasmid stability and expression of cloned cellulase gene in recombinant *Bacillus megaterium*. Biotech. Letters 9(12), 821-824.

Steiert, J.G., Pogell, B.M., Speedie, M.K., Laredo, J. (1989). A gene coding for a membrane-bound hydrolase is expressed as a secreted, soluble enzyme in *Streptomyces lividans*. Biotech. 7, 65-68.

Stein, D.S., Kendall, K.J., Cohen, S.N. (1989). Identification and analysis of transcriptional regulatory signals for the *kil* and *kor* loci of *Streptomyces* plasmid pIJ101. J. Bact.171(11), 5768-5775.

Stoichev, M., Ognyanov, I., Djejeva, G., (1981). Investigation of some conditions for the biosynthesis of the enzyme glucose isomerase by two *Streptomyces* strains. Proceedings of Conference on Chemistry, Biotechnology, Biological Activity of Natural Products Vol. I (Meet. 3,2), 443-453.

Stowe, R.S., Mayer, R.P. (1966). Efficient Screening of process variables. Ind. Eng. Chem. 58(2), 36-40.

Strauch, E., Takano, E., Baylis, H.A., Bibb, M.J. (1991). The stringent response in *Streptomyces coelicolor* A3(2). Mol. Micro. 5(2), 289-298.

Straus, D.B., Walter, W.A., Gross, C.A. (1987). The heat shock response of *Escherichia coli* is regulated by changes in the concentration of σ^{32}. Nature 329,348-351.

Strnadova, M., Prasad, R., Kucerova, H., Chaloupka, J. (1986). Effect of temperature on growth and protein turnover in *Bacillus megaterium*. J. Basic Micro. 26(5), 289-298.

Strohl, W.R. (1992). Compilation and analysis of DNA sequences associated with apparent streptomycete promoters. Nuc. Acid. Res. 20(5), 961-974.

Surowitz, K.G., Pfister, R.M. (1985). Glucose metabolism and pyruvate excretion by *Streptomyces alboniger*. Can. J. Micro. 31, 702-706.

Suzuki, I., Kataoka, M., Seki, T., Yoshida, T. (1997a). Three single-strand origins located on both strands of the *Streptomyces* rolling circle plasmid pSN22. Plasmid 37, 51-64.

Suzuki, I., Seki, T., Yoshida, T. (1997b). Nucleotide sequence of a nicking site of the *Streptomyces* plasmid pSN22 replicating by the rolling circle mechanism. FEMS Micro. Letters 150, 283-288.

Taguchi, S., Nishiyama, K.I., Kumagai, I., Miura, K.I. (1989a). Analysis of transcriptional control regions in the *Streptomyces* subtilisin-inhibitor encoding gene. Gene 84, 279-286.

Taguchi, S., Kumagai, I., Nakayama, J., Suzuki, A., Miura, K.I. (1989b). Efficient extracellular expression of a foreign protein in *Streptomyces* using secretory protease inhibitor (*ssi*) gene fusions. Biotech. 7, 1063-1066.

Taguchi, S., Maeno M., Momose, H. (1992). Extracellular production system of a heterologous peptide driven by a secretory protease inhibitor of *Streptomyces*. Appl. Micro. Biotech. 36, 749-753.

Taguchi, S., Yoshida, Y., Kumagai, I., Miura, K., Momose, H. (1993). Effect of downstream message secondary structure on the secretory expression of the *Streptomyces* subtilisin inhibitor. FEMS. Micro. Letts. 107, 185-190.

Tan, H., Yang, H., Tian, Y., Wu, W., Whatling, C.A., Chamberlin, L.C., Buttner, M.J., Nodwell, J., Chater, K.F. (1998). The *Streptomyces coelicolor* sporulation-specific σ^{WhiG} form of RNA polymerase transcribes a gene encoding a ProX-like protein that is dispensable for sporulation. Gene 212, 137-146.

Tang, L., Hutchinson, C.R. (1995). Regulation of expression of the valine (branched-chain-amino acid) dehydrogenase encoding gene from *Streptomyces coelicolor*. Gene 162, 69-74.

te Riele, H., Michel, B., Ehrlich, S.D. (1986a). Are single-stranded circles intermediates in plasmid DNA replication? EMBO J. 5(3), 631-637.

te Riele, H., Michel, B., Ehrlich, S.D. (1986b). Single-stranded plasmid DNA in *Bacillus subtilis* and *Staphylococcus aureus*. Proc. Natl. Acad. Sci. USA 83, 2541-2545.

Ternel, M.L.A., Gontier, E., Bienaime, C., Saucedo, N., Barbotin, J.N. (1997). Response surface analysis of chlortetracycline and tetracycline production with K-carrageenan immobilised *Streptomyces aureofaciens*. Enz. Micro. Tech. 21, 314-320.

Thompson, J., Cundliffe, E. (1981). Purification and properties of an RNA-methylase produced by *Streptomyces azureus* and involved in resistance to thiostrepton. J. Gen. Micro. 124, 291-297.

Thompson, C.J., Ward, J.M. Hopwood, D.A. (1982). Cloning of antibiotic resistance and nutritional genes in *Streptomyces*. J. Bact. 151, 668-677.

Tilly, K., Erickson, J., Sharma, S., Georgopoulos, C. (1986). Heat shock regulatory gene *rpoH* mRNA level increases after heat shock in *Escherichia coli*. J. Bact. 168(3), 1155-1158.

Titgemeyer, F., Walkenhorst, J., Reizer, J., Stuiver, M.H., Cui, X., Saier, M.H. (1995). Identification and characterisation of phosphoenolpyruvate:fructose phosphotransferase systems in three *Streptomyces* species. Microbiology 141, 51-58.

Ueda, Y., Taguchi, S., Nishiyama, K.I., Kumagai, I., Miura, K.I. (1993a). Effect of a rare leucine codon, TTA, on expression of a foreign gene in *Streptomyces lividans*. Biochim. Biophys. Acta 1172, 262-266.

Ueda, Y., Tsumoto, K., Watanabe, K., Kumagai, I. (1993b). Synthesis and expression of a DNA encoding the FV domain of an anti-lysozyme monoclonal antibody, HyHEL10, in *Streptomyces lividans*. Gene 129, 129-134.

Ueshima, R., Fujitat, N., Ishihama, A. (1989). DNA supercoiling and temperature shift affect the promoter activity of the *Escherichia coli rpoH* gene encoding the heat-shock sigma subunit of RNA polymerase. Mol. Gen. Genet. 215, 185-189.

Vanek, Z., Janecek, J., Spizek, J. (1995). Early stages of strain development and production of secondary metabolites in *Streptomyces*. Bioteknologiya 7-8, Proceedings of the 9th Symposium on the Actinomycetes, 175-182.

Vecht-Lifshitz, S.E., Magdassi, S., Braun, S. (1990). Pellet formation and cellular aggregation in *Streptomyces tendae*. Biotech. Bioeng. 35, 890-896.

Veronese, F.M., Boccu, E., Schiavon, O., Grandi, C., Fontana, A. (1984). General stability of thermophilic enzymes: studies on 6-phosphogluconate dehydrogenase from *Bacillus stearothermophilus* and yeast. J. Appl. Biochem. 6, 39-47.

Vigal, T., Gil, J.A., Daza, A., Garcia-Gonzalez, M.D., Martin, J.F. (1991a). Cloning, characterisation and expression of an α-amylase gene from *Streptomyces griseus* IMRU 3570. Mol. Gen. Genet. 225, 278-288.

Vigal, T., Gil, J.A., Daza, A., Garcia-Gonzalez, M.D., Villadas, P., Martin, J.F. (1991b). Effects of replacement of promoters and modification of the leader peptide region of the amy gene of *Streptomyces griseus* on synthesis and secretion of α-amylase by *Streptomyces lividans*. Mol. Gen. Genet. 231, 88-96.

Vining, L.C., Doull, J.L. (1988). Catabolite repression of secondary metabolites in actinomycetes. . In Biology of Actinomycetes '88 (Okami, Y., Beppu, T., Ogawara, H. eds.) Japan Scientific Societies, Tokyo. 406-411.

Virolle, M.J., Bibb, M.J. (1988). Cloning, characterisation and regulation of an α-amylase gene from *Streptomyces limosus*. Mol. Micro. 2(2), 197-208.

Virolle, M.J., Long, C.M., Chang, S., Bibb, M.J. (1988). Cloning, characterisation and regulation of an α-amylase gene from *Streptomyces venezuelae*. Gene 74, 321-334.

Virolle, M.J., Gagnat, J. (1994). Sequences involved in growth-phase-dependent expression and glucose repression of a *Streptomyces* α-amylase gene. Microbiology 140, 1059-1067.

Von Heijne, G., Abrahmsen, L. (1989). Species-specific variation in signal peptide design: implications for protein secretion in foreign hosts. FEBS Letters 244(2), 439-446.

Votruba, J., Vanek, Z. (1989). Physicochemical factors affecting actinomycete growth and secondary metabolism. In Regulation of Secondary Metabolism in Actinomycetes (Shapiro, S. ed.), CRC Press, Boca Raton.

Votruba, J., Pazlarova, J., Drovakova, M., Vachova, L., Strnadova, M., Kucerova, H., Vinter, V., Zourabian, R., Chaloupka, J. (1991). External factors involved in the regulation of synthesis of an extracellular proteinase in *Bacillus megaterium*: effect of temperature. Appl. Micro. Biotech. 35, 352-357.

Vujaklija, D., Ueda, K., Hong., S.K., Beppu, T., Horinouchi, S. (1991). Identification of an A-factor dependent promoter in the streptomycin biosynthetic gene cluster of *Streptomyces griseus*. Mol. Gen. Genet. 229, 119-128.

Vukelic, B., Ritonja, A., Renko, M., Pokorny, M., Vitale, L. (1992). Extracellular α-amylase from *Streptomyces rimosus*. Appl. Micro. Biotech. 37, 202-204.

Wahl, M.F., An, G., Lee, J.M. (1995). Effects of dimethyl sulphoxide on heavy chain monoclonal antibody production from plant cell culture. Biotech. Letters 17(5), 463-468.

Wallace, K.K., Payne, G.F., Speedie, M.K. (1992). *Streptomyces* bioprocessing: from secondary metabolites to heterologous proteins. Frontiers Bioprocess. 2, 168-180.

Washizu, K., Ando, K., Koikeda, S., Hirose, S., Matsuura, A., Takagi, H., Motoki, M., Takeuchi, K. (1994). Molecular cloning of the gene for microbial transglutaminase from *Streptoverticillium* and its expression in *Streptomyces lividans*. Biosci. Biotech. Biochem. 58(1), 82-87.

Weinberg, E.D. (1989). Roles of micronutrients in secondary metabolism of actinomycetes. In Regulation of Secondary Metabolism in Actinomycetes (Shapiro, S. ed.), CRC Press, Boca Raton. 239-261.

Westpheling, J., Ranes, M., Losick, R. (1985). RNA polymerase heterogeneity in *Streptomyces coelicolor*. Nature 313(3), 22-27.

Whitaker, A. (1992). Actinomycetes in submerged culture. Appl. Biochem. Biotech. 32, 23-35.

White, J., Bibb, M.J. (1997). *bldA* dependence of undecylprodigiosin production in *Streptomyces coelicolor* A3(2) involves a pathway-specific regulatory cascade. J. Bact. 179(3), 627-633.

Williams, S.T., Goodfellow, M., Alderson, G., Wellington, E.M., Sheath, P.H., Sackin, M.J. (1983). Numerical classification of *Streptomyces* and related genera. J. Gen. Micro. 129, 1743-1813.

Wilson, G.C., Bushell, M.E. (1995). The induction of antibiotic synthesis in *Saccharopolyspora erythraea* in *Streptomyces hygroscopicus* by growth rate decrease is accompanied by a down-regulation of protein synthesis rate. FEMS Micro. Letters 129, 89-96.

Wright, F., Bibb, M.J. (1992). Codon usage in the G and C rich *Streptomyces* genome. Gene 113, 55-65.

Wrigley-Jones, C.A. (1990). Plasmid vector stability in *Streptomyces lividans* fermentations. Ph.D. Thesis, University College London.

Wrigley-Jones, C., Richards, H., Thomas, C., Ward, J. (1992). A method for plasmid copy number determination in recombinant *Streptomyces*. J. Micro. Met. 16, 69-80.

Wrigley-Jones, C.A., Richards, H., Thomas, C.R., Ward, J. (1993). Stability of plasmid vector pIJ303 in *Streptomyces lividans* TK24 during laboratory-scale fermentations. Biotech. Bioeng. 41, 148-155.

Yamamori, T., Yura, T. (1980). Temperature induced synthesis of specific proteins in *Escherichia coli*: evidence for transcriptional control. J. Bact. 142(3), 843-851.

Yanish-Perron, C., Vieira, J., Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33, 103-119.

Yatvin, M.B. (1987). Influence of membrane-lipid composition on translocation of nascent proteins in heated *Escherichia coli*. Biochim. Biophys. Acta 901, 147-156.

Yin, X.H., Gagnat, J., Gerbaud, C., Guerineau, M., Virolle, M.J. (1997). Cloning and characterisation of a new α-amylase gene from *Streptomyces lividans* TK24. Gene 197, 37-45.

Yoon, Y.J., Choi, C.Y. (1997). Nutrient effects on FK-506, a new immunosuppressant, production by *Streptomyces* species in a defined medium. J. Ferm. Bioeng. 83(6), 599-603.

Zaman, S. (1991). Genetics and molecular biology of the *Streptomyces lividans* plasmid pIJ101. Ph.D. Thesis, University College London.

Zaman, S., Radnedge, L., Richards, H., Ward, J.M. (1993a). Analysis of the site for second strand initiation during plasmid replication of the *Streptomyces* plasmid pIJ101. J. Gen. Micro. 139, 669-676.

Zaman, S., Richards, H., Ward, J. (1993b). Identification of the minimal replicon of the *Streptomycete* plasmid pIJ101. Plasmid 29, 57-62.

Zhang, J., Marcin, C., Shifflet, M.A., Salmon, P., Brix, T., Greasham, R., Buckland, B., Chartrain, M. (1996). Development of a defined medium fermentation process for physostigmine production by *Streptomyces griseofuscus*. Appl. Micro. Biotech. 44, 568-575.