LIVERPOOL JOHN MOORES UNIVERSITY
SCHOOL OF BIOMOLECULAR SCIENCES

A PHYSIOLOGICAL STUDY OF *STREPTOMYCES CAPREOLUS* AND FACTORS GOVERNING GROWTH AND CAPREOMYCIN BIOSYNTHESIS

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Figure 1 on page 14 has been excluded at the request of the awarding university.

DECLARATION

I declare that while registered as a candidate for the degree of Doctor of Philosophy, no part of this thesis has been submitted in support of an application for any degree or qualification of Liverpool John Moores University or any other University or Professional institution.

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ABSTRACT

Streptomyces capreolus is an industrially important actinomycete responsible for the production of the aminoglycosidic antibiotic capreomycin. Capreomycin is of special interest since it is effective against multi-drug resistant strains of *Mycobacterium tuberculosis* that do not respond to standard anti-tubercular drugs.

Limited research has been carried out on *S. capreolus* since its discovery in 1960, and so this research encompassed a physiological study of *S. capreolus* that aimed to elucidate the factors governing growth, cellular metabolism and morphology, and their impact on capreomycin biosynthesis.

Media conditions were primarily divided into two categories, complex and minimal media allowing a fundamental comparative study. A propensity for fragmentation into rod-like structures was observed in many instances, a common trait of the genus *Nocardia*, and would reinforce its recent taxonomic classification. Fragmentation was induced by altering media composition, C/N ratio, and increasing pH and NaCl concentration. A rapid technique for the estimation of culture viability was developed using fluorimetric analysis to support this work, and associated fragmentation with a high degree of viability. This was found to be synonymous with poor antibiotic titres, therefore pelleted morphology appeared to be a prerequisite for capreomycin biosynthesis.

The importance of carbon and nitrogen metabolism and their interconnection was highlighted, alongside their influence over morphological status and antibiotic producing capacity. This was compounded by continuous culture studies based around a defined medium under both carbon and nitrogen limited conditions.

Substrate uptake studies primarily showed a repressive effect of particular amino acids on glucose uptake. It was also observed that *S. capreolus* exhibits a particularly low affinity for glucose uptake, but actively transports fructose without a lag period under conditions of carbon limitation, suggesting that the enzymes responsible for fructose uptake are constitutive as opposed to inducible in *S. capreolus*. A preferential utilisation profile of amino acids was described and results identified *S. capreolus* as an amino acid producer under defined media conditions. Alanine and phenylalanine appeared to be synthesised early on in the fermentation, followed consequently by their consumption.

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PUBLICATIONS

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1.0 INTRODUCTION

1.1 THE ACTINOMYCETES

The actinomycetes are a complex and diverse group of gram-positive, spore forming, mycelium producing bacteria, but are intermixed with organisms displaying a variety of simpler morphologies (Embley & Stackebrandt, 1994). The actinomycete family are characterised by their high G + C content (typically 60-70 %) within their DNA (Goodfellow & Cross, 1983). Some members of this family are termed sporoactinomycetes due to their ability to develop true mycelium as a means of colonization and spores as a means of reproduction, that are differentiated either singly or in chains at the tips of hyphae e.g., *Streptomyces*, *Streptosporangium* and the *Micromonospora*. In other actinomycetes, usually termed proactinomycetes, mycelial development is less complete and may occur during active growth but is quickly followed by fragmentation giving rise to unicellular structures that are rod or coccoid in form from which active growth may continue e.g., *Nocardia*, *Dermatophilus* and the *Mycobacterium*. In general, the actinomycetes can be divided into different genera on the basis of their morphological, physical and chemical characteristics and are a particularly diverse group in nature.

Streptomyces capreolus, the study organism in this research was originally assigned to the family Streptomycetaceae in 1962 (Stark *et al.*, 1962) following comparisons made with other *Streptomyces* including *Streptomyces canescus* to which it was found to be closely related. It is also known by various synonyms including *Saccharothrix mutabilis* subsp. *capreolus*, and *Nocardia capreola*. Recently the production strain has been classified following 16S ribosomal RNA sequencing as *Saccharothrix australiensis*. The genus *Saccharothrix* belonging to the actinomycetes has been found to be related to *Nocardiopsis* (Labeda *et al.*, 1984).

Since the *Streptomyces* species have been studied in more depth than any of the other actinomycetes leading to a greater understanding of their complexity, it is not unreasonable to compare *S. capreolus* to this higher range of actinomycetes.

1.1.1 STREPTOMYCES – AN INGENIOUS GENUS?

The unique developmental characteristics of members of the streptomycetes have generated considerable interest in their genetics and physiology, in particular *Streptomyces coelicolor* whose life cycle has been most extensively studied and whose genome has recently been fully sequenced (Bentley *et al.*, 2002). This development has provided an intrinsic overview of the genetic elements responsible for metabolic and structural diversity in this genus, there is still however a vast plenitude of research to be carried out in order to gain an explicit understanding of this genus.

Streptomycetes are among the most numerous and ubiquitous soil dwelling bacteria, normally encountering a nutritionally, physically and biologically variable environment where degradation of soluble and insoluble organic material is vital for survival. Indeed under nutrient rich conditions such as in the laboratory, streptomycetes can have generation times as short as one hour whilst in their natural soil environment this can be in the magnitude of days and so it is fitting that streptomycetes be termed facultative oligotrophs (Hodgson, 2000). These organisms thus exhibit a complex and morphologically unusual response to nutrient starvation including the sequential production of substrate and aerial mycelia followed by the emergence of spores, an intricate process termed differentiation (Chater & Hopwood, 1984). Growth media composition can profoundly affect the growth and stability of substrate and aerial mycelium (Kalakoutskii & Agre, 1976), thereby interfering with differentiation and the biochemical events associated with it.

Commercially, *Streptomyces* species are responsible for the biosynthesis of a number of medically important antibiotics and other chemotherapeutic agents via advanced metabolic pathways which include the generation of primary and secondary

metabolites. These compounds generally exhibit unusual structures and their formation is regulated by nutrients, growth rate, feedback control, enzyme inactivation and enzyme induction (Demain, 1998). Approximately 70 % of all antibiotics known today are produced by *Actinomycetales* and around 90 % of them are related to *Streptomyces* species (Bushell, 1988), and although thousands of antibiotics have been described, these are believed to represent only a small fraction of the repertoire of bioactive compounds produced by *Streptomyces* (Bérdy, 1995; Watve *et al.*, 2001). Indeed, prior to the full genomic sequencing of *S. coelicolor* by Bentley *et al.*, (2002), gene clusters specifying only four antibiotics were identified, these clusters encoded antibiotics including the pigmented compounds actinorhodin and undecylprodigiosin together with a cluster of genes coding for a type II polyketide synthase for a grey spore pigment. It has now been elucidated that over 7,000 genes arranged in a further 18 clusters are likely to code for many more secondary metabolites than previously contemplated. It is therefore highly likely that the same genetic information regarding encoding of secondary metabolites will emerge for other streptomycetes and become a widespread genomic phenomenon.

Empirical screening has revealed that streptomycetes produce other pharmaceutically active compounds such as anti-viral and anti-cancer compounds, modulators of immune responses, and various enzyme inhibitors, as well as herbicides, insecticides and anti-parasitic compounds (Vining, 1990; Sanglier *et al.*, 1993; Bérdy, 1995). The importance of such natural metabolites has led to the screening of tens of thousands of *Streptomyces* strains annually by pharmaceutical companies as potential sources of novel chemical compounds, and high-throughput chromatographic analyses have revealed that each strain often has a unique profile of secondary metabolites each showing their own unique activity. Despite the

industrial importance of *Streptomyces* species however, there are still many aspects of their remarkable metabolic processes and complex structural diversity that are yet to be fully elucidated.

1.1.2 ACTINOMYCETE DIFFERENTIATION

Streptomycetes undergo complex morphological differentiation during their life cycle which can be defined as a series of biochemical and structural changes in the physiology of the microorganism (Sebastine *et al.*, 1999), and includes the sequential production of substrate (vegetative) mycelium, aerial (reproductive) mycelium, and spores (Chater, 1984). Differentiation characteristics also accompany the onset of secondary metabolite production or chemical differentiation and in recent years it has been postulated that the two are closely regulated (Chater, 2001; Challis & Hopwood, 2003). Mendez *et al.*, (1985) analysed colonies of *Streptomyces antibioticus* growing on cellophane membranes placed on the surface of a variety of nutrient agars and results indicated that particular compounds present in the substrate mycelium were used as nutrients during the development of aerial mycelium. The morphological diversity of actinomycetes is exemplified primarily by their reproductive strategies (Chater & Hopwood, 1973). Unlike unicellular bacteria, where daughter cells separate, *Streptomyces* cells primarily remain associated thus forming long mycelial filaments that grow initially as substrate mycelia branching out into a complex intertwined network of hyphae similar to growth of many fungi. Indeed it is believed that together with some other genera of the *Actinomycetales*, *Streptomyces* species exhibit strong evidence of parallel evolution with filamentous fungi (Kalakoutskii & Agre, 1976; Ensign, 1978). This filamentous mode of growth allows full utilization of the solid materials in soil, their natural habitat, and enables streptomycetes to colonize solid substrates more efficiently than non-motile unicellular microorganisms. *Streptomyces coelicolor* exhibits a typical life cycle of a streptomycete, preceeding at substrate mycelial development, followed by aerial mycelial growth, subsequently followed by spore generation by differentiation of the

filamentous mycelium. Spore maturation occurs and dispersal of the structures ensues. Figure 1 illustrates *S. coelicolor* life cycle, the intricate stages of which are detailed in this chapter.

Figure 1. *Streptomyces coelicolor* life cycle (taken from Angert, 2005).

The morphology of mycelium is determined by mechanisms which regulate the polarity and direction of growth of the hyphae and the frequency with which they branch. On solid substrata, vegetative mycelia grow radially outward and branch from a single cell or inoculum. Substrate mycelium, which is assumed to grow into the medium, has a mean diameter of 0.7 μm and is bound by a 0.01-0.02 μm thick mucopeptide cell wall, reviewed by Hodgson, (1992). Such mycelia elongate by apical growth at the tips whilst at the same time branching into a complex network of interconnected hyphae that effectively spans a substrate. This type of mycelium is

assumed to be present in different stages of cellular degeneration during all growth phases. Early reports suggested that aerial mycelia were simply the result of branching of substrate hyphae (Hopwood & Glauert, 1961) and were preceded by a short period of decreased macromolecular synthesis (Granozzi *et al.*, 1990). Indeed, this biphasic growth exhibited by *Streptomyces* is correlated with a developmental switch (Granozzi *et al.*, 1990; Süsstrunk *et al.*, 1998) and thus an initial phase of growth is followed by a brief interruption; biomass accumulation then resumes, accompanied by the emergence of aerial hyphae on the colony surface and the onset of secondary metabolism.

Substrate mycelium have a characteristic lack of cross-walls or septa during the vegetative phase, however as the colony ages and nutrients become exhausted, specialized branches emerge from the surface of the colony, originating the reproductive aerial mycelium that grows upwards, vertically into the air (Miguélez, *et al.*, 1994). The generation of aerial mycelium is believed to coincide with a response to unknown signals involving nutrient limitation and causes lysis of the substrate hyphae, a phenomenon synonymous with programmed cell death and would explain the brief interruption in the growth cycle. Nucleases and other hydrolytic enzymes govern the hyphae and DNA degradation process in a highly conserved manner, yielding nutrients now available for translocation. Fernandez & Sanchez (2002) suggest that a change in nutritional environment or growth rate transmits a signal to *Streptomyces* cells which may activate a cytoplasmic effector thus triggering mycelial cell death and lytic processes, an event that is analogous to apoptosis in eukaryotic cells. Like most, if not all multicellular biological systems, colony development in streptomycetes depends on the relationships between cell proliferation, cell differentiation, and cell death during the life cycle. Many hyphae

degenerate and die primarily providing nutrients for aerial mycelium formation (Méndez *et al.*, 1985; Nicieza *et al.*, 1999), by an indiscriminate process of autolysis (Wildermuth, 1970). Recent studies suggest that during the streptomycete life cycle, the hyphae die by following two distinct sequences of degeneration: they either undergo autolysis or physiological cell death (Miguélez *et al.*, 1999). Autolytic hyphal death affects a minority of the hyphae throughout the colony and is characterized by the early degradation of the cell wall as a consequence of uncontrolled, poorly regulated, lytic action of murein hydrolases and usually occurs fairly rapidly, but is believed to release nutrients for continued hyphal growth (Méndez *et al.*, 1985), a crucial event for a densely packed hyphal population that will be experiencing nutrient limitation. In contrast, physiological cell death demonstrates hyphae that undergo an orderly process of internal cell dismantling that takes time to reach total cell death (Miguélez *et al.*, 1999; Miguélez *et al.*, 2000). Fragmentation and/or sporulation follows this event by the synchronous formation of septa along the hyphae which has been found to be dependent in part upon the expression level of at least one important actinomycete-specific protein, SsgA, whereby enhanced SsgA protein levels result in mycelial fragmentation, a process discovered in many *Streptomyces* species (Kawamoto *et al.*, 1997; van Wezel *et al.*, 2000a, 2000b). Members of the family of SsgA-like proteins (SALPs) are found exclusively in sporulating actinomycetes and it has been demonstrated that these proteins have a chaperonin-like role in supporting the function of enzymes involved in peptidoglycan maintenance (penicillin binding proteins (PBPs), and autolysins), (van Wezel *et al.*, 2000a). Most recently, it has been shown that SsgA localizes dynamically during development, and marks the sites where changes in local cell-

wall morphogenesis are required, in particular septum formation and germination (Noens *et al.*, 2007).

While the substrate mycelium has a primarily vegetative function, the role of the aerial mycelium appears to be mainly reproductive, forming spores and placing them in a favourable position to be dispersed, thus solving the problem raised by the immobility of the substrate mycelium.

The signals that trigger the formation of aerial mycelium however are not well understood (Willey *et al.*, 2006). Horinouchi & Beppu, (1994) stated that the formation of aerial hyphae can be triggered by extracellular factors, a phenomenon first observed in *Streptomyces griseus*. More recently, the γ-butyrolactone known as A-factor has been identified as the trigger of a well characterized pathway leading to morphogenesis and streptomycin biosynthesis in *S. griseus* (Ohnishi *et al.*, 1999; Yamazaki *et al.*, 2003), and is the subject of several reviews (Horinouchi *et al.*, 2001; Ohnishi *et al.*, 2002; Chater & Horinouchi, 2003).

Many mutants of *S. coelicolor* are defective in aerial mycelia formation and are known as bald (*bld*) mutants where colonies acquire their name from their smooth appearance as opposed to a regular powdery phenotype. These mutants are interestingly unable to produce actinorhodin and undecylprodigiosin (Chater, 1998; Kelemen & Buttner, 1998).

There is good reason to believe that the distinction between substrate (vegetative) and aerial (reproductive) hyphae is more than their locations. During the vegetative phase, aerial mycelium of *Streptomyces* is reported to be thicker and branches less than substrate mycelium and also contains a dark pigment not found in the substrate mycelia, hydrophobicity is also prevalent in the aerial hyphae (Locci & Sharples, 1984; Hodgson, 1992). Recent research indicates the existence of another regulatory

pathway in addition to the bld cascade. This new pathway operates after aerial hyphae have started to grow into the air and is known as the sky pathway (Claessen *et al.*, 2006). This pathway controls the expression of the chaplin and rodlin genes, the genes encoding proteins that assemble into a rodlet layer that provides surface hydrophobicity to aerial hyphae and spores. Research continues to determine the signals that govern pathways responsible for morphological development and antibiotic production. A novel type of metabolic regulator has recently been characterized that directly links carbon and nitrogen metabolism to development in *S. coelicolor* (Rigali *et al.*, 2006). This metabolic regulator senses the nutritional state of the habitat and maintains vegetative growth until changing circumstances trigger the switch to sporulation.

Following the emergence of aerial hyphae, the mycelia eventually septate into chains of grey-pigmented spores that give rise to new mycelium.

Again, *S. coelicolor* mutants have also been isolated that are impaired in the formation of grey-pigmented spores and are phenotypically white, hence their name (*whi* mutants) where septation, cell wall thickening and spore pigmentation are specifically affected. The *whi* genes are further subdivided into early and late *whi* genes, depending on the developmental state of the aerial hyphae. The early *whi* genes, including *whiA*, *whiB*, *whiG* and *whiH*, are involved in the regulatory cascade concerning the early stages of sporulation and fail to produce spore compartments (Flardh *et al.*, 1999; Ryding *et al.*, 1999). The late *whi* genes including *whiD* and *sigF* are involved in the final stages of sporulation and spore maturation (Potuckova *et al.*, 1995)

The recent acquisition of genome sequences for 16 species of actinobacteria, including two streptomycetes, makes it possible to attempt to reconstruct the

evolution of *Streptomyces* differentiation by a comparative genomic approach. Research suggests that *Streptomyces* development has evolved through the stepwise lateral transfer of DNA, whereby each successive acquisition has given rise to regulatory changes that affect the conditions under which development is initiated, or to changes in cellular structure or morphology (Chater & Chandra, 2006).

Electron microscope studies clearly show that young aerial hyphae develop a sheath before completing sporulation, which confers a characteristic configuration to the outer surface of spores of different streptomycete species, as a result of this, spores are fiercely hydrophobic. A good summary of the properties of the surface structures of aerial mycelia and spores is presented by Kalakoutskii & Agre, (1976).

Sporogenesis in *Streptomyces venezuelae* involves simultaneous septation at regular intervals of aerial hyphae (Bradley & Ritzi, 1968), and so the cross-wall of each spore is an independent structure where the inner hyphal wall, now forming the spore wall, thickens and the spores round up. The process for *S. coelicolor* was considered to entail several stages including coiling of aerial hyphae, septa formation at spore-size (1-2 μm) intervals and addition of new cell wall material to the septa and inner spore wall, forming a thickened spore wall. This segments and finally the outer hyphal wall disintegrates enclosing the spore chains in a fibrous sheath.

The resistance of spores to heat and radiation, a result of this hydrophobic fibrous sheath enables them to survive extreme conditions in particular dehydration that would otherwise render the colony non-viable. These dormant structures in the sense of being less metabolically active than vegetative cells, are able to survive for decades or longer. Germination of spores occurs quite rarely in nature, however within laboratory conditions, sporulation may be induced with factors such as heat or the addition of nutrients in a matter of hours.

1.1.3 ANTIBIOTICS: NATURE'S ELIXIR

Streptomycetes and related actinomycetes continue to be prolific sources of novel secondary metabolites with a range of biological activities that may ultimately find application as anti-infectives (including anti-fungal and anti-parasitic agents), anti-cancer or immunosuppressant agents or other pharmaceutically useful compounds (Bibb, 2005). The taxonomic distribution of antibiotics is restricted to spore-forming groups of bacteria, namely Bacilli and Actinomycetes and in contrast to primary metabolites, are not essential for growth of the organism (Beppu, 1992), but still play a vital role in the life cycle of the organism. A characteristic feature of secondary metabolism is that any given organism usually produces a group of related compounds that belong to the same class, and are relatively low molecular weight compounds (Maplestone *et al.*, 1992).

The actinomycetes produce approximately two-thirds of all known antibiotics of microbial origin, with over 6,000 of these being produced by *Streptomyces* species (Butler *et al.*, 2003). However, the recently increased development of resistance to older antibacterial and antifungal drugs has led to the use or clinical testing of older, underutilized or previously non-developed narrow-spectrum antibiotics as well as semisynthetic antifungal agents (Demain, 1999).

Despite the commercial exploitation of antibiotics, there is still no universally accepted definition for their chemical structure or role in nature. Indeed a definition based on chemical structure is not possible due to the great variety of structures and often complex nature of these secondary metabolites (Trilli, 1990).

It has been suggested that an antibiotic is a substance that is not essential for the vegetative growth of the producing organism in pure culture (Beppu, 1992), however the extent to which this statement holds true is controversial since it has been

discovered that peptide antibiotics produced by bacilli are involved in the sporulation process of the organism (Katz & Demain, 1977; Piret & Demain, 1981), whilst the antibiotic methlenomycin produced by *Streptomyces coelicolor* has been found to be associated with the formation of aerial mycelium (Wright & Hopwood, 1976). Indeed, recent work into gene expression during antibiotic synthesis has discovered that the commonly known *bld* genes are closely associated with the gene clusters necessary for secondary metabolite production and are required for both spore formation and initiation of aerial mycelium (Chater & Horinouchi, 2003; Elliot & Talbot, 2004). These examples do not necessarily typify vegetative growth, however they suggest that such compounds are still necessary for the overall survival of the bacterium in nature. This leads to the hypothesis that antibiotics possess an antagonistic role in nature between an organism and its environment, whereby the antibiotic producer holds a selective advantage over other competing organisms. In this case the producing organism may inhibit the growth of other surrounding organisms, enabling it to dominate a particular nutrient niche. This is a particularly useful function since the major habitation of antibiotic producing organisms is soil which presents a markedly hostile environment in terms of nutrient status, and would explain why a producing organism is not sensitive to its own secondary metabolites. Indeed, the protective mechanisms possessed by many of the actinomycetes against their own secondary metabolites are now being linked to antibiotic resistance in pathogenic organisms (Hopwood, 2007). This is believed to occur via horizontal gene transfer from non-pathogenic bacteria, the ultimate source of many of these genes is assumed to be the actinomycetes that make the antibiotics. Three major ways in which bacteria protect themselves from antibiotics have been identified and include antibiotic modification, modification of the normally sensitive antibiotic

target, thus rendering it insensitive, and the third mechanism, exemplified by the *Streptomyces* producers of the tetracyclines depends upon a trans-membrane export pump that removes intracellular antibiotic by coupling antibiotic excretion with proton import (Hopwood, 2007).

A wider generalisation may be that these compounds offer protection to cells in the resting phase that may otherwise encounter adverse conditions. Chater & Merrick (1979) have suggested that during autolytic hyphal death, when nutrients are readily released from the degrading cells, antibiotics protect these vital nutrients from other organisms that may colonize the surrounding area. A characteristic feature of secondary metabolism is that any given organism usually produces a group of related compounds that belong to the same class (Maplestone *et al.*, 1992) to which the producing organism yields a resistance to.

A more compelling and intriguing prospect for antibiotic production was put forward by Zähner (1979), whereby such metabolites were simply part of the process of evolution, and the case that the majority of them do not have a reasonable shared function now, suggests that over time and interaction with other substances such as enzymes and other complex compounds, a new structure may arise for which the producing bacterial cell eventually finds a use.

Earlier explanations provided by Zähner & Maas (1972) relating to the role of antibiotics include the proposal that they are waste products of metabolism or breakdown products of macromolecules that just happen to be toxic to selected organisms, or indeed antibiotics are a means of preventing the accumulation of primary metabolites that may be harmful to the cell during the resting phase, since it is widely postulated that primary metabolites such as amino acids, fatty acids and sugars form the basis of secondary metabolite synthesis.

There is no correct or incorrect answer to the origin and function of antibiotics and a central role for them will probably never be evident, however it remains that probably the most widely accepted and non-controversial definition for an antibiotic is that they are low-molecular weight organic natural products (secondary metabolites) made by microorganisms which are active against other microorganisms at low concentrations (Demain, 1999). Although this is a generalised assumption it has the ability to apply to a wide range of explanations without entering into specifics.

Whatever their true role, antibiotics have been therapeutically vital for over fifty years, and still to this day very little is known about the regulation of their synthesis.

1.1.4 *STREPTOMYCES* IN SUBMERGED CULTURE

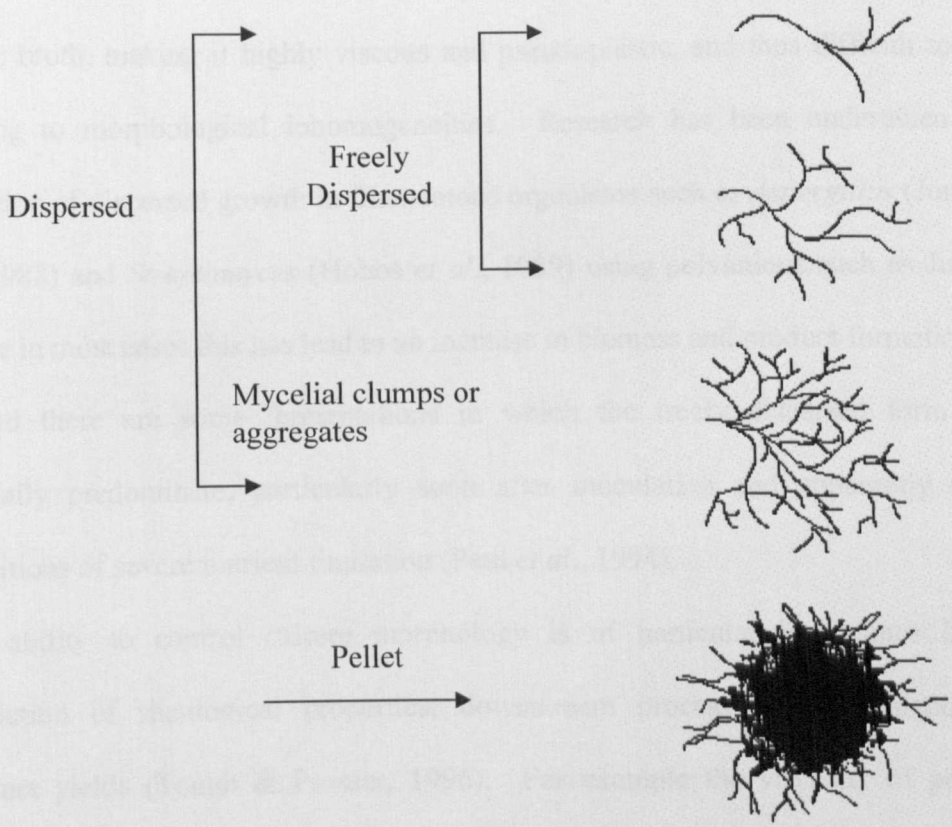
Many fermentations of industrial importance exploit the metabolism of filamentous microorganisms such as fungi and actinomycetes, however for economical production, most processes require submerged culture in large fermenters together with intense agitation and aeration under which conditions a variety of morphological states can be observed, the pelleted form of which often prevailing.

Sporulation can be induced in *Streptomyces* in submerged liquid culture following nutrient shift-down, whereby cells are subjected to growth in a defined minimal medium followed by subsequent growth in a rich medium (Kendrick & Ensign, 1983; Koepsel & Ensign, 1984; Daza *et* al., 1989; Glazebrook *et al.*, 1990) during which, a stage corresponding to aerial mycelium formation appears to be absent. Relatively little is known about submerged sporulation, but the 34 kDa protein named factor C has been found to restore submerged sporulation in a *S. griseus* mutant. It has also been found that the spores produced from submerged fermentations often have different properties to those formed on solid media (Koepsel & Ensign, 1984).

In general, growth in submerged culture gives rise to states varying from freely dispersed mycelial filaments to spherical colonies of densely interwoven mycelia known as pellets, which can become as large as one centimetre in diameter. Cox *et al.*, (1998) have fully reviewed image analysis tools for morphological characterization of filamentous organisms including advantages and disadvantages of various methodologies.

The dispersed mycelial forms can be further divided into freely dispersed and aggregated or 'clumped' forms (Fig. 2).

Figure 2. Morphological forms found in typical submerged cultures of filamentous fungi and actinomycetes (Reproduced from Cox *et al.*, 1998).



Although dispersed mycelial growth could be seen as the 'ideal' morphological state for mycelia, exhibiting no diffusional restriction in the exchange of substrates and metabolites within a culture, secondary metabolism can be directly affected as a result. In addition, the dispersed form of growth can lead to hyphal entanglement in a fermentation culture which can have an adverse effect on the rheological properties of the broth, making it highly viscous and pseudoplastic, and thus difficult to mix leading to morphological inhomogeneities. Research has been undertaken into selection of dispersed growth in filamentous organisms such as *Aspergillus* (Jones *et al.*, 1988) and *Streptomyces* (Hobbs *et al.*, 1989) using polyanions such as Junlon, where in most cases this has lead to an increase in biomass and product formation. Indeed there are some fermentations in which the freely dispersed form may naturally predominate, particularly soon after inoculation and apparently under conditions of severe nutrient limitation (Paul *et al.*, 1994).

The ability to control culture morphology is of particular importance in the prediction of rheological properties, downstream processing requirements and product yields (Tough & Prosser, 1996). For example the viscosity of pelleted cultures is low in comparison to highly dispersed mycelia thereby facilitating mixing of the culture and reducing the power input to the fermentation. Downstream processing is often easier for pelleted cultures since dispersed cultures are more prone to cause blockages of filtration equipment.

With pellet formation accompanies mass transfer limitations that form the basis of solute gradients through the hyphal pellets (Hobbs *et al.*, 1989). This phenomenon leads to nutrient limitation in addition to oxygen limitation of the cells within the core of the pellet and therefore differing physiological and metabolical states can be observed within the culture. As the pellet increases in size, mycelial viability is

confined to the periphery of the pellet because the diffusion rate of oxygen and nutrients is insufficient to maintain growth of the inner pellet (Pirt, 1967), and it is this degree of nutrient limitation that often triggers secondary metabolism (Martin & Demain, 1980). However in severe cases of oxygen limitation, a lowered productivity can be observed (Pinto *et al.*, 2004).

There are many factors that influence mycelial aggregation, these include inoculum size, type and age, genetic factors, medium composition, biosynthesis or addition of polymers (polyanions reduce aggregation, polycations induce aggregation), surfactants and chelators, shear forces, dissolved oxygen tension, pH, temperature, pressure and medium viscosity (Stanbury *et al.*, 2000), all can result in varying pellet morphologies and broth rheologies.

In many cases, it has been shown that morphology has a direct influence on productivity particularly for fermentations using pelleted organisms (Metz & Kossen, 1977; Martin & Bushell, 1996). Upper size limits of 400 μm have been suggested for *Penicillium chrysogenum* pellets to maintain viability and productivity (Schügerl *et al.*, 1983), whilst *Amycolatopsis mediterranei* shows greatest productivity of rifamycin B when pellets range between 2-3 mm in diameter and are devoid of a hollow centre (El-Tayeb *et al.*, 2004). The complex interactions between morphology, productivity and process conditions within a submerged fermentation are all inter-linked, with one factor directly influencing the other. To appreciate this relationship is to gain a fundamental understanding towards the potential control and regulation of a fermentation.

1.2 METABOLISM OF STREPTOMYCETES

The remarkable feature of streptomycetes to produce such diverse secondary metabolites has meant that sophisticated systems of primary metabolism are vital to this success. Primary metabolites serve as substrates for secondary metabolism and are building blocks for macromolecules, intermediates in reactions generating energy-rich compounds such as ATP, co-enzymes and vitamins (Zähner & Maas, 1972). Studies of primary metabolism are limited and many problems present themselves when studying this field. Hodgson (2000) has highlighted many of these issues which generally relate to the physiological inhomogeneities observed in liquid cultures of streptomycetes, namely the formation of mycelial pellets. Further research in this area would thus help to elucidate the complex regulatory networks that govern the switch from primary to secondary metabolism.

Carbon and nitrogen metabolism is fundamental in primary metabolism for precursor synthesis of antibiotics, and work to date has specified several transport systems for each. An excellent review concerning streptomycete metabolism has been given by Hodgson (2000). Hodgson discusses the wide range of catabolic pathways observed and studied in streptomycetes and the control that they exert over carbohydrate metabolism. These inducible and constitutive systems are fundamental when the normal competitive and largely nutrient limited environment that they inhabit is taken into consideration. Indeed soil environments are noted for being carbon-rich and nitrogen-poor places and so the implicated need for coordinate control of carbohydrate metabolism appears to be reflected in the carbon catabolite repression systems demonstrated in streptomycetes. Constitutive permeases of carbohydrate transport largely show low affinity, high capacity systems, as opposed to high affinity inducible permeases, with the former group most likely reflecting the

relatively carbon-rich conditions normally encountered. The nitrogen deficient conditions normally encountered in soil serve to rationalize the high affinity transport systems involved in amino acid transport and the lack of feedback regulation demonstrated in amino acid metabolism. Indeed if amino acids are not available, then a streptomycete will synthesise them. It is clear that many amino acid biosynthetic pathways and amino acid metabolism is constitutive (Hodgson, 2000). Throughout primary metabolism, the biochemical pathways involved are accurately tuned to ensure maximum growth rate and maximum efficiency under a given set of environmental conditions. To enable such bacterial growth, a complex set of regulatory mechanisms must therefore be in place to control the synthesis and activity of enzymes vital to these processes.

1.2.1 CARBON METABOLISM

Analysis of carbon metabolism in bacteria has shown that sugars enter the cell via inducible or constitutive uptake systems (Sabater *et al.*, 1972; Sabater & Asensio, 1973; Hodgson, 1982). The phosphoenolpyruvate: sugar phosphotransferase system (PEP:PTS), which was first identified in 1964 by Kundig *et al.*, catalyses the uptake of more than 20 different carbohydrates in Gram-positive and Gram-negative bacteria (Reizer *et al.*, 1988; Titgemeyer, 1993; Nothaft *et al.*, 2003). In *Streptomyces coelicolor* A3(2) it has been found that glucose is responsible for repressing the expression of many genes involved in the utilization of alternative carbon sources, such as arabinose and glycerol (Hodgson, 1982; Smith & Chater, 1988). This is known as carbon catabolite repression (CCR) and differs fundamentally in mechanism from that found in *Escherichia coli*, whereby glucose kinase is involved as opposed to cyclic AMP (Hodgson, 1982; Angell *et al.*, 1994).

In Gram-negative bacteria, glucose-specific PEP:PTS proteins are believed to play regulatory roles in nitrogen utilization (Reizer *et al.*, 1992), and several research groups have concluded that a glucose-specific PEP:PTS is lacking in streptomycetes (Sabater *et al.*, 1972; Novotna & Hostálék, 1985; Titgemeyer *et al.*, 1995). It has been found however, that *S. coelicolor* possesses a fructose-specific PEP:PTS (Titgemeyer *et al.*, 1994; Titgemeyer *et al.*, 1995). The PEP:PTS has also been reported in low G + C Gram-positive bacteria including *Corynebacterium glutamicum* (Reizer *et al.*, 1988).

Titgemeyer *et al.*, (1995) have identified Enzyme I, HPr and fructose-specific Enzyme II (II^{Fru}) activities of the PEP:PTS in *S. lividans*, *S. griseofuscus* and *S. coelicolor*.

Other PTS enzymes including HPr (Ser) kinase and HPr (Ser-P) phosphatase that characterize low G + C Gram-positive bacteria, were absent.

As well as the ability of streptomycetes to utilize a wide range of sugars, it has also been demonstrated that certain amino acids present in the medium can 'spare' glucose utilization. This has been demonstrated in *Streptomyces pristinaespirilis* (Voelker & Altaba, 2001) and *Micromonospora echinospora* (Hoskisson *et al.*, 2003), and suggests that carbon and nitrogen metabolism are intrinsically linked.

1.2.2 NITROGEN METABOLISM

The assimilatory metabolic pathways of nitrogen metabolism can be divided into two classes: the pathways necessary for utilization of nitrogen from the extracellular medium and the biosynthetic pathways for intracellular production of nitrogen-containing compounds (Merrick & Edwards, 1995). The precise pathways involved depend upon the organism in question. Most research into nitrogen metabolism has been carried out on the enteric bacteria, including *Escherichia coli*, *Salmonella typhimurium*, *Klebsiella aerogenes* and *Klebsiella pneumoniae* and has been extensively reviewed (Tyler, 1978; Magasanik, 1982; Reitzer & Magasanik, 1987). Nitrogen source regulation of primary metabolism has also been investigated in fungi (Marzluf, 1981).

1.2.3 METABOLIC PATHWAYS

Most bacteria and many other organisms have two primary pathways to facilitate the incorporation of ammonium into the key nitrogen donors for biosynthetic reactions, glutamate and glutamine (Merrick & Edwards, 1995). Indeed glutamate is an important precursor since its amino group provides nitrogen for the synthesis of most amino acids (Reitzer & Magasanik, 1987). Research on the *Enterobacteriaceae* family has shown that two enzymic pathways are responsible for nitrogen assimilation (Merrick & Edwards, 1995). The first pathway is probably the most important pathway and is ubiquitous in bacteria. It involves the enzymes glutamine synthetase (GS), which converts glutamate and ammonia to glutamine, as shown below, and glutamate synthase (GOGAT), which transfers the amide group from glutamine to 2-ketoglutarate producing two glutamate molecules. This predominantly occurs under ammonium limitation at medium concentrations of less than 0.1 mM.

$$NH_3 + \text{glutamate} + ATP \xrightarrow{GS} \text{glutamine} + ADP + P_i$$

$$\text{Glutamine} + \text{2-ketoglutarate} + NADPH \xrightarrow{GOGAT} 2\ \text{glutamate} + NADP^+$$

The second enzymic pathway of nitrogen metabolism utilizes glutamate dehydrogenase (GDH), and is bioenergetically more favourable since it ensures that

the organism acquires the optimal supply of nitrogen to the cell using the lowest consumption of energy. This pathway catalyses the reductive amination of 2-ketoglutarate to glutamate directly, as shown below and in most microorganisms, GDH is active under a high ammonium supply (greater than 1 mM). GDH and GOGAT activities have been detected in *Streptomyces venezuelae* (Shapiro & Vining, 1983) and *S. coelicolor* (Fisher, 1989), whereas only GOGAT activity has been shown in *Streptomyces clavuligerus* (Brana *et al.*, 1986).

$$NH_3 + \text{2-ketoglutarate} + NADPH \xrightarrow{GDH} \text{glutamate} + NADP^+$$

Proteins also serve as a valuable source of nitrogen and require proteases to solubilize them into free amino acids or peptides. Hodgson (2000) has suggested that streptomycetes should possess mechanisms to transport short peptide molecules since they will be the initial products of protease action, and this has already been demonstrated in many other bacteria and fungi. Organisms that can transport peptides would have a competitive advantage over one that simply must wait for free amino acids to be released.

Carbon and nitrogen metabolism can be controlled to some extent by the choice of carbon and nitrogen sources presented to an organism. This is especially important industrially, since the rate at which a nutrient source is metabolised can often influence the formation of biomass or production of secondary metabolites, this particularly applies to carbon sources and high concentrations of rapidly metabolizable sugars. Of course choice of nutrient source is largely dictated by cost,

availability and other economic factors and so empirical design of media supporting secondary metabolite formation is often employed together with complex carbon and nitrogen sources.

It is noted to date however, that carbon and nitrogen metabolism and regulation are much less well understood in the high G + C content, Gram-positive bacteria including the streptomycetes, and so further research into this area is required for a developed understanding.

1.3 *STREPTOMYCES CAPREOLUS* & CAPREOMYCIN

Capreomycin is a secondary metabolite produced by *Streptomyces capreolus* and was first described by Herr *et al.*, (1960). This antibiotic is active against a number of gram-positive and gram-negative organisms but is primarily effective against mycobacteria (Stark *et al.*, 1962). Treatment of pulmonary tuberculosis with capreomycin in combination with sodium para-aminosalicylate yields results similar to such treatment with isoniazid and sodium para-aminosalicylate (Popplewell *et al.*, 1962). The importance of capreomycin in the treatment of tuberculosis cannot be emphasized enough particularly with the emergence of new resistant strains of the disease.

Capreomycin can be classified as a cyclic peptide antibiotic related to the aminoglycosides whose mode of action is unclear, although it is believed to share similar chemical and biological characteristics to those of viomycin (Yoshioka *et al.*, 1971; Noda *et al.*, 1972). The proposed mode of action of viomycin is to block protein synthesis at 30S ribosomal initiation complex although the exact role of capreomycin in target cell disruption is mere speculation.

In 1962 it was originally proposed that capreomycin was composed of two components; capreomycins I and II, (Stark *et al.*, 1962), but by 1965 it had been established that four water soluble species were actually present, including capreomycins IA, IB, IIA and IIB (Stark & Boeck, 1965). Capreomycins IA and IIA (Fig. 3a, Fig. 3b) contain a hydroxyl group that is absent from IB and IIB (Fig. 3c, Fig. 3d) and also possess serine, whereas capreomycins IB and IIB contain alanine. Capreomycins IA and IB contain β-lysine, which is absent from IIA and IIB.

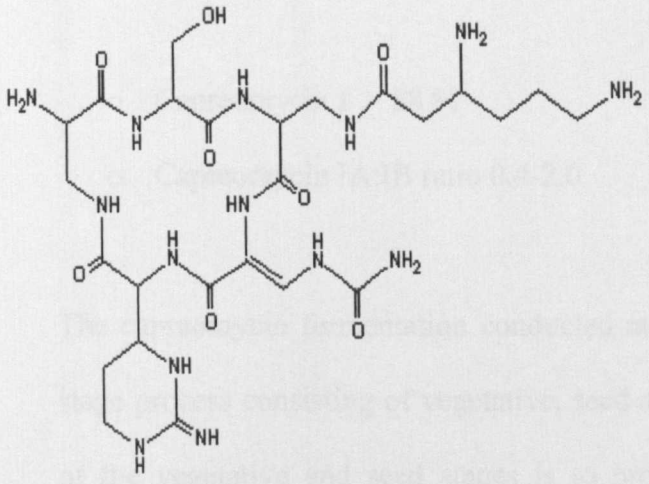
All components are based around a common cyclic peptide, have maximum ultraviolet absorbance at 266 nm and contain two moles of α, β-diaminopropionic

acid (DAPA) and one mole of α (2-iminohexahydro-4-pyrimidyl) glycine (PYR-GLY) (Herr & Redstone, 1966), with the latter being unique to the capreomycins. In addition to the amino acids detected in the total hydrolysates of the capreomycins, each component on acid hydrolysis releases ammonia (Herr, 1962).

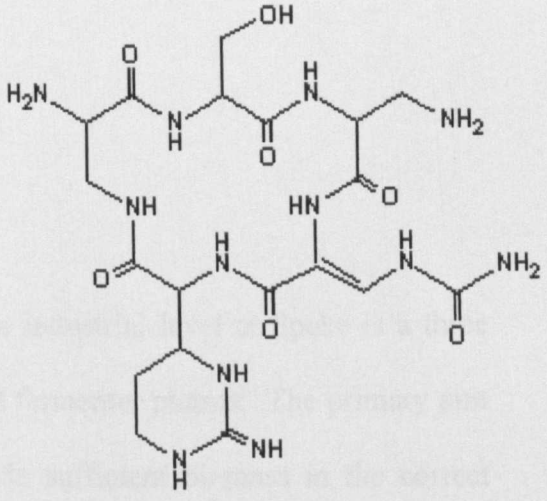
Herr & Redstone (1966) made various physical and chemical measurements on the four species and discovered that the percentages of carbon and hydrogen are similar in all. In addition, capreomycins IA and IB have similar nitrogen contents as do IIA and IIB, however, the latter contain more nitrogen overall. Elucidation of the molecular weights suggests that capreomycins IA and IB are larger molecules than IIA and IIB. With the information concerning the amino acid distribution among the species, it is possible to estimate the percentage of each of the four components of the capreomycin complex. Herr & Redstone (1966) calculated the percentage of each component in a representative lot of antibiotic to be around 25 % IA, 66 % IB, 3 % IIA and 6 % IIB and elucidated that capreomycins IA and IB are approximately two and a half times more active against *Mycobacterium butyricum* and *Klebsiella pneumoniae* than IIA and IIB, hence the clinical agent used for treatment of tuberculosis is a combination of the more active components of the capreomycin complex.

Figure 3. Structure of the capreomycins found in fermentation broth.

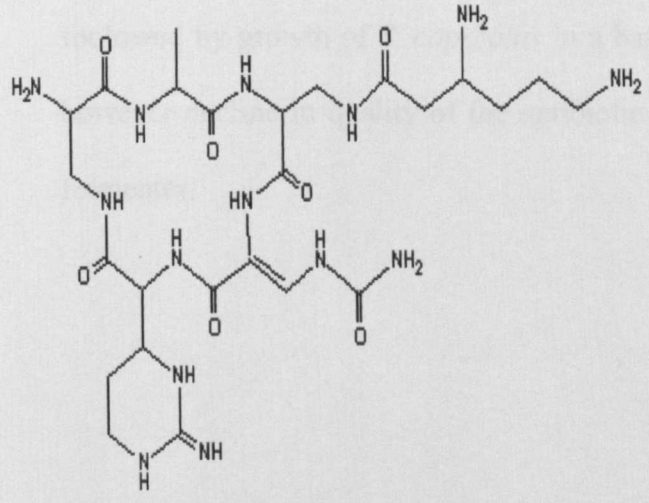
3.a) Capreomycin IA



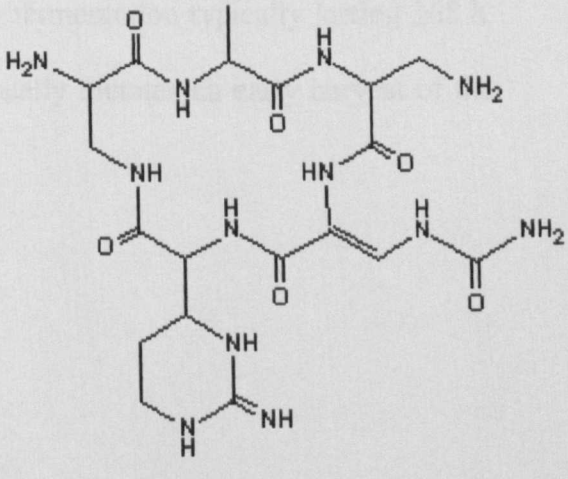
3.b) Capreomycin IIA



3.c) Capreomycin IB



3.d) Capreomycin IIB



Eli Lilly and Company Limited manufacture capreomycin on behalf of the World Health Organization (WHO) and must meet criteria set out by the Food and Drug Administration (FDA). These guidelines govern the quality of the antibiotic produced and typically they specify that:

- Capreomycin I > 88 %
- Capreomycin IA:IB ratio 0.4-2.0

The capreomycin fermentation conducted at an industrial level at Speke is a three stage process consisting of vegetative, seed and fermenter phases. The primary aim of the vegetative and seed stages is to provide sufficient biomass in the correct physiological state to maximise productivity in the final 7000 L fermenter stage. During growth in the fermenter vessel, agitation and aeration are ramped up and down to satisfy the oxygen demand of the organism thus avoiding oxygen limitation and to ensure vigorous growth.

A typical vegetative culture is grown for 72 h, followed by a 30 – 40 h seed stage foolowed by growth of *S. capreolus* in a batch fermentation typically lasting 168 h, however decline in quality of the antibiotic usually dictates an early harvest of the fermenter.

1.3.1 TUBERCULOSIS & MULTI-DRUG RESISTANT TUBERCULOSIS (MDR-TB)

Tuberculosis has re-emerged as one of the leading causes of death in the world today (Bloom & Murray, 1992), with an estimated 8.89 million new cases emerging every year of which there are 52,000 deaths per week or more than 7,000 each day (WHO, 1997a). This astonishing figure is overshadowed by the fact that one-third of the global population of humans is asymptomatically infected with tubercle bacilli (Sudre *et al.*, 1992). Such high death rates are surprising since treatments for this disease, in the form of vaccines or chemotherapy via anti-tuberculosis drugs have been around for more than fifty years (Kramnik *et al.*, 2000).

Many cases of clinically active tuberculosis arise from reactivation of an infection acquired years before the onset of symptoms and these reflect the re-emergence of actively growing organisms from an apparently non-replicative state (Barnes *et al.*, 1996; Le & Davidson, 1996). *Mycobacterium tuberculosis*, the causative agent of tuberculosis is renowned for being a particularly slow growing organism and can persist in a latent form in many individuals. It is these attributes that dictate the length of chemotherapy treatments: typically six to nine months (Bloom & McKinney, 1999). This prolonged treatment can cause increased problems of side effects and toxicity, and many un-monitored patients do not adhere to the proposed regime for their treatment, this in turn has lead to the development of multi-drug resistant tuberculosis (MDR-TB) infections. Chemotherapy is thus achieved by the administration of directly observed treatment short-course programme (DOTS) and appears to be the most effective way to ensure a decrease in primary resistance, acquired resistance and relapses (WHO, 1997b). This particular form of treatment however also has its downfalls, particularly in Asian countries such as Indonesia, China and India where poor treatment practices in the private sector lessen the

impact of the DOTS programme and contribute to a growing incidence of MDR-TB. This problem is further escalated by the availability of anti-tuberculosis drugs without prescription, and the rapid emergence of HIV-TB co-infection cases which often result in adverse drug effects, which in turn leads to incompliance of DOTS programmes and consequently increased cases of drug resistance (Phyu *et al.*, 2003). Cases of MDR-TB are further intensified through patients who fail to disclose previous exposures to anti-tuberculosis drugs, indeed a World Health Organization/International Union Against Tuberculosis and Lung Diseases global survey in the year 2000 showed that the prevalence of resistance to at least one anti-tuberculosis drug including isoniazid, rifampicin, ethambutol and streptomycin, among new cases ranged from 1.7 % to 36.9 %. Unless adequate measures are enforced to contain outbreaks and treatments of MDR-TB, then this disease has the capacity to become a global killer, it is therefore out of necessity that we must begin to understand the impact of the remaining tuberactinomycins, the group of cyclic peptide antibiotics which include capreomycin and viomycin that still possess the properties to successfully treat tuberculosis. The importance of this family of antibiotics, especially since they comprise the final line in defence of MDR-TB is reflected in their inclusion in the World Health Organization's list of Essential Medicines (2002). In addition to their importance in the treatment of tuberculosis, the tuberactinomycins have become of interest for their use in the treatment of other bacterial infections including those caused by methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (Dirlam *et al.*, 1997: Linde *et al.*, 1997: Lyssikatos *et al.*, 1997). The obvious advantages of these findings unfortunately outweigh the implications that will prevail at a later time, when resistance to these drugs eventuates.

1.3.2 CAPREOMYCIN & TREATMENT OF MDR-TB

Capreomycin has recently become a vital therapeutic agent for the treatment of MDR-TB, and is of special interest for the treatment of tuberculosis as it is active against streptomycin, rifampicin, amikacin, and viomycin resistant organisms (Herr *et al.*, 1962). It is prominently used as a secondary anti-tuberculous drug given intramuscularly with other appropriate drugs in severe cases, in patients with resistant forms of the tubercle bacilli (Donomae, 1966). This is largely due to adverse reactions often experienced with its use which include tinnitus, and vertigo with a lesser risk of deafness. Kidney damage may occur with elevation of serum and urine creatine. Hypokalaemia, hypocalcaemia and hypomagnesaemia have also been reported (WHO, 2003). It must be remembered however, that these side effects are fairly rare and are noted in most sources as necessity, indeed they are similar to those that may be experienced with use of the common classic antibiotic streptomycin and do not appear to be as toxic as the other aminoglycosides neomycin and kanamycin.

In 2006 the emergence of a critical new global threat, XDR-TB (Extensive/Extreme Drug Resistant Tuberculosis) has called for tuberculosis treatment measures to be strengthened and implemented to control the spread of this disease that has the potential to claim millions of lives. XDR-TB has emerged from poorly managed TB care. The problems that led to the emergence of MDR-TB in the first instance such as non-adherence to treatment programmes, incorrect drug prescribing practices, poor quality drugs or erratic supply of these drugs has further evolved to a new critical level. In cases of XDR-TB, patients may be resistant to three or more of the six classes of second-line drugs which leaves them with a condition that is virtually

untreatable. The six classes of second-line are those listed in the WHO Guidelines for the Management of Drug Resistant Tuberculosis (WHO, 1997b), and include:

- Aminoglycosides (kanamycin, amikacin)
- Polypeptides (viomycin, capreomycin)
- Fluoroquinolines (levofloxacin, ofloxacin, ciprofloxacin)
- Cycloserine (terizidone)
- Thioamides (ethionamide, prothionamide)
- Para-aminosalicylic acid (PAS)

Recent findings from a survey conducted by WHO and the US Centres for Disease Control and Prevention (CDC), published in the CDC Morbidity and Mortality Weekly Report (MMWR) on World TB day (24th March, 2006) on data from 2000-2004, found that XDR-TB has been identified in all regions of the world but is most frequent in the countries of the former Soviet union and in Asia (MMWR, 2006). XDR-TB poses a grave public health threat, especially in populations with high rates of HIV and where there are few health care resources.

The WHO has outlined recommendations for the treatment and control of this new form of TB in Guidelines for the Programmatic Management of Drug Resistant Tuberculosis (WHO, 2006). These include plans to:

- Strengthen basic TB care to prevent the emergence of drug-resistance.
- Ensure prompt diagnosis and treatment of drug resistant cases to cure existing cases and prevent further transmission.

- Increase collaboration between HIV and TB control programmes to provide necessary prevention and care to co-infected patients.
- Increase in investment in laboratory infrastructures to enable better detection and management of resistant cases.

2.0 MOTIVATION & AIMS

Production of capreomycin on an industrial scale can often be limited due to variability issues surrounding the process. This coupled with the distinct lack of research carried out on *S. capreolus* does not afford a consistently stable, predictable and reliable process. This presents serious issues in light of the escalating problem of MDR-TB throughout the world and is significant more so now than ever.

The lack of physiological and biochemical knowledge surrounding *S. capreolus* prevents development of an improved high yielding, stable process that would provide much needed antibiotic to those countries affected by this disease.

The aim of this research is thus to gain a better understanding of the physiology of *S. capreolus* and the factors which govern its growth in submerged fermentation. These findings will be linked to the impact that they have on capreomycin synthesis. Ultimately, novel research into this poorly understood process will highlight organism/media interactions in the current complex medium and form the basis to enable the development of a chemically defined medium at a later stage.

The current medium employed at Eli Lilly & Company to produce capreomycin at large scale consists of complex carbon and nitrogen sources and selected minerals which are believed to introduce variability into the process but have been vital for many years for capreomycin production. It is therefore of consequence that these raw materials present considerable variation between batches at the biochemical level leading to frequent deviations in biomass formation and productivity. Use of such ill-defined raw materials can also prove problematic at the downstream recovery and purification level of a process, thereby increasing costs at the end stages of a fermentation. Economic constraints in large-scale processes make cheap

by-products, such as beet molasses from the sugar industry and corn steep liquor from the starch industry favourable sources of carbon, nitrogen, vitamins and minerals, however residual components can interfere and even inhibit growth and metabolite production.

It is the presence of these poorly defined components which may enhance or inhibit antibiotic production, it is therefore of great importance to understand the benefits that they impart on the process and the way in which they drive the metabolic processes of *S. capreolus*.

The following criteria will be investigated to aid in the physiological study of *S. capreolus* and capreomycin:

- Determine the importance of media components and understand the interactions they exhibit with *S. capreolus*, both on a biochemical and morphological level. Are media components used sequentially or preferentially and do they have the potential to inhibit metabolism?

- The nature and the quantity of the carbon and nitrogen source largely governs antibiotic production. What is the significance of these components to *S. capreolus*, and how are they related to capreomycin synthesis?

- What are the contributory factors that regulate growth of *S. capreolus* and capreomycin production, and to what extent can they be regulated?

3.0 MATERIALS & METHODS

3.1 ORGANISM

Streptomyces capreolus master production strain C460.0 1999 was acquired from Eli Lilly and used throughout this study. The development cell bank was maintained primarily as a frozen suspension of mycelial broth culture contained within sealed straws and kept at -80 °C.

3.2 GROWTH ON SOLID MEDIA

A number of media were tested to determine the ability of the organism to sporulate as a means of gaining frozen spore stocks for the sub-culture of the organism as opposed to returning to the master strain for subsequent inoculations.

All media used were prepared using 1 litre distilled water and autoclaved at 121 °C for 15 min. Solid media plates contained a volume of 20 ml per plate and were incubated at 30 °C.

MANNITOL SOYA AGAR (MS AGAR) (HOBBS *et al.*, 1989)

20.0 $g.L^{-1}$	D-Mannitol (Sigma)
20.0 $g.L^{-1}$	Soya Flour (Holland and Barratt)
20.0 $g.L^{-1}$	Bacteriological Agar No. 1 (Oxoid)

YEAST EXTRACT MALT EXTRACT MEDIUM (YEME) (HOPWOOD *et al.*, 1985)

4.0 $g.L^{-1}$	Yeast Extract (Oxoid)
10.0 $g.L^{-1}$	Malt Extract (Sigma)
4.0 $g.L^{-1}$	Glucose (BDH)
20.0 $g.L^{-1}$	Bacteriological Agar No. 1 (Oxoid)

BENNETT'S MODIFIED AGAR (JONES, 1949)

1.0 $g.L^{-1}$	Yeast extract (Oxoid)
1.0 $g.L^{-1}$	Beef extract (Oxoid)
2.0 $g.L^{-1}$	NZ-Amine
10.0 $g.L^{-1}$	Glucose
15.0 $g.L^{-1}$	Bacteriological Agar No. 1 (Oxoid)

3.3 INOCULUM PREPARATION

3.3.1 SHAKEFLASK CULTURE

For subsequent inoculations, one master straw was used to inoculate vegetative liquid medium. This was prepared in 1.0 litre of distilled water and 50 ml was dispensed into 250 ml conical shakeflasks which were then autoclaved at 121 °C for 15 min. Following incubation at 30 °C, at 250 rpm on an orbital shaker for 72 h, the culture was divided into aliquots (1 ml) into sterile eppendorf tubes and stored at -80 °C using 10 % glycerol as the cryopreservant. Subsequent vegetative shakeflasks were inoculated from this cell bank using a 1 ml eppendorf, and after 72 h 1 ml was transferred to each shakeflask.

VEGETATIVE LIQUID MEDIUM (ELI LILLY)

15.0 $g.L^{-1}$	Glucose (Eli Lilly)
15.0 $g.L^{-1}$	Dried yeast (Eli Lilly)
0.22 $ml.L^{-1}$	Polyglycol P2000 (Eli Lilly)

pH adjusted to 6.8 prior to autoclaving using 1.0 M sodium hydroxide.

3.4 MEDIA & GROWTH CONDITIONS

Investigations were carried out on *S. capreolus* in a variety of liquid media, each was inoculated using 1 ml from a 72 h vegetative flask.

COMPLEX CAPREOMYCIN PRODUCTION MEDIUM (ELI LILLY)

30.0 $g.L^{-1}$	Glucose (Eli Lilly)
4.0 $g.L^{-1}$	*NZ-Amine Type A (Eli Lilly)
0.3 $g.L^{-1}$	$CaCl_2$ (Eli Lilly)
2.5 $g.L^{-1}$	$MgSO_4.7H_2O$ (Eli Lilly)
29.2 $g.L^{-1}$	†Gelita Sol P (Eli Lilly)
7.0 $g.L^{-1}$	Beet Molasses (Eli Lilly)
0.5 $g.L^{-1}$	Sodium Edetate (Eli Lilly)
0.2 $g.L^{-1}$	Polyglycol P2000 (Eli Lilly)

* Complex porcine derived peptone hydrolysate

† Complex bovine derived peptone hydrolysate

pH adjusted to 8.0 prior to autoclaving using 1.0 M sodium hydroxide.

Eli Lilly has recently provided a manufacturing firm in China (Hisun) with the technology to convert existing facilities over to the production of capreomycin, thereby increasing the supply of the product to the Far East and Russia. The medium used at the manufacturing facilities is different to the complex medium employed by Eli Lilly, and in most cases gives rise to a higher antibiotic yield, however due to raw material availability and stringent FDA regulations it has only been reconstructed using Eli Lilly raw materials at a developmental level on site.

PORCINE PEPTONE LIQUID MEDIUM (ELI LILLY/HISUN)

30.0 $g.L^{-1}$	Glucose (Eli Lilly)
4.0 $g.L^{-1}$	NZ-Amine Type A (Eli Lilly)
0.3 $g.L^{-1}$	$CaCl_2$ (Eli Lilly)
2.5 $g.L^{-1}$	$MgSO_4.7H_2O$ (Eli Lilly)
29.2 $g.L^{-1}$	Porcine Peptone (Hisun)
15.0 $g.L^{-1}$	Starch (Eli Lilly)
0.5 $g.L^{-1}$	Sodium Edetate (Eli Lilly)
0.2 $g.L^{-1}$	Polyglycol P2000 (Eli Lilly)

pH adjusted to 8.0 prior to autoclaving using 1.0 M sodium hydroxide.

Due to the complexity of the production medium employed by Eli Lilly, it was deemed necessary to encourage growth of the organism in a basal minimal medium to provide a basis for analytical work where the carbon and nitrogen source is known.

MINIMAL LIQUID MEDIUM (MODIFIED FROM; MEIWES *et al.*, 1990)

20.0 $g.L^{-1}$	Glucose (Sigma)
2.0 $g.L^{-1}$	NZ-Amine (Eli Lilly)
0.5 $g.L^{-1}$	NaCl (BDH)
0.2 $g.L^{-1}$	$MgSO_4.7H_2O$ (Prolabo)
0.1 $g.L^{-1}$	$CaCl_2.2H_2O$ (BDH)
0.002 $g.L^{-1}$	$ZnSO_4.7H_2O$ (BDH)
1.0 $g.L^{-1}$	KH_2PO_4 (BDH)
0.01 $g.L^{-1}$	$FeSO_4.7H_2O$ (BDH)

pH adjusted to 7.5 prior to autoclaving using 1.0 M sodium hydroxide.

DEFINED LIQUID MEDIUM + 20 AMINO ACIDS

20.0 $g.L^{-1}$	Glucose (Sigma)		
0.5 $g.L^{-1}$	NaCl (BDH)		
0.2 $g.L^{-1}$	$MgSO_4.7H_2O$ (Prolabo)		
0.1 $g.L^{-1}$	$CaCl_2.2H_2O$ (BDH)		
0.002 $g.L^{-1}$	$ZnSO_4.7H_2O$ (BDH)		
1.0 $g.L^{-1}$	KH_2PO_4 (BDH)		
0.01 $g.L^{-1}$	$FeSO_4.7H_2O$ (BDH)		
0.2 $g.L^{-1}$	L-Alanine	L-Leucine	
	L-Arginine HCl	L-Lysine HCl	
	L-Asparagine	L-Methionine	
	L-Aspartic Acid	L-Phenylalanine	
	L-Cysteine	L-Proline	(Sigma)
	L-Glutamic Acid	L-Serine	
	L-Glutaine	L-Threonine	
	Glycine	L-Tryptophan	
	L-Histidine HCl	L-Tyrosine	
	L-Isoleucine	L-Valine	

pH adjusted to 7.5 prior to autoclaving using 1.0 M sodium hydroxide.

CONTINUOUS CULTURE

S. capreolus was grown in continuous culture as a means of maintaining the cell population in exponential growth for long periods of time.

Within the continuous culture (chemostat), the rate at which the broth is diluted governs the growth rate, and the population size is governed by the concentration of the growth limiting nutrient entering the fermentation vessel.

S. capreolus has never before been grown as a continuous culture and so a novel insight would be gained into the behaviour of the organism under such conditions.

The contents of a vegetative flask (50 ml) grown for 72 h was used to inoculate a 2.0 litre fermenter (Applikon, UK) with a 1.8 litre working volume (vessel height 25 cm, vessel diameter 10 cm). Agitation was maintained at 300 rpm facilitated by a rushton type impeller (6 blade, diameter 5 cm), which was 3 cm from the base of the fermenter and 13 cm below a single marine impeller (3 blade, diameter 5 cm). The temperature of the broth culture was maintained at 30 °C and pH was continuously controlled at 7.5 by the addition of 1.5 M sodium hydroxide or 1.5 M sulphuric acid via an ADI 1030 Biocontroller (Applikon, UK).

Following a batch fermentation of 5 days, a continuous supply of fresh, sterile media was pumped into the fermenter.

The dilution rate of the culture was determined by the following equation:

$$D = \frac{F}{V}$$

$$D = \mu$$

$$td = \frac{\ln 2}{\mu}$$

Where: D = dilution rate

F = medium flow rate (L. h^{-1})

V = volume of fermenter (L)

μ = growth rate (h^{-1})

td = doubling time (h^{-1})

CARBON LIMITED GROWTH

9.0 $g.L^{-1}$	Glucose (Sigma)
2.1 $g.L^{-1}$	Serine (Sigma)
0.5 $g.L^{-1}$	NaCl (BDH)
0.2 $g.L^{-1}$	$MgSO_4.7H_2O$ (Prolabo)
0.1 $g.L^{-1}$	$CaCl_2.2H_2O$ (BDH)
0.002 $g.L^{-1}$	$ZnSO_4.7H_2O$ (BDH)
1.0 $g.L^{-1}$	KH_2PO_4 (BDH)
0.01 $g.L^{-1}$	$FeSO_4.7H_2O$ (BDH)

pH adjusted to 7.5 prior to autoclaving using 1.0 M sodium hydroxide.

Carbon limited growth was found to occur at a C/N ratio of 18:1 and below.

NITROGEN LIMITED GROWTH

18.0 $g.L^{-1}$	Glucose (Sigma)
2.1 $g.L^{-1}$	Serine (Sigma)
0.5 $g.L^{-1}$	NaCl (BDH)
0.2 $g.L^{-1}$	$MgSO_4.7H_2O$ (Prolabo)
0.1 $g.L^{-1}$	$CaCl_2.2H_2O$ (BDH)
0.002 $g.L^{-1}$	$ZnSO_4.7H_2O$ (BDH)
1.0 $g.L^{-1}$	KH_2PO_4 (BDH)
0.01 $g.L^{-1}$	$FeSO_4.7H_2O$ (BDH)

pH adjusted to 7.5 prior to autoclaving using 1.0 M sodium hydroxide.

Nitrogen limited growth was found to occur at a C/N ratio of 33:1 and above.

BIOMASS DETERMINATION

Biomass concentration was determined as a dry cell weight value whereby, 1 ml of culture was added to a pre-weighed cellulose nitrate 0.45 μm, 25 mm filter membrane (Whatman, UK) and mycelia washed twice with 1 ml distilled water. Filters were then dried to a constant weight in a 700-W microwave oven set to defrost for 2 min before finally being reweighed.

IMPRESSION MOUNT

In order to visualize spore formation using light microscopy, impression mounts were carried out. A coverslip was gently placed on top of the culture grown on solid medium and lowered onto a microscope slide on which a drop of distilled water had been placed. Cells were examined at x 400 magnification (Phaco 2 lens) using a Leitz, Laborlux 12 phase-contrast microscope (Leitz Wetzlar, Germany).

SCANNING ELECTRON MICROSCOPY

A segment of solid culture was removed and added to a bijoux bottle containing 25 % v/v glutaraldehyde (BDH) and left at room temperature for 1 hour. The sample was washed 10 times in succession with distilled water and the concentration of ethanol (BDH) was gradually increased by 20 % v/v until 100 % v/v ethanol concentration was reached.

The sample was then dried under vacuum and coated using a Polaron E5000 Gold sputter coater then examined using a Jeol JSM 840 scanning electron microscope (Jeol Instruments, Japan). Images were captured using PGT Spirit image capture and x-ray analysis software.

FLUORESCENT MYCELIAL STAINING

*Bac*Light™ LIVE/DEAD bacterial viability stain (Molecular Probes, USA) can be used to determine hyphal viability in actinomycetes (Sebastine *et al.*, 1999). It comprises two nucleic acid stains; SYTO 9 and propidium iodide (PI). The former is a green intercalating membrane permeant fluorescing dye that stains all nucleic acid and emits green light under blue excitation, whilst the latter is a well established and accepted membrane impermeant stain (Davey & Kell, 1996; Nebe-von-Caron *et al.*, 2000), that is excluded by healthy cells, and stains cells that are considered to have failed to maintain membrane integrity due to loss of viability, emitting red light under green excitation (Stocks & Thomas, 2001).

PREPARATION OF STAIN

The working stock of the *Bac*Light solution contained the following components;

1.5 µl SYTO 9

1.5 µl Propidium iodide

500 µl Distilled water

This solution was stored at -20 °C and used within one week.

MICROSCOPIC IMAGE ANALYSIS

S. capreolus was stained with the LIVE/DEAD® *Bac*Light™ bacterial viability kit (Molecular Probes, L-7012), whereby the two fluorescent stains (SYTO 9 and propidium iodide) are both present separately at 1.67 mM in anhydrous DMSO allowing the staining conditions to be adjusted accordingly. For fluorescence microscopy observations the concentrations of the stains were as above.

For image analysis, samples were aseptically removed from the shakeflasks, and 2.5 µl of broth was added to a microscope slide to which 2.5 µl *Bac*Light stain was also added. A coverslip was applied and the slide kept in the dark for 5 min before microscopic image analysis.

Excitation of the two dyes in the samples occurred at 490 nm, and emissions were detected at 517 nm for SYTO 9 and 605 nm for propidium iodide. Image analysis was performed using an Olympus BX51 microscope, connected to a Hamamatsu ORCA-ER (Japan) digital camera. An image was taken for each sample at each emission and the photographs merged using OpenLab analysis software by Improvision.

Photographs were further digitally analysed using Jasc Paint Shop Pro 8 to determine the percentage viability of the 2D image on a number of fields of view.

FLUORESCENCE VIABILITY ASSAY

Quantitative measurements of viability were carried out every 24 h in microtitre plates. Each well contained 40 µl broth sample and 60 µl *Bac*Light stain (comprising SYTO 9 and propidium iodide, at the same concentration as used for microscopic image analysis as outlined previously). Samples were allowed to equilibrate in the dark for 5 min before being analysed within 15 min in a Varian Cary Eclipse fluorometer (UK). Optimal incubation times have previously been determined and state that samples should be incubated between 15 and 20 min, at room temperature in the dark (Boulos *et al.*, 1999).

Both dyes were excited at 485 nm, and emission 1 (SYTO 9) occurred at 530 nm, whilst emission 2 (propidium iodide) occurred at 630 nm. Figure 4 shows typical emission spectra for SYTO 9 and propidium iodide.

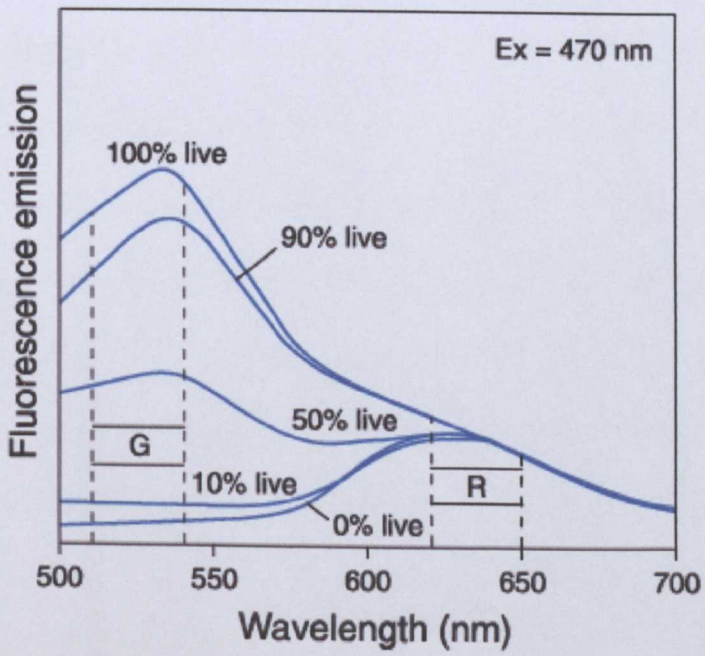
The excitation and emission slit width was set to 5 nm throughout the study as this was found to be the optimum setting for spectra analysis.

Results for both emissions for each well were used to calculate the ratio of green to red based on a calculation recommended by Molecular Probes;

$$\text{Ratio}_{\text{Green/Red}} = \frac{\text{Fluorescence intensity}^{\text{Emission 1}}}{\text{Fluorescence intensity}^{\text{Emission 2}}}$$

Whereby, emission 1 accounts for the green (viable) cells and emission 2 accounts for the red (non-viable) cells. The higher the ratio, the higher the proportion of viable mycelia to non-viable mycelia.

Figure 4. Typical emission spectra for *Bac*Light bacterial viability stain, comprising SYTO 9 and propidium iodide, used with *Escherichia coli* as the sample organism. Diagram taken from handbook of technical recommendations for *Bac*Light by Molecular Probes.



VIABILITY RESPONSE TO NaCl & pH

The viability response of *S. capreolus* to factors including pH and NaCl addition were investigated.

NaCl was included within the Complex medium at the following concentrations: 2.0 M, 1.75 M, 1.5 M, 1.25 M, 1.0 M, 0.75 M, 0.5 M, 0.25 M, 0.125 M and 0 M. Flasks were pH adjusted to 8.0 with 1 M NaOH prior to autoclaving. They were then inoculated as described in media and growth conditions and incubated at 30 °C on an orbital shaker at 250 rpm for 168 h. Image analysis was performed and dry cell weights were measured at 168 h. The live/dead fluorescence viability assay was carried out on samples at 168 h together with analysis of capreomycin concentration.

RADIOACTIVE UPTAKE STUDIES

Glucose is an important source of carbon for many organisms in industrial processes, and the growth of *S. capreolus* is no exception to this. Indeed it is believed to be an important structural precursor for the carbon backbone of capreomycin, and so has always been vital to the process to date. Hoskisson *et al.*, (2003) have found that the uptake of glucose is significantly reduced in the presence of certain amino acids by the actinomycete *Micromonospora echinospora*, and so it would be of significant value to the existing process if the uptake of glucose was examined along with potential inhibitory factors of uptake. This would help to identify any components within the complex production medium that would discourage sugar uptake.

UL-^{14}C-glucose (Sigma, UK) was used as the substrate in the following studies:

Biomass was collected and centrifuged at 4,000 g for 5 min at 18 °C and subsequently washed three times in the equivalent volume of Minimal medium (MM) base (Table. 1), containing no carbon or nitrogen source. The biomass was then re-suspended in 2.0 ml of MM base supplemented with carbon in the form of sugars at 2.0 g. L^{-1} or nitrogen in the form of amino acids at 0.2 g. L^{-1} and the cells were then equilibrated for 5 min on a heated stirrer at 30 °C. An aliquot (20 µl) of UL-^{14}C-glucose (Sigma, UK) was added at 0.01 µCi/µl. At 2 min, 250 µl was removed and immediately quenched in 2.3 ml of 1 M, ice-cold non-radiolabelled glucose. Samples were filtered under low pressure through 0.2 µm nitrocellulose membranes (Whatman, UK), washing once in 2.3 ml of MM base. Membranes were then added to 4.0 ml of Optiphase scintillation fluid (Fisher, UK) and counted for 5 min. For radiolabelled experiments whereby the effect on glucose uptake in the

presence of other sugars was examined, the unlabelled sugar was balanced (w/v) with unlabelled glucose at a ratio of 1:1.

For the determination of K_s, the biomass was re-suspended in 1800 µl of MM base containing no carbon or nitrogen source to which 200 µl of sugar at a known concentration was added. Cells were equilibrated for 5 min followed by the addition of UL-^{14}C-glucose at the appropriate concentration. Every 30 s for 2.5 min, 250 µl was removed and immediately quenched in 1 M ice-cold non-radiolabelled glucose as before, prior to being filtered and counted.

Table 1. Minimal Medium Base (MM Base) for radioactivity studies.

NaCl (BDH)	0.5 g. L^{-1}
$MgSO_4.7H_2O$ (Prolabo)	0.2 g. L^{-1}
$CaCl_2.2H_2O$ (BDH)	0.1 g. L^{-1}
$ZnSO_4.7H_2O$ (BDH)	0.002 g. L^{-1}
KH_2PO_4 (BDH)	1.0 g. L^{-1}
$FeSO_4.7H_2O$ (BDH)	0.01 g. L^{-1}

BEET MOLASSES REPLACEMENT

Beet molasses contributes an economical source of carbohydrate for many industrial processes, including capreomycin production. This ill-defined by-product of the sugar refining industry provides an economically feasible substrate affording sugars, vitamins and minerals to a process.

Due to the nature of the production of this complex substrate, variability between batches is common and thus presents the same problem for the routine production of capreomycin at large scale as the complex animal derived peptones NZ-Amine and Gelita Sol P. It is therefore beneficial for Eli Lilly to move away from such raw materials, replacing them ultimately with a chemically defined medium in a bid to reduce process variability and to enable the better prediction of antibiotic yield.

A beet molasses replacement medium was obtained (Godfrey Schmidt – Kerry Bioscience) that was used to replace beet molasses in a yeast production medium at large-scale. A 20 run Plackett-Burman design (Plackett & Burman, 1946), for 13 variables and 6 dummy variables has been devised based on values of x suggested by Bull *et al.*, (1990). This is shown in Table 2. and enables the effects of each component within the suggested media to be examined independently at high and low levels in order to determine the variables most important for capreomycin production.

It thereby follows that capreomycin yield for each run was determined in order to calculate the effect of each independent variable.

Table 2. A 20 (x) run Plackett-Burman design for 13 variables and 6 dummy variables (x-1=19). High levels denote levels suggested in beet molasses replacement medium whilst low levels represent concentrations at a tenth of these.

	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*A*	*B*	*C*	*D*	*E*	*F*
1	H	H	L	L	H	H	H	H	L	H	L	H	L	L	L	L	H	H	L
2	L	H	H	L	L	H	H	H	H	L	H	L	H	L	L	L	L	H	H
3	H	L	H	H	L	L	H	H	H	H	L	H	L	H	L	L	L	L	H
4	H	H	L	H	H	L	L	H	H	H	H	L	H	L	H	L	L	L	L
5	L	H	H	L	H	H	L	L	H	H	H	H	L	H	L	H	L	L	L
6	L	L	H	H	L	H	H	L	L	H	H	H	H	L	H	L	H	L	L
7	L	L	L	H	H	L	H	H	L	L	H	H	H	H	L	H	L	H	L
8	L	L	L	L	H	H	L	H	H	L	L	H	H	H	H	L	H	L	H
9	H	L	L	L	L	H	H	L	H	H	L	L	H	H	H	H	L	H	L
10	L	H	L	L	L	L	H	H	L	H	H	L	L	H	H	H	H	L	H
11	H	L	H	L	L	L	L	H	H	L	H	H	L	L	H	H	H	H	L
12	L	H	L	H	L	L	L	L	H	H	L	H	H	L	L	H	H	H	H
13	H	L	H	L	H	L	L	L	L	H	H	L	H	H	L	L	H	H	H
14	H	H	L	H	L	H	L	L	L	L	H	H	L	H	H	L	L	H	H
15	H	H	H	L	H	L	H	L	L	L	L	H	H	L	H	H	L	L	H
16	H	H	H	H	L	H	L	H	L	L	L	L	H	H	L	H	H	L	L
17	L	H	H	H	H	L	H	L	H	L	L	L	L	H	H	L	H	H	L
18	L	L	H	H	H	H	L	H	L	H	L	L	L	L	H	H	L	H	H
19	H	L	L	H	H	H	H	L	H	L	H	L	L	L	L	H	H	L	H
20	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L

BEET MOLASSES REPLACEMENT MEDIUM

The following components were incorporated into the Complex capreomycin production medium as an alternative to beet molasses, currently used at 7.0 g. L^{-1} in the Complex medium.

The following is based on the replacement of 500 kg of beet molasses and component amounts were adjusted accordingly via the following calculations;

471 ml water to replace 1 kg beet molasses, therefore in order to incorporate the 12 components (excluding glucose), but assess them individually, 471/12 = ~ 40 ml water per component.

The amounts of the components as outlined below were each added to 40 ml water. This volume would replace 1 kg of beet molasses, however, beet molasses is currently used at 7.0 g. L^{-1} in the Complex medium, therefore, 1000/7 = 142.85. So, 40/142.85 = 0.28 ml of each component (suspended in 40 ml water) is required in 1 L Complex medium to replace 7.0 g. L^{-1} beet molasses.

For the Plackett-Burman design, high levels were at the concentrations outlined on the following page. A 1 in 10 dilution of these components constituted the low level concentrations.

REPLACEMENT MEDIUM (SCHMIDT)

471 g	Water
500 g	[1]Glucose (Sigma)
15 g	[2]$MgSO_4.7H_2O$ (Prolabo)
12 g	[3]K_2SO_4 (BDH)
360 mg	[4]$CaCl_2.2H_2O$ (BDH)
240 mg	[5]$(NH_4)_2\,Fe(SO_4)_2.6H_20$ (BDH)
90 mg	[6]$ZnSO_4.7H_2O$ (BDH)
240 µg	[7]$CuSO_4.5H_2O$ (BDH)
250 mg	[8]Nicotinate (Sigma)
100 mg	[9]Pantothenate (Sigma)
220 mg	[10]Inositol (Sigma)
20 mg	[11]Thiamine (Sigma)
22 mg	[12]Pyridoxine (Sigma)
170 µg	[13]Biotin (Sigma)

*Numbers next to components signify variable number in Table 2.

pH adjusted to 8.0 prior to autoclaving using 1.0 M sodium hydroxide.

PLACKETT-BURMAN FOR COMPLEX PRODUCTION MEDIUM

Following the Plackett-Burman design to look at the variables in the replacement medium it seemed appropriate to investigate the importance of the major variables within the current Complex production medium and the impact that they have on capreomycin yield, quality and IA:IB, therefore a 12 run Plackett-Burman design for 6 variables and 5 dummy variables was carried out (Table 3).

For the Plackett-Burman design, high levels were at the concentrations outlined as below. A 1 in 10 dilution of these components constituted the low level concentrations.

COMPLEX MEDIUM VARIABLES

30.0 $g.L^{-1}$	[1]Glucose (Eli Lilly)
4.0 $g.L^{-1}$	[2]NZ-Amine Type A (Eli Lilly)
0.3 $g.L^{-1}$	[3]$CaCl_2$ (Eli Lilly)
2.5 $g.L^{-1}$	[4]$MgSO_4.7H_2O$ (Eli Lilly)
29.2 $g.L^{-1}$	[5]Gelita Sol P (Eli Lilly)
7.0 $g.L^{-1}$	[6]Beet Molasses (Eli Lilly)

*number next to component signifies variable number in Table 3.

pH adjusted to 8.0 prior to autoclaving using 1.0 M sodium hydroxide.

Table 3. A 12 (x) run Plackett-Burman design for 6 variables and 5 dummy variables (x-1=11). High levels denote levels used in current Complex production medium whilst low levels represent concentrations at a tenth of these.

	1	*2*	*3*	*4*	*5*	*6*	*A*	*B*	*C*	*D*	*E*
1	H	H	L	H	H	H	L	L	L	H	L
2	L	H	H	L	H	H	H	L	L	L	H
3	H	L	H	H	L	H	H	H	L	L	L
4	L	H	L	H	H	L	H	H	H	L	L
5	L	L	H	L	H	H	L	H	H	H	L
6	L	L	L	H	L	H	H	L	H	H	H
7	H	L	L	L	H	L	H	H	L	H	H
8	H	H	L	L	L	H	L	H	H	L	H
9	H	H	H	L	L	L	H	L	H	H	L
10	L	H	H	H	L	L	L	H	L	H	H
11	H	L	H	H	H	L	L	L	H	L	H
12	L	L	L	L	L	L	L	L	L	L	L

3.5 ANALYTICAL PROCEDURES

CAPREOMYCIN DETERMINATION

For the preparation of samples for capreomycin determination, 2.0 g of broth was weighed into a 100 ml volumetric flask before being filled to 100 ml with HPLC water (HiperSolv for HPLC, BDH). The flask was inverted several times to mix, before an aliquot of sample was filtered through a 0.45 μm nylon filter membrane (Whatman, UK). The initial eluent from filtration was discarded before collection of the prepared sample for HPLC analysis. This was to allow saturation of the filter with broth and capreomycin.

Capreomycin yield was determined at Eli Lilly using reversed-phase HPLC at 40 °C and wavelength 268 nm on a UV detector (Waters, USA). The injection volume was 10 μl with a flow rate of 1.0 ml. min^{-1} and the column a Spherisorb, 250 x 4.6 mm, CN, 5 μm analysis column (Phenomenex, USA). The chromatography was isocratic and utilised 60 % v/v of a 0.05 % w/v ammonium hydrogen sulphate solution (Biochemika, BDH) and 40 % v/v methanol (HiperSolv for HPLC, BDH) as the mobile phase. Samples were degassed by filtration through a 0.45 μm nylon filter membrane (Whatman, UK) and the mobile phase was helium sparged. Results were compared to a capreomycin sulphate standard (Eli Lilly).

GLUCOSE ASSAY (BARHAM & TRINDER, 1972)

Glucose concentrations were determined spectrophotometrically at 500 nm in a Uvicon 930, dual beam spectrophotometer (Kontron Instruments, USA) using the Boehringer-Mannheim Glucose Oxidase (GOD) assay (Sigma). This method is based on the following reaction to yield an indicator dye:

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{Glucose Oxidase}} \text{Gluconic acid} + H_2O_2$$

$$2H_2O_2 + \text{4-aminophenazone} + \text{Phenol} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine} + 4H_2O$$

An aliquot of reagent (1000 µl) was added to 10 µl of suitably diluted sample supernatant (which had previously been spun at 3,000 g in an Eppendorf microcentrifuge) in a cuvette, vortexed to mix for 10 sec, and left for 30 min at room temperature before reading at 500 nm against a distilled water/reagent blank in a Uvicon 930 dual beam spectrophotometer (Kontron Instruments, USA).

Glucose concentration :

$$\frac{\text{Absorbance (Sample)}}{\text{Absorbance (Standard)}} \times 5.55 = \text{mM}$$

AMMONIA ASSAY (FAWCETT & SCOTT, 1960)

The formation of ammonia was spectrophotometrically determined by the following reaction:

NH_4 + Phenol + Hypochlorite ⟶ Indophenol blue

Sodium Phenate: 25 g of phenol was dissolved in 80 ml of distilled water. 7.5 ml of 4 M sodium hydroxide was added and the volume made up to 100 ml with distilled water. This was stable at 4 °C for up to 1 month.

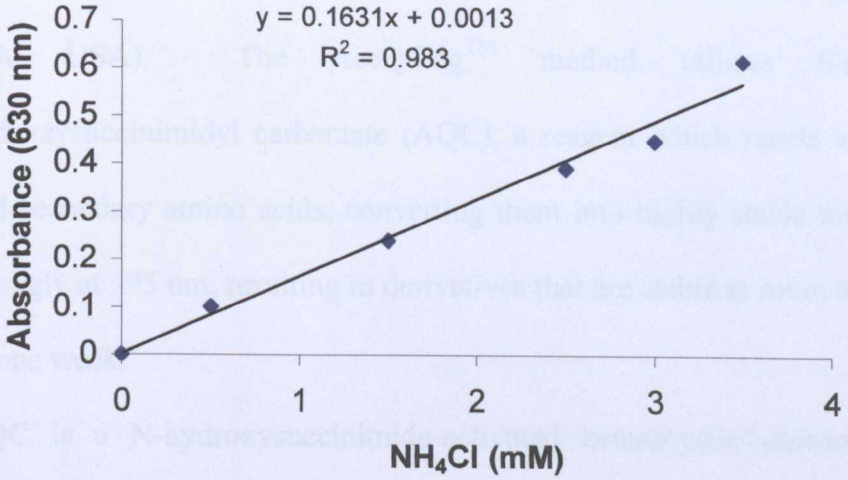
Sodium Nitroprusside: A stock solution was prepared of 0.1 % (w/v) in distilled water and diluted 1 in 10 prior to use.

Sodium Hypochlorite: This was diluted 1 in 5 prior to use.

Ammonium Chloride Standards: 0 – 350 µl of a 10 mM stock solution, made up to 1000 µl gave a range of standards with the concentrations 0 – 3.5 mM. These were stored at -20 °C.

An aliquot (20 µl) of suitably diluted supernatant was added to a cuvette followed by the 200 µl of sodium phenate, 300 µl of sodium nitroprusside and finally 300 µl of sodium hypochlorite. The samples were then vortexed for 10 sec and the reaction allowed to proceed at room temperature for 30 min. The absorbance readings for the samples and standards were determined at 630 nm in a Uvicon 930 dual beam spectrophotometer (Kontron Instruments, USA) against a distilled water/reagent blank.

Figure 5. Standard curve for ammonia assay.



AMINO ACID ANALYSIS

Free amino acids in the fermentation broth were analysed using reversed-phase HPLC coupled with a pre-column fluorescence derivatization technique, (Waters, MA, USA). The AccQ.Tag™ method utilises 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC), a reagent which reacts with both primary and secondary amino acids, converting them into highly stable ureas that fluoresce strongly at 395 nm, resulting in derivatives that are stable at room temperature for up to one week.

AQC is a N-hydroxysuccinimide-activated heterocyclic carbamate, and excess reagent hydrolyzes to produce 6-aminoquinoline (AMQ), N-hydroxysuccinimide (NHS) and carbon dioxide. The destruction of excess reagent is complete within one minute. The major hydrolysis product, AMQ, fluoresces weakly at 395 nm and produces a small peak that is easily resolved at the beginning of the chromatogram. NHS and carbon dioxide do not interfere with the chromatographic analysis. Using this technique, amino acid detection limits can be as low as 100-200 fmol.

The Waters reagent kit comprises:

AccQ.Fluor Borate Buffer (Sodium Tetraborate)

AccQ.Fluor Reagent Powder (AQC)

AccQ.Fluor Reagent Diluent (Acetonitrile)

AccQ.Tag Eluent A Acetate-phosphate buffer concentrate (Sodium Acetate, 19 % v/v, Phosphoric acid, 6 -7 % v/v) Triethylamine, 2 % v/v, Water, 72 -73 % v/v) 100 ml of concentrate is diluted in 1.0 L water (HiperSolv for HPLC, BDH).

FLUORESCENT DERIVATIZATION METHOD FOR AMINO ACIDS

The pre-formulated reagents in the Waters reagent kit were reconstituted before use by adding 1 ml of the reagent diluent to the reagent powder. This was mixed by vortexing and heated in a water bath at 55 °C for 10 min. This was stored in a dessicator for up to 1 week.

To derivatize the samples and amino acid hydrolysate standard, 60 µl of AccQ.Fluor borate buffer was added to 20 µl of sample and vortexed briefly. To this 20 µl of reconstituted AccQ.Fluor reagent was added and vortexed immediately for several seconds. This was allowed to stand at room temperature for 1 minute followed by heating in a water bath at 55 °C for 10 min.

A gradient controller (Waters, USA) connected to two 510 HPLC pumps (Waters, USA) utilised Eluent A (Acetate-phosphate buffer concentrate) and Eluent B (60 % v/v Acetonitrile, HiperSolv for HPLC, BDH) as the mobile phase, with a 5 µl injection volume, to resolve the peaks on a Jupiter, 250 x 4.60 mm, 4 µm Proteo 90A analysis column (Phenomenex, USA) at 37 °C and 395 nm on a scanning fluorescence detector (Waters, USA). All samples and mobile phases were degassed by sparging with helium.

4.0 RESULTS & DISCUSSION

4.1 GROWTH OF *STREPTOMYCES CAPREOLUS* ON SOLID CULTURE MEDIA

Over the years growth of *Streptomyces capreolus* has largely been confined to liquid media due to the demand for capreomycin production. It was therefore of great interest to investigate growth on solid media, particularly as a means of creating an inoculum, whereby agar plugs taken from plates would potentially be used. Regular re-plating of the organism would ensure continual cell lines and the constant availability of *S. capreolus* in exponential growth phase.

Mannitol soya agar, yeast extract malt extract agar and Bennett's modified agar has previously been used for growth and sporulation of *S. capreolus* on solid media (Stark & Boeck, 1965).

Growth proved to be problematic on all media examined, showing very slow or absent growth, and no apparent sporulation (Table 4). Bennett's agar supported the best growth, however confluency of the culture only occurred after approximately two weeks

Table 4. Growth characteristics of *S. capreolus* on solid media grown at 30 °C.

CULTURE MEDIUM	GROWTH CHARACTERISTICS
Mannitol Soya Agar	Poor growth, raised colonies, no sporulation
Yeast Extract Malt Extract Agar	Growth sparse, no sporulation
Bennett's Modified Agar	Growth confluent, powdery white appearance, no sporulation – mycelia highly fragmented

A primary observation of growth on Bennett's modified agar suggested signs of sporulation (Fig. 6), an attribution of the powdery white appearance of the plate that would normally characterize the appearance of aerial mycelia. This theory was disproved following an impression mount of the culture (Fig. 7), a method widely used as a means of spore detection on solid culture. Instead, mycelia appeared to be highly fragmented with cells ranging from filamentous to rod-like in structure. The absence of spores was verified by electron microscopy examination of the culture (Fig. 8).

Sporulation has so far been elusive in the production strain of *S. capreolus* both on solid and in liquid media, and would explain the storage and sub-culture of the organism as frozen mycelial stocks by Eli Lilly. A further benefit of using a mycelial starter inoculum is the reduction in the initial lag phase common with using spore stock suspensions. Mycelial inoculum was therefore used throughout this study for growth on both solid and liquid media.

Figure 6. Growth of *Streptomyces capreolus* on Bennett's modified agar at 336 h, following incubation at 30 °C.

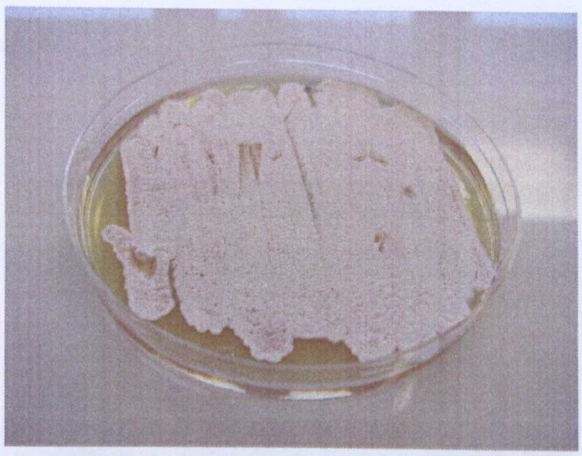


Figure 7. Impression mount taken from Bennett's modified agar (x 40 magnification) at 336 h, following incubation at 30 °C.

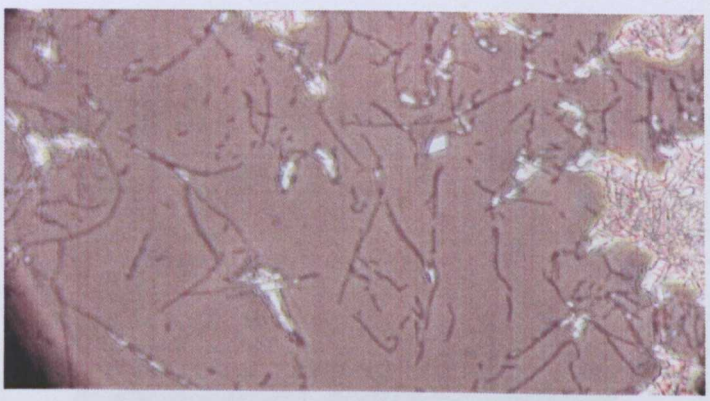
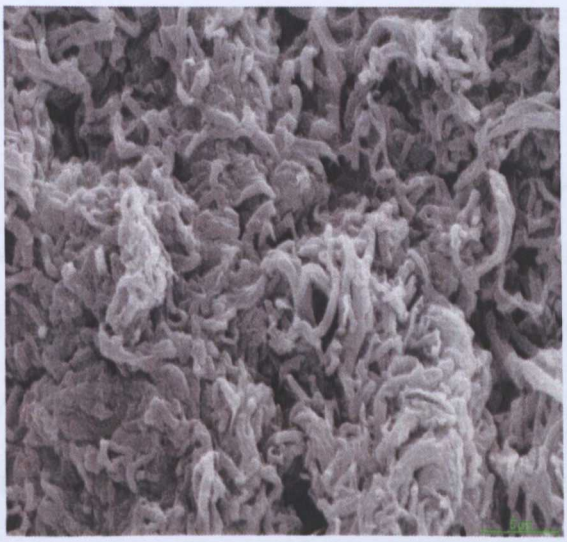
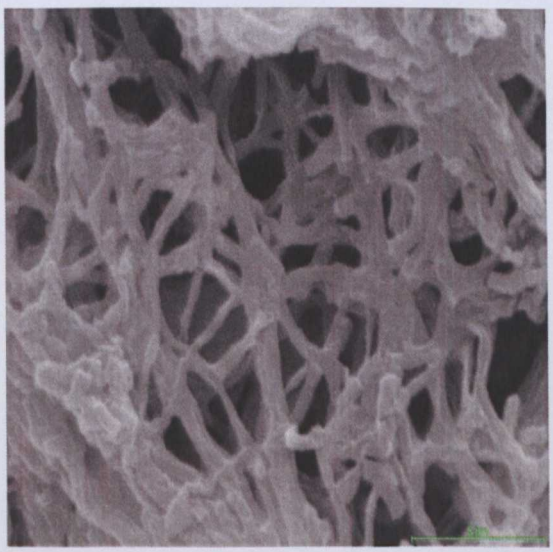


Figure 8. Scanning electron micrograph of a *S. capreolus* colony at 336 h, taken from Bennett's modified agar following incubation at 30 °C.

a) x 2,000 magnification



b) x 10,000 magnification



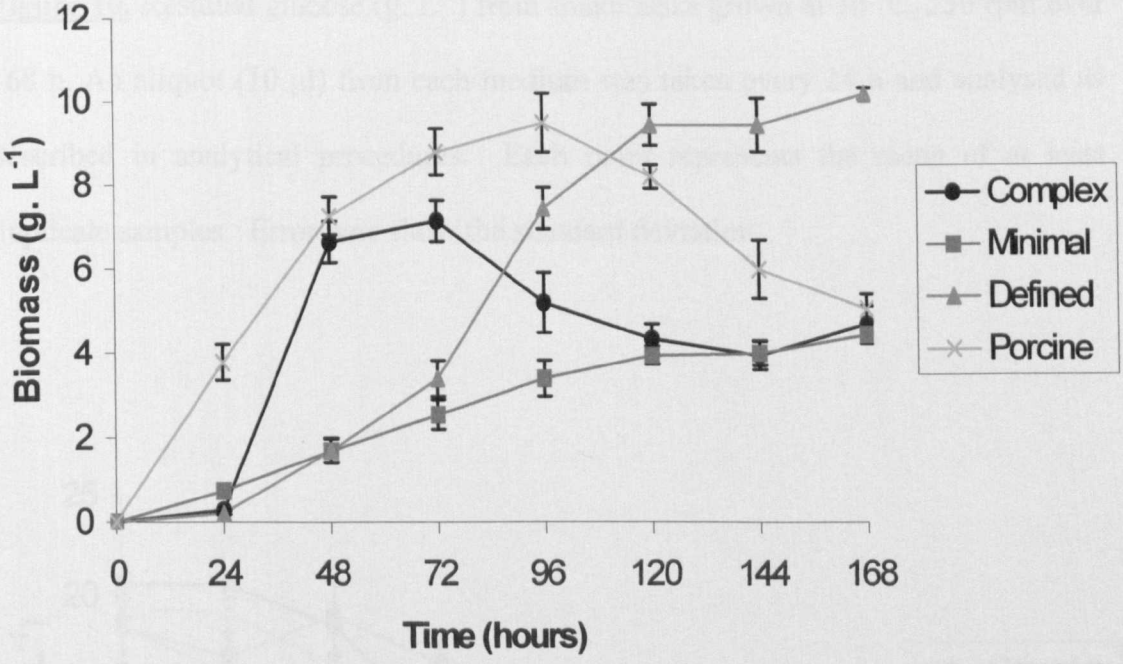
4.2 GROWTH CHARACTERISTICS IN LIQUID MEDIA

Growth of *S. capreolus* was predominantly investigated in four liquid media over the duration of a typical fermentation of 168 h. These media included the complex production medium (Complex), the porcine peptone Hisun medium (Porcine), a minimal medium (Minimal) and a defined medium containing 20 of the commonly occurring amino acids (Defined).

Over 168 h the major growth characteristics of *S. capreolus* were determined in order to gain an insight into the biochemical and physiological properties of the organism. This allowed a comparison to be made between growth in complex and minimal media, highlighting differences in metabolism in the two media categories.

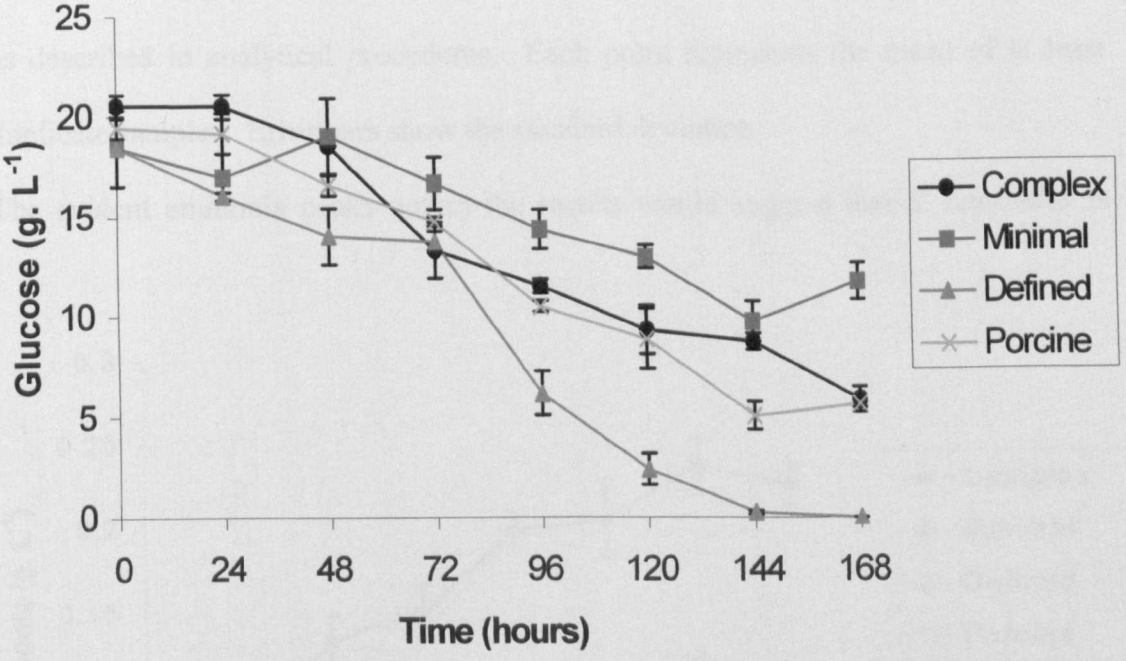
Biomass accretion was studied (Fig. 9), and results for the Complex and Porcine media showed a characteristic decline in dry weight biomass formation between 72 h and 96 h, decreasing throughout the remainder of the fermentation. This was in contrast to biomass formation in the Minimal and Defined media where biomass continued to increase throughout the fermentation, but at a slower rate.

Figure 9. Biomass production (g. L^{-1}) from shakeflasks grown at 30 °C, 250 rpm over 168 h. An aliquot (1 ml) from each medium was taken every 24 h and analysed as described in materials and methods. Each point represents the mean of duplicate samples. Error bars show the standard deviation.



Residual glucose was measured (Fig. 10), and showed a continual decline over 168 h, only reaching zero in the Defined medium. Final glucose concentrations remained between 5 and 12 g. L^{-1}, a significant surplus to the media even at the final stages of the fermentation.

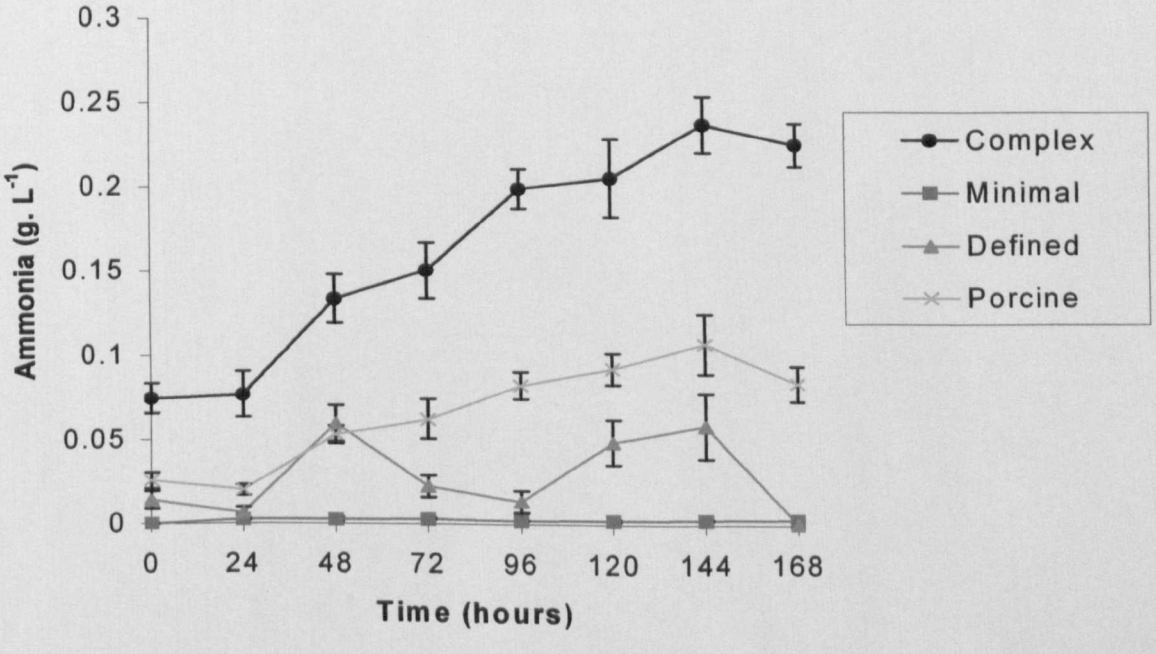
Figure 10. Residual glucose (g. L^{-1}) from shakeflasks grown at 30 °C, 250 rpm over 168 h. An aliquot (10 µl) from each medium was taken every 24 h and analysed as described in analytical procedures. Each point represents the mean of at least duplicate samples. Error bars show the standard deviation.



Ammonia production (Fig. 11) is significantly higher in the Complex medium suggesting that the plentiful supply of complex nitrogen sources within the medium are being actively metabolised yielding ammonia as a by-product of degradation. Indeed, it is not unusual that an excess of nitrogen source, especially ammonium salts, causes a decrease in the levels of antibiotic produced in many microorganisms (Brana, *et al.*, 1986). High levels of ammonia have been found to repress enzymes belonging to β-lactam antibiotic synthesis in the constitutive organisms (Shen *et al.*, 1984; Brana *et al.*, 1985; Castro *et al.*, 1985, Zhang *et al.*, 1989; Demain & Vaishnav, 2006). The final ammonia concentration in the Complex medium does not appear to inhibit capreomycin biosynthesis.

Figure 11. Ammonia production (g. L^{-1}) from shakeflasks grown at 30 °C, 250 rpm over 168 h. An aliquot (20 µl) from each medium was taken every 24 h and analysed as described in analytical procedures. Each point represents the mean of at least duplicate samples. Error bars show the standard deviation.

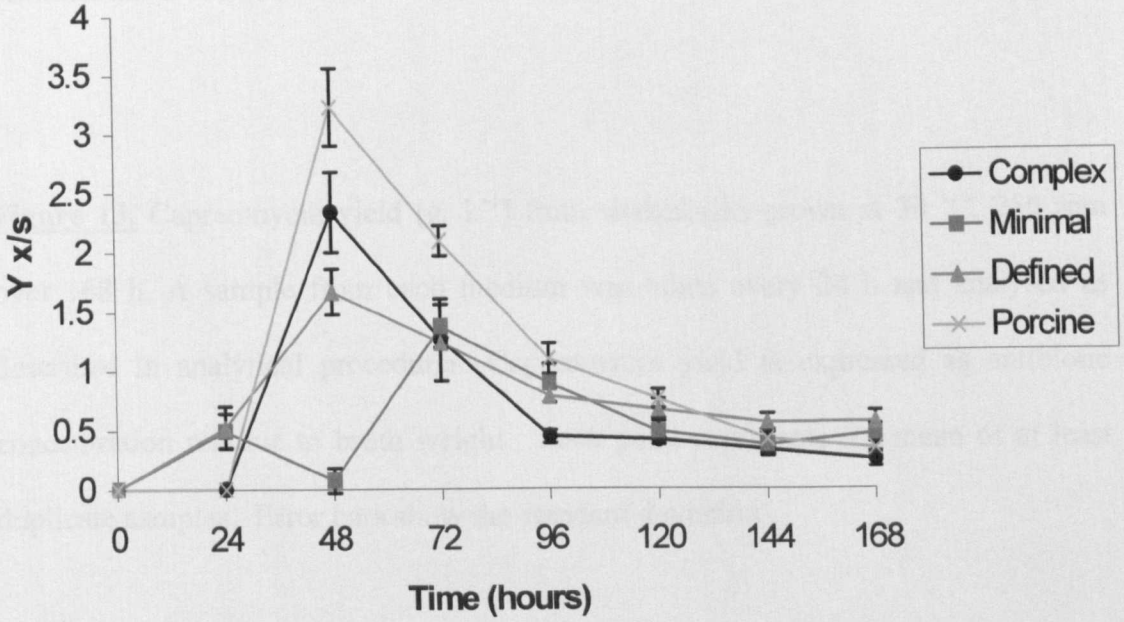
The evident ammonia peaks within the results would suggest that *S. capreolus* is



switching between the various nitrogen components present in the media. This phenomenon appears to be more profound in the Defined medium, whereby two significant peaks may be observed. This suggests a degree of preferential utilization of the amino acids present. Indeed, the growth yield in this instance (Fig. 12) is used to express the relationship between biomass formation and substrate utilisation. This has been calculated for glucose and all media examined demonstrate values over 1, markedly at 48 h which suggests that an alternative carbon source to glucose is being consumed early on to synthesize biomass. It would appear most likely that the alternative carbon source is being derived from the nitrogen component of the media. This is particularly the case for the Complex and Porcine media since the nitrogen portion is significantly larger than that of the carbon component.

The utilization of amino acids as carbon sources by *Streptomyces* species has been demonstrated in early papers (Gottlieb & Ciferri, 1956; Romano & Nickerson, 1958). More recently, the importance of amino acids as carbon sources for *Micromonospora echinospora* has been elucidated by Hoskisson *et al.*, (2003), and Bapat *et al.*, (2006a) have found that certain amino acids can be assimilated as the sole carbon source in *Amycolatopsis mediterranei*.

Figure 12. Growth yield coefficient relating biomass production (g. L^{-1}) to glucose utilization (g. L^{-1}). Each point represents the mean of at least duplicate samples. Error bars show the standard deviation.

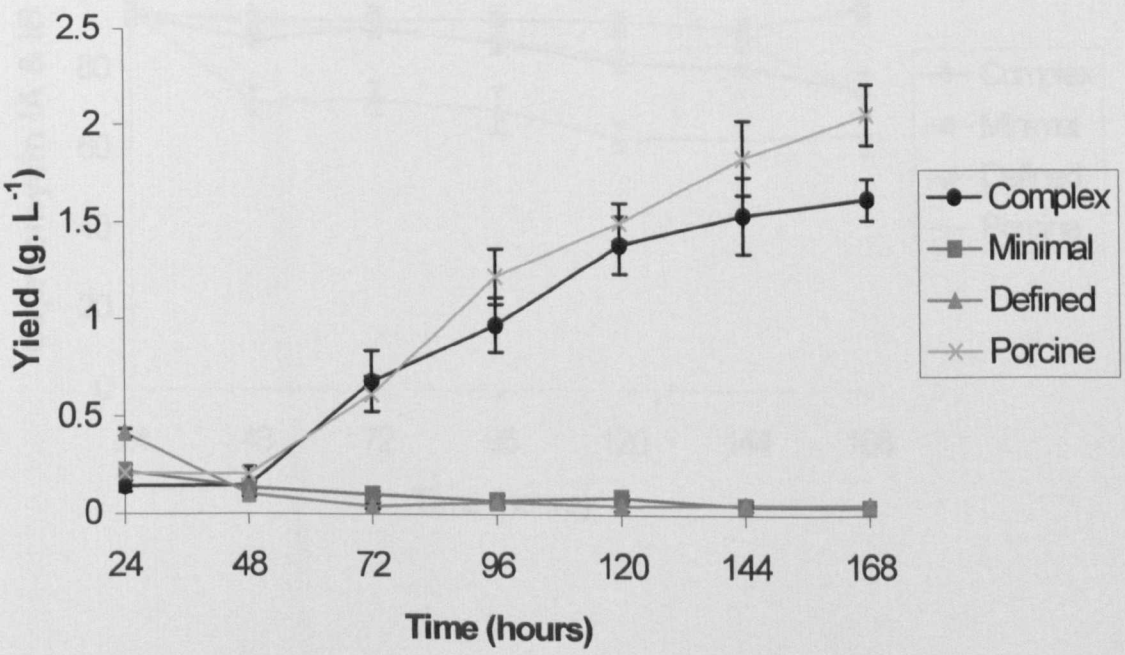


4.2.1 CAPREOMYCIN PRODUCTION

Capreomycin production was determined for each of the media examined (Fig. 13) and is expressed as yield which describes antibiotic concentration relative to broth weight.

Capreomycin synthesis proceeded in the Complex and Porcine media after 48 h and demonstrated a continual rise towards 168 h, whereas synthesis was negligible in the Minimal and Defined media.

Figure 13. Capreomycin yield (g. L^{-1}) from shakeflasks grown at 30 °C, 250 rpm over 168 h. A sample from each medium was taken every 24 h and analysed as described in analytical procedures. Capreomycin yield is expressed as antibiotic concentration relative to broth weight. Each point represents the mean of at least duplicate samples. Error bars show the standard deviation.



Capreomycin quality of the clinically active agent (IA and IB) is expressed as a percentage of the two relative to IIA and IIB (Fig. 14). The importance of high capreomycin quality is paramount to the process and is readily demonstrated in the producing media, showing only a small decline towards 168 h as the culture ages. The required ratio of capreomycin IA:IB must lie between 0.4 and 2.0 and is suitably demonstrated in the Complex and Porcine media (Fig. 15).

Figure 14. Percentage capreomycin IA and IB from shakeflasks grown at 30 °C, 250 rpm over 168 h. A sample from each medium was taken every 24 h and analysed as described in analytical procedures. Each point represents the mean of at least duplicate samples. Error bars show the standard deviation.

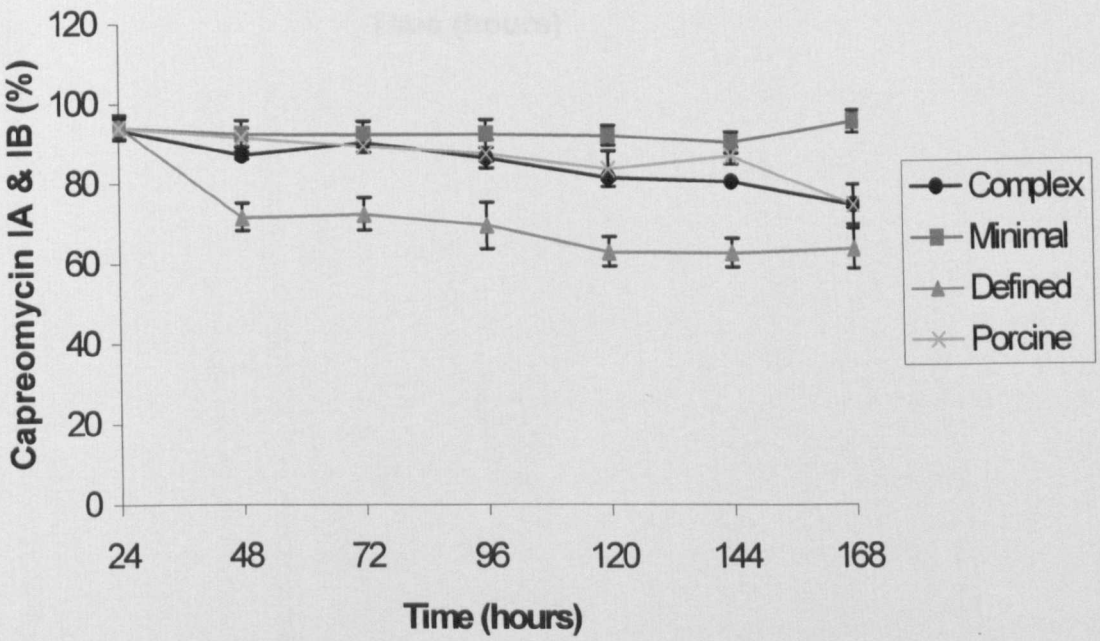
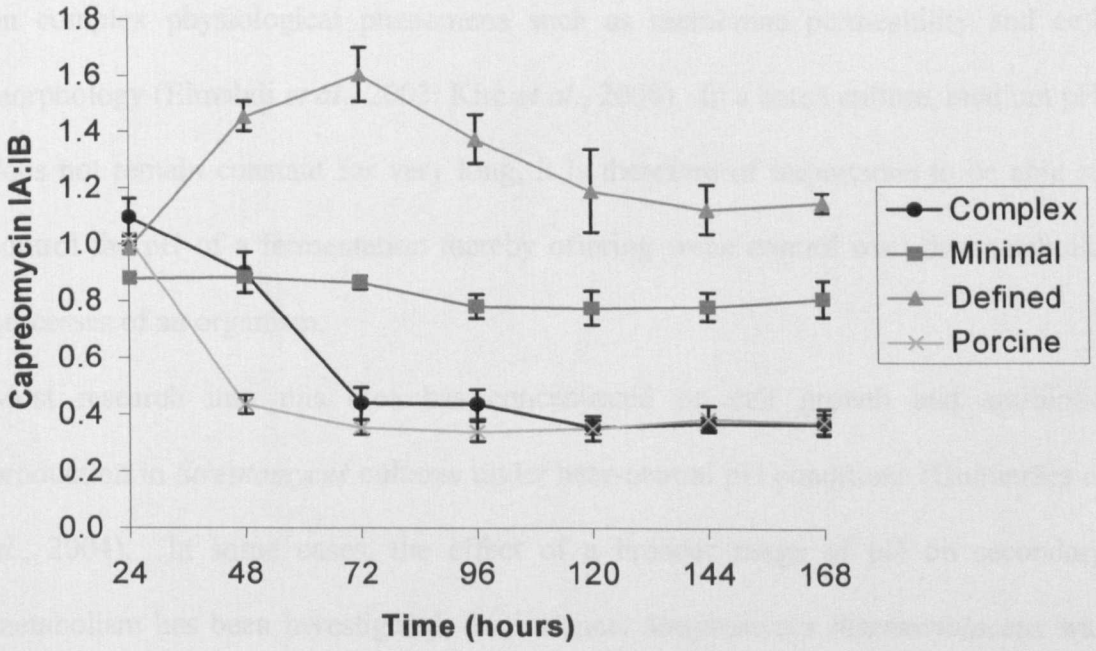


Figure 15. Capreomycin IA:IB from shakeflasks grown at 30 °C, 250 rpm over 168 h. A sample from each medium was taken every 24 h and analysed as described in analytical procedures. Each point represents the mean of at least duplicate samples. Error bars show the standard deviation.



4.3 EFFECT OF pH ON GROWTH & CAPREOMYCIN BIOSYNTHESIS

Several variables are important for cell growth and the production of bioactive compounds such as antibiotics, namely nutrient sources, oxygen, pH and temperature (Demain & Fang, 1995). The pH of the culture medium is one of the most important environmental factors, because it exerts a marked effect on the activity of several enzymes that catalyse metabolic reactions, as well as exerting significant influence on complex physiological phenomena such as membrane permeability and cell morphology (Elmahdi *et al.*, 2003; Kim *et al.*, 2000). In a batch culture, medium pH does not remain constant for very long, it is therefore of importance to be able to control the pH of a fermentation thereby offering some control over the metabolic processes of an organism.

Most research into this area has concentrated on cell growth and antibiotic production in *Streptomyces* cultures under near-neutral pH conditions (Guimarães *et al.*, 2004). In some cases, the effect of a broader range of pH on secondary metabolism has been investigated; for instance, *Streptomyces thermoviolaceus* was cultured over the pH range 5.5-8.0, whilst *Streptomyces olindensis* was studied over the range 6.0-8.0 (James *et al.*, 1991; Guimarães *et al.*, 2004). Both papers indicate optimum pH values around neutral for secondary metabolite production.

The effect of pH in batch culture was studied over the range 5.5-8.5 for *S. capreolus* in Complex and Porcine media with the addition of TES buffer to maintain pH at the desired value. Results taken at 168 h show the effect of pre-autoclave pH values on the main growth characteristics as studied earlier. Results are shown for biomass (Fig. 16), residual glucose (Fig. 17), ammonia production (Fig. 18) and growth yield calculated based on glucose utilization (Fig. 19).

Figure 16. The effect of pH prior to autoclaving on biomass concentration (g. L^{-1}) at 168 h from shakeflasks grown at 30 °C, 250 rpm. An aliquot (1 ml) from each medium was taken at 168 h and analysed as described in materials and methods. Each point represents the mean of at least duplicate samples. Error bars show the standard deviation.

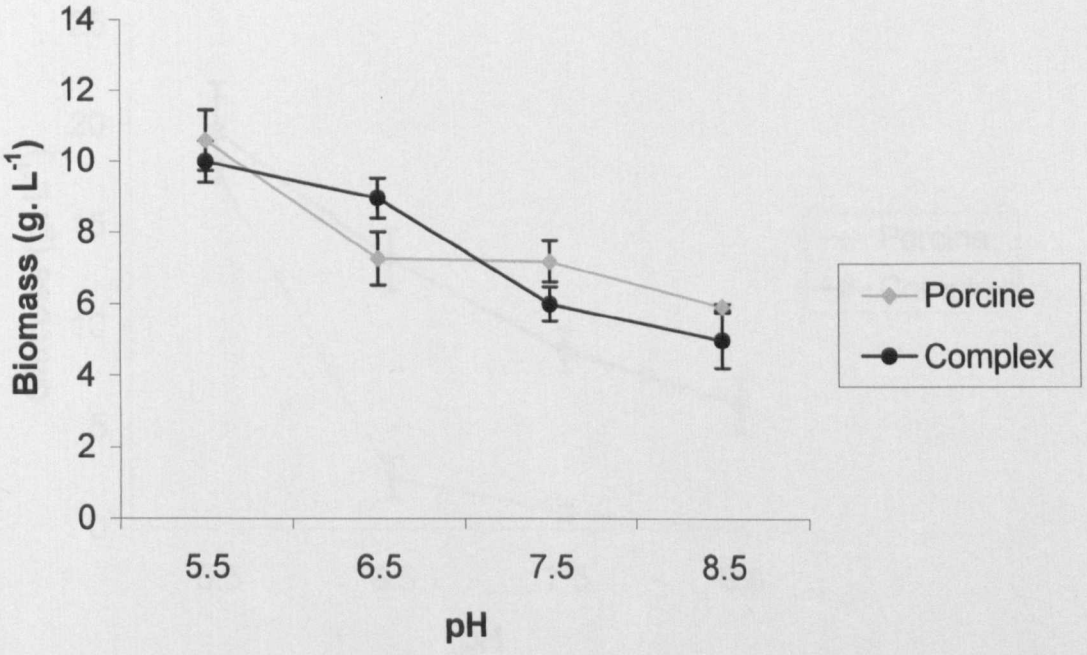


Figure 17. The effect of pH prior to autoclaving on residual glucose concentration (g. L^{-1}) at 168 h from shakeflasks grown at 30 °C, 250 rpm. An aliquot (10 µl) from each medium was taken at 168 h and analysed as described in analytical procedures. Each point represents the mean of at least duplicate samples. Error bars show the standard deviation.

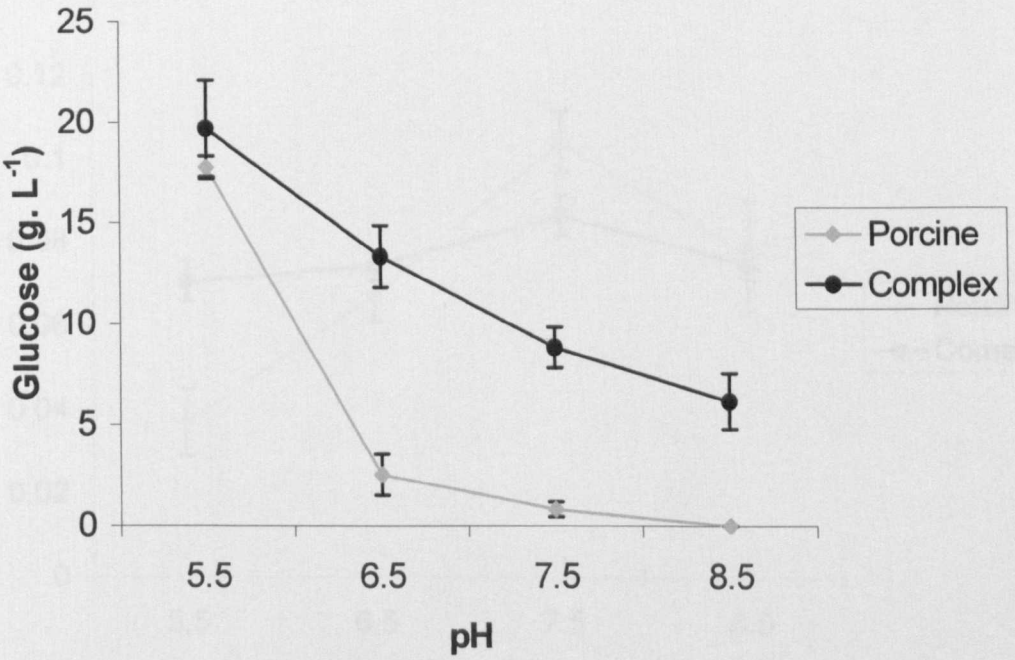


Figure 18. The effect of pH prior to autoclaving on ammonia production (g. L^{-1}) at 168 h from shakeflasks grown at 30 °C, 250 rpm. An aliquot (20 µl) from each medium was taken at 168 h and analysed as described in analytical procedures. Each point represents the mean of at least duplicate samples. Error bars show the standard deviation.

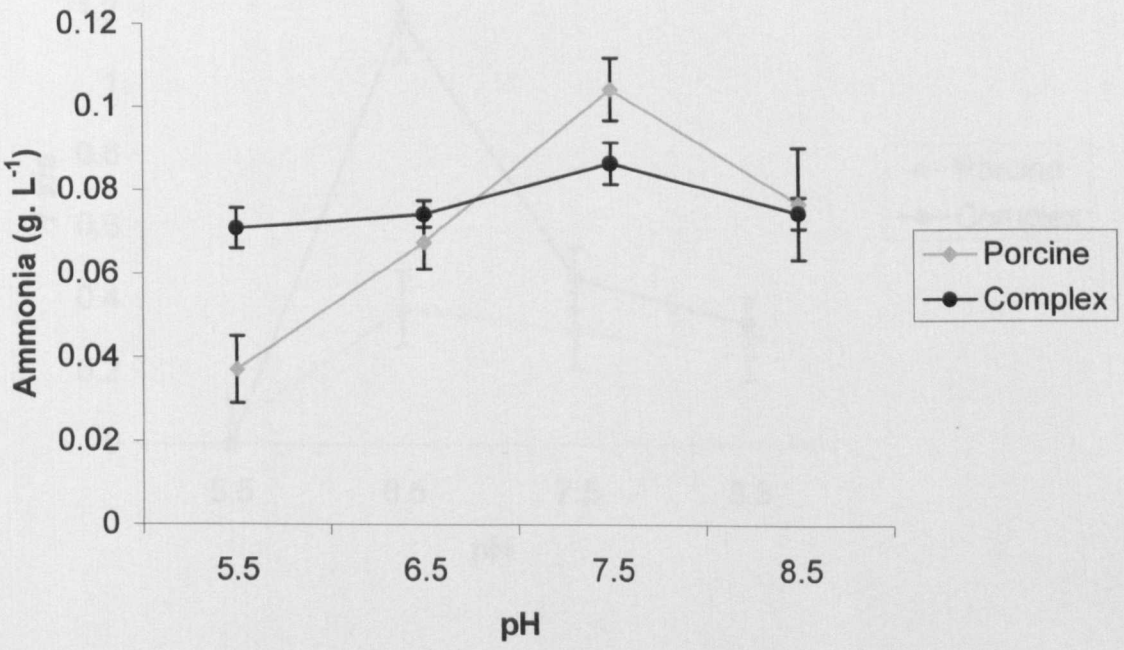
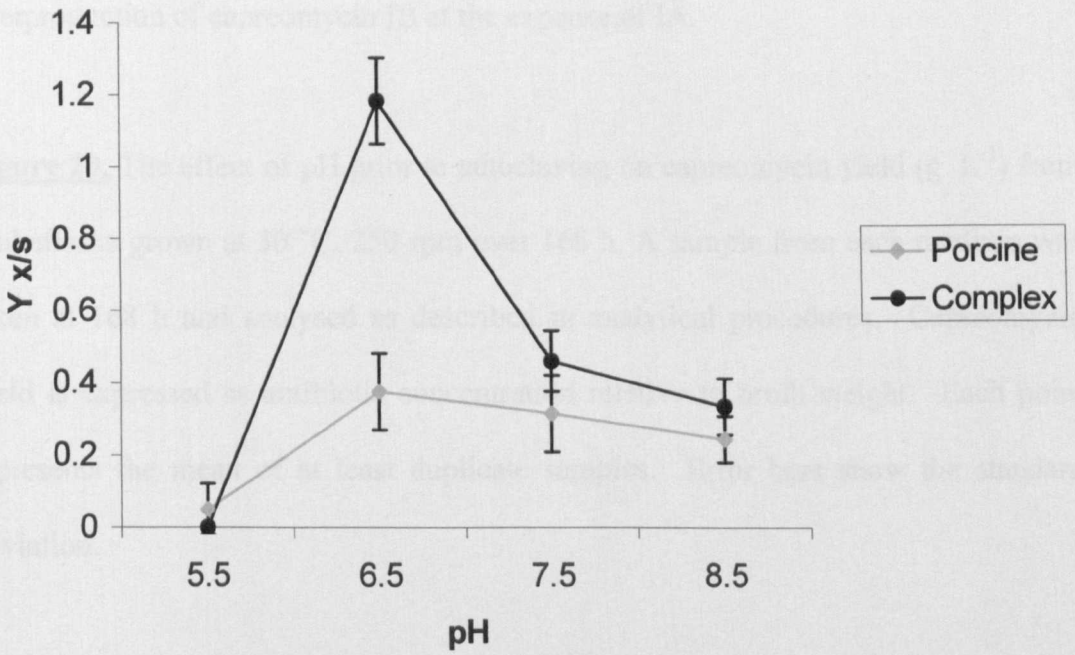


Figure 19. The effect of pH prior to autoclaving on the growth yield coefficient, relating biomass production (g. L^{-1}) to glucose utilization (g. L^{-1}). Each point represents the mean of at least duplicate samples. Error bars show the standard deviation.



Biomass decreases as the pH increases along with residual glucose. This suggests that glucose uptake is dramatically affected at low pH values. At pH 5.5 glucose uptake is totally inhibited since the glucose concentration is identical to 0 h of a typical fermentation. Carbon derived from glucose is an important structural precursor for capreomycin and at such low pH values is not readily available, therefore highlighting a link between nutrient status and productivity.

In terms of capreomycin yield (Fig. 20), a low pH (~ 5.5) appears to be detrimental to capreomycin yield. Capreomycin quality shows a deleterious decline with an

increase in pH (Fig. 21), whilst the effect on IA:IB is more prominent, showing a significant decrease (Fig. 22).

At large scale fermentation, it is vital to keep the IA:IB within the limits required and in some circumstances pH is used as a tool to maintain the species ratio between the compulsory limits.

Results from this study suggest that an alkaline pH drives the fermentation towards overproduction of capreomycin IB at the expense of IA.

Figure 20. The effect of pH prior to autoclaving on capreomycin yield (g. L^{-1}) from shakeflasks grown at 30 °C, 250 rpm over 168 h. A sample from each medium was taken at 168 h and analysed as described in analytical procedures. Capreomycin yield is expressed as antibiotic concentration relative to broth weight. Each point represents the mean of at least duplicate samples. Error bars show the standard deviation.

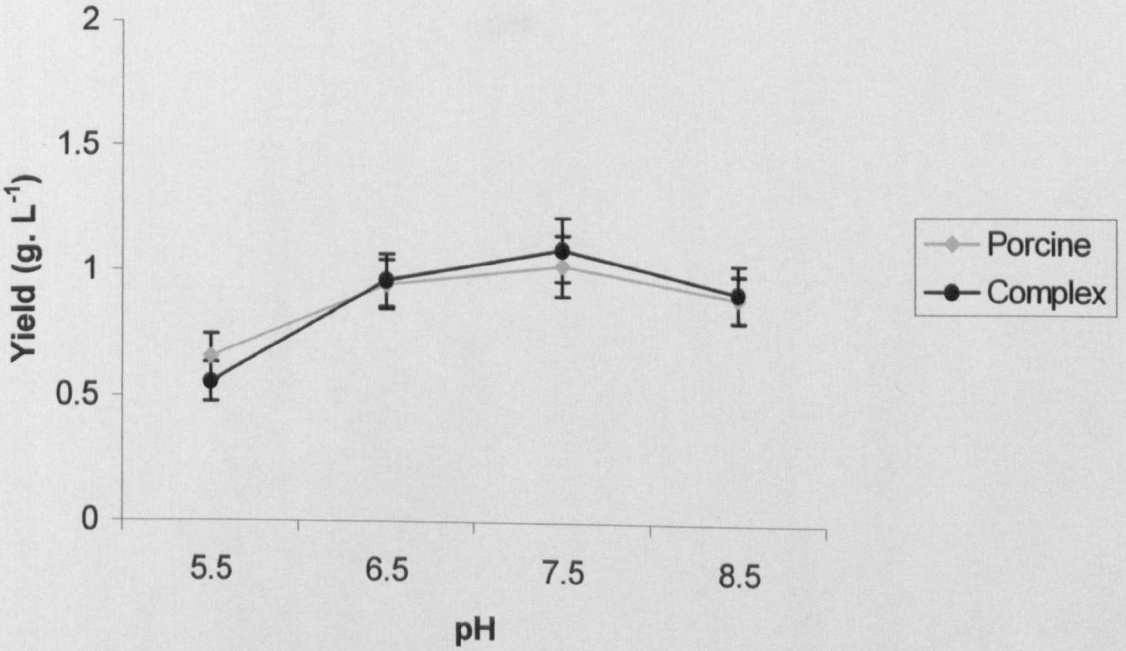


Figure 21. The effect of pH prior to autoclaving on percentage capreomycin IA and IB from shakeflasks grown at 30 °C, 250 rpm over 168 h. A sample from each medium was taken at 168 h and analysed as described in analytical procedures. Each point represents the mean of at least duplicate samples. Error bars show the standard deviation.

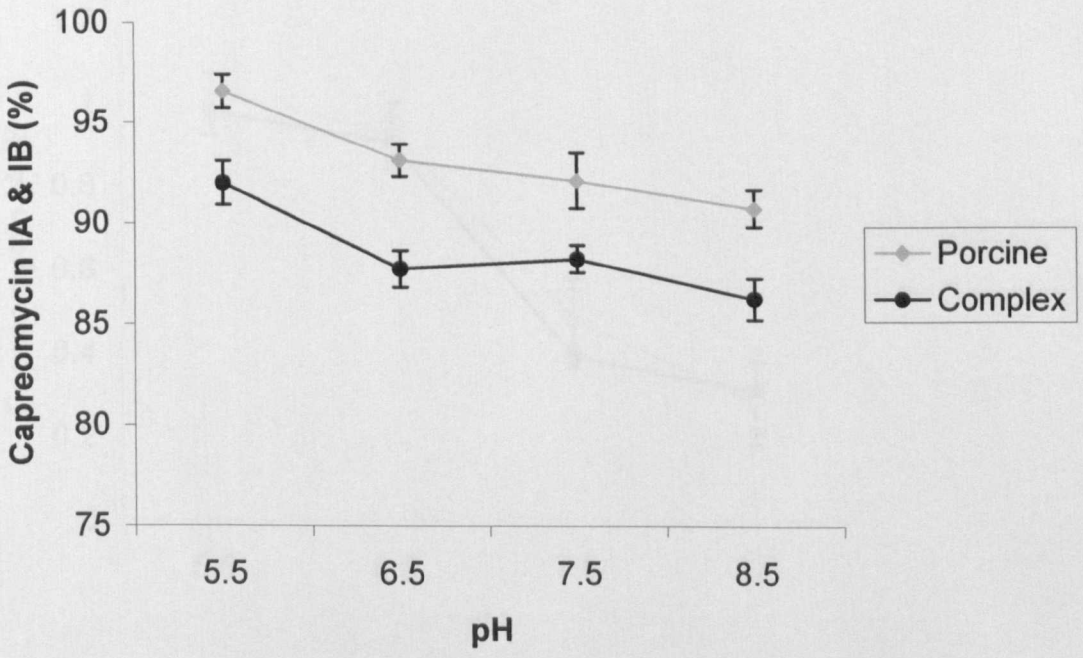
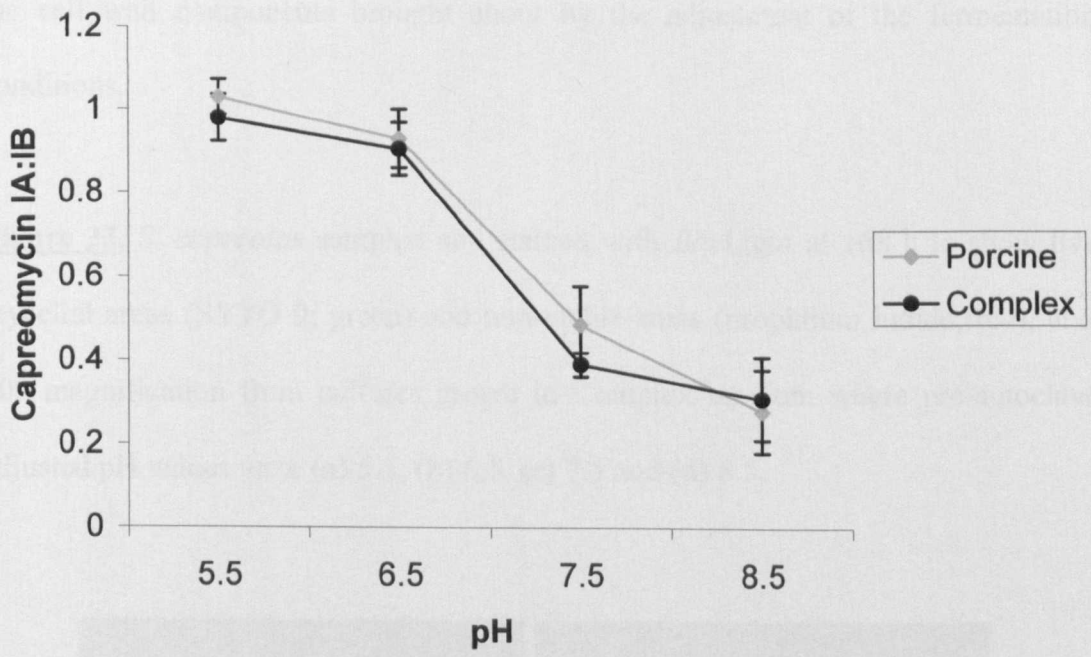


Figure 22. The effect of pH prior to autoclaving on capreomycin IA:IB from shakeflasks grown at 30 °C, 250 rpm over 168 h. A sample from each medium was taken at 168 h and analysed as described in analytical procedures. Each point represents the mean of at least duplicate samples. Error bars show the standard deviation.



Morphologically, the image analysis results for *S. capreolus* at 168 h in both the Complex (Fig. 23) and Porcine (Fig. 24) media show that at low pH values, pellet size appears to be larger, whilst an increase in media pH (above 7.5) brings about fragmentation of the culture. These results are in correlation with those reported for *Streptomyces tendae* (Vecht-Lifshitz *et al.*, 1990), and correspond to the increase in viability ratios (Fig. 25).

An explanation for this phenomenon is the modification of the adhesive properties of the cell wall components brought about by the adjustment of the fermentation conditions.

Figure 23. *S. capreolus* sampled and stained with *Bac*Light at 168 h to show live mycelial areas (SYTO 9; green) and non-viable areas (propidium iodide; red), at x 200 magnification from cultures grown in Complex medium where pre-autoclave adjusted pH values were **(a)** 5.5, **(b)** 6.5, **(c)** 7.5 and **(d)** 8.5.

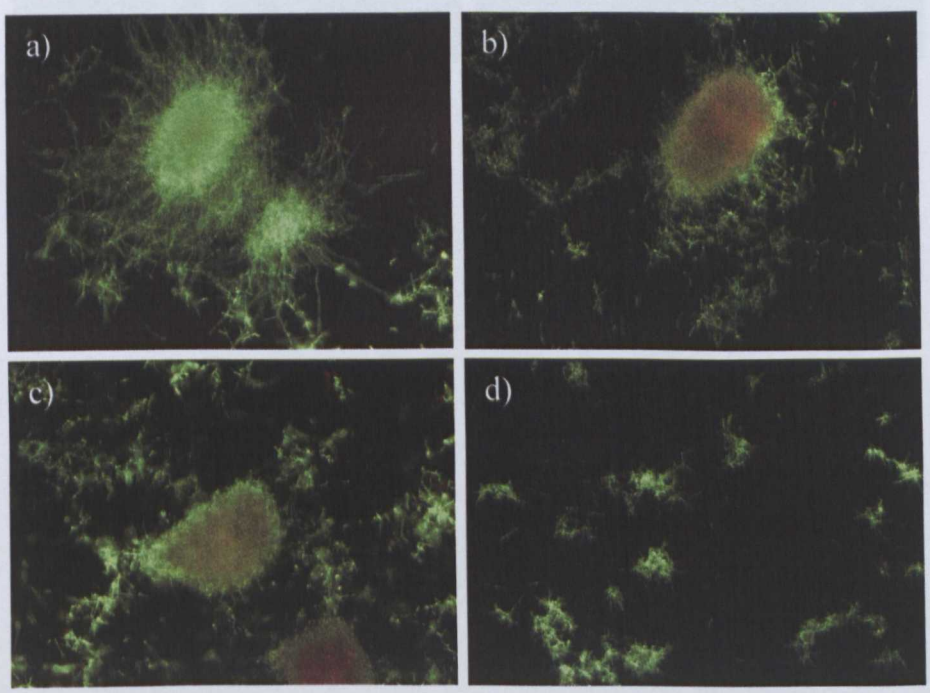


Figure 24. *S. capreolus* sampled and stained with *Bac*Light at 168 h to show live mycelial areas (SYTO 9; green) and non-viable areas (propidium iodide; red), at x 200 magnification from cultures grown in Porcine medium where pre-autoclave adjusted pH values were **(a)** 5.5, **(b)** 6.5, **(c)** 7.5 and **(d)** 8.5.

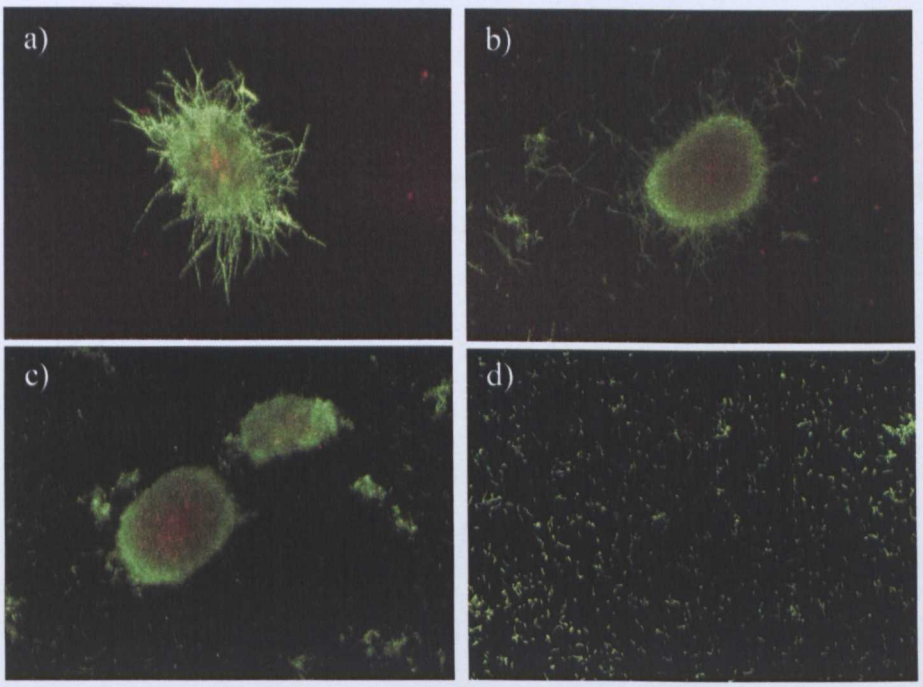
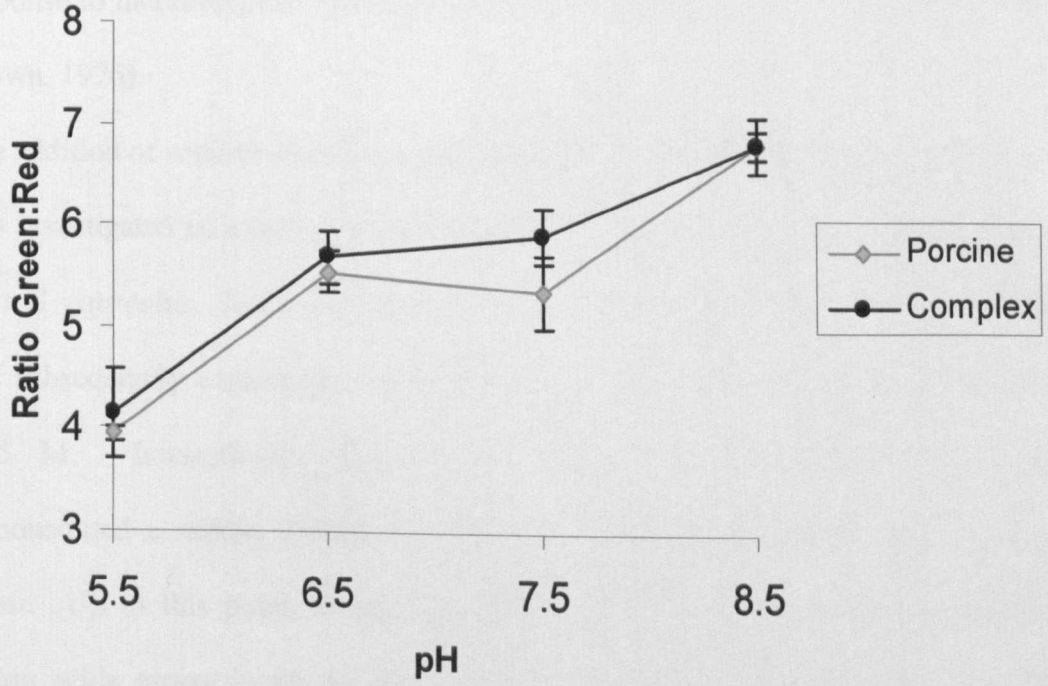


Figure 25. The effect of pH on green/red (live/dead) fluorescence ratio of *S. capreolus* grown in Complex and Porcine media. Each point represents the mean of 6 samples. Error bars show the standard deviation.



4.4 RESPONSE OF *S. CAPREOLUS* TO NaCl CONCENTRATION

Actinomycetes are relatively accustomed to salinity in their natural soil habitat. Research into the effects of salt stress has mainly been driven from an agricultural aspect and work has concentrated in particular on streptomycetes. The physiological response to salt stress appears to be species specific (Killham & Firestone, 1984), with many gram-positive bacteria showing an accumulation of certain amino acids in response to increasing salt concentration (Tempest & Meers, 1970; Measures, 1975; Brown, 1976).

The addition of sodium chloride to the Complex medium at a range of concentrations was investigated as a means of evoking a physiological or morphological response from *S. capreolus*. NaCl was added up to a concentration of 2 M, however growth and subsequently capreomycin production ceased to occur at concentrations above 0.75 M. Interestingly, *Streptomycs californicus* and *Streptomyces griseus* demonstrated a salinity threshold of 0.75 M, above which growth also ceased to occur. Up to this point, it was shown that the two streptomycetes accumulated amino acids proportionate to the size of the salt gradient (Killham & Firestone, 1984).

With increasing NaCl concentration came a decrease in specific growth yields of both streptomycetes and after 0.75 M, intracellular amino acid accumulation, noted prior to this point also declined.

Figure 26 shows the effect of NaCl on biomass concentration at 168 h. It was observed that biomass at 168 h did not radically change over the range of NaCl concentrations examined, and remained between 3.0 to 5.0 g. L^{-1}. Likewise, there did not appear to be a dramatic change in pH at 168 h as a consequence of NaCl addition (Fig. 27).

Figure 26. The effect of NaCl concentration (M) on dry weight biomass (g. L^{-1}) of *S. capreolus* at 168 h in Complex medium, with concentrations of NaCl; 0 M, 0.125 M, 0.25 M, 0.5 M and 0.75 M.

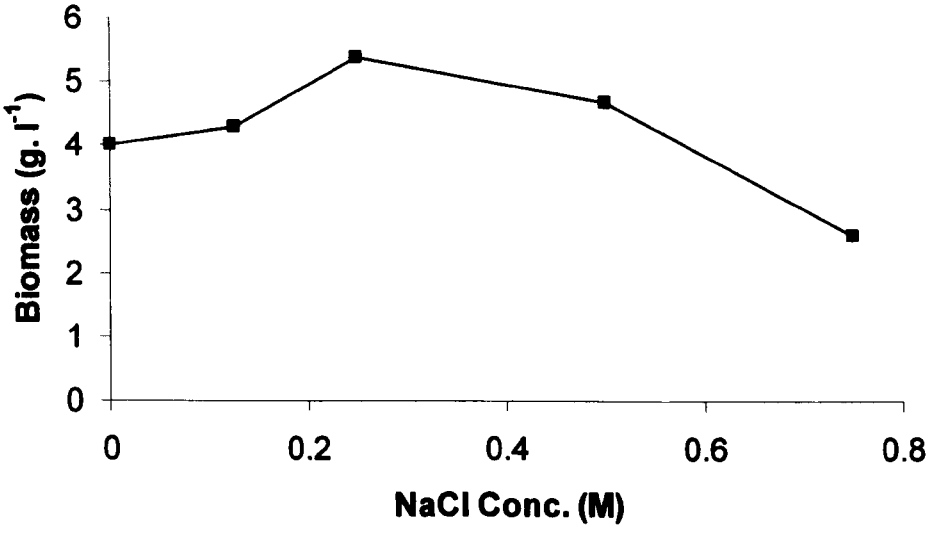
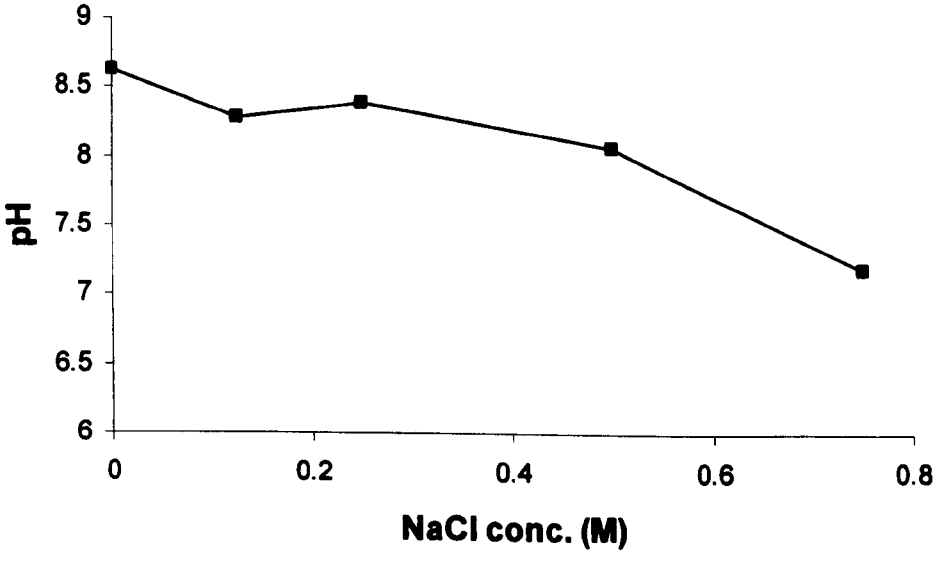


Figure 27. The effect of NaCl concentration (M) on pH measured at 168 h in Complex medium, with concentrations of NaCl; 0 M, 0.125 M, 0.25 M, 0.5 M and 0.75 M.



Although it may be true that antibiotic synthesis is linked to stress of the organism, there appears to be a certain level of stress after which it becomes unfavourable for secondary metabolite production. Capreomycin yield decreased following an increase in NaCl concentration (Fig. 28) to a point where growth of the organism was no longer feasible.

Results for the spectrophotometric viability assay performed at 168 h showed that an increase in NaCl concentration lead to an increase in the viability ratio which then declined sharply after 0.5 M NaCl (Fig. 29).

Figure 28. The effect of NaCl concentration (M) on capreomycin yield (g. L^{-1}) of *S. capreolus* at 168 h in Complex medium, with concentrations of NaCl; 0 M, 0.125 M, 0.25 M, 0.5 M and 0.75 M.

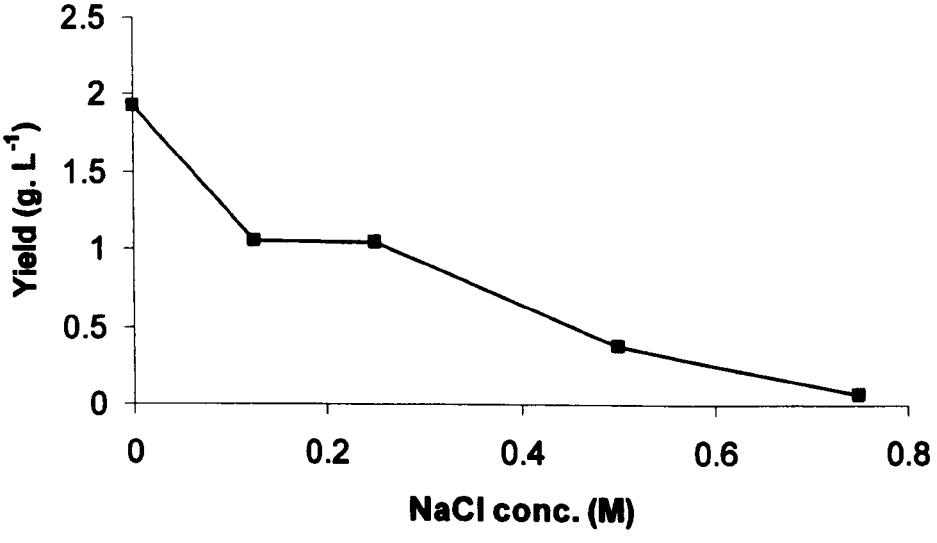


Figure 29. Green/red (live/dead) fluorescence ratio of *S. capreolus* at 168 h in Complex medium, with concentrations of NaCl; 0 M, 0.125 M, 0.25 M, 0.5 M and 0.75 M. Each point represents the mean of 10 samples. Error bars show the standard deviation.

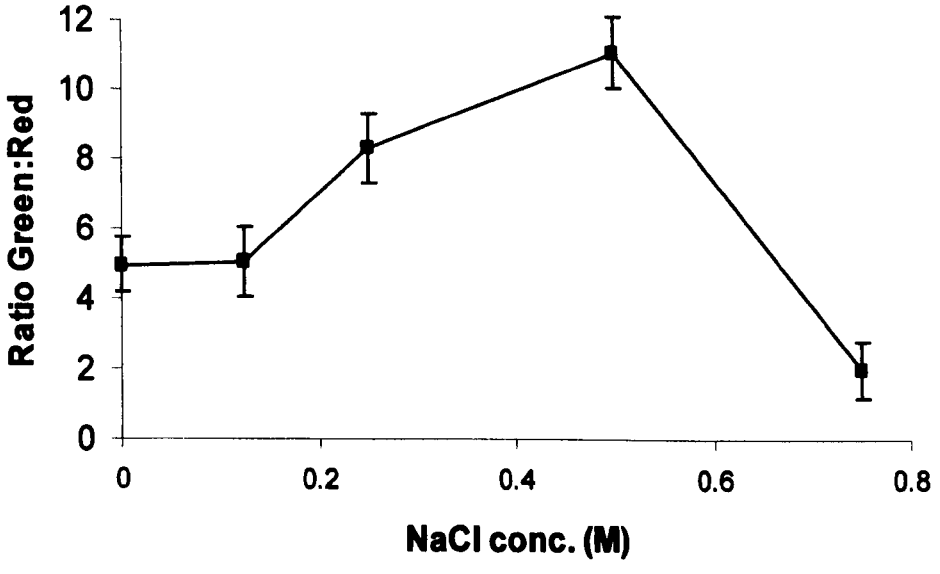


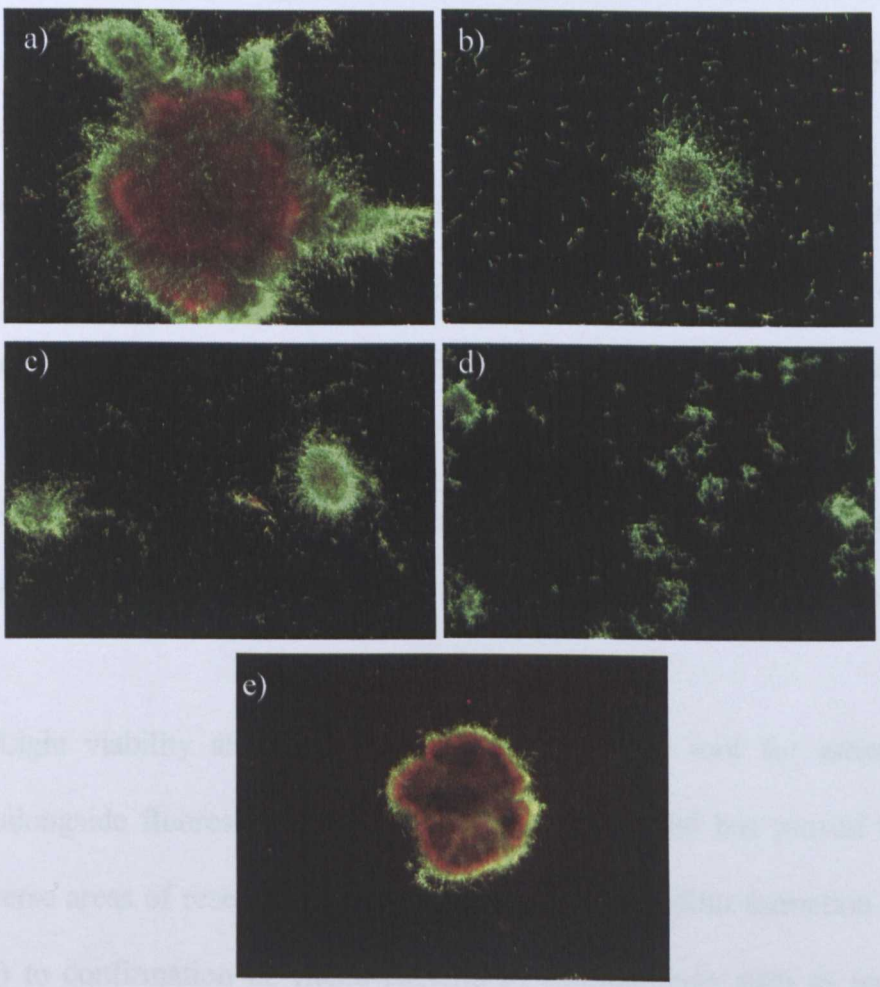
Image analysis of the culture (Fig. 30) showed an increase in fragmentation of *S capreolus* up until 0.5 M NaCl. The culture ceased to have a fragmented appearance after 0.5 M NaCl and pellets remained that appeared to be clumped into larger pellets via a process of agglutination, which were predominantly red (dead). A change in the cell wall properties would provide an explanation for this mass clumping phenomenon and would indicate a higher degree of hydrophobicity than is normally demonstrated.

The ability of *S. capreolus* to withstand the increase in osmotic pressure would suggest that there must be an accumulation of solutes within the cells to create a balanced gradient that would otherwise lead to cell death in such high salinity.

Proline was found to account for approximately 50 % of the free amino acid pool in *S. griseus* and *S. californicus* during salinity experiments and due to the nature of its neutral molecule, would make a particularly efficient osmoprotectant under such conditions (Killham & Firestone, 1984). Glutamine and alanine were consequently the next significant amino acids to be accumulated as opposed to glutamate and aspartate which predominate under relatively normal, non saline conditions.

Between 0 M and 0.75 M NaCl, biomass at 168 h did not radically change and remained between 3.0 to 5.0 g. L^{-1}. This indicates that an increase in fragmentation does not necessarily bring about an increase in biomass, which further advocates the spectrophotometric viability assay whereby ratios calculated are a true representation of the proportion of live/dead mycelia and not simply a measure of an increase in biomass.

Figure 30. *S. capreolus* sampled and stained with *Bac*Light at 168 h to show live mycelial areas (SYTO 9; green) and non-viable areas (propidium iodide; red), at x 100 magnification and at the following initial NaCl concentrations in Complex medium; 0 M **(a)**, 0.125 M **(b)**, 0.25 M **(c)**, 0.5 M **(d)** and 0.75 M **(e)**. Growth was absent in higher concentrations of NaCl.



4.5 MORPHOLOGICAL CHARACTERISTICS OF *S. CAPREOLUS*

Pelleted growth produces cultures with viability inconsistencies making the estimation of viability in such a culture particularly difficult. It is only recently that the characterisation of filamentous microorgansms grown in submerged cultures has seen significant progress, due largely to image analysis technology (Durant *et al.*, 1994).

Traditionally, bacterial viability has been determined by serial dilution of a sample suspension followed by plating of a known volume onto a solid medium and thus counting of the colonies, a method that relies solely on the enumeration of culturable bacteria but more significantly is restricted to unicellular organisms (Stocks, 2004).

The LIVE/DEAD® *Bac*Light™ bacterial viability kit (Molecular Probes) has previously been used to characterise hyphal viability in *Streptomyces clavuligerus* (Sebastine *et al.*, 1999), however this method relies upon image analysis techniques, requiring semiautomatic image capturing and pixel evaluation, which gives rise to techniques that are limited to 2 dimensional estimations. Not only are these techniques time consuming but they are labour intensive to perform on a routine basis.

The *Bac*Light viability stain has become a widely used tool for estimation of viability alongside fluorescence microscopy techniques, and has proved useful in many diverse areas of research from characterisation of biofilm formation (Sarró *et al.*, 2005) to confirmation of viable bacteria in environments such as sea surface water (Naganuma & Miura, 1997), glaciers (Miteva *et al.*, 2004) and the stratosphere, where viable but non-culturable bacteria were identified (Wainwright *et al.*, 2004).

Previous research has largely been limited to qualitative measurements of viability, whilst quantitative aspects have been confined to unicellular organisms such as *Escherichia coli* where analysis via flow cytometry can be achieved due to the single celled nature of the samples examined.

The *Bac*Light bacterial viability stain comprises two nucleic acid stains; SYTO 9 and propidium iodide (PI). The former is a green intercalating membrane permeant fluorescing dye that stains all nucleic acid and emits green light under blue excitation, whilst the latter is a well established and accepted membrane impermeant stain (Davey & Kell, 1996; Hewitt *et* al., 1998, 1999; Nebe-von-Caron *et al.*, 2000), and is excluded by healthy cells but stains cells that are considered to have failed to maintain membrane integrity, resulting in a decrease in viability and emitting red light under green excitation (Stocks & Thomas, 2001).

During this research, a rapid technique for the estimation of culture viability for filamentous organisms was developed. This high throughput method based on 96 well microtitre plates, utilizes fluorimetric analysis methods and requires only microlitre amounts of sample which are then coupled with the *Bac*Light fluorescent bacterial viability stain. Ratios of green:red channel wavelengths were calculated as outlined in Materials and Methods.

4.5.1 VIABILITY RATIO AS AN INDICATOR OF MORPHOLOGICAL STATUS

It can be observed from Fig. 31(a) and Fig. 31(b) that the viability ratios for both the Complex and Porcine media started at around 7 at 24 h and continued to gradually decrease throughout the 240 h fermentation, showing final ratios of around 3. These ratios suggest that the pelleted morphology common to both of these media is largely green (live), with the red regions associated with the inner pellets becoming more pronounced as the fermentation proceeds, thus leading to a decrease in the measured viability ratios. This was confirmed on image analysis of the cultures, whereby Fig. 32(a) and Fig. 32(b) show that fragmentation is limited in these media, and the overall gross morphological status of the culture is pelleted in appearance which remains fairly stable throughout.

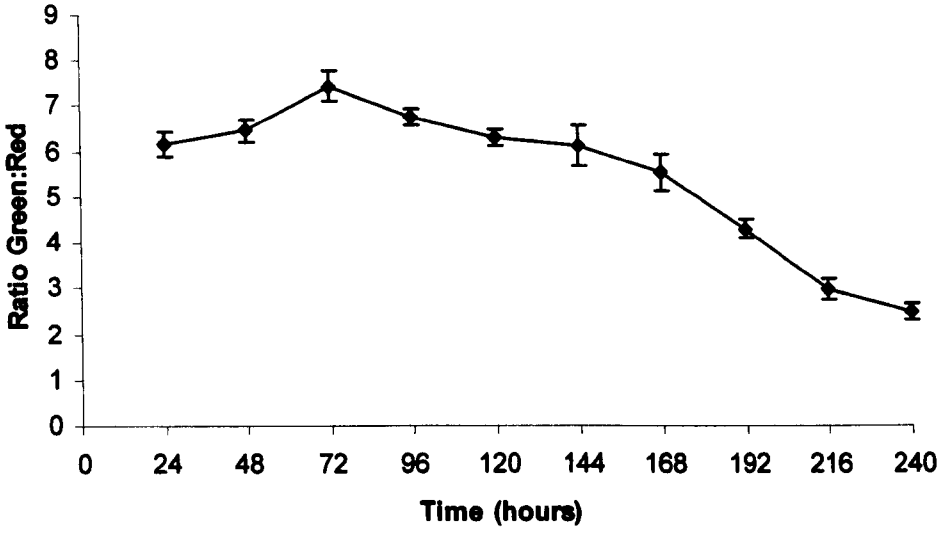
These results are in stark contrast to those observed in Fig. 31(c) for the Minimal medium and Fig. 31(d) for the Defined medium. The initial viability ratios at 24 h for these media were comparable to those observed in the production media. This was rapidly followed by an increase in both ratios very early on (~48 h) which remained high (above 17) throughout the 240 h fermentation. The high ratio is an indication of more live to dead cells, and is indicative from the image analysis results for the Minimal medium and the Defined medium as shown in Fig. 32(c) and Fig. 32(d) respectively. Collectively, these results were consistent with computer automated image analysis that was carried out to support the new method for spectrophotometric viability quantification. It can be observed from Fig. 33, that the percentage mycelial viability decreases towards 168 h for the Complex and Porcine media, whilst an increase after 24 h is observed in the Minimal and Defined media, which remains high throughout the fermentation and appears to be indicative of culture fragmentation.

Fragmentation is the major contributor to high viability ratios in that it gives rise to dispersed hyphae which are abundant and particularly viable and would confer the high viability ratios observed in both media very early on in the fermentation.

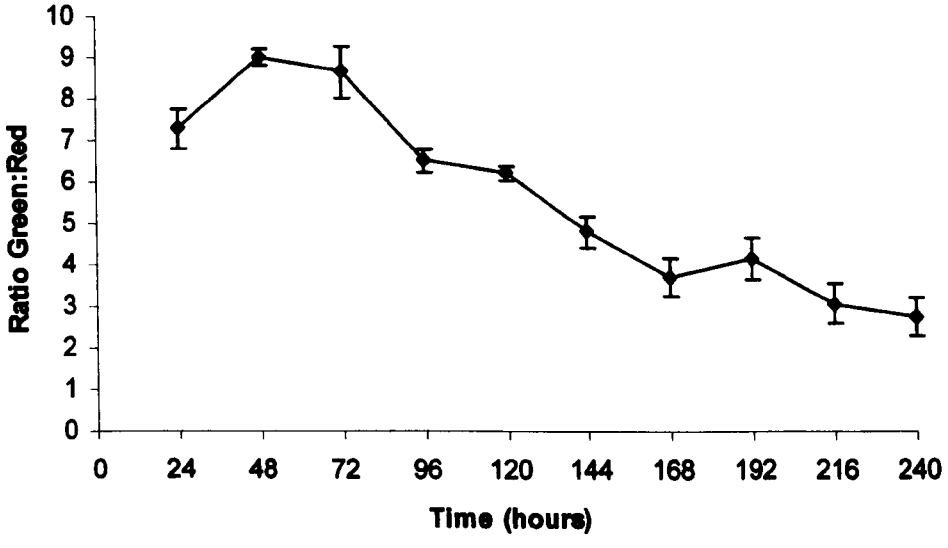
The Complex and Porcine media investigated suggest a predilection for pelleted morphology under conditions optimal for capreomycin biosynthesis. This is by no means a direct link between complexity and antibiotic production, but is merely suggestive that pelleted morphology is favoured under such conditions whilst fragmentation is largely absent.

Figure 31. Green/red (live/dead) fluorescence ratio of *S. capreolus* grown in Complex medium **(a)**, Porcine medium **(b)**, Minimal medium **(c)** and Defined medium **(d)** over 240 h. Each point represents the mean of 12 samples. Error bars show the standard deviation.

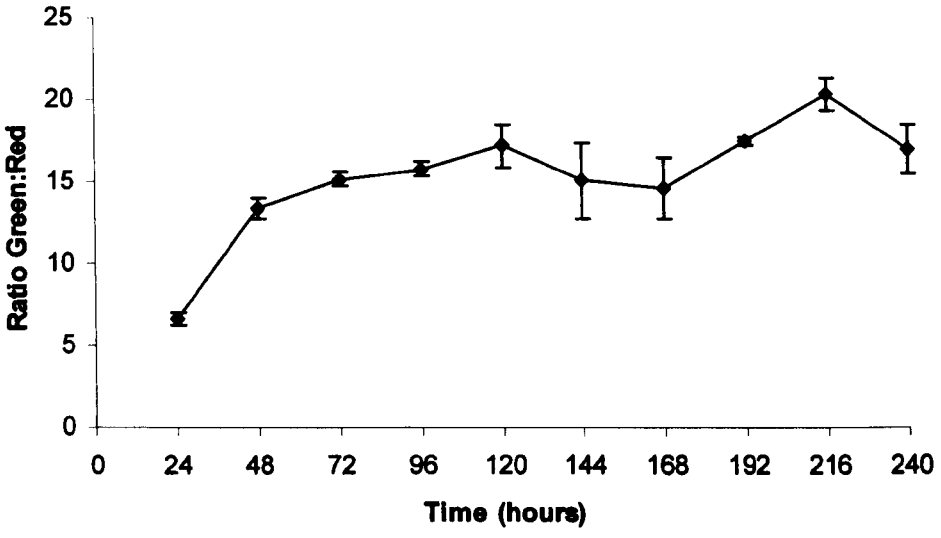
a)



b)



c)


Ratio Green:Red
25
20
15
10
5
0
0
24
48
72
96
120
144
168
192
216
240
Time (hours)

d)

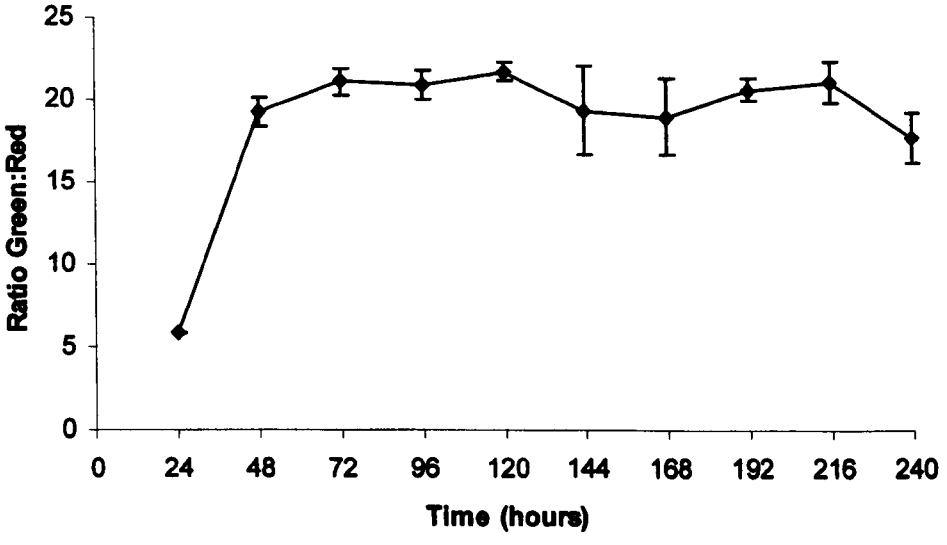
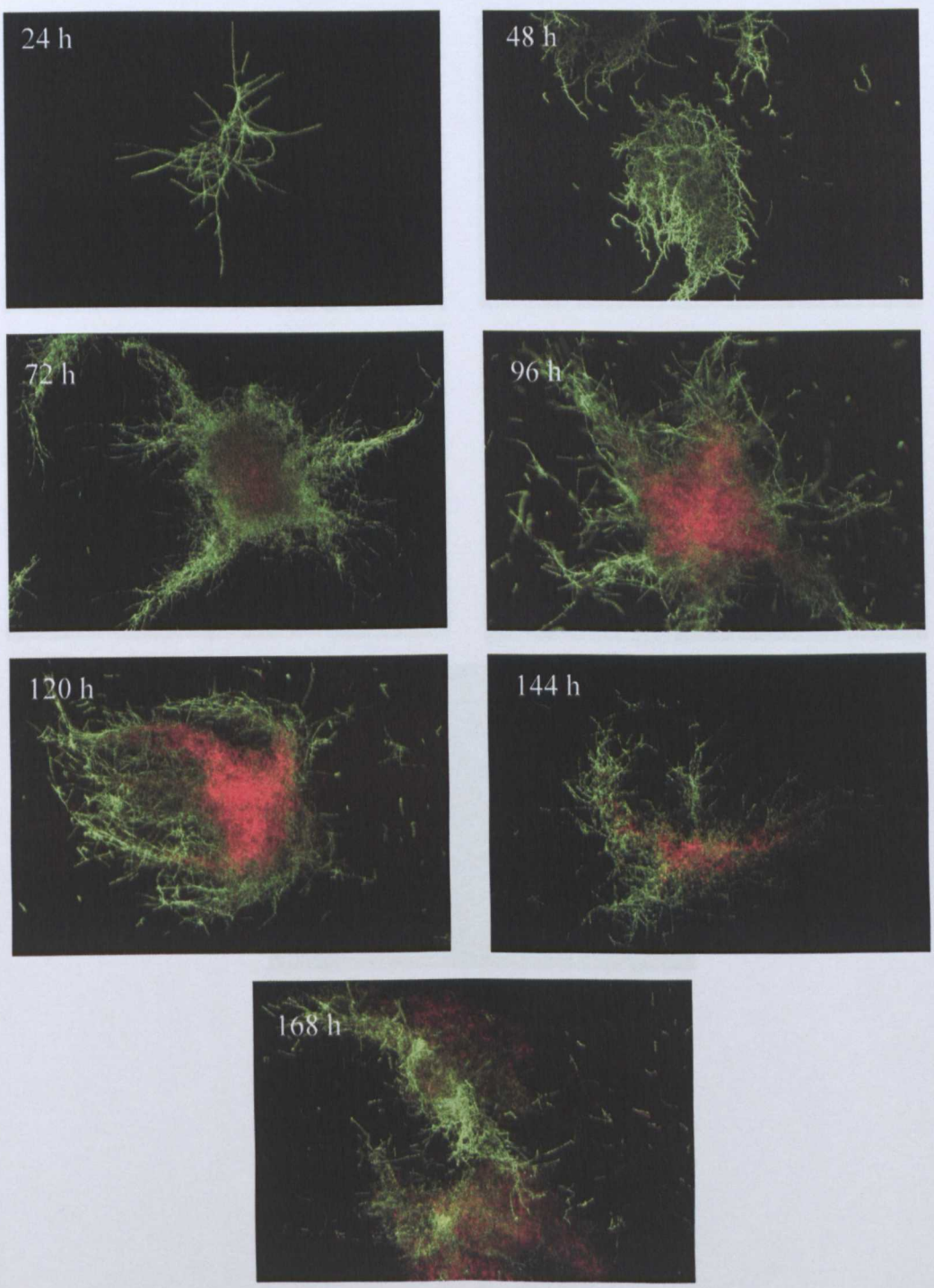
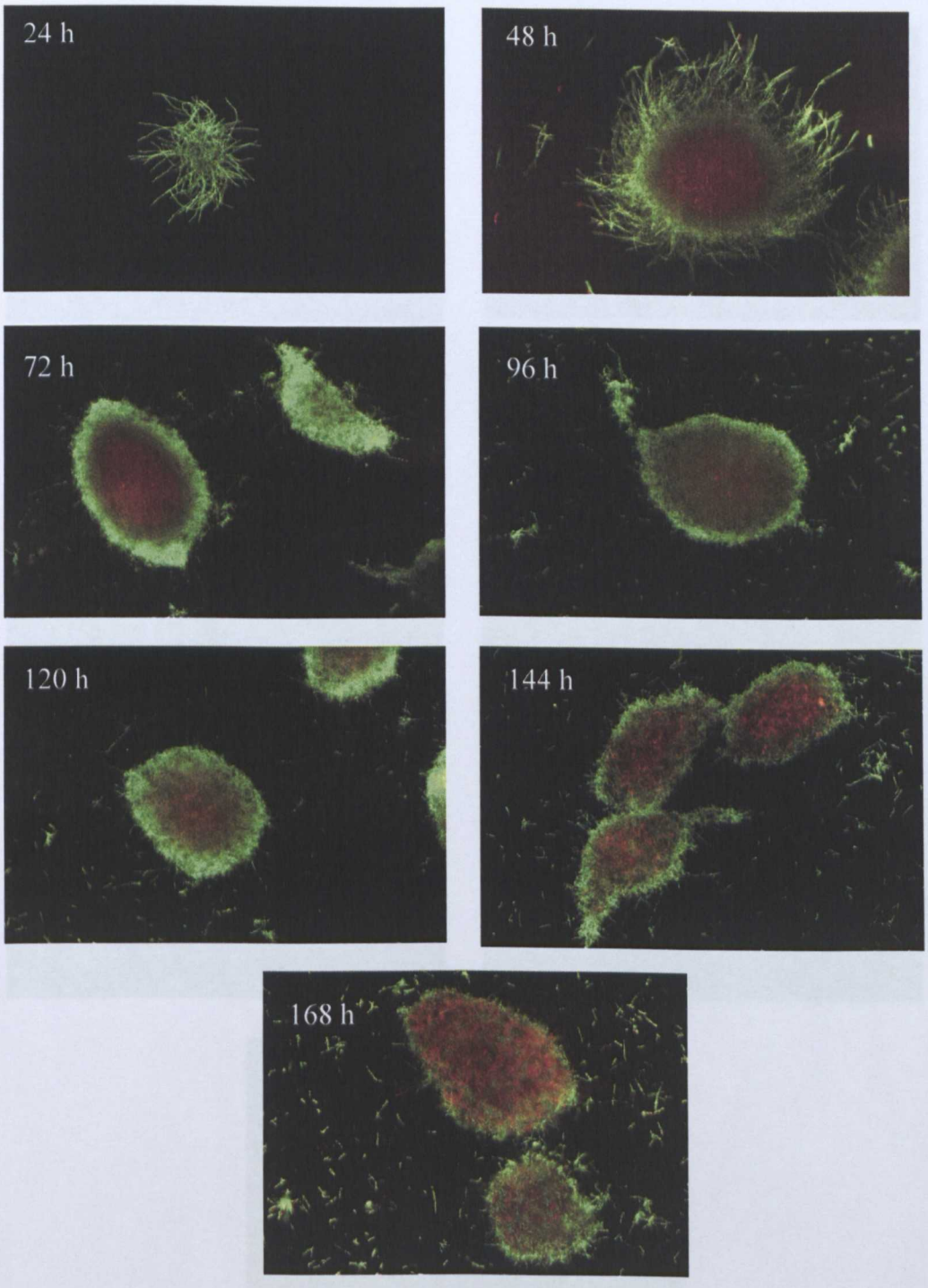

Ratio Green:Red
25
20
15
10
5
0
0
24
48
72
96
120
144
168
192
216
240
Time (hours)

Figure 32. *S. capreolus* sampled and stained with *Bac*Light at 24 h intervals to show live mycelial areas (SYTO 9; green) and non-viable areas (propidium iodide; red), at x 200 magnification, in Complex medium **(a)**, Porcine medium **(b)**, Minimal medium **(c)** and Defined medium **(d)**.

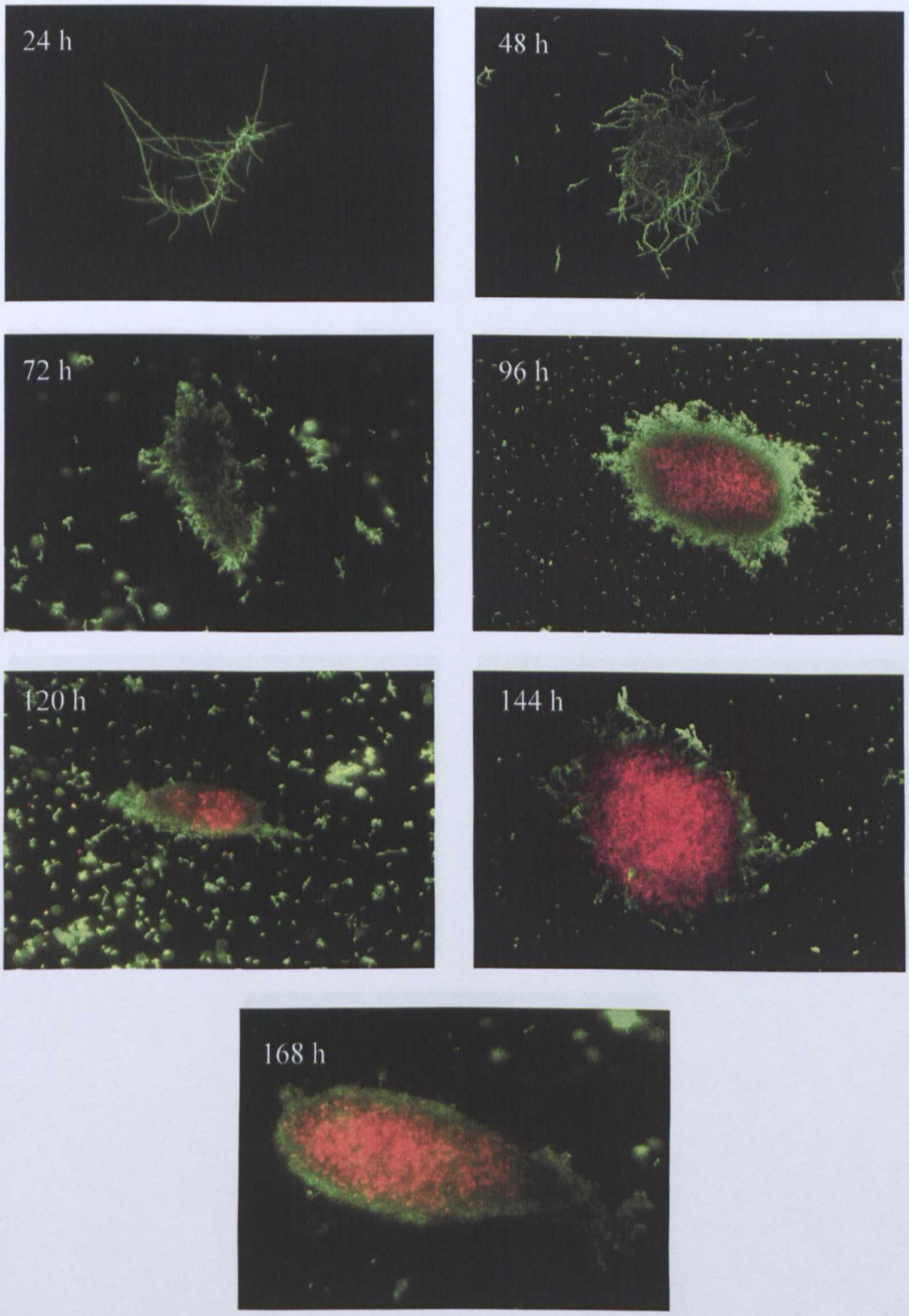
a) Complex Medium



b) Porcine Medium



c) Minimal Medium



d) Defined Medium

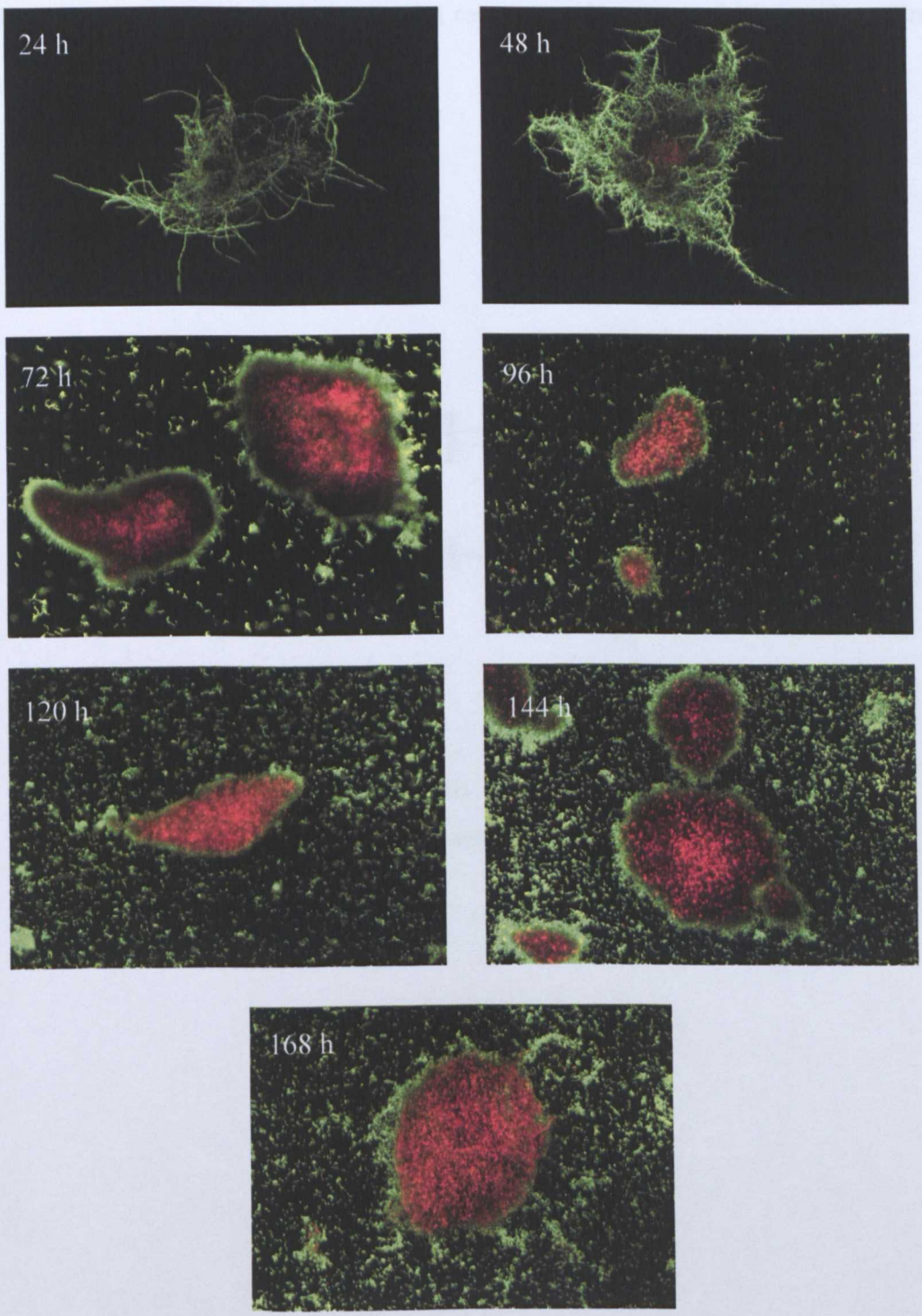
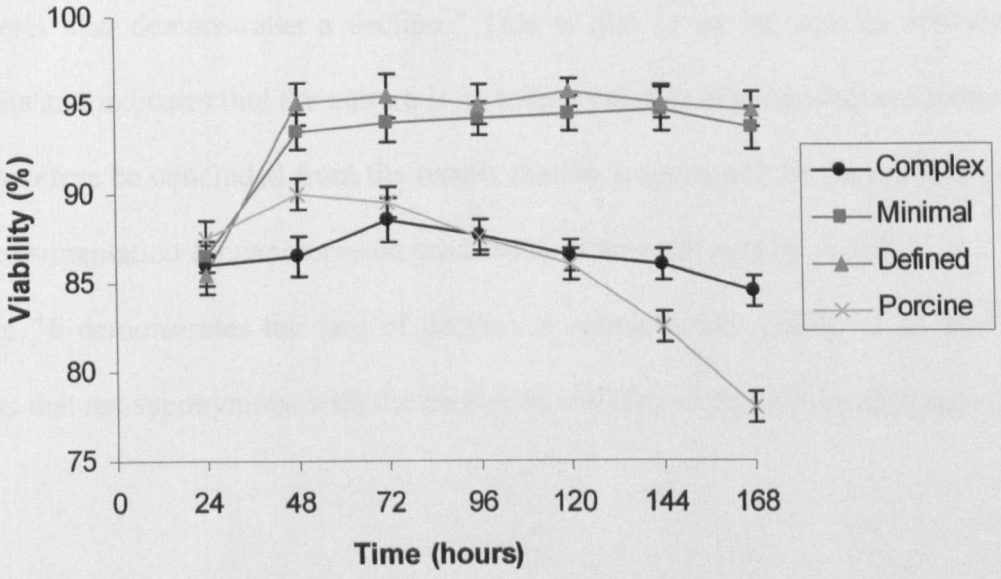


Figure 33. Image analysis quantification expressing percentage viability of mycelia based on growth in Complex medium, Porcine medium, Minimal medium and Defined medium over 168 h. Each point represents the mean of 10 samples. Error bars shown the standard deviation.



4.5.2 RELATIONSHIP BETWEEN VIABILITY & CAPREOMYCIN PRODUCTION

The rate of antibiotic production for the Complex and Porcine media can be observed in Fig. 34, whereby capreomycin production increases up until 96 h followed by a fairly rapid decrease from this point onwards until the end of the fermentation. These results show an agreement with those for culture viability and demonstrate a relationship between viability and antibiotic yield (Fig. 35). As culture viability declines, indicated by a decrease in green/red ratio, capreomycin synthesis also demonstrates a decline. This is due to an increase in non-viable mycelia and indicates that the culture is no longer capable of producing antibiotic. It can therefore be concluded from the results that 96 h appears to be the optimal point of the fermentation for capreomycin production in terms of culture viability.

Figure 36 demonstrates the rate of decline in capreomycin quality of IA and IB, results that are synonymous with the decline in viability of the culture as it ages (Fig. 37).

Figure 34. Specific capreomycin production rate (qp), measured as (g. L^{-1} h^{-1}) against time for Complex medium and Porcine medium over 168 h. Capreomycin yield is expressed as antibiotic concentration relative to broth weight.

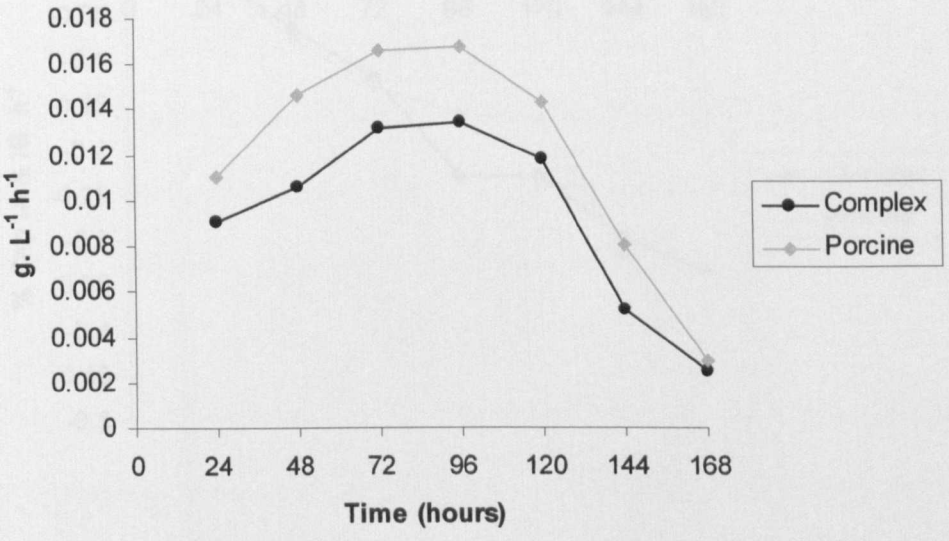


Figure 35. Relationship between capreomycin production (g. L^{-1} h^{-1}) and green:red mycelial viability rate of change of *S. capreolus* grown in Complex medium and Porcine medium over 168 h. Results are expressed as antibiotic production rate divided by green/red ratio.

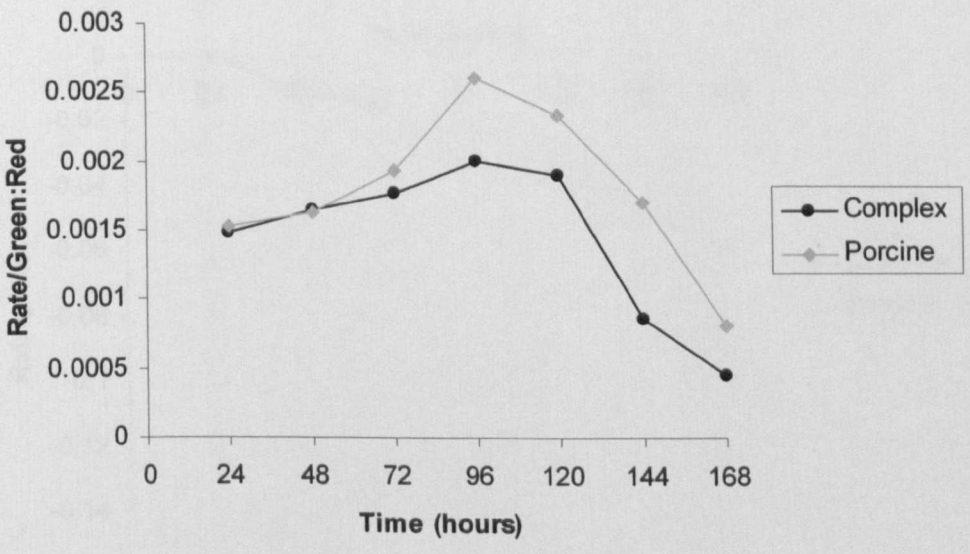


Figure 36. Rate of capreomycin quality decline (% Cap 1A & 1B. h^{-1}) against time for Complex medium and Porcine medium over 168 h.

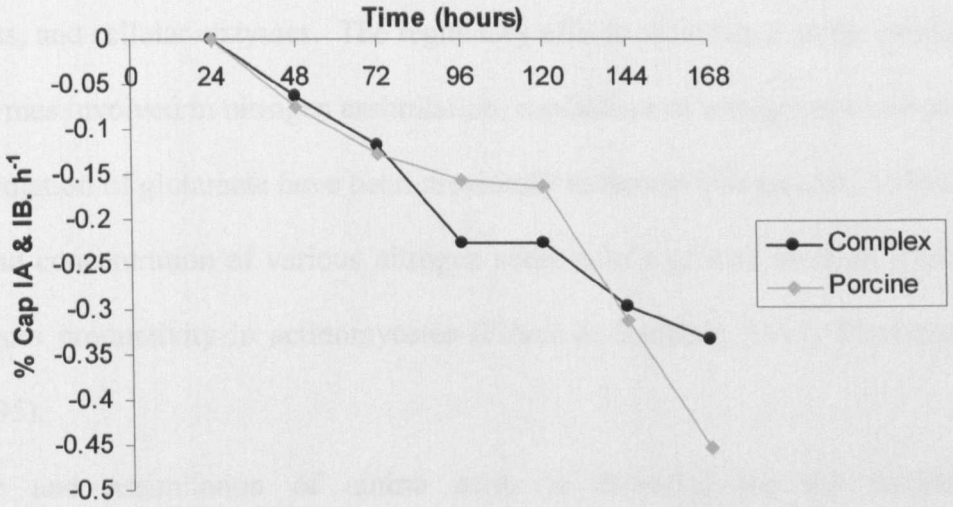
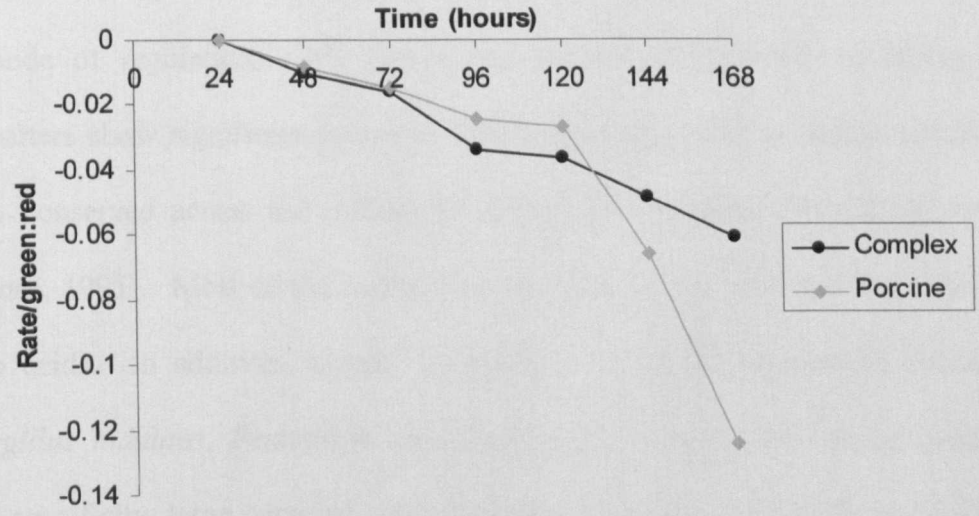


Figure 37. Relationship between capreomycin quality (% Cap 1A & 1B. h^{-1}) and gren:red mycelial viability rate of change of *S. capreolus* grown in Complex medium and Porcine medium over 168 h. Results are expressed as antibiotic quality rate divided by green/red ratio.



4.6 PREFERENTIAL UTILIZATION OF AMINO ACIDS

Nitrogen is one of the major components of living materials and plays a key role in biological regulation. The nitrogen source can govern cellular growth, formation of products, and cellular enzymes. The regulatory effects of nitrogen on the production of enzymes involved in nitrogen assimilation, catabolism of nitrogenous compounds, and formation of glutamate have been previously reviewed (Magasanik, 1976). The type and concentration of various nitrogen sources in a growth medium ultimately influences productivity in actinomycetes (Flores & Sanchez, 1985; Benslimane *et al.*, 1995).

Uptake and assimilation of amino acids is essential for the survival of microorganisms. They not only serve as the building blocks for proteins but also as the starting points for the synthesis of many important cellular molecules including vitamins and precursors. In antibiotic synthesis their role in the associated metabolic pathways of secondary metabolism is highly complex and occurs via an intricate network of substrate pathways. In most cases the coordinated expression of the enzymes of nitrogen metabolism appears to respond primarily to the intracellular nitrogen pool (Merrick & Edwards, 1995).

Amino acid transporters differ in their substrate specificities, uptake capacities and the mode of regulation. All fungal and several of the bacterial amino acid transporters show significant sequence similarities, suggesting a unique transporter family conserved across the prokaryotic-eukaryotic boundary (Sophianopoulou & Diallinas, 1995). Most of the transporters are specific for one or a few related L-amino acids. In addition, several organisms such as *Saccharomyces cerevisiae*, *Aspergillus nidulans*, *Penicillum chrysogenum* and *Neurospora crassa* possess a broad specificity, large capacity, general amino acid permease (GAP) mediating the

uptake of most L- and D-amino acids, non-proteinogenic amino acids such as citrulline, ornithine and a number of amino acid analogs. Most microorganisms thus possess multiple transport systems with partially overlapping specificities (Bapat *et al.*, 2006a).

Most of the industrial fermentations involving actinomycetes employ a mixture of inorganic and organic nitrogen substrates as is the case with capreomycin production by *S. capreolus*. For the commercially important actinomycete fermentations, the sequence of uptake of amino acids and the underlying mechanism of regulation has not been reported. It is of interest to predict the sequence of uptake of an amino acid and its implication on product formation under various nitrogen substrate combinations (Bapat *et al.*, 2006a). We therefore investigated amino acid uptake in contrasting media, where capreomycin production was present and absent to allow a comparison to be made between the two and also with a view to rationale medium design.

The pathways associated with capreomycin production are not well characterized and the following results aim to acquire a better understanding of the uptake of free amino acids in a medium that supports capreomycin production and one that fails to support production. A preferential utilization profile of extracellular amino acids present in both the Complex medium and the Defined medium has been demonstrated. Samples were taken from *S. capreolus* grown in shakeflask culture over 168 h and analysed as described in Analytical Procedures, whereby reversed-phase HPLC coupled with a pre-column fluorescence derivatization technique was employed.

Samples were analysed up to 168 h of growth, however it was apparent that all amino acid concentrations had reached zero by 120 h, therefore the latter results are not included.

In terms of amino acid utilization in the non-capreomycin supporting Defined medium, amino acid consumption can be divided into four hierarchical groups: fastest reduction (Fig. 38a), moderate reduction (Fig. 38b), slowest reduction (Fig. 38c) and one that shows an increase in amino acid concentration (Fig. 39).

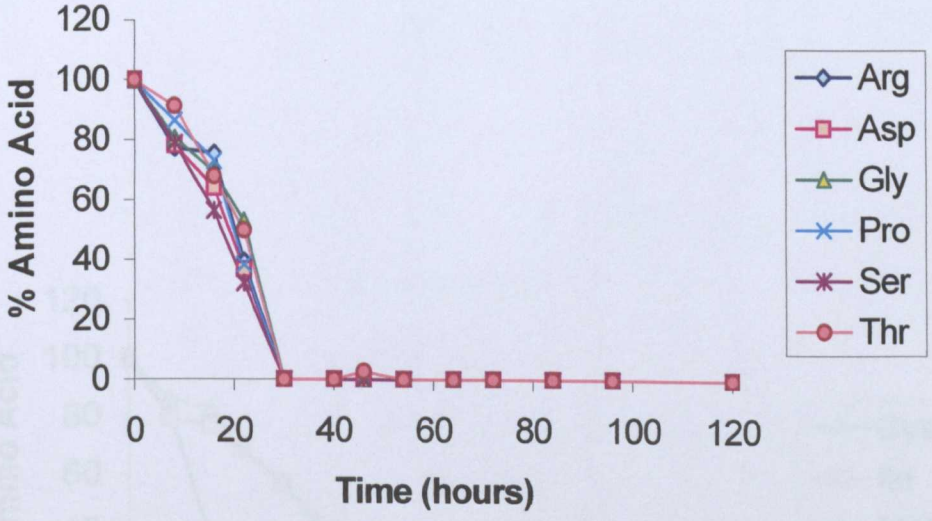
A reduction in initial amino acid concentrations is detected early on in the fermentation and arginine, asparagine, glycine, proline, serine and threonine are exhausted rapidly before 30 h. Indeed it is apparent that all of the free amino acids measured in the Defined medium are exhausted by approximately 60 h, whilst from 0 h it can be observed that there is an increase in alanine and phenylalanine concentration followed by their consequent consumption before 60 h.

The Complex production medium gives rise to three noticeable groups of amino acid consumption: fastest amino acid reduction (Fig. 40a), slowest reduction (Fig. 40b) and again an increase in certain amino acids (Fig. 41).

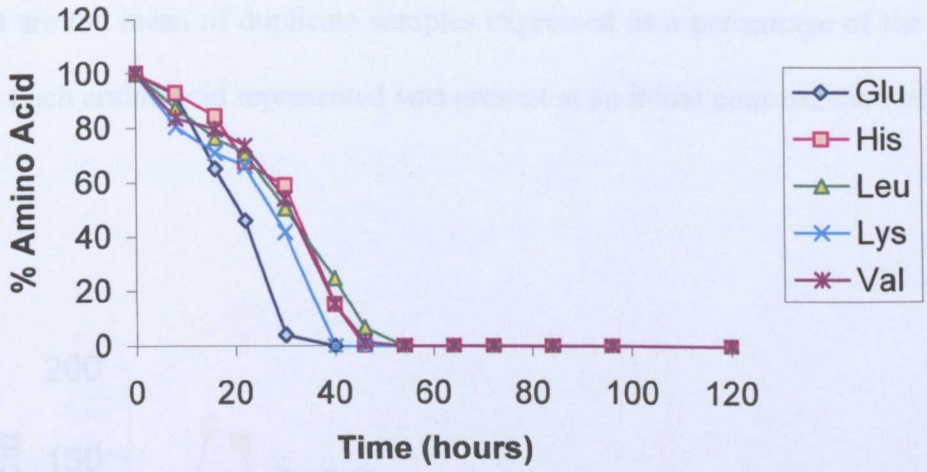
A rapid consumption of histidine, isoleucine and phenylalanine is observed followed by the more gradual reduction of the other amino acids in the medium throughout the fementation. In contrast, alanine and threonine are shown to increase over the course, whilst proline increases by more than 600 % of its initial concentration by 24 h.

Figure 38. Amino acid consumption expressed as degree of utilization; fastest **(a)**, moderate **(b)**, and slowest **(c)** in shakeflasks contanining *S. capreolus* and Defined medium, grown at 30 °C, 250 rpm over 120 h. An aliquot (20 µl) from each medium was taken and analysed as described in analytical procedures. Values obtained are the mean of duplicate samples expressed as a percentage of the initial amount. Each amino acid represented was present at an initial concentration of 0.2 g. L^{-1}.

a)



b)


% Amino Acid
120
100
80
60
40
20
0
0
20
40
60
80
100
120
Time (hours)
Glu
His
Leu
Lys
Val

c)

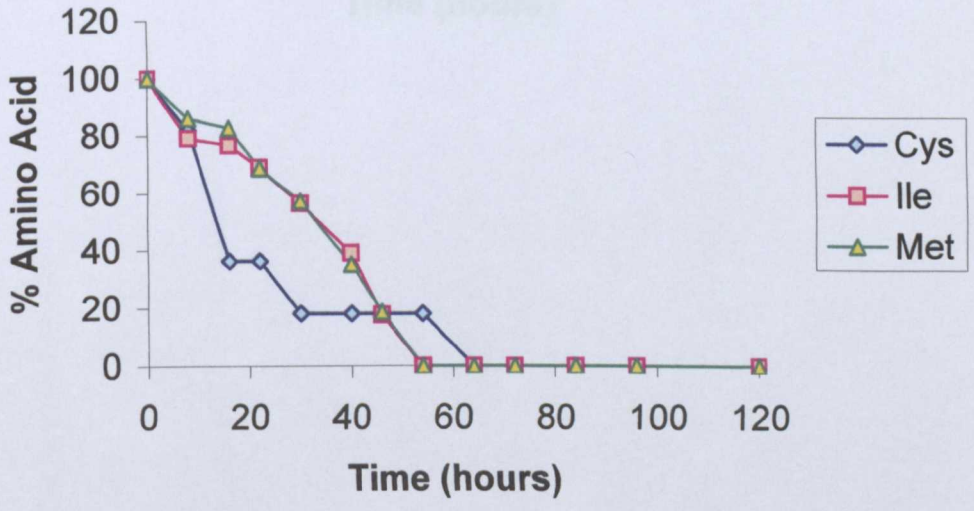

% Amino Acid
120
100
80
60
40
20
0
0
20
40
60
80
100
120
Time (hours)
Cys
Ile
Met

Figure 39. Increase in alanine and phenylalanine in shakeflasks contanining *S. capreolus* and Defined medium, grown at 30 °C, 250 rpm over 120 h. Values obtained are the mean of duplicate samples expressed as a percentage of the initial amount. Each amino acid represented was present at an initial concentration of 0.2 g. L^{-1}.

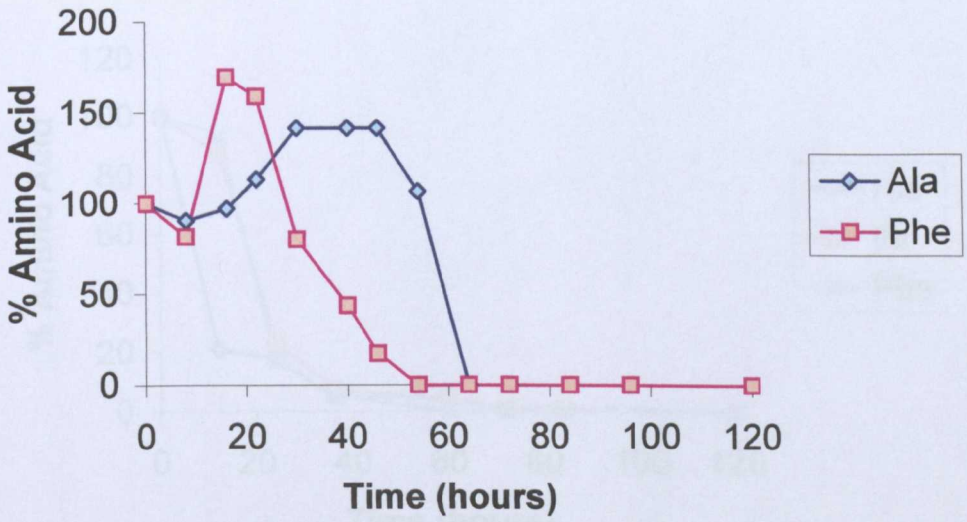
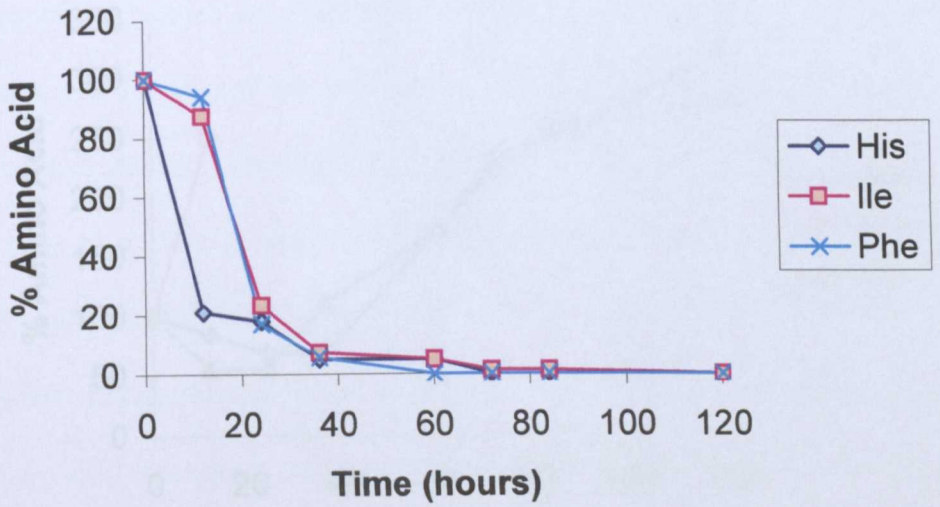


Figure 40. Amino acid consumption expressed as degree of utilization; fastest **(a)**, and slowest **(b)** in shakeflasks contanining *S. capreolus* and Complex medium grown at 30°C, 250 rpm over 120 h. An aliquot (20 µl) from each medium was taken and analysed as described in analytical procedures. Values obtained are the mean of duplicate samples expressed as a percentage of the initial amount.

a)



b)

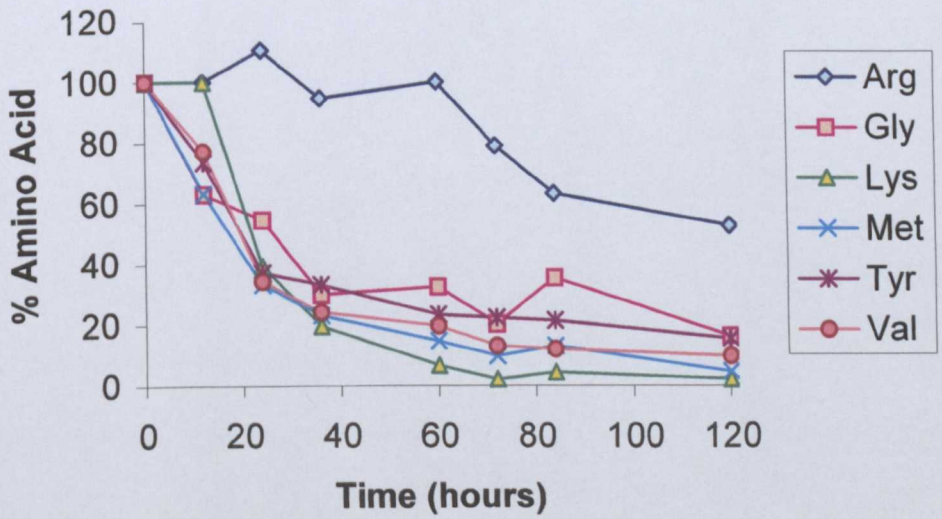
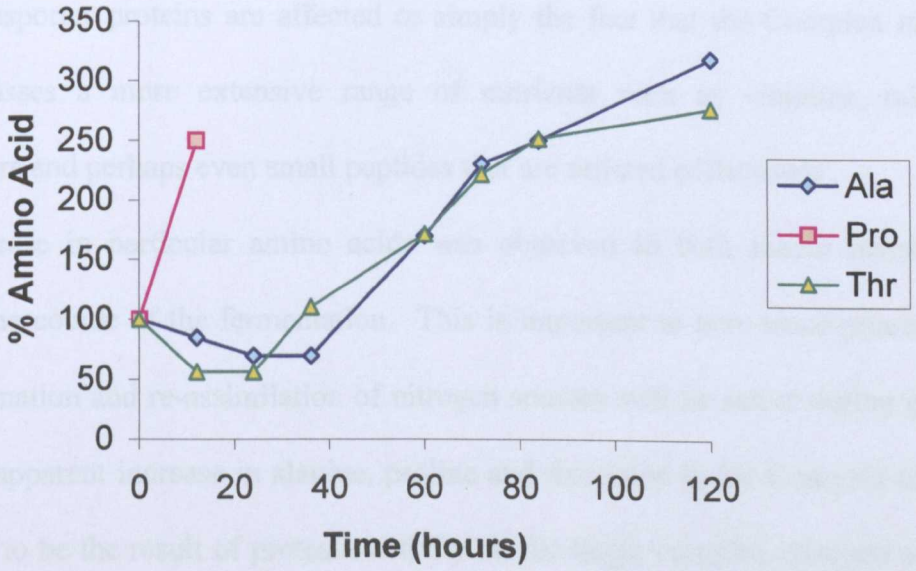


Figure 41. Increase in alanine, proline and threonine in shakeflasks contanining S. capreolus and Complex medium, grown at 30 °C, 250 rpm over 120 h. Values obtained are the mean of duplicate samples expressed as a percentage of the initial amount. Proline increases above 600 % of the initial concentration by 20 h (not indicated on graph).



The results for the Defined medium are in significant contrast to utilization in the Complex medium, whereby histidine, isoleucine and phenylalanine are the first amino acids to be exhausted, this only occurs by approximately 60 h in contrast to 30 h as observed in the Defined medium, and the remaining amino acids are progressively utilized throughout 120 h. This slow and progressive utilization of free amino acids suggests that other factors may potentially be slowing the uptake process in the Complex medium. This may be at a biochemical level whereby amino acid transporter proteins are affected or simply the fact that the Complex medium encompasses a more extensive range of nutrients such as vitamins, minerals, precursors and perhaps even small peptides that are utilized collectively.

An increase in particular amino acids was observed in both media investigated during the course of the fermentation. This is important to note since processes of transamination and re-assimilation of nitrogen sources will be active during growth, and the apparent increase in alanine, proline and threonine in the Complex medium is likely to be the result of protease activity on the large, complex nitrogen portions of the medium, yielding free amino acids from polypeptides. It is likely that *S. capreolus* transports the necessary compounds required for growth, whilst others may be left to accumulate in the medium. Explanantion of the apparent increase in alanine and phenylalanine in the Defined medium is more complicated and may reflect the ability of the organism to synthesize these compounds. There is an unexplainable increase above initial alanine and phenylalanine concentrations and may suggest transamination and re-assimilation processes occurring in *S. capreolus*. The ability of the organism to synthesize amino acids is a rare occurrence in streptomycetes. Other actinomycetes are favoured for their amino acid production at

large scale, such as *Corynebacterium glutamicum* (Schmid *et al.*, 2000), which produces alanine, and proline.

Alanine and phenylalanine are shown to increase in the Defined medium early on followed by their rapid depletion. This suggests that these amino acids are primarily synthesized in order to sustain growth of the organism since no capreomycin biosynthesis is noted in this medium. Amino acid synthesis is most likely a reflection of nutrient levels within the growth medium. Although an increase in measured free amino acids is observed in the Complex medium, there is no evidence that the mentioned amino acids are utilized following their creation. Indeed, their concentration increases throughout and beyond 120 h.

The extensive release or indeed production of proline very early on in the Complex medium is significant, and may suggest the importance of proline in the growth of *S. capreolus*, notably since it is rapidly utilized in the Defined medium. An increase in proline in the Defined medium is not observed however. This may be apparent for several reasons including the absence of the relevant precursors to allow proline synthesis to proceed. Although there is no measured decrease of the amino acids generated in the Complex medium which would indicate their consumption, this may in fact be overshadowed by their extensive increase in concentration. Synthesis may occur at a much higher rate than their consumption and thus may not be evident from the results.

It has been previously stated in the section outlining the response of *S. capreolus* to NaCl concentration, that proline has been shown to act as an osmoprotectant alongside alanine in times of osmotic stress. This may be coincidental or may in fact provide an explanation for the increase of these amino acids in the Defined and Complex media. A surplus of these amino acids to the cells would lead to their

extracellular excretion and would provide a hypothesis as to why a measured increase in their concentration was observed.

Chen & Kaiser (2002) have also demonstrated a hierarchical utilization of particular amino acids for *Saccharomyces cerevisiae*, whilst Bapat *et al.*, (2006a) have recently presented a preferential amino acid utilization profile for *Amycolatopsis mediterranei* using a similar amino acid derivatization method, and found that growth occurred in both the presence and absence of glucose, indicating that amino acids were being utilized as the carbon source but did not support rifamycin B production during glucose absence. Similarly a sudden arrest in the utilization of amino acids was observed at approximately 60 h, which the authors suggest is attributed to inhibition by ammonia accumulation. This explanation may support the decrease in amino acid utilization in the Complex medium where ammonia production increases substantially throughout a typical 168 h fermentation as demonstrated in the section on growth characteristics, but ammonia accumulation in the Defined medium is relatively low in comparison and arrest of amino acid utilization may simply be due to their exhaustion, which may ultimately lead to nitrogen limitation.

4.7 CARBON & NITROGEN METABOLISM

Microorganisms are able to synthesize a great variety of metabolites that are required for growth, development, and survival. To gain selective advantages, highly developed mechanisms for the regulation of metabolic processes have evolved that enable organisms to respond to and to utilize as efficiently as possible the nutrients that are normally available in the environment. Thus, the presence of a required metabolite in excess, generally results in the repression and/or inhibition of enzymes leading to its own synthesis, with the concomitant decrease in the concentration of intermediates that are part of its biosynthetic pathway (Aharonowitz, 1980).

When bacteria are exposed to a combination of carbon sources, they select the substrate that yields a maximum profit for growth (Gunnewijk *et al.*, 2001; Brückner & Titgemeyer, 2002). Therefore they have developed sophisticated mechanisms that enable them to sense the nutritional situation and adjust their catabolic capacities by regulatory responses that fall under the term carbon catabolite repression (Titgemeyer & Hillen, 2002), which is the result of global transcriptional control and inducer exclusion.

Nitrogen also has a major role in regulatory mechanisms in bacterial metabolism. The form in which a nitrogen source is provided to a cell determines the pattern of formation of enzymes, intermediates, and end products (Aharonowitz & Demain, 1979). The assimilatory metabolic pathways of nitrogen metabolism can be divided into two classes: the pathways necessary for utilization of nitrogen from the extracellular medium and the biosynthetic pathways for intracellular production of nitrogen-containing compounds (Merrick & Edwards, 1995).

The effect of carbon and nitrogen sources on growth and biosynthesis of the cephalosporins in the actinomycete *Streptomyces clavuligerus* have been studied. It

was found that all the cephalosporins have a common β-lactam structure and are assembled from the primary metabolites L-cysteine, L-valine and L-α-aminoadipic acid. Indeed, the repression of enzymes belonging to the specific pathway of β-lactam antibiotic synthesis by ammonium has been demonstrated extensively (Shen *et al.*, 1984; Brana *et al.*, 1985 Castro *et al.*, 1985; Zhang *et al.*, 1989; Demain & Vaishnav, 2006). The central role of ammonia in nitrogen metabolism and the associated metabolic pathways have been reviewed and illustrated at the level of enzyme activity, translation and transcription by ter Schure *et al.*, (2000).

Nitrogen substrate is assumed to be present in two forms in a typical complex medium as employed by many industrial processes; available nitrogen such as free amino acids, and unavailable nitrogen such as peptides and proteins. Although available nitrogen is required for growth and product formation, high concentrations have been found to inhibit antibiotic synthesis, such as rifamycin production (Bapat *et al.*, 2006b). It has therefore been assumed that complex media sources with lower available nitrogen encourage higher antibiotic productivity. Minimization of the effects of nitrogen catabolite repression is thought to be responsible for this phenomenon (Bapat *et al.*, 2006b).

The availability of such primary metabolic precursors for antibiotic biosynthesis depends mainly on the regulatory processes that control both the anabolic reactions and the catabolic pathways of nitrogen metabolism, including the degradation of proteins and amino acids. Furthermore, the four capreomycin species are based around a common peptide containing α, β-diaminopropionic acid and α (2-iminohexahydro-4-pyrimidyl) glycine, thereby it is reasonable to suggest that the absence of these directly in the growth media is indicative of anabolic and catabolic nitrogen metabolism in *S. capreolus*. The active excretion of ammonia into the

Complex and Porcine media is a further example of such nitrogen metabolism, and in particular, is indicative of amino acid degradation.

4.7.1 CARBON & NITROGEN LIMITATION IN CONTINUOUS CULTURE

Traditionally, most studies into the metabolism of microorganisms have relied on growth under batch culture conditions. Although useful for many purposes, batch growth suffers from a number of drawbacks. The levels of substrates in a batch culture, for the majority of the growth phase are very high, only becoming low during a short phase between exponential and stationary state (Small, 1994). Even during exponential growth, the internal physiology of the microorganism may be constantly changing due to the response to changing levels of substrate. Chemostats provide two major advantages over batch growth conditions. Firstly the use of continuous culture allows steady-state conditions to be set up, such as substrate levels, biomass and productivity. In addition, it is possible to obtain a wide range of growth rates and substrate concentrations by manipulation of the experimental conditions. A continuous process may be more economic than a batch process if higher productivities at higher efficiencies can be achieved. Unfortunately, in some processes, the final product concentration in the effluent broth from a continuous culture will be less than that obtained in a batch process, which will create a need for greater concentration at the recovery stage (Stanbury *et al.*, 2000).

The principal advantage of continuous culture for the following study of microbial metabolism in *S. capreolus* is the guarantee of reproducible biomass in a constant physiological state for a maintained period under a controlled set of conditions, thereby allowing comparability of results between experiments and reducing experimental variability overall. Hoskisson & Hobbs, (2005) have reviewed the experimental benefits of continuous culture. Growth of *S. capreolus* has not been previously investigated in continuous culture and so the approach including the nutrient composition was largely empirical in nature. Indeed, *S. capreolus* was

successfully grown as a continuous culture in a defined medium, both under carbon and nitrogen limited conditions. The provision of a chemically defined medium enables the exact analysis of the physiological response of an organism to nutrients and other key factors affecting growth and product formation.

For the establishment of continuous culture, *S. capreolus* was initially grown as a batch fermentation until ~96 h when sterile medium was added at a dilution rate of 0.0073 h^{-1}. This dilution rate was found to balance cell loss from the vessel and new biomass formation, thereby achieving a steady state. Investigation of substrate uptake was performed during this steady state.

S. capreolus exhibited particularly slow growth characteristics, with a dilution rate of 0.0073 h^{-1} and a doubling time of 94.5 h^{-1}. Biomass was successfully generated however at approximately 4.0 g. L^{-1} during steady-state under both carbon and nitrogen limited conditions for further experiments.

The molar C/N ratios for *S. capreolus* that result in carbon-limited and nitrogen-limited growth were investigated using serine as the sole nitrogen source and glucose as the sole carbon source.

This was achieved by varying the concentration of one nutrient, whilst maintaining the other at a constant level. The carbon content of serine was included in the subsequent calculations.

Serine was chosen due to its large presence in the capreomycin species and also due to the fact that it was rapidly utilized during the amino acid studies.

Carbon limited growth was found to occur at a C/N ratio of 18:1 and below (Fig. 42), whilst nitrogen limitation was found to occur at a ratio of 33:1 and above (Fig. 43). Subsequent nutrient limited conditions were carried out under these conditions.

Figure 42. The effect of increasing levels of carbon on biomass formation by *S. capreolus* grown in shakeflasks at 30 °C, 250 rpm for 72 h. Initial nitrogen concentration was 20 mM.

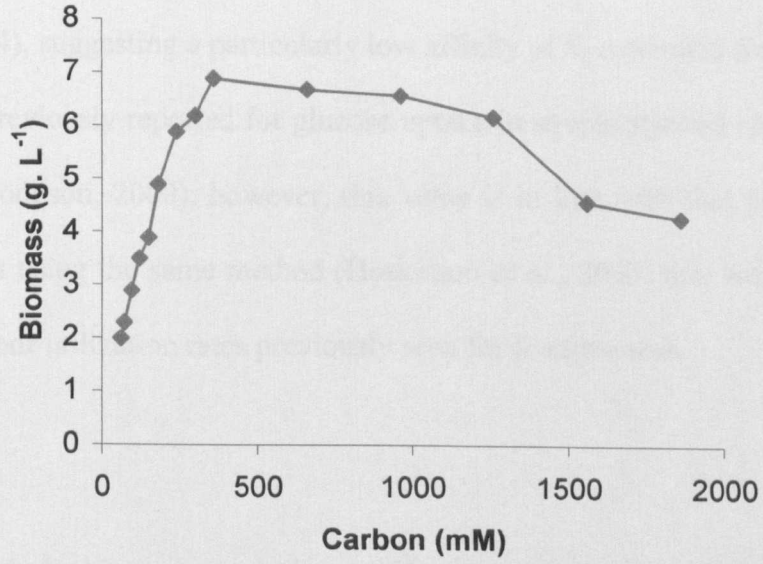
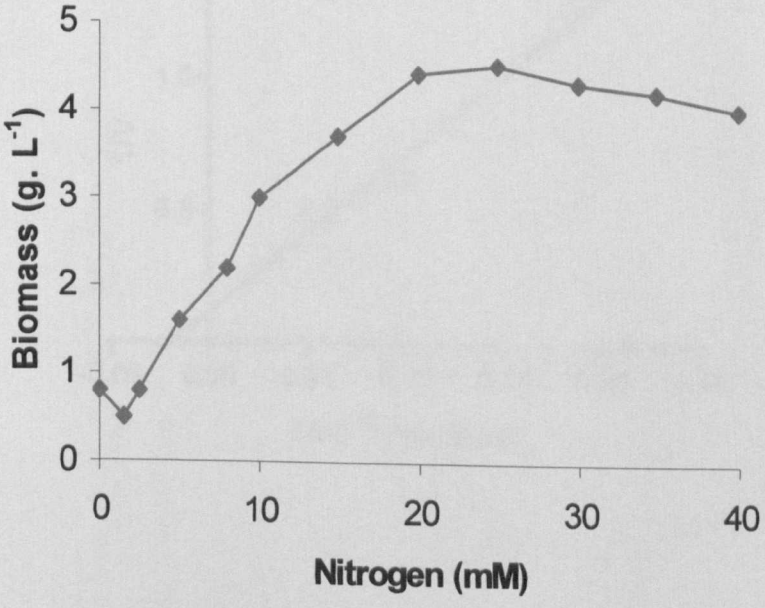


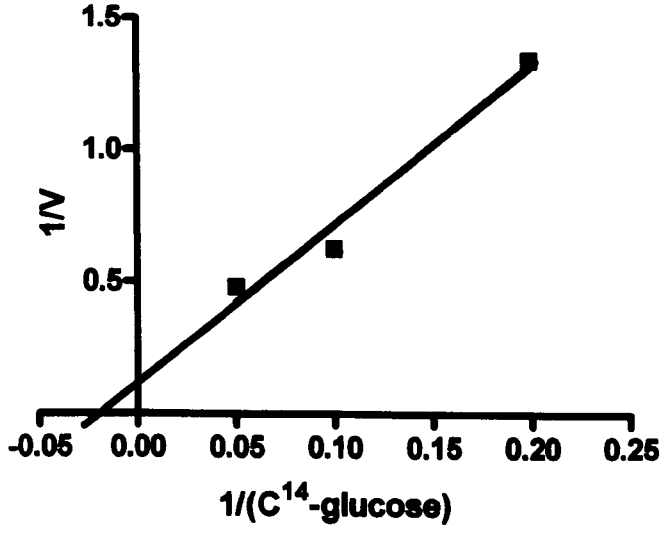
Figure 43. The effect of increasing levels of nitrogen on biomass formation by *S. capreolus* grown in shakeflasks at 30 °C, 250 rpm for 72 h. Initial carbon concentration was 600 mM.



4.7.2 SUBSTRATE AFFINITY CONSTANT

Radioactive substrate uptake studies, using mycelia grown in minimal medium in a nitrogen-limited continuous culture were used to determine the substrate affinity constant (K_s) for glucose. Under these conditions the K_s was determined to be 46 mM (Fig. 44), suggesting a particularly low affinity of *S. capreolus* for this substrate. K_s values previously reported for glucose uptake in streptomycetes range from 0.05-6.2 mM (Hodgson, 2000), however, this value is in line with that proposed for *M. echinospora* using the same method (Hoskisson *et al.*, 2003) and would explain the relatively poor utilization rates previously seen for *S. capreolus*.

Figure 44. A double reciprocal plot showing glucose uptake rate (mM min^{-1} g. dry wt^{-1}) against glucose concentration (mM). Biomass used for this analysis was harvested from a nitrogen-limited chemostat culture of *S. capreolus* and the K_s value was calculated to be 46 mM after duplicate results.



4.7.3 EFFECT OF CARBON & NITROGEN LIMITATION ON MORPHOLOGICAL STATUS

Control of mycelial morphology is often a prerequisite for industrial application, particularly since it has been established that productivity is predominantly determined by morphology (Atkinson & Daoud, 1976; Hermersdörfer *et* al., 1987; Braun & Vecht-Lifshitz, 1991; Nielsen *et al.*, 1995).

Many factors are attributed to culture morphology, including the nutritional availability within a fermentation.

The complex interactions between morphology, productivity and process conditions in fermentations of filamentous microorganisms are shown in Fig. 45.

The morphological appearance of *S. capreolus* under conditions of carbon limitation was in stark contrast to that during nitrogen-limited growth as shown in Fig. 46(a) and Fig. 46(b) respectively. It appears that nitrogen limitation leads to a predisposition for culture fragmentation, giving rise to a highly branched and largely fragmented culture in comparison to a highly pelleted culture with minimal branching under carbon limitation conditions, suggesting that carbon and nitrogen exert a very strong constraint over culture morphology. Thus there is potential to select and control bacterial morphology by altering the C/N ratio of the media.

Figure 45. Complex interactions between morphology, productivity, and process conditions in submerged fermentations of filamentous microorganisms (Reproduced from Cox *et al.*, 1998).

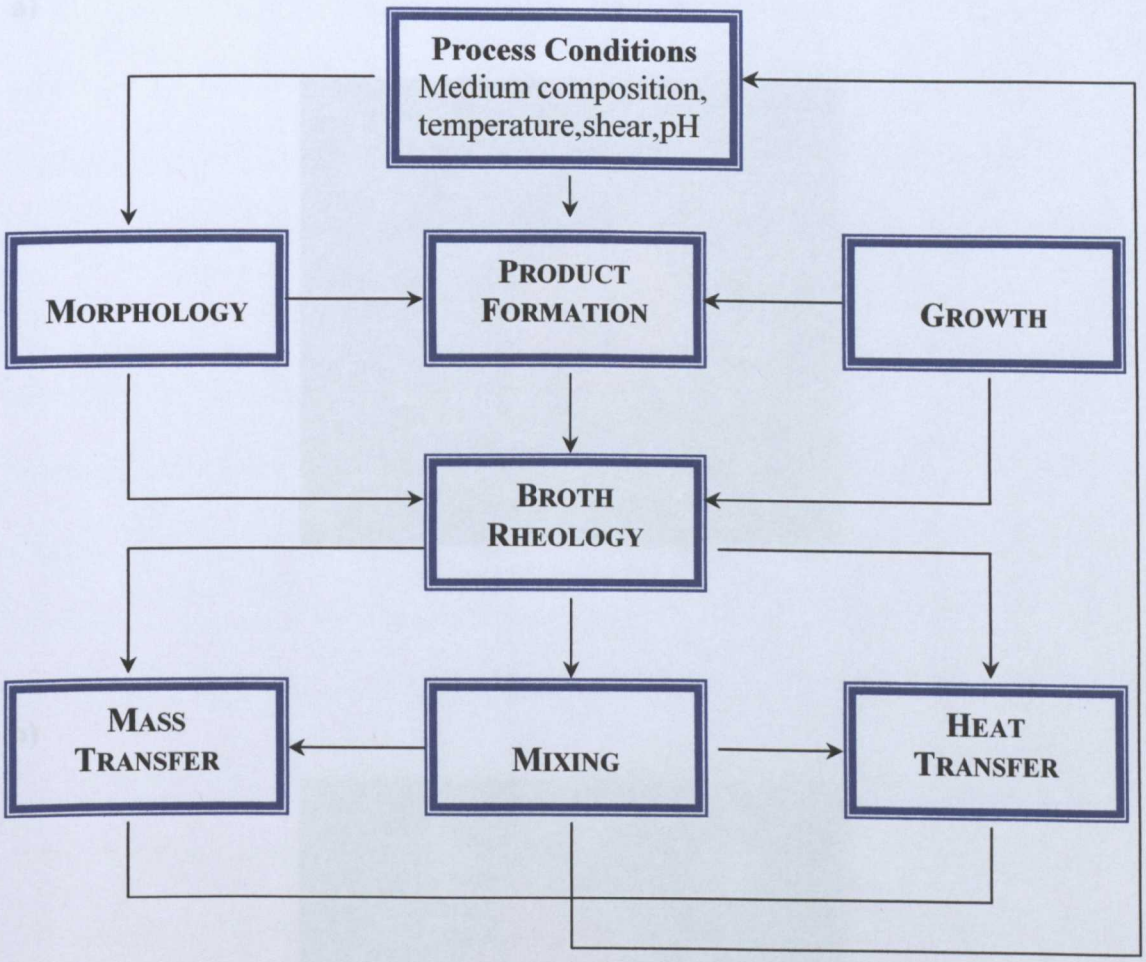
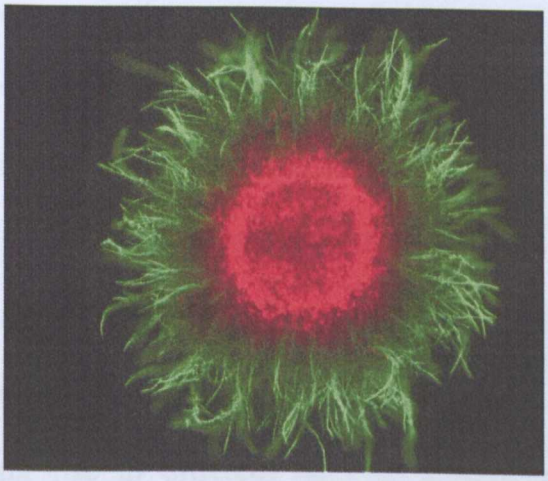
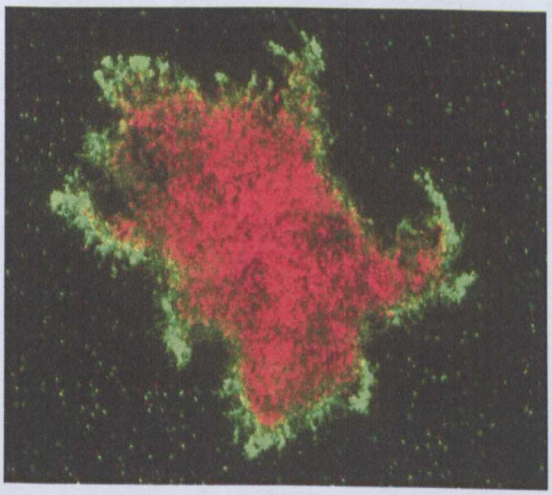


Figure 46. Morphology of *S. capreolus* from continuous culture under carbon-limited conditions **(a)**, and nitrogen-limited conditions **(b)**, stained with *Bac*Light bacterial viability stain showing viable mycelial areas as green (SYTO 9) and non-viable areas as red (propidium iodide) at x 400 magnification.

a)



b)



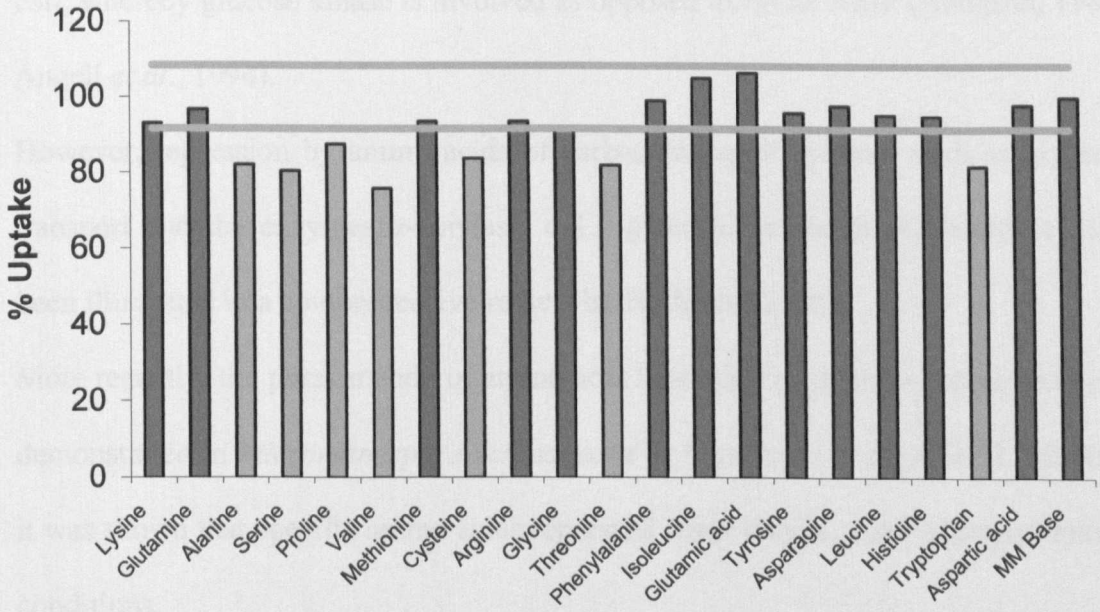
4.7.4 REPRESSION OF GLUCOSE UPTAKE BY AMINO ACIDS & SUGARS

The importance of glucose as a carbon source is paramount to many industrial processes to date, it is surprising therefore that relatively little is known about sugar transport in streptomycetes and the factors which govern its uptake and utilisation. Structurally, glucose is believed to be vital in the formation of the carbon backbone of capreomycin and thus forms a large portion of the complex media composition necessary to permit capreomycin production. It is thus of great importance to understand the factors which may potentially inhibit efficient uptake of glucose thereby reducing this phenomenon by maximizing media composition and thus directing metabolism towards antibiotic biosynthesis. The impact of catabolite repression in an industrial process is heightened with the use of undefined raw materials, since the variability of the integral components between batches can differ greatly, thereby giving rise to different rates of catabolite repression between batches. A result of this is inconsistent antibiotic productivity between fermentations thus giving rise to unstable and largely variable processes.

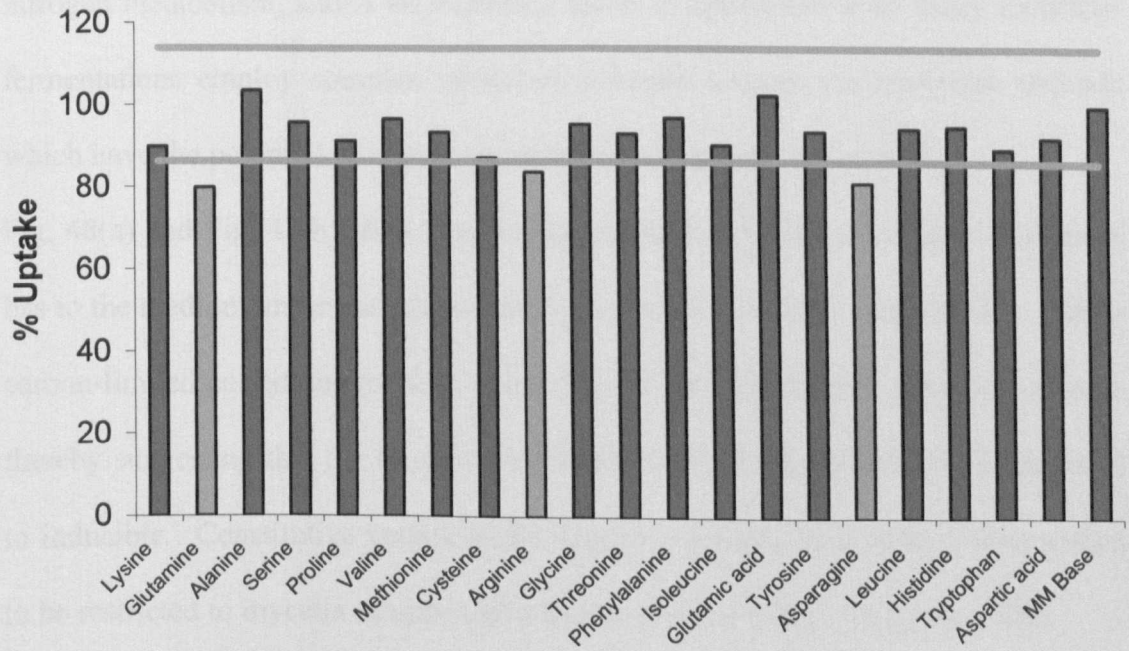
The response that *S. capreolus* exhibits when confronted with amino acids as potential carbon sources in the presence of glucose has been demonstrated, an effect that appears to be more pronounced during carbon-limited conditions (Fig. 47a) in comparison to nitrogen-limited conditions (Fig. 47b).

Figure 47. Repression of ^{14}C-glucose uptake by amino acids (0.2 g. L^{-1}) using carbon limited (a) and nitrogen limited (b) chemostat cultures of *S. capreolus*. Results shown as percentages of the control (highlighted column) and amino acids with values below the standard error bars show a significant effect on glucose uptake.

a)



b)



Indeed, it is widely recognised that glucose is responsible for repression of uptake systems in many streptomycetes. In *Streptomyces coelicolor* A3(2) it has been found that glucose is responsible for repressing the expression of many genes involved in the utilization of alternative carbon sources, such as arabinose and glycerol (Hodgson, 1982; Smith & Chater, 1988). This is known as carbon catabolite repression and differs fundamentally in mechanism from that found in *Escherichia coli*, whereby glucose kinase is involved as opposed to cyclic AMP (Hodgson, 1982; Angell *et al.*, 1994).

However, repression by amino acids of carbon transport systems such as glycerol transport and the enzymes α-amylase, α-1,4-glucosidase and β-galactosidase have been illustrated in a comprehensive review by Hodgson (2000).

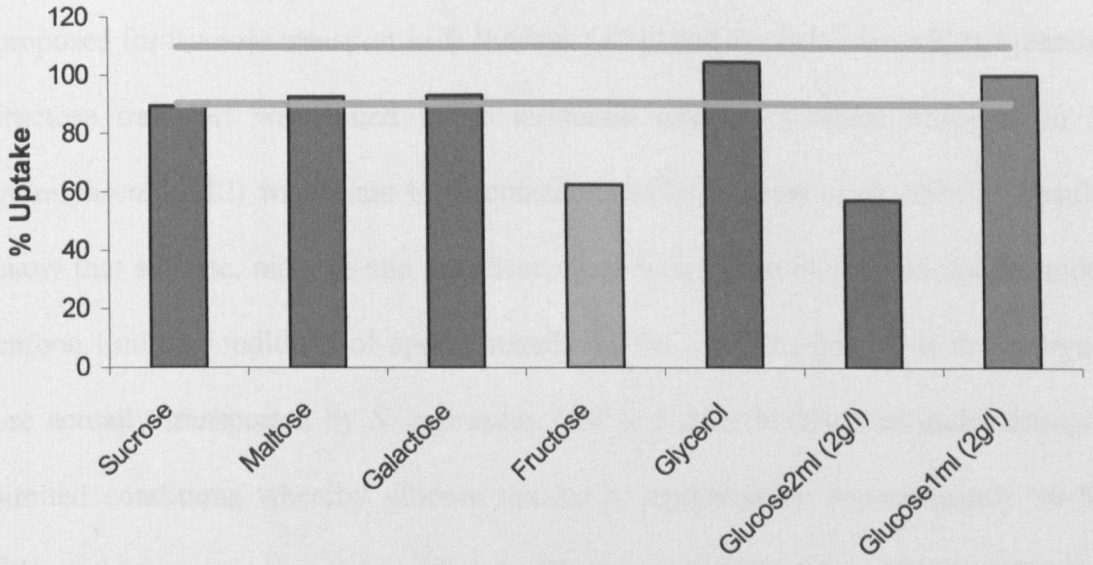
More recently, the phenomenon of amino acid inhibition of glucose uptake has been demonstrated in *Micromonospora echinospora* by Hoskisson *et al.*, (2003), whereby it was shown that specific amino acids repressed sugar uptake under nitrogen limited conditions.

This further compounds the intrinsic link that appears to exist between carbon and nitrogen metabolism, and is an important factor to appreciate since many industrial fermentations employ complex undefined nitrogen sources via empirical methods which have the potential to impact on other carbon sources in the media.

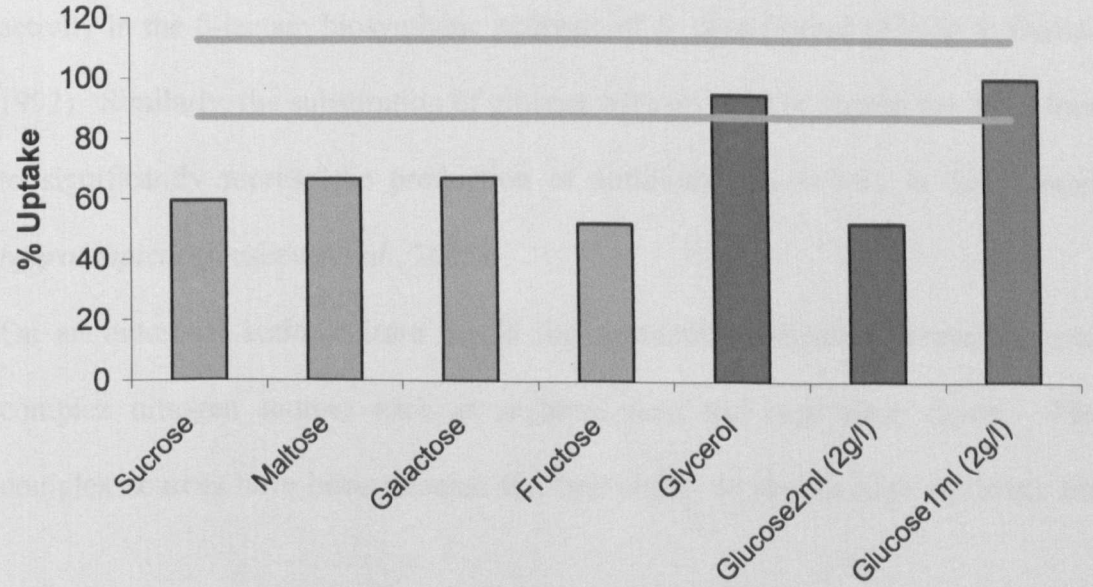
Fig. 48(a) and Fig. 48(b) show the impact that adding sugars alternative to glucose has to the medium under carbon and nitrogen limited conditions respectively. Under carbon-limited conditions fructose appears to be actively utilized alongside glucose thereby suggesting that the enzymes for fructose uptake are constitutive as opposed to inducible. Constitutive carbohydrate transport systems have indeed been shown to be restricted to mycelia as opposed to spores in many cases (Hodgson, 2000).

Figure 48. Repression of ^{14}C-glucose uptake by sugars (2.0 g. L^{-1}) using carbon limited **(a)** and nitrogen limited **(b)** chemostat cultures of *S. capreolus*. Results shown as percentages of the control (highlighted column), whereby mycelia was re-suspended in 1.0 ml (2.0 g. L^{-1}) glucose plus 1.0 ml MM base. Sugars with values below the standard error bars show a significant effect on glucose uptake.

a)



b)



These results suggest that fructose may be a more suitable carbon source for capreomycin production, however limited data has shown that capreomycin production at 168 h is 50 % of the yield with fructose, than when glucose is employed as the sole sugar. It is therefore not advantageous and inexplicable as to why fructose is not suitable for the formation of the secondary metabolite capreomycin particularly since it appears that the enzymes for its metabolism exist. Despite there being no evidence to suggest that a phosphoenolpyruvate-phosphotransferase (PEP-PTS) system exists for glucose transport, one has been proposed for fructose transport in *S. lividans* (A21) and *S. coelicolor* A3(2), whereby fructose transport was found to be inducible whereas fructose transport in *S. griseofuscus* (A12) was found to be constitutive (Titgemeyer *et al.*, 1995). Results show that sucrose, maltose and galactose cause a decrease in glucose uptake under carbon limited conditions of approximately 10 %. This suggests that these sugars are actually transported by *S. capreolus*, and is further highlighted under nitrogen limited conditions whereby glucose uptake is repressed by approximately 50 %. Glycerol however, does not exert any affect on glucose uptake indicating that this sugar is not readily utilizable by the organism. Indeed, it has been found that glycerol represses δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) synthetase activity in the β-lactam biosynthetic pathway of *S. clavuligerus* (Zhang & Demain, 1992). Similarly, the substitution of glucose with glycerol or lactose has been found to significantly repress the production of antibiotic AK-111-81 in *Streptomyces hygroscopicus* (Gesheva *et al.*, 2005).

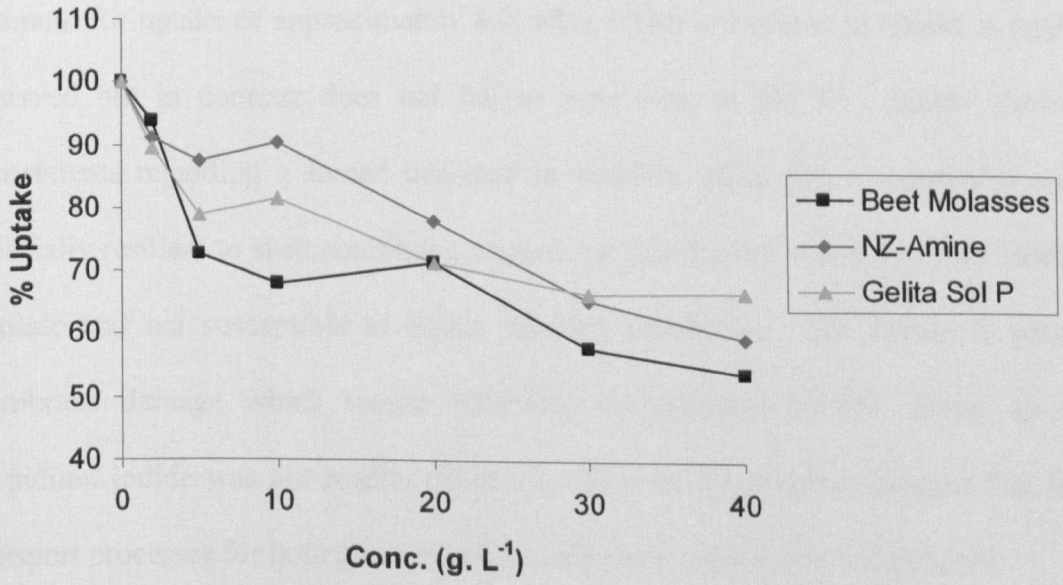
On an industrial scale, culture media for antibiotic production generally contain complex nitrogen sources such as soybean meal and corn steep liquor. These complex sources have been selected for their ability to sustain high antibiotic titres

primarily due to their slow release of nitrogenous components during the course of the fermentation (Voelker & Altaba, 2001). The capreomycin Complex production medium employs NZ-Amine and Gelita Sol P as the undefined nitrogenous portion of the medium and Fig. 49 shows the effect of increasing the concentration of these nitrogenous sources on glucose uptake. The complex carbohydrate, beet molasses is also present within the Complex medium, therefore as an undefined component, analysis of the effect on glucose uptake was also investigated. It can be observed from Fig. 49, that NZ-Amine and Gelita Sol P repress glucose uptake, a phenomenon that becomes more pronounced with an increase in their concentration. The effect of these complex nitrogenous sources is somewhat expected, and is more marked since they encompass a higher degree of total nitrogen in comparison to the nitrogen component of the free amino acids investigated. NZ-Amine is present in the current Complex production medium at 4.0 g. L^{-1}, whilst Gelita Sol P is present at 29.2 g. L^{-1}. These results have shown that independently they repress glucose uptake by 20 % and 30 % respectively. The effect on uptake when combined at these concentrations has not been investigated but there is the potential for up to a 50 % decrease in glucose uptake to be experienced. Indeed, this does not take into account the repressive impact that beet molasses has in the medium. At 7.0 g. L^{-1}, beet molasses causes a 30 % decrease in glucose uptake. Indeed, beet molasses is largely composed of fructose together with glucose, and the effect of sugars on glucose uptake has indicated that fructose is a significant repressor of glucose uptake. This may thus explain the marked effect that beet molasses has on sugar uptake.

This research further illustrates the intrinsic link between carbon and nitrogen metabolism, an area relatively little is known about, particularly in streptomycetes. When altering one entity it is important to begin to understand the influence and

impact that is had on the other. This is vital in the formulation of fermentation media, particularly when undefined complex raw materials such as peptones are employed which can ultimately vary in composition between batches. As suggested by Hoskisson *et al.*, (2003), a solution to overcome such problems of inhibition of glucose uptake would be to move towards chemically defined media. Such a move however would be costly but would improve process variability and downstream processing, thus recovering the cost in the latter stages of production. A more cost effective route would be to employ fed-batch fermentations thereby keeping nutrient levels below those that would induce repression of the carbon source. The importance of carbon and nitrogen sources within a fermentation is paramount to product biosynthesis, it is therefore necessary to understand their interactions with each other and assess the advantages of employing a substrate feeding regime.

Figure 49. Repression of ^{14}C-glucose uptake by beet molasses, NZ-Amine and Gelita Sol P using carbon limited chemostat cultures of *S. capreolus*. Results shown as percentages of the control (MM base containing no supplement).



4.7.5 EFFECT OF pH ON SUBSTRATE UPTAKE

The response of *S. capreolus* to uptake of glucose, fructose and glutamate in buffered Minimal Medium base over a pH range of 2-12 has been investigated using radiolabelled uptake studies as described in Materials and Methods.

Figure 50 shows the effect of pH on glucose uptake and indicates a pH optimum of approximately 9.0, higher than this causes glucose uptake by *S. capreolus* to decrease dramatically to virtually zero, an explanation for which may be the repression of metabolic uptake systems at such a high pH or disturbance of the cell membrane leading to leakage of glucose which in fact has been taken up. Given the importance of glucose to the process of capreomycin production, an obvious recommendation would be to keep the pH during a fermentation below 9.0, particularly towards the end of a pH non-controlled process when highly alkaline conditions are routinely observed. Highly alkaline conditions have been found to encourage capreomycin IB formation, this is unfavourable if a decrease in IA:IB ratio to below 0.4 is observed as this would fall outside of the recommended guidelines set by the FDA.

The effect of pH on fructose uptake is illustrated in Fig. 51 and similarly shows a pH optimum for uptake of approximately 8.0, after which a decrease in uptake is again observed but in contrast does not fall to zero even at pH 12. Indeed during experiments regarding a forced decrease in viability using pH, *S. capreolus* was especially resilient to such conditions, suggesting that the cell membrane was indeed resolute and not susceptible to highly alkaline conditions. The failure to cause membrane damage which would otherwise be indicated by the taking up of propidium iodide was not readily observed. This would therefore suggest that the transport processes for both glucose and fructose were affected by alkaline pH.

This has also been shown to be the case for glutamate transport (Fig. 52), whereby a more stringent effect to pH is observed. A pH optimum for this amino acid is again approximately 9.0, showing decreases in uptake at either side of this value.

Figure 50. The effect of pH on ^{14}C-glucose uptake using nitrogen limited chemostat cultures of *S. capreolus*. Results are expressed as disintegrations per minute (dpm).

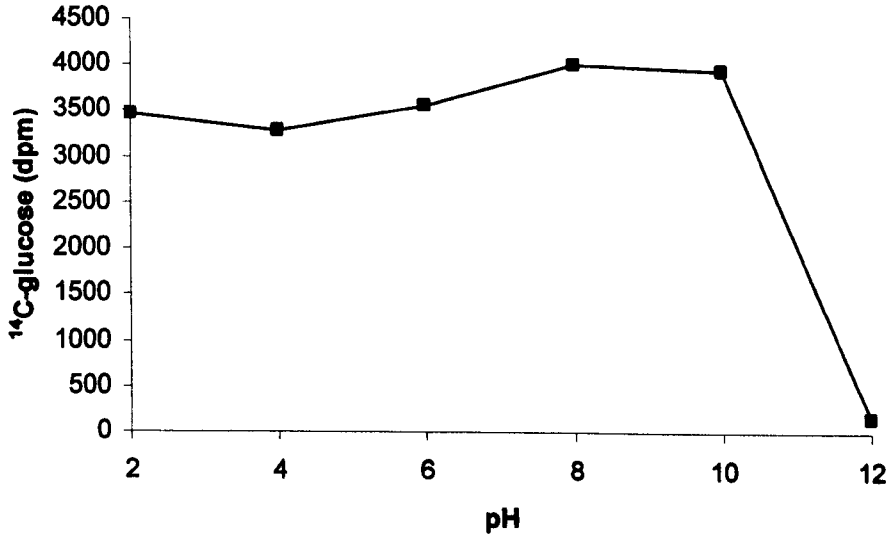


Figure 51. The effect of pH on ^{14}C-fructose uptake using nitrogen limited chemostat cultures of *S. capreolus*. Results are expressed as disintegrations per minute (dpm).

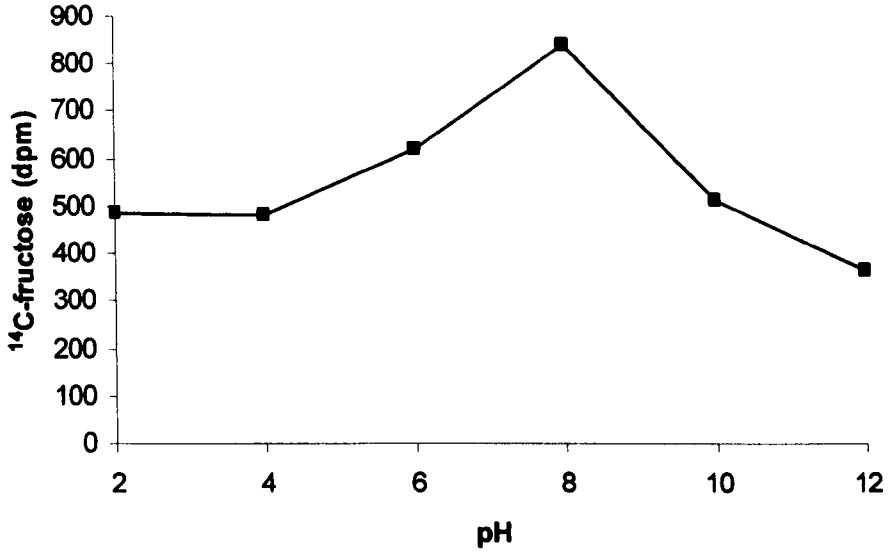
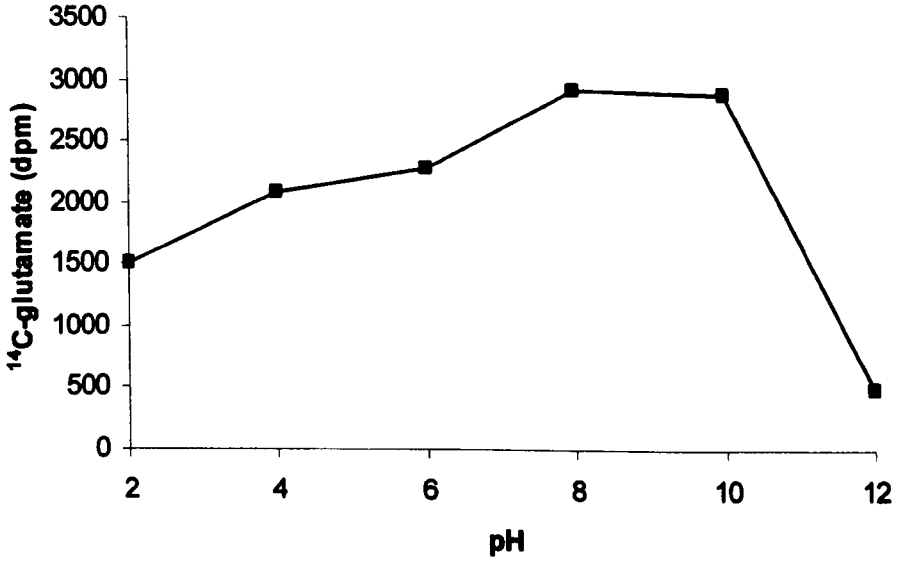


Figure 52. The effect of pH on ^{14}C-glutamate uptake using nitrogen limited chemostat cultures of *S. capreolus*. Results are expressed as disintegrations per minute (dpm).



4.7.6 EFFECT OF METAL IONS ON SUBSTRATE UPTAKE

Many heavy metals are essential for microbial growth and are therefore required in low concentrations as microelements. However, they may be toxic at high concentrations (Gadd & Griffiths, 1978; Trevors *et al.*, 1985). Some, such as mercury and cadmium, which have no function in cellular metabolism, are extremely toxic even at low concentrations (Babich & Stotzky, 1980).

The presence of metals in a growth medium can generate many metal-microbe interactions, including inhibition of enzymic activities, competition with essential microelements, binding to cell structures such as the cell membrane, and precipitation of nutrients such as phosphate resulting in their unavailability to the cell (Abbas & Edwards, 1990).

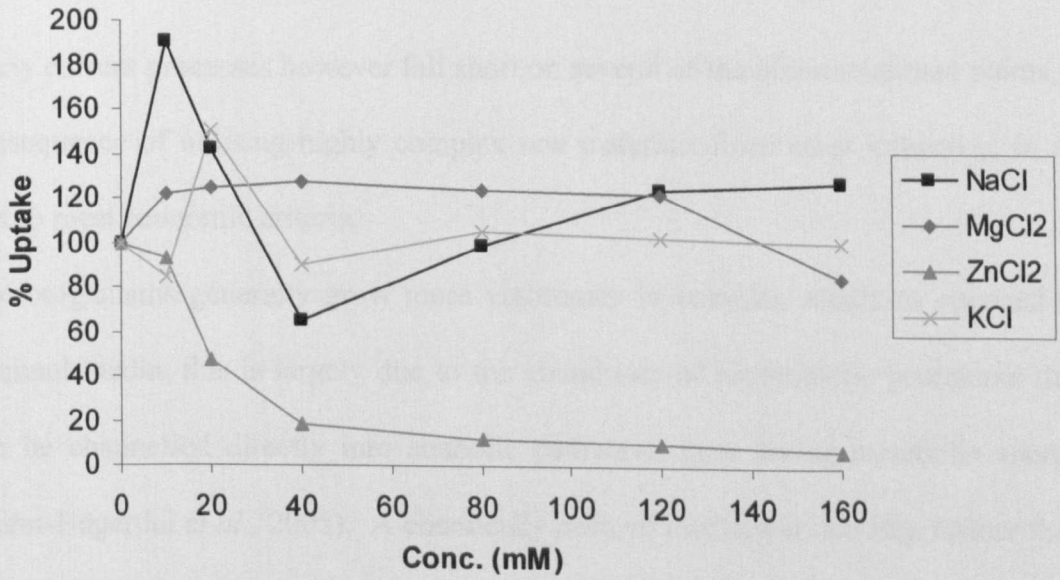
Secondary metabolism is also known to be affected by the presence or absence of metals, as they are potentially responsible for the activation of some biosynthetic pathways (Babich & Stotzky, 1979; Summers, 1985). The regulatory effects of trace metals or medium mineral content on microbial secondary metabolism has been recorded for a variety of species (Summers, 1985), but little work has been reported for streptomycetes.

Figure 53 shows that zinc causes an approximate 80 % reduction in glucose uptake, even at low concentrations, whilst magnesium appears to enhance glucose uptake until its concentration reaches 120 mM. A low concentration of sodium also appears to enhance glucose uptake followed by a sharp decline which may symbolize a change in the osmotic properties of the cell membrane. Potassium appears to have little effect on glucose uptake at the concentrations investigated. Indeed Abbas and Edwards (1990) also reported complex effects that in general resulted in some enhancement of growth yield, but a reduction in antibiotic titres. Gesheva *et al.*,

(2005) found that manganese, copper and iron stimulates production of the antibiotic AK-111-81 by *S. hygroscopicus*.

These results for *S. capreolus* represent an important finding since mineral content within complex media components is likely to change between batches, especially within the millimolar range as investigated. This may further explain the reproducibility issues often experienced between fermentations and although a minimal concentration of ions appears to promote glucose uptake and ultimately capreomycin production, it is necessary in aiming to maintain this at a constant level.

Figure 53. Repression/stimulation of ^{14}C-glucose uptake by NaCl, $MgCl_2$, $ZnCl_2$ and KCl using carbon limited chemostat cultures of *S. capreolus*. Results shown as percentages of the control (MM base containing no additional ions).



4.8 BEET MOLASSES REPLACEMENT

For many years the development of a suitable medium for large scale fermentations has dictated the selection of raw materials largely based on the following criteria as outlined by Stanbury *et al.*, (2000).

1. It will produce the maximum yield of product or biomass per gram of substrate used.
2. It will produce the maximum concentration of product or biomass.
3. It will permit the maximum rate of product formation.
4. There will be the minimum yield of undesired products.
5. It will be of a consistent quality and be readily available throughout the year.
6. It will cause minimal problems during media making and sterilisation.
7. It will cause minimal problems in other aspects of the production process particularly aeration and agitation, extraction, purification and waste treatment.

Many current processes however fall short on several of the aforementioned points, a consequence of utilising highly complex raw materials from other industries, in an aim to meet economic criteria.

Microorganisms generally grow more vigorously in complex media as opposed to minimal media, this is largely due to the abundance of biosynthetic precursors that can be channelled directly into anabolic pathways, thus saving metabolic energy (Hahn-Hägerdal *et al.*, 2005). A chemically defined medium is one step further than a minimal medium but offers less process variability which is often encountered when inconsistencies between raw material batches is met. At the laboratory scale, chemically defined media are vital for the investigation of many factors, including

physiological and biochemical assessment during growth of an organism in continuous culture and are by all means relatively inexpensive at such low utilisation levels, however for many years undefined complex media components have largely dictated large scale fermentation processes due their inexpensive nature and ready availability, and are usually by-products of other industries.

Availability of some raw materials may be influenced by government legislation. An example of this is beet sugar and beet molasses, whereby a minimum price is controlled within the European Economic Community (EEC) to encourage their use, whilst the prices of other sugar derivatives are maintained in a non-competitive manner (Coombs, 1987). It is therefore of consequence that these raw materials present considerable variation between batches at the biochemical level leading to deviations in biomass formation and productivity. Use of such ill-defined raw materials can also prove problematic at the downstream recovery and purification level of a process, thereby increasing costs at the end stages of a fermentation. Economic constraints in large-scale processes make cheap by-products, such as beet molasses from the sugar industry and corn steep liquor from the starch industry favourable sources of carbon, nitrogen, vitamins and minerals, however residual components can interfere and even inhibit growth and metabolite production.

It is true that many organisms used at an industrial level have an extensive biosynthetic capacity and grow well on defined media for example *Saccharomyces cerevisiae* and *Escherichia coli*, whilst in contrast many lactic acid producing bacteria require complex or extensively supplemented media for efficient growth (van Niel & Hahn-Hägerdal, 1999). With many industries governed by quantity and more importantly quality of their products, chemically defined media are now becoming a more sought after way of delivering growth predictability and

reproducibility, and ultimately recovery, purification and effluent treatment become much simpler and therefore cheaper as a result. Any changes to a process, whether they be at an operator level or an analytical level will become more apparent and easier to detect if the variable nature of the most important elements of a process (the raw materials) is eliminated. The current complex nature of the medium required for capreomycin production highlights all of the above problems and fails to meet several of the aforementioned criteria, it is therefore of increasing importance that capreomycin be produced by a stable and predictable process, a factor that can only be achieved by movement towards a chemically defined medium. Movement from a complex medium however to a chemically defined medium is a complicated process and requires the identification of components fundamental to the current established process.

Ultimately, it is advantageous for Eli Lilly to move away from undefined substrates for many of the aforementioned reasons. This means an eventual replacement of the animal-sourced substrates NZ-Amine and Gelita Sol P, and also the complex carbohydrate beet molasses.

This research has included work on the replacement of beet molasses with a chemically defined alternative in a move to improve product recovery and purification that is otherwise affected by this variable and highly viscous component. Beet molasses constitutes an economical source of carbohydrate for many industrial processes to grow yeasts, moulds and bacteria, to manufacture products such as citric acid (Soccol *et al.*, 2006), lysine (Hadj-Sassi *et al.*, 1990) and alcohol (Goksungur & Zorlu, 2001). It is also widely used in many antibiotic fermentations including capreomycin biosynthesis by *S. capreolus*, however beet molasses is merely a by-product of the sugar refining industry providing a more economically feasible

substrate than pure defined media components that are generally more expensive. Beet molasses is in effect a very thick, dark syrup that comes from the final boiling and crystallisation stage of sugar cane and sugar beets and contains around 50 % dry weight of sugar, predominantly glucose, fructose and sucrose. The non-sugar content is largely composed of vitamins and minerals. The largest concern regarding this complex natural substrate is the variability that it can introduce to a process predominantly between batches, particularly since its quality depends on the maturity of the sugar cane or beet, the amount of sugar extracted and the method of extraction.

4.8.1 PLACKETT-BURMAN DESIGN FOR BEET MOLASSES REPLACEMENT

A 20 run Plackett-Burman design for 13 variables and 6 dummy variables was carried out. This enabled the effects of each component within the suggested beet molasses replacement medium (outlined in Materials and Methods) to be examined independently at high and low levels in order to determine the variables that support and are most important for capreomycin production.

Capreomycin yield, quality and the ratio of capreomycin IA:IB for each run at 168 h was determined (Table 7), in order to calculate the effect of each independent variable. Calculations were performed as outlined here and all results appear in Appendix I.

The effect of each independent variable (1-13) was calculated as described by Bull *et al.*, (1990). Each variable appears at its high level 10 times and at its low level 10 times. The effect of an individual response is the difference between the average value of the response for the experiments at the high value and the average value for the experiments at the low level;

$$E(x) = \frac{R \text{ at (H)}}{10} - \frac{R \text{ at (L)}}{10}$$

Where;

E = Effect of variable (x)

R = Response

H = High level

L = Low level

The mean effect of the dummy variables was calculated using the effect of each one, divided by the total number of dummy variables included.

$$E\,(DV) = \frac{E(\mathrm{A}) + E(\mathrm{B}) + E(\mathrm{C}) + E(\mathrm{D}) + E(\mathrm{E}) + E(\mathrm{F})}{6}$$

Where;

E = Effect of variable (x)

DV = Dummy variable

This allowed a comparison to made between the effects of each of the variables and the mean of the dummy variables, thus determining if an effect was significant or due to experimental error. All raw materials were ranked according to their effects on each of the parameters examined. Those ranked highly in the table show an effect that is significantly higher than the effect of the associated dummy variables. By ranking the variables in such a manner, a hierarchy of their importance is presented which forms a basis for further work into a beet molasses replacement medium.

Table 5 shows the effects of each individual variable on capreomycin yield, quality and ratio in the beet molasses replacement medium. In terms of capreomycin yield and quality, zinc sulphate, potassium sulphate and thiamine appear to be important components of the beet molasses replacement medium, followed by pantothenate, nicotinate and biotin. The latter may be influential precursors in the pathways for antibiotic synthesis. Potassium sulphate and thiamine have significant effects on capreomycin quality in that they encourage capreomycin species IA and IB synthesis.

The elements important to the ratio of the capreomycin species IA:IB are based upon high ratio values. High ratios are indicative of high capreomycin IB and guidelines state that this cannot be above 2.0, so the importance of these compounds is of less significance when further developing the beet molasses replacement medium as high IB content is not desirable, indeed ratio values may also be adjusted using pH as a control.

Table 6 shows final capreomycin yield, quality and ratio of capreomycin IA:IB measured at 168 h. For observational purposes, wall growth and shakeflasks with a red pigmented appearance were noted. For many years, it has been speculated that a red pigmented broth colour is synonymous with a culture actively producing capreomycin, this experiment thus provided an ideal opportunity to assess this theory due to the large number of shakeflasks whereby slight changes in the Complex production medium were made, coupled with capreomycin measurements. This phenomenon is discussed further in the following section.

Table 5. The effects of each individual variable on capreomycin yield, quality and ratio in the beet molasses replacement medium. Variables are ranked into a hierarchy of their effects on the aforementioned results. The mean effect for the dummy variables is included.

YIELD		QUALITY (% IA+IB)		RATIO (IA:IB)	
0.45	$ZnSO_4$	**42.42**	K_2SO_4	**3.03**	$MgSO_4$
0.36	K_2SO_4	**20.06**	Thiamine	**2.92**	$CuSO_4$
0.31	Thiamine	**17.13**	$ZnSO_4$	**2.81**	$(NH_4)_2$ $Fe(SO_4)_2$
0.19	Pantothenate	**8.56**	Nicotinate	**2.75**	Pyridoxine
0.17	Biotin	**6.22**	Inositol	**2.66**	Biotin
0.13	Nicotinate	**3.77**	Biotin	**2.06**	Glucose
0.11	$(NH_4)_2$ $Fe(SO_4)_2$	**-4.67**	Pantothenate	**1.73**	K_2SO_4
-0.01	$CaCl_2$	**-4.79**	Glucose	**-2.52**	$CaCl_2$
-0.10	Pyridoxine	**-6.00**	$(NH_4)_2$ $Fe(SO_4)_2$	**-2.68**	Pantothenate
-0.02	Inositol	**-7.15**	Pyridoxine	**-2.94**	Nicotinate
-0.04	Glucose	**-7.71**	$CaCl_2$	**-3.01**	Inositol
-0.09	$MgSO_4$	**-8.08**	$MgSO_4$	**-3.35**	Thiamine
-0.42	$CuSO_4$	**-19.99**	$CuSO_4$	**-3.96**	$ZnSO_4$
-0.04	Dummy variable	**1.23**	Dummy variable	**-0.03**	Dummy variable

Table 6. Final capreomycin yield, quality and ratio IA:IB measured from shakeflasks containing beet molasses replacement medium grown at 30 °C, 250 rpm over 168 h.

SHAKEFLASK NUMBER	YIELD ($g.L^{-1}$)	% IA & IB	IA:IB
1*	0.46	7.91	0.88
2	1.67	70.07	0.04
3	0.61	6.56	1.17
4	1.56	7.61	1.52
5*	1.81	62.88	0.05
6	1.46	59.46	0.04
7	0.65	4.98	2.34
8	1.53	4.2	1.41
9	0.93	6.67	0.75
10	0.59	5.49	2.21
11	1.53	76.86	0.10
12	0.72	4.97	2.67
13*†	1.18	71.59	0.06
14	1.04	7.94	0.68
14	0.95	2.08	28.86
16†	1.48	61.19	0.05
17*†	0.53	3.44	2.66
18*	1.83	71.91	0.05
19*†	1.31	7.36	0.68
20	0.69	4.76	2.68

* Denotes flasks with red pigmented broth appearance.

† Denotes flasks with significant wall growth.

Table 7 shows the effects of each individual variable on pH, biomass production, residual glucose and residual ammonia based on their measurement at 168 h, in the beet molasses replacement medium.

A number of the analysed variables have been shown to encourage alkalinity of the broth, where a high effect is linked to a high pH value. This is not intrinsically vital to optimization of the medium since pH may ultimately be controlled externally. Potassium sulphate, magnesium sulphate and the precursors inositol, thiamine, biotin and nicotinate have been shown to have significant effects on biomass concentration, although it has already been shown that high biomass yields are not necessarily indicative of high capreomycin yields. The variable effects on ammonia production are diminutive and do not vary significantly due to the small concentration differences between the variables and in their entirety within the medium as a whole. For reference and comparison purposes, the actual values for all of the aforementioned parameters are tabulated in Table 8.

Table 7. The effects of each individual variable on pH, biomass production, residual glucose and residual ammonia based on their measurement at 168 h, in the beet molasses replacement medium. Variables are ranked into a hierarchy of their effects on the aforementioned results. The mean effect for the dummy variables is included.

pH		Biomass Production		Residual Glucose		Residual Ammonia	
0.08	K_2SO_4	**0.84**	K_2SO_4	**4.75**	Glucose	**0.02**	$MgSO_4$
0.08	Thiamine	**0.66**	Inositol	**1.66**	Pantothenate	**0.02**	$ZnSO_4$
0.07	Inositol	**0.64**	$MgSO_4$	**0.94**	Pyridoxine	**0**	Glucose
0.07	Biotin	**0.58**	Thiamine	**0.72**	$CuSO_4$	**0**	Inositol
0.06	$ZnSO_4$	**0.33**	Biotin	**0.15**	$(NH_4)_2$ $Fe(SO_4)_2$	**0**	Thiamine
0.06	Nicotinate	**0.3**	Nicotinate	**-0.37**	Nicotinate	**-0.01**	$(NH_4)_2$ $Fe(SO_4)_2$
0.03	$CuSO_4$	**0.28**	$ZnSO_4$	**-1.03**	$MgSO_4$	**-0.02**	K_2SO_4
0.03	Pantothenate	**0.08**	Pyridoxine	**-1.11**	$CaCl_2$	**-0.02**	$CaCl_2$
0.02	$MgSO_4$	**0.06**	Pantothenate	**-1.23**	Inositol	**-0.02**	$CuSO_4$
0.02	$CaCl_2$	**-0.08**	$CuSO_4$	**-1.65**	Biotin	**-0.02**	Nicotinate
0	Pyridoxine	**-0.28**	Glucose	**-1.74**	Thiamine	**-0.02**	Biotin
-0.06	Glucose	**-0.32**	$CaCl_2$	**-2.46**	$ZnSO_4$	**-0.03**	Pyridoxine
-0.07	$(NH_4)_2$ $Fe(SO_4)_2$	**-0.68**	$(NH_4)_2$ $Fe(SO_4)_2$	**-5.69**	K_2SO_4	**-0.04**	Pantothenate
0.01	Dummy variable	**0.05**	Dummy variable	**0.62**	Dummy variable	**-0.03**	Dummy variable

Table 8. Final pH, biomass, residual glucose and ammonia measured from shakeflasks containing beet molasses replacement medium grown at 30 °C, 250 rpm over 168 h.

SHAKEFLASK NUMBER	pH	BIOMASS ($g.L^{-1}$)	GLUCOSE ($g.L^{-1}$)	AMMONIA ($g.L^{-1}$)
1	8.46	4.3	17.50	0.54
2	8.79	6.2	4.81	0.47
3	8.66	4.3	15.23	0.41
4	8.73	4.9	14.38	0.43
5	8.75	6.3	5.50	0.48
6	8.86	5.7	4.22	0.42
7	8.64	4.1	11.92	0.43
8	8.60	3.2	15.44	0.39
9	8.76	5.0	16.52	0.45
10	8.73	5.4	13.35	0.42
11	8.63	5.5	15.52	0.38
12	8.51	5.0	13.53	0.44
13	8.51	4.2	12.09	0.49
14	8.61	4.1	15.47	0.48
14	8.54	4.7	12.67	0.40
16	8.67	5.4	8.47	0.51
17	8.60	3.7	11.48	0.45
18	8.67	5.0	3.86	0.43
19	8.41	3.0	18.21	0.45
20	8.43	3.6	14.47	0.59

4.8.1 RESPONSE SURFACE METHODOLOGY

Following the Plackett-Burman analysis, the key independent variables for capreomycin production, in terms of yield and quality, were incorporated into a response surface technique (Box & Wilson, 1951), thereby allowing the concentration of the key variables to be optimized.

Zinc sulphate and potassium sulphate were identified as the key variables for capreomycin biosynthesis in the beet molasses replacement medium, therefore the concentrations of these were adjusted in order to optimize them at the shakeflask level. Nine shakeflasks were run to investigate three concentrations of each, in all pair wise combinations. Concentrations were chosen to reflect those used during the Plackett-Burman analysis, and were based around the high levels since it was found that capreomycin yield was greatest when these variables were present at the higher level. The concentrations for zinc sulphate were as follows; 0.315 mg. L^{-1}, 0.63 mg. L^{-1} and 1.26 mg. L^{-1}, whilst potassium sulphate was present at the following three concentrations; 0.042 g. L^{-1}, 0.084 g. L^{-1}, and 0.168 g. L^{-1}. Capreomycin yield and quality was measured at 168 h and results were incorporated into a response surface plot (Fig. 54) and (Fig. 55) respectively. Results for the optimization of the above variables indicate that less zinc and more potassium in comparison with the concentrations employed in the Plackett-Burman analysis, were required for good capreomycin yields, of good quality. This coincides with concentrations of approximately 0.3 mg. L^{-1} zinc sulphate and 0.17 g. L^{-1} potassium sulphate based upon their inclusion in the beet molasses replacement medium. Indeed high concentrations of zinc have previously been shown in this research to repress glucose uptake and would explain the requirement of a lower zinc concentration.

Figure 54. Response surface plot for capreomycin yield (g. L^{-1}) measured at 168 h for optimization of zinc sulphate and potassium sulphate concentrations.

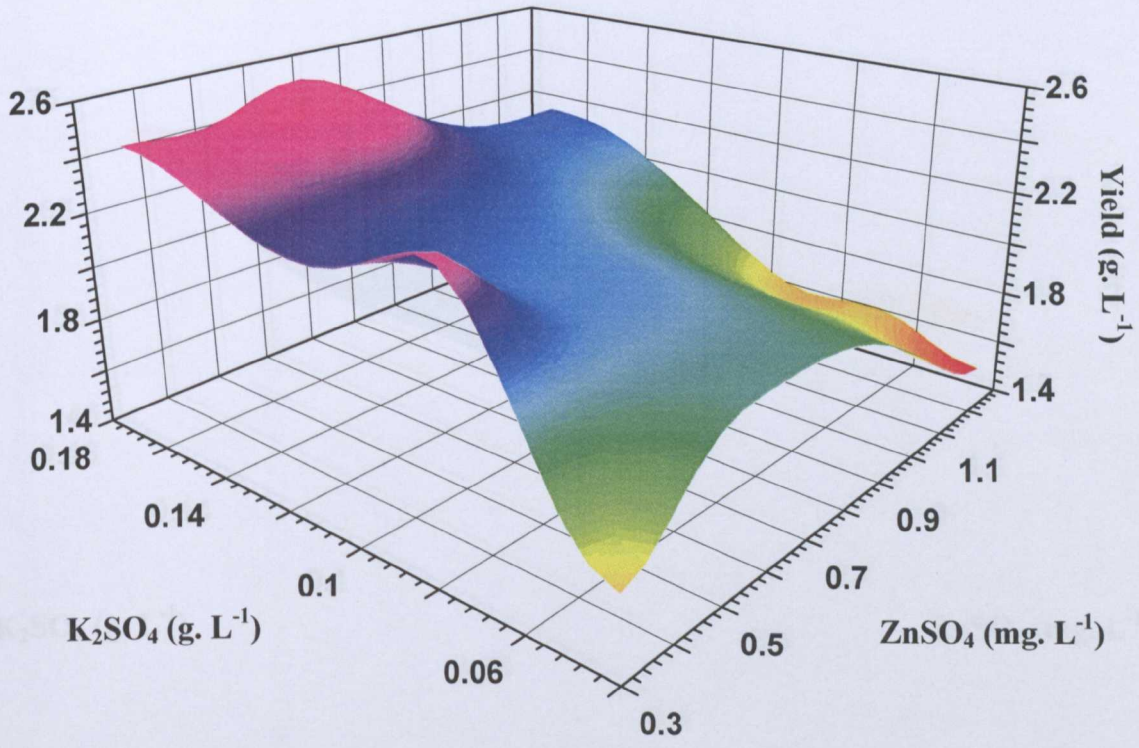
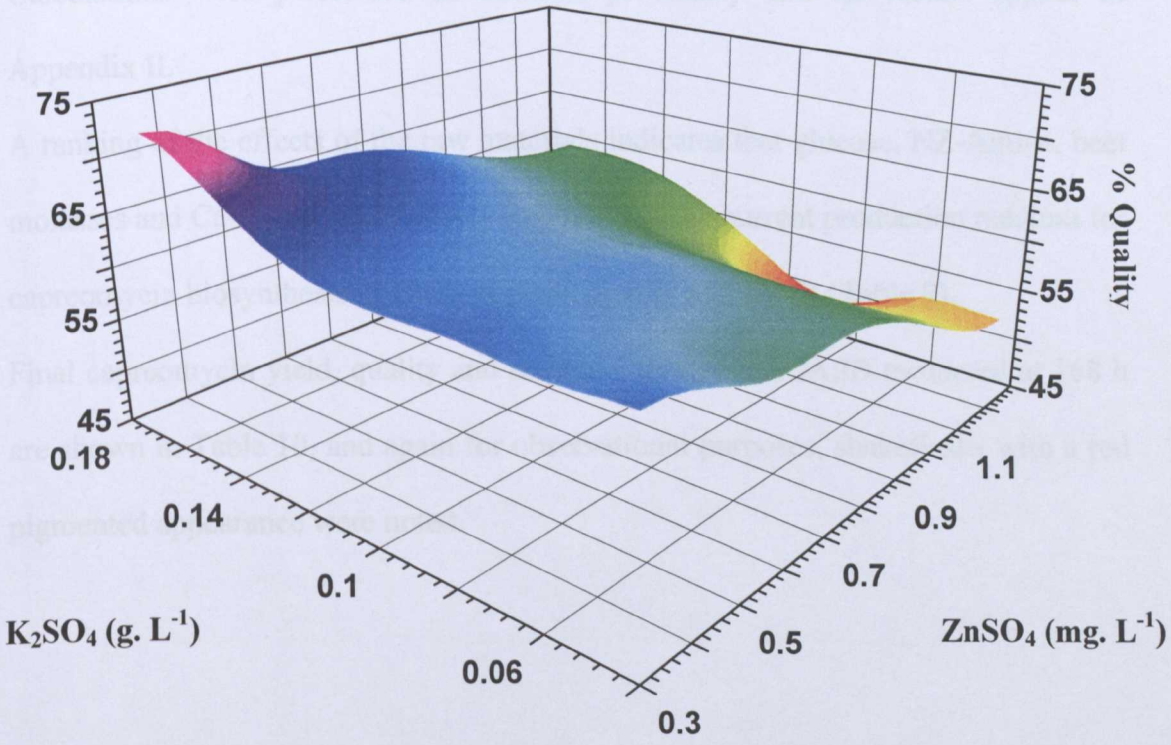


Figure 55. Response surface plot for capreomycin quality (percentage IA + IB) measured at 168 h for optimization of zinc sulphate and potassium sulphate concentrations.



4.9 PLACKETT-BURMAN DESIGN FOR CURRENT PRODUCTION MEDIUM

Following the Plackett-Burman design to look at the variables in the replacement medium it seemed appropriate to investigate the importance of the major variables within the current Complex production medium and the impact that they have on capreomycin yield, quality and IA:IB, therefore a 12 run design for 6 variables and 5 dummy variables was carried out.

Calculations were performed as outlined previously and all results appear in Appendix II.

A ranking of the effects of the raw materials indicates that glucose, NZ-Amine, beet molasses and $CaCl_2$ are cumulatively important in the current production medium for capreomycin biosynthesis in terms of yield, quality and IA:IB (Table 9).

Final capreomycin yield, quality and ratio of capreomycin IA:IB measured at 168 h are shown in Table 10, and again for observational purposes, shakeflasks with a red pigmented appearance were noted.

Table 9. The effects of each individual variable on capreomycin yield, quality and ratio in the beet molasses replacement medium. Variables are ranked into a hierarchy of their effects on the aforementioned results. The mean effect for the dummy variables is included.

YIELD		QUALITY (% IA+IB)		RATIO (IA:IB)	
0.55	$CaCl_2$	**40.06**	Glucose	**3.92**	NZ-Amine
0.26	Glucose	**4.93**	NZ-Amine	**3.60**	Beet molasses
0.11	Beet molasses	**-1.91**	$CaCl_2$	**3.38**	Glucose
0.10	NZ-Amine	**-7.12**	Beet molasses	**-2.12**	$MgSO_4$
-0.05	$MgSO_4$	**-10.63**	$MgSO_4$	**-3.22**	Gelita Sol P
-0.22	Gelita Sol P	**-18.29**	Gelita Sol P	**-4.21**	$CaCl_2$
-0.01	Dummy variable	**4.55**	Dummy variable	**0.89**	Dummy variable

Table 10. Final capreomycin yield, quality and ratio IA:IB measured from shakeflasks containing Complex production medium grown at 30 °C, 250 rpm over 168 h.

SHAKEFLASK NUMBER	YIELD ($g.L^{-1}$)	QUALITY (% IA+IB)	RATIO (IA:IB)
1*	0.1205	29.62	2.13
2*	0.6735	5.43	0.13
3	1.1559	71.88	2.04
4*	0.0779	33.03	3.75
5	0.2883	13.41	0.13
6	0.0504	11.49	1.51
7	0.0196	59.97	0
8*	0.1976	73.59	20.90
9	1.1077	81.46	0.87
10	0.2689	18.43	0.02
11	0.3175	30.44	0.22
12	0.0186	24.80	0.36

* Denotes flasks with red pigmented broth appearance

Table 11 shows the effects of each individual variable on pH and biomass measured at 168 h. Results suggest that biomass is by no means indicative of good antibiotic titres. Results for shakeflasks 3 and 9 (Table 11) have final biomass concentrations of 3.3 g. L^{-1} and 7.8 g. L^{-1} respectively, whereas capreomcyin yields for both shakeflasks are the highest in the experiment at approximately 1.1 g. L^{-1} with similar quality and IA:IB ratios (Table 10). Theoretically, the culture in flask 3 has a greater antibiotic producing efficiency with relation to biomass concentration than flask 9, however the actual results show that this is not necessarily the case.

In terms of the effects of the raw materials on pH, Gelita Sol P and magnesium sulphate are ranked highly, signifying that they induce alkalinity within the medium. Again, this is not a vital factor since the medium pH can ultimately be controlled throughout a fermentation if necessary, but it is important to note since it has already been shown previously in this research that alkalinity induces fragmentation of *S. capreolus*, an effect that has been shown to be deleterious to antibiotic biosynthesis. These results merely place the substrates for the current process in a hierarchical order in terms of their effects on particular parameters, and will aid in the development of a chemically defined medium at a later stage.

For reference and comparison purposes, the actual values for pH and biomass concentration measured at 168 h are tabulated in Table 12.

Table 11. The effects of each individual variable on pH and biomass production, based on their measurement at 168 h, in Complex production medium. Variables are ranked into a hierarchy of their effects on the aforementioned results. The mean effect for the dummy variables is included.

pH		Biomass Production	
0.59	Gelita Sol P	**3.18**	NZ-Amine
0.58	$MgSO_4$	**3.12**	Glucose
-0.04	Beet molasses	**0.88**	Beet molasses
-0.06	$CaCl_2$	**-0.92**	$CaCl_2$
-0.40	NZ-Amine	**-0.95**	Gelita Sol P
-0.69	Glucose	**-1.18**	$MgSO_4$
-0.09	Dummy variable	**-0.03**	Dummy variable

Table 12. Final pH and biomass measured from shakeflasks containing Complex production medium grown at 30 °C, 250 rpm over 168 h.

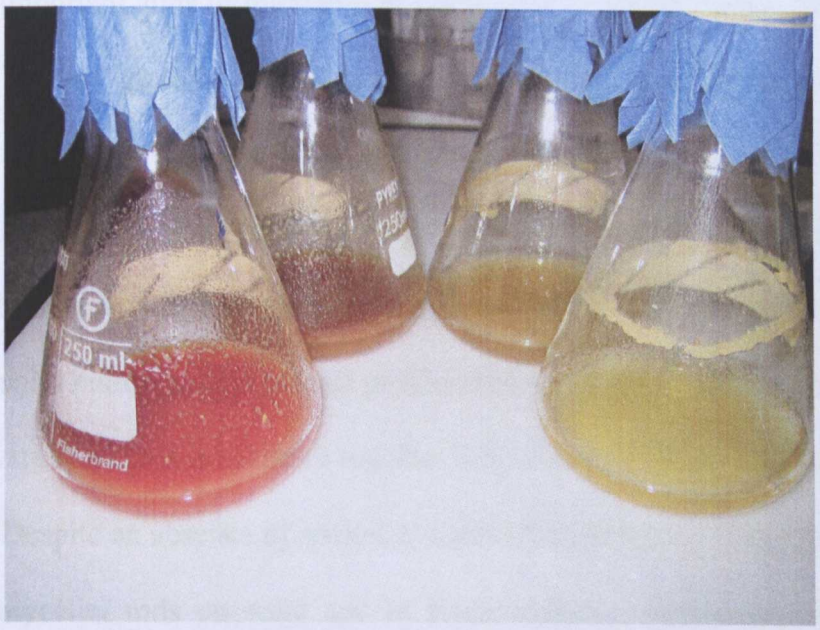
SHAKEFLASK NUMBER	pH	BIOMASS ($g.L^{-1}$)
1	8.47	6.4
2	8.45	3.5
3	8.06	3.3
4	8.45	3.3
5	8.34	0.8
6	8.44	1.6
7	8.41	2.8
8	6.70	8.7
9	6.65	7.8
10	8.66	1.5
11	8.23	2.0
12	8.30	1.6

4.10 BROTH COLOUR & CAPREOMYCIN

It has been speculated that a red pigmented broth colour is synonymous with a culture actively producing capreomycin. This was observed routinely in the Complex medium and to a lesser degree in the Porcine medium, although still obvious (Fig. 56), but the theory was ultimately tested when the appearance of red coloured flasks was noted during the Plackett-Burman experiments. Shakeflask numbers 1, 5, 13, 17, 18 and 19, identified in Table 7, all exhibited a distinct red broth colour, as did shakeflasks 1, 2, 4 and 8 in Table 10. Following the comparison with the capreomycin yield results, it was not apparent that a pigmented broth was a sole indicator of antibiotic productivity. Indeed, samples from shakeflasks 1 (red colour) and 2 (no red colour) from the beet molasses replacement experiment were taken and scanned for peaks between 300 nm and 800 nm to determine any differences between the two spectra, consequently no differences were apparent.

An initial explanation for this phenomenon was that capreomycin was a pH-sensitive pigmented compound, similarly to the pigmented antibiotic granaticin produced by *S. thermoviolaceus* (Brabban & Edwards, 1997), and actinorhodin produced by *S. coelicolor*. The latter is in effect a pH indicator in that it turns red below a pH of approximately 8.5 and blue above. The blue colour was ascribed to actinorhodin, but it is questionable whether it is in fact identical to the red pigmented compound when absorption spectra differ in the literature for both pigments (Bystrykh *et al.*, 1996). This pH dependent theory for capreomycin however was dispelled when a range of pH values was observed at 168 h for the aforementioned flasks. The pigmented colour must be attributed to some degree to capreomycin biosynthesis as no pigment was observed in the simple, non-producing Minimal and Defined media.

Figure 56. Broth colour in Complex medium, Porcine medium, Minimal medium and Defined medium (left to right).



5.0 CONCLUSION

A physiological study of *Streptomyces capreolus* was carried out that aimed to elucidate the factors governing growth, cellular metabolism and morphology, and the impact that they have on capreomycin biosynthesis.

One of the important aims of microbial physiology is to acquire quantitative data on the effect of the growth environment on microbial metabolism and growth energetics. The major findings in this research are not only novel for this organism, but they will provide a good basis for further investigation of *S. capreolus* and will aid in the ultimate development of a chemically defined production medium.

Growth on solid culture media proved problematic for *S. capreolus* and poor growth dynamics were exhibited in all cases together with a noticeable absence of spore-like structures. Despite an absence of spores, *S. capreolus* did appear to fragment readily into short mycelial rods on solid and in liquid culture. The event whereby the mycelia branch frequently, followed by subsequent fragmentation into short rod like structures has been reported by several authors to be characteristic of the genus *Nocardia* (Thiemann *et al.*, 1969; Lee & Rho, 1994). It may therefore be fitting to affiliate *S. capreolus* with *Nocardia* spp. largely based upon the morphological characteristics exhibited in this research. The initial morphological appearance of *S. capreolus* would suggest that this species is synonymous to the streptomycetes, but closer investigation including the readiness at which the culture fragments under a variety of conditions would imply a resemblance to the taxonomic category *Nocardia*. The lack of ability to sporulate may have resulted from the numerous sub-culturing of the organism over the years, a downfall of an industrial strain, especially since it has become apparent that *S. capreolus* is much more suited to

growth in liquid culture as has been observed in these studies. The property of antibiotic production in liquid culture has most likely been selected in the production strain during isolation techniques of *S. capreolus* many years ago. It could also be eluded that sporulation would be a futile event in such a complex medium used for antibiotic production, where severe nutrient limitation is rare.

Strong evidence has emerged from this research to suggest a link between morphological status and antibiotic production. Mycelia in the Complex medium appears to remain pelleted in form throughout a typical 168 h fermentation, and is unique in morphology to other media investigated, including the secondary production medium; Porcine peptone medium. Both Minimal and Defined media induce a high degree of fragmenation that cannot be attributed solely to pH since they exhibited a final pH similar to that experienced in both complex media, of approximately pH 8.0.

A continuous culture of *S. capreolus* was also established, although a very low dilution rate was applied. This however, provided a steady-state model based around a defined medium which allowed physiology to be investigated under carbon and nitrogen limited conditions.

Nitrogen limitation appeared to evoke a high degree of fragmentation of the culture in contrast to the morphological status during carbon limited growth. In *Penicillium chrysogenum*, nutrient limitation causes hyphae to become heavily vacuolated which leads to fragmentation (Paul *et al.*, 1994), whilst *Streptomyces* cultures exhibiting a high degree of fragmentation have been linked to a lack of antibiotic production (Shomura *et al.*, 1979; Roth & Noack, 1982). Nutrient limitation may result in poor cell wall structure and potentially lead to frequent vacuolation and septation of the hyphae, which would aid formation of fragmented filaments, which in most cases

appear coccoid in structure. Indeed, a streptomycete cell wall consists of N-acetylglucosamine, N-acetylmuramic acid, and some amino acids notably L-alanine, D-alanine, and D-glutamic acid, therefore if these components or precursors for the formation of these structural units are not in place, then this could be detrimental to cell wall integrity and maintenance.

Indeed, fragmentation is the major contributor to high viability ratios in that it gives rise to dispersed hyphae which are abundant and particularly viable and would confer the high viability ratios observed in the Minimal and Defined media very early on in the fermentation. The Complex and Porcine media investigated in this research suggest a predilection for pelleted morphology under conditions optimal for capreomycin biosynthesis. This is by no means a direct link between complexity and antibiotic production, but is merely suggestive that pelleted morphology is favoured under such conditions whilst fragmentation is largely absent. Indeed, it has ultimately been shown that fragmentation may be induced in a complex medium when certain environmental factors are altered to extreme limits for growth, such as pH or NaCl addition. It so far stands that dispersed growth of *S capreolus* is unfavourable for capreomycin production but does not preclude that a re-formulation of a chemically defined medium that does not favour such an event would not be capable of capreomycin biosynthesis.

Biomass accretion in all media investigated was > 3.0 g. L^{-1} during exponential growth and in most cases higher than that observed for the Complex medium used routinely to produce capreomycin. This implies that high biomass concentrations do not necessarily guarantee antibiotic synthesis. It may be suggested that nutrient availability in the Defined medium is alternatively directed primarily towards biomass formation, whilst the limited supply of nitrogen in the Minimal medium

primarily restricts growth of the organism and cannot direct metabolites towards capreomycin production. As expected, capreomycin production was highest in the nitrogen rich Complex and Porcine media, suggesting that nitrogen largely governs antibiotic formation in *S. capreolus*, whether it be from accumulation of primary metabolites or the direct incorporation of amino acids within the aminoglycosidic structures of capreomycin.

Amino acid uptake was investigated for the Complex medium (capreomycin producing) and also the Minimal medium (non-capreomycin producing). The uptake of specific amino acids differed between the two types and it cannot necessarily be stated that those actively taken up in the Complex medium are required directly for antibiotic synthesis. This is important to note since the processes of transamination and re-assimilation of nitrogen sources will be active during growth, and the ability of the organism to synthesize particular amino acids is a rare occurrence in streptomycetes, whereas other actinomycetes are favoured for their amino acid production at large scale, such as *Corynebacterium glutamicum* (Schmid *et al.*, 2000), which produces alanine, and proline, similarly to *S. capreolus*. Amino acid synthesis is not surprising as this ability is likely to reflect nutrient levels within the growth medium. Indeed, following the apparent synthesis of alanine and phenylalanine in the Defined medium, there is a fairly rapid decrease in their concentrations signifying their active utilization by *S. capreolus*. In the Complex medium, alanine, proline and threonine appear to increase early on in the fermentation and may simply suggest their accumulation due to the activity of proteases on polypeptides in the complex nitrogenous portion of the Complex medium. Further investigation based around a profile of total amino acid

concentration throughout a fermentation would provide explanantion to the results observed.

Little is known about sugar transport in *Streptomyces* species (Romano, 1986), but specific constitutive or inducible sugar transport systems are used (Sabater & Asensio, 1973; Chatterjee & Vining, 1981; Hodgson, 1982). Hexoses are thought to be taken up by an active transport process which does not appear to proceed via the phosphoenolpyruvate: sugar phosphotransferase group translocation system (Novotna & Hostálék, 1985).

Radiolabelled studies using ^{14}C-glucose have shown that uptake of this hexose is significantly reduced in the presence of certain amino acids, indeed substrate affinity experiments showed that *S. capreolus* exhibits a low affinity for glucose. This is an important factor to appreciate, as many of the nutrients examined are present in the current Complex medium employed by Eli Lilly, and may be suggestive of the fact that this organism derives a great expanse of its carbon from nitrogenous sources, a factor reinforced by growth yield results which indicate that an alternative carbon source to glucose is being utilized, particularly early on in the fermentation. It is noted however that those affecting glucose uptake in carbon-limited mycelia, do not necessarily have the same effect on nitrogen-limited mycelia, perhaps because they are rapidly being utilized by the organism. This data suggests a prominent link between carbon and nitrogen metabolism, and in order to overcome the inhibitory effects of particular amino acids, it would be advisable to incorporate these nitrogen sources as a feed, at concentrations that are not inhibitory to sugar transport. The effects of other sugars was also investigated and it was found that fructose is actively taken up along with glucose by carbon limited mycelia. The absence of a lag period suggests that the enzymes for fructose uptake in *S. capreolus* are constitutive as

opposed to inducible due to the rapid uptake mechanism exhibited in a short period of time in the study. A similar phenomenon has been shown in *Escherichia coli* grown as a continuous culture with glucose as the carbon source, whereby several other sugars are actively metabolised without lag (Lendenmann & Egli, 1995).

It may be concluded that all conditions of growth that *S. capreolus* may be subjected to can have profound effects on its growth characteristics and morphology, as well as secondary metabolite production, an important factor industrially. It has become apparent from this research that the current process would be better suited as a fed-batch process, this is due to the potential inhibitory effects that the complex substrates possess and their impact on metabolism. The high concentration of glucose present at the start of the fermentation is under the potential repression of the amino acids in the complex nitrogenous sources. The results indicate that *S. capreolus* is deriving its carbon from an alternative carbon source to glucose, and this is further compounded by the high concentration of residual glucose at 168 h.

The physiological response to nutrient status is still not fully understood and is likely to be a combination of many factors including the up-regulation and down-regulation of selected genes and protein pathways, but *S. capreolus* is more prone to nutrient limitation in defined media, and this research has shown the morphological response to such conditions, including the capreomycin producing capabilities, in comparison to complex media.

If we can understand what affects and regulates the processes necessary for antibiotic production then we can optimise and potentially control these metabolic pathways that dictate production.

5.1 RECOMMENDATIONS

The ideal conclusion for this work would be to recommend a chemically defined medium that supports capreomycin biosynthesis that is equal to, if not better than the current complex production medium. The sheer complexity of the production medium serves to complicate such media development, however this research highlights specifications for key parameters which would aid future design.

In morphological terms, it appears vital to promote pellet formation as opposed to fragmentation of the culture in submerged fermentation in order to ensure capreomycin production. Mycelial viability is intrinsically linked with this phenomenon and total culture viability appears to have a deleterious effect on capreomycin production. Viability is likely to be promoted by fragmentation of mycelium into rod-like structures, and is found to be regulated by factors such as pH and C/N ratio.

Based empirically on work done so far, a medium rich in histidine, isoleucine, phenylalanine, arginine, glycine, lysine, methionine, tyrosine and valine would be recommended, optimal concentrations of which should be further investigated. These amino acids have been found to be readily utilised in the Complex medium, particularly histidine, isoleucine and phenylalanine. Due to the apparent repressive effect of particular amino acids on glucose, it may be beneficial to incorporate glucose as a feed. Indeed a surplus of glucose at 168 h was noted, but it is apparent that glucose is crucial in capreomycin production.

The key components from the beet molasses replacement medium that were found to be significantly important in terms of capreomycin yield and quality include ZnSO4, K2SO4, thiamine, pantothenate, biotin, nicotinate and (NH4)2Fe(SO4)2. Further

work is required to determine the optimal amounts for their incorporation, but there is potential for them to replace beet molasses entirely.

5.2 FURTHER WORK

This physiological study has provided a fundamental basis for further work to be carried out on *S. capreolus*.

To further characterise this organism, particularly in terms of biochemical metabolism during growth and capreomycin biosynthesis, it would be desirable to investigate the following criteria as ameans of progressing the work presented in this thesis.

- In terms of nitrogen metabolism, further work should include investigation into the accumulation/potential production of particular amino acids. This should be linked to total amino acid concentrations and also investigation into intracellular amino acid pools would be beneficial, noting changes during a fermentation and responses to certain induced factors.

- Protease activity, identification and factors regulating their expression should be investigated. Are they up-regulated or down-regulated under certain conditions, and to what extent can this be controlled for beneficial reasons?

- Further radiolabelled studies would be desirable to determine substrate affinity constants for other components, typically those present within the current production medium. Fructose is actively utilised by *S. capreolus*, but what is its actual K_s value? Does *S. capreolus* show a high affinity for this sugar, despite it not supporting capreomycin biosynthesis?

- Measurement of key metabolic enzymes such as glutamate dehydrogenase (GDH), glutamine synthetase (GS) and glutamate synthase (GOGAT), and

also extracellular organic acid excretion e.g pyruvate and α-ketoglutarate, in order to characterise biochemical pathways involved in the synthesis of capreomycin.

- Further optimisation of the beet molasses replacement medium, investigating optimal levels for the hierarchical components, with a view to removing the raw material indefinitely. The move towards animal sourced media free components is becoming paramount in media development and so the chemical analysis of Gelita Sol P and NZ-Amine with a view to replacing them with chemically defined alternatives would be advantageous. This would also improve the overall experimental variability of the process which is currently observed.

- Supplying substrates to the fermentation, thus shifting the process from a batch to a fed-batch fermentation. This would reduce potential carbon catabolite repression that would be expected with such high amounts of glucose present from 0 h onwards. Identifying and feeding key metabolic precursors may prolong antibiotic synthesis, thus extending the industrial process of capreomycin production and increasing productivity. A fed-batch fermentation can often be more successful than batch or continuous culture since the generation of by-products of fermentation are usually synonymous with high starting concentrations of substrates, which in turn can have toxic effects on the process.

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7.0 APPENDIX I

CAPREOMYCIN YIELD (g. L^{-1})

Yield	H Level	L Level
Variable 1	0.47	1.68
	0.62	1.82
	1.56	1.47
	0.94	0.66
	1.54	1.54
	1.18	0.59
	1.05	0.73
	0.95	0.54
	1.48	1.84
	1.32	0.70
sum	11.10	11.55
sum/10	1.11	1.16

EFFECT -0.04

	H	L
Variable 2	0.47	0.62
	1.68	1.47
	1.56	0.66
	1.82	1.54
	0.59	0.94
	0.73	1.54
	1.05	1.18
	0.95	1.84
	1.48	1.32
	0.54	0.70
sum	10.86	11.80
sum/10	1.09	1.18

EFFECT -0.09

	H	L
Variable 3	1.68	0.47
	0.62	1.56
	1.82	0.66
	1.47	1.54
	1.54	0.94
	1.18	0.59
	0.95	0.73
	1.48	1.05
	0.54	1.32
	1.84	0.70
sum	13.11	9.54
sum/10	1.31	0.95

EFFECT 0.36

	H	L
Variable 4	0.62	0.47
	1.56	1.68
	1.47	1.82
	0.66	1.54
	0.73	0.94
	1.05	0.59
	1.48	1.54
	0.54	1.18
	1.84	0.95
	1.32	0.70
sum	11.25	11.40
sum/10	1.13	1.14

EFFECT -0.01

	H	L
Variable 5	0.47	1.68
	1.56	0.62
	1.82	1.47
	0.66	0.94
	1.54	0.59
	1.18	1.54
	0.95	0.73
	0.54	1.05
	1.84	1.48
	1.32	0.70
sum	11.87	10.78
sum/10	1.19	1.08

EFFECT 0.11

	H	L
Variable 6	0.47	0.62
	1.68	1.56
	1.82	0.66
	1.47	0.59
	1.54	1.54
	0.94	0.73
	1.05	1.18
	1.48	0.95
	1.84	0.54
	1.32	0.70
sum	13.59	9.07
sum/10	1.36	0.91

EFFECT 0.45

	H	L
Variable 7	0.47	1.56
	1.68	1.82
	0.62	1.54
	1.47	1.54
	0.66	0.73
	0.94	1.18
	0.59	1.05
	0.95	1.48
	0.54	1.84
	1.32	0.70
sum	9.22	13.44
sum/10	0.92	1.34

EFFECT -0.42

	H	L
Variable 8	0.47	1.82
	1.68	1.47
	0.62	0.94
	1.56	0.73
	0.66	1.18
	1.54	1.05
	0.59	0.95
	1.54	0.54
	1.48	1.32
	1.84	0.70
sum	11.96	10.69
sum/10	1.20	1.07

EFFECT 0.13

	H	L
Variable 9	1.68	0.47
	0.62	1.47
	1.56	0.66
	1.82	0.59
	1.54	1.18
	0.94	1.05
	1.54	0.95
	0.73	1.48
	0.54	1.84
	1.32	0.70
sum	12.27	10.38
sum/10	1.23	1.04

EFFECT 0.19

	H	L
Variable 10	0.47	1.68
	0.62	0.66
	1.56	1.54
	1.82	1.54
	1.47	1.05
	0.94	0.95
	0.59	1.48
	0.73	0.54
	1.18	1.32
	1.84	0.70
sum	11.21	11.44
sum/10	1.12	1.14

EFFECT -0.02

	H	L
Variable 11	1.68	0.47
	1.56	0.62
	1.82	1.54
	1.47	0.94
	0.66	0.73
	0.59	0.95
	1.54	1.48
	1.18	0.54
	1.05	1.84
	1.32	0.70
sum	12.86	9.79
sum/10	1.29	0.98

EFFECT 0.31

	H	L
Variable 12	0.47	1.68
	0.62	1.56
	1.82	0.94
	1.47	0.59
	0.66	1.18
	1.54	1.48
	1.54	0.54
	0.73	1.84
	1.05	1.32
	0.95	0.70
sum	10.83	11.83
sum/10	1.08	1.18

EFFECT -0.10

	H	L
Variable 13	1.68	0.47
	1.56	0.62
	1.47	1.82
	0.66	0.59
	1.54	1.54
	0.94	1.05
	0.73	0.54
	1.18	1.84
	0.95	1.32
	1.48	0.70
sum	12.19	10.47
sum/10	1.22	1.05

EFFECT 0.17

Dummy Variable 1	H	L
	0.62	0.47
	1.82	1.68
	0.66	1.56
	1.54	1.47
	0.94	1.54
	0.59	0.73
	1.18	0.95
	1.05	1.84
	1.48	1.32
	0.54	0.70
sum	*10.41*	*12.25*
sum/6	1.73	2.04

EFFECT -0.31

Dummy Variable 2	H	L
	1.56	0.47
	1.47	1.68
	1.54	0.62
	0.94	1.82
	0.59	0.66
	1.54	0.73
	1.05	1.18
	0.95	1.48
	0.54	1.32
	1.84	0.70
sum	12.01	10.64
sum/6	2.00	*1.77*

EFFECT 0.23

Dummy Variable 3	H	L
	1.82	0.47
	0.66	1.68
	0.94	0.62
	0.59	1.56
	1.54	1.47
	0.73	1.54
	0.95	1.18
	1.48	1.05
	1.84	0.54
	1.32	0.70
sum	11.86	10.79
sum/6	1.98	1.80

EFFECT 0.18

Dummy Variable 4	H	L
	0.47	1.6762
	1.47	0.6158
	1.54	1.5637
	0.59	1.8183
	1.54	0.6574
	0.73	0.9383
	1.18	1.0463
	1.48	0.9536
	0.54	1.8390
	1.32	0.6993
sum	10.85	11.8079
sum/6	1.81	1.967983

EFFECT -0.16

Dummy Variable 5	H	L
	0.4654	0.6158
	1.6762	1.5637
	0.6574	1.8183
	0.9383	1.4684
	1.5384	1.5375
	0.7286	0.5905
	1.1845	0.9536
	1.0463	1.4801
	0.5370	1.3164
	1.8390	0.6993
sum	10.6111	12.0436
sum/6	1.768517	2.007267

EFFECT -0.23875

Dummy Variable 6	H	L
	1.6762	0.4654
	0.6158	1.5637
	1.5375	1.8183
	0.5905	1.4684
	0.7286	0.6574
	1.1845	0.9383
	1.0463	1.5384
	0.9536	1.4801
	1.8390	0.5370
	1.3164	0.6993
sum	11.4884	11.1663
sum/6	1.914733	1.86105

EFFECT 0.053683

CAPREOMYCIN QUALITY (% IA & IB)

% cap	H Level	L Level		H	L
Variable 1	7.91	70.07	Variable 2	7.91	6.56
	1.17	62.88		70.07	59.46
	1.52	59.46		7.61	4.98
	6.67	4.98		62.88	4.20
	76.86	4.20		5.49	6.67
	71.59	5.49		4.97	76.86
	7.94	4.97		7.94	71.59
	2.08	3.44		2.08	71.91
	61.19	71.91		61.19	7.36
	7.36	4.76		3.44	4.76
sum	244.29	292.16	sum	233.58	314.35
sum/10	24.43	29.22	sum/10	23.36	31.44

EFFECT -4.79 EFFECT -8.08

	H	L		H	L
Variable 3	70.07	7.91	Variable 4	6.56	7.91
	6.56	7.61		7.61	70.07
	62.88	4.98		59.46	62.88
	59.46	4.20		4.98	4.20
	76.86	6.67		4.97	6.67
	71.59	5.49		7.94	5.49
	2.08	4.97		61.19	76.86
	61.19	7.94		3.44	71.59
	3.44	7.36		71.91	2.08
	71.91	4.76		7.36	4.76
sum	486.04	61.89	sum	235.42	312.51
sum/10	48.60	6.19	sum/10	23.54	31.25

EFFECT 42.42 EFFECT -7.71

	H	L
Variable 5	7.91	70.07
	7.61	6.56
	62.88	59.46
	4.98	6.67
	4.20	5.49
	71.59	76.86
	2.08	4.97
	3.44	7.94
	71.91	61.19
	7.36	4.76
sum	243.96	303.97
sum/10	24.40	30.40

EFFECT -6.00

	H	L
Variable 6	7.91	6.56
	70.07	7.61
	62.88	4.98
	59.46	5.49
	4.20	76.86
	6.67	4.97
	7.94	71.59
	61.19	2.08
	71.91	3.44
	7.36	4.76
sum	359.59	188.34
sum/10	35.96	18.83

EFFECT 17.13

	H	L
Variable 7	7.91	7.61
	70.07	62.88
	6.56	4.20
	59.46	76.86
	4.98	4.97
	6.67	71.59
	5.49	7.94
	2.08	61.19
	3.44	71.91
	7.36	4.76
sum	174.02	373.91
sum/10	17.40	37.39

EFFECT -19.99

	H	L
Variable 8	7.91	62.88
	70.07	59.46
	6.56	6.67
	7.61	4.97
	4.98	71.59
	4.20	7.94
	5.49	2.08
	76.86	3.44
	61.19	7.36
	71.91	4.76
sum	316.78	231.15
sum/10	31.68	23.12

EFFECT 8.56

	H	L
Variable 9	70.07	7.91
	6.56	59.46
	7.61	4.98
	62.88	5.49
	4.20	71.59
	6.67	7.94
	76.86	2.08
	4.97	61.19
	3.44	71.91
	7.36	4.76
sum	250.62	297.31
sum/10	25.06	29.73

EFFECT -4.67

	H	L
Variable 10	7.91	70.07
	6.56	4.98
	7.61	4.20
	62.88	76.86
	59.46	7.94
	6.67	2.08
	5.49	61.19
	4.97	3.44
	71.59	7.36
	71.91	4.76
sum	305.05	242.88
sum/10	30.51	24.29

EFFECT 6.22

	H	L
Variable 11	70.07	7.91
	7.61	6.56
	62.88	4.20
	59.46	6.67
	4.98	4.97
	5.49	2.08
	76.86	61.19
	71.59	3.44
	7.94	71.91
	7.36	4.76
sum	374.24	173.69
sum/10	37.42	17.37

EFFECT 20.06

	H	L
Variable 12	7.91	70.07
	6.56	7.61
	62.88	6.67
	59.46	5.49
	4.98	71.59
	4.20	61.19
	76.86	3.44
	4.97	71.19
	7.94	7.36
	2.08	4.76
sum	237.84	309.37
sum/10	23.78	30.94

EFFECT -7.15

	H	L
Variable 13	70.07	7.91
	7.61	6.56
	59.46	62.88
	4.98	5.49
	4.20	76.86
	6.67	7.94
	4.97	3.44
	71.59	71.91
	2.08	7.36
	61.19	4.76
sum	292.82	255.11
sum/10	29.28	25.51

EFFECT 3.77

Dummy Variable 1	H	L
	6.56	7.91
	62.88	70.07
	4.98	7.61
	4.20	59.46
	6.67	76.86
	5.49	4.97
	71.59	2.08
	7.94	71.91
	61.19	7.36
	3.44	4.76
sum	234.94	312.99
sum/6	39.16	52.17

EFFECT -13.01

Dummy Variable 2	H	L
	7.61	7.91
	59.46	70.07
	4.20	6.56
	6.67	62.88
	5.49	4.98
	76.86	4.97
	7.94	71.59
	2.08	61.19
	3.44	7.36
	71.91	4.76
sum	245.66	302.27
sum/6	40.94	50.38

EFFECT -9.44

Dummy Variable 3	H	L
	62.88	7.91
	4.98	70.07
	6.67	6.56
	5.49	7.61
	76.86	59.46
	4.97	4.20
	2.08	71.59
	61.19	7.94
	71.91	3.44
	7.36	4.76
sum	304.39	243.54
sum/6	50.73	40.59

EFFECT 10.14

Dummy Variable 4	H	L
	7.91	70.07
	59.46	6.56
	4.20	7.61
	5.49	62.88
	76.86	4.98
	4.97	6.67
	71.59	7.94
	61.19	2.08
	3.44	71.91
	7.36	4.76
sum	302.47	245.46
sum/6	50.41	40.91

EFFECT 9.50

Dummy Variable 5	H	L
	7.91	6.56
	70.07	7.61
	4.98	62.88
	6.67	59.46
	76.86	4.20
	4.97	5.49
	71.59	2.08
	7.94	61.19
	3.44	7.36
	71.91	4.76
sum	326.34	221.59
sum/6	54.39	36.93167

EFFECT 17.46

Dummy Variable 6	H	L
	70.07	7.91
	6.56	7.61
	4.20	62.88
	5.49	59.46
	4.97	4.98
	71.59	6.67
	7.94	76.86
	2.08	61.19
	71.91	3.44
	7.36	4.76
sum	252.17	295.76
sum/6	42.02833	49.29333

EFFECT -7.265

CAPREOMYCIN RATIO (IA:IB)

Ratio	H Level	L Level
Variable 1	0.88	0.04
	1.17	0.05
	1.52	0.04
	0.75	2.34
	0.10	1.41
	0.06	2.21
	0.68	2.67
	28.86	2.66
	0.05	0.05
	0.68	2.68
sum	34.75	14.15
sum/10	3.48	1.42

EFFECT 2.06

	H	L
Variable 2	0.88	1.17
	0.04	0.04
	1.52	2.34
	0.05	1.41
	2.21	0.75
	2.67	0.10
	0.68	0.06
	28.86	0.05
	0.05	0.68
	2.66	2.68
sum	39.62	9.28
sum/10	3.96	0.93

EFFECT 3.03

	H	L
Variable 3	0.04	0.88
	1.17	1.52
	0.05	2.34
	0.04	1.41
	0.10	0.75
	0.06	2.21
	28.86	2.67
	0.05	0.68
	2.66	0.68
	0.05	2.68
sum	33.08	15.82
sum/10	3.31	1.58

EFFECT 1.73

	H	L
Variable 4	1.17	0.88
	1.52	0.04
	0.04	0.05
	2.34	1.41
	2.67	0.75
	0.68	2.21
	0.05	0.10
	2.66	0.06
	0.05	28.86
	0.68	2.68
sum	11.86	37.04
sum/10	1.19	3.70

EFFECT -2.52

	H	L
Variable 5	0.88	0.04
	1.52	1.17
	0.05	0.04
	2.34	0.75
	1.41	2.21
	0.06	0.10
	28.86	2.67
	2.66	0.68
	0.05	0.05
	0.68	2.68
sum	38.51	10.39
sum/10	3.85	1.04

EFFECT 2.81

	H	L
Variable 6	0.88	1.17
	0.04	1.52
	0.05	2.34
	0.04	2.21
	1.41	0.10
	0.75	2.67
	0.68	0.06
	0.05	28.86
	0.05	2.66
	0.68	2.68
sum	4.63	44.27
sum/10	0.46	4.43

EFFECT -3.96

	H	L
Variable 7	0.88	1.17
	0.04	1.52
	1.17	1.41
	0.04	0.10
	2.34	2.67
	0.75	0.06
	2.21	0.68
	28.86	0.05
	2.66	0.05
	0.68	2.68
sum	39.63	10.39
sum/10	3.96	1.04

EFFECT 2.92

	H	L
Variable 8	0.88	0.05
	0.04	0.04
	1.17	0.75
	1.52	2.67
	2.34	0.06
	1.41	0.68
	2.21	28.86
	0.10	2.66
	0.05	0.68
	0.05	2.68
sum	9.77	39.13
sum/10	0.98	3.91

EFFECT -2.94

	H	L
Variable 9	0.04	0.88
	1.17	0.04
	1.52	2.34
	0.05	2.21
	1.41	0.06
	0.75	0.68
	0.10	28.86
	2.67	0.05
	2.66	0.05
	0.68	2.68
sum	11.05	37.85
sum/10	1.11	3.79

EFFECT -2.68

	H	L
Variable 10	0.88	0.04
	1.17	2.34
	1.52	1.41
	0.05	0.10
	0.04	0.68
	0.75	28.86
	2.21	0.05
	2.67	2.66
	0.06	0.68
	0.05	2.68
sum	9.40	39.50
sum/10	0.94	3.95

EFFECT -3.01

	H	L
Variable 11	0.04	0.88
	1.52	1.17
	0.05	1.41
	0.04	0.75
	2.34	2.67
	2.21	28.86
	0.10	0.05
	0.06	2.66
	0.68	0.05
	0.68	2.68
sum	7.72	41.18
sum/10	0.77	4.12

EFFECT -3.35

	H	L
Variable 12	0.88	0.04
	1.17	1.52
	0.05	0.75
	0.04	2.21
	2.34	0.06
	1.41	0.05
	0.10	2.66
	2.67	0.05
	0.68	0.68
	28.86	2.68
sum	38.20	10.70
sum/10	3.82	1.07

EFFECT 2.75

	H	L
Variable 13	0.04	0.88
	1.52	1.17
	0.04	0.05
	2.34	2.21
	1.41	0.10
	0.75	0.68
	2.67	2.66
	0.06	0.05
	28.86	0.68
	0.05	2.68
sum	37.74	11.16
sum/10	3.77	1.12

EFFECT 2.66

Dummy Variable 1	H	L
	1.17	0.88
	0.05	0.04
	2.34	1.52
	1.41	0.04
	0.75	0.10
	2.21	2.67
	0.06	28.86
	0.68	0.05
	0.05	0.68
	2.66	2.68
sum	11.38	37.52
sum/6	1.90	6.25

EFFECT -4.36

Dummy Variable 2	H	L
	1.52	0.88
	0.04	0.04
	1.41	1.17
	0.75	0.05
	2.21	2.34
	0.10	2.67
	0.68	0.06
	28.86	0.05
	2.66	0.68
	0.05	2.68
sum	38.28	10.62
sum/6	6.38	1.77

EFFECT 4.61

Dummy Variable 3	H	L
	0.05	0.88
	2.34	0.04
	0.75	1.17
	2.21	1.52
	0.10	0.04
	2.67	1.41
	28.86	0.06
	0.05	0.68
	0.05	2.66
	0.68	2.68
sum	37.76	11.14
sum/6	6.29	1.86

EFFECT 4.44

Dummy Variable 4	H	L
	0.88	0.04
	0.04	1.17
	1.41	1.52
	2.21	0.05
	0.10	2.34
	2.67	0.75
	0.06	0.68
	0.05	28.86
	2.66	0.05
	0.68	2.68
sum	10.76	38.14
sum/6	1.79	6.36

EFFECT -4.56

Dummy Variable 5	H	L
	0.88	1.17
	0.04	1.52
	2.34	0.05
	0.75	0.04
	0.10	1.41
	2.67	2.21
	0.06	28.86
	0.68	0.05
	2.66	0.68
	0.05	2.68
sum	10.23	38.67
sum/6	1.71	6.45

EFFECT -4.74

Dummy Variable 6	H	L
	0.04	0.88
	1.17	1.52
	1.41	0.05
	2.21	0.04
	2.67	2.34
	0.06	0.75
	0.68	0.10
	28.86	0.05
	0.05	2.66
	0.68	2.68
sum	37.83	11.07
sum/6	6.31	1.85

EFFECT 4.46

EFFECT OF VARIABLES ON FINAL pH

pH	H Level	L Level
Variable 1	8.46	8.79
	8.66	8.75
	8.73	8.86
	8.76	8.64
	8.63	8.60
	8.51	8.73
	8.61	8.51
	8.54	8.60
	8.67	8.67
	8.41	8.43
sum	85.98	86.58
sum/10	8.60	8.66

EFFECT -0.06

	H	L
Variable 2	8.46	8.66
	8.79	8.86
	8.73	8.64
	8.75	8.60
	8.73	8.76
	8.51	8.63
	8.61	8.51
	8.54	8.67
	8.67	8.41
	8.60	8.43
sum	86.39	86.17
sum/10	8.64	8.62

EFFECT 0.02

	H	L
Variable 3	8.79	8.46
	8.66	8.73
	8.75	8.64
	8.86	8.60
	8.63	8.76
	8.51	8.73
	8.54	8.51
	8.67	8.61
	8.60	8.41
	8.67	8.43
sum	86.68	85.88
sum/10	8.67	8.59

EFFECT 0.08

	H	L
Variable 4	8.66	8.46
	8.73	8.79
	8.86	8.75
	8.64	8.60
	8.51	8.76
	8.61	8.73
	8.67	8.63
	8.60	8.51
	8.67	8.54
	8.41	8.43
sum	86.36	86.20
sum/10	8.64	8.62

EFFECT 0.02

	H	L
Variable 5	8.46	8.79
	8.73	8.66
	8.75	8.86
	8.64	8.76
	8.60	8.73
	8.51	8.63
	8.54	8.51
	8.60	8.61
	8.67	8.67
	8.41	8.43
sum	85.91	86.65
sum/10	8.59	8.67

EFFECT *-0.07*

	H	L
Variable 6	8.46	8.66
	8.79	8.73
	8.75	8.64
	8.86	8.73
	8.60	8.63
	8.76	8.51
	8.61	8.51
	8.67	8.54
	8.67	8.60
	8.41	8.43
sum	86.58	85.98
sum/10	8.66	8.60

EFFECT 0.06

	H	L
Variable 7	8.46	8.73
	8.79	8.75
	8.66	8.60
	8.86	8.63
	8.64	8.51
	8.76	8.51
	8.73	8.61
	8.54	8.67
	8.60	8.67
	8.41	8.43
sum	86.45	86.11
sum/10	8.65	8.61

EFFECT 0.03

	H	L
Variable 8	8.46	8.75
	8.79	8.86
	8.66	8.76
	8.73	8.51
	8.64	8.51
	8.60	8.61
	8.73	8.54
	8.63	8.60
	8.67	8.41
	8.67	8.43
sum	86.58	85.98
sum/10	8.66	8.60

EFFECT 0.06

	H	L
Variable 9	8.79	8.46
	8.66	8.86
	8.73	8.64
	8.75	8.73
	8.60	8.51
	8.76	8.61
	8.63	8.54
	8.51	8.67
	8.60	8.67
	8.41	8.43
sum	86.44	86.12
sum/10	8.64	8.61

EFFECT 0.03

	H	L
Variable 10	8.46	8.79
	8.66	8.64
	8.73	8.60
	8.75	8.63
	8.86	8.61
	8.76	8.54
	8.73	8.67
	8.51	8.60
	8.51	8.41
	8.67	8.43
sum	86.64	85.92
sum/10	8.66	8.59

EFFECT 0.07

	H	L
Variable 11	8.79	8.46
	8.73	8.66
	8.75	8.60
	8.86	8.76
	8.64	8.51
	8.73	8.54
	8.63	8.67
	8.51	8.60
	8.61	8.67
	8.41	8.43
sum	86.66	85.90
sum/10	8.67	8.59

EFFECT 0.08

	H	L
Variable 12	8.46	8.79
	8.66	8.73
	8.75	8.76
	8.86	8.73
	8.64	8.51
	8.60	8.67
	8.63	8.60
	8.51	8.67
	8.61	8.41
	8.54	8.43
sum	86.26	86.30
sum/10	8.63	8.63

EFFECT 0.00

	H	L
Variable 13	8.79	8.46
	8.73	8.66
	8.86	8.75
	8.64	8.73
	8.60	8.63
	8.76	8.61
	8.51	8.60
	8.51	8.67
	8.54	8.41
	8.67	8.43
sum	86.61	85.95
sum/10	8.66	8.60

EFFECT 0.07

Dummy Variable 1	H	L
	8.66	8.46
	8.75	8.79
	8.64	8.73
	8.60	8.86
	8.76	8.63
	8.73	8.51
	8.51	8.54
	8.61	8.67
	8.67	8.41
	8.60	8.43
sum	86.53	86.03
sum/6	14.42	14.34

EFFECT 0.08

Dummy Variable 2	H	L
	8.73	8.46
	8.86	8.79
	8.60	8.66
	8.76	8.75
	8.73	8.64
	8.63	8.51
	8.61	8.51
	8.54	8.67
	8.60	8.41
	8.67	8.43
sum	86.73	85.83
sum/6	14.46	14.31

EFFECT 0.15

Dummy Variable 3	H	L
	8.75	8.46
	8.64	8.79
	8.76	8.66
	8.73	8.73
	8.63	8.86
	8.51	8.60
	8.54	8.51
	8.67	8.61
	8.67	8.60
	8.41	8.43
sum	86.31	86.25
sum/6	14.39	14.38

EFFECT 0.01

Dummy Variable 4	H	L
	8.46	8.79
	8.86	8.66
	8.60	8.73
	8.73	8.75
	8.63	8.64
	8.51	8.76
	8.51	8.54
	8.67	8.67
	8.60	8.41
	8.41	8.43
sum	85.98	86.38
sum/6	14.33	14.40

EFFECT -0.07

Dummy Variable 5	H	L
	8.46	8.66
	8.79	8.73
	8.64	8.75
	8.76	8.86
	8.63	8.60
	8.51	8.73
	8.51	8.54
	8.61	8.67
	8.60	8.41
	8.67	8.43
sum	86.18	86.38
sum/6	14.36	14.40

EFFECT -0.03

Dummy Variable 6	H	L
	8.79	8.46
	8.66	8.73
	8.60	8.75
	8.73	8.86
	8.51	8.64
	8.51	8.76
	8.61	8.63
	8.54	8.67
	8.61	8.60
	8.41	8.43
sum	85.97	86.53
sum/6	14.33	14.42

EFFECT -0.09

EFFECT OF VARIABLES ON FINAL BIOMASS CONCENTRATION

Biomass	H Level	L Level		H	L
Variable 1	4.30	6.20	Variable 2	4.30	4.30
	4.30	6.30		6.20	5.70
	4.90	5.70		4.90	4.10
	5.00	4.10		6.30	3.20
	5.50	3.20		5.40	5.00
	4.20	5.40		5.00	5.50
	4.10	5.00		4.10	4.20
	4.70	3.70		4.70	5.00
	5.40	5.00		5.40	3.00
	3.00	3.60		3.70	3.60
sum	45.40	48.20	sum	50.00	43.60
sum/10	4.54	4.82	sum/10	5.00	4.36
EFFECT	-0.28		EFFECT	0.64	

	H	L		H	L
Variable 3	6.20	4.30	Variable 4	4.30	4.30
	4.30	4.90		4.90	6.20
	6.30	4.10		5.70	6.30
	5.70	3.20		4.10	3.20
	5.50	5.00		5.00	5.00
	4.20	5.40		4.10	5.40
	4.70	5.00		5.40	5.50
	5.40	4.10		3.70	4.20
	3.70	3.00		5.00	4.70
	5.00	3.60		3.00	3.60
sum	51.00	42.60	sum	45.20	48.40
sum/10	5.10	4.26	sum/10	4.52	4.84
EFFECT	0.84		EFFECT	-0.32	

	H	L
Variable 5	4.30	6.20
	4.90	4.30
	6.30	5.70
	4.10	5.00
	3.20	5.40
	4.20	5.50
	4.70	5.00
	3.70	4.10
	5.00	5.40
	3.00	3.60
sum	43.40	50.20
sum/10	4.34	5.02

EFFECT -0.68

	H	L
Variable 6	4.30	4.30
	6.20	4.90
	6.30	4.10
	5.70	5.40
	3.20	5.50
	5.00	5.00
	4.10	4.20
	5.40	4.70
	5.00	3.70
	3.00	3.60
sum	48.20	45.40
sum/10	4.82	4.54

EFFECT 0.28

	H	L
Variable 7	4.30	4.90
	6.20	6.30
	4.30	3.20
	5.70	5.50
	4.10	5.00
	5.00	4.20
	5.40	4.10
	4.70	5.40
	3.70	5.00
	3.00	3.60
sum	46.40	47.20
sum/10	4.64	4.72

EFFECT -0.08

	H	L
Variable 8	4.30	6.30
	6.20	5.70
	4.30	5.00
	4.90	5.00
	4.10	4.20
	3.20	4.10
	5.40	4.70
	5.50	3.70
	5.40	3.00
	5.00	3.60
sum	48.30	45.30
sum/10	4.83	4.53

EFFECT 0.30

	H	L
Variable 9	6.20	4.30
	4.30	5.70
	4.90	4.10
	6.30	5.40
	3.20	4.20
	5.00	4.10
	5.50	4.70
	5.00	5.40
	3.70	5.00
	3.00	3.60
sum	47.10	46.50
sum/10	4.71	4.65

EFFECT 0.06

	H	L
Variable 10	4.30	6.20
	4.30	4.10
	4.90	3.20
	6.30	5.50
	5.70	4.10
	5.00	4.70
	5.40	5.40
	5.00	3.70
	4.20	3.00
	5.00	3.60
sum	50.10	43.50
sum/10	5.01	4.35

EFFECT 0.66

	H	L
Variable 11	6.20	4.30
	4.90	4.30
	6.30	3.20
	5.70	5.00
	4.10	5.00
	5.40	4.70
	5.50	5.40
	4.20	3.70
	4.10	5.00
	3.60	3.60
sum	50.00	44.20
sum/10	5.00	4.42

EFFECT 0.58

	H	L
Variable 12	4.30	6.20
	4.30	4.90
	6.30	5.00
	5.70	5.40
	4.10	4.20
	3.20	5.40
	5.50	3.70
	5.00	5.00
	4.10	3.00
	4.70	3.60
sum	47.20	46.40
sum/10	4.72	4.64

EFFECT 0.08

	H	L
Variable 13	6.20	4.30
	4.90	4.30
	5.70	6.30
	4.10	5.40
	3.20	5.40
	5.00	4.10
	5.00	3.70
	4.20	5.00
	4.70	3.00
	5.40	3.60
sum	48.40	45.10
sum/10	4.84	4.51

EFFECT 0.33

Dummy Variable 1	H	L
	4.30	4.30
	6.30	6.20
	4.10	4.90
	3.20	5.70
	5.00	5.50
	5.40	5.00
	4.20	4.70
	4.10	5.00
	5.40	3.00
	3.70	3.60
sum	45.70	47.90
sum/6	7.62	7.98

EFFECT -0.37

Dummy Variable 2	H	L
	4.90	4.30
	5.70	6.20
	3.20	4.30
	5.00	6.30
	5.40	4.10
	5.50	5.00
	4.10	4.20
	4.70	5.40
	3.70	3.00
	5.00	3.60
sum	47.20	46.40
sum/6	7.87	7.73

EFFECT 0.13

Dummy Variable 3	H	L
	6.30	4.30
	4.10	6.20
	5.00	4.30
	5.40	4.90
	5.50	5.70
	5.00	3.20
	4.70	4.20
	5.50	4.10
	5.00	3.70
	3.00	3.60
sum	49.50	44.20
sum/6	8.25	7.37

EFFECT 0.88

Dummy Variable 4	H	L
	4.30	6.20
	5.70	4.30
	3.20	4.90
	5.40	6.30
	5.50	4.10
	5.00	5.00
	4.20	4.10
	5.40	4.70
	3.70	5.00
	3.00	3.60
sum	45.40	48.20
sum/6	7.57	8.03

EFFECT -0.47

Dummy Variable 5	H	L
	4.30	4.30
	6.20	4.90
	4.10	6.30
	5.00	5.70
	5.50	3.20
	5.00	5.40
	4.20	4.70
	4.10	5.40
	3.70	3.00
	5.00	3.60
sum	47.10	46.50
sum/6	7.85	7.75

EFFECT 0.10

Dummy Variable 6	H	L
	6.20	4.30
	4.30	4.90
	3.20	6.30
	8.73	5.70
	5.00	4.10
	4.20	5.00
	4.10	5.50
	4.70	5.40
	5.00	3.70
	3.00	3.60
sum	48.43	48.50
sum/6	8.07	8.08

EFFECT -0.01

EFFECT OF VARIABLES ON FINAL RESIDUAL GLUCOSE CONCENTRATION

Glucose	H Level	L Level		H	L
Variable 1	17.50	4.81	Variable 2	17.50	15.23
	15.23	5.50		4.81	4.22
	14.38	4.22		14.38	11.92
	16.52	11.92		5.50	15.44
	15.52	15.44		13.35	16.52
	12.09	13.35		13.53	15.52
	15.47	13.53		15.47	12.09
	12.67	11.48		12.67	3.86
	8.47	3.86		8.47	18.21
	18.21	14.47		11.48	14.47
sum	146.06	98.58	sum	117.16	127.48
sum/10	14.61	9.86	sum/10	11.72	12.75
EFFECT	4.75		EFFECT	-1.03	

	H	L		H	L
Variable 3	4.81	17.50	Variable 4	15.23	17.50
	15.23	14.38		14.38	4.81
	5.50	11.92		4.22	5.50
	4.22	15.44		11.92	15.44
	15.52	16.52		13.53	16.52
	12.09	13.35		15.47	13.35
	12.67	13.53		8.47	15.52
	8.47	15.47		11.48	12.09
	11.48	18.21		3.86	12.67
	3.86	14.47		18.21	14.47
sum	93.85	150.79	sum	116.77	127.87
sum/10	9.39	15.08	sum/10	11.68	12.79
EFFECT	-5.69		EFFECT	-1.11	

	H	L
Variable 5	17.50	4.81
	14.38	15.23
	5.50	4.22
	11.92	16.52
	15.44	13.35
	12.09	15.52
	12.67	13.53
	11.48	15.47
	3.86	8.47
	18.21	14.47
sum	123.05	121.59
sum/10	12.31	12.16

EFFECT 0.15

	H	L
Variable 6	17.50	15.23
	4.81	14.38
	5.50	11.92
	4.22	13.35
	15.44	15.52
	16.52	13.53
	15.47	12.09
	8.47	12.67
	3.86	11.48
	18.21	14.47
sum	110.00	134.64
sum/10	11.00	13.46

EFFECT -2.46

	H	L
Variable 7	17.50	14.38
	4.81	5.50
	15.23	15.44
	4.22	15.52
	11.92	13.53
	16.52	12.09
	13.35	15.47
	12.67	8.47
	11.48	3.86
	18.21	14.47
sum	125.91	118.73
sum/10	12.59	11.87

EFFECT 0.72

	H	L
Variable 8	17.50	5.50
	4.81	4.22
	15.23	16.52
	14.38	13.53
	11.92	12.09
	15.44	15.47
	13.35	12.67
	15.52	11.48
	8.47	18.21
	3.86	14.47
sum	120.48	124.16
sum/10	12.05	12.42

EFFECT -0.37

	H	L
Variable 9	4.81	17.50
	15.23	4.22
	14.38	11.92
	5.50	13.35
	15.44	12.09
	16.52	15.47
	15.52	12.67
	13.53	8.47
	11.48	3.86
	18.21	14.47
sum	130.62	114.02
sum/10	13.06	11.40

EFFECT 1.66

	H	L
Variable 10	17.50	4.81
	15.23	11.92
	14.38	15.44
	5.50	15.52
	4.22	15.47
	16.52	12.67
	13.35	8.47
	13.53	11.48
	12.09	18.21
	3.86	14.47
sum	116.18	128.46
sum/10	11.62	12.85

EFFECT -1.23

	H	L
Variable 11	4.81	17.50
	14.38	15.23
	5.50	15.44
	4.22	16.52
	11.92	13.53
	13.35	12.67
	15.52	8.47
	12.09	11.48
	15.47	3.86
	14.47	14.47
sum	111.73	129.17
sum/10	11.17	12.92

EFFECT -1.74

	H	L
Variable 12	17.50	4.81
	15.23	14.38
	5.50	16.52
	4.22	13.35
	11.92	12.09
	15.44	8.47
	15.52	11.48
	13.53	3.86
	15.47	18.21
	12.67	14.47
sum	127.00	117.64
sum/10	12.70	11.76

EFFECT 0.94

	H	L
Variable 13	4.81	17.50
	14.38	15.23
	4.22	5.50
	11.92	13.35
	15.44	15.52
	16.52	15.47
	13.53	11.48
	12.09	3.86
	12.67	18.21
	8.47	14.47
sum	114.05	130.59
sum/10	11.41	13.06

EFFECT -1.65

Dummy Variable 1	H	L
	15.23	17.50
	5.50	4.81
	11.92	14.38
	15.44	4.22
	16.52	15.52
	13.35	13.53
	12.09	12.67
	15.47	3.86
	8.47	18.21
	11.48	14.47
sum	125.47	119.17
sum/6	20.91	19.86

EFFECT 1.05

Dummy Variable 2	H	L
	14.38	17.50
	4.22	4.81
	15.44	15.23
	16.52	5.50
	13.35	11.92
	15.52	13.53
	15.47	12.09
	12.67	8.47
	11.48	18.21
	3.86	14.47
sum	122.91	121.73
sum/6	20.49	20.29

EFFECT 0.20

Dummy Variable 3	H	L
	5.50	17.50
	11.92	4.81
	16.52	15.23
	13.35	14.38
	15.52	4.22
	13.53	15.44
	12.67	12.09
	8.47	15.47
	3.86	11.48
	18.21	14.47
sum	119.55	125.09
sum/6	19.93	20.85

EFFECT -0.92

Dummy Variable 4	H	L
	17.50	4.81
	4.22	15.23
	15.44	14.38
	13.35	5.50
	15.52	11.92
	13.53	16.52
	12.09	15.47
	8.47	12.67
	11.48	3.86
	18.21	14.47
sum	129.81	114.83
sum/6	21.64	19.14

EFFECT 2.50

Dummy Variable 5	H	L
	17.50	15.23
	4.81	14.38
	11.92	5.50
	16.52	4.22
	15.52	15.44
	13.53	13.35
	12.09	12.67
	15.47	8.47
	11.48	18.21
	3.86	14.47
sum	122.70	121.94
sum/6	20.45	20.32

EFFECT 0.13

Dummy Variable 6	H	L
	4.81	17.50
	15.23	14.38
	15.44	5.50
	13.35	4.22
	13.53	11.92
	12.09	16.52
	15.47	15.52
	12.67	8.47
	3.86	11.48
	18.21	14.47
sum	124.66	119.98
sum/6	20.78	20.00

EFFECT 0.78

EFFECT OF VARIABLES ON FINAL RESIDUAL AMMONIA CONCENTRATION

Ammonia	H Level	L Level		H	L
Variable 1	0.54	0.47	Variable 2	0.54	0.41
	0.41	0.48		0.47	0.42
	0.43	0.42		0.43	0.43
	0.45	0.43		0.48	0.39
	0.38	0.39		0.42	0.45
	0.49	0.42		0.44	0.38
	0.48	0.44		0.48	0.49
	0.40	0.45		0.40	0.43
	0.51	0.43		0.51	0.45
	0.45	0.59		0.45	0.59
sum	4.54	4.52	sum	4.62	4.44
sum/10	0.45	0.45	sum/10	0.46	0.44
EFFECT	0.00		EFFECT	0.02	

	H	L		H	L
Variable 3	0.47	0.54	Variable 4	0.41	0.54
	0.41	0.43		0.43	0.47
	0.48	0.43		0.42	0.48
	0.42	0.39		0.43	0.39
	0.38	0.45		0.44	0.45
	0.49	0.42		0.48	0.42
	0.40	0.44		0.51	0.38
	0.51	0.48		0.45	0.49
	0.45	0.45		0.43	0.40
	0.43	0.59		0.45	0.59
sum	4.44	4.62	sum	4.45	4.61
sum/10	0.44	0.46	sum/10	0.45	0.46
EFFECT	-0.02		EFFECT	-0.02	

	H	L
Variable 5	0.54	0.47
	0.43	0.41
	0.48	0.42
	0.43	0.45
	0.39	0.42
	0.49	0.38
	0.40	0.44
	0.45	0.48
	0.43	0.51
	0.45	0.59
sum	4.49	4.57
sum/10	0.45	0.46

EFFECT -0.01

	H	L
Variable 6	0.54	0.41
	0.47	0.43
	0.48	0.43
	0.42	0.42
	0.39	0.38
	0.45	0.44
	0.48	0.49
	0.51	0.40
	0.43	0.45
	0.45	0.59
sum	4.62	4.44
sum/10	0.46	0.44

EFFECT 0.02

	H	L
Variable 7	0.54	0.43
	0.47	0.48
	0.41	0.39
	0.42	0.38
	0.43	0.44
	0.45	0.49
	0.42	0.48
	0.40	0.51
	0.45	0.43
	0.45	0.59
sum	4.44	4.62
sum/10	0.44	0.46

EFFECT -0.02

	H	L
Variable 8	0.54	0.48
	0.47	0.42
	0.41	0.45
	0.43	0.44
	0.43	0.49
	0.39	0.48
	0.42	0.40
	0.38	0.45
	0.51	0.45
	0.43	0.59
sum	4.41	4.65
sum/10	0.44	0.47

EFFECT -0.02

	H	L
Variable 9	0.47	0.54
	0.41	0.42
	0.43	0.43
	0.48	0.42
	0.39	0.49
	0.45	0.48
	0.38	0.40
	0.44	0.51
	0.45	0.43
	0.45	0.59
sum	4.35	4.71
sum/10	0.44	0.47

EFFECT -0.04

	H	L
Variable 10	0.54	0.47
	0.41	0.43
	0.43	0.39
	0.48	0.38
	0.42	0.48
	0.45	0.40
	0.42	0.51
	0.44	0.45
	0.49	0.45
	0.43	0.59
sum	4.51	4.55
sum/10	0.45	0.46

EFFECT 0.00

	H	L
Variable 11	0.47	0.54
	0.43	0.41
	0.48	0.39
	0.42	0.45
	0.43	0.44
	0.42	0.40
	0.38	0.51
	0.49	0.45
	0.48	0.43
	0.59	0.59
sum	4.59	4.61
sum/10	0.46	0.46

EFFECT 0.00

	H	L
Variable 12	0.54	0.47
	0.41	0.43
	0.48	0.45
	0.42	0.42
	0.43	0.49
	0.39	0.51
	0.38	0.45
	0.44	0.43
	0.48	0.45
	0.40	0.59
sum	4.37	4.69
sum/10	0.44	0.47

EFFECT -0.03

	H	L
Variable 13	0.47	0.54
	0.43	0.41
	0.42	0.48
	0.43	0.42
	0.39	0.38
	0.45	0.48
	0.44	0.45
	0.49	0.43
	0.40	0.45
	0.51	0.59
sum	4.43	4.63
sum/10	0.44	0.46

EFFECT -0.02

Dummy Variable 1	H	L
	0.41	0.54
	0.48	0.47
	0.43	0.43
	0.39	0.42
	0.45	0.38
	0.42	0.44
	0.49	0.40
	0.48	0.43
	0.51	0.45
	0.45	0.59
sum	4.51	4.55
sum/6	0.75	0.76

EFFECT -0.01

Dummy Variable 2	H	L
	0.43	0.54
	0.42	0.47
	0.39	0.41
	0.45	0.48
	0.42	0.43
	0.38	0.44
	0.48	0.49
	0.40	0.51
	0.45	0.45
	0.43	0.59
sum	4.25	4.81
sum/6	0.71	0.80

EFFECT -0.09

Dummy Variable 3	H	L
	0.48	0.54
	0.43	0.47
	0.45	0.41
	0.42	0.43
	0.38	0.42
	0.44	0.39
	0.40	0.49
	0.51	0.48
	0.43	0.45
	0.45	0.59
sum	4.39	4.67
sum/6	0.73	0.78

EFFECT -0.05

Dummy Variable 4	H	L
	0.54	0.47
	0.42	0.41
	0.39	0.43
	0.42	0.48
	0.38	0.43
	0.44	0.45
	0.49	0.48
	0.51	0.40
	0.45	0.43
	0.45	0.59
sum	4.49	4.57
sum/6	0.75	0.76

EFFECT -0.01

Dummy Variable 5	H	L
	0.54	0.41
	0.47	0.43
	0.43	0.48
	0.45	0.42
	0.38	0.39
	0.44	0.42
	0.49	0.40
	0.48	0.51
	0.45	0.45
	0.43	0.59
sum	4.56	4.50
sum/6	0.76	0.75

EFFECT 0.01

Dummy Variable 6	H	L
	0.47	0.54
	0.41	0.43
	0.39	0.48
	0.42	0.42
	0.44	0.43
	0.49	0.45
	0.48	0.38
	0.40	0.51
	0.43	0.45
	0.45	0.59
sum	4.38	4.68
sum/6	0.73	0.78

EFFECT -0.05

8.0 APPENDIX II

CAPREOMYCIN YIELD (g. L^{-1})

Yield	H Level	L Level
Variable 1	0.12	0.67
	1.16	0.08
	0.02	0.29
	0.20	0.05
	1.11	0.27
	0.32	0.02
sum	2.92	1.38
sum/6	0.49	0.23

EFFECT 0.26

	H	L
Variable 2	0.12	1.16
	0.67	0.29
	0.08	0.05
	0.20	0.02
	1.11	0.32
	0.27	0.02
sum	2.45	1.85
sum/6	0.41	0.31

EFFECT 0.10

	H	L
Variable 3	0.67	0.12
	1.16	0.08
	0.29	0.05
	1.11	0.02
	0.27	0.20
	0.32	0.02
sum	3.81	0.48
sum/6	0.64	0.08

EFFECT 0.55

	H	L
Variable 4	0.12	0.67
	1.16	0.29
	0.08	0.02
	0.05	0.20
	0.27	1.11
	0.32	0.02
sum	1.99	2.31
sum/6	0.33	0.38

EFFECT -0.05

	H	L
Variable 5	0.12	1.16
	0.67	0.05
	0.08	0.20
	0.29	1.11
	0.02	0.27
	0.32	0.02
sum	1.50	2.80
sum/6	0.25	0.47

EFFECT -0.22

	H	L
Variable 6	0.12	0.08
	0.67	0.02
	1.16	1.11
	0.29	0.27
	0.05	0.32
	0.20	0.02
sum	2.49	1.81
sum/6	0.41	0.30

EFFECT 0.11

Dummy Variable 1	H	L
	0.67	0.12
	1.16	0.29
	0.08	0.20
	0.05	0.27
	0.02	0.32
	1.11	0.02
sum	3.09	1.21
sum/5	0.62	0.24

EFFECT 0.37

Dummy Variable 2	H	L
	1.16	0.12
	0.08	0.67
	0.29	0.05
	0.02	1.11
	0.20	0.32
	0.27	0.02
sum	2.01	2.29
sum/5	0.40	0.46

EFFECT -0.06

Dummy Variable 3	H	L
	0.08	0.12
	0.29	0.67
	0.05	1.16
	0.20	0.02
	1.11	0.27
	0.32	0.02
sum	2.04	2.26
sum/5	0.41	0.45

EFFECT -0.04

Dummy Variable 4	H	L
	0.12	0.67
	0.29	1.16
	0.05	0.08
	0.02	0.11
	1.11	0.32
	0.27	0.02
sum	1.86	2.35
sum/5	0.37	0.47

EFFECT -0.10

Dummy Variable 5	H	L
	0.67	0.12
	0.05	1.16
	0.02	0.08
	0.20	0.29
	0.27	1.11
	0.32	0.02
sum	1.53	2.77
sum/5	0.31	0.55

EFFECT -0.25

CAPREOMYCIN QUALITY (% IA & IB)

%	H Level	L Level
Variable 1	29.62	5.43
	71.88	33.03
	59.97	13.41
	73.59	11.49
	81.46	18.43
	30.44	24.80
sum	346.96	106.59
sum/6	57.83	17.77

EFFECT 40.06

	H	L
Variable 2	29.62	71.88
	5.43	13.41
	33.03	11.49
	73.59	59.97
	81.46	30.44
	18.43	24.80
sum	241.56	211.99
sum/6	40.26	35.33

EFFECT 4.93

	H	L
Variable 3	5.43	29.62
	71.88	33.03
	13.41	11.49
	81.46	59.97
	18.43	73.59
	30.44	24.80
sum	221.05	232.50
sum/6	36.84	38.75

EFFECT -1.91

	H	L
Variable 4	29.62	5.43
	71.88	13.41
	33.03	59.97
	11.49	73.59
	18.43	81.46
	30.44	24.80
sum	194.89	258.66
sum/6	32.48	43.11

EFFECT -10.63

	H	L
Variable 5	29.62	71.88
	5.43	11.49
	33.03	73.59
	13.41	81.46
	59.97	18.43
	30.44	24.80
sum	171.90	281.65
sum/6	28.65	46.94

EFFECT -18.29

	H	L
Variable 6	29.62	33.03
	5.43	59.97
	71.88	81.46
	13.41	18.43
	11.49	30.44
	73.59	24.80
sum	205.42	248.13
sum/6	34.24	41.36

EFFECT -7.12

Dummy Variable 1	H	L
	5.43	29.62
	71.88	13.41
	33.03	73.59
	11.49	18.43
	59.97	30.44
	81.46	24.80
sum	263.26	190.29
sum/5	52.65	38.06

EFFECT 14.59

Dummy Variable 2	H	L
	71.88	29.62
	33.03	5.43
	13.41	11.49
	59.97	81.46
	73.59	30.44
	18.43	24.80
sum	270.31	183.24
sum/5	54.06	36.65

EFFECT 17.41

Dummy Variable 3	H	L
	33.03	29.62
	13.41	5.43
	11.49	71.88
	73.59	59.97
	81.46	18.43
	30.44	24.80
sum	243.42	210.13
sum/5	48.68	42.03

EFFECT 6.66

Dummy Variable 4	H	L
	29.62	5.43
	13.41	71.88
	11.49	33.03
	59.97	73.59
	81.46	30.44
	18.43	24.80
sum	214.38	239.17
sum/5	42.88	47.83

EFFECT -4.96

Dummy Variable 5	H	L
	5.43	29.62
	11.49	71.88
	59.97	33.03
	73.59	13.41
	18.43	81.46
	30.44	24.80
sum	199.35	254.20
sum/5	39.87	50.84

EFFECT -10.97

CAPREOMYCIN RATIO (IA:IB)

IA:IB	H Level	L Level		H	L
Variable 1	2.13	0.13	Variable 2	2.13	2.04
	2.04	3.75		0.13	0.13
	0.00	0.13		3.75	1.51
	20.90	1.51		20.90	0.00
	0.87	0.02		0.87	0.22
	0.22	0.36		0.02	0.36
sum	26.16	5.90	sum	27.80	4.26
sum/6	4.36	0.98	sum/6	4.63	0.71
EFFECT	3.38		EFFECT	3.92	

	H	L		H	L
Variable 3	0.13	2.13	Variable 4	2.13	0.13
	2.04	3.75		2.04	0.13
	0.13	1.51		3.75	0.00
	0.87	0.00		1.51	20.90
	0.02	20.90		0.02	0.87
	0.22	0.36		0.22	0.36
sum	3.41	28.65	sum	9.67	22.39
sum/6	0.57	4.78	sum/6	1.61	3.73
EFFECT	-4.21		EFFECT	-2.12	

	H	L		H	L
Variable 5	2.13	2.04	Variable 6	2.13	3.75
	0.13	1.51		0.13	0.00
	3.75	20.90		2.04	0.87
	0.13	0.87		0.13	0.02
	0.00	0.02		1.51	0.22
	0.22	0.36		20.90	0.36
sum	6.36	25.70	sum	26.84	5.22
sum/6	1.06	4.28	sum/6	4.47	0.87
EFFECT	-3.22		EFFECT	3.60	

Dummy Variable 1	H	L
	0.13	2.13
	2.04	0.13
	3.75	20.90
	1.51	0.02
	0.00	0.22
	0.87	0.36
sum	8.30	23.76
sum/5	1.66	4.75

EFFECT -3.09

Dummy Variable 2	H	L
	2.04	2.13
	3.75	0.13
	0.13	1.51
	0.00	0.87
	20.90	0.22
	0.02	0.36
sum	26.84	5.22
sum/5	5.37	1.04

EFFECT 4.32

Dummy Variable 3	H	L
	3.75	2.13
	0.13	0.13
	1.51	2.04
	20.90	0.00
	0.87	0.02
	0.22	0.36
sum	27.38	4.68
sum/5	5.48	0.94

EFFECT 4.54

Dummy Variable 4	H	L
	2.13	0.13
	0.13	2.04
	1.51	3.75
	0.00	20.90
	0.87	0.22
	0.02	0.36
sum	4.66	27.40
sum/5	0.93	5.48

EFFECT -4.55

Dummy Variable 5	H	L
	0.13	2.13
	1.51	2.04
	0.00	3.75
	20.90	0.13
	0.02	0.87
	0.22	0.36
sum	22.78	9.28
sum/5	4.56	1.86

EFFECT 2.70

EFFECT OF VARIABLES ON FINAL pH

pH	H Level	L Level		H	L
Variable 1	8.47	8.45	Variable 2	8.47	8.06
	8.06	8.45		8.45	8.34
	8.41	8.34		8.45	8.44
	6.70	8.44		6.70	8.41
	6.65	8.66		6.65	8.23
	8.23	8.30		8.66	8.30
sum	46.52	50.64	sum	47.38	49.78
sum/6	7.75	8.44	sum/6	7.90	8.30
EFFECT	-0.69		EFFECT	-0.40	

	H	L		H	L
Variable 3	8.45	8.47	Variable 4	8.47	8.45
	8.06	8.45		8.06	8.34
	8.34	8.44		8.45	8.41
	6.65	8.41		8.44	6.70
	8.66	6.70		8.66	6.65
	8.23	8.30		8.23	8.30
sum	48.39	48.77	sum	50.31	46.85
sum/6	8.07	8.13	sum/6	8.39	7.81
EFFECT	-0.06		EFFECT	0.58	

	H	L		H	L
Variable 5	8.47	8.06	Variable 6	8.47	8.45
	8.45	8.44		8.45	8.41
	8.45	6.70		8.06	6.65
	8.34	6.65		8.34	8.66
	8.41	8.66		8.44	8.23
	8.23	8.30		6.70	8.30
sum	50.35	46.81	sum	48.46	48.70
sum/6	8.39	7.80	sum/6	8.08	8.12
EFFECT	0.59		EFFECT	-0.04	

Dummy Variable 1	H	L
	8.45	8.47
	8.06	8.34
	8.45	6.70
	8.44	8.66
	8.41	8.23
	6.65	8.30
sum	48.46	48.70
sum/5	9.69	9.74

EFFECT -0.05

Dummy Variable 2	H	L
	8.06	8.47
	8.45	8.45
	8.34	8.44
	8.41	6.65
	6.70	8.23
	8.66	8.30
sum	48.62	48.54
sum/5	9.72	9.71

EFFECT 0.02

Dummy Variable 3	H	L
	8.45	8.47
	8.34	8.45
	8.44	8.06
	6.70	8.41
	6.65	8.66
	8.23	8.30
sum	46.81	50.35
sum/5	9.36	10.07

EFFECT -0.71

Dummy Variable 4	H	L
	8.47	8.45
	8.34	8.06
	8.44	8.45
	8.41	6.70
	6.65	8.23
	8.66	8.30
sum	48.97	48.19
sum/5	9.79	9.64

EFFECT 0.16

Dummy Variable 5	H	L
	8.45	8.47
	8.44	8.06
	8.41	8.45
	6.70	8.34
	8.66	6.65
	8.23	8.30
sum	48.89	48.27
sum/5	9.78	9.65

EFFECT 0.12

EFFECT OF VARIABLES ON FINAL BIOMASS CONCENTRATION

Biomass	H Level	L Level
Variable 1	6.40	3.50
	3.30	3.30
	2.80	0.80
	8.70	1.60
	7.80	1.50
	2.00	1.60
sum	31.00	12.30
sum/6	5.17	2.05

EFFECT 3.12

Dummy	H	L
Variable 2	6.40	3.30
	3.50	0.80
	3.30	1.60
	8.70	2.80
	7.80	2.00
	1.50	1.60
sum	31.20	12.10
sum/6	5.20	2.02

EFFECT 3.18

Dummy	H	L
Variable 3	3.50	6.40
	3.30	3.30
	0.80	1.60
	7.80	2.80
	1.50	8.70
	2.00	1.60
sum	18.90	24.40
sum/6	3.15	4.07

EFFECT -0.92

Dummy	H	L
Variable 4	6.40	3.50
	3.30	0.80
	3.30	2.80
	1.60	8.70
	1.50	7.80
	2.00	1.60
sum	18.10	25.20
sum/6	3.02	4.20

EFFECT -1.18

Dummy	H	L
Variable 5	6.40	3.30
	3.50	1.60
	3.30	8.70
	0.80	7.80
	2.80	1.50
	2.00	1.60
sum	18.80	24.50
sum/6	3.13	4.08

EFFECT -0.95

Dummy	H	L
Variable 6	6.40	3.30
	3.50	2.80
	3.30	7.80
	0.80	1.50
	1.60	2.00
	8.70	1.60
sum	24.30	19.00
sum/6	4.05	3.17

EFFECT 0.88

Dummy Variable 1	H	L
	3.50	6.40
	3.30	0.80
	3.30	8.70
	1.60	1.50
	2.80	2.00
	7.80	1.60
sum	22.30	21.00
sum/5	4.46	4.20

EFFECT 0.26

Dummy Variable 2	H	L
	3.30	6.40
	3.30	3.50
	0.80	1.60
	2.80	7.80
	8.70	2.00
	1.50	1.60
sum	20.40	22.90
sum/5	4.08	4.58

EFFECT -0.50

Dummy Variable 3	H	L
	3.30	6.40
	0.80	3.50
	1.60	3.30
	8.70	2.80
	7.80	1.50
	2.00	1.60
sum	24.20	19.10
sum/5	4.84	3.82

EFFECT 1.02

Dummy Variable 4	H	L
	6.40	3.50
	0.80	3.30
	1.60	3.30
	2.80	8.70
	7.80	2.00
	1.50	1.60
sum	20.90	22.40
sum/5	4.18	4.48

EFFECT -0.30

Dummy Variable 5	H	L
	3.50	6.40
	1.60	3.30
	2.80	3.30
	8.70	0.80
	1.50	7.80
	2.00	1.60
sum	20.10	23.20
sum/5	4.02	4.64

EFFECT -0.62