LIVERPOOL JOHN MOORES UNIVERSITY
SCHOOL OF BIOMOLECULAR SCIENCES

Micromonospora echinospora (ATCC 15837) growth,

differentiation and antibiotic production.

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy to the School of Biomolecular Sciences, Liverpool John Moores University.

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The following figures and appendix has been omitted on request of the university –

Figs 1 & 2 (p. 5)

Figs 3 (p. 7)

Figs 4 & 5 (p. 17)

Appendix

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

Members of the genus *Micromonospora* exhibit complex developmental cycles, differentiating both morphologically and physiologically. The physiology of the genus is poorly understood when compared with other actinomycete genera such as *Streptomyces*. It is believed that a greater understanding of *Micromonospora* physiology will lead to increased industrial exploitation of the genus.

In the present study the growth and physiology of *Micromonospora echinospora* were investigated under a range of conditions. Growth on complex solid and liquid medium allowed the general aspects of the life cycle to be characterized. The development of a chemically defined medium, resulting in reproducible growth and sporulation, facilitated the detailed examination of the physiological and morpholopgical responses to each nutrient limitation. A relationship between the molar C/N ratio of the medium and spore formation was determined. Stoichiometric limitation of growth by nitrogen was found to be a prerequisite for sporulation.

Studies on substrate uptake rates and primary metabolism indicate *M. echinospora* has a preference for amino acids as carbon sources, in the presence of glucose. The estimated Km for this organism was almost 10-fold higher than that of other actinomycetes. These data suggest that this group possesses a low affinity, high capacity sugar transport system. A discussion of this in relation to the ecology of the organism is presented.

A feature of the biology of *M. echinospora* is its ability to accumulate large amounts of storage carbohydrate, such as glycogen, trehalose and lipid (including triacylglycerols). The accumulation of these polymeric storage compounds coincided with the depletion of the nitrogen source. Trehalose, on the other hand, was found to accumulate during nutrient sufficiency, suggesting a role as a carbon sink in this organism. Examination of extracellular protease production found that proteolytic enzymes are produced in response to nitrogen depletion indicating a role in the nitrogen nutrition. Additionally a profile of proteolytic enzymes produced under carbon-limiting conditions revealed at least two proteases produced that were found under both carbon- and nitrogen-limiting conditions, coinciding with sporulation.

The factors affecting the formation of gentamicin were examined. It was found that formation of antibiotic was subject to growth rate and nutritional control. Continuous culture studies revealed that gentamicin formation corresponded with increased pentose phosphate pathway activity.

Transcriptional studies on glycogen biosynthetic and sporulation gene homologues were performed, revealing interesting similarities with the genetically well characterised actinomycete, *Streptomyces coelicolor*.

Acknowledgements

Table of Contents

List of publications

Material contained in this thesis had previously been published in the following journals and proceedings (Reprints are included in the appendix):

Hoskisson, P. A., Hobbs, G., & Sharples, G. P. (2001). PHYSIOLOGICAL STUDIES ON MICROMONOSPORA ECHINOSPORA IN CONTINUOUS CULTURE. Abstract at the 12th International symposium on the Biology of Actinomycetes, Vancouver, 5-9th August 2001.

Hoskisson, P. A., Hobbs, G., & Sharples, G. P. (2001). DIFFERENTIAL PROTEASE PRODUCTION IN *MICROMONOSPORA ECHINOSPORA* IN CARBON AND NITROGEN LIMITING CULTURES. *Abstract at the 148th meeting of the society for General Microbiology, Heriot-Watt University, 26th-30th March 2001.*

Hoskisson, P. A., Hobbs, G., & Sharples, G. P. (2001). GLUTAMINE SYNTHETASE ACTIVITY IN *MICROMONOSPORA ECHINOSPORA*. *Abstract at the 148th meeting of the society for General Microbiology, Heriot-Watt University, 26th-30th March 2001.*

Hoskisson, P. A., Hobbs, G., & Sharples, G. P. (2001). Antibiotic production, accumulation of intracellular carbon reserves and sporulation in *Micromonospora echinospora* (ATCC 15837). *Can. J. Microbiol.* **47**, 148-152.

Hoskisson, P. A., Makin, V., Taylor, H., Sharples, G. P. & Hobbs, G. (2001). Intracellular Protease Activity during the Growth of *Streptomyces coelicolor*. *Biotech. Lett.* **23**, 189-193.

Hoskisson, P. A., Hobbs, G., & Sharples, G. P. (2000). PHYSIOLOGICAL RESPONSES TO NUTRIENT LIMITATION IN THE ACTINOMYCETE *MICROMONOSPORA ECHINOSPORA*. *Abstract at the 147th meeting of the society for General Microbiology, University of Exeter, 12th-15th September 2000.*

Hoskisson, P. A., Hobbs, G., & Sharples, G. P. (2000). PRODUCTION OF PROTEOLYTIC ENZYMES DURING DEVELOPMENT OF THE ACTINOMYCETE, *MICROMONOSPORA ECHINOSPORA*. *Abstract at the 146th meeting of the society for General Microbiology, University of Warwick, 10th-14th April 2000.*

Hoskisson, P. A., Hobbs, G., & Sharples, G. P. (2000). Sensitivity of *Micromonospora echinospora* (NCIMB 12744) spores to heat treatment with further evidence of a heat activation phenomenon. *Letters in Applied Microbiology*, **30**, 114-117.

Hoskisson, P. A., Hobbs, G., & Sharples, G. P. (1999). GROWTH AND DEVELOPMENT OF MICROMONOSPORA ECHINOSPORA IN A COMPLEX MEDIUM. *Abstract at the 11th International symposium on the Biology of Actinomycetes, Crete. 24-28th October 1999.*

Hoskisson, P. A., Hobbs, G., & Sharples, G. P. (1999). ACCUMULATION OF INTRACELLULAR RESERVES IN *MICROMONOSPORA ECHINOSPORA*. *Abstract at the 144th meeting of the society for General Microbiology, University of Leeds7-10th September 1999.*

Hoskisson, P. A., Hobbs, G., & Sharples, G. P. (1999). THE EFFECT OF HEAT TREATMENT ON THE SPORES OF *MICROMONOSPORA ECHINOSPORA*. *Abstract at the 143rd meeting of the society for General Microbiology, University of Edinburgh.12-16th April 1999.*

Aims

The aims of this research programme were to:

1) characterise the growth of *Micromonospora echinospora* and to elucidate the general characteristics of the developmental cycle.

2) determine a set of chemically defined medium conditions, which promote full morphological and physiological development.

3) conduct a detailed and comprehensive analysis of temporal changes in the physiological state of the organism in association with the appearance of spores and antibiotic.

4) investigate the changes in primary metabolism during growth in continuous culture in relation to conditions permissive and non-permissive of antibiotic production.

5) Identify the key developmental gene homologues and perform RT-PCR transcriptional analysis of these genes, attempting to correlate these with development.

The main aim of this study was to generate sufficient physiological data to characterise growth and differentiation in *Micromonospora*. The industrial potential of this genus is enormous. Our lack of understanding of their basic physiological processes have restricted the commercial exploitation of these organisms.

1. Introduction

1.1 Differentiation in Prokaryotes

In *Escherichia coli*, the most extensively studied bacterium, the life cycle and cell cycle of each cell are virtually synonymous (Chater 1989). The ability of certain bacteria to extend their life cycle beyond the cell cycle, by the formation of spores for example is of considerable interest from a developmental biology aspect with *Bacillus subtilis* and *Myxococcus xanthus* being particularly well studied.

The formation of the *Bacillus* endospore is probably the best-characterised bacterial developmental process. The exhaustion of an essential nutrient results in the initiation of a number of stationary growth-phase responses and the initiation of sporulation (Moir, 1992). Such as complex process requires extensive *de novo* protein synthesis and takes up to 7-8 hours at 37°C. The *Bacillus* sporulation process can be classified in to a number of distinct micromorphological stages. During the vegetative cell cycle replicated chromosomes separate and more to opposite poles of the cell. The first visible changes occur with the formation of sporulation septa (prespore body) and the engulfment of the prespore by the septation membrane. The synthesis of cortex and wall peptidoglycan characterise the subsequent developmental stages, with the former believed to be involved in the dehydration of the spore and its heat resistance (Moir, 1992). The laying down of a spore coat and its maturation result in the full resistance properties of the spore being realised, prior to the release of the spore following lysis of the mother cell. This developmental process has been shown to involve in excess of 100 genes, including a cascade of at least six sigma factors (Kroos & Yu, 2000).

In the myxobacterium, *Myxococcus xanthus* the developmental process leading to the formation of the fruiting body is also induced by nutritional shiftdown. Cells glide to a central location, where a fruiting body, up to 100μm tall, is formed. Development is mediated by at least two distinct types of communication processes: those involved in inducing and regulating development and those involved in bringing the cells together (Shimkets, 1992). The aggregation of the cells results in the formation of myxospores which are essentially encysted vegetative cells. These are surrounded by a thick capsule of polysaccharide and confer resistance to deleterious agents (Parish, 1979).

The cell cycle of *Caulobacter* is characterised by a series of well-defined changes in cell structure and function. These changes appear to be independent of growth rate and as such are an essential stage in the life cycle of the organism. The cell cycle of *Caulobacter* is of significant interest because it involves the process of unequal binary fission. Elongation of the stalked cell and the formation of a flagellum on the daughter cell are followed by cell division, which results in the release of a motile swarmer cell. The immature swarmer cell is considered as a dispersal agent, and is incapable of reproduction. The swarmer cell locates a suitable surface and the flagellum is shed. The formation of a stalk coincides with the synthesis of DNA, and is essential to cell division (Parish, 1979; Brock *et al.*, 1994).

The ability of certain bacteria to form differentiated structures is a fascinating aspect of their life cycle. The brief discussion above reveals the diversity of reproductive and dispersal mechanisms employed by some bacteria to ensure survival in

conditions that are unfavourable for growth. The variety of reproductive strategies exhibited by bacteria is exemplified by the actinomycetes.

1.2 Actinomycetes

Actinomycetes are Gram-positive bacteria that form elongated cells or filaments and usually exhibit some degree of true branching. They are characterised by a high G + C (above 55 mol %) content within their DNA (Goodfellow & Cross, 1983). Two broad morphological groups are recognised i.e. the nocardioform- and sporo-actinomycetes, of which *Micromonospora* belongs to the latter. The sporo-actinomycetes exhibit morphological complexity, with spores produced in or on definite parts of the mycelium, whereas the nocardioform-actinomycetes produce fragmenting mycelia, which form coccoid or rod like elements, giving rise to new mycelia.

1.2.1 Actinomycete differentiation

All but the simplest actinomycetes exhibit complex colony structures, representing vegetative and reproductive roles that have attracted interest in their developmental biology (See Fig. 1).

Actinomycete genera display a variety of growth and differentiation processes:

- Unicellular growth e.g. *Arthrobacter*, *Corynebacterium* and *Mycobacterium*.

- Fragmentation of vegetative hyphae, such as in species of *Nocardia* and *Rhodococcus*.

- Spores formed directly on the substrate mycelium e.g. *Micromonospora*, and *Micropolyspora*.

- Spores formed on aerial hyphae such as *Streptomyces*, *Streptoverticillium* and *Saccharopolyspora*.

1.2.2 *Micromonospora*

The genus *Micromonospora* was first proposed in 1923 by Ørskov, as a subgroup of the Actinomycetales. The genus was created for the monosporic species *Streptothrix chalcea* (Foulerton, 1905), which Ørskov considered distinct from the other known actinomycetes. This was due to the absence of aerial mycelium and the formation of single spores. Chemotaxonomic studies have grouped the micromonosporas with the genera *Actinoplanes*, *Amorphosporangium*, *Ampullariella*, and *Dactylosporangium* (Lechevalier & Lechevalier 1970). Subsequently, *Micromonospora* were classified, along with the above, under the suprageneric group, the Actinoplanetes (Goodfellow, 1989). Additional work by Goodfellow *et al.* (1990) proposed the family *Micromonosporaceae* Krassilnikov 1938 based on numerical and chemical methods. This classification utilised the chemically homogeneous cell wall composition of these organisms, with peptidoglycan containing *meso-* and hydroxy-diaminopimelic acid and glycine and whole cell hydrolysates rich in arabinose and xylose. Koch *et al.* (1996) attempted to determine the intrageneric relationship of the micromonosporas based upon their 16S ribosomal DNA (rDNA) sequences. They found that micromonosporas exhibited sequence similarity in their 16S rDNA of 98 %, this indicated a phylogenetically close grouping. Recent work (Kasai *et al.*, 2000) examined the phylogenetic structure within *Micromonospora* based on *gyrB* sequences. Their work agreed with Koch *et al.* (1996), regarding the phylogenetic

Fig. 1. Reproductive cycles in selected actinomycetes. Numbers refer to life cycle stages, solid lines represent the presence of a life cycle stage, broken lines denote the absence of a stage. 1) Motile cells; 2) Non-motile cells; 3) Mycelium; 4) Fragmenting mycelium; 5) Spores formed on the substrate mycelium; 6) Spores formed on aerial mycelium; 7) Motile spores. Taken from Kalakoutskii & Agre, 1976.

Fig. 2. A). A schematic representation of *Micromonospora* morphology. Taken from Goodfellow & Cross (1983). **B).** Electron micrograph of sporulating *M. echinospora*.

The use of protein encoding genes, such as *gyrB* provides a more reliable phylogenetic classification than 16S rDNA in micromonosporas. Aminoglycoside antibiotics, produced by many members of the genus, are known to bind to the A-site of the 16S rRNA, which may result in increased selective pressure on ribosomal genes (Kasai *et al.*, 2000).

Morphologically, the genus *Micromonospora* is characterised by well-developed, branched, septate hyphae averaging 0.5 μm in diameter. Non-motile spores are usually borne singly and are sessile, on short or long sporophores that often occur in branched clusters (See Fig 2 a & b). Aerial hyphae are usually absent (Kawamoto, 1989). On agar medium most species of *Micromonospora* produce colonies that are initially orange, maturing to a progressively dark hue, accompanied by the production of spores (Mullins, 1993).

Members of the genus *Micromonospora* have attracted attention as producers of many anti-bacterial compounds. Two thirds of the known aminoglycoside antibiotics are produced by non-*Streptomyces* species, with a significant number of these being isolated from the genus *Micromonospora* (Tilley *et al.*, 1990). Gentamicin is currently the only *Micromonospora* antibiotic commercially exploited, from nearly 150 aminoglycoside antibiotics isolated from the genus, highlighting the industrial potential of this genus. Additionally, representatives of practically every chemical family of antibiotics have been isolated from the genus *Micromonospora* (Wagman & Weinstein, 1980), with novel therapeutic compounds, such as calicheamicin and megalomicin receiving significant recent interest (Thorson *et al.*, 2000 and Alarcon *et al.*, 1988)..

1.2.3 Gentamicin.

Gentamicin is a complex of small, basic, water-soluble, relatively stable aminoglycoside antibiotics, exhibiting broad-spectrum activity against both Gram-positive and Gram-negative bacteria. The aminoglycosides are characterised by having an aminocyclitol moiety connected to various sugar/amino sugar units by glycosidic linkages (Berdy & Jarai, 1986). Gentamicin is a representative of the group containing a 2-deoxystreptamine moiety (Pipersberg, 1997).

Fig.3. Gentamicin (from Berdy & Jarai, 1986). R-groups differ in up to 45 gentamicin types.

1.2.4 *Micromonospora echinospora* (ATCC 15837)

Micromonospora echinospora subsp. *echinospora* (Luedemann & Brodsky, 1963) was named according to its spore morphology (*echinos* Greek; spiny appearance: *spora* Greek; seed), and characteristically produce antibiotics of the gentamicin complex. The organism was isolated from soil collected from Jamesville, New York State, USA. Luedemann & Brodsky (1963) further characterised this and several other *Micromonospora* species on the basis of carbohydrate usage pattern and macroscopic morphology. The species *M. echinospora* has now been emended to include three further taxa based on the results of DNA-DNA hybridisation (Kasai *et al.*, 2000); namely *M. rhodorangea*, *M. echinospora* subsp. *ferruginea* and *M.*

purpurea. This molecular analysis of *M. purpurea* confirms the observations of

Kawamoto (1989) that *M. purpurea* is a non-sporulating variant of *M echinospora*.

1.2.5 Ecology of micromonosporas

Actinomycetes represent a significant part of the microflora of soils and their ability

to form bioactive molecules has led to interest in their isolation from the natural

environment (Goodfellow & O'Donnell, 1989). Jensen (1930) first noted that soil

was the natural habitat for micromonosporas, having isolated 67 strains from a range

of soils. Kriss (1939), isolated further micromonosporas from soils in the Saratov

region of Russia. They have long been considered as aquatic organisms, however,

due to the occurrence of spores in sea and lake sediments. The work of Umbreit &

McCoy (1941) demonstrated that micromonosporas formed a large part of the

microbial population (10-50 %) of the lake bottom and mud of Lake Wisconsin.

Umbreit & McCoy (1941) suggested that micromonosporas act as mineralisers of

lake bottom deposits, fulfilling a role similar to that of streptomycetes on land. It

should be noted however that the total bacterial numbers isolated by Umbreit &

McCoy (1941) were low (250 cfu/ml), possibly giving a false impression of the

actual number of micromonosporas (Cross, 1981). Micromonosporas have also been

isolated from marine environments, such as beach sand, and deep marine sediments

(Kawamoto, 1989). It appears, however, that the spores of micromonosporas are

easily washed into water reservoirs from soil where owing to their resistance

properties, they can remain dormant for extended periods (Johnston & Cross, 1976).

It has been shown that micromonosporas are comparable in terms of their abundance

to *Streptomyces* in certain terrestrial ecosystems, such as the rhizosphere of forest

moss (Zenova & Zvyagintsev, 1997).

The isolation of non-streptomycete actinomycetes has recently become of interest as a source of industrially-useful metabolites. The isolation of these actinomycetes from soil by conventional dilution plate techniques is generally hindered by the presence of other bacteria and the faster growing *Streptomyces* species, with efficient recovery being reliant upon the use of specific media and selective pre-treatments to depress the growth of competitive species (Hayakawa *et al.*, 1991; Mullins, 1993). Such treatments to increase selectivity of isolation media include the addition of novobiocin and tunicamycin (Wakisaka *et al.*, 1982), cyclohexamine, nalidixic acid and phenol (Hayakawa *et al.*, 1991; Seong *et al.*, 2001). Selective pre-treatments exploiting the ability of spores to withstand dry heat (120°C for 1 h) or wet heat (55°C for 1 h) have also allowed the isolation of micromonosporas from soils (Nonomura & Ohara, 1969; Mullins, 1993).

1.3 Spores, germination and hyphal growth

The life cycle of a sporoactinomycete begins with spore germination. Germination has typically been defined as the process leading to the emergence of a visible germ tube in actinomycetes (Attwell & Cross, 1973). Ensign *et al.*, (1978), Hardisson *et al.*, (1978) and Suarez *et al.*, (1980), have shown that initiation of germination, demonstrated by a loss of refractility when viewed by phase contrast microscopy. Three morphological stages of germination – phase-darkening (loss of refractility), swelling and germ tube emergence, as specified by Hardisson *et al.*,(1978), have been adopted for characterising germination in actinomycetes. The mobilisation of intracellular storage compounds such as trehalose may result in the darkening of spores during the early stages of germination (McBride & Ensign, 1987). Suarez *et al.*, (1980) demonstrated the above mechanism of germination in *Micromonospora*

chalcea. The organism did not show the same degree of swelling as demonstrated in *Streptomyces* species (Hardisson *et al.,* 1978 & Suarez *et al.,* 1980). The reduced swelling in *Micromonospora* may be attributed to the inflexibility of the relatively thicker spore wall present in micromonosporas (Sharples & Williams, 1976).

Requirements for germination are variable (Chater & Losick, 1997). The loss of refractility can be initiated be the provision of divalent cations (Hardisson, 1978). Eaton & Ensign (1980) demonstrated that Ca^{2+} ions are important for the germination of *Streptomyces viridochromogenes*. The requirement of only divalent cations to induce the loss of refractility (Suarez *et al.,* 1980), coupled with an increase in respiration may indicate the utilisation of an endogenous carbon source. The presence of exogenous carbon and nitrogen sources was shown to be required for the emergence of a germ tube in *M. chalcea* (Suarez *et al.,* 1980).

It has been demonstrated that spores of the genus *Streptomyces* are less metabolically active than vegetative cells, yet they contain metabolic enzyme activities and a functional respiratory system (Ensign, 1978). It has been suggested that dormancy is 'any rest period or reversible interruption of the phenotypic development of an organism' (Sussman, 1969). Keynan & Evanchik (1969) assumed that the breaking of dormancy required the provision of an activating factor. It has been suggested that several conditions result in the disruption of dormancy, leading to germination, such as temperature and chemical activators (Sussman, 1969). Mild stress has been shown to initiate germination in actinomycetes. This appears not to be a universal phenomenon. Heat stress has been shown to affect the rate of germination in *S. viridochromogenes* (Hirsch & Ensign, 1976), yet Suarez *et al.,* (1980) found no requirement for heat activation of germination in *M. chalcea*. Grund

& Ensign (1982) demonstrated that spores of *S. viridochromogenes* could also be activated by treatment with various detergents.

Various deleterious agents have been used to assess the resistance properties of actinomycetes spores, with the aim of characterising the spore population, such as mild acid, lysozyme, sonication and heat (Lee & Rho, 1993; McBride & Ensign, 1987; Suarez *et al.*, 1980; Koepsel & Ensign, 1984; & Glazebrook *et al.*, 1990). It has been demonstrated, however, that *Streptomyces viridochromogenes* spores are exogenously dormant, with spores harvested after 10 days of growth germinating when provided with a defined medium containing divalent cations, and amino acids. The incubation of these spores in nutrient-free conditions resulted in the spores becoming less able to germinate (Hirsch & Ensign, 1976). This would suggests that dormancy is an intrinsic property of spores exposed to conditions unsuitable for germination,

During swelling the thickness of the spore wall becomes reduced by approximately 50 % (Hardisson & Suarez, 1979). Germ tube wall formation varies in actinomycetes, with three categories recognised (Sharples & Williams, 1976),

- The germ tube is synthesised *de novo* during germination e.g. *Microellobosporia*

- The germ tube arises from the inner wall layer. It is not visible until after germination is initiated e.g. *Streptomyces*.

- The germ tube arises from an existing inner layer of the spore wall, distinguishable in the dormant spore e.g. *Micromonospora*.

The emergence of a germ tube from a spore is followed later by branching of hyphae, which eventually leads to the formation of substrate mycelium. This

extension and branching has been shown to be by apical extension in *Streptomyces*, during which, rounds of DNA synthesis occur (Miguelez *et al.,* 1992). The majority of our knowledge concerning the physiology of growth and morphogenesis in bacteria is concerned with unicellular prokaryotes such as *Bacillus* and *Escherichia*. This is mainly due to the rapid formation of physiologically heterogeneous cell types in mycelial prokaryotes (Chater & Losick, 1997).

Hyphal growth is linear. Exponential colony growth, however, is achieved by branch formation (Allen & Prosser, 1983, 1985). Miguelez *et al.,* (1992) proposed a model of apical growth for streptomycetes. They considered the apex as a multilayered wall that expands by inside to out incorporation of cell wall precusors. Kretschmer (1991) suggested that accumulation of cell wall precursors beyond those levels that can be consumed by the growing tip might be a critical signal in the formation of hyphal branches. The formation of branches is frequently close to septa (Chater & Losick, 1997). The septation of cells leads to the formation of two cell types, an actively extending apical cell and a non-extending sub-apical cell. The formation of cross walls is frequently associated with older areas of the mycelium. In the filamentous fungi, the concept of the hyphal growth unit (HGU) provides information of the density of hyphal branching. It is defined as the ratio of total mycelium length to the total number of branches and is therefore the mean hyphal length associated with a growing tip (Prosser, 1995). In the fungi the HGU is relatively constant, with values increasing as the branching becomes sparse. Kretschmer (1987) postulated that the maintenance of a specific genome/cytoplasm ratio might result in the regulation of septation and branching, hence their formation in older parts of the mycelium. Although the formation of branches is frequently associated with septa, they are

normally found in front of the septa, rather than behind, as in the filamentous fungi (Prosser & Tough, 1991). This led to speculation by these authors that the environmental factors governing growth of these organisms in the natural environment may result in the formation of septa as a mechanism of isolating the rapidly growing apical hyphae from distal hyphal regions where conditions may not be favourable for growth.

1.4.1 Sporulation

The mycelial actinomycetes are by nature organisms containing physically attached differentiated cell types in which physiological and biochemical analyses have proved problematical (Hodgson 1992; 2000). Genetically the most studied mycelial actinomycete *Streptomyces coelicolor* has been used as a model for the sporulation process and the factors governing its initiation. Much of the work has been conducted on surface grown cultures owing to the suppression of aerial mycelium formation and sporulation in liquid culture in this organism. The development of a system, which provides dispersed growth of these organisms in liquid culture, has long been a goal of actinomycete physiologists. Such a system would simplify the study their developmental biology (Hobbs *et al.*, 1989; Hodgson 2000).

1.4.2 Environmental factors in control of sporulation

It has been suggested that the development of aerial mycelium and the formation of spores occurs following a point in colony development when nutrients become limiting (Chater 1984). These are suggestions based on a number of reports in which sporulation was promoted by nutrient-limitation or nutrient shiftdown. The most compelling of these was that of Kendrick & Ensign (1993), in which submerged

cultures of *S. griseus*, actively growing, were placed into conditions depleted in either nitrogen or phosphorus. Such conditions greatly stimulated sporulation. The provision of growth-limiting amounts of arginine to cultures of *S. viridochromogenes* resulted in micro-cycle sporulation, i.e. the germination of a spore to form a primary hypha, which then developed in to a spore chain (Koepsel & Ensign, 1984). This microcycle growth phenomenon is observed in many streptomycetes during growth on soil particles (Hodgson 1992). Physiological studies carried out on *S. antibioticus* (Brana *et al.*, 1986) indicated that the amount of nitrogen source in the culture medium influenced the timing of aerial mycelium formation. In contrast, Allen & Prosser (1987) reported that the continuous supply of nutrients to surface cultures still resulted in the formation of aerial mycelium.

The levels of nutrients present and the nature of limiting substrate are seldom reported. It is therefore apparent that little evidence is available confirming the role of nutrient limitation on the formation of spores in actinomycetes.

1.4.3 The role of catabolite repression. The formation of aerial mycelium in *S. alboniger* was shown to be sensitive to glucose repression, reversible by the addition of adenine (Redshaw *et al.*, 1976). Hodgson (1992) suggested that the addition of adenine restored the 'balance' within the catabolic pathways involved. The secretion of organic acids (mainly pyruvate) during 'unbalanced' growth was due to glucose-stimulated glycolysis, without the subsequent induction of the citric acid cycle, resulting in primary metabolite overflow. The accumulation of organic acids has previously been reported in nitrogen-limited growth, under carbon-sufficient conditions (Wanner & Egli, 1990). Coleman & Ensign (1982) reported that inhibition of aerial mycelium formation could be achieved by the addition of casein

hydrolysate to cultures of *S. viridochromogenes*. In both cases the 'glucose repression' and 'casein hydrolysate repression' of development were examples of nutritional inhibition of development rather than starvation-induced development (Hodgson, 1992). *S. coelicolor* mutants which are impaired in their ability to form aerial hyphae and spores (*bld*) have been shown to be defective in their ability to regulate carbon metabolism and, as a secondary consequence, are unable to sense/signal starvation and therefore initiate development (Pope *et al.,*1996). It has been suggested that such mutants may reveal the presence of dual pathways of development, one nutritionally linked, and one that is not (Hodgson, 1992).

1.4.4 Amino acid starvation and the stringent response

Many bacteria exhibit the stringent response (See Fig 4.). In *E. coli*, depletion of amino acids leads to the stringent response, which is characterised by an immediate reduction in the rate of synthesis of RNA (see Cashel & Rudd, 1987; Chatterji & Ojha 2001), and is apparently mediated by the highly phosphorylated guanosine nucleotide, ppGpp (guanosine 5'-diphosphate-3'-diphosphate). As the rate of RNA synthesis correlates with the growth rate of a organism, ppGpp can be considered as a global transcriptional regulator (Chatterji & Ojha, 2001). In *E. coli*, ppGpp is made from ATP and GTP, by the product of the *relA* gene, ppGpp-synthetase which is activated by non-aminoacylated tRNA binding to a translating ribosome (Cashel *et al.,* 1996). In addition to the reduction in the overall synthesis of RNA, ppGpp is believed to mediate increased synthesis of the alternative sigma factor RpoS, which is a major regulator of stationary phase gene expression and the upregulation of certain amino acid biosynthetic operons (Burkovski *et al.,* 1995; Shand *et al.,* 1989).

The stringent response appears to be a conserved pathway in bacteria and it influences diverse pathways in distantly related bacteria (Chatterji & Ojha, 2001). The sporulation cascade of *Myxococcus xanthus* has been shown to be mediated early-on by ppGpp, with *relA*⁻ mutants unable to sporulate (Harris *et al.*, 1998). Ochi (1988) demonstrated that a number of streptomycetes exhibited a classic stringent response, with ppGpp accumulation associated with secondary metabolism, and a concomitant fall in GTP resulting in the initiation of sporulation. These interpretations are considered controversial (Hodgson, 1992) and it is considered more likely that ppGpp acts as a general transcriptional mediator (Kang *et al.*, 1999). The constructed *relA* null mutants of *S. coelicolor* were delayed in the onset of secondary metabolism and aerial mycelium formation (Chakraburtty *et al.*, 1996). In *E. coli* there is a second route for ppGpp synthesis, via ppGpp synthetase II, the product of the *SpoT* gene. The activity of *SpoT* is mediated by carbon and energy source deprivation (Cashel *et al.*, 1996). The second function of *SpoT* is to catalyse the degradation of ppGpp, indicating the presence of dual catalytic domains (Sun *et al.*, 2001). Indeed, the *relA* gene of *S. coelicolor* has been shown to possess both ppGpp synthetase and hydrolase activity (Martinez-costa *et al.*, 1998). It has recently been demonstrated (Sun *et al.*, 2001) that there are two *relA/SpoT* homologues present in *S. coelicolor*.

Fig. 4. Activation of ppGpp synthesis in *E. coli*. The majority of knowledge is about how amino acid starvation causes ppGpp synthesis. The effects of carbon and phosphate starvation are little studied. Adapted from Chater & Bibb, 1997.

Fig. 5. Factors potentially determining the onset of antibiotic production in streptomycetes. Thinner lines represent possible interactions where there is no direct evidence. Adapted from Chater & Bibb, 1997.

1.4.5 Sporulation factors

Bacteria respond to, and process, external signals from neighbouring cells, with such intercellular communication being mediated by small diffusible signal molecules (See Fig. 5.). Such bacterial 'cross-talk' has been shown to be coordinated by a wide range of signalling compounds, such as the acyl-homoserine lactones in Gram-negative bacteria and small thiolactone peptide pheromones in *Staphylococcus aureus* and *Bacillus* (Williams *et al.,* 1999). These signals can result in the expression of genes encoding various phenotypes such as protease production, competence, sporulation, motility and virulence factors (Williams *et al.,* 1999).

1.4.6 Butyrolactone autoregulators. The involvement of low molecular weight signalling molecules involved in streptomycete differentiation has been known for nearly thirty years (Yamada, 1999). These substances are known to appear during exponential growth at around 10 nM concentrations where they can induce morphogenesis and secondary metabolite production. A-factor was the first butyrolactone autoregulator identified (Khokhlov 1980). It was found that mutants of *Streptomyces griseus*, deficient in streptomycin production and sporulation, had this ability restored when complemented with wild type *S. griseus*. The production of A-factor by wild type organisms was shown to be responsible for the restoration of aerial hyphae formation. A-factor is the best-characterised sporulation factor in actinomycetes, with production being temporally regulated. It is speculated that A-factor triggers developmental gene cascades presumably to co-ordinate development within colonies (Hodgson, 1992). This view appears to be an over simplified view-point, as recently a series of *S. griseus* mutants defective in the A-factor receptor

protein, have been found to be able to produce streptomycin and form aerial mycelium (Kang *et al.*, 1999).

1.4.7 Cyclic AMP (cAMP). A role for cAMP in morphogenesis and antibiotic production has been implicated in several *Streptomyces* species (Botsford & Harman, 1992). Ragan & Vining (1978) noticed a casual relationship between cAMP and streptomycin production. In addition Susstrunk *et al.*, (1998), demonstrated pleiotrophic effects of cAMP in *S. coelicolor* on germination, aerial mycelium formation and antibiotic production. Further to this work Kang *et al.*, (1999) showed that cAMP has a regulatory role in the synthesis of ppGpp and the interaction of cAMP with the A-factor regulatory cascade.

1.4.8 Virginiae butanolides. The virginiae butanolides (VB) of *S. virginiae* have been shown to promote antibiotic production. The appearance of VB in late exponential phase leads to the production of the antibiotic virginiamycin. Introduction of VB early in culture, however, results in reduced antibiotic production. This suggests that VB is involved in promotion and suppression of antibiotic genes (Yamada, 1999). There is no evidence that VB plays a role in morphogenesis (Hodgson, 1992).

1.4.9 Pamamycin. At least one antibiotic has been shown to have a stimulatory effect on morphological differentiation (Chater, 1989). Pamamycin, a complex of at least 8 homologous compounds was produced by *S. alboniger*. Kondo *et al.*, (1988) demonstrated that formation of aerial mycelium was promoted in the presence of pamamycin concentrations in the region of 0.1 µg. Subsequently one single homologue (pamamycin-607) was shown to promote the formation of aerial mycelium. Kondo *et al.*, (1988) provided evidence that this molecule could act as an

anion transporter, as MnO_4^- could be transferred from water in to benzene. The exact role and mode of action within the cell of pamamycin have still to be elucidated.

1.4.10 Factor C. A 34.5 kDa hydrophobic protein in conditioned media from *S. griseus* cultures, which exhibited a high specific activity (8 ng. ml^{-1}) restored sporulation in deficient mutants. Non-sporulating mutants however have been shown to produce small amounts of Factor C (Biro *et al.*, 1980). The role of Factor C has yet to be fully elucidated. The ability of Factor C to repair developmental defects may suggest a role similar to the hydrophobic, spore associated protein (SapB) of *Streptomyces coelicolor*, reflecting a structural role rather than a signalling one (Hodgson, 1992).

Despite the evidence for extracellular signalling and sensing systems in the majority of prokaryotic organisms studied, there is a no literature indicating the presence of such mechanisms in *Micromonospora*. Given the ubiquitous nature of these pathways, it is possible that such mechanism occur in this genus.

1.5.1 Developmentally regulated biochemical markers of actinomycetes

Prior to the initiation of morphological differentiation, such as the erection of aerial hyphae in *S. coelicolor*, substrate hyphae undergo various physiological and biochemical changes (Chater, 1989).

1.5.2 Carbohydrate storage metabolism.

The accumulation of granular deposits of glycogen within the cytoplasm has been demonstrated in over forty species of bacteria (Preiss, 1984). The accumulation of glycogen is usually in response to the limitation of nitrogen in the presence of excess carbon. Other responses are known however such as accumulation during exponential growth and the accumulation of glycogen during carbon-limited continuous culture (Dawes, 1992). The synthesis of glycogen is achieved via the polymerisation of ADPglucose, derived from glucose-1-phosphate, catalysed by ADPglucose pyrophosphorylase (product of the *glgC* gene). The resulting ADPglucose is enzymatically coupled to an α-1,4-glucan primer, by the *glgA* gene product glycogen synthase. The subsequent branching of the glycogen structure is achieved by the branching enzyme (*glgB*), which transfers glucosyl units to the 6-position of certain units within the chain, creating α-1,6 branching points. The physiological role of glycogen is presumed to allow increased survival potential in the accumulating organism during unfavourable conditions and has been shown to play an important role in sporulation of certain bacteria (Dawes, 1992).

Trehalose has been implicated in the stress and storage metabolism of a multitude of prokaryotes and several eukaryotic organisms (Thevelein, 1984). This disaccharide is constitutively produced by mycobacteria and is subject to continuous turnover within the cells (De Smet *et al.*, 2000). Three pathways are available for the

biosynthesis of trehalose, via the condensation of glucose-6-phosphate and UDPglucose, the mobilsation of polyglucans such as glycogen and the enzymatic modification of maltose. De Smet *et al.*, (2000) demonstrated the presence and activity of each of these pathways in a variety of mycobacteria, indicating the importance of this compound to these organisms.

The two forms of accumulated carbon have been implicated in having a role in storage metabolism in actinomycetes (Schneider *et al.*, 2000; De Smet *et al.*, 2000; Belanger & Hatfull, 1999 & Preiss & Romeo, 1989). Glycogen accumulates biphasically in *S. coelicolor* during growth (Karandikar *et al.*, 1997; Martin *et al.*, 1997) correlating with the observed accumulation in two locations within the colony. Ultrastructural studies (Plaskitt & Chater, 1995; Brana *et al.*, *1986*) of glycogen accumulation in *S. coelicolor* and *S. antibioticus* indicate that the first phase of glycogen accumulation occurs prior to the onset of aerial mycelium formation, close to the area where aerial hyphae emerge from the surface of solid media. The second phase of accumulation occurs within the developing spore chains (Plaskitt & Chater, 1995). Morphological mutants such as *bldA39* failed to produce phase I glycogen deposits but when sporulation was induced, phase II deposits were detected. It has since been discovered that *glgB* is duplicated in *S. coelicolor* and is present on a large operon containing genes for trehalose metabolism in addition to glycogen synthesis. The observation that *glgC* is essential only for the first phase of accumulation (Martin *et al.*, 1997) and that it is unlinked to the duplicated operons containing *glgB* raises interesting questions regarding the regulation of glycogen and trehalose metabolism in these organisms. This situation contrasts with that seen in *E.*

coli where all the genes for glycogen biosynthesis are found on the same operon (Dawes, 1992).

The complex nature of storage metabolism in other actinomycetes, such as *Mycobacterium* and *Streptomyces*, would suggest a similar pattern of accumulation might be present in the micromonosporas. In the present study we have shown that *M. echinospora* accumulates both glycogen and trehalose in a complex manner. The importance of these compounds to the life cycle of *Streptomyces* is well established yet the exact physiological role of each of the various phases of accumulation has not been proven. The accumulation pattern during the life cycle of *Micromonospora* may provide comparative evidence for the role of storage carbohydrate in actinomycetes in a non-aerial hyphae forming filamentous species.

1.5.3 Role of proteases & proteolysis in differentiation.

Proteolysis is an important process in both cell differentiation and progression through the cell cycle. The timed expression of proteins during developmental pathways requires precise regulation. The role of proteolysis as a switching mechanism during development has been revealed by studies in *Bacillus* and *Caulobacter*. The close association of nitrogen nutrition and the regulation of differentiation in streptomycetes may also reveal a role for proteases in regulation of growth and differentiation (Kang et al., 1995). These authors investigated the growth of *Streptomyces albidoflavus*, in relation to protease production, and found that this organism produced proteolytic enzymes essential for the hydrolysis of complex nitrogen sources, the formation of aerial hyphae and spore maturation. The presence of specific inhibitors for serine proteases and metalloproteases were found to block

development at each morphological stage in this organism. *Bacillus subtilis* has been shown to produce a number of intracellular and extracellular proteases soon after the end of exponential growth, with a putative essential role in the initiation of sporulation (Ochi, 1985). Ochi (1985) speculated that an intracellular serine protease produced at the initiation of sporulation was involved in the degradation or inactivation of enzyme(s) involved in synthesis of purine nucleotides. Tentative links between a serine protease expressed in stationary phase cultures of *S. peucetius* and *S. lactamdurans* and a putative role in secondary metabolism and morphogenesis have been suggested (Gibb & Strohl, 1987; Ginther, 1978). Cleavage of peptide bonds is one of the most important and frequent modifications of proteins (Neurath, 1989). Four classes of proteolytic enzyme are recognised each differing subtly in their catalytic mechanism. These enzymes are the serine proteases, cysteine proteases, aspartic proteases and metallo-proteases (Dunn, 1989). The mechanism of proteolytic action can be determined based on the susceptibility of a protease to inhibition by a group of specific inhibitors (Dunn, 1989).

1.6.1 Antibiotic production

Antibiotics are secondary metabolites, nonessential for primary vegetative growth of the producing organism. The nonessential function of these compounds does not, however, mean that they have no function in the cellular activities of the producer (Beppu, 1992).

1.6.2 Ecological & evolutionary role of antibiotics. It has been speculated that the production of secondary metabolites, conferring a selective advantage on the producing organism through the interaction of the compounds with specific receptors

in other organisms, has evolved as part of the producers strategy for survival (Maplestone *et al.,* 1992). The production of secondary metabolites is common amongst organisms lacking an immune system, such as bacteria, algae, corals, sponges and plants. This reinforces the concept that the production of secondary metabolites increases the fitness of the producing organism. The model of Chater & Merrick (1979) has been widely quoted as an ecological role for antibiotics. These authors suggested that extensive lysis of the substrate hyphae observed during the erection of aerial hyphae, which is believed to provide nutrients for aerial hyphae growth, releases significant nutrients in to the environment. The production of antibiotics by these organisms provides a mechanism of protecting these nutrients from competitor organisms that could invade the colony.

Vining (1992) listed the features of secondary metabolites:-

- Secondary metabolites are not essential for growth and tend to be strain specific.

The synthesis of secondary metabolites can be divided in to two processes, synthesis of primary metabolites, the building blocks of the secondary metabolite, and the specific biosynthetic steps of secondary metabolite formation (Drew & Demain, 1977). The possibility of competition between primary and secondary metabolism has been raised (Drew & Demain, 1977), since the primary metabolites may be used for growth and maintenance, or diverted to production of a secondary metabolite. There is also the possible role of metabolic imbalance influencing the production of secondary metabolites. The excretion of organic acids and the production of methylenomycin appear to be intimately linked (Hobbs *et al.,* 1992).

- Secondary metabolites have a wide range of chemical structures and biological activities.

Secondary metabolites are derived by unique biosynthetic pathways from primary intermediates and metabolites. These pathways are often long and complex, catalysed by specific enzymes different from those of primary metabolism (Martin & Liras, 1989). The multiplicity of chemical structures found in actinomycetes is testimony to this biosynthetic diversity observed in the producing organisms. This diversity is exemplified by the genus *Micromonospora*, which is capable of producing structurally diverse antibiotics such as aminoglycosides e.g. gentamicin; macrolides e.g. megalomicin; isonitriles e.g. YM-47515; enediynes e.g. calicheamicin; and ansamysins e.g. halomycin. Additionally the actinomycetes in general can produce antibiotics such as polyketides, prodigiosins and cyclopentanones. (Wagman & Weinstein, 1980; Sugawara *et al.,* 1997; Thorson *et al.,* 2000; Kieser *et al.,* 2000). The spectrum of these molecules is diverse also, with activity exhibited against Gram-positive and Gram-negative bacteria, antifungal, anti-tumour, herbicidal and growth promoting activities.

- Secondary metabolite formation is directed by organised sets of genes associated with special regulatory mechanisms that control the timing and level of gene expression.

The genes for the biosynthesis of secondary metabolites are organised in clusters, with the resistance genes often associated with those for biosynthesis (Martin & Liras, 1989). The resistance is conferred by a number of mechanisms, such as target modification, efflux systems or by modification of the antibiotic (Cundliffe, 1989).

The biosynthetic genes of many secondary metabolites seem to be organised into several transcription units, of varying complexity. The presence of pathway specific regulatory genes has been identified within many of these clusters (Bibb, 1996). Much of the work concerning the regulation of antibiotic biosynthesis in *S. coelicolor* has been concentrated on the blue-pigmented polyketide antibiotic, actinorhodin (*Act*). The *Act* biosynthetic cluster contains a transcriptional activator, *act*II-ORF4, the transcription of which is activated during the transition to stationary phase (Chater & Bibb, 1997). The activity of *act*II-ORF4 is believed to be regulated by a putative activator protein, which, when over-expressed results in premature *Act* production.

1.6.3 Aminoglycoside production. Despite the clinical importance of aminoglycoside antibiotics, very little is understood of their production and description of microbial resistance mechanisms. Only one system has been studied in detail which is that of streptomycin, produced by *S. griseus*. The mechanism of regulation of gentamicin production is hitherto unknown, however hybridisation experiments of various aminoglycoside pathways in *Streptomyces* have identified biosynthetic cluster similarities (Pipersberg, 1997). The biosynthetic genes of streptomycin are clustered in one genomic region of about 30-40 kbp, containing functionally mixed, subpathway-specific operon regions. The order of these genes is not conserved in other streptomycin producing strains, although the sub-pathway operon regions appear to be conserved, this would suggest that coordinated supply of activated precursors is required (Pipersberg, 1997). Regulation of streptomycin production is believed to be mediated by the DNA-binding protein *strR*, which has been shown to positively regulate an amidinotransferase found within the cluster.

The *strR* gene also contains a TTA codon, which is regulated by the action of the developmentally regulated tRNA gene, *bldA*, with mutants in this gene being deficient in streptomycin production (McCue *et al.*,1992; Pipersberg, 1997).

1.6.4 Physiology of antibiotic production. The production of antibiotics is strongly linked to the regulatory networks present within the cell and are subject to external and inherent physiological programmes (Pipersberg, 1997). Antibiotics are generally produced in a growth-phase-dependant manner (Bibb 1996). The appearance of antibiotics normally occurs after vegetative growth has finished, as indicated by the cessation of DNA replication (Martin & McDanial, 1975). Biomass accretion is known to continue and this is assumed to be a result of the accumulation of storage compounds, such as glycogen (Bibb, 1996). The production of antibiotics has been shown to be confined to the substrate mycelium (Sun *et al.*, 1999) and, in the case of oleandomycin production, synthesis was completed prior to the emergence of aerial hyphae (Mendez *et al.*, 1985). When growth is rapid, usually when the presence of readily utilised, nitrogen, carbon and phosphorus sources are in abundance, and it is believed that these metabolites act as repressors/inhibitors of antibiotic biosythesis (Chater & Bibb, 1996). There are many examples of metabolite interference with antibiotic production, yet the underlying mechanisms are generally not understood. Phosphate has been shown to repress the transcription of the genes responsible for actinorhodin and gentamicin production (Hobbs *et al.*, 1992; Obregon *et al.*, 1994). The negative effect exerted by the type and concentration of carbon source upon antibiotic biosynthesis is well documented in actinomycetes (Hu, *et al.*, 1984), including a negative correlation between gentamicin production and medium glucose concentration (Escalante *et al.*, 1992). Many studies have demonstrated a

relationship between the formation of secondary metabolites and the status of nitrogen within the medium (Aharonowitz, 1980), with the nature and concentration of the nitrogen source being shown to affect timing and magnitude of production in a number of actinomycetes (Hobbs *et al.*, 1990; Lilley *et al.*, 1981; & Shapiro & Vining, 1983). Much of this work pointed to the growth requirement of the organism. No link was made, however, to the actual product yield ($Y_{x/p}$) of antibiotic under the studied conditions. Liao *et al.*, (1995) linked the production of antibiotic to growth rate and indicated that nutrient-limitation during growth allowed secondary metabolite production during the growth phase.

1.6.4 Primary metabolism and antibiotic production

Primary metabolism involves the integration of catabolic and anabolic reactions resulting in an increase in biomass; the production of energy and reducing power with which to synthesise proteins, nucleic acids, lipids, and structural and storage polysaccharides. Secondary metabolism often gives rise to compounds that are not essential to growth of a microorganism, with the synthesis of compounds and enzymes unique to pathways that appear to be of secondary importance to the organism. In the actinomycetes the pathways of primary metabolism supply the precursors of secondary metabolism and, therefore, factors affecting primary metabolism may impact upon secondary metabolism. The fluxes between intermediary carbon and nitrogen metabolism are of primary importance in the understanding of aminoglycoside production, especially the origin of the nitrogenous groups (Pipersberg, 1997). There is a strong correlation between pentose phosphate pathway flux and the formation of neomycin and methylenomycin (Pipersberg, 1997; Obanye *et al.*, 1996). The onset of secondary metabolism has been correlated

with flux through the citric acid cycle and, during chlortetracycline production by *S. aureofaciens*, is subject to significant shift in activity (Hostalek *et al.*, 1969). Enzymatic activity shifts, however, are not universal phenomena, with little differences observed in primary metabolic enzyme activities, between production and non-production phases of anthracycline in *Streptomyces* C5 (Dekleva & Strohl, 1988). Additionally, the preferred nitrogenous donor for streptomycin production is not asparagine, when supplied as the sole nitrogen source, but glutamine, which must be derived from metabolism (Pipersberg, 1997).

1.7.1 Bacterial growth.

The batch growth curve of a non-filamentous microorganism is shown in Figure 6. An initial lag phase is followed by an exponential growth phase, during which the specific growth rate reaches its maximum value. Growth deceleration usually occurs before the organism enters a stationary phase, where there is no net increase in biomass. Thereafter the cell viability and biomass levels drop, mainly due to lysis. It is obvious that the exponential growth phase cannot continue indefinitely, as one or more essential nutrients will become depleted (Wanner & Egli, 1990).
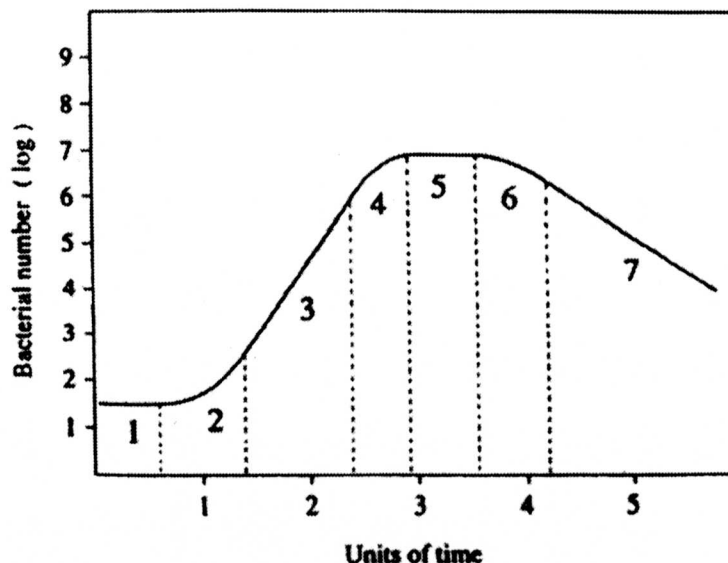
Fig. 6. A typical batch growth curve. 1) Initial stationary phase; 2) lag phase; 3) Logarithmic growth; 4) growth deceleration phase; 5) Stationary phase; 6) accelerated death phase; 7) logarithmic death phase.

The growth of many unicellular bacteria follows the above pattern of batch growth. The growth of filamentous organisms however, does not necessarily follow this model (Stanbury *et al.,* 1995). A consequence of growing mycelial organisms in stirred or shaken culture is the aggregation of hyphae, resulting in a heterogeneous

mixture of mycelial clumps (Prosser & Tough, 1991). These responses are all observations from unicellular bacteria such as *Escherichia, Klebsiella* and *Bacillus*. It has been demonstrated that the nature of the limiting-substrate can affect the cellular response to nutrient limitation, thus affecting the growth curve observed. The exhaustion of carbon usually results in the absence of or a reduced deceleration phase coupled with a relatively constant biomass during stationary phase. The exhaustion of nitrogen from the growth medium normally results in a prolonged deceleration phase, characterised by significant accumulation of polymeric storage compounds (Wanner & Egli, 1990).

1.7.2 Growth under nutrient limitation. Microorganisms are commonly exposed to nutrient-limitation in the natural environment. Such selection pressures have resulted in the evolution of responses to such conditions, allowing an organism to exhibit a competitive advantage over sympatric organisms. A microorganism may respond to nutrient limitation in a number of ways:-

- increase the rate of transport, and/or synthesise an alternative uptake pathway for a substrate

- increase the rate of initial metabolism of the limiting substrate, once it has been accumulated,

- respond to the limitation by the alteration of the cellular composition, by the redirection of metabolic fluxes (Harder & Dijkhuizen, 1983).

The responses to nutrient limitation discussed above can be considered as strategies to enable an organism to survive a changing environment. The ability of actinomycetes to differentiate morphologically and physiologically has long been considered as responses to nutrient limitation or a changing environment (Chater

1993), thus providing the organism with a selective advantage over competitor organisms in the soil environment.

It is apparent from the lack of literature that we do not fully understand how filamentous actinomycetes behave under specific nutrient-limited conditions. The difficulty in growing certain members of this group in reproducible, homogeneous cultures has restricted our progress in this area of actinomycete biology. One such problem affecting culture homogeneity is the formation of mycelial aggregates and pellet formation. Much attention has been focussed on the physical factors affecting the formation of pellets and aggregates during growth such as agitation, shear, viscosity, aeration and pH (Prosser & Tough, 1991). The effect of nutrients upon culture morphology has received little attention in actinomycetes. Published works used undefined media such as those containing casamino acids and nucleic acid bases to produce relatively homogeneous dispersed growth in liquid cultures (Caslavska et al., 1978). The evidence in fungi suggests that media permitting rapid and abundant growth (Prosser & Tough, 1991), and increasing C:N ratios (Byrne & Ward, 1989) result in dispersed growth.

Growth on surfaces in culture vessels is a significant problem in many fungal cultures, adding to the heterogeneity of the growth form (Prosser & Tough, 1991). Such surface growth has also posed similar problems in conducting physiologically meaningful experiments with actinomycetes in batch and continuous culture. Mycelial pellets are inherently nutrient-limited (Stanbury et al., 1995; Prosser & Tough, 1991), with a gradient of nutrient and oxygen supply from the external surface of a pellet to the centre (Huang & Bungay, 1973). These gradients of nutrient supply obviously affect the overall physiological response of the culture to nutrient

limitation, leading to asynchronous cellular responses throughout the pellet. Much attention has been focussed on methods to remove or minimise the effect of pelleting, including the use of disruptive baffles and polymeric compounds such as polyethylene glycol or Junlon (Hobbs *et al.*, 1989; Hodgson, 1982 & 2000).

1.7.3 Microbial growth and metabolism.

The rate at which an organism grows is dependant upon the rate at which the limiting nutrient is transported in to the cell and metabolised within the cell. In both instances, it is not only the amount of each intracellular metabolite, derived from the exogenous nutrient, but also the relative levels of ATP, ADP and AMP that are crucial in determining metabolic balance. The adenosine nucleotides (ATP, ADP, & AMP) exhibit an interdependency that can be expressed by the concept of the adenylate energy charge (EC). The total energy stored within the adenylate system is proportional to the number of anhydride-bound phosphate groups per adenosine moiety, and can be expressed as

$$\text{Energy charge} = \frac{[ATP] + 0.5\,[ADP]}{[ATP] + [ADP] + [AMP]}$$

The half ADP was introduced to ensure a linear scale (0-1). When all the adenylate within a system is present as AMP the EC is 0, and when all is present as ATP the EC is 1 (Atkinson, 1968; 1977). A metabolic process utilising ATP (anabolism) results in a decreasing EC, whereas, a catabolic process regenerating ATP results in an increase in EC (Dawes, 1986). During exponential growth, the EC is maintained at a level around 0.80-0.95. An EC below 0.5 in *Escherichia coli*, corresponds to a zero ATP level, resulting in cell death (Gottschalk, 1986). When grown in minimal

medium with a single carbon source, the growth rate and biomass yield of *E.coli* is dependant upon that carbon source. The work of Bauchop & Elsden (1960), who measured the biomass formed by a range of organisms, with a range of carbon sources under anaerobic conditions indicated the relationship between growth and biomass yield. They concluded that there is a linear relationship between biomass formation and the ATP derived from a carbon source, resulting in the concept of a yield coefficient for microbial growth, Y_{ATP} of 10.5 (g of biomass per mol of ATP generated by substrate catabolism). It has since been shown that Y_{ATP} is not a biological constant, and the changing environments of batch culture affects the yield calculated (Dawes, 1986). Thus, the rates at which a precursor metabolite is supplied to a system and the monomers are derived from it govern the growth rate rather than the flux of ATP (Marr, 1991). The growth rate of an organism has been shown to affect cellular parameters such as cell size, macromolecular composition, secondary metabolite production and morphology (Wanner & Egli, 1990; Shahab *et a.,*1996).

The literature regarding systematic studies of the growth characteristics of *Micromonospora* is scarce, and restricted to batch studies focussed on antibiotic formation in the non-sporulating species *M. purpurea* (Escalante *et al.,* 1992; Obregon *et al.,* 1994).

1.7.4 The chemostat as a tool in microbial physiology.

The chemostat is a continuous culture system where the growth of an organism is controlled by the availability of one or more growth-limiting substrates in the medium. The basic chemostat is a homogeneously mixed, single stage reactor, with an inflow of sterile medium and an outflow of residual medium, biomass and microbial products. The flow of medium in and out of the vessel are equal thus

maintaining a constant culture volume. The addition of fresh medium, fed constantly into the system, results in continuous cell production described as the steady state (Stanbury *et al.,* 1995; Pirt, 1975).

The flow of medium into the vessel is related to the volume of the vessel by the term dilution rate, D, defined as:

$$D = F/V$$

Where F is the flow rate (L. h^{-1}), and V is the volume (L), thus D is expressed as h^{-1}.

The net change in cell concentration over time can be expressed as dx/dt = growth – output

$$\text{Or} \quad dx/dt = \mu x - Dx$$

Under steady state biomass remains constant, thus dx/dt = 0:

Therefore D = μ

The steady state growth of an organism in a chemostat culture therefore allows the physiological responses of that organism to be investigated under nutrient-limiting conditions at a fixed growth rate. This system overcomes the difficulty of studying physiology in batch cultures, which are characterised by transient, constantly changing growth rates offering the potential to study the effect of nutrient-limiting conditions and growth rate as independent variables.

The study of growth and development of filamentous actinomycetes has proved problematic due to factors such as wall growth and culture heterogeneity. Additionally a minority of streptomycetes sporulate in liquid culture (Lee, 1998). The genus *Micromonospora,* despite being relatively poorly studied physiologically offers advantages over other filamentous actinomycetes in respect to their ability to

sporulate readily in liquid culture. In the present study, a defined set of culture conditions providing dispersed growth in specific nutrient-limited conditions was developed. Under these conditions, *M. echinospora* underwent full differentiation, with little or no growth on the surfaces of the culture vessel. The organism was studied in batch and continuous culture enabling the investigation of nutrient-limitation responses.

2. 0 Materials & Methods

2.1 *Organism*

Micromonospora echinospora **ATCC 15837 (NCIMB 12744)** was used throughout this study.

The strain was maintained primarily as a frozen spore stock in 20 % (v/v) glycerol solution at − 20 °C. Cultures were also maintained on slopes of modified Yeast Extract Malt Extract (YEME) Agar (Hopwood *et al.* 1985) at + 4 °C, these being sub-cultured every six months to fresh medium.

2.2 *Media & Culture conditions*

Yeast Extract Malt Extract Medium (YEME)
Modified from Hopwood *et al.*, (1985).

3 g.L^{-1}	Yeast Extract (Oxoid)
3 g.L^{-1}	Malt Extract (Sigma)
5 g.L^{-1}	Bacteriological Peptone (Oxoid)
10 g.L^{-1}	Glucose (Sigma)
20 g.L^{-1}	Agar, select (Sigma), if required for solid medium.

Mannitol Soya Agar (MS)
(Hobbs *et al.*, 1989).

20 g.L^{-1}	D – Mannitol
20 g.L^{-1}	Soya Bean Meal
16 g.L^{-1}	Bacteriological Agar No. 1 (Oxoid).

Minimal Medium (Hopwood *et al.*, 1985).

0.5 g.L^{-1} Asparagine

0.5 g.L^{-1} K_2HPO_4

0.3 g.L^{-1} KOH

0.2 g.L^{-1} $MgSO_4$

0.01 g.L^{-1} $FeSO_4$

Dissolved in 800 ml of distilled H_2O. Once autoclaved 200 ml of 50 g.L^{-1} glucose solution and 2.5 ml of sterile trace salt solution were added.

Trace salts solution contains

2 g.L^{-1} $ZnCl_2$

1 g.L^{-1} $MnCl_2$

0.3 g.L^{-1} H_3BO_3

0.43 g.L^{-1} $CuCl_2$

0.43 g.L^{-1} Na_2MoO_4

8.7 g.L^{-1} $FeCl_3$

0.42 g.L^{-1} NaI

Trace salts solution was sterilized by autoclaving for 15 minutes at 121 °C.

Preparation of Spore Suspension

Sporulating plates (YEME) were flooded with 9 ml of sterile distilled water and allowed to stand for 10 min. Spores were suspended by gentle agitation with a glass spreader, harvested into universal bottles and centrifuged (3000g, 10 min). The pellet was then resuspended in 9 ml of sterile distilled water. The spore suspension was appropriately diluted to give an optical density of 0.7 at 600nm. This suspension gave rise to 5×10^8 colony forming units per ml.

Membrane Cultures

Cultures were grown on cellophane membranes (325 P Courtaulds-Cannings, Bristol, UK). These were prepared by boiling for 15 minutes in distilled water to remove plasticising compounds. The washed cellophane membranes were then layered between moist Whatman No. 1 filter paper (Whatman, Maidstone, UK) in glass petri dishes before sterilising. Cellophane membranes were aseptically overlaid on to 20 ml of solidified YEME agar. Plates were inoculated with 100 μl of spore suspension. Biomass was removed from the membranes with a razor blade and subjected to biomass determinations. Removal of the membrane facilitated the removal of agar plugs using a 5 mm cork borer, which were subjected to the "freeze squeeze" technique of Shahab *et al.,* (1994). Microfuge tubes (0.5 ml) were filled with glass wool, to a depth of 5 mm. Agar plugs are added to the microfuge tube and then frozen. The frozen tubes were defrosted, the bottoms pierced, with a hot dissecting needle, placed within a 1.5ml tube, and centrifuged for 10 minutes at 13, 000rpm. The liquid in the 1.5 ml tube was collected for analysis.

Shake-Flask Culture

Micromonospora echinospora was grown in shake flasks (Ehrlenmeyer flasks) containing 20% of their volume in media (i.e. 50 ml in a 250 ml flask). These were incubated in an orbital shaker (Gallenkamp) at 175 rpm, at 30 °C.

Fermentation (Batch and continuous culture)

Spores (5 x 10^9 colony forming units) were inoculated into a 2 L fermenter (Applikon Ltd., UK) with 1.5 L working-volume. The fermenter was maintained at 30°C by an external jacket heater. Agitation was at 700 rpm, facilitated by a single marine type impeller at the base of the shaft, and double, rushton-type agitators spaced along the impeller shaft. Aeration was provided at 1 v/v per min. The pH was continuously monitored and controlled (pH 7.2) from a Biocontroller ADI 1030 (Applikon Ltd.,UK) by the addition of 1M NaOH.

Batch fermentation was also carried out in 10 L LH 2000 series fermenter (LH Fermentation). All conditions were as specified above.

Continuous culture (chemostat) was performed from initial batch fermentations by the addition of fresh media from 20L reservoirs of sterile filtered medium. The dilution rate was established from the measurement of flow of new media in to the fermenter via a peristaltic pump according to the expression below:

$$D = \frac{f}{v} \qquad\qquad D = \mu$$

where D = dilution rate; f = flow rate (L.h^{-1}); v = fermenter volume (L); μ= growth rate (h^{-1}).

Oxygen uptake.

Oxygen uptake rates were determined, at 30 °C, in a Rank Digital Oxygen System, model 10 (Rank Brothers, Bottisham, Cambridge, UK). Aliquots (100μl) of mycelium or spore suspension were added to 3.9 ml of air saturated buffer (Chappell, 1964), previously equilibrated in the reaction chamber for 5 minutes prior to addition of the sample. The reaction was allowed to proceed for 30 minutes. The reaction was mixed by magnetic agitation.

Numbers of colony forming units (cfu's)

Aliquots (100μl) of spore suspensions were serially diluted into sterile distilled water. Duplicate samples (100μl) of the dilutions were spread onto the surface of a YEME agar plate, using a sterile glass spreader. The plates were incubated for seven days at 30°C, before counting the visible colonies.

Total cell count.

Total spore counts were conducted using a Thoma haemocytometer after suitable dilution in distilled water.

Biomass determination.

Biomass was determined by reduced pressure filtration of culture sample over pre-weighed glass fibre filter papers (Whatman, GF/A), followed by washing with twice the original volume of culture with distilled water. Filters were dried to constant weight in a microwave on defrost setting for 15 minutes.

2.3 *Analytical Procedures*

2.3.1 Ninhydrin Positive Amino Compound Assay

The method of Rosen (1957) was used to quantify ninhydrin-positive amino compounds.

Method

Reagent: Ninhydrin Reagent (3 % w/v Ninhydrin, dissolved in absolute ethanol), was stored at + 4 °C in a dark bottle.

Standard solution of 0 - 0.3 g.L^{-1} of D L – Alanine.

Alanine provides a useful standard amino acid solution due to its 100 % colour reaction with the reagent. Furthermore, with the exception of proline and hydroxyproline all amino acids yield 100 % reactions with ninhydrin, therefore ninhydrin is suitable for the quantification of compounds containing an amino group (Rosen, 1957).

Aliquots (20 μl) of standards or appropriately diluted sample were added to 1 ml of ninhydrin reagent, mixed by vortexing and incubated at 37 °C for 30 minutes. The absorbance at 570 nm was measured for the samples and standards against distilled water blank. All absorbances were determined in a Uvicon 930 dual beam spectrophotometer (Kontron Instruments, USA).

2.3.2 HPLC analysis of asparagine

Culture samples were filtered through 0.2 μm pore nylon filters (Acrodisc, Whatman, Maidstone, UK). The filtrates were loaded on to an ODS C18 HPLC column (Jones Chromatography), with 95/5 % Water/methanol with 0.01 % orthophosphoric acid (v/v) as the mobile phase. Samples were eluted at 0.5 ml per minute, and asparagine detected by absorbance at 210 nm. Asparagine was identified and quantified by comparison with authentic standards (see Fig. 8).
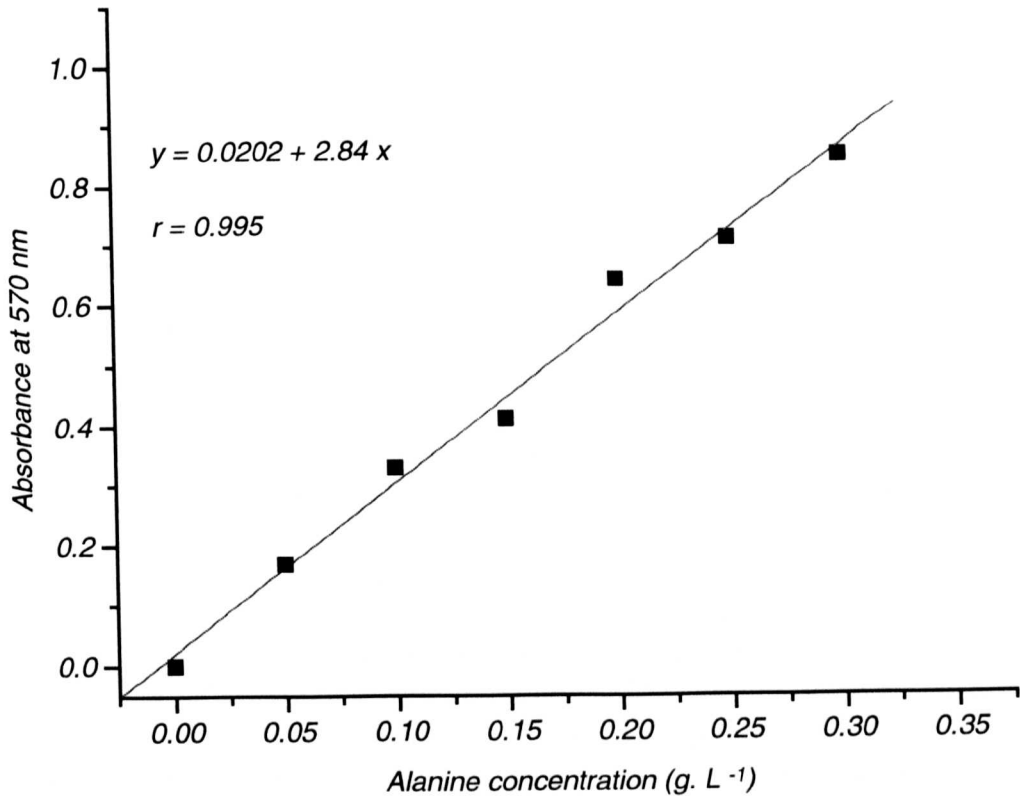
Fig. 7. Standard curve for Ninhydrin-positive amino compounds
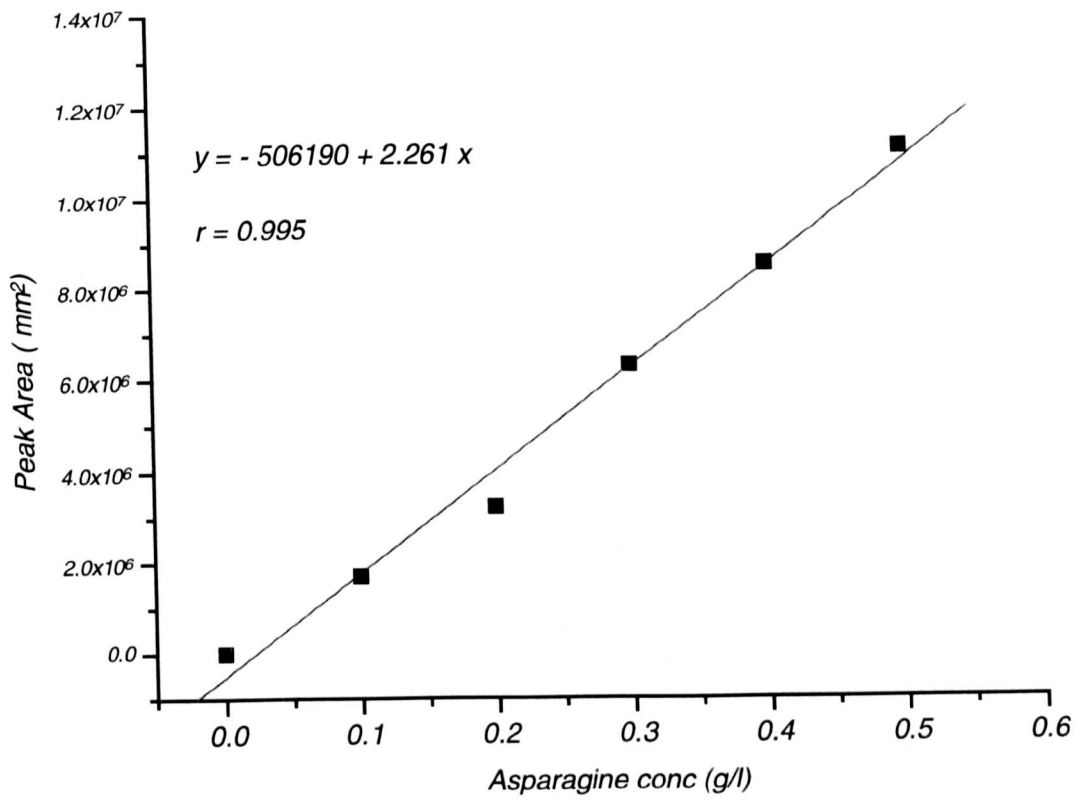
Fig 8. Standard curve for the Asparagine (HPLC method)

2.3.3 Glucose Assay

The concentration of glucose was measured spectrophotometrically using the Boehringer-Mannheim Glucose Oxidase assay (GOD Method). All absorbances were determined in a Uvicon 930 dual beam spectrophotometer (Kontron Instruments, USA). Suitably diluted samples (200 µl) were added to 5 ml of freshly prepared glucose oxidase reagent. The tubes were mixed by vortexing and the reaction allowed to proceed at room temperature for 30 minutes. The absorbances of samples and standard were determined at 600 nm against a distilled water/reagent blank.

2.3.4 Ammonia assay

The presence of ammonia was determined spectrophotometrically based on the method of Fawcett & Scott (1960). The reaction relies on the following,

Ammonium + Phenol + Hypochlorite ⟶ Indophenol blue

<u>Method</u>

Sodium phenate, 25 g of phenol was dissolved in 80 ml of distilled water, to which 7.5 ml of 4 M sodium hydroxide was added and the solution was made up to 100 ml with distilled water. This solution was Stable for 1 month at + 4 °C.

Sodium nitroprusside Stock solution of 0.1 % (w/v) in distilled water. Stable for 1 month at + 4 °C. Diluted 1 in 10 prior to use.

Sodium hypochlorite (Sigma) – Diluted 1 in 5 prior to use.

Ammonium chloride standards 0 – 350 μl of 10 mM stock solution, made up to 1 ml giving a standard range of 0 – 3.5 mM.

Aliquots (20 μl) of suitably diluted sample were added to a 1.5 ml microfuge tube, and in the following order, 200 μl of sodium phenate, 300 μl of sodium nitroprusside and 300 μl of sodium hypochlorite were added. The tubes were mixed, and incubated for 30 minutes at room temperature. Following incubation the absorbance were determined for the samples and standards against distilled water blanks at 630 nm. All absorbances were determined in a Uvicon 930 dual beam spectrophotometer (Kontron Instruments, USA).

Fig. 9. Standard curve for ammonia

2.3.5 Phosphorus Assay

Reagents.

- Ammonium molybdate 50 g.L^{-1} (a small amount of ammonia solution was added to aid solubilisation).

- Copper acetate buffer (pH 4); 25 g of copper sulphate and 46 g of sodium acetate dissolved in 1 L of 2.0 M Acetic acid.

- Reducing agent: 20 g p- methylaminophenol sulphate dissolved in a solution of Na_2SO_3. 7 H_2O (100 g.L^{-1}), made up to 1 L. Store in a dark bottle.

- Standard Phosphorus stock (0-10 μg.ml^{-1}) using K_2HPO_4 with respect to phosphorus.

Method (Plummer, 1987).

Aliquots (200 μl) of sample and standards were added to 600 μl of copper acetate buffer, 100 μl of ammonium molybdate, and 100 μl of reducing agent. Following each addition the tubes were mixed thoroughly and incubated for 10 minutes at room temperature. Following incubation the absorbance was determined for the samples and standards against distilled water blank at 880 nm. All absorbances were determined in a Uvicon 930 dual beam spectrophotometer (Kontron Instruments, USA).
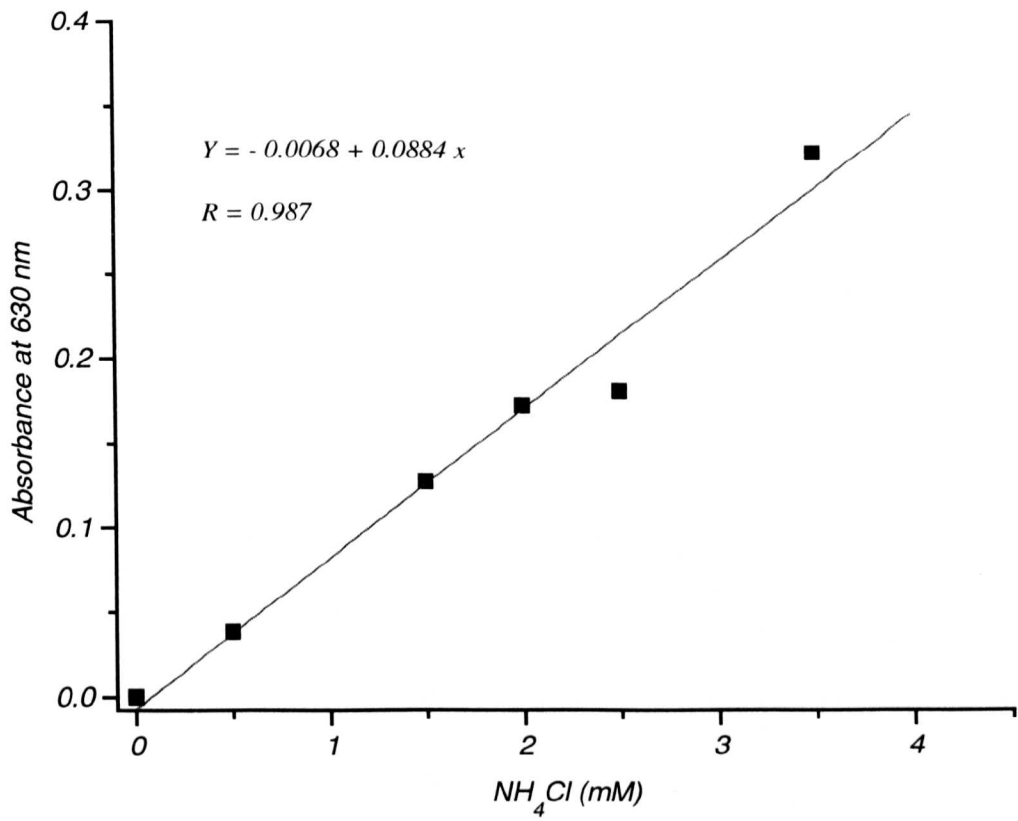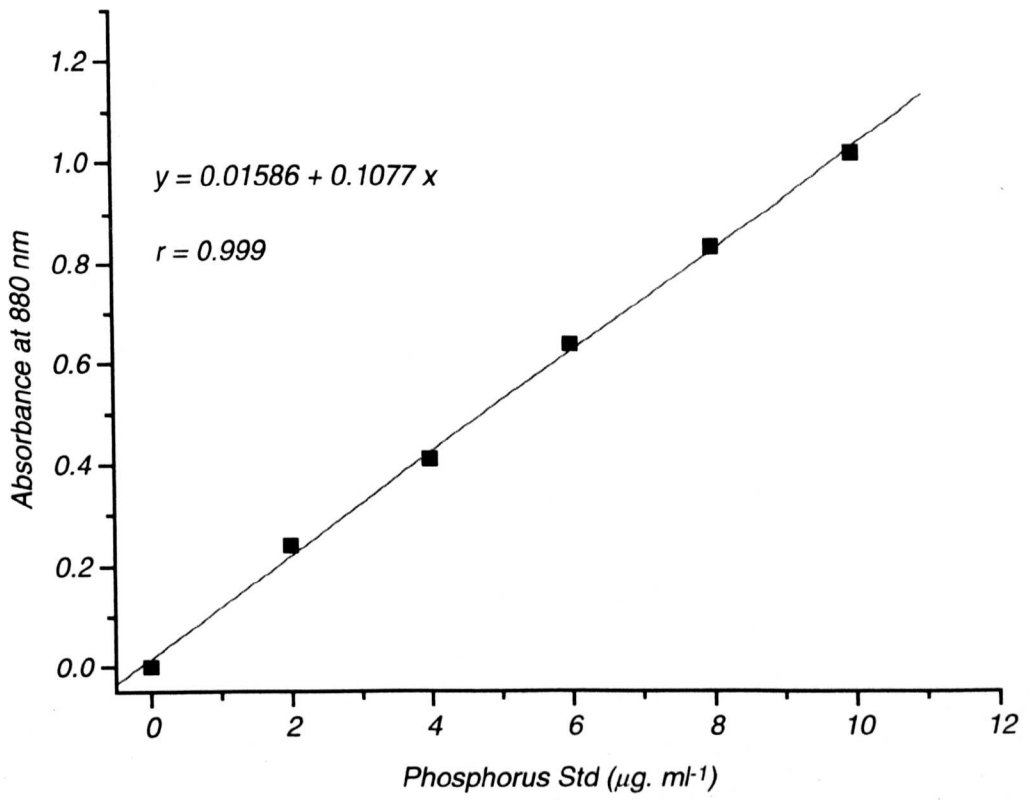
Fig. 10. Standard curve for phosphorus

2.3.6 Protein assay (Bradford, 1976).

The concentration of protein was determined by the method of Bradford (1976) using the Bio-Rad Laboratories (USA) protein assay reagent against a Bovine Serum albumin (Sigma) standard (0-1 mg. ml^{-1}). Aliquots (100 μl) of sample were added to 5 ml of pre-diluted reagent (1:5). These were mixed and allowed to stand for 10 minutes, before a final mixing and measurement spectrophotometrically at 595 nm against distilled water blank. All absorbances were determined in a Uvicon 930 dual beam spectrophotometer (Kontron Instruments, USA).

2.3.7 Gentamicin Bioassay

The gentamicin concentration of the culture medium was measured by plate bioassay, using *Staphylococcus epidermidis* (ATCC 12228) as the test organism according to the method of Arret *et al.*, (1971). The test organism was maintained on slopes of Nutrient agar (Oxoid Ltd, UK) at + 4 °C. Inoculum for bioassay plates was prepared by removing a loop of *S. epidermidis* growth from a slope, and inoculating 50 ml of nutrient broth (Oxoid Ltd, UK) in a 250 ml conical flask. The culture was incubated overnight at 37 °C resulting in an inoculum absorbance of approximately 0.1 OD units (600 nm). Aliquots (100 μl) were spread over the surface of nutrient agar plates (20 ml), and allowed to dry (1 hour) in a laminar flow hood (Baker and sons, USA). Samples and authentic gentamicin sulphate (Sigma) standards were applied to 5mm diameter antibiotic assay discs (Whatman) and placed on the surface of inoculated plates. Zones of inhibition were measured following incubation at 37 °C overnight, with gentamicin concentrations being calculated from a standard curve (Fig.13).
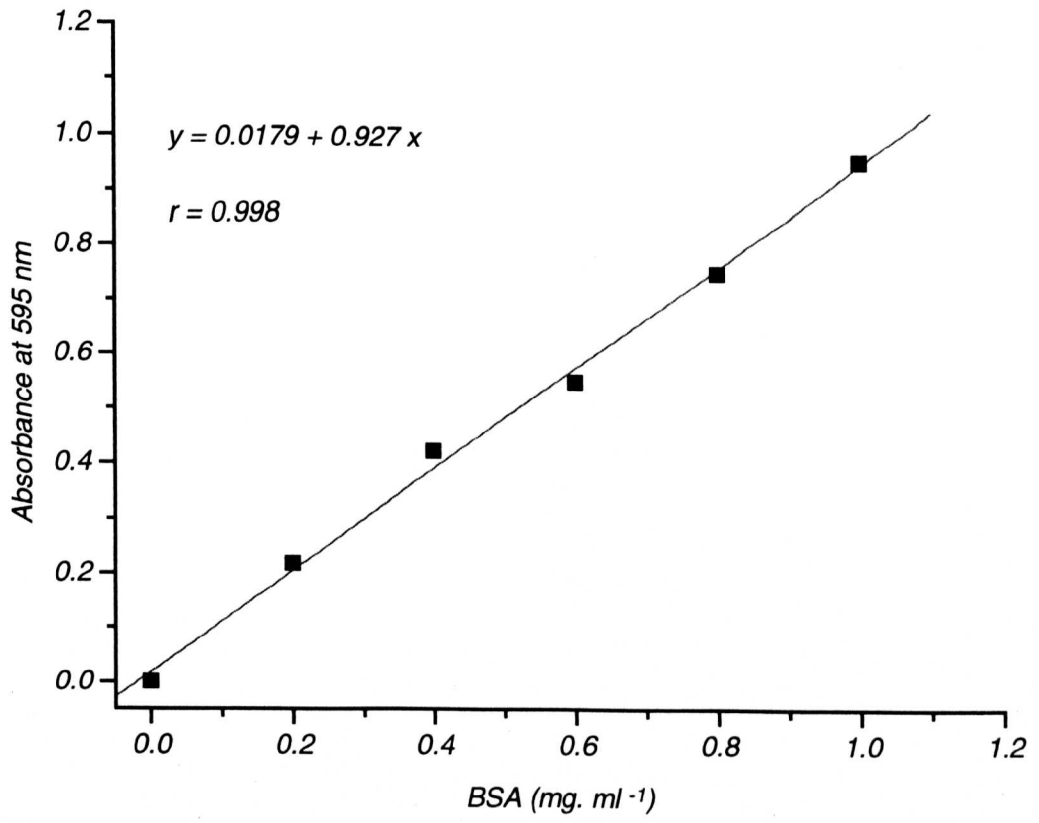
Fig. 11 Standard curve for Protein (Bradford assay).
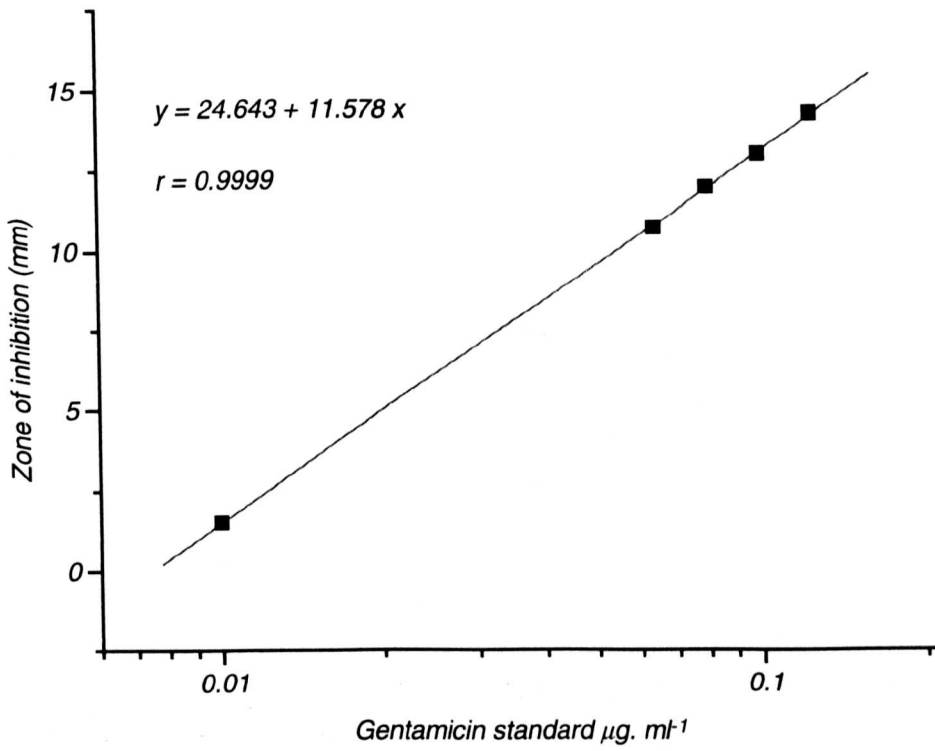
Fig 12. Standard curve for Gentamicin bioassay

2.3.8 *Analysis of Proteolytic activities*

2.3.8.1 Protease assay

Protease activity was assayed using Azocasein (Sigma) as the substrate according to the method of Brock *et al.,* (1982). Azocasein was suspended at a final concentration of 5mg/ml in an appropriate buffer.

Method

Buffers.

Molarity (mM)	Buffer	pH range
300	Sodium acetate – acetic acid	4 - 6
300	Sodium phosphate – sodium hydrogen orthophosphate	6.5 - 7
300	Tris - HCl	7.5 - 9

The buffer (500 μl) was placed in a 1.5 ml microfuge tube, the sample to be assayed (50 μl) was added followed by the 500 μl Azocasein solution (10 mg/ml in distilled water). The tubes were mixed thoroughly, and incubated for 2-18 hours dependant upon the activity of the sample at 30°C. The reaction was stopped by the addition of 70 % (w/v) TCA (final concentration 6 %), mixed and protein was allowed to precipitate for 30 minutes. The tubes were centrifuged at 3000 g for 5 minutes. An aliquote of the supernatant (500μl) was removed and added to a cuvette containing

500µl of 1M sodium hydroxide. One unit of proteolytic activity was defined as the increase in absorbancy at 340 nm of 0.001 per minute (Gibb & Strohl, 1987).

The absorbance of this solution was read against a blank (buffer, azocasein, and distilled water instead of sample) at 450 nm in a Uvicon 930 dual beam spectrophotometer (Kontron Instruments, USA).

2.3.8.2 Protease inhibitor studies.

The classification of proteolytic mechanism was investigated by the susceptibility of a protease to a group of protease inhibitors. To establish the proteolytic class of *Micromonospora echinospora* proteases the method of Dunn (1989) was followed. Protease inhibitors active against each class of proteases were prepared as stocks. Serine protease inhibitors (Tosyl lysyl chloromethyl ketone TLCK active against Trypsin-like serine proteases and Tosyl phenylalanyl chloromethyl ketone TPCK active against Chymotrypsin-like serine proteases) were prepared at 100mM concentrations. TPCK was made up in methanol. Cysteine protease inhibitor, E-64, was prepared at a concentration of 1mM. Metallo-protease inhibitor (Ethylenediaminetetraacetic acid, EDTA) was prepared at 100mM concentrations. Pepstatin was used as an inhibitor of aspartic proteases at a 1mM concentration.

The protease inhibitor assays were set-up according to the method stated above (Section X.x.x) with the following modifications. Aliquots of protease sample (25 μl) were added to 25 μl of either the inhibitor, or buffer (controls), and incubated for 10 minutes at 30°C for 10 minutes. The protease and inhibitor or protease and buffer were added to the reaction mixture and incubated as specified above. The results were calculated as percentage inhibitions of the control samples.

2.3.8.3 Bacitracin-Sepharose Affinity Chromatography of proteases

The peptide antibiotic bacitracin, acts as an inhibitor of all classes of proteases (Makinen, 1972). Immobilised bacitracin has previously been used to purify a number of individual proteases (van Noort *et al.*, 1991). It is believed that bacitracin binds the active site of Proteolytic enzymes by either electrostatic or hydrophobic-interaction, however, the exact mechanism has not been unequivocally established (Makinen, 1972). The method of van Noort *et al.*, (1991) was used to purify the extracellular proteases from the culture filtrates of *Micromonospora echinospora*. Purified proteases were subsequently visualised by SDS-PAGE, and silver staining.

Method

Bacitracin was coupled to the cyanogen bromide-activated Sepharose 4B (Pharmacia, Sweden) according to the manufacturers instructions and the conditions specified by van Noort *et al.*, (1991). Briefly, 0.3 g of bacitracin was suspended in 30 ml of 0.1 M $NaHCO_3$/0.5 M NaCl, pH 8.3, and added to 4 g of previously swelled sepharose 4B, the mixture was incubated at 4 °C overnight. Following incubation, the gel was blocked, and uncoupled bacitracin removed by treatment with 0.1 M Tris-HCl, pH 8.0, for 150 min at room temperature. At this point the bacitracin-sepharose was loaded onto a 10 x 2.5 cm Econo-column (Bio-rad), and washed with 500 ml of 0.1 M $NaHCO_3$/0.5 M NaCl, pH 8.3. Following the wash the column was stored in 0.1 M Tris-HCl/0.5 M NaCl, pH 8.5, containing 0.1 % sodium azide at 4 °C until required.

Affinity chromatography was performed at 4 °C. Before sample application to the column, it was equilibrated with 100 ml of 25 mM NaAc-Hac, pH 4. Samples were applied (10 ml), at a flow rate of 0.1 ml per min. The applied sample was collected following exit from the column, and was reapplied to the column twice more to ensure maximum binding. The bacitracin-sepharose was washed with 10 ml of 25 mM NaAc-Hac, pH 4 to remove all unbound material. Finally the retained proteins were eluted with 10 ml of 25 % (v/v) Iso-propanol/ 1M NaCl in 200 mM Tris-HCl, pH 7.8. Throughout the procedure, fractions (1 ml) were collected at each stage to establish binding, and activity. These fractions were assayed for proteolytic activity and protein content.

After use the column was regenerated with alternating washes of 25 % (v/v) Iso-prpanol/ 0.5 M NaCl in 0.1M Tris-HCl, pH 8; 0.1 mM NaAc-Hac/0.5 M NaCl, pH 4.5, and finally stored in 0.1 M Tris-HCl/0.5 M NaCl, pH 8.5, containing 0.1 % sodium azide at 4 °C until required.

Following chromatography, purified proteases were visualised by sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmeli (1970) with 5 % (w/v) acrylamide stacking gel and 10 % (w/v) acrylamide resolving gel. Gels were silver stained according to the method of Merril *et al.,* (1981).

2.3.9 Cellular composition assays

2.3.9.1 Assay for Glycogen (Based on Brana *et al.* 1982).

Intracellular glycogen content was determined by enzymatic hydrolysis of glycogen to glucose using *Aspergillus niger* amyloglucosidase (2 mg. ml^{-1} ; Sigma). Tubes were set up as indicated below:-

- **Tube 1** (1.5 ml eppendorff)

    50 µl sonicated cells

    50 µl 0.1 M acetate buffer (pH 4.5).

    25 µl of Amyloglucosidase (2 mg/ml).

- **Tube 2** (1.5 ml eppendorff)

    50 µl sonicated cells

    50 µl 0.1 M acetate buffer (pH 4.5).

    25 µl of distilled water.

The reaction tubes were incubated for 2 hrs at 37°C, and centrifuged (4000 x g for 10 minutes), to pellet cellular debris. The glucose content of each tube was measured as previously described. The difference in glucose content of each tube represents glucose derived from hydrolysis of glycogen. A previous set of experiments established that 2 hours incubation was sufficient to hydrolyse the glycogen present using 5 mg. ml^{-1} Oyster glycogen (Sigma).

2.3.9.2 Assay for Trehalose (Based on Brana *et al.*, 1982).

Intracellular trehalose content is determined by enzymatic hydrolysis of cellular trehalose to glucose using trehalase from porcine kidney (Sigma). Tubes were set up as indicated below:-

- **Tube 1** (1.5 ml eppendorff)

    100 µl sonicated cells

    200 µl 0.05 M citrate buffer (pH 5.7).

    100 µl of trehalase

- **Tube 2** (1.5 ml eppendorff)

    100 µl sonicated cells

    200 µl 0.05 M citrate buffer (pH 5.7).

    100 µl of distilled water.

The reaction tubes are incubated for 1 hr at 37°C, and centrifuged (4000 x g for 10 minutes), to pellet cellular debris. The glucose content of each tube was measured as previously stated. The difference in glucose content of each tube represents glucose derived from hydrolysis of cellular trehalose. A previous set of experiments established that a 1 hour incubation is sufficient to hydolyse the trehalose present in a 5 mg. ml^{-1} solution of trehalose from *Saccharomyces cerevisae* (Sigma).
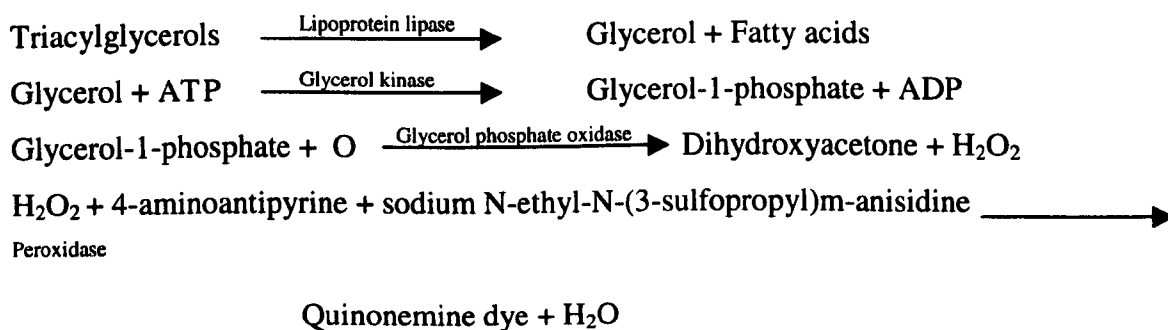
2.3.9.3 Extraction of Total lipids (Folch *et al.*, 1957).

Total lipid content of *M. echinospora* cells was carried out gravimetrically by the method of Folch *et al.*, (1957). Fresh cells were harvested and frozen at –80 °C, and subsequently freeze-dried. The cells were weighed, followed by the addition of 20 x cell mass of 2:1 chloroform/methanol (v/v), and mixed vigorously for 5 minutes. The mixture was incubated at room temperature for 4 hours on a rotary shaker. Following incubation, the mixture was filtered through whatman No. 1 filter paper, to remove cell debris. The filtrate was mixed with 0.2 x its volume of distilled water and following settling, the upper phase was removed (non-lipid material). Any remaining distilled water was recombined with the lower phase by the addition of absolute methanol. The solvent/lipid mixture was then added to pre-weighed glass vials and dried to constant weight.

2.3.9. 4 Triacylglcerol Assay

Triacylglycerols were quantified enzymatically using a commercial kit (Sigma; Triacylglycerides GPO-trinder). This method based on that of McGowen *et al.*, (1983), is as follows:-

Triacylglycerols $\xrightarrow{\text{Lipoprotein lipase}}$ Glycerol + Fatty acids

Glycerol + ATP $\xrightarrow{\text{Glycerol kinase}}$ Glycerol-1-phosphate + ADP

Glycerol-1-phosphate + O $\xrightarrow{\text{Glycerol phosphate oxidase}}$ Dihydroxyacetone + H_2O_2

H_2O_2 + 4-aminoantipyrine + sodium N-ethyl-N-(3-sulfopropyl)m-anisidine $\xrightarrow{\hspace{3cm}}$
Peroxidase

Quinonemine dye + H_2O

Sonicated cells, standard (Glycerol 250 mg/100ml; Sigma), or distilled water blank (10µl) were added to 1 ml of reconstituted triacylglycerides (GPO-trinder) reagent.

was read at 540 nm in a Uvicon 930 dual beam spectrophotometer (Kontron Instruments, USA). The concentration of triacylglycerides was calculated as follows:-

TAG   =   Sample   x   Concentration of standard (250 mg/100ml)
          —————
          Standard

2.3.9.5 hydroxybutyrate assay

Hydroxybutyrate was quantified enzymatically using a commercial kit (Sigma; β-HBA). The method based on the oxidation of β-hydroxybutyrate to acetoacetate in the presence of β-hydroxybutyrate dehydrogenase (β-HBDH) is as follows:-

β-hydroxybutyrate + NAD $\xrightarrow{\text{β-HBDH}}$ Acetoacetate + NADH
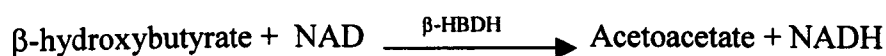
Sonicated cells, standard (D-β-hydroxybutyrate 25 mg/ml; Sigma), or distilled water blank (50μl) was added to 3 ml of β-HBA reagent, and incubated for 15 minutes at 37 °C. Following incubation the absorbances of the samples and standard were determined in a Uvicon 930 dual beam spectrophotometer (Kontron Instruments, USA). The concentration of D-β-polyhydroxybutyrate was calculated as follows,

D-β-polyhydroxybutyrate   =   Sample   X   Conc.   of   standard (50mg/100ml)
                              ——————

                              Standard

2.3.9.6 Extraction of Total Protein (Herbert *et al*, 1971)

Total cellular protein was quantified according to the method of Herbert *et al.*, (1971). Fresh cells of known mass, were harvested directly into universal bottles. The biomass suspended in 2 ml of distilled water, and mixed thoroughly to disperse the cells. To each bottle 1 ml of 3 M NaOH was added, followed by boiling, in a water bath for 5 minutes. The bottles were removed and allowed to cool, followed by the addition of 3 ml of 1M HCl to neutralise the NaOH. The extract was centrifuged (4000g, 10 minutes) to remove cell debris, and the protein concentration determined according to the Bradford method.

2.3.9.7 Extraction of total DNA (Herbert *et al*, 1971).

Fresh cells of known mass harvested were collected in universal bottles. Aliquotes (5 ml) of ice cold $HClO_4$ (0.25 M) was added to each bottle and allowed to stand for 30 min on ice, shaking occasionally. The bottles are centrifuged (3000g, 10 minutes) at +4 °C, and resuspended in 3 ml of ice cold 0.5 M $HClO_4$, and incubated at 70 °C for 15 min with occasional shaking. The bottles are centrifuged again (3000g, 10 minutes) at +4 °C, and the supernatant pooled, followed by resuspension in 3 ml of ice cold 0.5 M $HClO_4$ a further three times. The total DNA content was determined from the combined extracts (12 ml). Preliminary experiments demonstrated that four extractions results in total recovery of the DNA. The total DNA was quantified according to the method of Burton (1956).

2.3.9.8 Determination of DNA (Burton 1956).

Total DNA was quantified by the reaction of DNA, following extraction, with diphenylamine in a mixture of acetic and sulphuric acids, and acetaldehyde.

Reagents.

• Diphenylamine reagent (Sigma): 1.5 g in 100 ml of glacial acetic acid, with 1.5 ml of conc. Sulphuric acid added. On the day of use 0.1 ml of aqueous Acetalaldehyde (16 g/l) for each 20 ml of reagent was added

• DNA Std. (calf thymus, Sigma) dissolved in 5 mM NaOH. Stock of 1 mg/ml, stable at +4°C for 6 months. Stock was diluted with equal volumes of 0.5 m $HClO_4$, and heated for 15 min at 70 °C, prior to dilution and use.

Aliquots (350µl) of sample or standard (0-100 µg.ml^{-1}) are added to 700 µl of Diphenylamine reagent, mixed thoroughly, and incubated at 30 °C overnight (16-24 h). The absorbance of the samples and standards were measured at 600 nm in a Uvicon 930 dual beam spectrophotometer (Kontron Instruments, USA).

2.3.9.9 Extraction of total RNA (Herbert *et al*, 1971).

Fresh cells of known mass were harvested directly into universal bottles. To each bottle 5 ml of ice cold 0.25 M $HClO_4$ is added and allowed to stand for 30 min on ice, shaking occasionally. The bottles were centrifuged (3000g, 10 minutes) at +4 °C, and resuspended in 3 ml of ice cold 0.5 M $HClO_4$, and incubated at 37 °C for 15 min with occasional shaking. The bottles were centrifuged again (3000g, 10 minutes) at +4 °C, and the supernatant pooled, followed by resuspension in 3 ml of ice cold 0.5 M $HClO_4$ a further four times. Total RNA content is determined from the combined extract (15 ml). Preliminary experiments demonstrated that five extractions results in total recovery of the RNA. Total RNA was determined in the extract according to the method of Herbert *et al.*, (1971).

2.3.9.10 Determination of RNA (Herbert *et al.*, 1971).

Total RNA content was determined by the reaction of the purine-ribulose content of RNA with orcinol.

Reagents.

- Ferric Chloride Reagent- 0.9 g Ferric Chloride in 1 L of Conc. HCl.

- Orcinol Solution- 1 g Orcinol per 100 ml of distilled water. Stable for 2 weeks at 0 °C.

- Orcinol Reagent- 1 volume of Orcinol solution is added to 4 Volumes of Ferric Chloride reagent. Prepared fresh.

- RNA Standard. (Torula yeast, Sigma) dissolved in 100 mM NaCl, 10 mM Tris-HCl, 1 mM Magnesium chloride. Stock of 1 mg/ml (stable at -20°C for 1 month) was diluted to 0-250 $\mu g.ml^{-1}$ in $HClO_4$ prior to use.

Method.

Aliquots (1 ml) of sample or standard were added to 3 ml of orcinol reagent in glass stoppered test tubes, and mixed thoroughly. Tubes were boiled for 20 minutes in a water bath. Following heating, 200 μl of boiled reagent, and sample or standard, were added to 800 μl of n-butanol, and mixed thoroughly. The absorbance of the samples and standards were measured at 672 nm in a Uvicon 930 dual beam spectrophotometer (Kontron Instruments, USA).
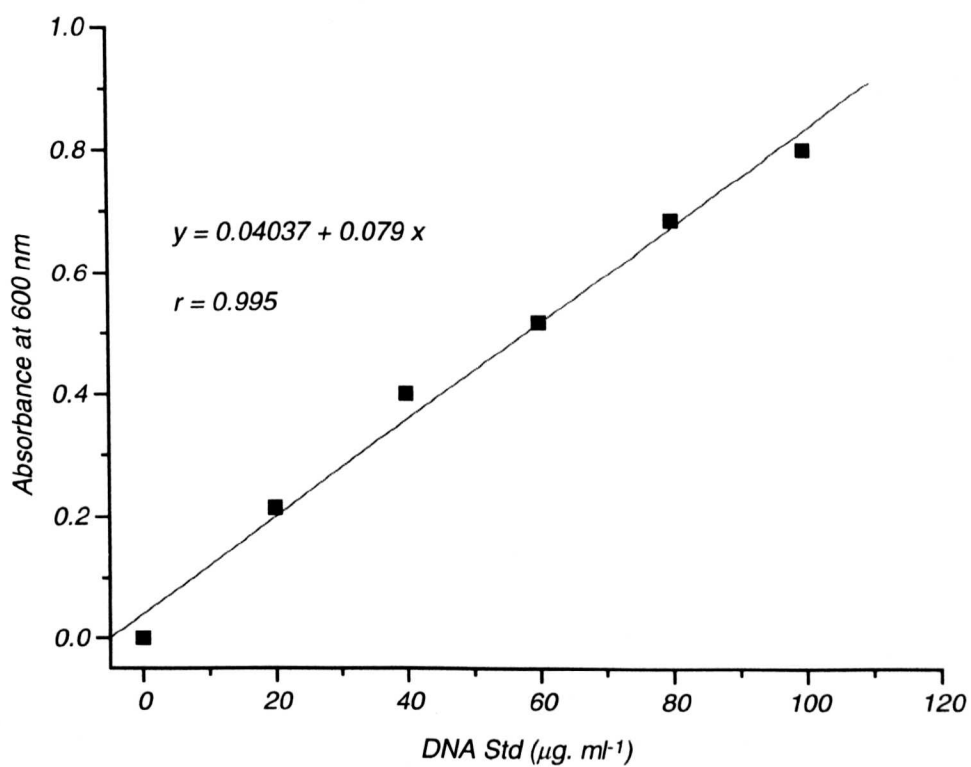
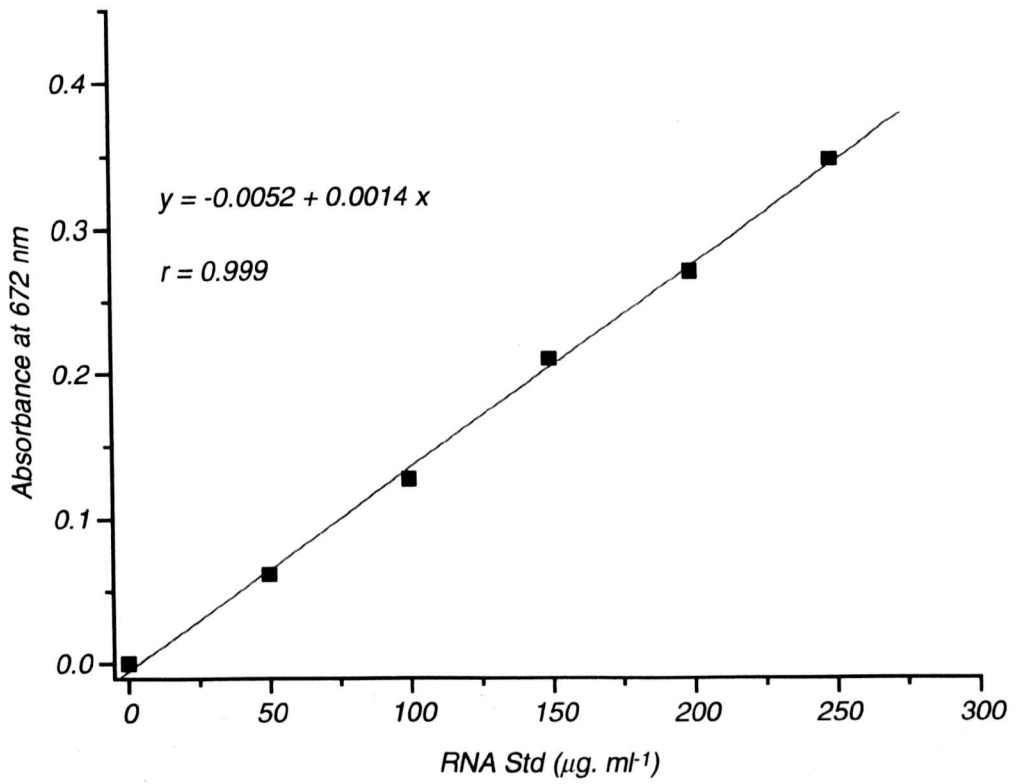Fig 14 . Standard curve for Total DNA (Calf thymus DNA; Sigma).

Fig. 15 Standard curve for total RNA (Torula yeast RNA; Sigma).

2.3.10 Enzyme assays and primary metabolic studies

2.3.10.1 Preparation of crude extracts for enzyme assays

Cells were collected by centrifugation (4000g, 10 minutes). Biomass was washed twice with 0.2 M sodium phosphate buffer (pH 7.2), and resuspended in the buffer. Cells were sonicated (Labplant???) for 3 minutes (6 x 30 s pulses, with cooling on ice). The sonicated cells were centrifuged (4000g, 10 minutes), to pellet cell debris, and the supernatant used for the assays. Protein concentration from the crude extracts was determined by the Bradford (1976) assay.

2.3.10.2 Assay procedures

Reaction mixtures were prepared with all components, except the substrate (all reactions were initiated by the addition of the substrate) and held in a water bath at 30°C. All reactions were performed at 30 °C in a Uvicon 930 dual beam spectrophotometer (Kontron Instruments, USA).. The basal absorbance was determined for five minutes before the substrate was added. The specific activity of the relevant enzyme in Units (IU) was determined using the formula:

$$IU = \frac{\Delta A \text{ in minutes} / \varepsilon}{\text{cuvette protein concentration}}$$

where ε is the extinction coefficient of the relevant metabolite or co-enzyme.

2.3.10.3 Alanine Dehydrogenase Assay (Aharonowitz & Friedrich, 1980).

The main physiological function of alanine dehydrogenase is degradation of L-alanine to produce pyruvate, for use as a carbon and energy source.

Assay principle

$$\text{Pyruvate} + NH_3 + NADH + H^+ \xrightleftharpoons{\quad ADH \quad} \text{alanine} + NAD + H_2O$$
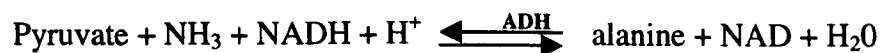
Reagents

Assay mixture. <u>Ammonium Assimilating Activity.</u>

750 µl	100 µM	Tris HCl buffer (pH 8.4)
50 µl	2 µM	Sodium Pyruvate
50 µl	0.25 µM	NADH
50 µl	100 µM	Ammonium Chloride

100 µl of crude cell extract in each assay.

The oxidation of NADH was monitored by the change in absorbance at 340 nm.

Assay mixture. <u>Oxidative deaminating reaction.</u>

750 µl	100 µM	Tris HCl buffer (pH 9.4)
50 µl	0.5 µM	NAD
50µl	50 µM	L-Alanine

100 µl of crude cell extract in each assay.

The reduction of NAD was monitored by the change in absorbance at 340 nm.

2.3.10.4 Alanine:2-Oxoglutarate Aminotransferase Assay (Segal & Matsuzawa, 1970).

Alanine:2-oxoglutarate aminotransferase catalyses the deamination alanine to form pyruvate and glutamate. The reaction is reversible.

Assay principle

Alanine:2-oxoglutarate aminotransferase catalyses the transfer of the amino group of alanine to 2-oxoglutarate to produce pyruvate and glutamate. The pyruvate is reduced to lactate in the presence of lactate dehydrogenase, with the simultaneous oxidation of NADH.

Alanine + 2-oxoglutarate $\rightleftharpoons$ pyruvate + glutamate

Reagents

4.1 mM	a-Ketoglutarate -	0.05 ml
17 mM	DL-Alanine -	0.2 ml
5 *units/3 ml*	Lactic Dehydrogenase	0.1 ml
~0.17 mM	NADH	0.1 ml
0.1 M	Sodium phosphate buffer (pH 7.2)	2.55 ml

100 µl of crude cell extract in each assay.

The oxidation of NADH is monitored by a change in absorbance at 340 nm.

2.3.10.5 Glutamate Dehydrogenase Assay (Meers & Tempest, 1970)

The main physiological role of this enzyme has been assumed to be biosynthetic but, due to the high K_m for ammonia of these enzymes, it appears unlikely that they function efficiently in ammonia assimilation, except when the environmental ammonia concentration is high (Brown *et al., 1974*).

Reagents

50 µl	2-oxoglutarate	5 mM	}
50 µl	NADPH	0.25 mM	}
50 µl	NH$_4$Cl	40mM	} Final Conc.
800 µl	Tris Buffer (pH 7.6)	50 mM	}

100 µl of crude cell extract in each assay.

The oxidation of NADPH is monitored by a change in absorbance at 340 nm.

2.3.10.6 Glutamate Synthase Assay (Meers & Tempest, 1970)

Glutamate synthase catalyses the reductive transfer of the Glutamine amide nitrogen to 2-oxoglutarate, forming two molecules of Glutamate in the presence of $NADPH_2$. The reaction is irreversible, but is inhibited by some metal ions and Glutamate.

Reagents

| 50 μl	2-oxoglutarate	5 mM	}
50 μl	NADPH	0.25 mM	}
50 μl	Glutamine	5mM	} Final Conc.
800 μl	Tris Buffer (pH 7.6)	50 mM	}

100 μl of crude cell extract in each assay.

The oxidation of NADPH is monitored by a change in absorbance at 340 nm.

2.3.10.7 Glutamine Synthetase (GS) Assay (Bender *et al.* 1977)

Glutamine synthetase is the key enzyme in the regulation of nitrogen metabolism and nitrogen assimilation, catalysing the incorporation of ammonium ion in to the glutamate molecule, to form Glutamine.

Assay principle

$$\text{Glutamate} + NH_4^+ + ATP \xrightleftharpoons{\text{GS}} \text{glutamine} + ADP + P_i + H^+$$
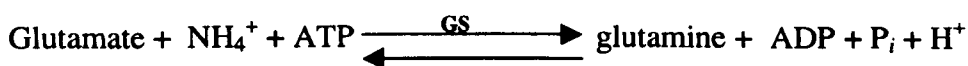
Reagents

The γGT assay is used to measure the amount of, both adenylated and unadenylated amount of Glutamine synthetase present, i.e. total amount. Prepare the following fresh daily, added in the following order.

9.03 ml	Water
2.25 ml	1.0 M Imidazole- hydrochloride, pH 7.15
0.37 ml	0.80 M Hydroxylamine hydrochloride
0.045 ml	0.10 M $MnCl_2$
1.5 ml	0.28 M Potassium arsenate, pH 7.15
0.15 ml	40 mM Sodium ADP, pH 7

Store at 4 °C.

Add the sample (0.05 ml) 0.40 ml of the assay mixture. Equilibrate assay tubes at 30°C for 5 minutes. Initiate the reaction by the addition of 0.05 ml of 0.20 M L-Glutamine. The reaction is terminated by the addition of 1.0 ml of stop mix (55 g $FeCl_3$. $6H_2O$, 20 g trichloroaceitic acid, and 21 ml Conc. HCl per litre.). Following the addition of stop mixture, the tubes are centrifuged (4000g, 5 minutes), and the absorbance is read at 540 nm. One unit of GS activity is defined as the amount of enzyme producing 1 μmol of glutamyl hydroxamate per min. One μmol of glutamyl hydroxamate gives 0.532 units of absorbance at 540 nm. Absorbance was measured in a Uvicon 930 dual beam spectrophotometer (Kontron Instruments, USA).

2.3.10.8 Glucose-6-Phosphate Dehydrogenase Assay (Uijita & Kimura, 1982).

Glucose-6-phosphate dehydrogenase is generally considered the control point for entry to the pentose phosphate pathway (Dawes, 1986).

Reagents

NAD Reaction:

D-Glucose-6-phosphate +NAD $\xrightarrow{\text{G-6-PDH}}$ 6-phosphoglucono-δ-lactone +NADH + H
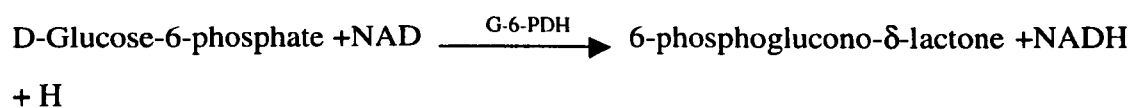
0.8 ml	0.1 M MOPS-KOH buffer (pH6.6) with 5mM MgCl$_2$
50μl	15mM NAD
50μl	15mM G-6-Phosphate
100μl	Protein sample

The reduction of NAD was monitored by the change in absorbance at 340 nm.

NADP Reaction:

D-Glucose-6-phosphate +NADP $\xrightarrow{\text{G-6-PDH}}$ 6-phosphoglucono-δ-lactone + NADPH + H
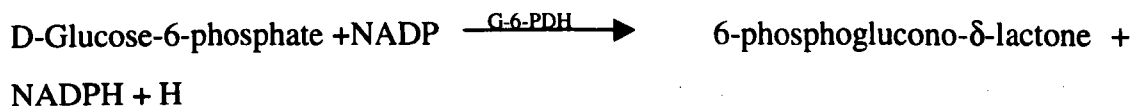
0.8 ml	0.1 M Tris-HCl buffer (pH 7.5) with 5mM MgCl$_2$
50μl	5mM NADP
50μl	5 mM G-6-Phosphate
100μl	Protein sample

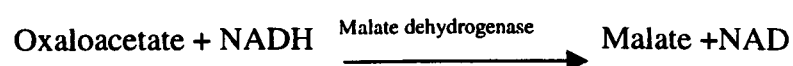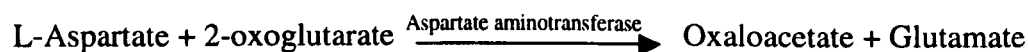The reduction of NADP was monitored by the change in absorbance at 340 nm.

2.3.10.9 Glutamate: Oxaloacetate aminotransferase (Sigma Kit)

Aspartate aminotransferase activity was assayed according to the method of Karmen (1955) using a commercially available kit (GOT-optimised aspartate aminotransferase kit; Sigma).

Assay priciple

$$\text{L-Aspartate + 2-oxoglutarate} \xrightarrow{\text{Aspartate aminotransferase}} \text{Oxaloacetate + Glutamate}$$

$$\text{Oxaloacetate + NADH} \xrightarrow{\text{Malate dehydrogenase}} \text{Malate +NAD}$$

Aspartate aminotransferase catalyses the transfer of the amino group of aspartate to 2-oxoglutarate to produce oxaloacetate and glutamate. The oxaloacetate is reduced to malate in the presence of malate dehydrogenase, with the simultaneous oxidation of NADH.

The assay was performed according to the manufacturers instructions.

2.5 ml GOT Assay reagent A

0.5 ml Sample

0.25 ml GOT Assay reagent B (to initiate reaction)

The oxidation of NADH is monitored by a change in absorbance at 340 nm.

2.3.10.10 Phosphofructokinase Assay (Ling *et al.,* 1966).

Phosphofructokinase is an essential enzyme in the Embden-Meyerhof glycolytic pathway. Activity of this enzyme is a key marker for glycolysis (Dawes, 1986).

Assay principle

$$\text{Fructose-6-phosphate} + \text{ATP} \xrightarrow{\text{PFK}} \text{Fructose 1,6-biphosphate} + \text{ADP}$$
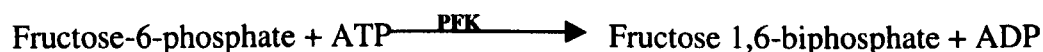
This assay is linked to the activity of aldolase, triose phosphate isomerase and α-glycerophosphate dehydrogenase.

Reagents

|		Final concentration
0.5 ml	0.2 M Tris- HCl, pH 8	33 mM
0.3 ml	0.02 M ATP	2 mM
0.075 ml	0.2 M MgSO$_4$	5 mM
0.75 ml	0.20 M KCl	0.05 M
0.3 ml	0.02 M Fructose-6-P	2 mM
0.2 ml	2.4 mM NADH	0.16 mM
0.03 ml	0.1 M Dithiothreitol	1 mM
0.2 ml	Aux. Enzymes	
	0.25 ml (10 mg/ml) Aldolase	
0.05 ml	Triose phosphate isomerase and α-glycerophosphate dehydrogenase (Sigma)(10 mg/ml) dissolved in 4.7 ml of 0.01 MTris-HCl (pH 8), containing 10 mg/ml Bovine serum albumin.	
0.45 ml	dH$_2$O	
0.2 ml	Crude cell extract.	

The rate of reduction of NAD during this reaction, catalysed by α-glycerophosphate dehydrogenase, was measured at 340 nm.

2.3.10.11 Citrate synthase (Srere, 1969)

The tricarboxylic acid cycle is principally controlled at the point of entry of Acetyl-CoA, and its conversion to citrate, catalysed by citrate synthase.

Assay principle

$$\text{Acetyl-CoA} + \text{oxaloacetate} + H_2O \xrightarrow{\ \ CS\ \ } \text{Citrate} + \text{CoASH} + H^+$$
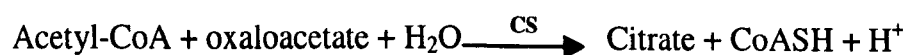
Reagents

0.1 ml	1 mM DTNB (3.9 mg in 10 ml 1M Tris HCL, pH 8.1)
0.03 ml	10 mM Acetyl CoA (10 mg/ml)
0.77 ml	dH$_2$O
0.05 ml	Crude cell extract

The reaction was started with the addition of 0.05 ml of 10 mM Oxaloacetate (1.32 mg in 1 ml Tris HCl) The change in absorbance was measured at 412 nm.

2.3.11 Extraction and assay of nucleotides

The extraction and quantification of the intracellular nucleotide pools were performed according to the method of Jones *et al.*, (1996). Samples of culture medium (200-1000 ml) were removed from the fermenter, and centrifuged (10 000 g, 10 minutes at 4 °C). The biomass was resuspended in ice cold 1 M Formic acid and incubated for 1 hour at 4 °C, with shaking. The biomass following extraction was pelleted by centrifugation (10 000 g, 10 minutes at 4 °C), and the supernantant filtered through 0.3 µm nitrocellulose filters (Gelman Ltd., UK). The filtrates were freeze dried, and resuspended in 50 µl of deionised distilled water. The intracellular concentrations of nucleotides were assayed by HPLC on a Partisil 10 SAX column (Whatman) with a gradient of 7 mM K_2HPO_4, pH 4, to 0.5 M K_2HPO_4/ 0.5 M Na_2SO_4, pH 5.4 at a flow rate of 1.5 ml min^{-1}. Detection of the nucleotides was at 254 nm, and concentrations of nucleotides were expressed relative to the dry cell weight of the culture.

2.3.12 Uptake studies

The uptake of nutrients by whole cells was studied using ^{14}C-Glucose, ^{3}H-2-Deoxyglucose, and ^{14}C-Glutamate (Sigma). Mycelium (20 ml) was removed from steady state chemostats (D = 0.046h^{-1}), and washed twice in MM containing no carbon or nitrogen (each centrifugation step 4000 g, 5 minutes). Biomass was resuspended in 2 ml of MM and placed on a heated stirrer at 30 °C, and agitated by magnetic stirrer. The radiolabelled nutrient was added at the required concentration (See results), and samples removed as required. Samples (250 µl) were removed and immediately quenched in ice cold, 1 M solution of unlabelled nutrient of being studied. Samples were filtered through 0.2 µm nitrocellulose filter discs under reduced pressure and washed with 10 ml of MM. Discs were removed from the filter apparatus and added to scintillation cocktail.

2.3.13 Morphology and microscopy

2.3.13.1 Phase Contrast Microscopy.

The morphology of the cultures was examined by phase contrast microscopy using a Leitz Laborlux 12 (Leitz Wetzlar, GmBH, Germany), at x 40 magnification (Phaco 2 lens).

2.3.13.2 Scanning Electron Microscopy.

Samples from liquid culture (1 ml) were centrifuged (4000g, 2 minutes), and resuspended in 5 % gluteraldehyde (v/v), for at least 24 hours to fix the biomass. The samples were washed three times in distilled water, by centrifugation at 4000g for 1 minute. Following the final wash the pellet was resuspended in 0.5 ml of distilled water, and allowed to settle by gravity (1-2 hours). Aliquotes (100 μl) of the resuspended sample was placed on to a round glass cover slip (10 mm), being left to air dry, with occasional teasing of specimen using a mounted needle during drying to separate the cell aggregates. The coverslip was mounted on to an aluminium stub using quick drying silver paint. The samples were splutter-coated with gold (Polaron Equipment Ltd. coating equipment). The coating chamber was flushed with argon, the high tension applied and argon again leaked into the chamber until 20 mA was achieved on the meter, high tension was then switched off and the timer set to 5 minutes, and coating allowed to proceed. Samples were examined using a Jeol JSM-840 scanning electron microscope (Jeol Instruments, Japan). The working distance was set to 8 mm, and the accelerating voltage to between 10 and 25 kV, depending upon the stability and cleanliness of the sample.

2.3.14 Molecular biology

2.3.14.1 Kirby mix procedure for the isolation of genomic DNA (Kieser *et al.*, 2000).

DNA extraction for PCR procedures was performed according to the method of Kieser *et al.*,(2000) using the modified Kirby procedure.

Reagents

TE25S buffer:	25mM Tris-HCl, pH 8, 25 mM EDTA pH 8, 0.3 M sucrose
Lysozyme (Sigma)	60 mg ml^{-1}
Phenol	500 g phenol (AnalaR), 0.5 g 8-hydroxyquinoline (AnalaR) saturated with 50 mM Tris-HCl, pH 8
Phenol/ Chloroform/ Isoamyl alchohol	50 ml equilibrated phenol, 50 ml chloroform, 1ml Isoamyl alcohol
2 x Kirby mix	2 g SDS, 12 g sodium 4-aminosalicilate, 5 ml 2M Tris-HCl pH 8, 6 ml equilibrated phenol pH 8, made up to 100 ml with distilled water
3M unbuffered sodium acetate	408 g sodium acetate made up to 1 L with distilled water.
RNase A	4 mg ml^{-1}
TE buffer	10mM Tris-HCl, pH 8 containing 1mM EDTA

Procedure

1. Centrifuge mycelium, 50 ml of 72 h YEME culture (4000 g, 10 minutes).

2. Resuspend mycelium in 3 ml of TE25S buffer, add 100 µl of lysozyme solution, and incubate for 10 minutes at 37°C.

3. Add 4 ml of 2 x Kirby mix, and agitate for 1 minute on a vortex mixer.

4. Add 8 ml of Phenol/Chloroform/ Isoamyl alchohol and agitate for 15 seconds as in 3.

5. Transfer upper (aqueous) phase to fresh tube, containing 3 ml of Phenol/Chloroform/ Isoamyl alchohol and 600 µl of 3M sodium acetate, agitate as in 3.

6. Add 0.6 volume of isopropanol, mix and spool DNA on to a sealed Pasteur pipette, wash DNA in 5 ml 70 % ethanol.

7. Redissolve the DNA pellet in 5 ml of TE buffer, add 50 µl of RNase A and incubate for 30 minutes at 37°C. Precipitate DNA as in step 5.

8. Air dry pellet, and redissolve in 0.5-1 ml TE.

2.3.14.2 Quantification of DNA

The amount of DNA harvested was quantified spectrophotometrically using a A_{260}. The quality of the DNA was checked using $A_{260/280}$ ratio.

Aliquots (25 µl) of extracted DNA were diluted in 475 µl of TE buffer (1 in 20 dilution).

1 A_{260} unit = 50 µg ml^{-1}

Therefore $A_{260} = x$ $= x$ x 50 x dilution factor (20)

$= $ DNA (µg ml^{-1})

$A_{260/280}$ ratio $= 1.8$ pure DNA

$= >2.0$ RNA contamination of sample

$= <1.8$ Phenol contamination of sample

2.3.14.3 RNA Extraction

The extraction of RNA for RT-PCR was performed using the Qiagen RNeasy mini kit for total RNA preparations. This method is based upon selective binding properties of a silica-gel-based membrane. Cells were first treated with Lysozyme (3 mg ml^{-1}) for 10 minutes, followed by lysis using a buffer supplied by the manufacturer based upon guanidine isothiocyanate and ethanol. The lysed cell sample wass applied to an RNeasy spin column. Total RNA binds to the membrane, contaminants are washed away by centrifugation, and RNA is eluted using distilled water (RNase free).

2.3.14.4 Quantification of RNA

The amount of RNA harvested was quantified spectrophotometrically using a A_{260}. The quality of the RNA was checked using $A_{260/280}$ ratio.

Aliquots (5 µl) of extracted RNA were diluted in 495 µl of distilled water (1 in 100 dilution).

1 A_{260} unit = 40 µg ml^{-1}

Therefore $A_{260} = x$ = x x 40 x dilution factor (100)

                           = RNA (µg ml^{-1})

$A_{260/280}$ ratio   = $\geq$ 2.0 pure RNA

                    = < 1.9 Protein or phenol contamination of sample

2.3.15 Polymerase chain reaction (PCR) of *Micromonospora echinospora* genes

2.3.15.1 Primers

PCR primers used to amplify *M. echinospora* were previously published in the literature and used to clone specific genes in *S. coelicolor*.

bldA (Leskiw & Mah, 1995). Approximate 297 bp product

Forward - 5'-CAT GGA TCC ACC CGG TAA CTG ATG CAC C-3'

Reverse – 5'-GCC GCT GAG TCG CAA CCA-3'

bldD (Kelemen *et al.*,2001)

Forward - 5'-ACC TTA AAG GAC GTC GGA TAT GCG-3'

Reverse – 5'-CGA GCT GTT TGG CGT ATT CGC TGG-3'

whiB (Soliveri *et al.*, 2000). Approximate 309 bp product

Forward - 5'-CGT CCT TCG TCC ACT CCC C -3'

Reverse – 5'-GTG CCC AGT GGA TCG CAG -3'

relA (Chakraburtty *et al.*, 1996) Approximate 400 bp product

Forward-5'-CAT GGA TCC AAC GG(CG) TAC GAG (AT)(CG)(CG) (AC)T(CG) CAC AC-3'

Reverse – 5'-CAT GGA TCC GTG TG(CG) A(CT)(CG) GCG TA(CG) GCG AAG TC -3'

glgB (Bruton *et al.*, 1997) Approximate 450 bp product

Forward - 5'-ATC GGC GCT ATC CTG GAC TGG CTC CCG GGC CAC TTC CCG AAG GAC -3'

Reverse – 5'-CAT CCA GCC CAT GTT CCA CTT GTA GAA GCC CAG GCC GCC-3'

glgC (Martin *et al.*, 1997) Approximate 500 bp product

Forward - 5'-CCG AAT TC(ATGC) GC(ATGC) GG(CT) TT(CT) TC -3'

Reverse – 5'-CTA AGC TT(CT) T(TCG)G C(ATGC)G G(ATGC)G -3'

Primers were custom synthesised (Life technologies Ltd., UK) to the manufacturers standard purity. Primers were reconstituted to a concentration of 100 pmol/µl in 10mM Tris-HCl, pH 8 containing 1mM EDTA, and frozen (-70°C) in 10 µl aliquots.

PCR reaction mixtures were set up as detailed below (50 µl final volume):-

45 µl   PCR Master mix (ABgene 1.1x Reddy mix)

(10 mM Tris-HCl pH 8.3, 1.5 mM MgCl$_2$, 50 mM KCl, 200 µM each of dATP, dCTP, dGTP and dTTP).

0.5 µl	Forward primer (50 pmol final concentration)
0.5 µl	Reverse primer (50 pmol final concentration)
3 µl	Sterile distilled water
1 µl	Template DNA (50 ng per reaction)

Cycle conditions were as follows:-

Initial denaturation: 95 °C for 5 minutes

PCR - 30 cycles of: denaturation at 95 °C for 30 seconds

Annealing at T_m – 5 °C for 30 seconds

Extension at 72°C for 1 minute

Final extension: 72°C for 10 minutes

PCR reactions were carried out in Techne Touchgene thermocycler (Techne (Cambridge)Ltd., UK).

2.3.16 Reverse transcription PCR (RT-PCR)

RT-PCR was performed using Qiagen one step RT-PCR Kit (Qiagen Ltd., UK) according to the manufactures instructions. Reaction were prepared as follows (50 µl Final volume):-

10 µl	5 x Qiagen One step RT-PCR buffer
2 µl	dNTP mix (containing 10 mM of each dNTP)
10 µl	5 x Qiagen Q-solution
1 µl	Forward primer (100 pmol final concentration)
1 µl	Reverse primer (100 pmol final concentration)

2 µl   Qiagen One step RT-PCR enzyme mix (containing Omniscript and Sensiscript reverse transcriptases and DNA polymerase).

22 µl   RNase-free water

2 µl   Template RNA (50 ng per reaction)

Cycle conditions were as follows:-

Reverse transcription: 50°C for 30 minutes

Initial denaturation and DNA polymerase activation: 95 °C for 15 minutes

PCR - 30 cycles of: denaturation at 95 °C for 30 seconds

Annealing at $T_m - 5$ °C for 30 seconds

Extension at 72°C for 1 minute

Final extension: 72°C for 10 minutes

PCR reactions were carried out in Techne Touchgene thermocycler (Techne (Cambridge)Ltd., UK).

2.3.16.1 Agarose gel electrophoresis

Polymerase chain reaction products were visualised by ethidium bromide stained 2 % (w/v) agarose gels in Tris acetate EDTA (TAE) buffer. TAE buffer was made at 10 X concentration (48.4 g. l^{-1} Tris base (0.4 M), 16.4 g. l^{-1} Sodium acetate (0.12 M), 8.32 g. l^{-1} Na EDTA (0.022 M), adjusted to pH 7.7 with glacial acetic acid.) and diluted to 1 X concentration as required (Sambrook *et al.*, 1989). The agarose was melted in the TAE buffer and 2 μl of 10 mg ml^{-1} ethidium bromide was added before the gel was cast. Routinely PCR products were loaded in 5 μl volumes containing 1μl of loading buffer (6 X; Promega Ltd, UK).

2.3.16.2 Sequencing of PCR products

The identity of the PCR products was assessed by sequencing, following purification using the Qiaquick gel extraction kit (Qiagen Ltd., UK). Sequencing was performed by Dr Margaret Hughes of the Liverpool School of Tropical Medicine, using the ABI Prism 377, automated DNA sequencer.

3.0 Results

3.1 Growth of *Micromonospora echinospora*

The cultivation of *Micromonospora* has previously proved to be problematic with strains adapting badly to laboratory culture conditions (Luedemann, 1970; 1971). The initial experiments conducted were aimed at establishing appropriate culture conditions that provide reproducible growth. Firstly studies were conducted in an complex, relatively undefined medium, using both surface and submerged growth conditions. These were then extended to establish chemically defined minimal media protocols to facilitate meaningful physiological studies. The characterisation of growth in rich liquid and on rich solid medium provided transferable knowledge for establishment of defined growth conditions to study physiological and morphological differentiation.

3.1.2 Membrane culture growth dynamics

In its environmental niche *M. echinospora* is associated with solid substrata. Studies were conducted using cellophane membrane grown cultures (Semorati, 1969) to examine how this organism grows in a spatially constrained surface culture.

3.1.2.1 Morphological development of cultures.

M. echinospora produced confluent growth on the surface of the cellophane membranes. The initial 120 hours was marked by biomass accumulation resulting in a distinct orange film of branched mycelium. From 120 hours onwards the culture darkened, coinciding with the appearance of spores (Plate 1).

3.1.2.2 Accumulation of storage compounds.

Actinomycetes have also been shown to accumulate storage compounds in response to nutrient imbalance. The synthesis of glycogen and trehalose has previously been shown to be regulated by nitrogen availability (Brana *et al.* 1986) with initiation of synthesis coinciding with nitrogen depletion. Lipids have also been shown to accumulate in actinomycetes (Karandikar 1997; Ranade and Vining 1992; Packter *et al.* 1985) where their augmentation has been shown to be associated with nitrogen deprivation (Olukoshi and Packter 1994). The exact roles of these carbonaceous reserves have yet to be clearly elucidated, although there have been reports linking them with morphological differentiation (Chater, 1994). There have been no previous studies of macromolecule accumulation in *Micromonospora*.

Glycogen, trehalose, total lipids, triacylglycerols and D-β-hydroxybutyrate contents of *M. echinospora* cultures were measured over a 240-hour period. A temporal profile relating the accumulation of these storage compounds with nutrient utilisation and culture morphology is shown in Figure 16. The major biomass accretion phase occurred over the period 50-160 hours.
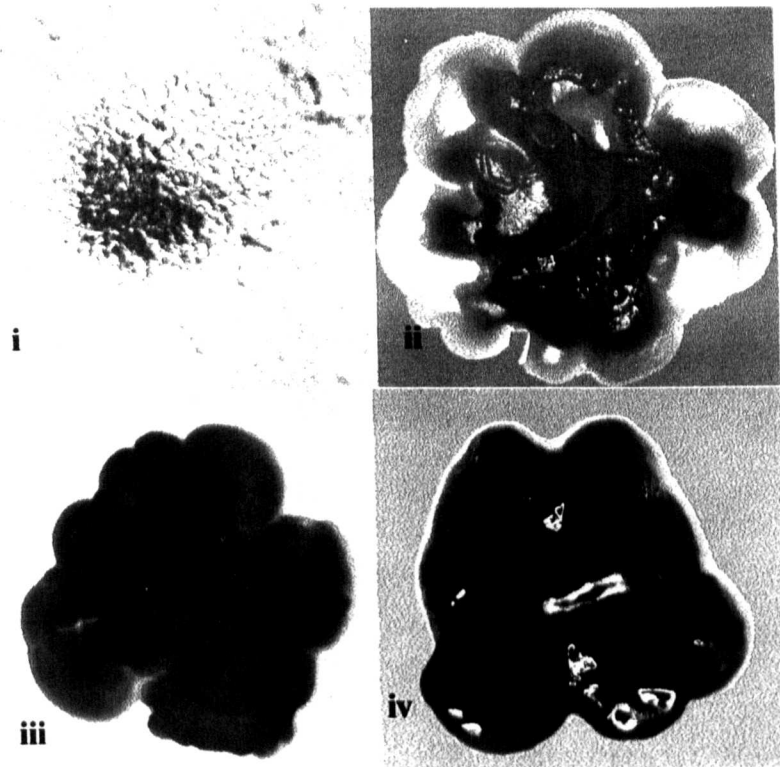
Plate 1. **Photomicrographs of surface cultures at selected time points on YEME medium demonstrating a temporal profile of colony development.** i) young colony at 48 h post-inoculation. ii) The darkening of the colony indicating the formation of spores at the colony surface after 96 h. iii) The dark appearance of the colony reveals extensive sporulation following 120 h of growth. iv) The whole colony darkens following 192 h, indicating that it consists mainly of spores.

Glycogen was found to be accumulated in two phases, initially during the early rapid growth phase, and again following the onset of sporulation at 120 hours (*Figure. 16*). Both glucose and ninhydrin positive compounds declined throughout the period of rapid biomass accretion (*Figure. 17*). The cessation of biomass accumulation coincided with a decline in cellular glycogen levels suggesting that glycogen reserves were being used as a carbon and energy source despite the availability of residual glucose in the medium. The exhaustion of ninhydrin-positive compounds within the medium coincides with the cessation of biomass accretion, indicating that this is limiting growth.

The pattern of trehalose accumulation differed from that of glycogen (*Figure. 16*). Trehalose was accumulated throughout the growth phase, reaching a steady level with the depletion of ninhydrin positive amino compounds (*Figure. 17*). At the onset of sporulation, trehalose content of cells remained constant. Trehalose was found to account for up to 5 % (w/w) of mycelial dry weight.

There was a marked accumulation of cellular lipid with values reaching as high as 25% (w/w) of the cell dry weight (Fig 18). Total lipid content of biomass was found to increase in a biphasic pattern (*Fig.18*), accumulating rapidly prior to sporulation (22 % w/w), and then accumulating more slowly, coinciding with depletion of ninhydrin-positive amino compounds. Lipid content of mycelia reached a maximum of 26 % (w/w) at 240 hours.

Of the lipid material measured, a significant proportion of it was found to be in the form of triacylglycerols. Triacylglycerols were found to accumulate markedly following the initiation of sporulation. These compounds were found to rapidly decline once biomass accretion had ceased. TAG content of the mycelium increased throughout growth in a biphasic pattern (*Figure. 19*). Prior to sporulation TAG content of the mycelia (1 % w/w) was low, however following depletion of the positive-ninhydrin compounds, rapid accumulation of TAG was observed, reaching a maximum of 8 % (w/w). The increase in TAG content of the mycelium correlated with the decrease in medium glucose. This pattern was similar to that seen for glycogen, suggesting a possible role for these compounds in carbon storage.

D-β-hydroxybutyrate could not be detected in any of the cell extracts of *Micromonospora echinospora*.

3.1.2 Antibiotic production

Antibiotic production was transient (*Fig. 20*), with bioactivity being first detected after 72 hours, peaking after 120 hours and rapidly declining to undetectable levels at 144 hours. The antibiotic biosynthetic phase preceded the onset of sporulation suggesting that the two processes are independently controlled.

Surface grown cultures of *M. echinospora* provided a useful model to study differentiation and antibiotic production which was reproducible, allowing insights in to the physiology of this organism.
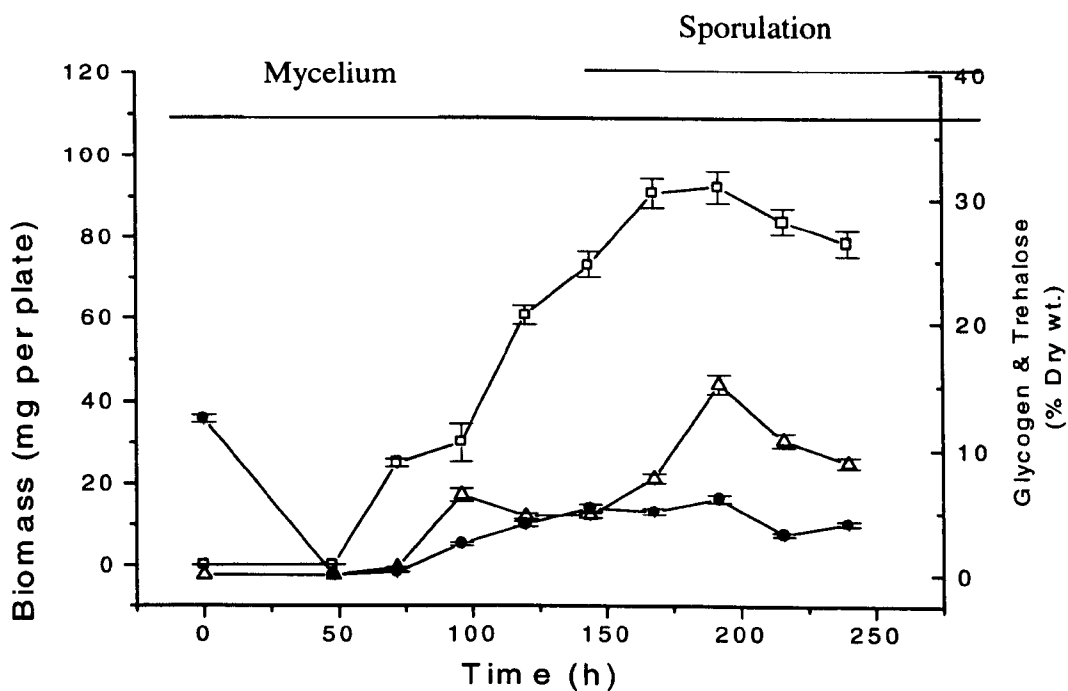
Figure 16. Growth and accumulation of intracellular carbohydrate in Micromonospora echinospora. Dry weight biomass (- -), Percentage glycogen of dry weight (-ρ-), and Percentage trehalose of dry weight (-●-). Error bars represent the standard deviation of the data.
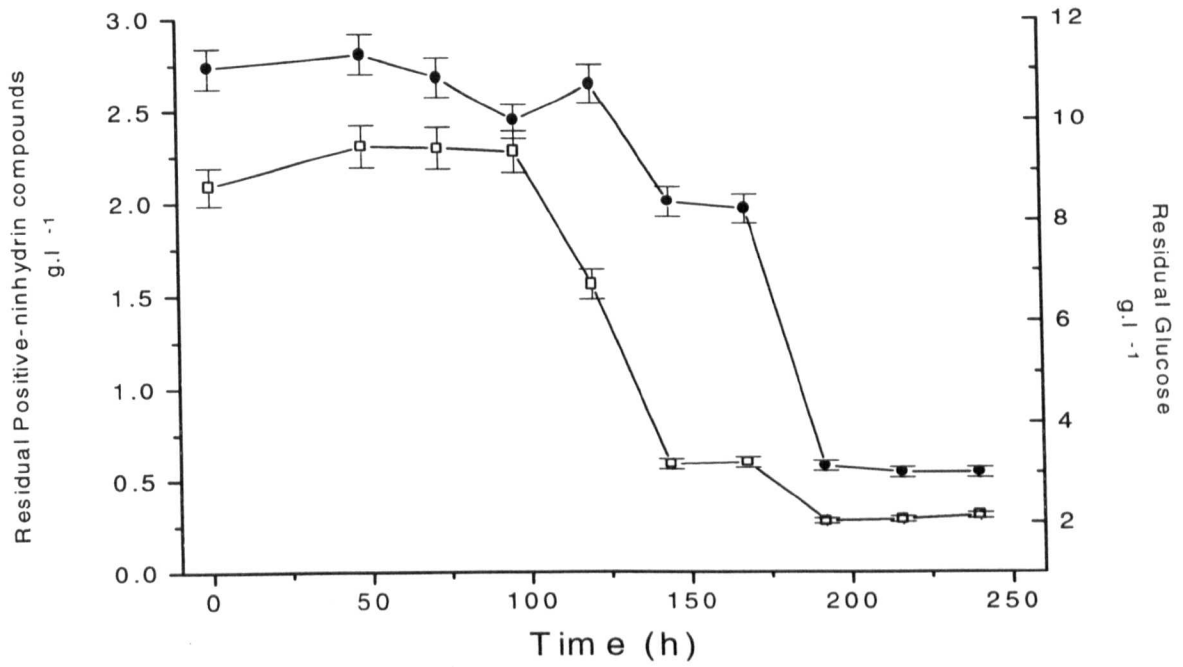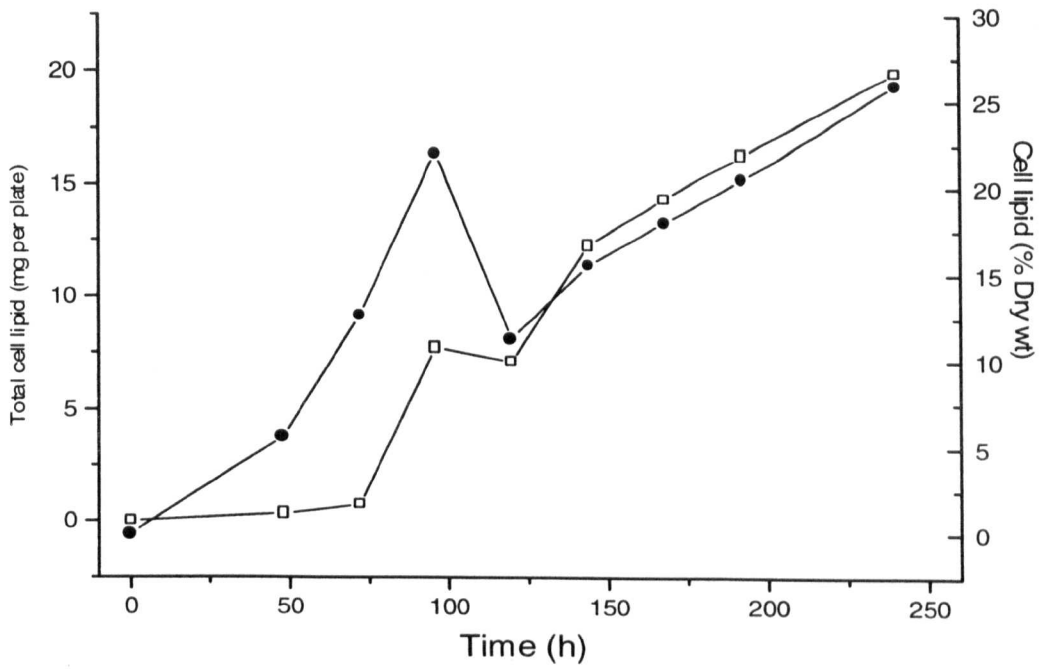
Figure 17. Residual nutrients. Residual glucose (-●-), and residual ninhydrin-

positive compounds (- -). Error bars represent the standard deviation of the data.

Figure 18. Total lipid content of the mycelium (-●-), and percentage lipid of the dry

weight (- -). Each sample point is the mean lipid content per plate, determined from
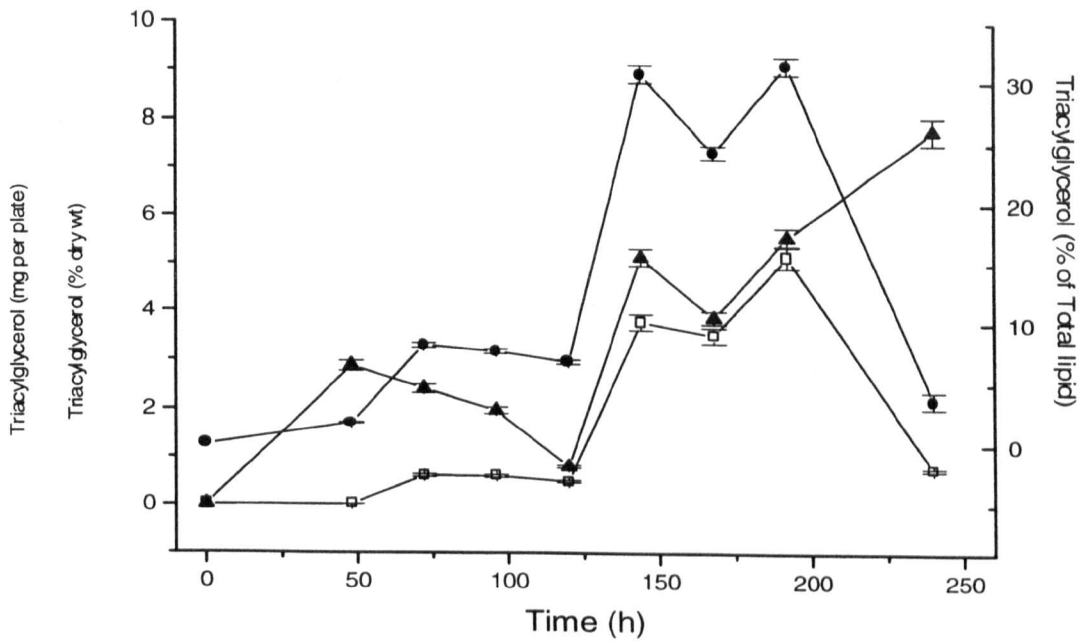
three plates of pooled biomass.

Figure 19. Total TAG content of the mycelium (- -), percentage TAG of the dry weight (-●-), and percentage TAG of total cell lipid (-π-). Error bars represent the standard deviation of the data.
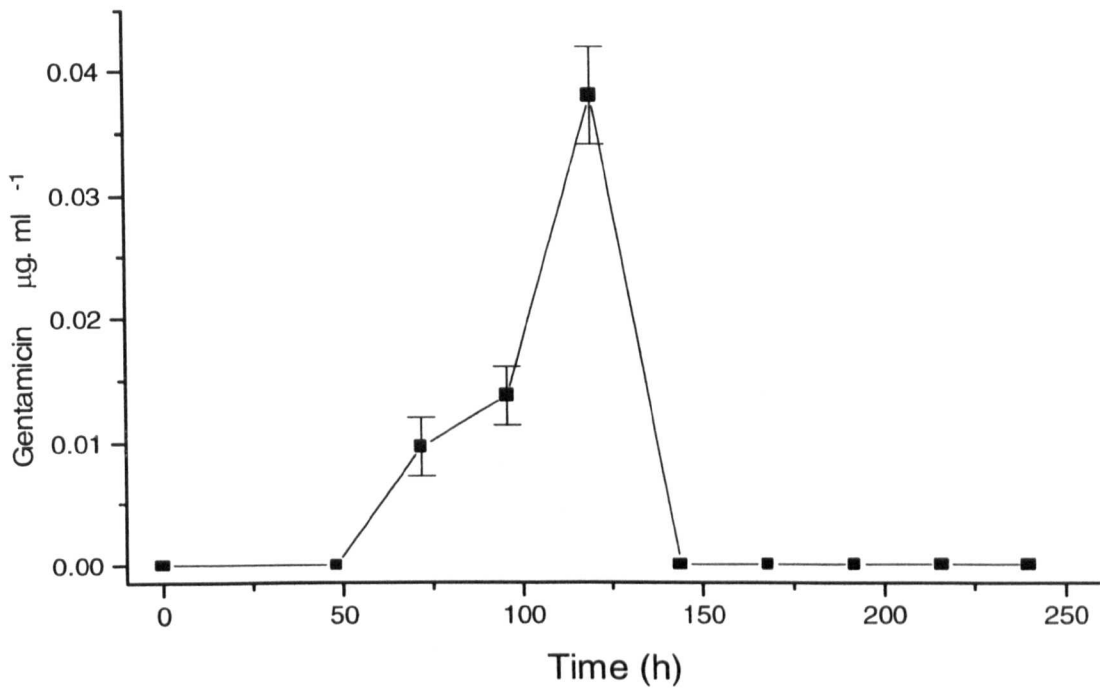
Figure 20. Antibiotic activity (-□-) quantified by plate bioassay. Error bars represent the standard deviation of the data.

3.2 Liquid Culture

In contrast to many streptomycetes, *Micromonospora* species exhibit full differentiation in liquid medium as well as on solid medium. Sporulation in submerged culture provides several advantages over solid culture when determining specific responses to environmental changes (Lee, 1998), due to the heterogeneity of the surface culture conditions. The previous study on solid medium indicated that antibiotic production and sporulation were mutually exclusive. To further study this phenomenon is was essential to establish a reliable assessment of sporulation. To achieve this end it was essential to distinguish between spores and mycelia within the medium.

3.2.1 Assessment of spore properties and development of sporulation assay

Previous reports of sporulation have relied upon the visual assessment of sporulation (Chater, 1972) and a method for the quantification of sporulation was required. Workers such as Daza *et al.*, (1989), Glazebrook *et al.*, (1990) and Lee & Rho (1993) have used the intrinsic resistance properties of the spores to gain a more exact assay of sporulation. Surface spores were initially studied to test the culturability of spores following treatment with various deleterious agents.

Total cell counts were made of freshly-harvested spore suspensions and these were compared with the number of CFU's generated from the suspensions following suitable dilution. It was found that approximately 20% of the original spore population gave rise to visible colonies. This level of culturability was consistent between batches of spores and all subsequent experiments were conducted with freshly-prepared spore suspensions.

Spore suspensions were treated with temperatures ranging from 50-90 $^{\circ}$C. Heat treatment at 50 $^{\circ}$C for 30 min did not affect the number of CFU's generated from the spore suspension when compared with control (unheated) suspensions. At temperatures of 60 $^{\circ}$C and 70°C the number of CFU's generated following treatment for 5 and 10 min was higher than that measured in the control samples. The effect of these short-term, mild, heat treatments produced a marked increase in CFU's with a five-fold increase following treatment at 60 $^{\circ}$C for 10 min (Fig. 21) and a three-fold increase at 70 $^{\circ}$C after 5 min (Fig. 22). At higher temperatures classic thermal death profiles were observed (Fig. 23, 24, 25) and decimal reduction times (D-values) were determined (Table 2). Some tailing of the curves was observed due to the natural heterogeneity of the spore population. This tailing has recently been the subject of reviews (Peleg & Cole, 1998; Smerage & Teixeira, 1993). The decimal reduction times were used to calculate a z-value of 12.6 $^{\circ}$C for this organism (Fig. 26).

Oxygen uptake rates were determined for the spores in nutrient-free buffer (100 mM sodium phosphate buffer), before and after heat treatment (Figs. 1 and 2). Consistent with previous reports (Ensign 1982) freshly-collected spores exhibited low-level endogenous respiration. Heat treatment of these spores resulted in a rapid (within 10 min following treatment) increase in spore respiration. This increase indicates that readily utilisable intracellular carbon reserves were available to fuel the increase in metabolic activity, as no exogenous carbon source was available.

Treatment with 0.1 M HCl and lysozyme also resulted in a reduction in spore viability, whilst sonic disruption of the spore suspensions resulted in the largest reduction in viability of spores (Fig. 29. 30, 31). The curve tailing observed during heat-treatment was also observed for these agents, again emphasising the natural heterogeneity of the spore population. It was therefore decided that the various treatments tested could not be utilised as a reliable and accurate assay for sporulation. Sporulation from liquid culture could, however, be quantified by dilution plating for CFU's, which gives an indication of sporulation, as a sudden burst of CFU's was indicative of spore formation.
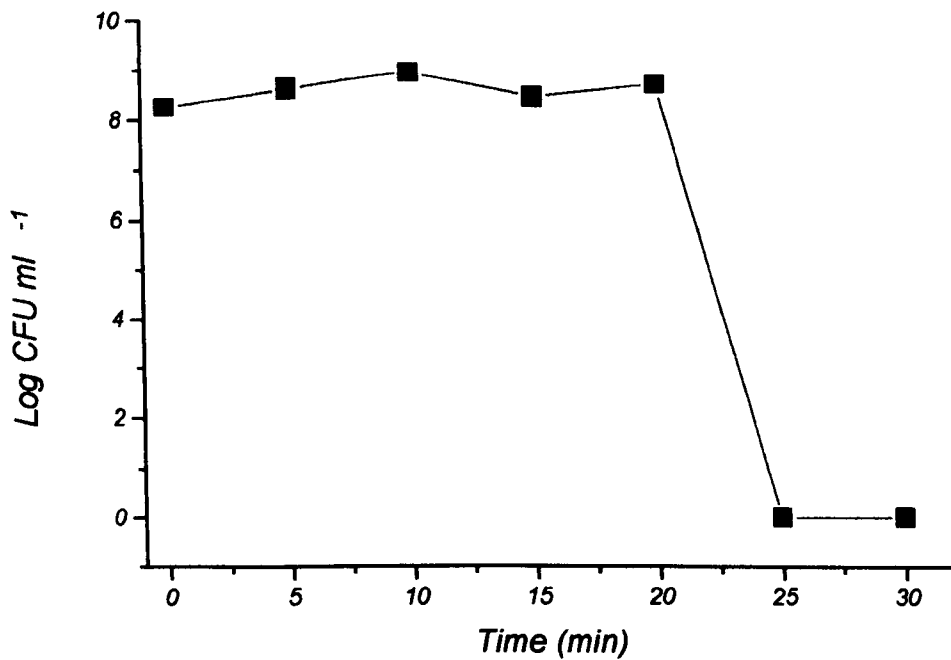
Figure 21. **Resistance of spores to heat treatment at 60 °C.** Values are the logarithm of the mean of at least three determinations. Heat treatment was conducted according to the method of Daza *et al.*, (1989).
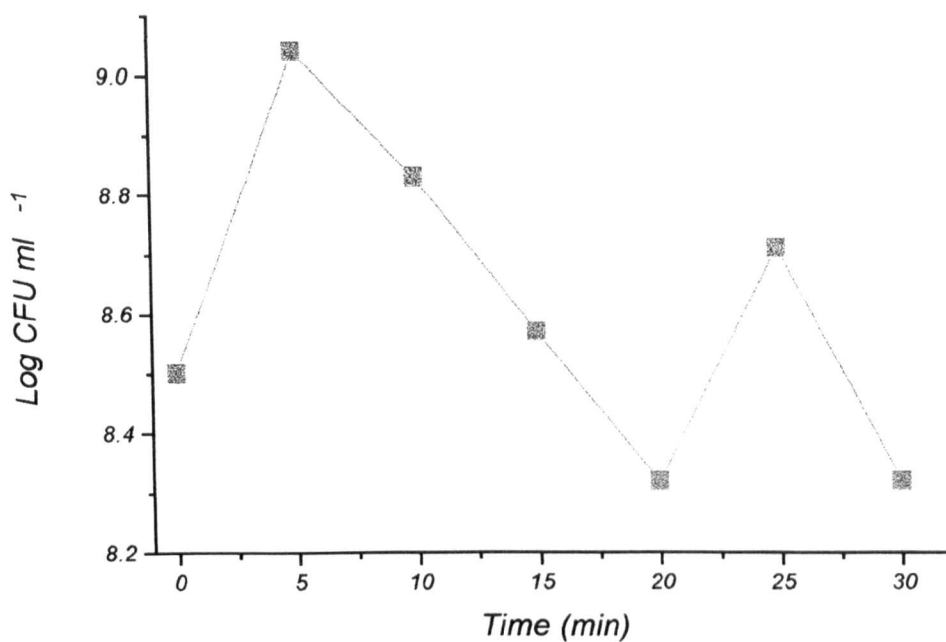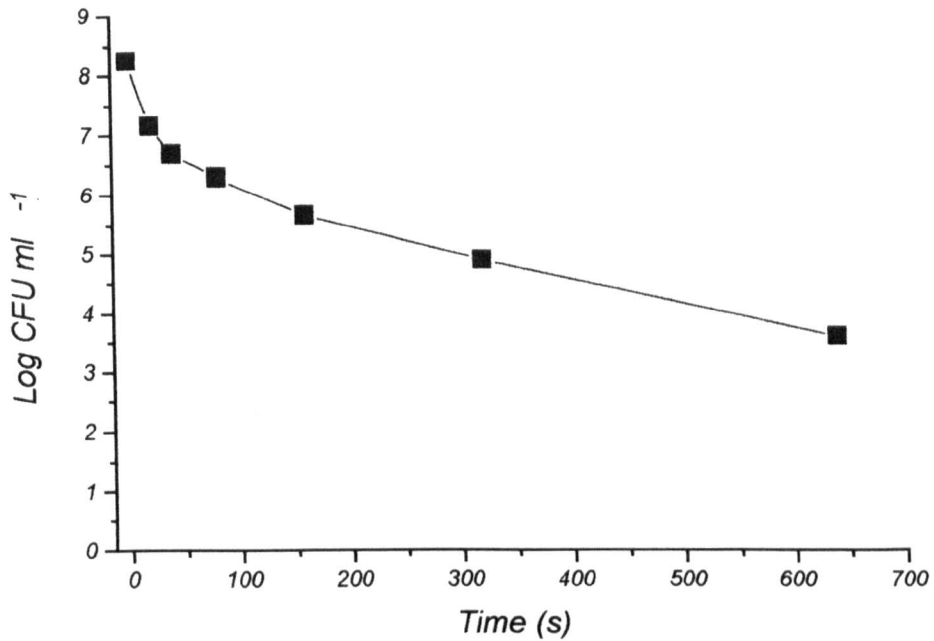
Figure 22. **Resistance of spores to heat treatment at 70 °C.** Values are the logarithm of the mean of at least three determinations. Heat treatment was conducted according to the method of Daza *et al.*, (1989).
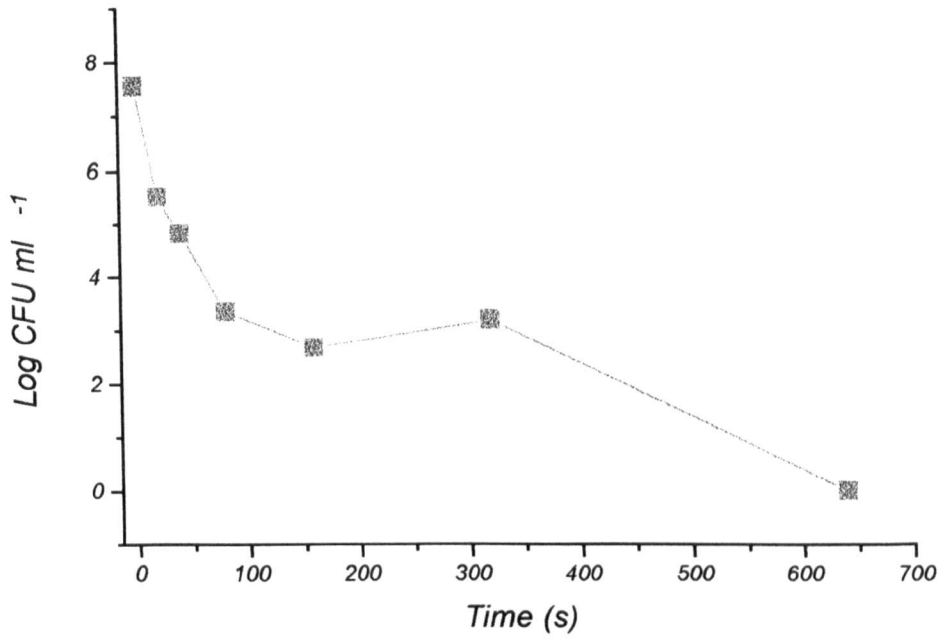
Figure 23. **Resistance of spores to heat treatment at 80 °C.** Values are the logarithm of the mean of at least three determinations. Heat treatment was conducted according to the method of Daza *et al.,* (1989).

Figure 24. **Resistance of spores to heat treatment at 90 °C.** Values are the logarithm of the mean of at least three determinations. Heat treatment was conducted according to the method of Daza *et al.,* (1989).
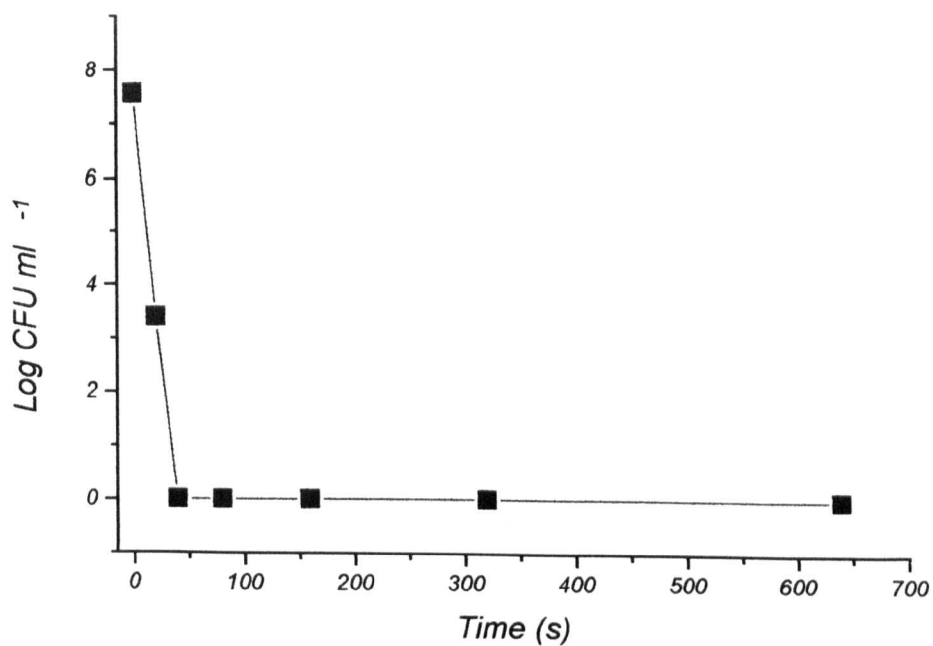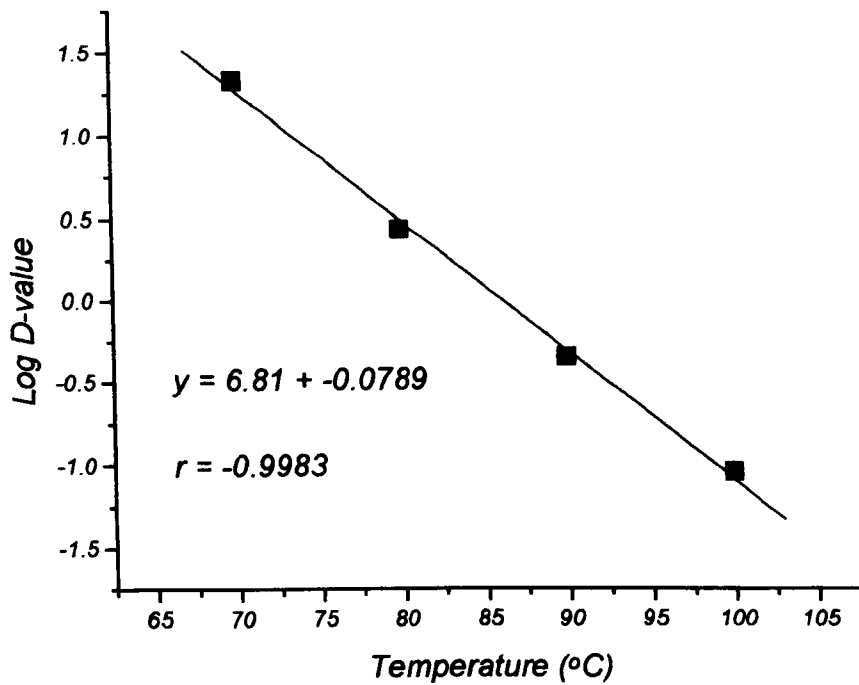
Figure 25. **Resistance of spores to heat treatment at 100 °C.** Values are the logarithm of the mean of at least three determinations. Heat treatment was conducted according to the method of Daza *et al.*, (1989).
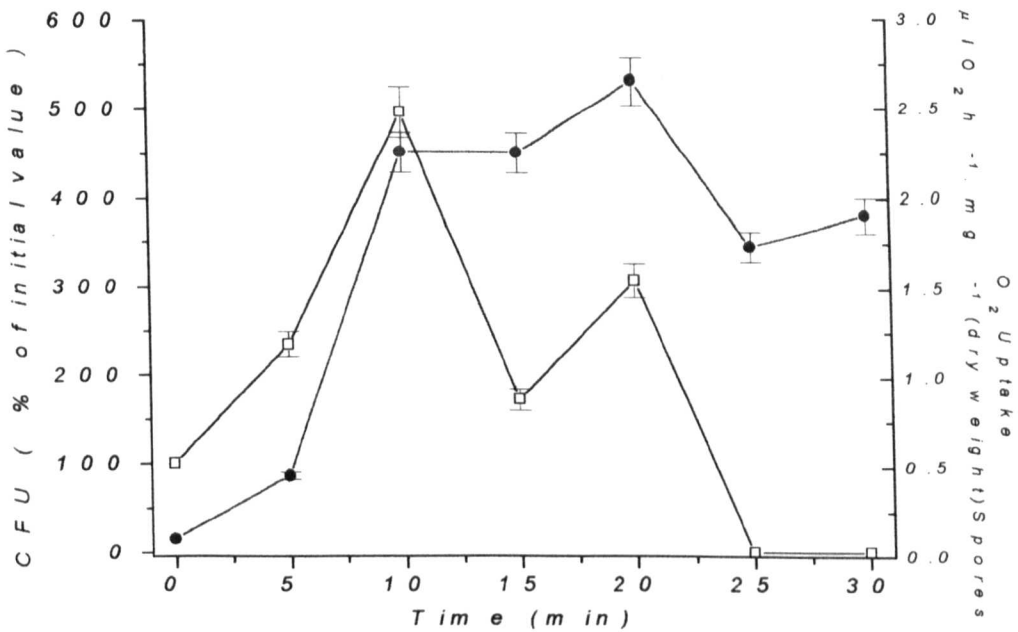
Table 2. Resistance of spores to heat treatment. Decimal reduction times were calculated according to Gould (1971). Where D-values are the time taken (min) for the survivor count to fall by one log cycle.

| Temperature of heat treatment (°C)	D-value (min)
70	21.43
80	2.67
90	0.45
100	0.09

Figure 26. **Z-Value.** Log D-values obtained at 70, 80, 90, and 100 °C plotted against temperature. The reciprocal of the slope represents the z-value for this organism, which was calculated to be 12.6 °C.

Figure 27. **Resistance of spores to heat treatment.** The effect of exposure of spores at 60 $^{\circ}$C for varying times on the number of colony forming units generated post heat-treatment, expressed as a percentage of the CFU's produced from unheated (control) suspensions (-□-). Respiration rates of spore populations following exposure to heat, measured 10 min following treatment (-●-). Error bars represent the standard deviation of the data.
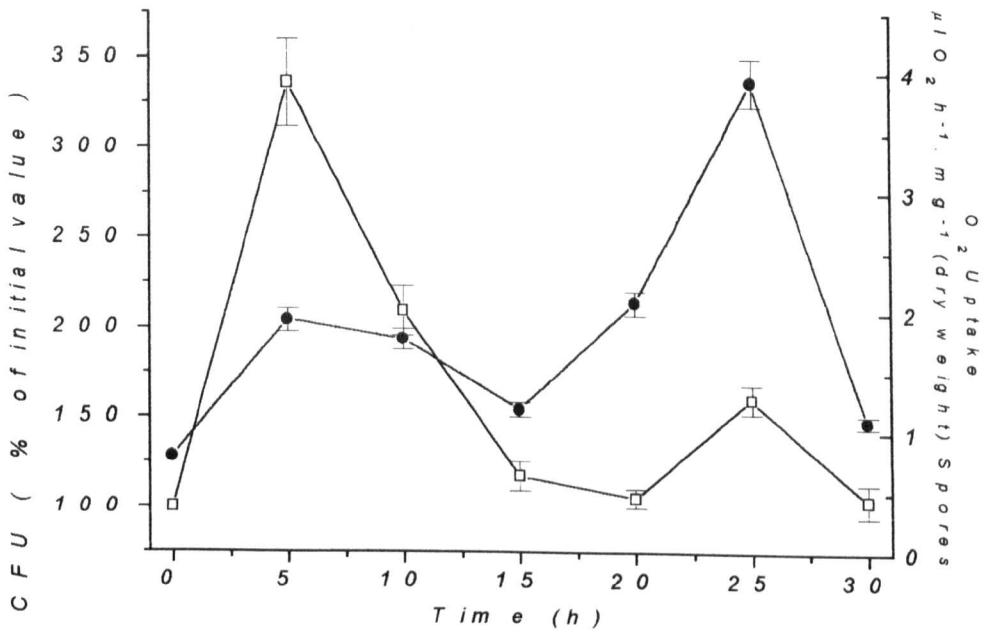
Figure 28. **Resistance of spores to heat treatment.** The effect of exposure of spores at 70 °C for varying times on the number of colony forming units generated post heat-treatment, expressed as a percentage of the CFU's produced from unheated (control) suspensions (-□-). Respiration rates of spore populations following exposure to heat, measured 10 min following treatment (-●-). Error bars represent the standard deviation of the data.

Figure 29. **Resistance of spores to mild-acid treatment (0.1 M HCl).** Values are the logarithm of the mean of at least three determinations. Acid treatment was conducted according to the method of Daza *et al.,* (1989).

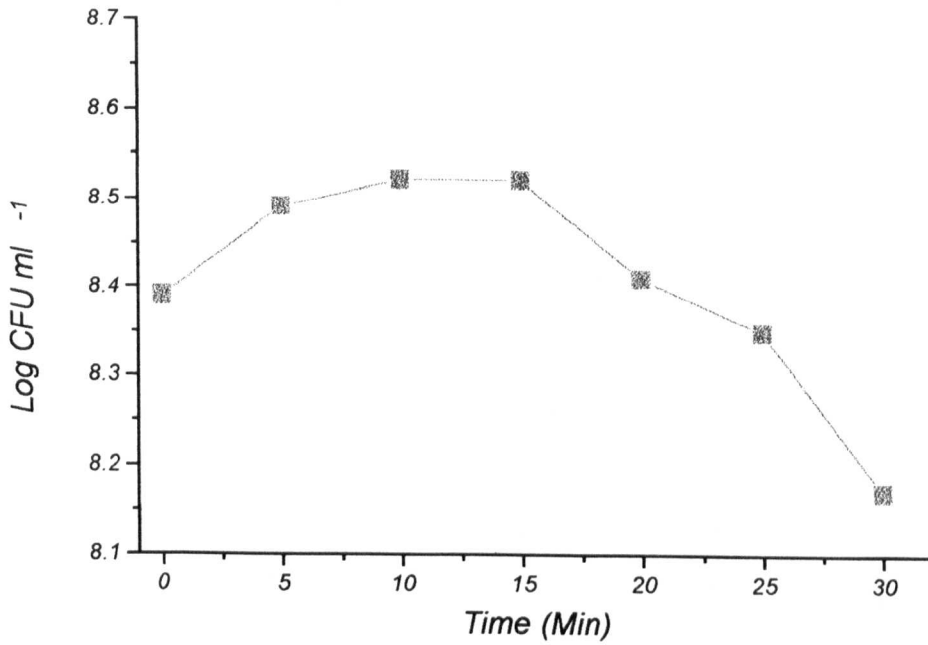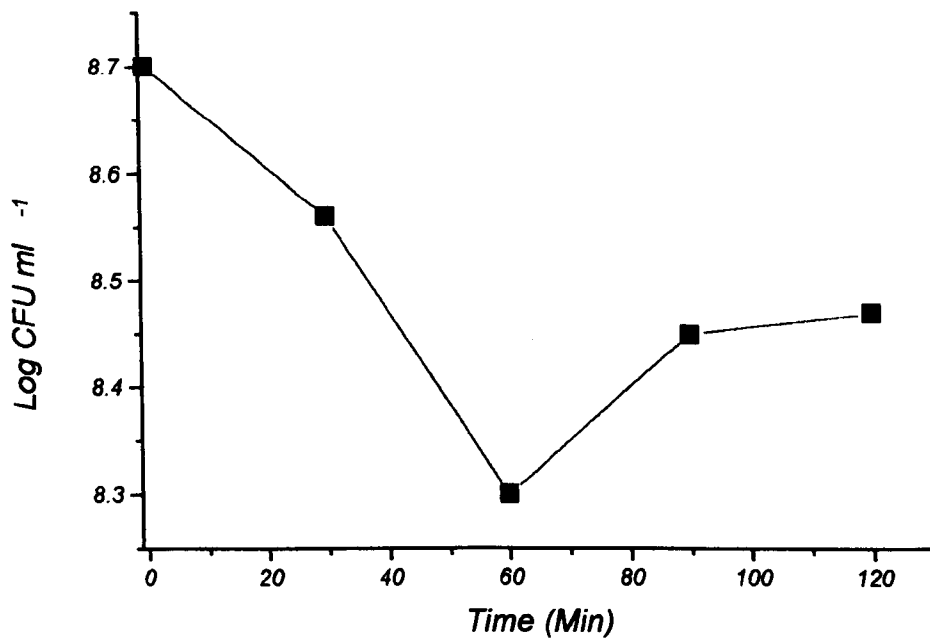**Figure 30** . **Resistance of spores to lysozyme treatment.** Values are the logarithm

of the mean of at least three determinations. Lysozyme treatment was conductèd

according to the method of Daza *et al.*, (1989).
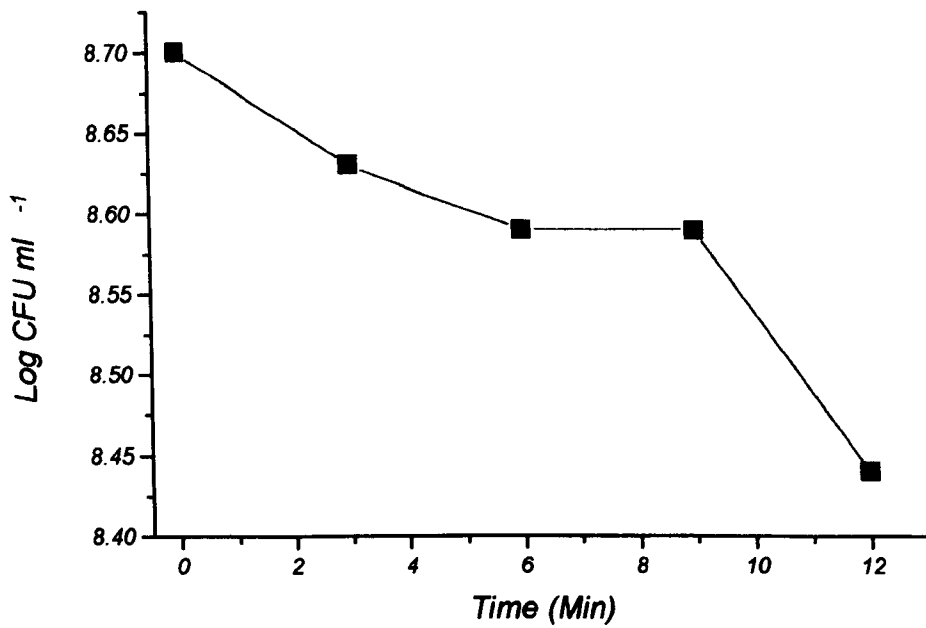
Figure 31. **Resistance of spores to sonic disruption.** Values are the logarithm of the mean of at least three determinations. Sonic disruption was conducted according to the method of Karandikar (1997).

3.3 Fermentation characteristics

The aim of this section was to establish the basic physiological and growth characteristics of *Micromonospora echinospora* in submerged culture. The medium of choice was mYEME, which had previously been shown to allow gentamicin production and full differentiation of this organism on surface grown cultures (Section 3.1.2).

3.3.1 Growth in liquid YEME

Fermenter cultures of *M. echinospora* grown in liquid mYEME medium exhibited multiple growth phases (Fig. 32). An initial slow growth phase of 48 h duration was followed, by a rapid increase in biomass over the period 48-120 h post inoculation. The growth phase slowed thereafter reaching a maximum biomass of 2.75 g. l^{-1} after 144 h incubation. The end of the rapid growth phase coincided with the appearance of spores in the culture (between 96-120 h), observed by phase contrast microscopy (Plate 2). The increase in CFU's during growth was also found to be pronounced after 96 h of incubation (Fig. 32). Extensive fragmentation of the mycelium was also observed 144 h post inoculation. A semi-logarithmic plot of biomass against time indicated that the major growth phase was exponential (Fig. 33). The growth rate of the culture was at its highest during this rapid growth period, reaching a maximum of 0.125 h^{-1} (Fig 33).

The rapid growth phase also corresponded with the consumption of ninhydrin-positive amino compounds (Fig 34) and phosphorus (Fig. 35). The rapid consumption of glucose was delayed until 96 h post-inoculation. The determination of specific substrate uptake rates for ninhydrin-positive amino compounds (q_{nin}) and

glucose ($q_{glucose}$) revealed that ninhydrin-positive amino compounds were consumed at a greater rate than glucose (Fig. 37). The appearance of ammonia in the media indicated that deamination of the amino compounds was occuring (Fig. 35). This suggested that the amino compounds were being used as the primary carbon source in preference to glucose in this medium during the early growth phase. In each case, the rate of substrate consumption was highest during the rapid growth phase. The phosphorus content of liquid YEME was depleted following 72 hours of growth, indicating that liquid YEME cultures were phosphate limited.

The growth yields for ninhydrin-positive amino compounds ($Y_{x/nin}$) and glucose ($Y_{x/glucose}$) were calculated during growth and were found change throughout (Fig. 36). This trend has previously been observed in the *Streptomyces coelicolor* (Karandikar *et al.*, 1997). The growth yield for ninhydrin-positive amino compounds was found to be higher than that of glucose, with a maximum growth yield ($Y_{x/nin}$) of 5 $g.g^{-1}$ compared with ($Y_{x/glucose}$) 0.45 $g.g^{-1}$. This coupled with the specific substrate uptake rates (q_s) enforces the concept of preferential amino-compound use as a carbon source resulting in low glucose consumption.

3.3.1.2 Kinetics of carbonaceous storage compound accumulation in liquid YEME

It has already been reported that during surface cultivation *M.* echinospora accumulates significant carbon reserves. To establish if this is a feature of surface cultivation, a series of experiments were conducted in submerged culture in fermenters. This was to determine if the later phases of biomass accretion in batch culture were associated with the accumulation of the polyglucan, glycogen, and the

disaccharide, trehalose, a temporal profile of these compounds was obtained during growth in liquid YEME.

Glycogen was accumulated in a bi-phasic manner in liquid YEME. Cellular glycogen levels reached their maximum level at 168 h post-inoculation (Fig 38). When considered as a percentage of the cellular dry weight, however, glycogen represented up to 12 % of the dry cell weight following 72 h of growth. The accumulation of trehalose in liquid culture (Fig. 39) followed a similar pattern to that of glycogen, in contrast, to that observed in surface cultures. The maximum level of trehalose accumulation, as a percentage of the mycelium (dry weight), was observed at 144 h, coinciding with extensive sporulation within the culture.

McBride & Ensign (1987a & b) have previously demonstrated that trehalose plays an important role as a storage carbon source in spores of several actinomycetes.

The examination of total lipid and TAG in liquid YEME remained low and constant throughout growth (less than 15 % and 1% respectively) suggesting that during liquid culture lipid is not modulated in the same manner as on solid medium (Data not shown). This is similar to the pattern observed in *S. coelicolor* and *S. albus* where TAG and phospholipids content of mycelium were not temporally controlled during growth (Olukoshi & Packer, 1995). D-β-hydroxybutyrate could not be detected in any extracts of *M. echinospora* from liquid YEME cultures.

Gentamicin production in liquid YEME

Unlike the previous studies conducted on solid medium liquid YEME fermenter batch cultures did not support the production of gentamicin. Escalante *et al.*, (1992) demonstrated that high levels of glucose (40 g. L^{-1}) repressed gentamicin production in *M. purpurea* in a chemically defined medium. Obregon *et al.*, (1994)

demonstrated the repressive effects of phosphate on the production of gentamicin. The limiting nature of phosphate under these conditions eliminates the sole role for phosphate repression of gentamicin production in this organism.

The cellular density and biomass levels of cultures have previously been shown to affect the production of antibiotics (Sanchez & Brana, 1996; McIntyre *et al.*, 1999). *M. echinospora* was grown for 96 h, and then subjected to medium and cell density changes (Table 3).

A two-fold increase in biomass concentration into either fresh YEME or conditioned YEME (YEME medium where cells were grown to 96 h) resulted in the production of gentamicin. A three-fold increase in biomass concentration when transferred into fresh or conditioned YEME did not permit gentamicin production. This suggests that antibiotic production is subject to complex regulation that is not easily explained by nutritional status, cell density, or growth rate suggesting interplay of all three variables may be involved.

Micromonospora echinospora undergoes full differentiation when grown as surface cultures or in submerged culture in YEME medium. The complete exhaustion of a single nutrient was not evident and changes in growth rate throughout growth did not easily correlate with the morphology of the organism. Important insights in to the physiology of *M. echinospora*, however, were established such as an apparent preference for amino compounds as a carbon source. It was therefore decided to conduct all further experiments in a defined minimal medium, which would allow the physiological responses to nutrient limitations to be quantified.

Having established reproducible cultures both on surfsce and submerged conditions,

attention was focussed on developing a chemically defined medium that would allow

physiologically phenomena to be correlated with nutrient consumption.
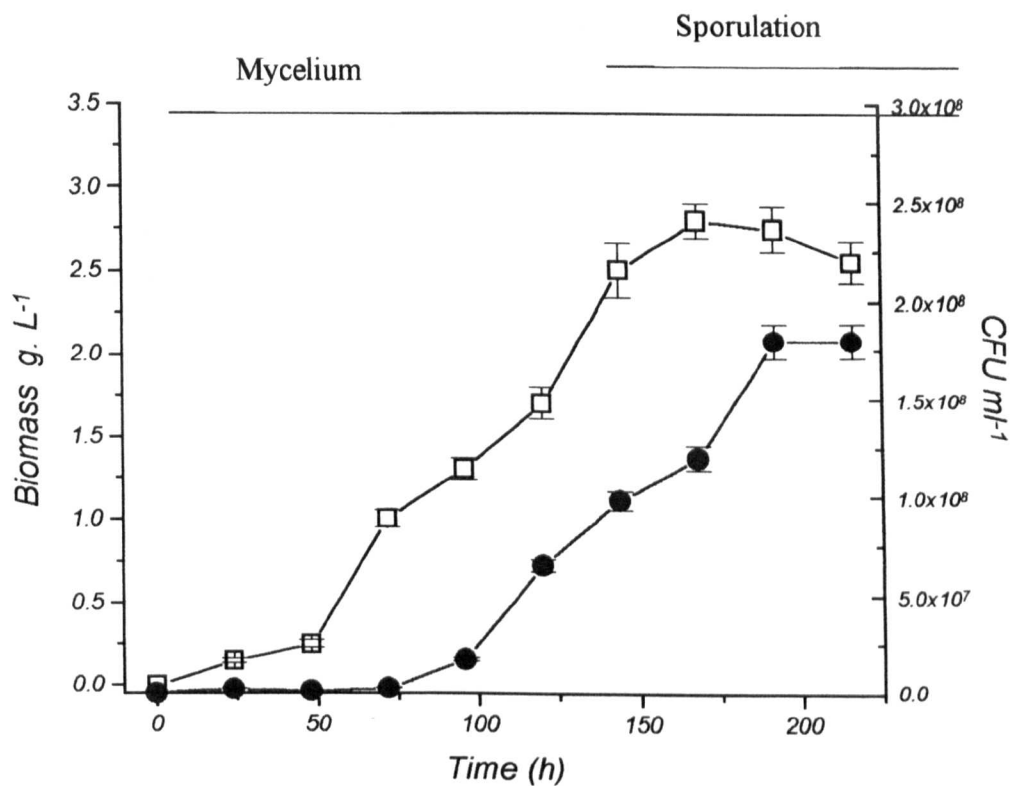
Figure 32. **Growth kinetics during batch culture in liquid YEME.** Biomass dry weight (-□-), and CFU (-●-). Values are the mean of at least three determinations. Error bars represent the standard deviation of the data.
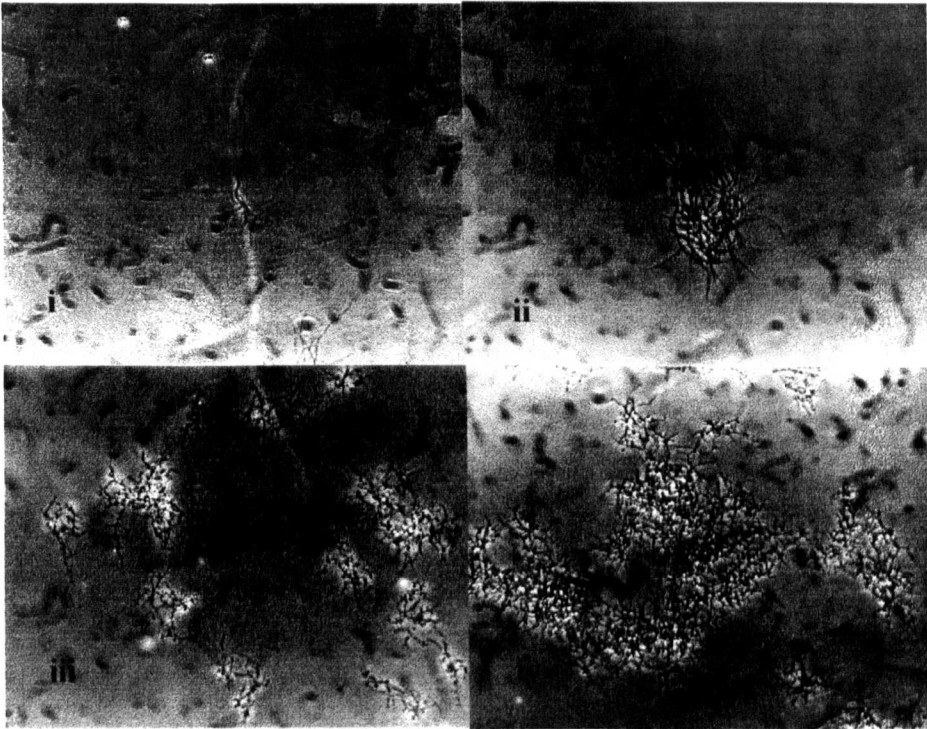
Plate 2. **Photomicrographs of mycelium at selected time points in liquid YEME medium demonstrating a temporal profile of development.** i) young hyphae at 48 h post-inoculation. ii) Formation of aggregates of interwoven hyphae following 72 h of growth. iii) The formation of immature spores at 96 h post inoculation. iv) The presence of mature spores on the mycelial aggregates following 144h of growth.
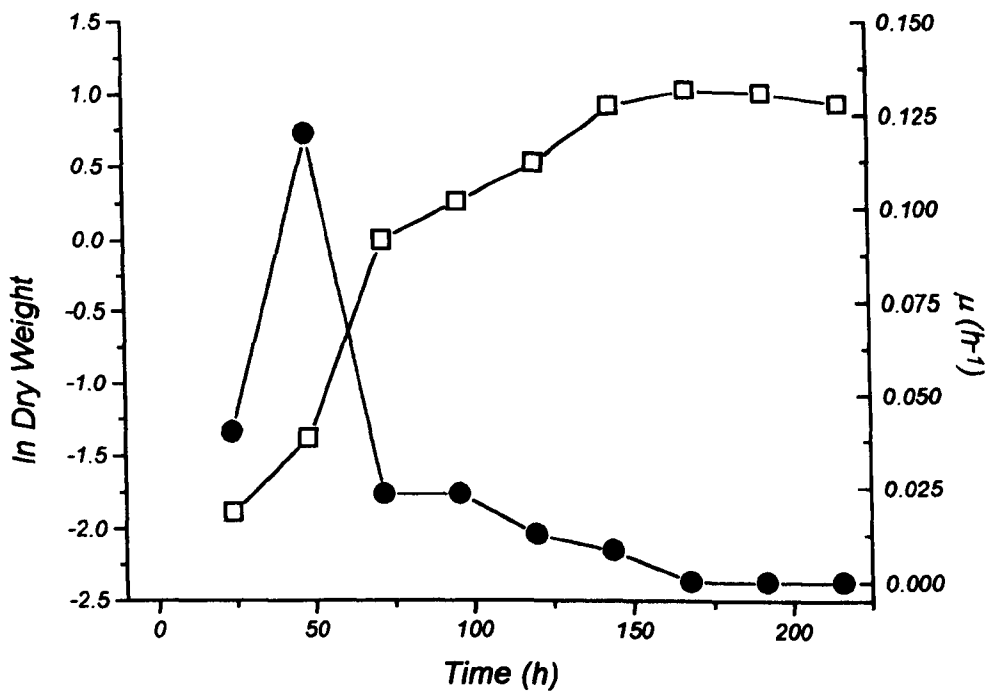
Figure 33. **Growth kinetics during batch culture in liquid YEME.** Natural log of Biomass (-□-), and specific growth rate, μ (-●-). Values are the mean of at least three determinations. Specific growth rates were calculated from the tangent of semi-logarithmic plot of the mean dry weight.
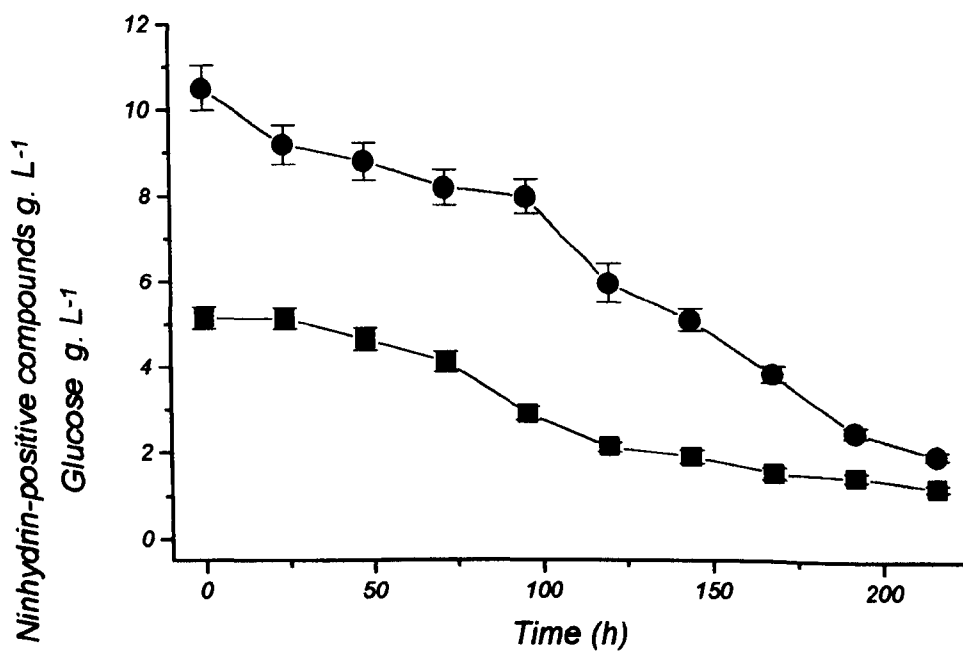
Figure 34. **Growth kinetics during batch culture in liquid YEME.** Residual ninhydrin-positive amino compounds (-■-), and glucose (-●-). Values are the mean of at least three determinations. Error bars represent the standard deviation of the data.

Figure 35. **Growth kinetics during batch culture in liquid YEME.** Residual phosphorus (-□-), and ammonia (-●-). Values are the mean of at least three determinations. Error bars represent the standard deviation of the data.
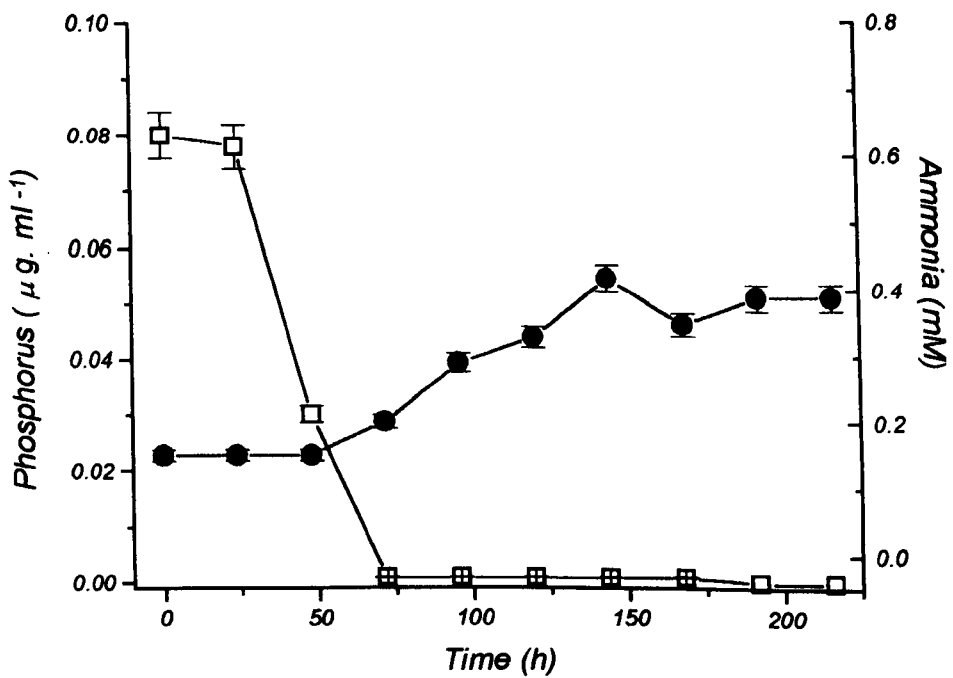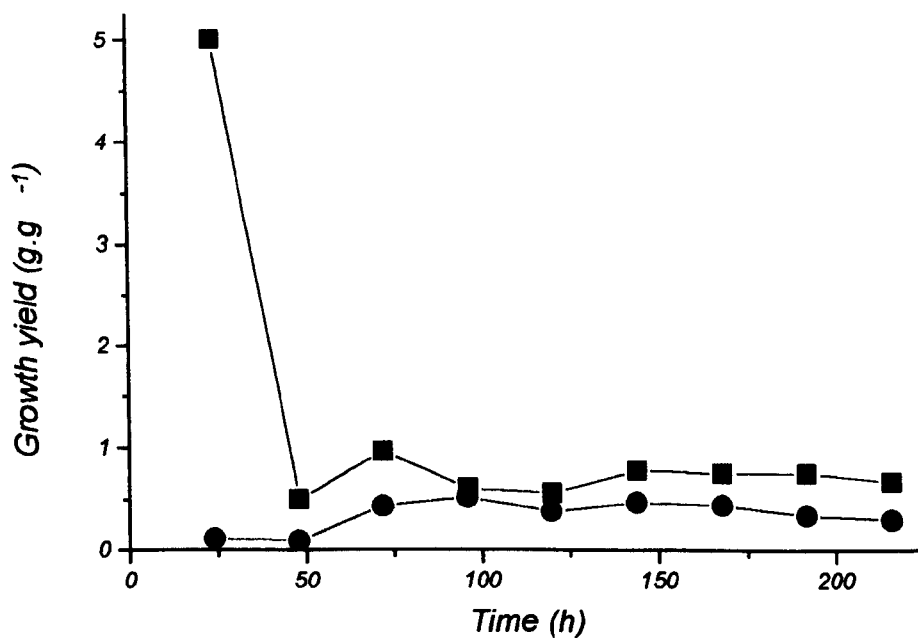
Figure 36. **Growth kinetics during batch culture in liquid YEME.** Growth yield

from glucose (-●-), and ninhydrin-positive amino compounds (-■-). Growth yield

values were determined from the mean biomass and mean residual substrate

concentration (g. L^{-1}).
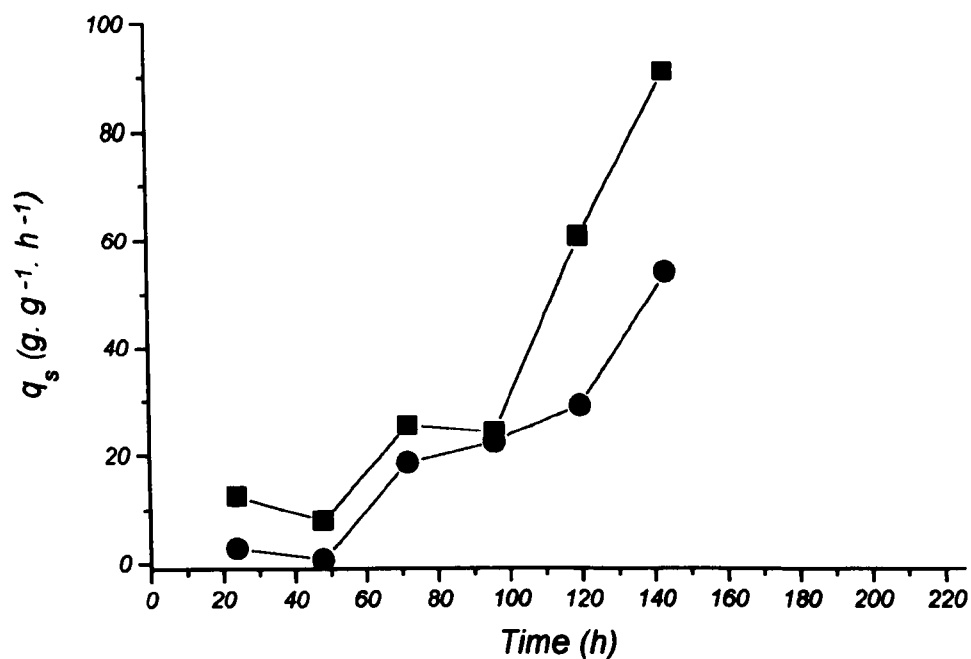
Figure 37. **Growth kinetics during batch culture in liquid YEME.** Specific substrate uptake rate for glucose (-●-), and ninhydrin-positive amino compounds (-■-). Values were determined from μ and the growth yield data shown in *Figure* X.

Figure 38. **Growth kinetics during batch culture in liquid YEME.** Accumulation of intracellular glycogen. Total cellular glycogen (-□-), and % glycogen of the dry cell weight (-●-). Values are the mean of at least three determinations. Error bars represent the standard deviation of the data.
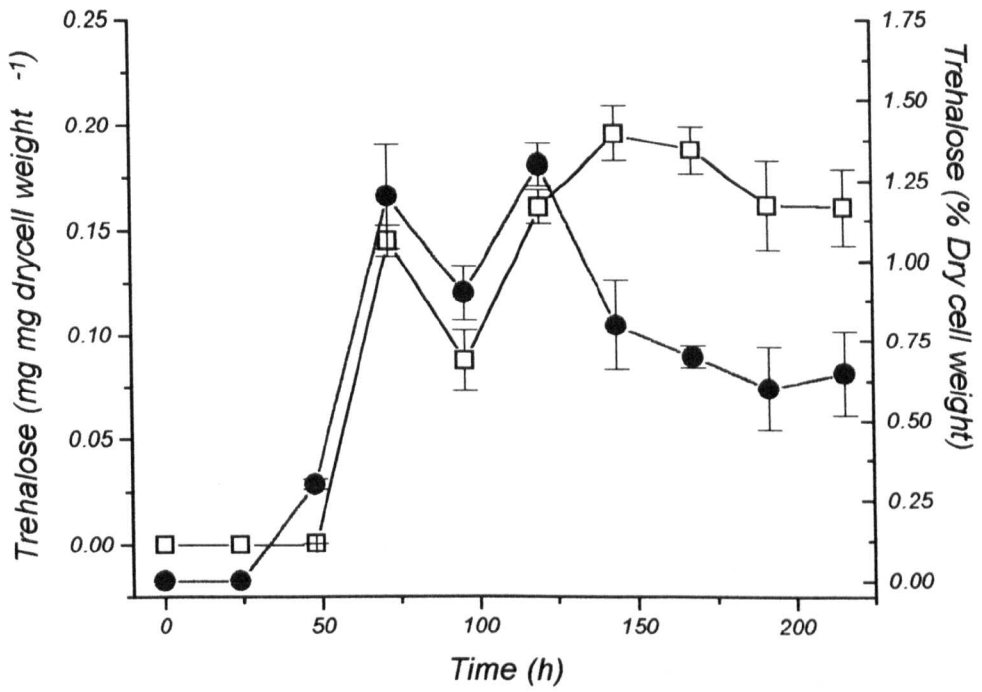
Figure 39. **Growth kinetics during batch culture in liquid YEME.** Accumulation of intracellular trehalose. Total cellular trehalose (-□-), and % trehalose of the dry cell weight (-●-). Values are the mean of at least three determinations. Error bars represent the standard deviation of the data.

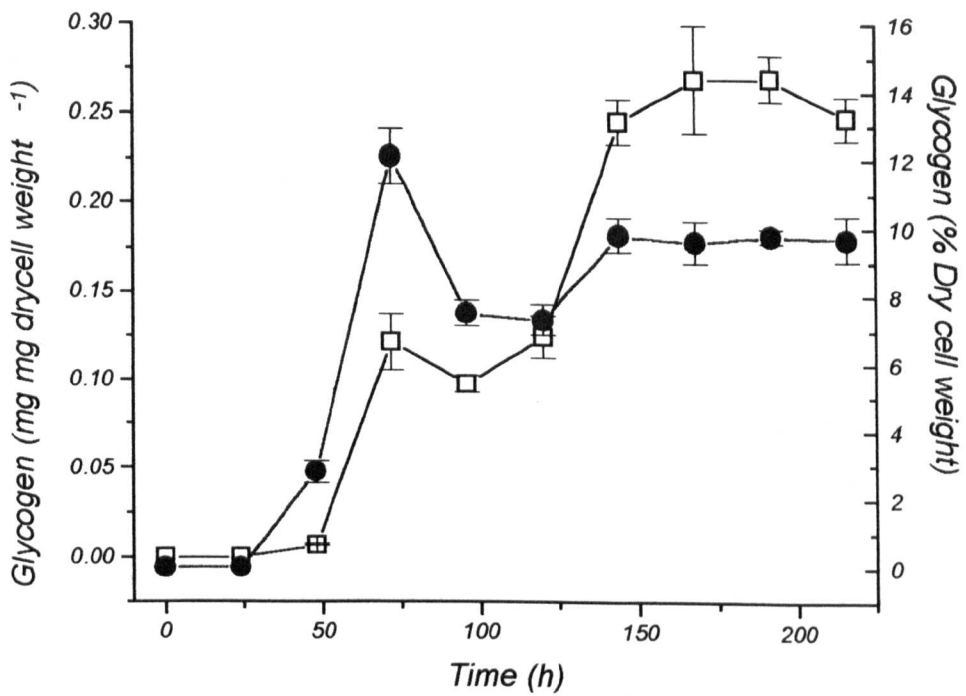Table 3. Production of gentamicin in liquid YEME following cell concentration.

Treatment	Final Biomass (144h) g. L^{-1}	Gentamicin titre at 120 h (μg. ml^{-1})	Gentamicin titre at 144 h (μg. ml^{-1})
Control*	2.38	0	0
2 X conc. Biomass into conditioned YEME	4.75	9.8	12.2
2 X conc. Biomass into fresh YEME	6.15	0	11.3
3 X conc. Biomass into conditioned YEME	5.85	0	0
3 X conc. Biomass into fresh YEME	7.65	0	0

*Control flask consisted of a batch run allowed to proceed without any treatment.

3.3.2 Determination of optimal carbon and nitrogen concentrations

A prerequisite for conducting physiological experiments is the provision of a chemically defined medium which subsequently enables exact analysis of nutrient consumption and product formation in correlation with culture growth (Karandikar, 1997).

The chemically defined medium of Hopwood (1967) was used as a basal medium and growth of *M.echinospora* was studied on various carbon and nitrogen sources (Table 4 & 5). It was found that asparagine and glucose permitted full differentiation and maximal biomass formation It was decided to use these nutrients as the principal carbon and nitrogen sources within the medium.

In order to examine the effects and interactions of glucose and asparagine at various initial concentrations, a matrix of concentrations was designed around the initial medium of Hopwood (1967). The initial glucose and asparagine concentrations were examined at eight levels and the levels of biomass formed and CFU's generated (a crude measure of sporulation) were quantified. The x, y and z data are shown in table 6 and 7. The data was analysed and interpolated using Stanford graphics software (Stanford University). The response surface for each growth parameter examined is presented in Figure X and Y.

Table 4. The effect of carbon source on biomass formation and CFU's in shake flask cultures.

Carbon source (Supplied at 10 g.L^{-1})	Stationary-phase biomass (g. L^{-1})	CFU (ml^{-1})
Glucose	1.3 ± 0.4	8.2 x 10^8
Sucrose	1.9 ± 0.5	3 x 10^6
Maltose	1.4 ± 0.3	2.4 x 10^6
Mannitol	1.9 ± 0.6	7.2 x 10^5
Mannose	1.1 ± 0.2	3 x 10^6

Ammonium sulphate was supplied as the nitrogen source in each case at 2 g. L^{-1}.

Table 5. The effect of nitrogen source on biomass formation and CFU's in shake flask cultures.

Nitrogen source (Supplied at 0.5 g.L^{-1})	Stationary-phase biomass (g. L^{-1})	CFU (ml^{-1})
Glutamate	0.9 ± 0.2	4×10^8
Glutamine	1.2 ± 0.5	2.5×10^7
Asparagine	1.45 ± 0.25	2.4×10^8
Alanine	1.2 ± 0.45	6×10^7
Ammonium sulphate	0.8 ± 0.15	4.2×10^5
Sodium nitrate	0.45 ± 0.2	3×10^5

Glucose was supplied as the principle carbon source in each case at 10 g. L^{-1}.

The data analysed by the response surface for biomass (Fig 40) indicates that optimal biomass is achieved when cultures are grown at high concentrations of carbon (>450 mM) and moderate to high levels of asparagine (>5 mM and <12 mM). The response surface constructed for sporulation (fig 41), assessed by the number of CFU's produced on each medium, demonstrated that levels of carbon greater than 200 mM, yet lower than 500 mM permitted significant sporulation. At higher levels of nitrogen, (> 8 mM) sporulation was much more pronounced, with the response surface revealing an effect directly related to the levels of carbon within the medium, with high levels (500mM) of carbon possibly repressing sporulation. This methodology does not allow the exact levels of nutrient required for maximum biomass or sporulation to be determined, however it does indicate that the general relationship between the levels of carbon and nitrogen influencing biomass formation and sporulation are important in the promotion of biomass formation and sporulation.

Table 6. Two-variable surface response design and biomass in shake flask cultures.

mM of Carbon	mM of Nitrogen	Biomass (g. L^{-1})
43.5	7.6	0.1667
87	7.6	0.3
174	7.6	0.233
261	7.6	0.3
348	0.945	0.0428
348	1.89	0.136
348	3.79	0.14
348	5.68	0.3347
348	9.47	0.5557
348	11.4	0.5918
348	13.25	0.54421
435	7.6	0.733
522	7.6	0.66
609	7.6	0.7
43.5	0.945	0.09595
609	1.89	0.1979
174	3.79	0.42
261	5.68	0.5808
43.5	13.26	0.0303
174	11.4	0.57979
522	3.79	0.4373
435	9.47	0.6979
522	11.4	0.806
609	13.26	0.533
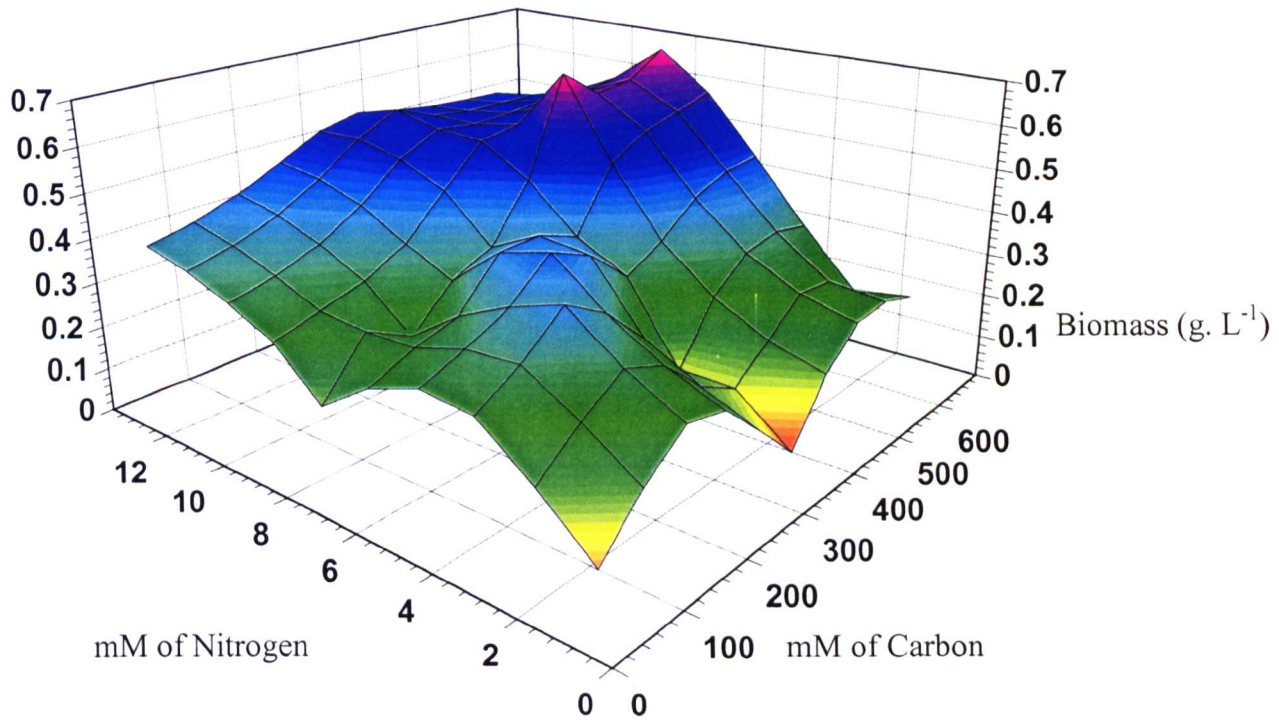
Figure 40. **3-D response surface plot of biomass.** Plot of biomass (z) using initial medium concentrations of carbon (x) and nitrogen (y) as variables. Data was recalculated from initial data (Table x) using Stanford graphics (Stanford Software).
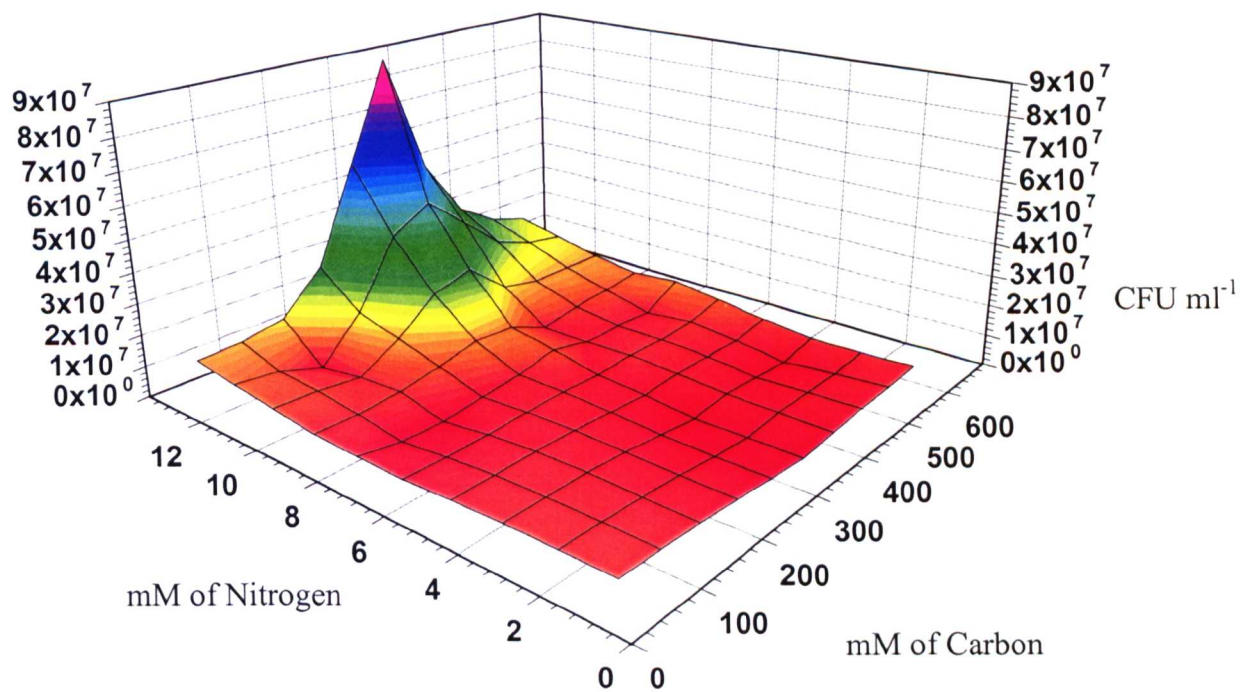
Figure 41. **3-D response surface plot of sporulation.** Plot of sporulation (z) using

initial medium concentrations of carbon (x) and nitrogen (y) as variables. Data was

recalculated from initial data (Table x) using Stanford graphics (Stanford Software).

Table 7. Two-variable surface response design and sporulation in shake flask cultures.

| mM of Carbon	mM of Nitrogen	CFU ml^{-1}
43.5	7.6	3.8×10^6
87	7.6	5.8×10^4
174	7.6	8.5×10^5
261	7.6	7.6×10^5
348	0.945	2.4×10^5
348	1.89	8.95×10^3
348	3.79	3.3×10^4
348	5.68	1.6×10^6
348	9.47	8.5×10^6
348	11.4	4.35×10^7
348	13.25	9×10^7
435	7.6	2.6×10^6
522	7.6	1.1×10^6
609	7.6	1×10^7
174	11.4	1.08×10^4
522	3.79	1.1×10^5
435	9.47	1.3×10^6

3.3.3 Determination of conditions supporting carbon and nitrogen limiting growth

It would appear from figure 40 and 41, that sporulation may be induced in response to limitation by a specific nutrient. The work of Kendrick & Ensign, (1983), Daza *et al.*, (1989) and Glazebrook *et al.*,(1990) indicated that nutrient shiftdown stimulates sporulation in liquid culture in certain streptomycetes.

The molar C/N ratios that result in carbon-limited and nitrogen-limited growth of *M. echinospora* in minimal medium were achieved by varying the concentration of one nutrient, whilst maintaining the other at a constant level. This rationale resulted in a linear response to increasing substrate concentrations. The carbon component of the asparagine molecule was accounted for in the calculation of the C/N ratio. The tailing of the linear response indicates that more than one nutrient is becoming limiting. Growth that was stoichiometrically carbon-limited was below a C/N ratio of 57:1, indicated by a non-linear response above that value (Figure 42). Stoichiometrically nitrogen-limited growth was found to occur above a C/N ratio of 37:1 (Fig. 43). This resulted in a transitional zone of apparent dual nutrient limitation, between 37 and 57:1. To ensure that nutrient-limiting conditions were within the single nutrient-limiting boundary, carbon-limiting conditions were set at 15:1, and nitrogen-limiting conditions were set at 80:1. Subsequent nutrient-limiting experiments were carried out under these conditions.

The level of phosphate within the cultures was found to remain above 10 μg. ml^{-1}, indicating that these cultures were not phosphate limited. The addition of trace salts was in excess, with no difference in biomass formation observed when 50 % less

trace salts were added to shake flask cultures. This indicated that only carbon and

nitrogen were limiting growth under the studied conditions.
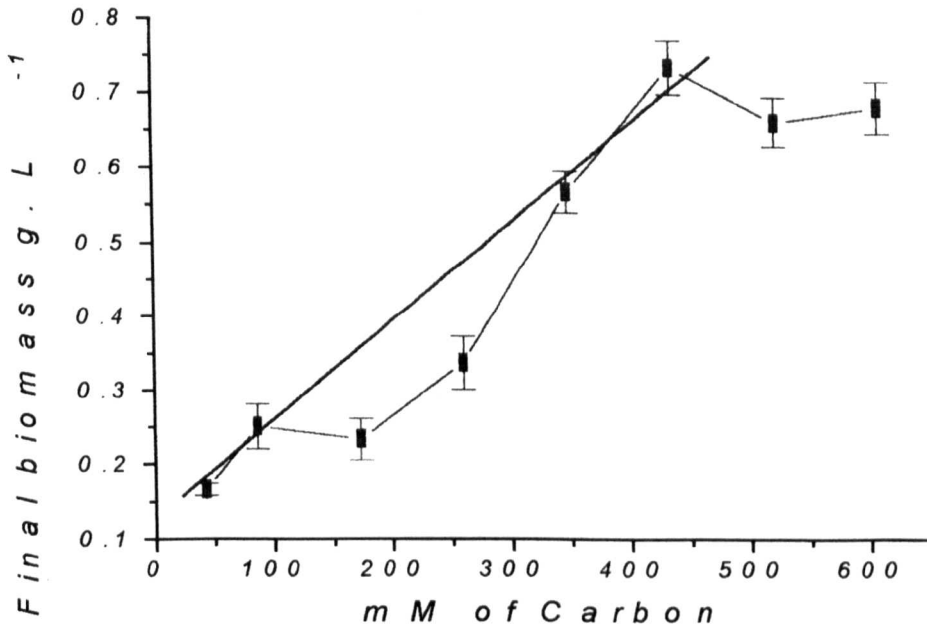
Figure 42. **The effect of increasing substrate levels**. The effect of increasing levels of carbon upon maximum biomass formation. In all cases the initial nitrogen concentration was 7.6 mM. Values are the mean of at least three determinations. Error bars represent the standard deviation of the data.
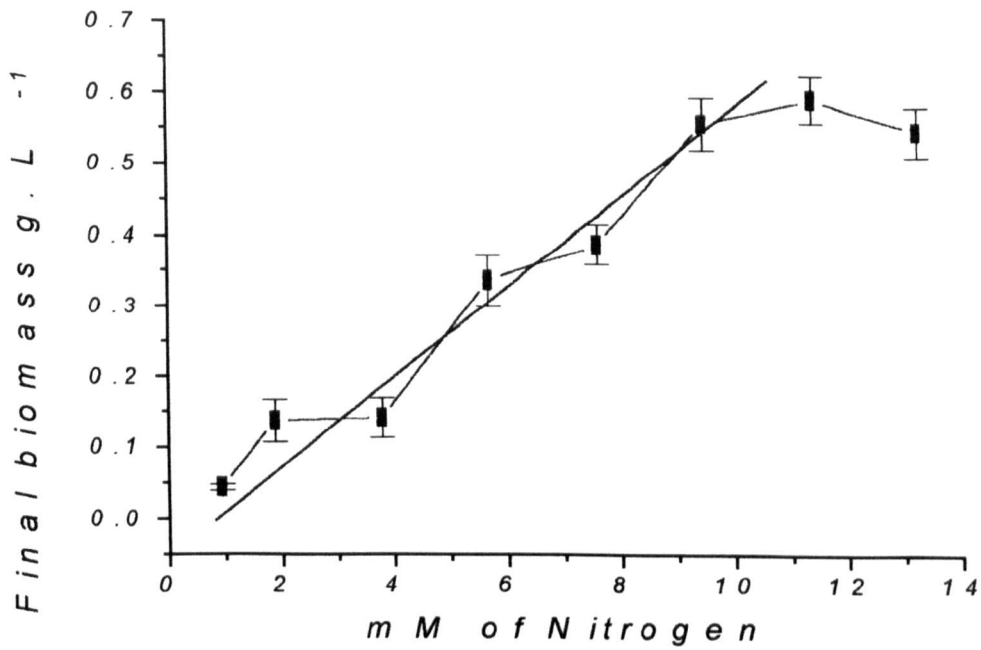
Figure 43. **The effect of increasing substrate levels**. The effect of increasing levels of nitrogen upon maximum biomass formation. In all cases the initial carbon concentration was 348 mM. Values are the mean of at least three determinations. Error bars represent the standard deviation of the data.

3.3.4 The effect of carbon to nitrogen molar ratio upon biomass and sporulation.

The molar carbon/nitrogen ratio of the growth medium has been shown to effect sporulation in fungi (Trinci & Collinge, 1974; Smith, 1978). The response surfaces created in this work demonstrated that C/N ratio of the growth medium had an effect upon biomass formation and sporulation. Further work was conducted (Fig 44 & 45), growing *M. echinospora* in chemically defined medium with varying initial C/N ratio's. The relationship between initial medium C/N ratio and biomass formation illustrates that maximum biomass is formed between C/N ratio of 15-138:1, whilst lower than 15:1 and higher than 138:1 resulted in low yields of biomass (<0.3 g. L^{-1}). The effect of C/N ratio upon sporulation, assessed by the level of CFU's formed, indicated a much closer relationship, with ratio's above 15:1 and less than 42:1 permitted maximum sporulation. Above and below these values the CFU's were greatly reduced, which may reflect the nature of the limiting nutrient. The limitation of carbon (C/N<15:1) results in low-level sporulation, however above this value, sporulation is abundant, until the C/N ratio becomes nitrogen limiting. This would suggest that in *M. echinospora* the abundance of carbon, and limitation of nitrogen (>37:1) results in abundant sporulation. Thus from a physiological perspective nitrogen limitation results in the promotion of sporulation, as observed in many differentiating microorganisms (Trinci & Collinge, 1974; Smith, 1978).
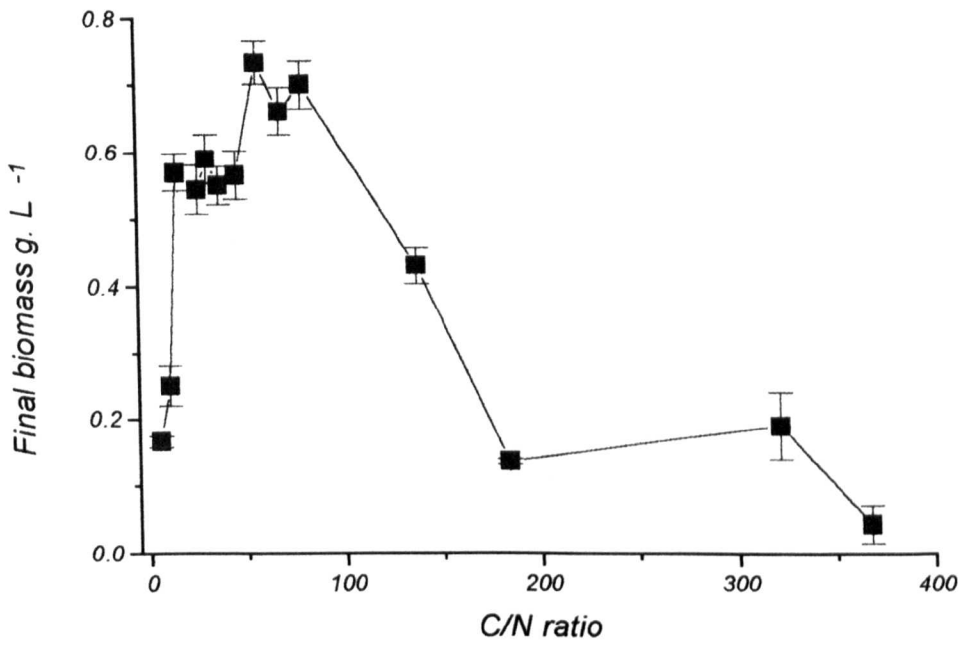
Figure 44. **The effect of molar C/N ratio on final biomass levels**. Values are the mean of at least three determinations. Error bars represent the standard deviation of the data.
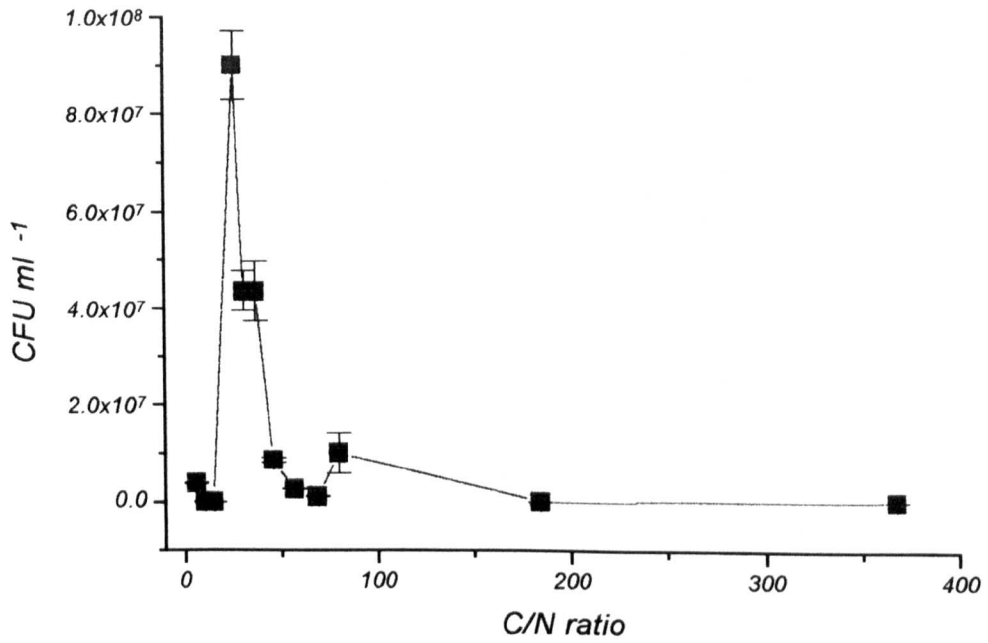
Figure 45. **The effect of molar C/N ratio on sporulation**. Values are the mean of at least three determinations. Error bars represent the standard deviation of the data.

3.3.5 Minimal medium

Previous results demonstrated that the nature and ratio of carbon and nitrogen has a profound effect upon growth and developmental of *M. echinospora*. To further investigate these relationships stoichiometrically limiting conditions for both carbon and nitrogen were established using chemically defined media.

3.3.5.1 Carbon-limited batch culture

Micromonospora echinospora was grown in chemically defined, stoichiometrically limiting for carbon. The cultures exhibited two main growth phases (Fig. 46). An initial slow growth period, extending up to 96 h post-inoculation, was followed by a rapid increase in the culture biomass, reaching maximum biomass of 1.2 g. L^{-1} at 144 h. Thereafter the biomass rapidly declined, with extensive mycelial fragmentation being observed. Carbon-limitation in actinomycetes has previously been shown to result in rapid cell lysis and biomass decline (Karandikar, 1998). A logarithmic plot of biomass against time revealed that the growth phase was exponential (Fig. 47), with a mean growth rate of 0.045 h^{-1} (t_d 15.4 h).

The appearance of spores within the medium was evident after 144 h post-inoculation (Plate 3). The number of CFU's within the culture was found to increase rapidly following the cessation of growth, it should be noted that this could be a reflection of the high degree of fragmentation observed, rather than a measurement of sporulation.

The rapid phase of growth under these conditions corresponded with the marked consumption of asparagine in the medium (Fig 48). The consumption of glucose was slow, and despite low-level consumption (0.25 g over the first 96 h of growth) the main glucose utilisation phase did not occur until 96 h post inoculation. These latter results are intriguing as the conditions were apparently stoichiometrically carbon-limiting, indicating that glucose may not be the primary carbon source under these conditions. The quantification of ammonia from the culture reveals that the rapid asparagine consumpion phase coincides with the appearance of ammonia in the medium. This suggests that deamination of asparagine is occurring, with concomitant ammonia excretion. The exhaustion of asparagine from the medium resulted in the utilization of the excreted ammonia, which remained detectable throughout the remainder of the fermentation indicating that nitrogen was not the factor limiting biomass production (fig. 49).

During batch growth, under carbon-limiting conditions, the growth yield was found to change (Fig 50) with the $Y_{x/glucose}$ being greater than that of $Y_{x/asp}$, indicating that the organism has a greater requirement for carbon derived from glucose under these conditions. The measurement of specific substrate uptake rates (q_s) for glucose and asparagine under carbon-limiting conditions reveals that uptake rates are similar during the rapid growth phase (Fig. 51), thereafter the rate of substrate uptake for glucose increases to a much higher degree ($q_{glucose} = 115$ g. g^{-1}. h^{-1}) than that of asparagine ($q_{asp.} = 68$ g. g^{-1}. h^{-1}).
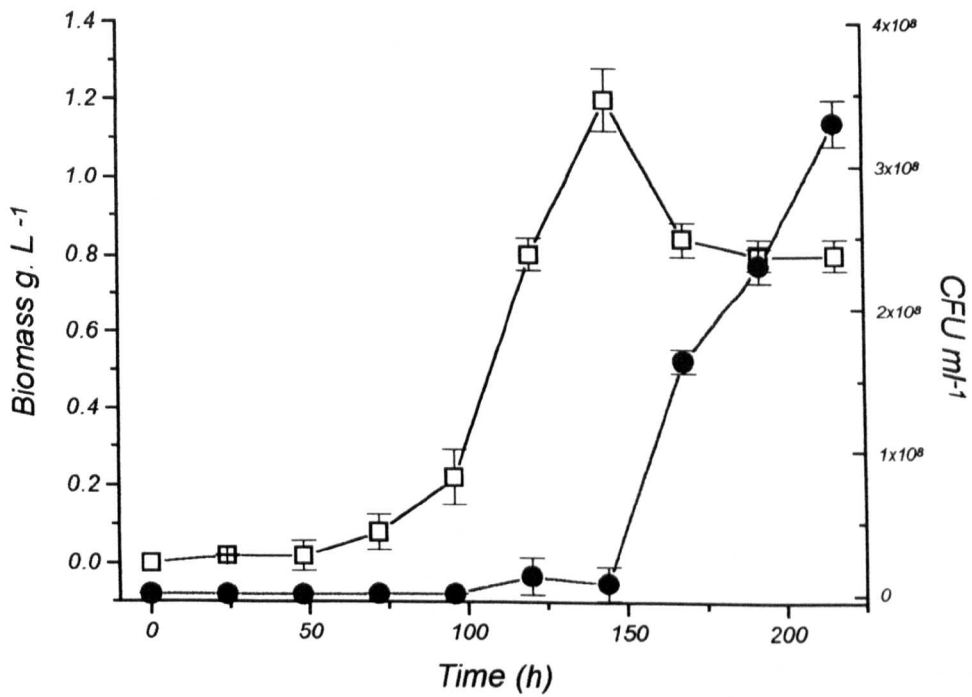
Figure 46. **Growth kinetics during batch culture in Carbon-limited minimal medium.** Biomass dry weight (-□-), and CFU (-●-). Values are the mean of at least three determinations. Error bars represent the standard deviation of the data.
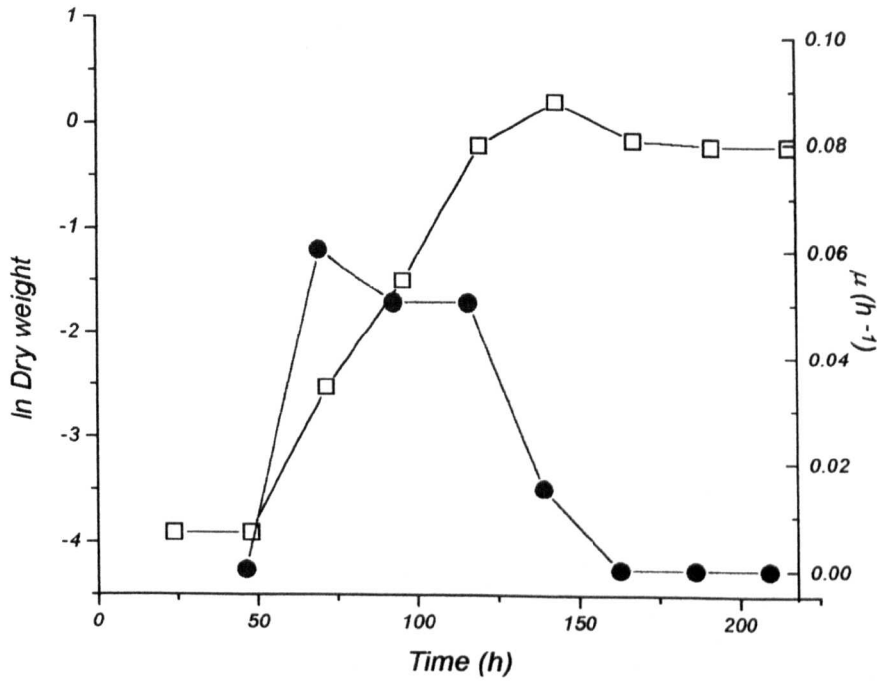
Figure 47. **Growth kinetics during batch culture in Carbon-limited minimal medium.** Natural log of Biomass (-□-), and specific growth rate, μ (-●-). Values are the mean of at least three determinations. Specific growth rates were calculated from the tangent of semi-logarithmic plot of the mean dry weight.
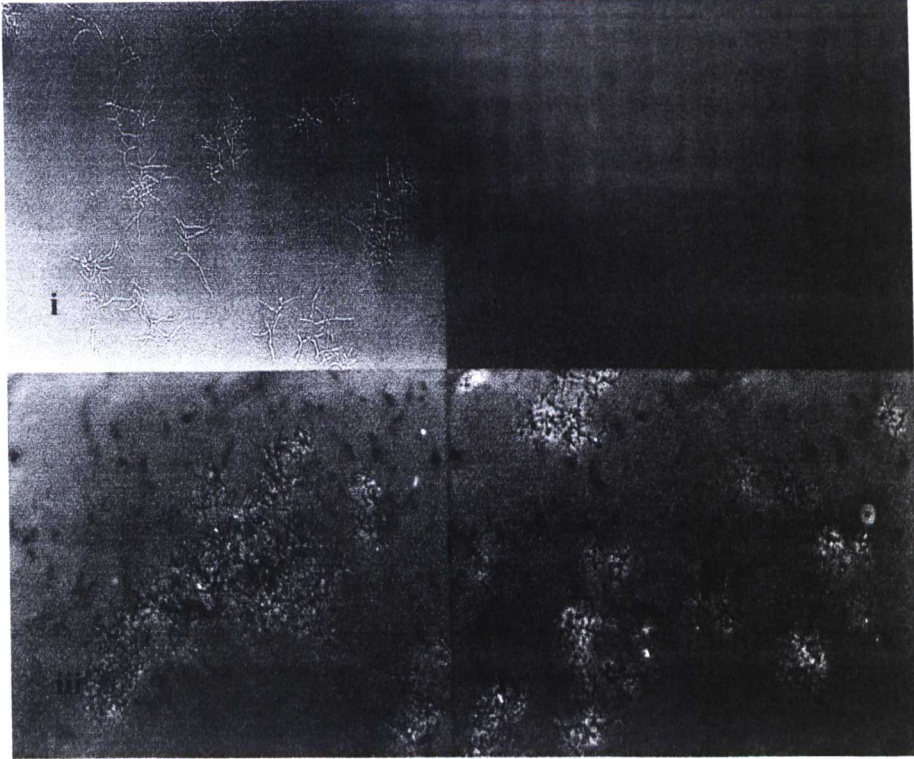
Plate 3. **Photomicrographs of mycelium at selected time points in carbon-limited minimal medium demonstrating a temporal profile of development.** i) young hyphae at 48 h post-inoculation. ii) Formation of aggregates of hyphae following 72 h of growth. iii) The formation of immature spores at 96 h post inoculation. iv) The presence of mature spores on the mycelial aggregates following 144h of growth.
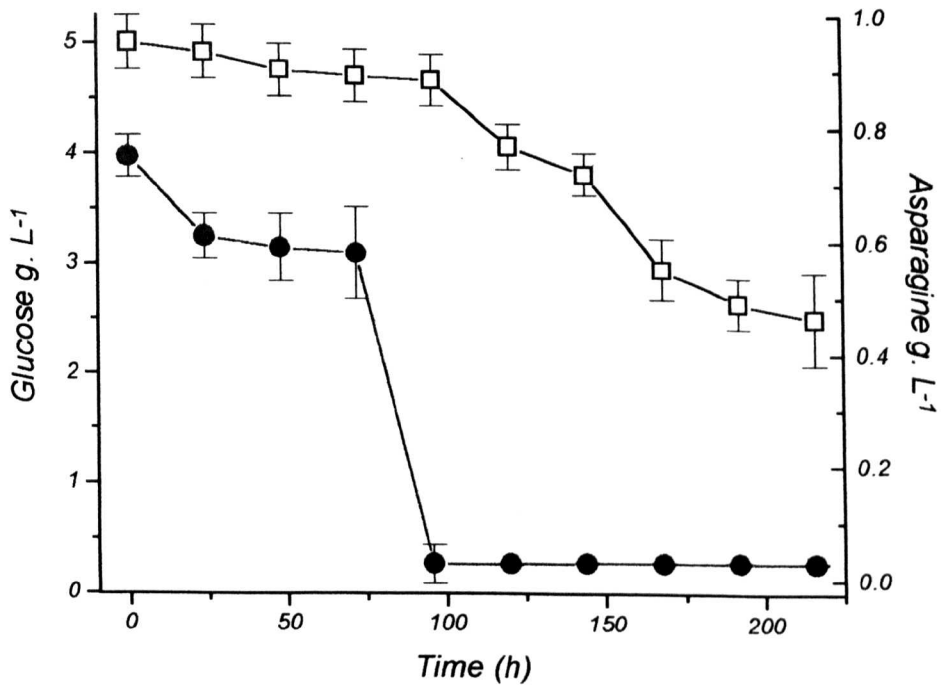
Figure 48. **Growth kinetics during batch culture in Carbon-limited minimal medium.** Residual glucose (-□-), and asparagine (-●-). Values are the mean of at least three determinations. Error bars represent the standard deviation of the data.
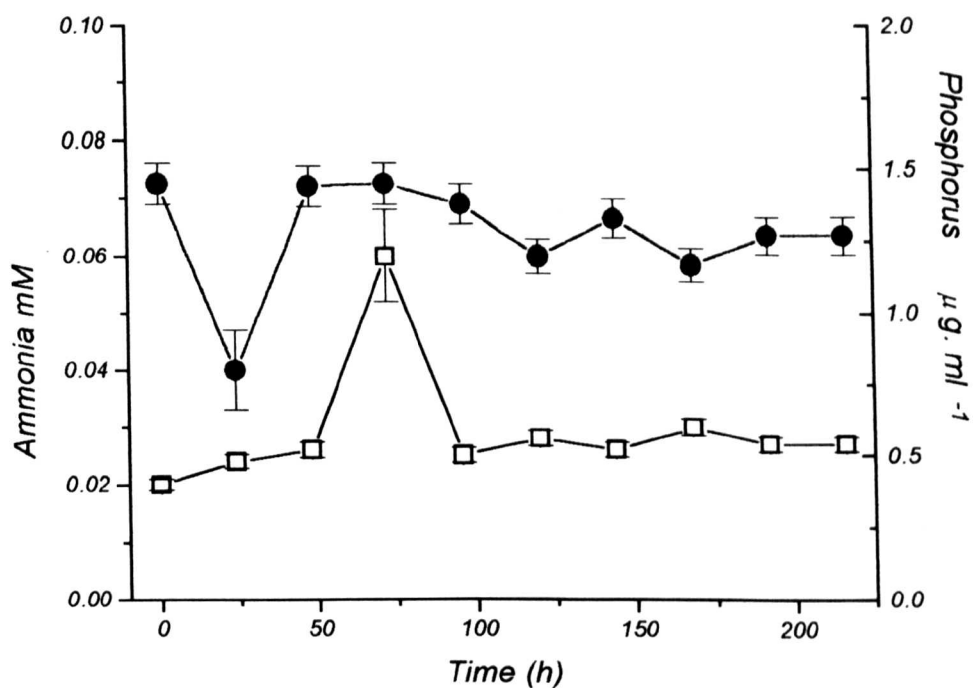
Figure 49. **Growth kinetics during batch culture in Carbon-limited minimal medium.** Residual phosphorus (-□-), and ammonia (-●-). Values are the mean of at least three determinations. Error bars represent the standard deviation of the data.
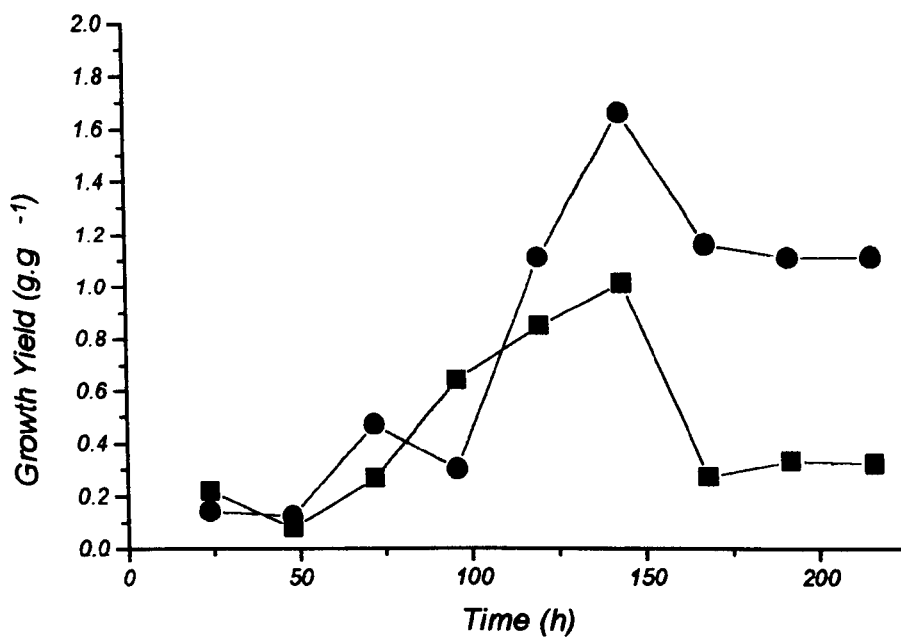
Figure 50. **Growth kinetics during batch culture in Carbon-limited minimal medium.** Growth yield from glucose (-●-), and asparagine (-■-). Growth yield values were determined from the mean biomass and mean residual substrate concentration (g. L^{-1}).
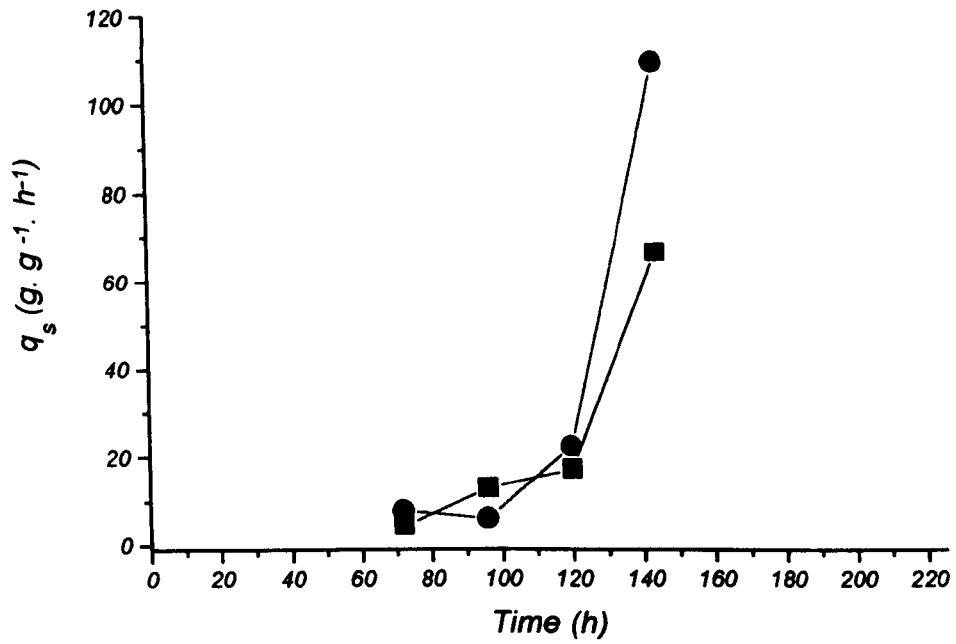
Figure 51. **Growth kinetics during batch culture in Carbon-limited minimal medium.** Specific substrate uptake rate for glucose (-●-), and asparagine (-■-). Values were determined from μ and the growth yield data shown in *Figure 50 & 47.*

3.3.5.2. Kinetics of carbonaceous storage compound accumulation in carbon-limited minimal medium.

Growth of *M. echinospora* in complex medium indicated the importance of carbon storage metabolism to this organism. Previous studies in other actinomycetes have revealed the roles of carbonaceous storage compounds to this group of bacteria (Brana *et al.*, 1986; Plaskitt & Chater 1995). The majority of this previous work has been carried out in complex medium, with the authors suggesting that conditions are nitrogen-limiting (Brana *et al.*, 1986). The accumulation of such compounds under carbon-limiting conditions is poorly studied and is generally believed not to occur (Dawes 1992).

Glycogen was found to be accumulated in a bi-phasic manner, with the initial phase of accretion occurring during the rapid growth phase of the organism (Fig. 52). This first phase of accumulation represented up to 2 % (w/w) of the biomass. The second phase of accumulation, resulted in the glycogen levels reaching 7 % of the biomass. This was unexpected as the levels of biomass were decreasing at this time suggesting cell lysis was occurring.

The accumulation of trehalose during carbon-limited culture was found to increase rapidly during the initial growth phase (96-120 h), and thereafter remained relatively constant (Fig. 53). The levels of trehalose present within the mycelium was low, representing up to 0.33 % (w/w) of the biomass. It has been suggested that trehalose may play a role in carbon flux within the cells of a range of organisms including *Mycobacterium* (De Smet *et al.*, 2000). The low level observed here may be a result of reduced carbon flux due to carbon-limitation.

Accumulation of storage compound during carbon-limitation indicates different roles for glycogen and trehalose. Trehalose appears to be accumulated during carbon-sufficiency and appears not to mobilised during carbon starvation, this is consistent with their role in sporulation. Glycogen is accumulated biphasically, suggesting that it is accumulated both during carbon sufficiency and under stress conditions.

3.3.5.3 Gentamicin production in carbon-limited minimal medium

Gentamicin could not be detected during carbon-limited batch culture. The availability of carbon within the culture may be the factor responsible for the lack of production under these conditions.
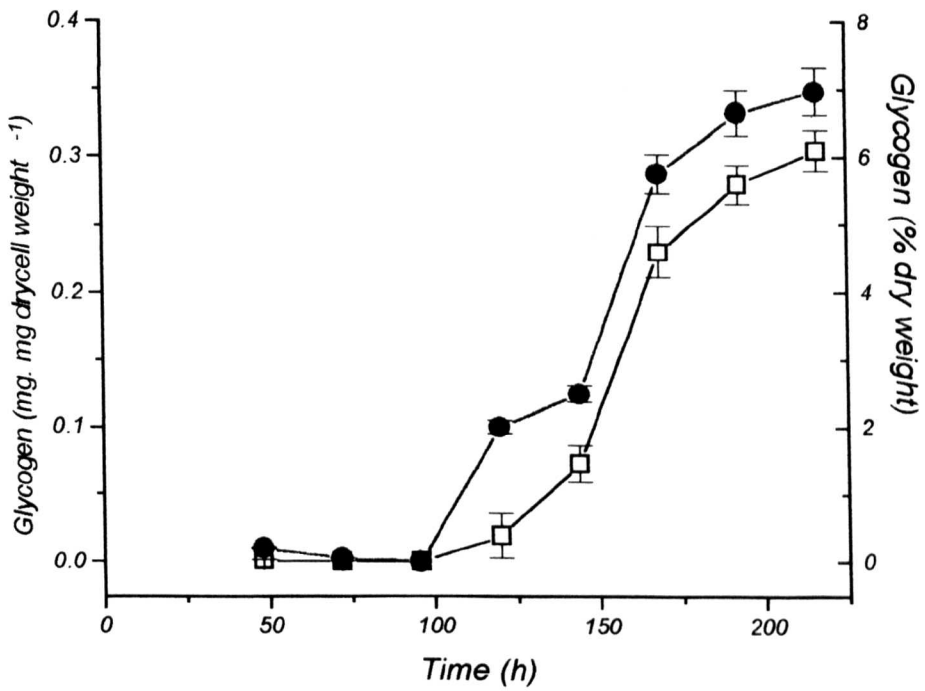
Figure 52. **Growth kinetics during batch culture in Carbon-limited minimal medium.** Accumulation of intracellular glycogen. Total cellular glycogen (-□-), and % glycogen of the dry cell weight (-●-). Values are the mean of at least three determinations. Error bars represent the standard deviation of the data.
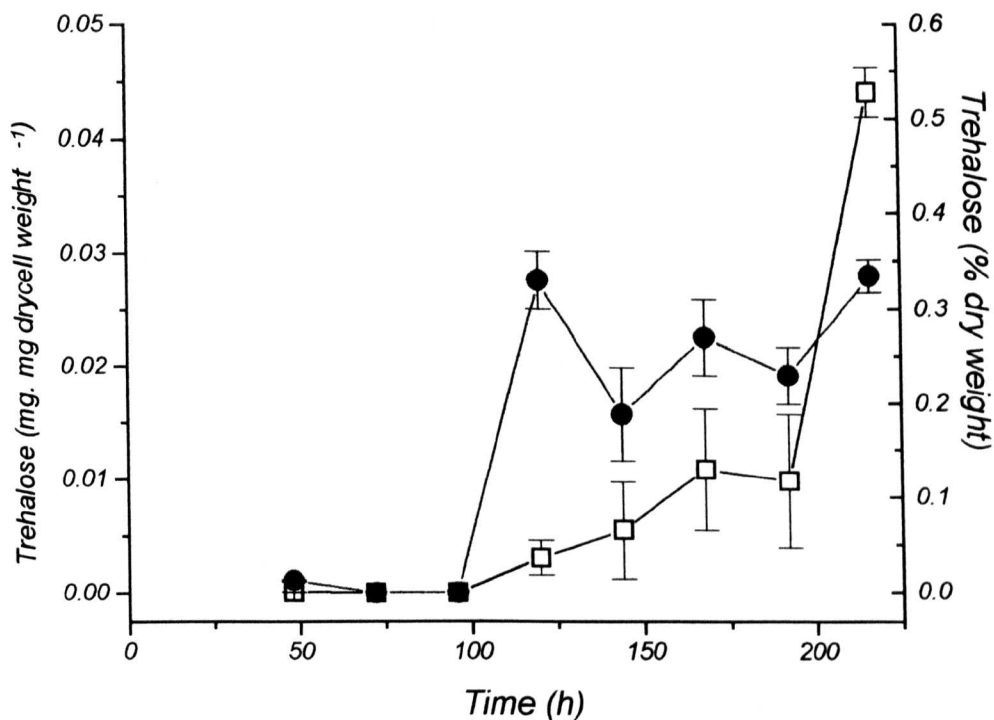
Figure 53. **Growth kinetics during batch culture in Carbon-limited minimal medium.** Accumulation of intracellular trehalose. Total cellular trehalose (-□-), and % trehalose of the dry cell weight (-●-). Values are the mean of at least three determinations. Error bars represent the standard deviation of the data.

3.3.6. Nitrogen limited batch culture

Micromonospora echinospora was grown in minimal medium, stoichiometrically limiting for nitrogen. Growth was multi-phasic under nitrogen-limiting conditions with an initial slow (lag) phase up to 48 h post-inoculation being succeeded by a rapid growth phase to 120 h (Fig. 54). The rapid growth phase was followed by a short period of growth arrest between 120 and 144 h, thereafter a second rapid accretion of biomass was observed, where maximum biomass (1.22 g. L^{-1}) was achieved after 168 h. The appearance of spores within the culture occurred at 120 h (Plate 4), with a steady increase in the CFU count from 96 h post-inoculation, and throughout the remaining of the growth period (Fig. 54).

A logarithmic plot of biomass against time reveals that growth is exponential up to 168 h. The mean specific growth rate was found to be 0.034 h^{-1}, allowing a mean culture doubling time of 20 h to be calculated.

The kinetics of nutrient utilisation during nitrogen-limited batch culture revealed that asparagine was completely utilised 72 h post-inoculation (Fig. 56). The appearance of ammonia in the culture medium was concurrent with the pattern observed in carbon-limited culture. The ammonia excreted being completely consumed following 96 h of culture, demonstrating the nitrogen-limiting nature of the medium (Fig. 57). Glucose utilisation during nitrogen-limited culture was low, with only 2.75 g of glucose being consumed throughout growth. The most rapid phase of glucose accumulation coincides with the second rapid biomass accretion phase following 120 h growth. These observations indicate that the carbon skeletons from amino acids may be used in preference to glucose under these conditions. Levels of phosphorus within the cultures remained relatively constant throughout growth (Fig. 57).

Figure 54. **Growth kinetics during batch culture in Nitrogen-limited minimal medium.** Biomass dry weight (-□-), and CFU (-●-). Values are the mean of at least three determinations. Error bars represent the standard deviation of the data.

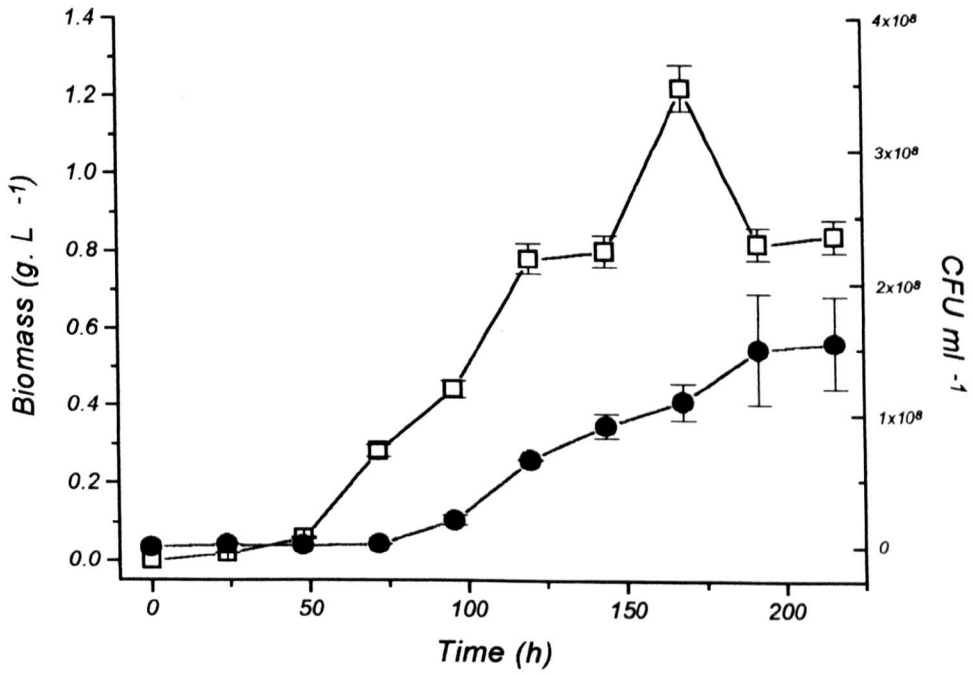*Figure 55.* **Growth kinetics during batch culture in Nitrogen-limited minimal medium.** Natural log of Biomass (-□-), and specific growth rate, μ (-●-). Values are the mean of at least three determinations. Specific growth rates were calculated from the tangent of semi-logarithmic plot of the mean dry weight.
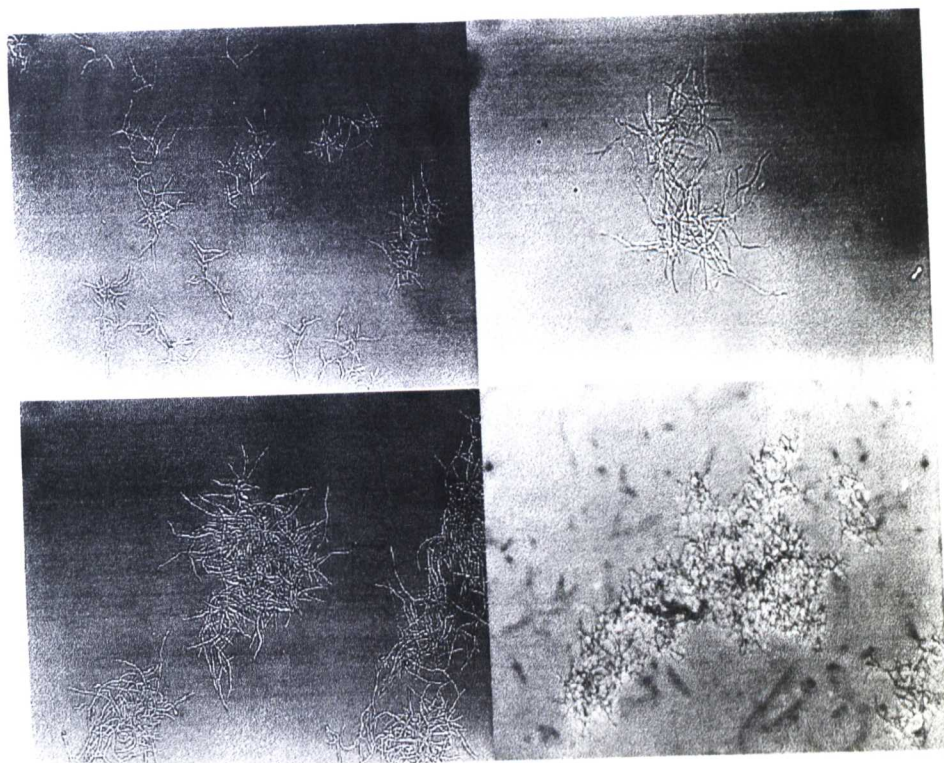
Plate 4. **Photomicrographs of mycelium at selected time points in nitrogen-limited minimal medium demonstrating a temporal profile of development.** i) young hyphae at 48 h post-inoculation. ii) Formation of aggregates of hyphae following 72 h of growth. iii) The formation of immature spores at 96 h post inoculation. iv) The presence of mature spores on the mycelial aggregates following 144h of growth.
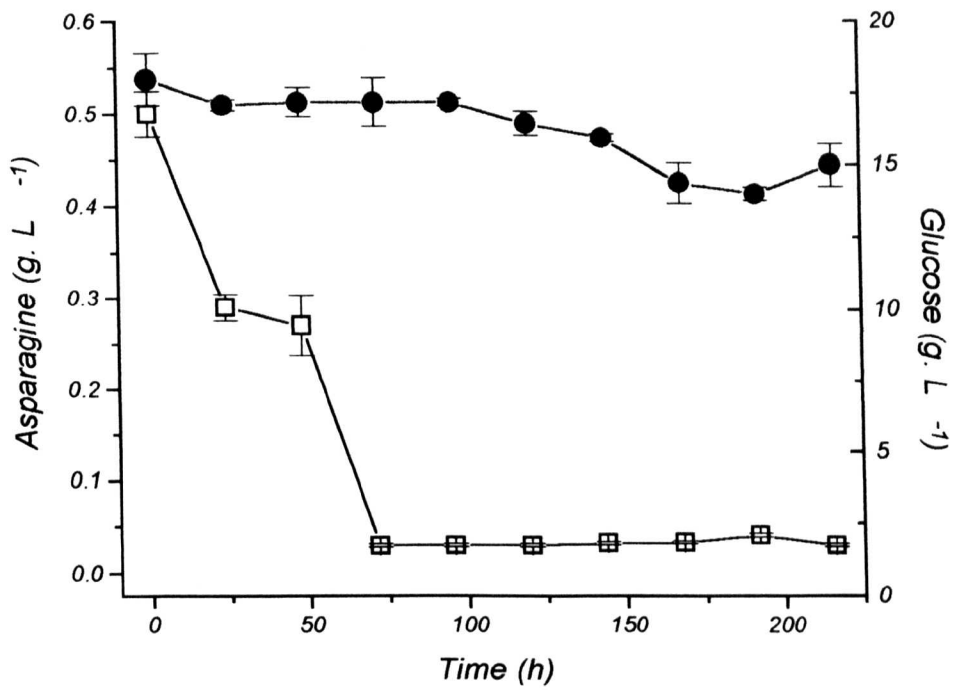
Figure 55. **Growth kinetics during batch culture in Nitrogen-limited minimal medium..** Residual asparagine (-■-), and glucose (-●-). Values are the mean of at least three determinations. Error bars represent the standard deviation of the data.
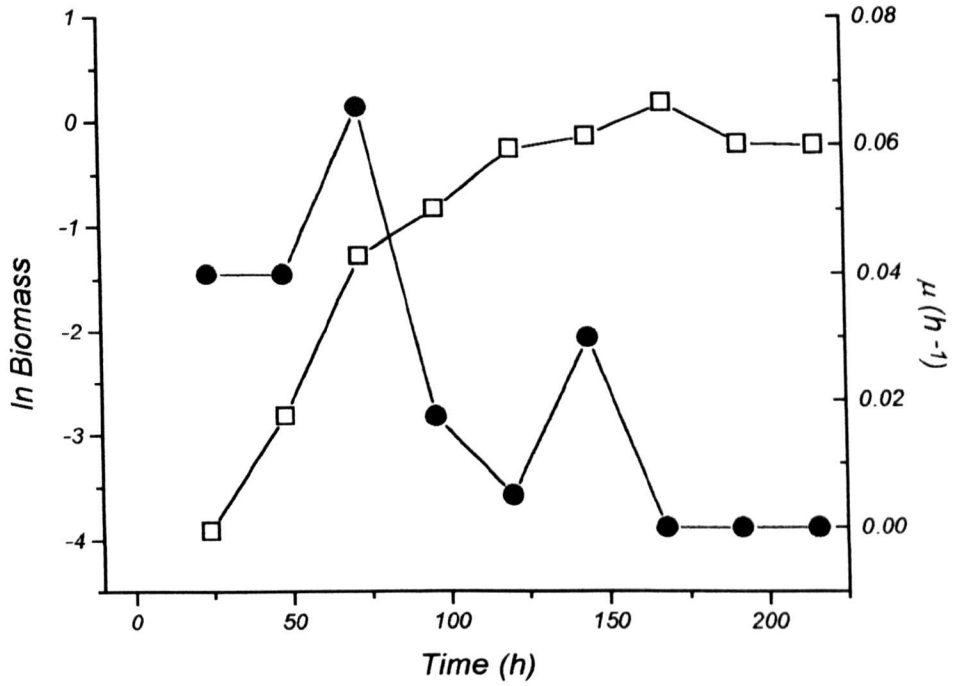
Figure 57. **Growth kinetics during batch culture in Nitrogen-limited minimal medium.** Residual phosphorus (-□-), and ammonia (-●-). Values are the mean of at least three determinations. Error bars represent the standard deviation of the data.

The growth yields for asparagine ($Y_{x/asp}$) and glucose ($Y_{x/glucose}$) were calculated during growth under nitrogen-limited batch culture (Fig. 58). The growth yield on asparagine was found to be higher than that of glucose throughout the culture period, suggesting a higher requirement for nitrogen under these conditions. This high $Y_{x/asp}$ (maximum = 2.75 g. g^{-1} compared to maximum $Y_{x/glucose}$ = 0.5 g. g^{-1}) could reflect a preference for the amino acid derived carbon within the system. The specific substrate uptake rates (q_s) for asparagine (q_{asp}) and glucose ($q_{glucose}$) also demonstrate that asparagine is consumed throughout growth to a higher degree (maximum q_{asp} = 160 g.g^{-1}.h^{-1}; 1.2 g. M of C. h^{-1}) than glucose (maximum $q_{glucose}$ = 90 g.g^{-1}.h^{-1}; 0.5 g. M of C. h^{-1}), reinforcing the notion that the primary carbon source for *M. echinospora* under these conditions is that derived from asparagine and glucose supplies only supplementary carbon to the system (fig. 59).
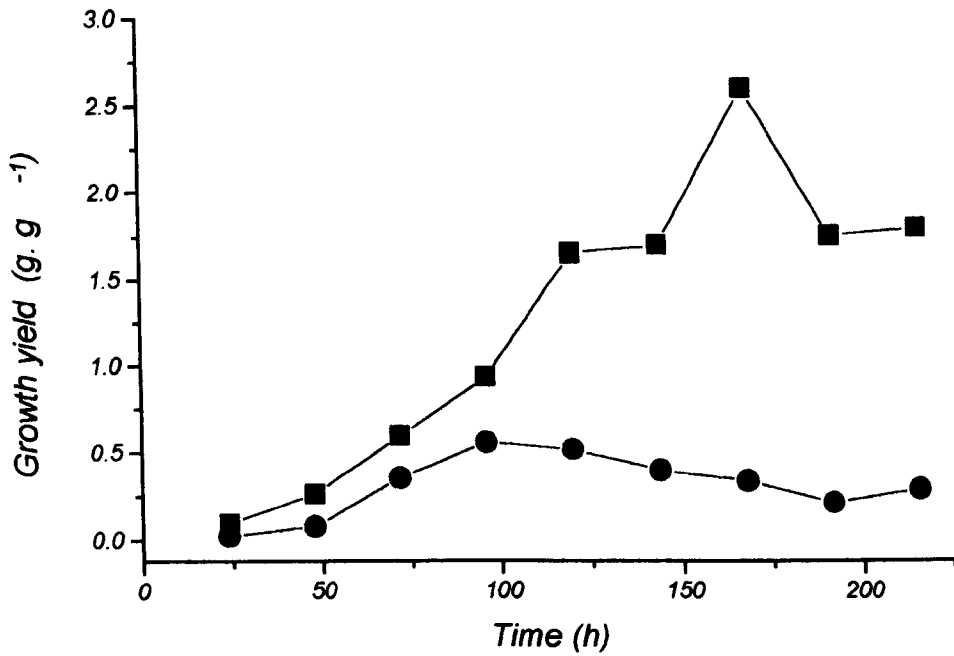
Figure 58 . **Growth kinetics during batch culture in Nitrogen-limited minimal medium** Growth yield from glucose (-●-), and asparagine (-■-). Growth yield values were determined from the mean biomass and mean residual substrate concentration (g. L^{-1}).

Figure 59. **Growth kinetics during batch culture in Nitrogen-limited minimal medium** Specific substrate uptake rate for glucose (-●-), and ninhydrin-positive amino compounds (-■-). Values were determined from μ and the growth yield data shown in *Figure* X.

3.3.6.1 Kinetics of carbonaceous storage compound accumulation in nitrogen-limited minimal medium.

Growth of *M. echinospora* under nitrogen-limiting conditions revealed a period of growth arrest between 120 and 144 h, prior to a rapid increase in biomass (Fig. 54). In order to establish if this final rapid biomass accretion phase was the result of increased cell proliferation, or a reflection of increased activity of storage metabolism, the levels of glycogen and trehalose were quantified during the growth period.

Glycogen exhibited a multi-phasic accumulation pattern (Fig. 60) with an initial accumulation phase after 48 h of growth accounting for 3 % (w/w) of the biomass. Thereafter the cellular glycogen levels decreased, before a rapid phase of accumulation which coincided with the rapid growth phase of the culture (72-96 h). A second rapid accumulation phase was observed between 120-144 h, coinciding with the period of growth arrest. It is possible that this second rapid accumulation phase, coinciding with growth arrest is linked with sporulation, similar to the observations of Chater & Plaskitt (1995) who demonstrated that intracellular glycogen is required for spore maturation in *Streptomyces coelicolor*. Evidence for this later observation is provided by the subsequent decrease in cellular glycogen levels (144-192 h), which coincides with the maximum levels of sporulation observed in the culture (Fig. 54 & 60).

Intracellular levels of trehalose were low throughout the early rapid growth phase (48-96 h), increasing during the early sporulation phase (96-144 h), accounting for up to 3 % of the biomass, thereafter the levels declined rapidly (fig. 61).
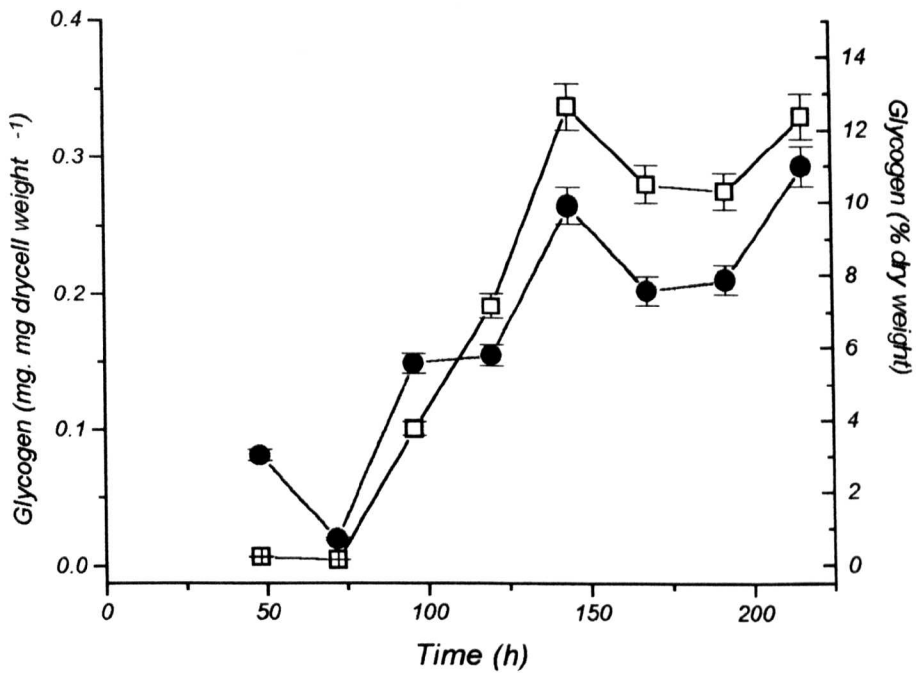
Figure 60. **Growth kinetics during batch culture in Nitrogen-limited minimal medium.** Accumulation of intracellular glycogen. Total cellular glycogen (-□-), and % glycogen of the dry cell weight (-●-). Values are the mean of at least three determinations. Error bars represent the standard deviation of the data.

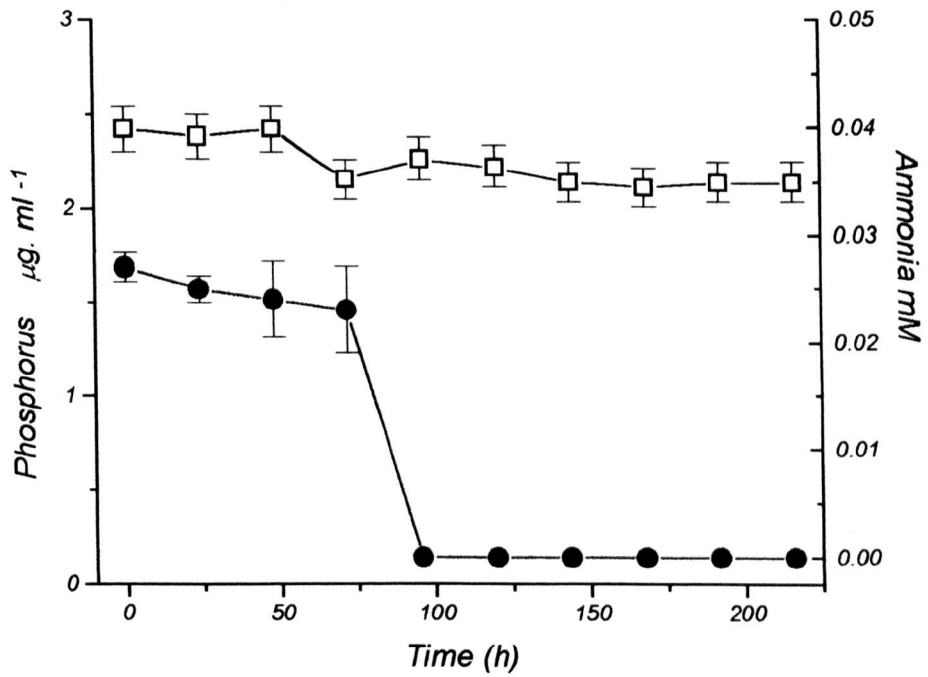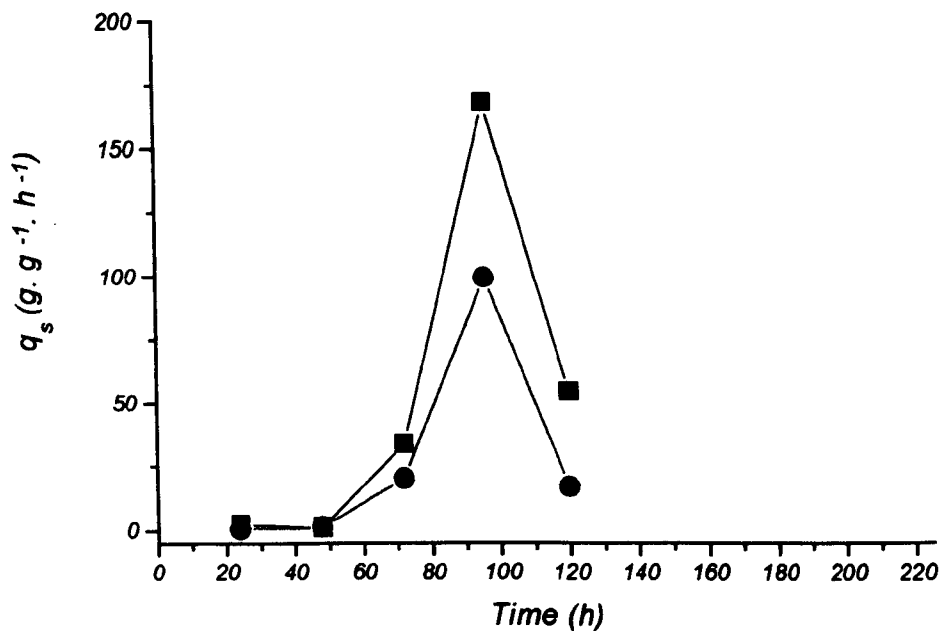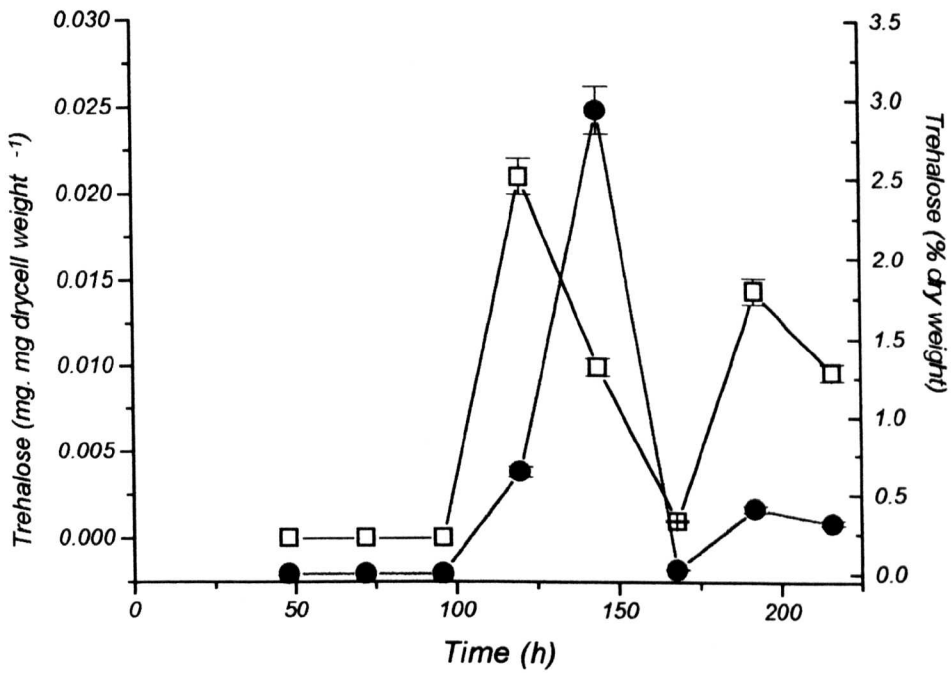*Figure 61.* **Growth kinetics during batch culture in Nitrogen-limited minimal medium** Accumulation of intracellular trehalose. Total cellular trehalose (-□-), and % trehalose of the dry cell weight (-●-). Values are the mean of at least three determinations. Error bars represent the standard deviation of the data.

3.3.6.2 Gentamicin production in nitrogen-limiting batch culture

Gentamicin was not detected during growth under nitrogen-limiting conditions in

batch culture.

3.3.6.3 Summary of carbon & nitrogen-limited batch growth

The establishment of conditions stoichiometrically limiting for carbon and nitrogen allowed the specific physiological responses to single nutrient limitations to be assessed and compared. Physiological parameters obtained during nutrient limited batch culture are summarised in table 8. The degree of sporulation, measured by CFU's generated under carbon-limitation was greater than that obtained under nitrogen-limitation. The degree of fragmentation observed, however, under carbon-limitation may have affected the CFU measurement under these conditions.

The growth yields obtained under each nutrient limiting condition reflected the nature of the limiting growth substrate and specific substrate uptake rates.

The accumulation of carbonaceous storage compounds, glycogen and trehalose, was greater under nitrogen-limiting conditions. This could be due to the lack of excess of the preferred carbon source (asparagine) under carbon-limiting conditions, rather than a true representation of the physiology of the organism under the given conditions. Glycogen biosynthesis has long been associated with nitrogen-limitation (Dawes, 1992). The role of trehalose is poorly understood, yet it has been implicated in the stress response and as an intracellular carbon sink in *Saccharomyces* and *Mycobacterium*, along with the well established role as a storage compound in spores of several actinomycetes (McBride & Ensign, 1987 a, b). Recent research has also implicated a complex interaction between glycogen and trehalose metabolism with a conserved, duplicated operon containing genes for the formation of both storage compounds in *Streptomyces coelicolor* (Schneider *et al.*, 2000).

Table. 8. Comparison of physiological responses under carbon and nitrogen-limiting conditions.

Physiological parameter	Carbon-limiting conditions	Nitrogen-limiting conditions
Maximum biomass (g. L^{-1})	1.20	1.22
Maximum CFU's (ml^{-1})	3.25×10^8	1.55×10^8
Mean specific growth rate (h^{-1})	0.045	0.034
Maximum growth yield – asparagine ($g.g^{-1}$)	1.0	2.6
Maximum growth yield – glucose ($g.g^{-1}$)	1.7	0.6
Maximum specific substrate uptake rate ($g.g^{-1}. h^{-1}$) – asparagine	65	170
Maximum specific substrate uptake rate ($g.g^{-1}. h^{-1}$) – glucose	110	100
Maximum cellular glycogen content (% dry wt.)	9	11
Maximum cellular trehalose content (% dry wt.)	0.35	3

3.3.7. Influence of carbon source on the production of gentamicin

Growth of *M. echinospora* in minimal medium, under both carbon and nitrogen limited conditions did not permit detectable gentamicin production. Previous experiments indicated that the level of biomass may influence the production of gentamicin. Systematic studies of the formation of gentamicin are scarce in the literature, and all relate gentamicin formation in the closely related organism *M. purpurea*. Escalante *et al.*, (1992) demonstrated that high levels of glucose exerted a negative effect on the formation of gentamicin in *M. purpurea*. Carbon catabolite repression of antibiotic formation has also been documented in *Streptomyces clavuligerus* (Hu *et al.*, 1984).

To investigate the effect of carbon source on gentamicin formation in *M. echinospora*, the organism was grown in the basal minimal medium with asparagine as the nitrogen source (0.5 g. L^{-1}) in shake flask culture with various carbon sources. Maltose, glucose, glycerol and fructose containing medium did not permit the formation of gentamicin. The growth of *M. echinospora* in medium containing sucrose permitted the formation of gentamicin (7.2 µg. ml^{-1}). The levels of biomass obtained from Maltose, glycerol and fructose were relatively low compared to sucrose maybe explaining the inability to detect gentamicin. These results do, however suggest that catabolite repression may play a role in the repression of antibiotic formation in glucose grown culture on *M. echinospora*.

3.3.8 Continuous culture study of nutrient limited growth

Chemostat cultures can be used to study substrate-limited growth at a fixed growth rate, which overcomes the difficulty of studying nutrient-limitation in batch-culture. Batch culture conditions are transient and are always accompanied by changing growth rates (Pirt, 1975) whereas chemostat culture offers the potential to study the effect of nutrient-limiting conditions and growth rate as independent variables in a stable environment.

Escherichia coli, is considered to be the model bacterium, yet differs fundamentally in growth habit to the filamentous actinomycetes. Shahab *et al.,* (1996) used chemostat culture to examine the role of growth rate in the synthesis of protein and nucleic acid in *Streptomyces coelicolor*, comparing their findings to published data on *E. coli*. The use of the chemostat to study physiology of antibiotic production has demonstrated a variety of behaviours (Trilli *et al.,* 1987), in relation to growth rate, nutrient repression (Pirt & Righelato, 1967) and nutrient limitation has been implicated in secondary metabolite production in a range of organisms (Lilley *et al.,* 1981).

In this section, the physiology of *M. echinospora* was examined under nutrient limiting conditions at three dilution rates, to examine the relationship between product formation, cellular composition and growth rate.

3.3.8.1 Determination of culture conditions

Previous experiments enabled the establishment of conditions stoichiometrically limiting for carbon and for nitrogen. The maximum specific growth rate (μ_{max}) of *M. echinospora* was determined under carbon and nitrogen-limiting conditions (Table 9). The values of μ_{max} were determined by the increasing the dilution rate (*D*) above the critical dilution rate (D_c) inducing biomass wash out from the chemostat. The slope of the logarithmic plot is equal to μ_{max}-*D* (Pirt, 1975). The maximum specific growth rate (μ_{max}) was found to be greater under carbon limitation than nitrogen limitation. At dilution rates higher than 70 % of μ_{max}, Shahab *et al.*, (1996) observed a rapid decrease in biomass. Comparisons of *M. echinospora* μ_{max} with published values for other organism indicate that it is slow growing. Published values for *S. coelicolor* are in the range 0.025-0.35 h^{-1} (Hobbs *et al.*, 1990; Shahab *et al.*, 1996) and in *Escherichia coli* from 0.10-0.5 h^{-1}. It should be noted that these values are in various chemically defined and rich media.

It was therefore decided to examine growth *M. echinospora* at three dilution rates below 70 % of μ_{max} under each limiting nutrient. The selected dilution rates were as follows 0.034, 0.046, and 0.093 h^{-1}.

Kinetics of nutrient limited growth in chemostat culture.

Chemostat cultures were initiated by the addition of sterile medium to batch cultures grown to late exponential phase (~96 h). Once steady state was achieved, residual nutrients, product formation and cellular composition was measured. Steady state was considered to have been reached after a minimum of three culture residence

times had elapsed, following initial feeding and after each dilution rate change (Pirt, 1975).

Steady state biomass levels in the chemostat under the studied nutrient limitations demonstrated an inverse relationship to the dilution rate (Fig. 62). Each nutrient limitation exhibited similar levels of biomass at each dilution rate.

Gentamicin was produced at only one of the examined growth rates (0.034 h^{-1}) under nitrogen-limiting conditions, resulting in a medium gentamicin concentration of 5 $\mu g. ml^{-1}$. The calculated product yield for gentamicin was found to be 0.0019 $g.g^{-1}$. These results would suggest that nitrogen-limitation and slower growth rates influence biosynthesis of this antibiotic.

Specific substrate uptake rates were calculated over a range of growth rates during carbon and nitrogen-limited continuous culture. A surprising relationship between glucose uptake, asparagine uptake and growth rate was found (Table 10). The results indicated that as growth rates were increased glucose utilisation declined while asparagine utilisation increased. It is generally accepted that specific substrate uptake rates increase with specific growth rate in microrganisms (Pirt, 1975). How can this relationship be explained?

One possibility is that the presence of asparagine in the medium was affecting the rate of glucose comsumption in a growth rate linked manner. To investigate this further radioactive uptake studies were conducted to examine the impact of asparagine upon glucose uptake in this organism.

Table. 9. Maximum specific growth rate (μ_{max}) and culture doubling time (T_d) determined from culture wash-out kinetics according to the method of Pirt (1975).

Nutrient status	Maximum specific growth rate (μ_{max}), h^{-1}	Culture doubling time (T_d) h
Carbon-limited	0.19	3.7
Nitrogen-limited	0.14	5.0
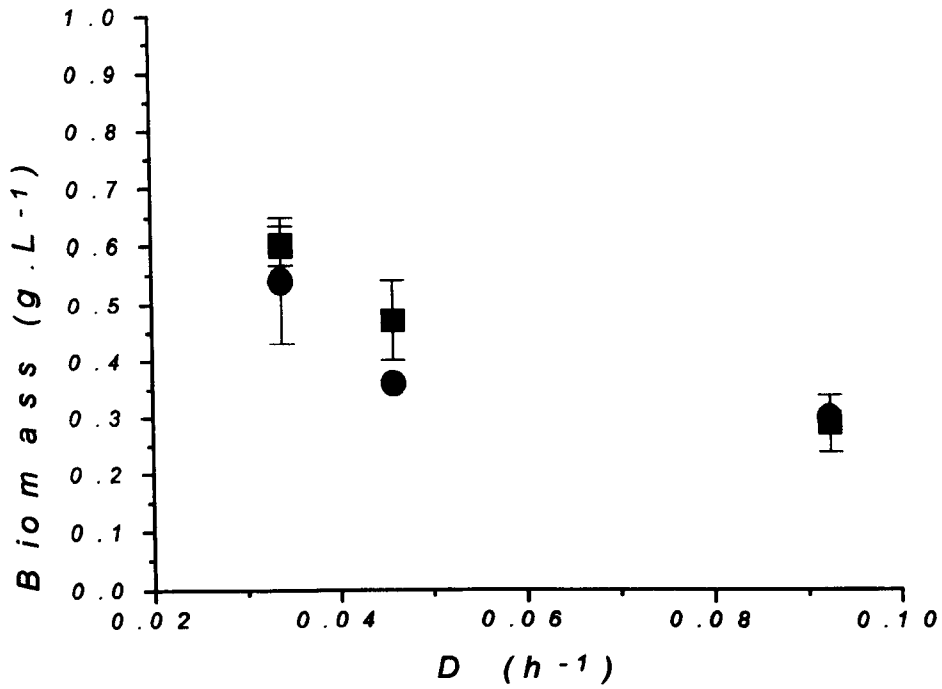
Figure 3. **Growth kinetics during continuous culture.** Biomass from carbon-limited chemostat (-●-), Biomass from nitrogen-limited chemostat (-■-). Values are the mean of at least three determinations. Error bars represent the standard deviation of the data.
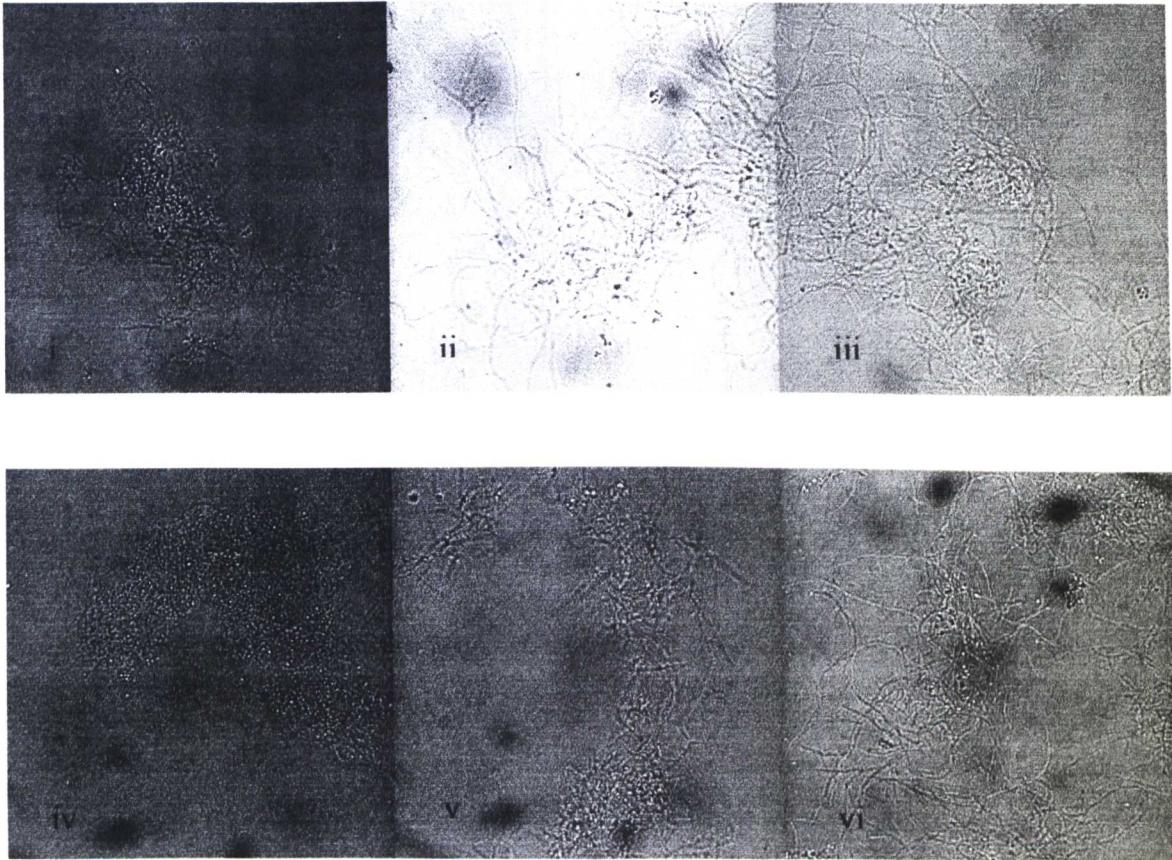
Plate 5. **Photomicrographs of mycelium at different in nutrient-limited minimal medium during continuous culture.** i) Nitrogen-limited chemostat at 0.034 h^{-1}. ii) Nitrogen-limited chemostat at 0.046 h^{-1}. iii) Nitrogen-limited chemostat at 0.093 h^{-1}. iv) Carbon-limited chemostat at 0.034 h^{-1}. v) Carbon-limited chemostat at 0.046 h^{-1}. vi) Carbon-limited chemostat at 0.093 h^{-1}.

Table 10. Specific substrate uptake rates and gentamicin yield during continuous culture growth under nutrient limiting conditions.

Nutrient status & specific growth rate (h⁻¹)	$q_{glucose}$ $(g.g^{-1}.h^{-1})$	q_{asp} $(g.g^{-1}.h^{-1})$	$Y_{gentamicin}$ $(g.g^{-1})$
Carbon-limited 0.034	68	19.5	0
Carbon-limited 0.046	26	14.5	0
Carbon-limited 0.093	11	31.0	0
Nitrogen-limited 0.034	69	0.01	0.0019
Nitrogen-limited 0.046	26	0.04	0
Nitrogen-limited 0.093	11	13.5	0

3.3.8.2 Uptake studies

Key to an organisms success in a growth medium is the ability to assimilate essential nutrients. Micromonosporas have evolved to exploite oligotrophic soil environments where the levels of free sugars and amino acids are low and growth limiting (Williams, 1985). It would not be unreasonable to assume that efficient transport systems for such nutrients exist in micromonosporas Previous experiments have indicated that *M. echinospora* has a poor uptake rate of glucose, and appears to utilise amino acids in preference to glucose. These experiments examined glucose and amino acid assimilation by *M. echinospora*.

During batch growth in mYEME , *M.echinospora* was found to utilize ninhydrin-positive amino compounds during the early stages of biomass accretion. Typical specific substrate uptake rates (q_s) revealed that glucose was not utilised until after 48h post-inoculation (Fig 34). Appearance of ammonia in the media provided evidence that deamination of the amino compounds had occurred (Fig. 35) indicating that the amino compounds were used as the primary carbon source in preference to glucose in this medium. This phenomenon was further characterised, by growing the organism in a defined minimal medium with asparagine (0.5 g.L^{-1}) as the sole nitrogen source and glucose (17.5 g.L^{-1}) as the major carbon source. Under these conditions the specific substrate uptake rate (q_s) of asparagine was greater than that of glucose (Fig 48) indicating that the amino acid constituted the major source of carbon in these fermentations.

$$y = 3.939 + 0.019\,x$$
$$r = 0.937$$
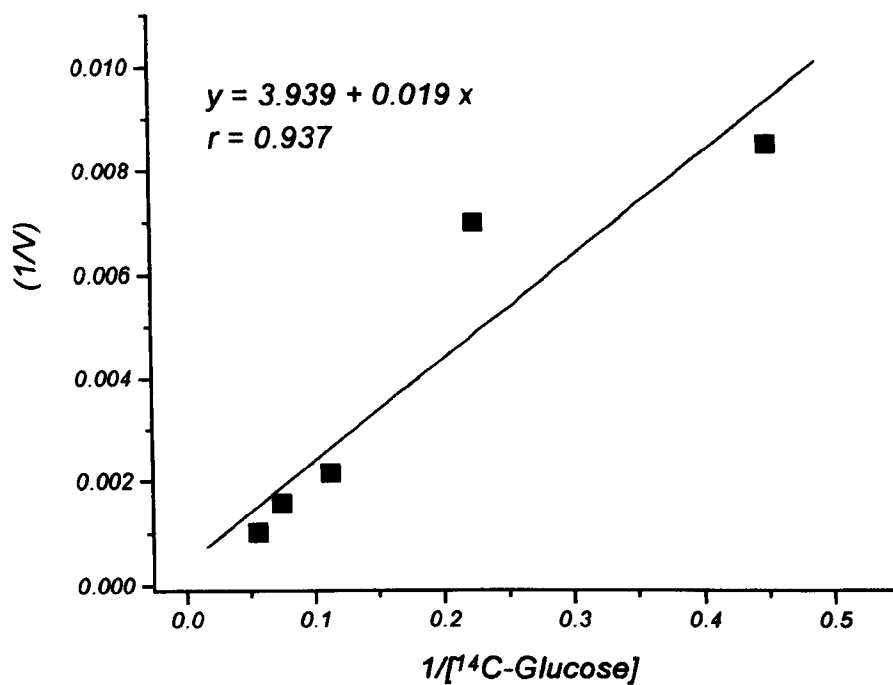
Figure. 63. Double reciprocal plot of glucose uptake rate (mM min^{-1}. g (Dry wt.)$^{-1}$ against glucose concentration (mM). The K_s for glucose in this organism was estimated at 53 mM.

Radioactive substrate uptake studies, using mycelia grown in minimal medium in a nitrogen-limited continuous culture (D = 0.046 h^{-1}), were used to determine the substrate affinity constant (Km) for glucose. Under these conditions the Km was determined to be 53 mM (Fig 63) . Km values previously reported for glucose uptake in streptomycetes range from 0.05-6.2mM (Hodgson, 2000), indicating that under the conditions examined, *M. echinospora* has a poor affinity for glucose. The Vmax for this system was determined to be 555 mM. Similarly high Vmax values have been reported for facilitator systems in bacteria, present during high substrate concentrations (Lengeler, 1993). This low affinity, high capacity uptake system may go part way toward explaining the relatively slow growth rates observed.

With such a low affinity, high capacity process transport process it is tempting to speculate that the uptake of glucose occurred by a passive mechanism. To examine this possibility, experiments were conducted with a range of inhibitors of active transport and in all cases glucose uptake was reduced (Table 12) clearly indicating that glucose uptake by *M.echinospora* is an active process. This process appears dependant upon a proton motive force, uptake having been inhibited by CCCP, DNP and gramicidin, and the supply of ATP as indicated by the inhibition of uptake by the respiratory inhibitor KCN. This is perhaps not surprising as passive diffusion by substrate specific facilitators is rare in bacteria, being mainly restricted to organisms growing in environments with exceptionally high nutrient levels (Lengeler, 1993).

The uptake rate of glucose was found to be affected by the presence of an amino acid in the assay mixture (Table 11). Each of the amino acids tested decreased the glucose uptake rate by greater than 90% of the control. This suggested that the amino acids, or a product of their metabolism, modulated glucose uptake. Uptake rates for the non-metabolisable glucose analogue 2-deoxy-glucose in the presence of asparagine

revealed a less pronounced effect of the amino acid on the uptake kinetics (Table 11). This latter result suggesting that the decrease in glucose uptake is mediated by products of metabolism.

The observed low affinity for glucose does not completely explain the kinetics of nutrient consumption by this organism as the depletion of the amino acid during growth resulted in increased glucose utilization rates. This suggests a direct influence of amino acid upon carbon metabolism and contrasts with the phenomenon described for enteric bacteria where amino acid catabolism is repressed by glucose (Magasanik & Neidhardt, 1987). This phenomenon in *M. echinospora* may reflect the organisms natural soil ecology, where soluble organic nitrogen compounds may also provide an energetically efficient source of carbon, necessitating only one energy requiring uptake process.

The uptake of ^{14}C-glutamate was examined to understand the process of amino acid uptake in *M. echinospora*. The preference of amino acids to glucose would indicate an energy dependant process was required for amino acid uptake. Mycelia grown in minimal medium in a nitrogen-limited continuous culture (D = 0.046 h^{-1}), were removed and the uptake of ^{14}C-glutamate measured in the presence of various inhibitors (Fig. 13). These results suggest the dependence on ATP, indicated by inhibition by KCN; this is consistent with previous reports in actinomycetes (Ring & Heinz, 1966) where amino acid transport has been shown to be an active process. The presence of the ionophores, CCCP and DNP did not result in greater than 90 % inhibition of ^{14}C-glutamate uptake, indicating a lesser role for proton-motive force in the transport of glutamate.

Table 11. Reduction of glucose transport by the addition of amino acids (equivelent to 0.5 g. L^{-1}), expressed as a percentage of the control uptake rate (Glucose only). The control value (100 % relative value) for ^{14}C-Glucose was 15 mM. min^{-1}. g (Dry wt.)$^{-1}$. The control value (100 % relative value) for ^{3}H-2-deoxyglucose (DOG) was 20 mM. min^{-1}. g (Dry wt.)$^{-1}$. Values are the mean of at least two determinations ± the standard deviation of the data.

Amino acid competition	% of Control
10 mM ^{14}C-Glucose Control	100
3.8 mM Asparagine + 10 mM ^{14}C-Glucose	8 ± 3
3.4 mM Glutamine + 10 mM ^{14}C-Glucose	6 ± 3
4.3 Mm Valine + 10 mM ^{14}C-Glucose	3 ± 3
5.6 mM Alanine + 10 mM ^{14}C-Glucose	7 ± 4
10 mM ^{3}H-DOG	100
3.8 mM Asparagine + 10 mM ^{3}H-DOG	83 ± 1

Table 12. Reduction of glucose transport by the addition of inhibitors, expressed as a percentage of the control uptake rate (Glucose only). The control value (100 % relative value) for ^{14}C-Glucose was 15 mM. min^{-1}. g (Dry wt.)$^{-1}$. Values are the mean of at least two determinations ± the standard deviation of the data.

Inhibitor	% of Control
10 mM ^{14}C-Glucose Control	100
10 mM ^{14}C-Glucose + 100 μM CCCP	6 ± 6
10 mM ^{14}C-Glucose + 10 μM CCCP	42 ± 3
10 mM ^{14}C-Glucose + 100 μM DNP	10 ± 5
10 mM ^{14}C-Glucose + 10 μM DNP	94 ± 2
10 mM ^{14}C-Glucose + 100 μM KCN	18 ± 4
10 mM ^{14}C-Glucose + 10 μM KCN	54 ± 3
10 mM ^{14}C-Glucose + 100 μM Gramicidin	15 ± 4
10 mM ^{14}C-Glucose + 10 μM Gramicidin	27 ± 4

Table 13. Reduction of glutamate transport by the addition of inhibitors, expressed as a percentage of the control uptake rate (Glutamate only). The control value (100 % relative value) for ^{14}C-Glutamate was 19 mM. min^{-1}. g (Dry wt.)$^{-1}$. Values are the mean of at least two determinations ± the standard deviation of the data.

Glutamate uptake	% of Control
10 mM ^{14}C-Glutamate Control	100
10 mM ^{14}C- Glutamate + 100 μM CCCP	83 ± 2
10 mM ^{14}C- Glutamate + 100 μM DNP	35 ± 2
10 mM ^{14}C- Glutamate + 100 μM KCN	8 ± 5

3.3.9. Cellular composition during nutrient-limited growth in continuous culture

Cellular composition of *M.echinospora* was examined during continuous culture under carbon and nitrogen-limiting conditions. Previous studies of cellular composition in bacteria have revealed modulation of macromolecular content by specific growth rate (Shahab *et al.,* 1996; Marr, 1991; Riesenberg & Bergter 1979). Studies of intracellular storage carbohydrates flux during continuous culture in bacteria are few. Gray (1976) found that carbon-limited cells of *Arthrobacter* when grown in continuous culture had decreased levels of storage carbohydrate at slower growth rates, conversely under nitrogen-limitation the cells accumulated storage carbohydrate during slower growth.

The accumulation of substantial amounts of glycogen by this organism has previously been demonstrated. The variation during nutrient limited growth revealed that under carbon-limitation at the studied growth rates, glycogen was not accumulated. This contrasts with the situation under nitrogen-limitation, where at slow growth rates glycogen forms less than 2 % of the dry cell weight. At a specific growth rate of 0.093 h^{-1} glycogen accounted for 5.5 % of the dry cell weight (Fig. 64). Glycogen certainly appears to be accumulated in response to nitrogen-limitation, yet it would appear that growth rate also affects the accretion of glycogen. Studies of glycogen accumulation in batch culture of streptomycetes suggests that it occurs in bi-phasic manner, and that gene duplication events have resulted in separate operons, differentially regulated with regards to the developmental stage of the culture (Karandikar *et al.,* 1997; Schneider *et al.,* 2000). The pattern of accumulation in continuous culture could therefore represent glycogen accumulation regulated by growth rate.

Trehalose levels during continuous culture contribute less than 1 % of the total cell mass. The highest levels were observed during nitrogen-limited growth at a growth rate of 0.046 h^{-1} (Fig. 65). During batch growth, trehalose is accrued as the growth rate slows and has been shown to be the major carbon source found within spores and the increased levels in chemostat culture at low growth rates, may be a reflection of this behaviour (Karandikar *et al.,*1997; McBride & Ensign 1987 a, b).

Growth of this organism in surface cultures demonstrated that lipid represents a significant proportion of the cell mass. Growth under nutrient-limited conditions in continuous culture indicated that although levels of total cellular lipid were found to change with increasing growth rate, the nature of the limiting nutrient did not affect the total cellular lipid content (Fig. 66).
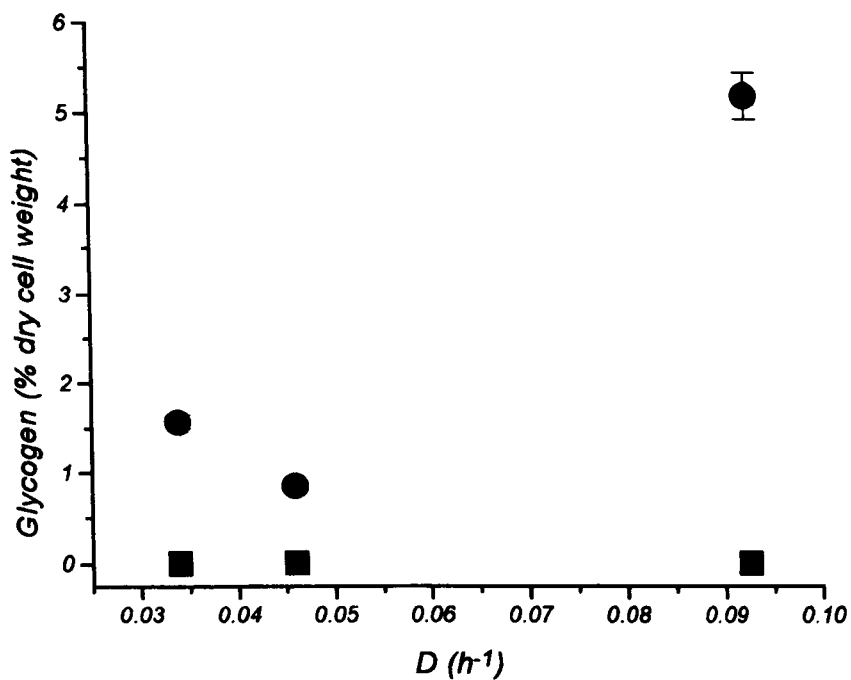
Figure 64. **Growth kinetics during continuous culture.** Percentage glycogen of the dry cell weight from nitrogen-limited chemostat (-●-), and from carbon-limited chemostat (-■-). Values are the mean of at least three determinations. Error bars represent the standard deviation of the data.
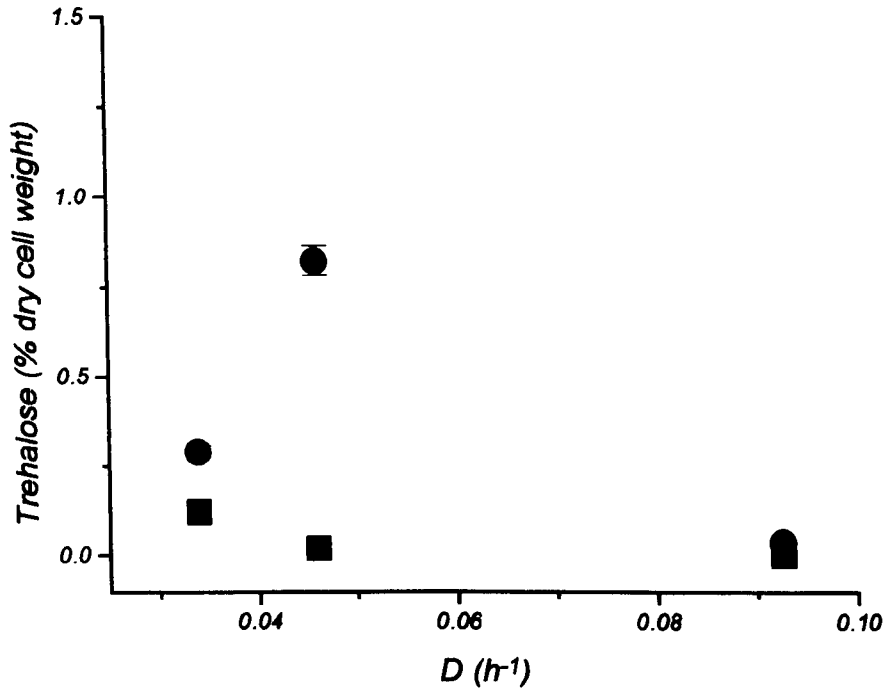
Figure 65. **Growth kinetics during continuous culture.** Percentage trehalose of the dry cell weight from nitrogen-limited chemostat (-●-), and from carbon-limited chemostat (-■-). Values are the mean of at least three determinations. Error bars represent the standard deviation of the data.
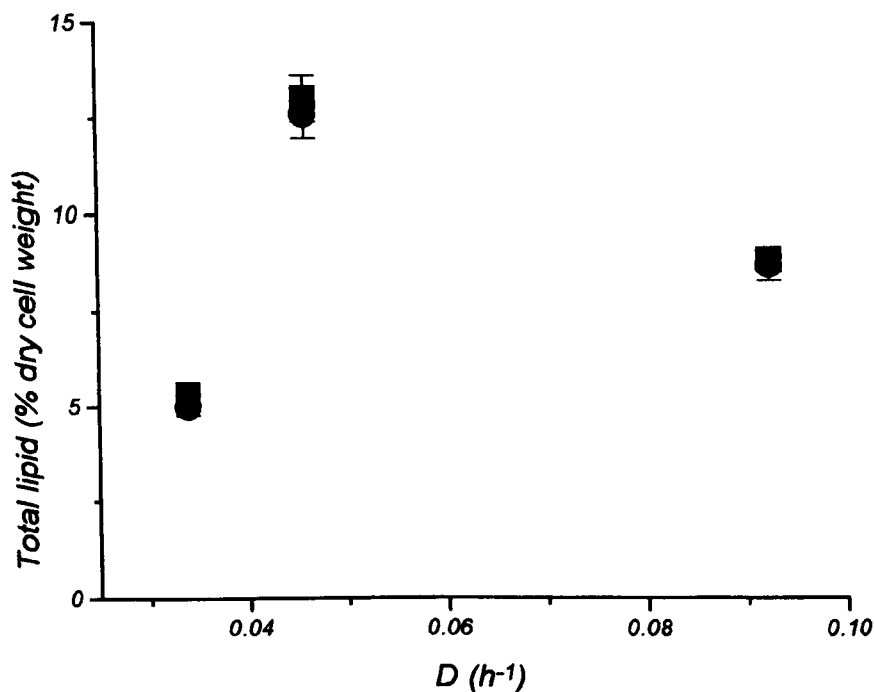
Figure 66. **Growth kinetics during continuous culture.** Percentage total lipid of the dry cell weight from nitrogen-limited chemostat (-●-), and from carbon-limited chemostat (-■-). Values are the mean of at least three determinations. Error bars represent the standard deviation of the data.

The content of nucleic acid was examined during nutrient limited continuous culture. The DNA content of the cells was found to remain relatively constant throughout growth, accounting for no greater than 7 % of the total cell mass, with the highest content being in nitrogen limited culture (Fig. 67). This observation is similar to the values obtained for *Streptomyces coelicolor* and *S. hygroscopicus* by Shahab *et al.*, (1996) and Riesenberg & Bergter (1979).

The content of RNA within the mycelium was modulated by specific growth rate. Under nitrogen limitation the RNA content increased proportionally with the specific growth rate, reaching a maximum of 53 % of the biomass. Carbon-limitation resulted in a similar relationship between growth rate and RNA content at the slower growth rates, however at the fastest growth rate the RNA content was found to be less than the slower growth rates. This may be a result of the limiting nutrient affecting growth. The increase of RNA proportionally to specific growth rate reflects the increased demand for RNA for protein synthesis at faster growth rates. Additionally the single greatest portion of the cellular RNA in *E.coli* represents rRNA (Shahab *et al.*, 1996).

The total protein content of the mycelium during nutrient limited continuous culture was found to be related to growth rate under carbon-limited conditions, with total protein increasing with increasing growth rate (Fig 68). When total protein was studied under nitrogen limitation, it was found to be relatively constant. This could be due to a greater conservation of nitrogen containing compounds under these conditions, or could reflect the increased RNA content of the mycelium under these conditions.
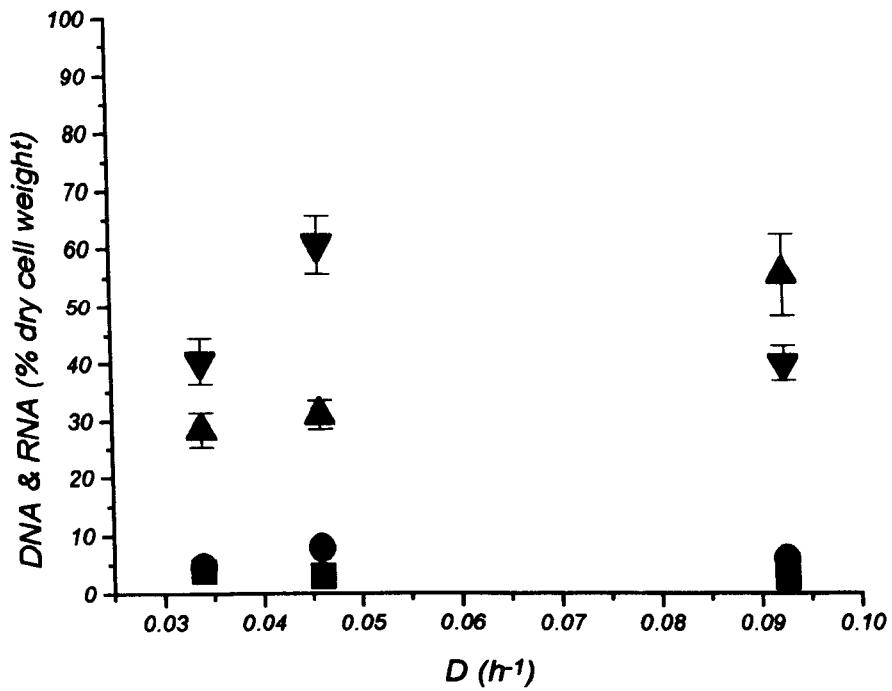
Figure 67. **Growth kinetics during continuous culture.** Percentage DNA of the dry cell weight from nitrogen-limited chemostat (●), and from carbon-limited chemostat (■). Percentage RNA of the dry cell weight from nitrogen-limited chemostat (▲), and from carbon-limited chemostat (▼). Values are the mean of at least three determinations. Error bars represent the standard deviation of the data.

Figure 68. **Growth kinetics during continuous culture.** Total protein from carbon-limited chemostat (-●-), and from nitrogen-limited chemostat (-■-). Values are the mean of at least three determinations. Error bars represent the standard deviation of the data.
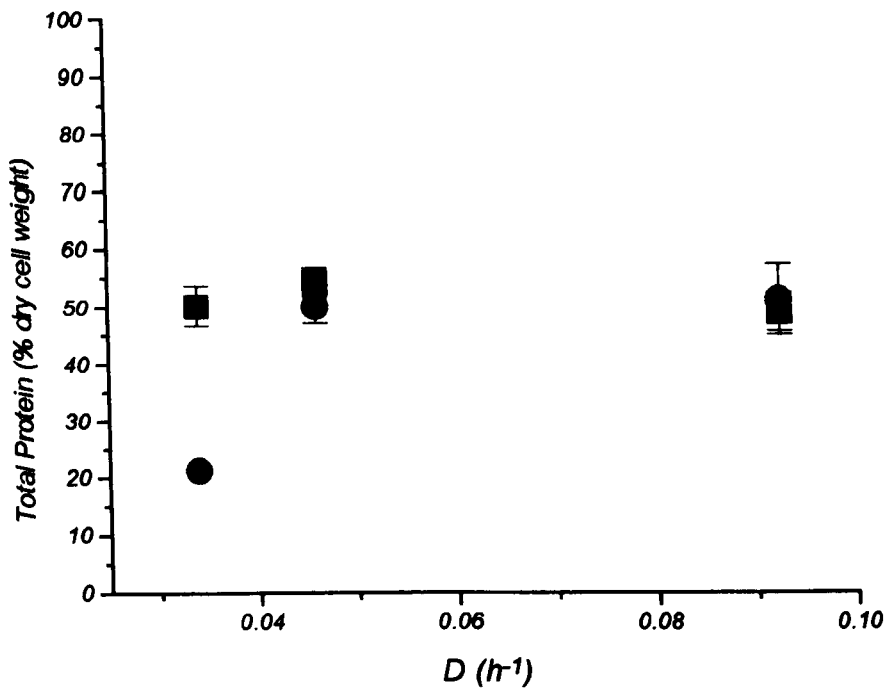
3.3.10 The stringent response and the influence of nucleotide pools upon differentiation

The stringent response and the role of highly phosphorylated guanosine nucleotides have been studied extensively in *Escherichia coli* and evidence suggests the stringent response plays a central role in starvation and control of growth rate. The function of these molecules in the life cycle of the actinomycetes *Streptomyces coelicolor* and *Mycobacterium tuberculosis* have also been the subject of considerable research (Ochi, 1986; 1987; Hesketh *et al.*, 2001; and Primm *et al.*, 2000). These studies have suggested a role for these highly phosphorylated molecules in the initiation of differentiation, antibiotic production and growth rate control.

The role of adenine nucleotides, specifically adenosine triphosphate (ATP), and their function in metabolism is well established as the universal energy carrier of living systems (Dawes 1986). The interdependency of adenine nucleotides within cells allows the energetic state of a system to be quantitatively expressed according to the adenylate energy charge of the system (Atikinson, 1968, and 1977). The total energy stored within the adenylate system is proportional to the average number of anhydride-bound phosphate groups per adenosine moiety; varying between zero for AMP to two for ATP. The adenylate energy charge (EC) of a system containing AMP is zero, and of just ATP is one. The assumption that equilibrium levels of each nucleotide are present. It is generally recognised that an EC of 0.8 and above is required for normal cell growth and metabolism (Dawes 1986). Published data for EC is scarce in the literature for actinomycetes. Vu-Trong & Gray (1982) however,

presented chemostat data for EC in the tylosin producer *S. fradiae*, indicating a relatively constant EC over a range of growth rates.

3.3.10.1 The stringent response of *Micromonospora echinospora*

To elicit amino acid starvation conditions *M. echinospora* mycelia grown for 72 h in YEME medium were transferred to minimal medium, containing sodium nitrate as the sole nitrogen source and glucose as the sole carbon source. Intracellular ppGpp was detected within 10 minutes of shiftdown (Fig. 69 & 70), and peaked 30 minutes following shiftdown at 26.5 pmol mg dry cell weight^{-1}.

To determine if the appearance of intracellular ppGpp coincides with morphological differentiation and gentamicin production, the organism was grown under nitrogen-limiting conditions, with sucrose as the principle carbon source. This allowed the formation of antibiotic by removing the possible repressive effects of glucose within the medium (see section 3.3.7).
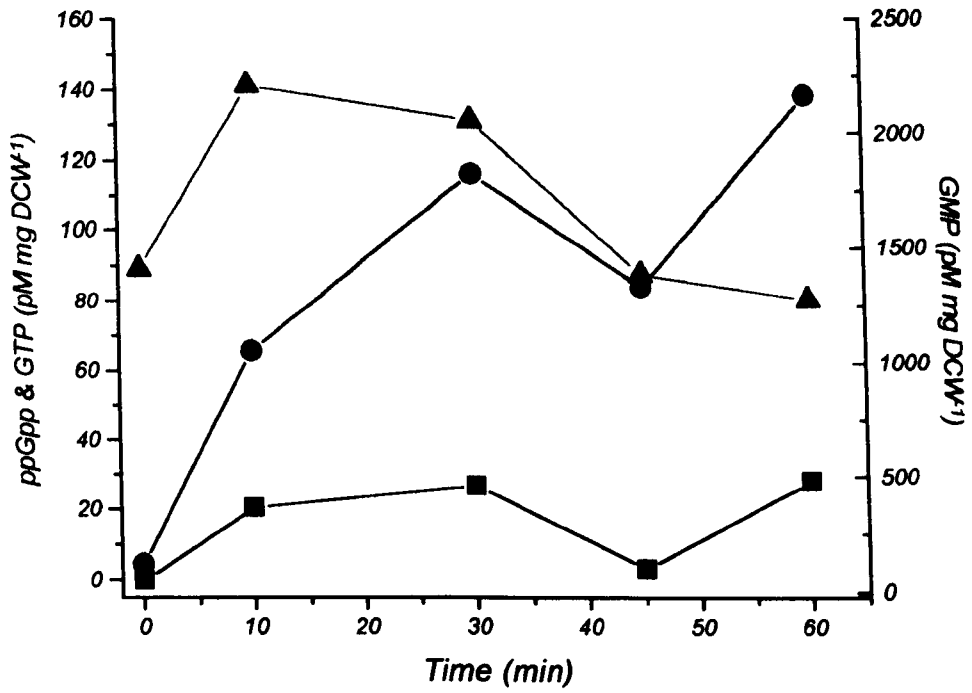
Figure 69. **Change in intracellular nucleotide pools following nitrogen downshift.** ppGpp (-■-), GMP (-●-), GTP (-▲-). Values are the mean of at least three determinations.
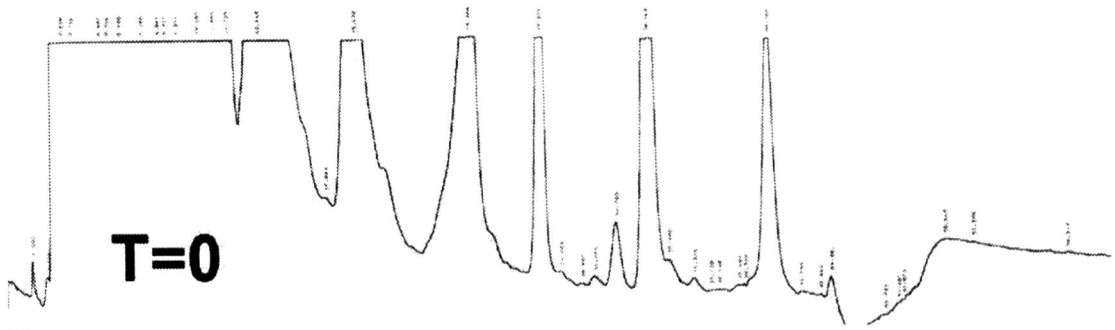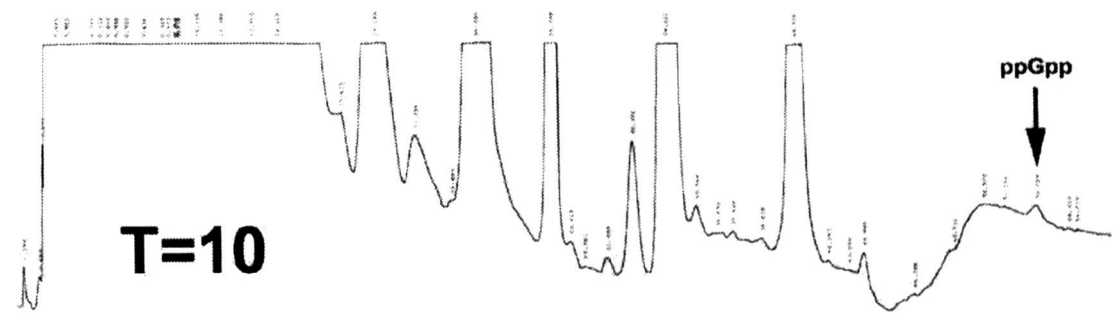
Figure 70. Demonstration of ppGpp synthesis in *M. echinospora* following nitrogen downshift. HPLC traces of intracellular nucleotides, A) illustrates the nucleotide pool prior to nitrogen downshift. B) illustrates the appearance of ppGpp within 10 minutes of nitrogen downshift.

Growth in nitrogen-limited medium containing sucrose as the primary carbon source (7 g. L^{-1} = 267 mM of total carbon) and asparagine as the sole nitrogen source, resulted in a profile similar to that obtained with glucose as the primary carbon source (17.5 g. L^{-1} = 267 mM of total carbon). Residual nitrogen was exhausted from the medium as previously demonstrated with glucose growth. Gentamicin was detected transiently at 96 h post-inoculation, at a level of 7.2 µg. ml^{-1}, but was undetectable throughout the remainder of the fermentation (Fig. 71). The appearance of spores within the culture was apparent from 120 h. Both gentamicin and sporulation occurred during a slower growth phase, prior to the attainment of maximum biomass under these conditions (Fig. 72). ppGpp was detected within the mycelium at 48 h post-inoculation (12 pmol mg dry cell weight^{-1}). The level of ppGpp was found to decrease rapidly thereafter, not being detected throughout the remainder of the culture. The presence of other highly phosphorylated guanosine nucleotides (ppGp, pppGpp) was not established during growth under these conditions. The transient appearance of ppGpp within the culture prior to the appearance of antibiotic and morphological differentiation may indicate a role for this molecule in differentiation (Fig. 72). Jones *et al.*, (1996) demonstrated the appearance of highly phosphorylated nucleotides between 2 and 12 h prior to the cephamycin C biosynthesis in *Streptomyces clavuligerus*. This study has shown a delay of up to 48 h between the detection of ppGpp and gentamicin. This may be as a result of the slow growth rate of this organism (T_d =25 h) in relation to *S.clavuligerus* (T_d=6.5 h) at the onset of ppGpp appearance.
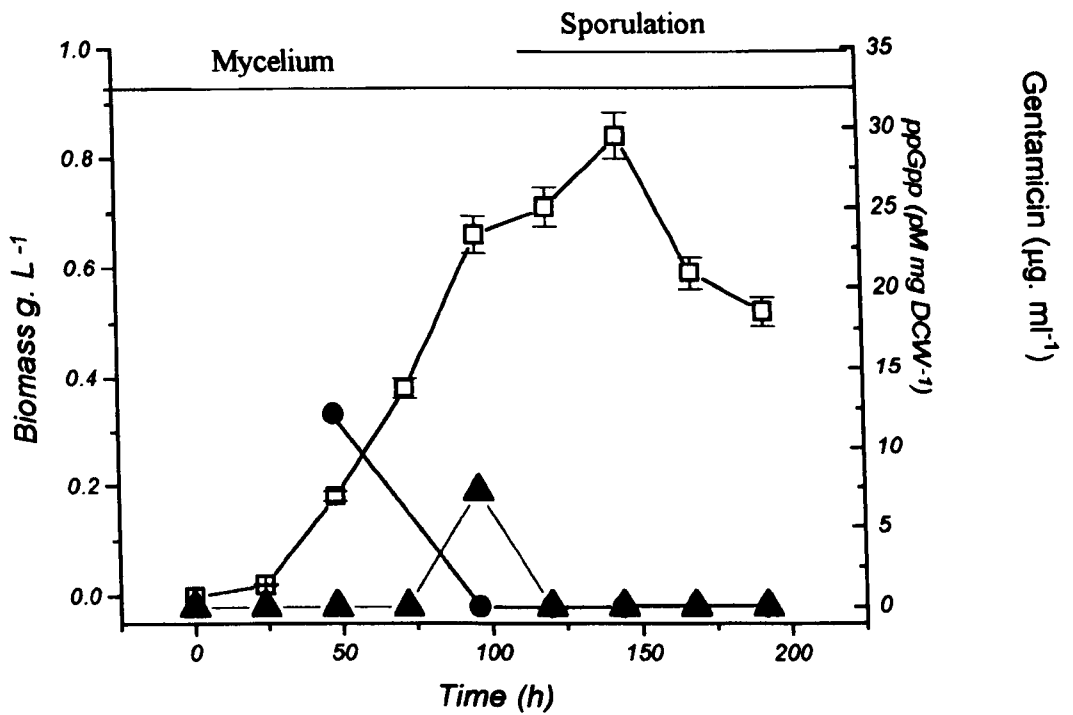
Figure 71. **Intracellular accumulation of ppGpp during nitrogen limited batch**

culture. ppGpp (-●-), gentamicin (-▲-) and biomass (-□-). Values are the mean of

at least three determinations. Error bars represent the standard deviation of the data.

3.3.10.2 Modulation of guanine and adenosine nucleotide pools

The phosphorylation of GDP and GTP to ppGpp and ppGppp respectively, with ATP acting as the phosphate donor, reveals the intimate link between the stringent response and nucleotide pools.

Amino acid shiftdown of mycelium, as previously described, resulted in a rapid increase in intracellular GTP and GMP over the first 10 minutes following shiftdown. When grown in batch culture the content of guanine nucleotide changed in a complex manner. GMP was found to be high during the early stages of growth (Fig. 72), declining rapidly thereafter. GDP increased in a growth associated manner (Fig. 73) declining rapidly during stationary phase. The intracellular GTP content were found to increase during the exponential phase of growth, up to 144 h post-inoculation (Fig. 72) followed by a rapid decline, observed following entry in to stationary phase. The profiles obtained for GTP and GMP (Fig. 72) appear to mirror each other emphasising the interdependency of purine metabolism.

The flux of adenosine nucleotides during nitrogen limited batch culture changed throughout growth. AMP and ADP were found to increase throughout growth in a growth phase associated manner (Fig. 74). The intracellular ATP content appeared to decrease rapidly prior to sporulation. This is also apparent from the adenylate energy charge (Fig76).
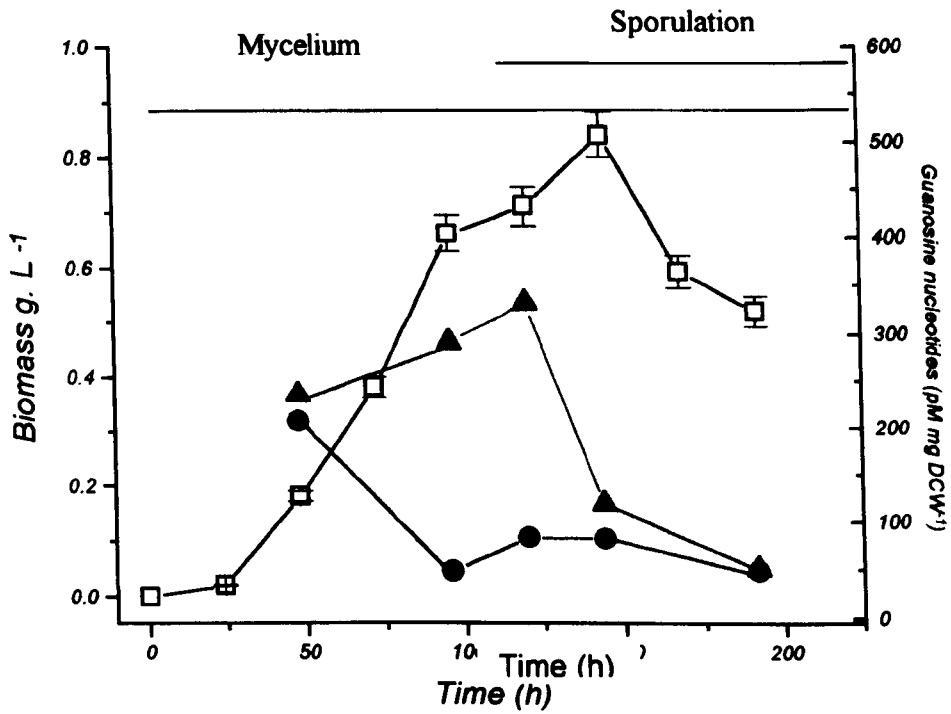
Figure 72. **Intracellular concentration of guanosine nucleotides during nitrogen limited batch culture. GTP (-▲-), GMP (-●-).** Biomass (-□-). Values are the mean of at least three determinations. Error bars represent the standard deviation of the data.
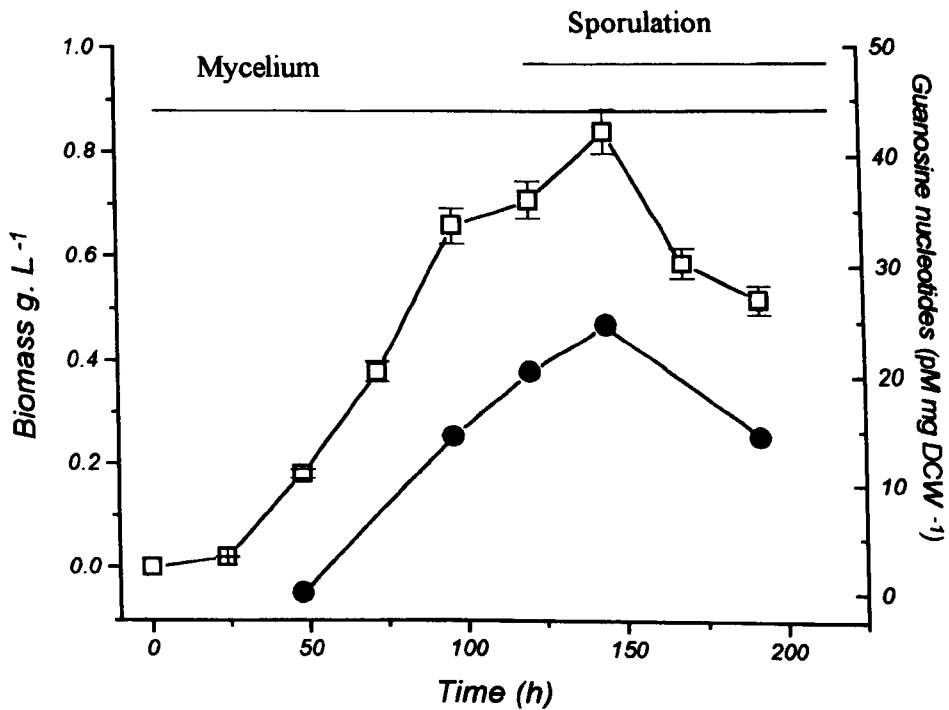
Figure 73. **Intracellular concentration of guanosine nucleotides during nitrogen limited batch culture.** GDP (-●-). Biomass (-□-). Values are the mean of at least three determinations. Error bars represent the standard deviation of the data.
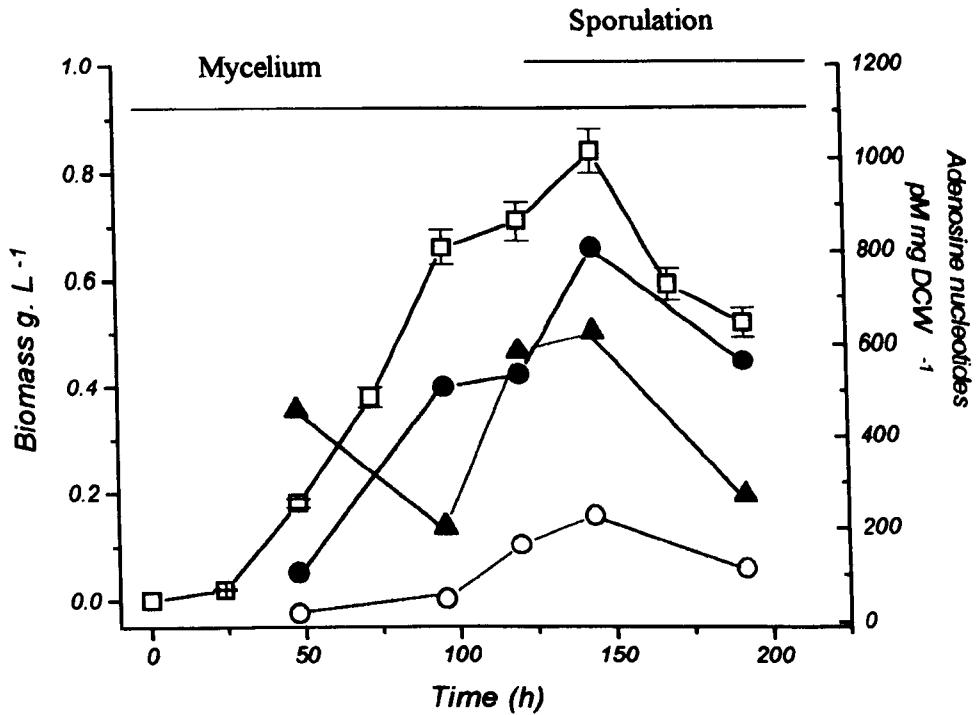
Figure 74. **Intracellular concentration of adenosine nucleotides during nitrogen limited batch culture.** Biomass (-□-), AMP (-●-), ADP (-O-), ATP (-▲-). Values are the mean of at least three determinations. Error bars represent the standard deviation of the data.

Intracellular content of cAMP has been shown to control transcription of catabolic enzymes, morphology, environmental response mediation (Chatterjee & Vining, 1982) and influence aerial mycelium formation in *Streptomyces* spp (Kang *et al.*, 1999), however the molecule does not appear to directly effect antibiotic biosynthesis (Botsford & Harman, 1992). The intracellular levels of cAMP during nitrogen-limited batch culture were found to decrease marginally prior to the appearance of gentamicin in the culture, and thereafter increased with growth, peaking on entry to stationary phase (Fig. 75).

The interdependence of AMP, ADP, and ATP can be unified, and their relationship expressed with the calculation of the adenylate energy charge (Atkinson, 1968), which reflects the total metabolic energy stored in the adenylate system. The calculation of adenylate energy charge (EC) throughout growth revealed a high EC (0.8) during the exponential phase of growth (Fig. 76). A rapid decrease in the EC at 96 h post-inoculation, coinciding with antibiotic biosynthesis and initiation of sporulation, resulted in entry in to a slower growth phase. This decrease also coincides with the exhaustion of asparagine in the medium and may represent a change in carbon source usage, switching from amino acid derived carbon to the medium sugar, resulting in the decrease in EC during the delay in catabolism. Following the decrease in EC the level increases transiently up to 120 h, and thereafter decreases during stationary phase.
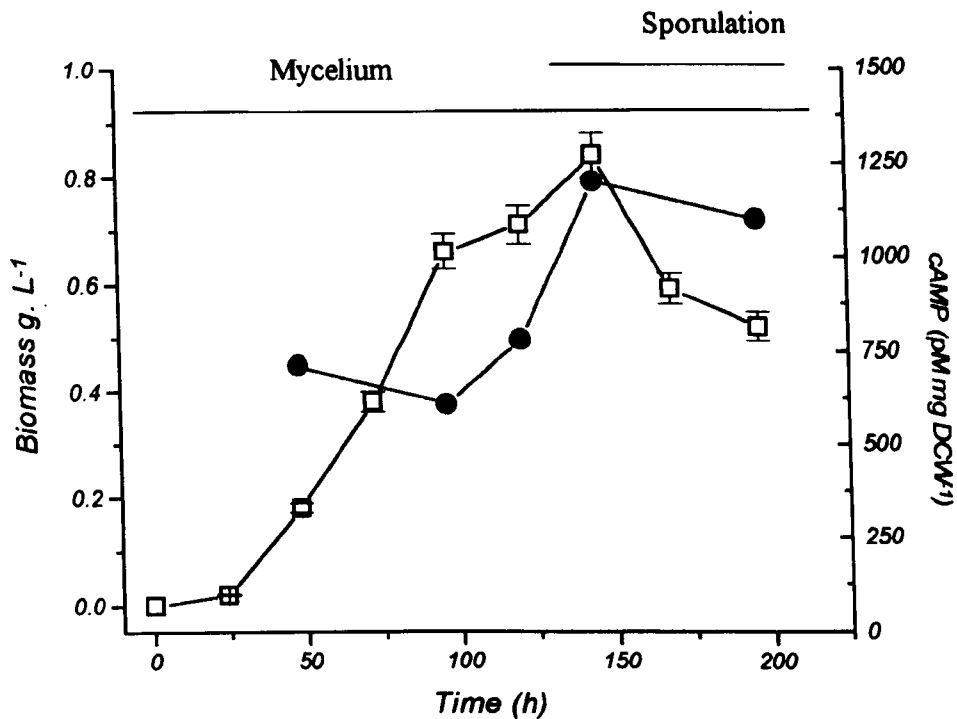
Figure 75. **Intracellular concentration of cAMP during nitrogen limited batch culture.** cAMP (-●-), and biomass (-□-). Values are the mean of at least three determinations. Error bars represent the standard deviation of the data.
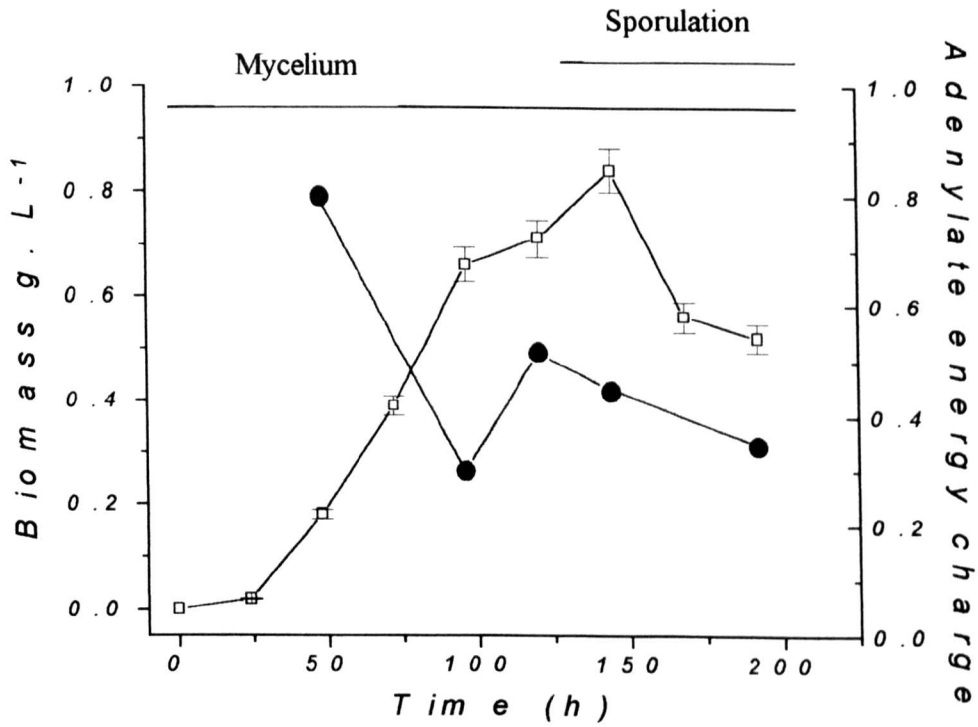
Figure 76. **Change in adenylate energy charge during nitrogen limited batch culture.** Adnylate energy charge (-●-), and biomass (-□-). Values are the mean of at least three determinations. Error bars represent the standard deviation of the data.

The interactions of the adenosine and guanine nucleotides during growth are complex, and result in rapid changes throughout the growth phases. A continuous culture approach was used to observe the interaction between growth rate and nutrient limitation upon the pools of intracellular nucleotides.

The adenosine nucleotide content of nutrient-limited, continuous culture grown mycelium indicates a growth rate dependant relationship. Carbon-limited culture, produced biomass with decreased adenosine pools, when compared to nitrogen-limitated cultures (Fig. 77 & 78). Under both carbon and nitrogen-limitation intracellular ATP levels were found to increase with dilution rate. The adenylate energy charge (EC) was calculated during nutrient limited continuous culture and under both carbon and nitrogen-limitation EC increased with the increasing growth rate. The increase in EC was found to be higher under carbon-limitation reflecting the dependency of EC upon carbon supply. A similar relationship was observed with intracellular cAMP levels, again reflecting the dependency upon energy supply (Fig. 79).

The highly phosphorylated guanosine nucleotides (ppGpp, pppGpp and ppGp) were not detected under carbon or nitrogen-limitation at any of the growth rates examined. GMP, GDP and GTP levels under nitrogen limitation revealed a complex relationship (Fig. 81 & 82). Intracellular guanine nucleotide content under carbon-limitation reveals a proportional relationship with increasing growth rate. These results are probably a reflection of the importance of nitrogen supply in purine biosynthesis, with glutamine and aspartate being important purine precursors (Stryer, 1995).
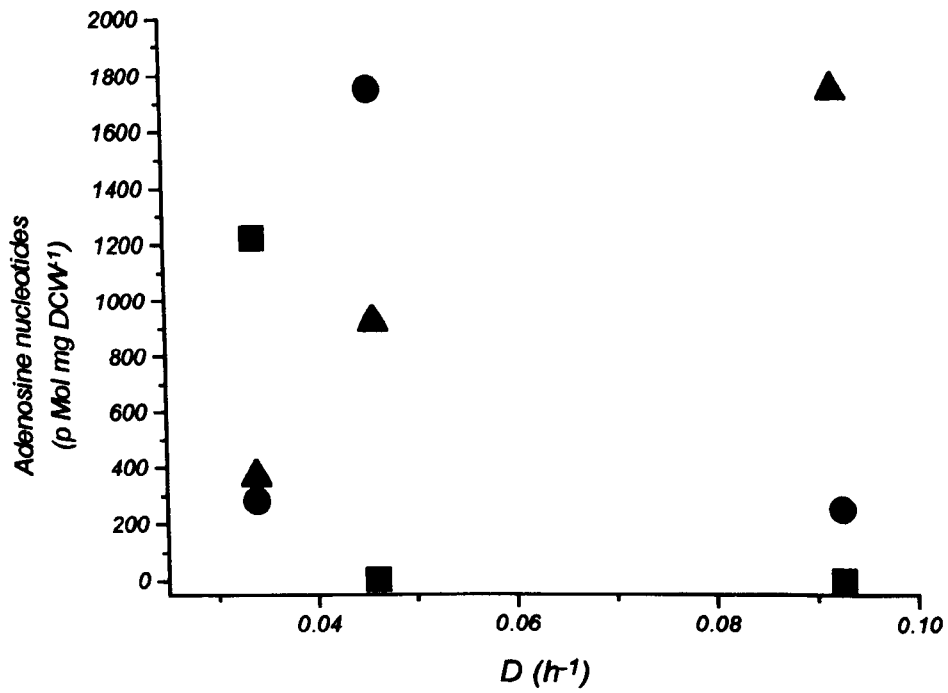
Figure 77. **Intracellular concentration of adenosine nucleotides during nitrogen-limited chemostat culture. AMP (■), ADP (●), ATP (▲).** Values are the mean of at least three determinations.
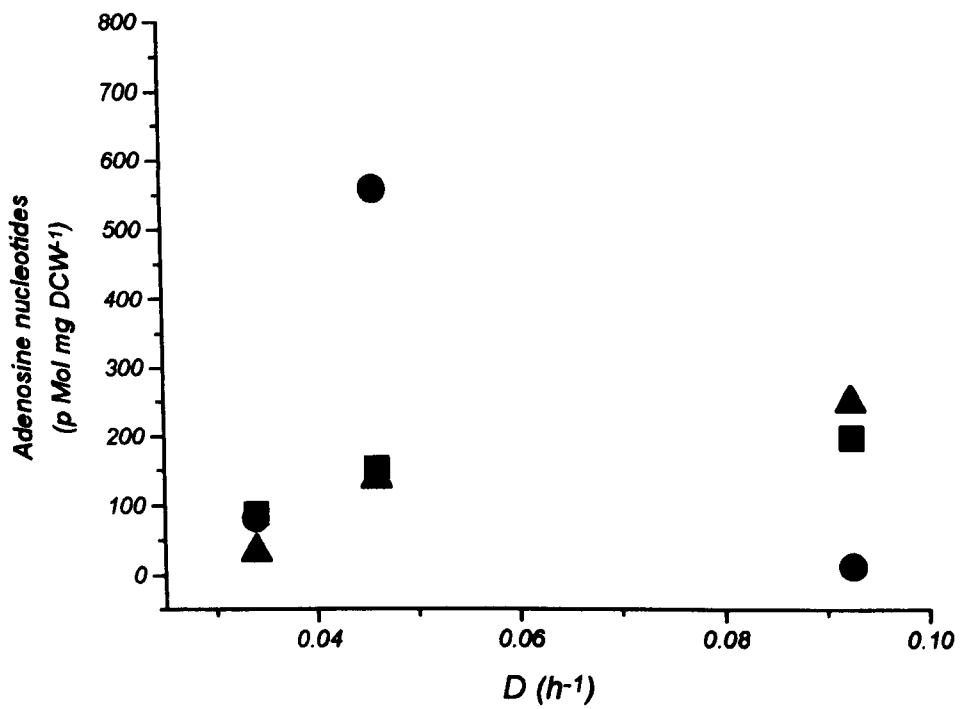
Figure 78. **Intracellular concentration of adenosine nucleotides during carbon-limited chemostat culture. AMP (■), ADP (●), ATP (▲). Values are the mean of at least three determinations.**
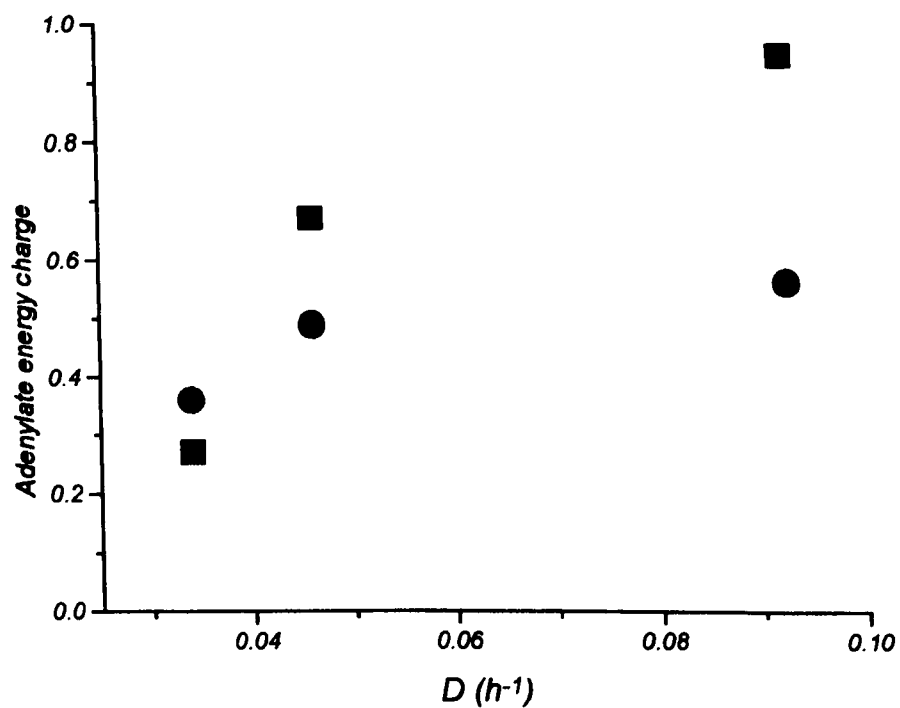
Figure 79. **Adenylate energy charges during carbon and nitrogen-limited chemostat culture.** Carbon-limited (■), Nitrogen-limited (●), Values are the mean of at least three determinations.
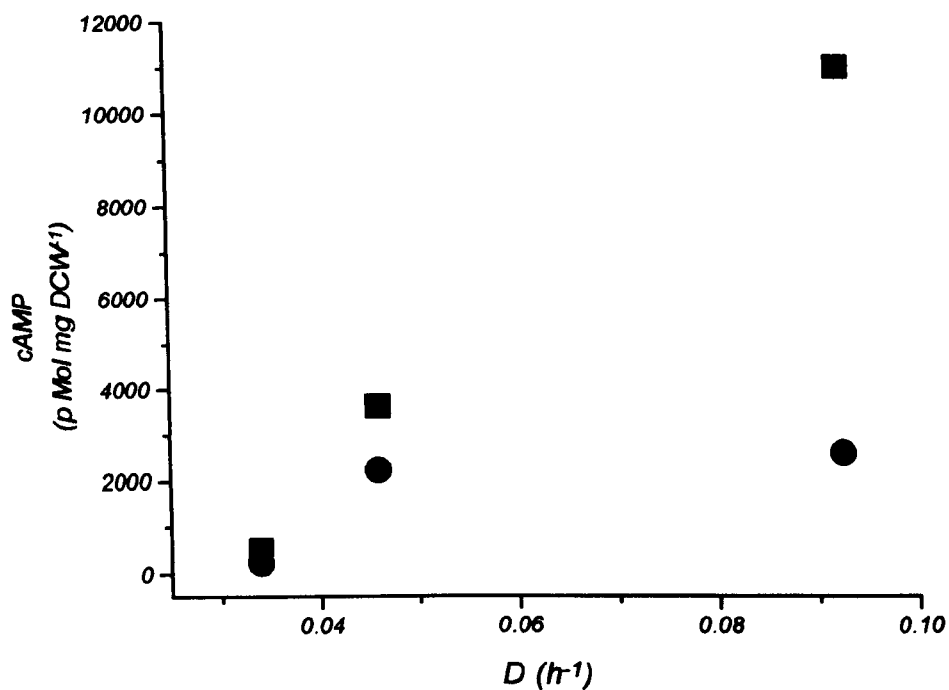
Figure 80. **Intracellular concentration of cAMP during carbon and nitrogen-limited chemostat culture.** Carbon-limited (■) Nitrogen-limited (●). Values are the mean of at least three determinations.
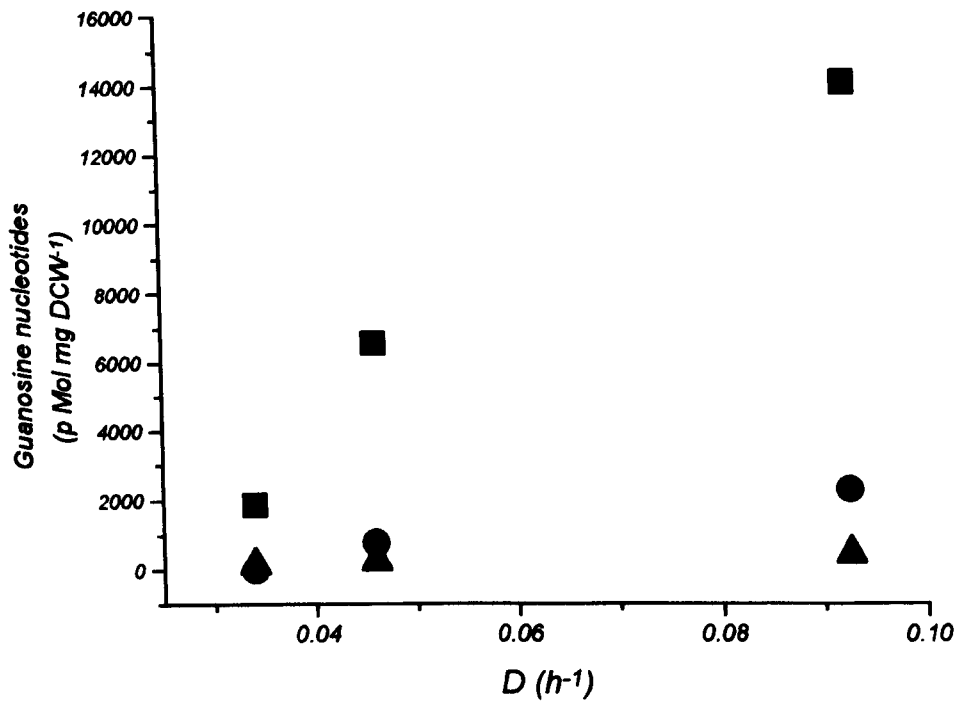
Figure 81. **Intracellular concentration of guanosine nucleotides during carbon-limited chemostat culture.** GMP (■), GDP (●), GTP (▲). Values are the mean of at least three determinations.
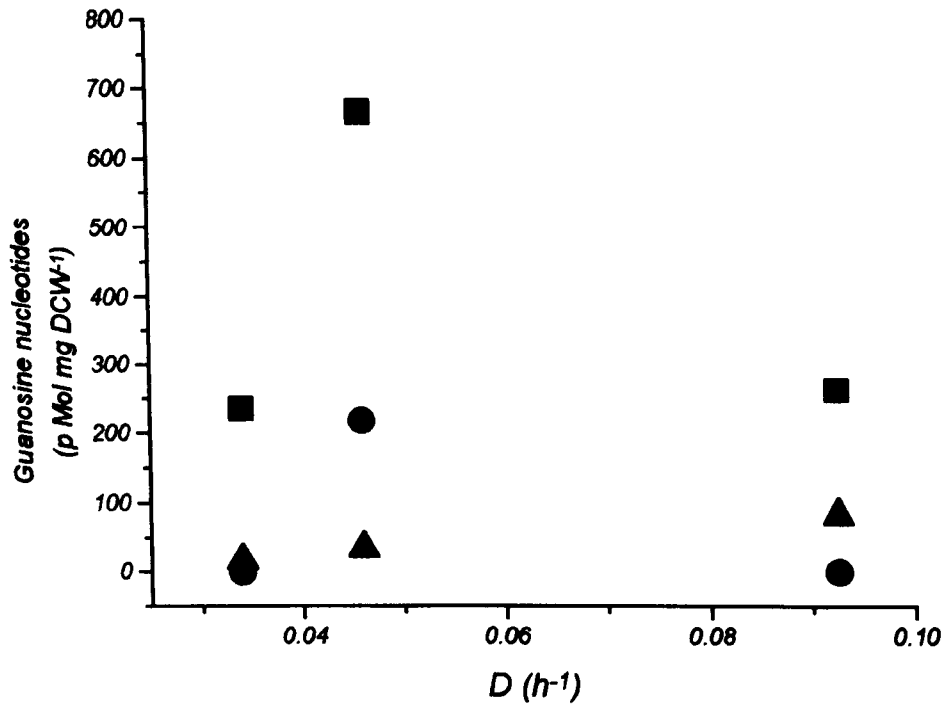
Figure 82. **Intracellular concentration of guanosine nucleotides during nitrogen-limited chemostat culture.** GMP (■), GDP (●), GTP (▲). Values are the mean of at least three determinations.

3.3.10.3 Summary of intracellular nucleotides & their influence upon differentiation and antibiotic production

The quantification of intracellular nucleotide pools during nutrient shiftdown, nutrient-limited batch and continuous culture indicated a complex interaction between the individual groups of nucleotides. Adenosine nucleotide content of the mycelium was influenced by the supply and nature of the carbon source, with the switch between amino acid derived carbon and medium sugar influencing cellular pool flux. The influence of growth rate also resulted proportional increases in adenosine nucleotides with growth rate. The interactions between adenosine nucleotides and the influence of these on growth, antibiotic biosynthesis, and morphogenesis were most easily characterised by the consideration of adenylate energy charge (EC). Rapid decreases in the EC coincided with the onset of antibiotic production, and occurred prior to sporulation, indicating that the decrease in EC correlates with the initiation of morphogenesis and antibiotic production.

Guanine nucleotide pools (GMP, GDP, &GTP) revealed little in relation to antibiotic production and morphological differentiation, however they did indicate the relationship between nitrogen metabolism and purine biosynthesis. The stringent response was demonstrated for *Micromonospora*, this being the first report, and was shown to be initiated in response to nutrient shiftdown , consistent with previous reports in actinomycetes (Ochi, 1986; 1987a; 1987b; Hesketh *et al.*, 2001). Additionally, the accumulation of ppGpp was demonstrated in batch culture prior to the appearance of gentamicin and spores within the culture. The presence of other highly phosphorylated guanosine nucleotides, such as ppGp, and pppGpp, was not verified in these studies.

3.3.11 Primary metabolic studies on nutrient limited continuous cultures

The majority of studies of actinomycetes have focused on the molecular biology and enzymology of antibiotic biosynthesis, with relatively little attention being paid to the enzymes and regulation of primary metabolism (Alves *et al.*, 1994). Although the majority of metabolic reactions are common to most organisms, there are certain pathways and regulatory functions that are specific to the supply of antibiotic precursors. There are a few reports of increased metabolic flux through certain primary metabolic pathways, which correlate with the expression secondary metabolites (Obanye *et al.*, 1996; Pipersberg 1997, and references therein).

There have been no previous studies of primary metabolism in *Micromonospora*. The previous studies of actinomycete primary metabolism have followed enzyme flux during batch culture and have attempted to characterise enzyme regulation in a constantly changing environment. This current study was initiated in continuous culture, under previously established, defined carbon and nitrogen-limiting conditions (section 3.3.2), to eliminate such variation due to changing physiological conditions.

The enzymes chosen to indicate the significance of primary metabolism in the production of gentamicin and morphogenesis, represent the recognised regulation/control enzymes of each pathway.

3.3.11.1 Enzymes of nitrogen metabolism

The levels of seven nitrogen metabolic enzymes were investigated during carbon and nitrogen-limited growth in continuous culture at three growth rates under each limitation.

Asparagine comprised the sole nitrogen source within the culture. The presence of glutamate dehydrogenase (GDH) could not be detected under either nutrient limitation or at any growth rate. This may reflect an absence of this enzyme in *Micromonospora* or more likely the lack of induction of this enzyme, as it has a low affinity for ammonium, and therefore only functions efficiently at high ammonium concentrations (Hodgson, 2000), which are not present in this system.

The absence of detectable glutamate synthase (GOGAT) activity, is not surprising as the activity of this enzyme is usually linked to the activity of GDH in high ammonium concentrations, and to glutamine synthetase (GS) in low ammonium concentrations. It can be seen from earlier work, that ammonia is excreted during growth on asparagine. Catabolism of amino acids results in the generation of organic acids and ammonia. The organic acids can thus be utilised as citric acid cycle intermediates, while the amino groups can be utilised in amination reactions to provide further biosynthetic intermediates.

Glutamine synthetase activity was detected under both nitrogen and carbon-limiting conditions. The activity of the enzyme was found to vary with the limiting nutrient and the growth rate of the organism (Fig. 83). The highest levels of GS activity were observed during nitrogen-limitation, at the slow growth rate ($0.034 \ h^{-1}$), being three-fold higher than the levels observed at faster growth rates, where activity was

relatively constant. The levels of GS under carbon-limiting conditions were relatively constant at each of the studied growth rates.

The effect of various nitrogen sources upon the activity of GS in mycelia removed from the nitrogen-limited chemostat (0.046 h^{-1}), was investigated (Table 14). Harvested mycelia was washed in basal minimal medium containing no nitrogen source, and resuspended medium containing amino acid or ammonium chloride (30 mM), or mixed ammonium and amino acid (30 mM total). Medium containing glutamate, asparagine and alanine as the nitrogen source resulted in the highest levels of GS observed. These nitrogen sources combined with ammonium resulted in decreased levels of GS activity, compared to cultures with amino acid only. Aspartate resulted · in the lowest level of GS activity, which may be due to Glutamate:oxaloacetate aminotransferase (GOAT) activities in this organism by-passing the GS system (see below). GS levels were low when glutamine was supplied as the sole nitrogen source, perhaps not surprisingly as glutamine is the end-point of the pathway. These results are consistent with previous reports in the literature, with GS levels being reduced in the presence of ammonium (Wray & Fisher, 1988; Streicher & Tyler, 1981).

The activity of GS has previously been shown to been regulated at the level of gene expression, and at the enzyme, by the availability of ammonium (Hodgson, 2000). GS activity in bacteria is known to be repressed by high concentrations of ammonia. *M. echinospora* GS activity can be inactivated by the addition of 0.2 % (w/v) ammonium chloride to growing cultures (Fig. 83). The response to the addition of ammonium chloride is an 85 % reduction in GS activity within 4 minutes. In other bacteria the mechanism of this inactivation has been shown to be post-translational adenylation. GS activity in *M, echinospora* can be partially recovered (up to 10 %)

by the addition of Snake venom phosphodiesterase (SVDPE) indicating that adenylation may play a role in this rapid inactivation mechanism.
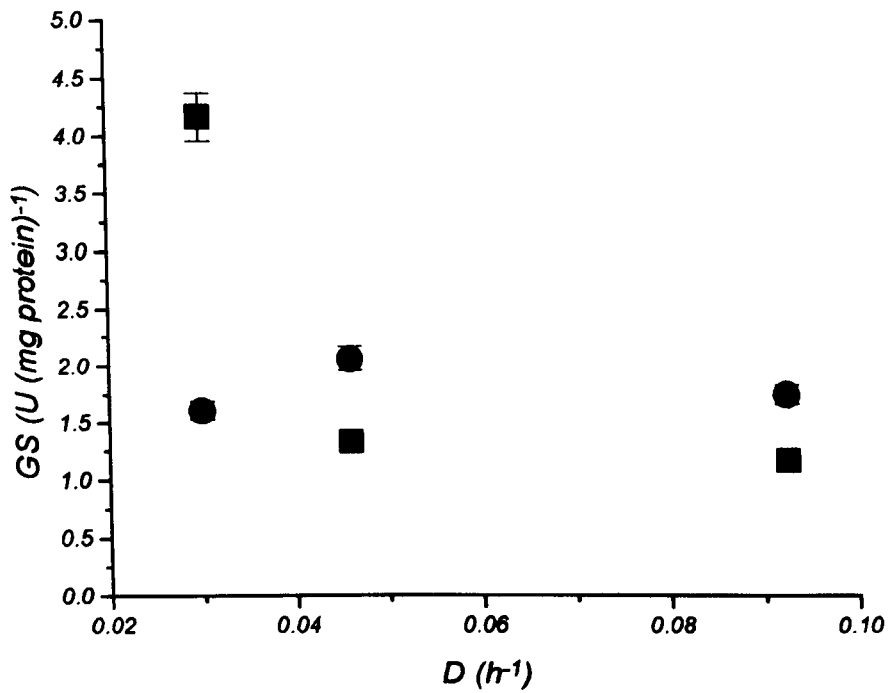
Figure 83. **Change in Glutamine synthetase (GS) levels during carbon and nitrogen-limited continuous culture.** Carbon-limited (-●-), and nitrogen-limited (-■-). Values are the mean of at least three determinations. Error bars represent the standard deviation of the data.
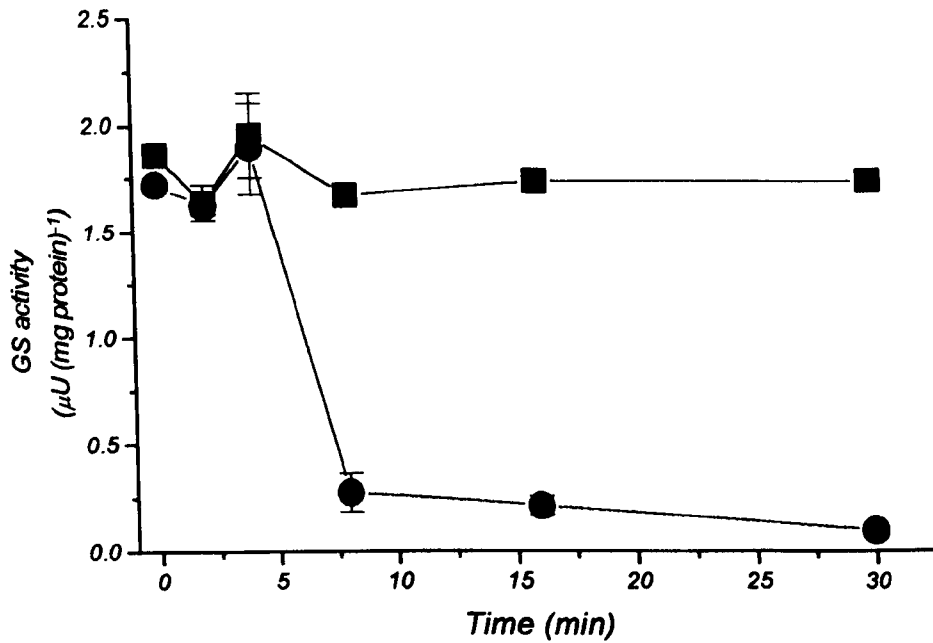
Figure 84. GS inactivation by NH$_4^+$ shock in whole cells. GS activity in control

cells (-■-); GS activity in NH$_4^+$-shocked cells (-•-). Values are the mean of at least

three determinations. Error bars represent the standard deviation of the data.

Table 14. **Change in Glutamine synthetase (GS) levels during growth on different nitrogen sources.** All nitrogen sources were supplied at a final concentration of 30 mM. Values are the mean of at least three determinations.

Nitrogen source	Specific activity U. ml. min. mg of protein^{-1}
Glutamate	0.6
Glutamine	0.1
Aspartate	0
Asparagine	0.7
Histidine	0.1
Alanine	1.4
Ammonium chloride	0.3
Glutamate + Ammonium chloride	0.4
Glutamine + Ammonium chloride	0.3
Aspartate + Ammonium chloride	0
Asparagine + Ammonium chloride	0.8
Histidine + Ammonium chloride	0.04
Alanine + Ammonium chloride	0.5

The inhibition of GS activity by aspartate and to a certain degree asparagine, indicates that glutamate:oxaloacetate aminotransferase (GOAT) may play a key role in nitrogen catabolism in this organism under the conditions studied. Hodgson (2000) suggested that AOAT or GOAT may provide an alternative mechanism of ammonium assimilation, via alanine or aspartate and the transamination of glutamate to yield glutamine and oxaloacetate or pyruvate respectively. The initial uptake of asparagine, via asparaginase, results in the formation of ammonium and aspartate. This would suggest that GOAT plays an important role in nitrogen metabolism in *M. echinospora*, enabling the energetically efficient generation of glutamine and oxaloacetate.

Under nitrogen-limited conditions, levels of GOAT were relatively low, probably a reflection of the low supply of aspartate in the system. When GOAT levels were examined during nitrogen-limited continuous culture, a proportional relationship between dilution rate and GOAT activity was found (Fig. 85). This latter result indicates that the carbonaceous skeleton of asparagine is acting as the major carbon source under these conditions. The effect of different nitrogen sources on the activity of GOAT was investigated (Table. 15). It was found that aspartate induced the highest levels of GOAT activity, consistent with previous reports of this enzyme in actinomycetes (Lee & Lee, 1993). The presence of ammonium in the cultures resulted in decreased GOAT levels. This would indicate ammonium repression of GOAT activity may have been occurring.
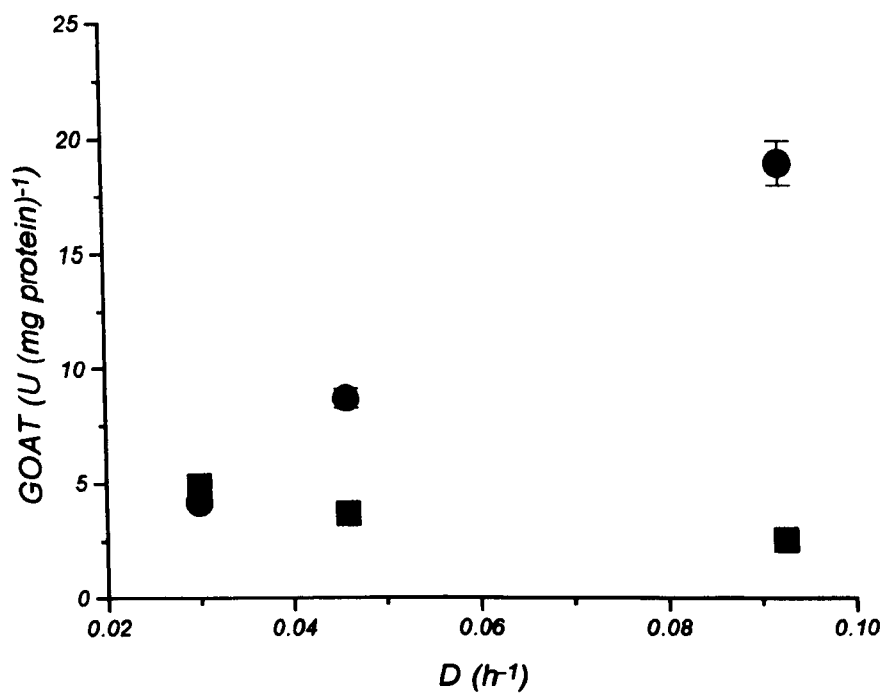
Figure 85. **Change in Glutamate:oxaloacetate aminotransferase (GOAT) levels during carbon and nitrogen-limited continuous culture.** Carbon-limited (-●-), and nitrogen-limited (-■-). Values are the mean of at least three determinations. Error bars represent the standard deviation of the data.

Table. 15. **Change in Glutamate:oxaloacetate aminotransferase (GOAT) levels during growth on different nitrogen sources.** All nitrogen sources were supplied at a final concentration of 30 mM. Values are the mean of at least three determinations.

Nitrogen source	Specific activity U. ml. min. mg of protein^{-1}
Glutamate	55
Glutamine	71
Aspartate	619
Asparagine	151
Histidine	306
Alanine	256
Ammonium chloride	429
Glutamate + Ammonium chloride	18
Glutamine + Ammonium chloride	21
Aspartate + Ammonium chloride	151
Asparagine + Ammonium chloride	85
Histidine + Ammonium chloride	28
Alanine + Ammonium chloride	19

The assimilation of ammonium by mechanisms other than GS-GOGAT and GDH-GOGAT, requires that enzymes such as alanine dehydrogenase be present. The assimilation of ammonium via ADH also requires alanine: 2-oxoglutarate aminotransferase (AOAT) activity. The presence of this enzyme was verified in *M. echinospora* under nutrient-limited continuous culture. Activities of AOAT appear to be relatively constant under both carbon and nitrogen-limited conditions at the studied growth rates (Fig. 88). This probably reflects the low levels of ammonium in the cultures. ADH-AOAT couples have been shown to act as the ammonium assimilation mechanism in *Streptomyces avermitilis* (Novack *et al.*, 1992).

The detection of GS activity without the presence of GDH, or GOGAT activity is surprising, however, GS activity can be coupled to the activity of an alanine dehydrogenase (ADH). ADH can play a role in the assimilation of ammonia or the deamination of alanine when either of these compounds serves as the nitrogen source (Aharonowitz & Friedrich, 1980). The coupling of ADH and GS activity requires the action of alanine aminotransferase (Hodgson, 2000).

The activity of assimilating and deaminating ADH was examined during growth under carbon and nitrogen-limiting conditions in continuous culture. Assimilating ADH levels during nitrogen-limitation were found to increase with increasing growth rate (Fig. 86), Whereas the deaminating ADH levels demonstrated an inverse relationship to increasing growth rate (Fig. 86). This reflects the general physiology of the organism under these conditions with deaminating ADH being expressed at higher levels when nitrogen is limiting, and growth rate is slow, indicating the catabolic role of this enzyme when coupled to GS. Assimilating ADH is higher at the faster growth rates due to the reductive amination of pyruvate implying an anabolic role for ADH under these conditions.

The activity of deaminating ADH under carbon-limiting conditions was found to be highest at slow growth rates, indicative of increasing carbon supply via the catabolism of amino acids under these conditions (fig. 87). The lack of alanine in the system, other than the endogenous formation by the organism, may reflect the scavenging of carbon under these conditions. Assimilating ADH activity was found to be relatively constant at the studied growth rates under carbon limitation, this is similar to the observations of Aharonowitz & Friedrich (1980), where asparagine, alanine and ammonium permitted both assimilation of ammonium to yield alanine, and the catabolism of alanine to yield pyruvate and ammonia.

3.3.11.2 Summary of nitrogen metabolism in *M. echinospora* under nutrient-limited continuous culture

The presence of GS, ADH, AOAT and GOAT was established in *M. echinospora*. Previously there have been no studies of nitrogen metabolic enzymes in this organism. The presence of GDH and GOGAT could not be established under the conditions studied. The main mechanism of ammonium assimilation under the experimental conditions appears to be via the deamination of asparagine to yield aspartate (fig. 93). Aspartate is transaminated resulting in the formation of glutamate and oxaloacetate catalysed by GOAT. The oxaloacetate generated appears to play an important role in the carbon metabolism of this organism, as GOAT activity is increased with the dilution rate under carbon-limited conditions. This would explain the lack of GDH and GOGAT activities. The presence of a highly regulated GS, allows the formation of glutamine from glutamate and ammonium. GS activity appears to be post-translationally regulated by adenylation, with SVPDE allowing recovery of GS activity following ammonium shock.
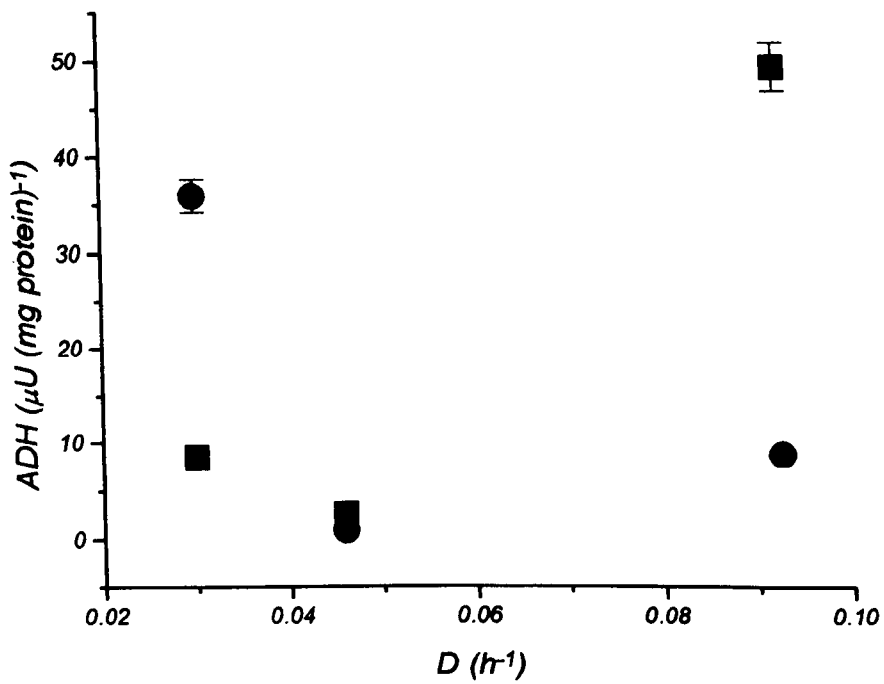
Figure 86. **Change in alanine dehydrogenase (ADH) levels during nitrogen-limited continuous culture.** Deaminating ADH (-●-), and assimilating ADH (-■-). Values are the mean of at least three determinations. Error bars represent the standard deviation of the data.
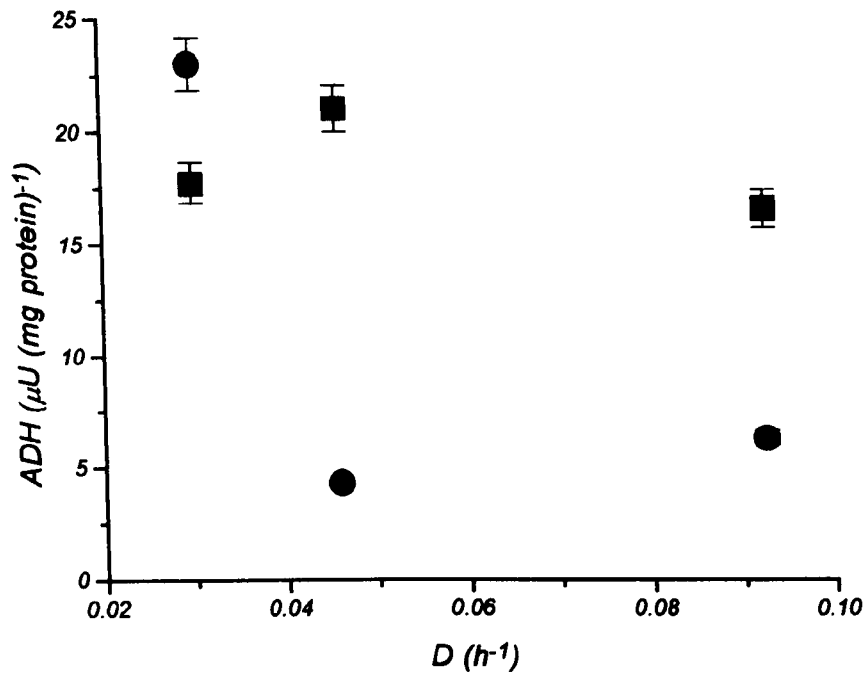
Figure 87. **Change in alanine dehydrogenase (ADH) levels during carbon-limited continuous culture.** Deaminating ADH (-●-), and assimilating ADH (-■-). Values are the mean of at least three determinations. Error bars represent the standard deviation of the data.
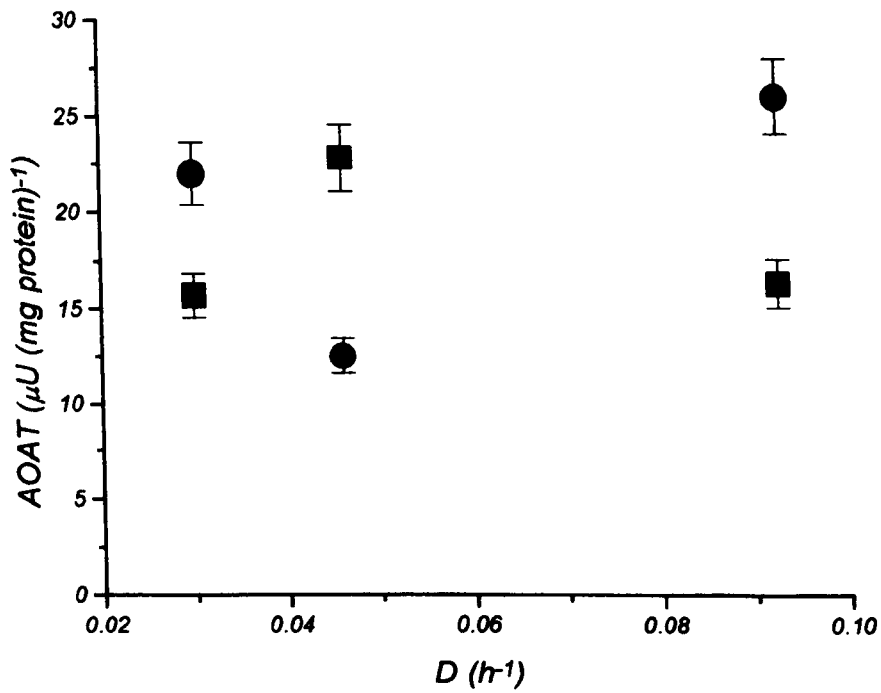
Figure 88. **Change in alanine:oxaloacetate aminotransferase (AOAT) levels during carbon and nitrogen-limited continuous culture.** Carbon-limited (-●-), and nitrogen-limited (-■-). Values are the mean of at least three determinations. Error bars represent the standard deviation of the data.

3.3.11.3 Enzymes of carbon metabolism

The levels of four carbon catabolic enzymes were investigated during carbon and nitrogen-limited growth in continuous culture at three growth rates under each limitation. Previous experiments demonstrated that nitrogen catabolic enzymes may play an important role in the supply of citric acid cycle intermediates (GOAT, AOAT, and ADH). The effect of these intermediates upon the catabolism of glucose was investigated via the relative activites of phosphofructokinase (the singularly most import glycolytic enzyme; Dawes, 1986), citrates synthase (known to be one of the main controlling enzymes of the citric acid cycle; Dawes, 1986). Additionally the pentose phosphate pathway (PPP) has been shown to function as an important pathway to the generation of reducing power (NADPH) for biosynthetic reactions and the formation of pentoses for synthesis of nucleic acids. The control of the PPP is at the entry to the pathway by the first enzyme, glucose-6-phosphate dehydrogenase (G-6-PDH). Obanye *et al.* (1996) demonstrated that antibiotic (methylenomycin) production correlated with increased flux through the PPP. The presence of NAD^+ and $NADP^+$ dependant G-6-PDH has been demonstrated in four streptomycetes species indicating that the PPP may also have a role in ATP-generating metabolism (Neuzil *et al.*, 1988).

The levels of citrate synthase (CS) were examined during nutrient-limited continuous culture (Fig. 89). During carbon-limited culture, the levels of CS activities were found to increase with the dilution rate. This is probably a reflection of increased carbon supply at the higher dilution rates. During nitrogen-limited culture at the faster growth rates (0.046 & 0.093 h^{-1}), CS levels were low (Fig. 89), however, the lowest studied growth rate (0.034 h^{-1}) under nitrogen-limited conditions resulted in CS activity seven-fold higher than that observed at the other nitrogen-limited growth rates.

Figure 89. **Change in citrate synthase (CS) levels during carbon and nitrogen-limited continuous culture.** Carbon-limited (-●-), and nitrogen-limited (-■-). Values are the mean of at least three determinations. Error bars represent the standard deviation of the data.

The levels of phosphofructokinase (PFK) were examined during nutrient-limited continuous culture (Fig. 90). The activities of PFK were found to be relatively constant during carbon-limited growth this reflecting the usage of glucose under these conditions, with increased glucose derived carbon within the organisms metabolism. Nitrogen-limited continuous culture resulted in low levels of PFK activity, at the faster growth rates studied (0.046 & 0.093 h^{-1}). The slowest growth rate under nitrogen-limitation resulted in a twelve-fold higher level of PFK activity. This would indicate that the carbon was derived from glucose rather than the catabolism of asparagine.

The level of glucose-6-phosphate dehydrogenase (G-6-PDH) was investigated during nutrient-limited continuous culture. Dual co-enzyme specificities of G-6-PDH have been reported from different species (Neuzil *et al.*, 1988). NADP linked G-6-PDH was found to exhibit an inversely proportional relationship to growth rate during nitrogen-limited growth (Fig. 91). The activity of this enzyme during carbon-limited growth was found to exhibit the opposite trend, with NADP linked G-6-PDH, increasing with the dilution rate. NAD linked G-6-PDH exhibited a complex profile during nutrient-limited continuous culture (Fig. 92). NAD linked G-6-PDH levels were lower during carbon-limited culture than nitrogen-limited culture. At a dilution rate of 0.046 h^{-1}, NAD linked G-6-PDH activities were similar under both nutrient-limitations. An increase in dilution rate resulted in increased activities of NAD linked G-6-PDH under nitrogen-limitation, yet under carbon-limitation no activity was detected.
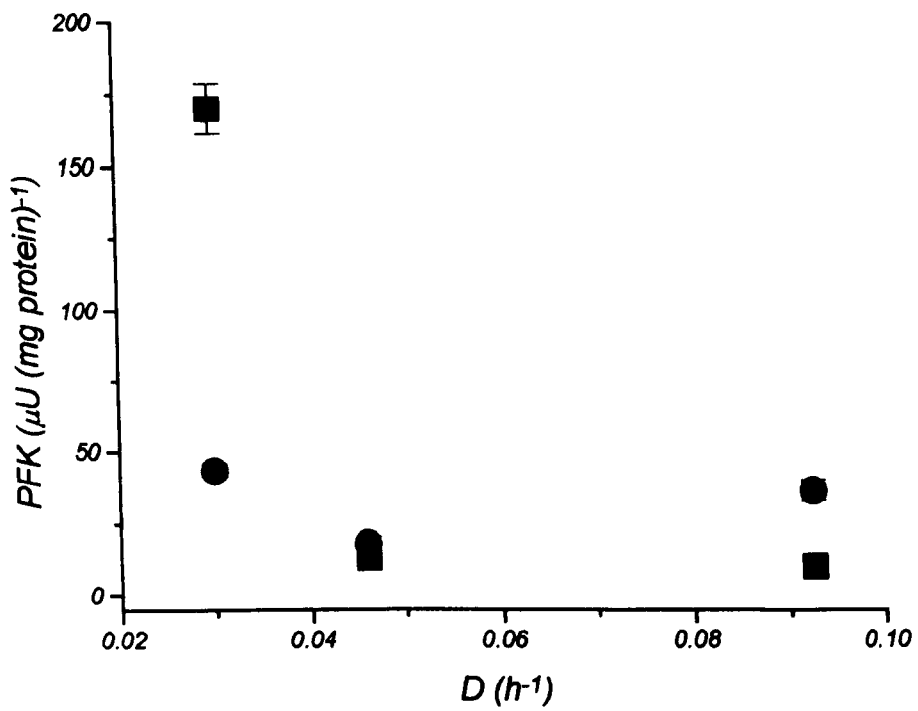
Figure 90. **Change in phosphofructokinase (PFK) levels during carbon and nitrogen-limited continuous culture.** Carbon-limited (-●-), and nitrogen-limited (-■-). Values are the mean of at least three determinations. Error bars represent the standard deviation of the data
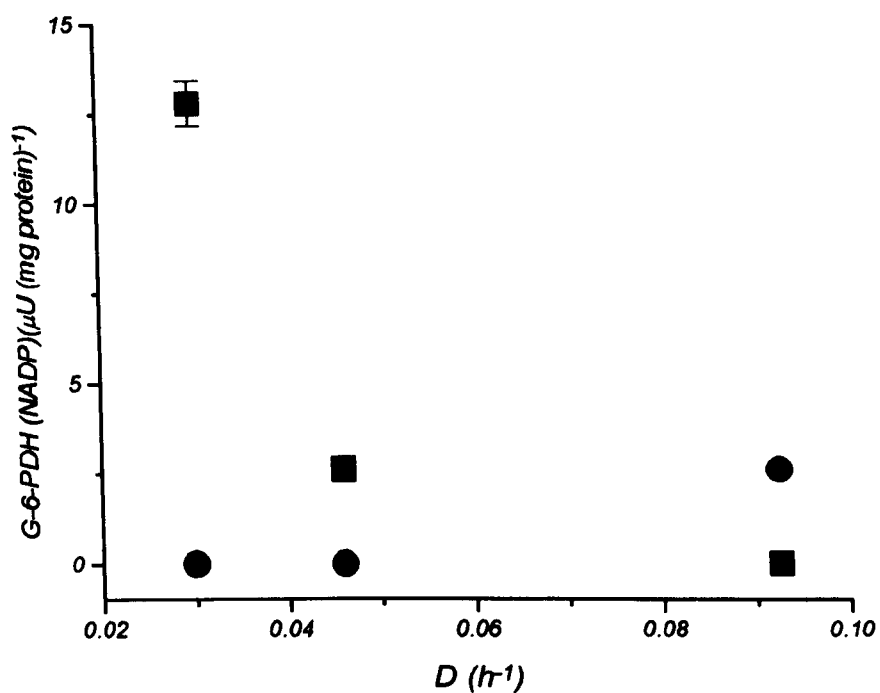
Figure 91. **Change in Glucose-6-phosphate dehydrogenase NADP-dependant (G-6-PDH) levels during carbon and nitrogen-limited continuous culture.** Carbon-limited (-●-), and nitrogen-limited (-■-). Values are the mean of at least three determinations. Error bars represent the standard deviation of the data.
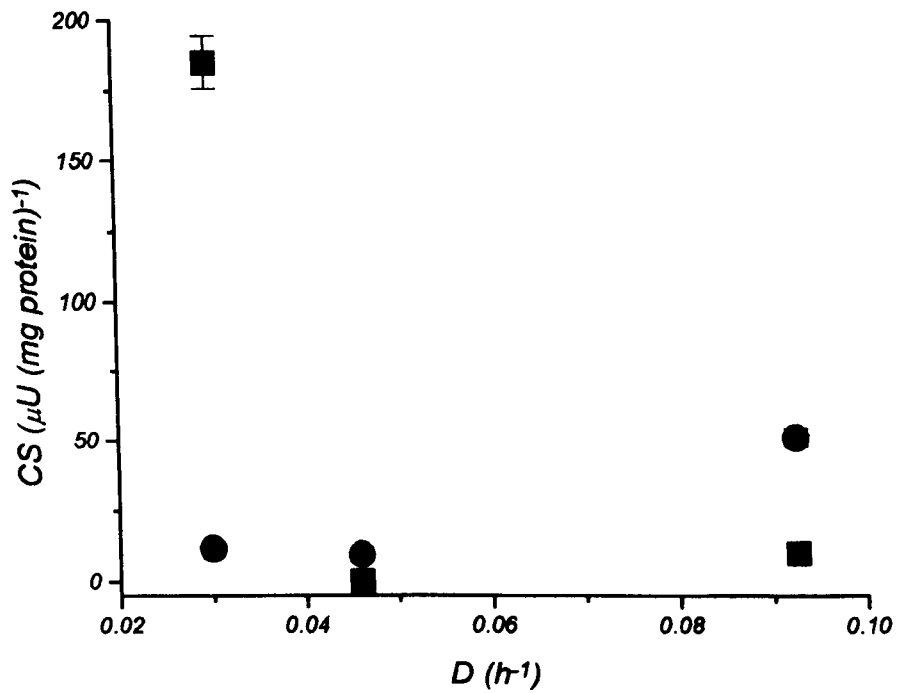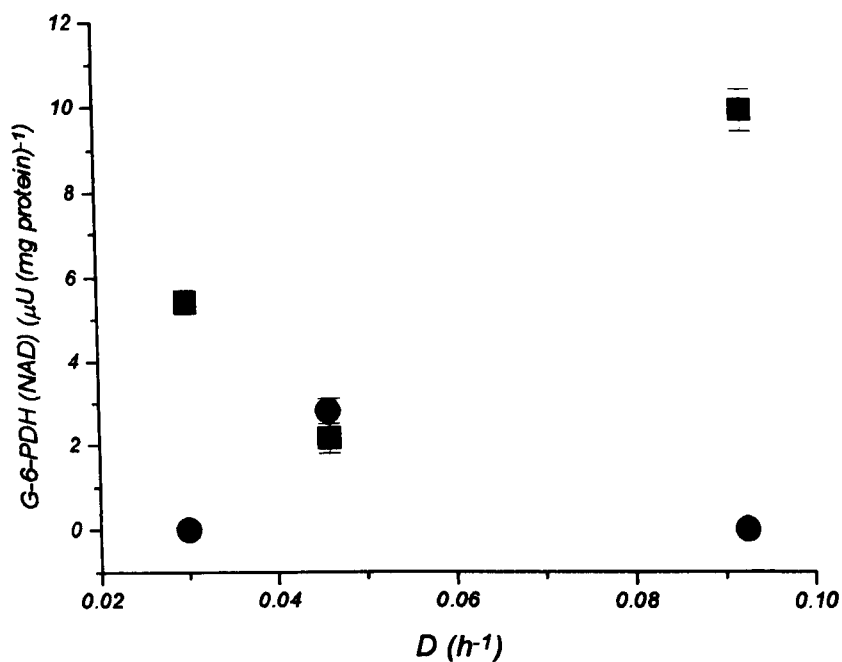
Figure 92. Change in Glucose-6-phosphate dehydrogenase NAD-dependant (G-6-PDH) levels during carbon and nitrogen-limited continuous culture. Carbon-limited (-●-), and nitrogen-limited (-■-). Values are the mean of at least three determinations. Error bars represent the standard deviation of the data.

3.3.11.4 Summary of carbon metabolism in *M. echinospora* under nutrient-limited continuous culture.

The presence of CS, PFK and NAD & NADP linked G-6-PDH were examined in *M. echinospora*. Previously there have been no studies of carbon metabolic enzymes in this organism. The main mechanism of carbon assimilation under the studied conditions appears to be via the deamination of asparagine to yield aspartate, which is subsequently deaminated to yield ammonium and oxaloacetate. The classic enzymes of glycolysis, the citric acid cycle, and the pentose phosphate pathway, appear to be peripheral to carbon metabolism when asparagine is present. Nitrogen-limitation results in the increase in the activities of all enzymes studied (Fig. 94).

Summary of primary metabolism in *M. echinospora* under nutrient-limited continuous culture and its relationship to gentamicin formation.

Previous experiments have revealed that nitrogen-limited, slow growth rates permit the formation of gentamicin. The flux through major primary metabolic pathways, indicates that asparagine, and its derivatives (aspartate, oxaloacetate, & ammonium) form an important role in this organisms primary metabolism. Previous studies of gentamicin biosynthesis have demonstrated that methionine, and deoxystreptomine are primary precursors (Rinehart & Stroshane, 1976). Methionine can be synthesised from aspartate, a product of asparagine catabolism, and deoxystreptamine can be formed from glucose.

The increase in glucose utilisation at the growth rate permitting gentamicin formation, and increased flux through the PPP may indicate a role for increased glucose catabolism under this growth rate. Glutamine has been shown to be an important source of nitrogenous groups for the biosynthesis of another aminoglycoside antibiotic, streptomycin, with nitrogen derived from asparagine being insignificant (Pipersberg, 1997). The increased activity of GS during culture may be a result of an increased requirement of glutamine for antibiotic biosynthesis.
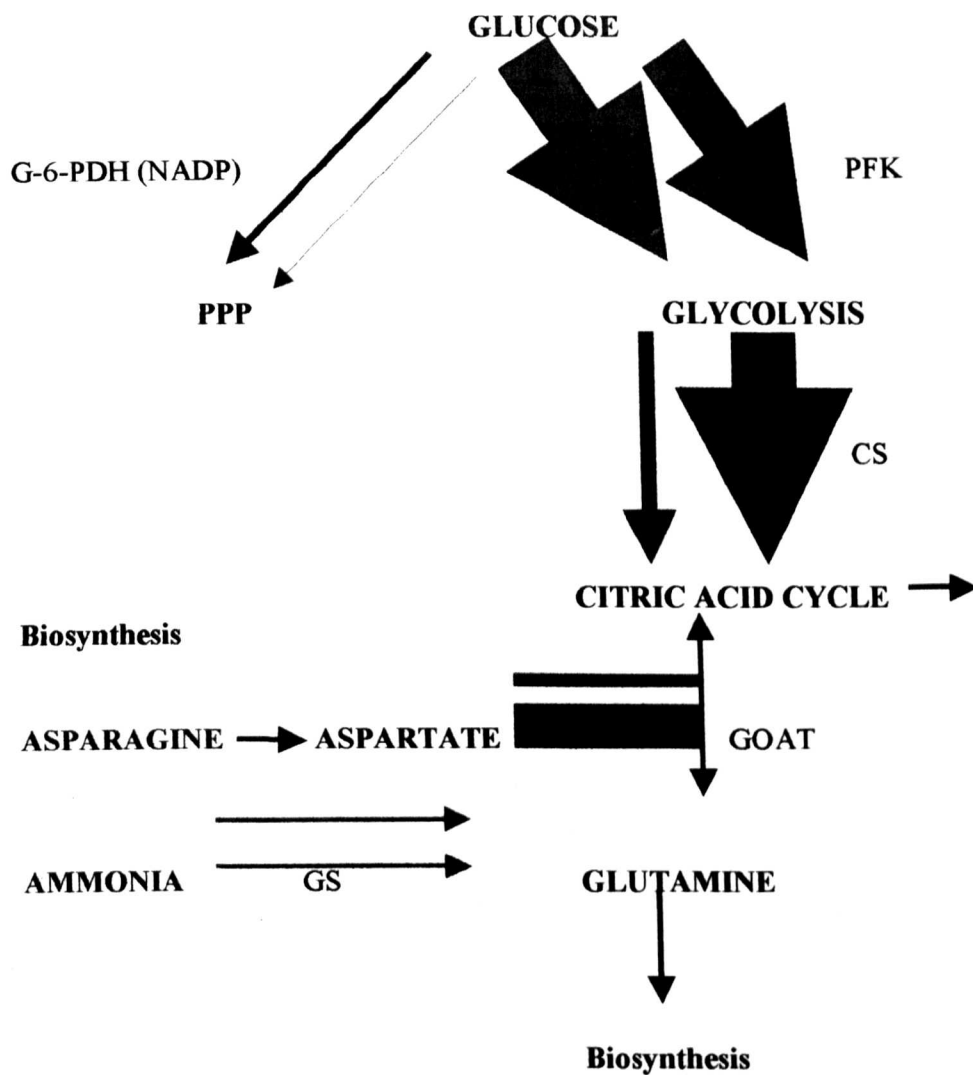
Figure 93. Diagrammatic representation of primary metabolic flux model for carbon-limited growth of *M. echinospora*. Line weight is relative to pathway activity. Black lines represent common pathways or hypothetical reactions. Green lines represent slow growth rate (0.034 h^{-1}). Red lines represent fast growth rate (0.093 h^{-1}). Blue text represents starting substrates.

GLUCOSE

G-6-PDH (NADP)

PFK

PPP

GLYCOLYSIS

CS

CITRIC ACID CYCLE

Biosynthesis

ASPARAGINE → **ASPARTATE**

GOAT

AMMONIA   GS

GLUTAMINE

Biosynthesis
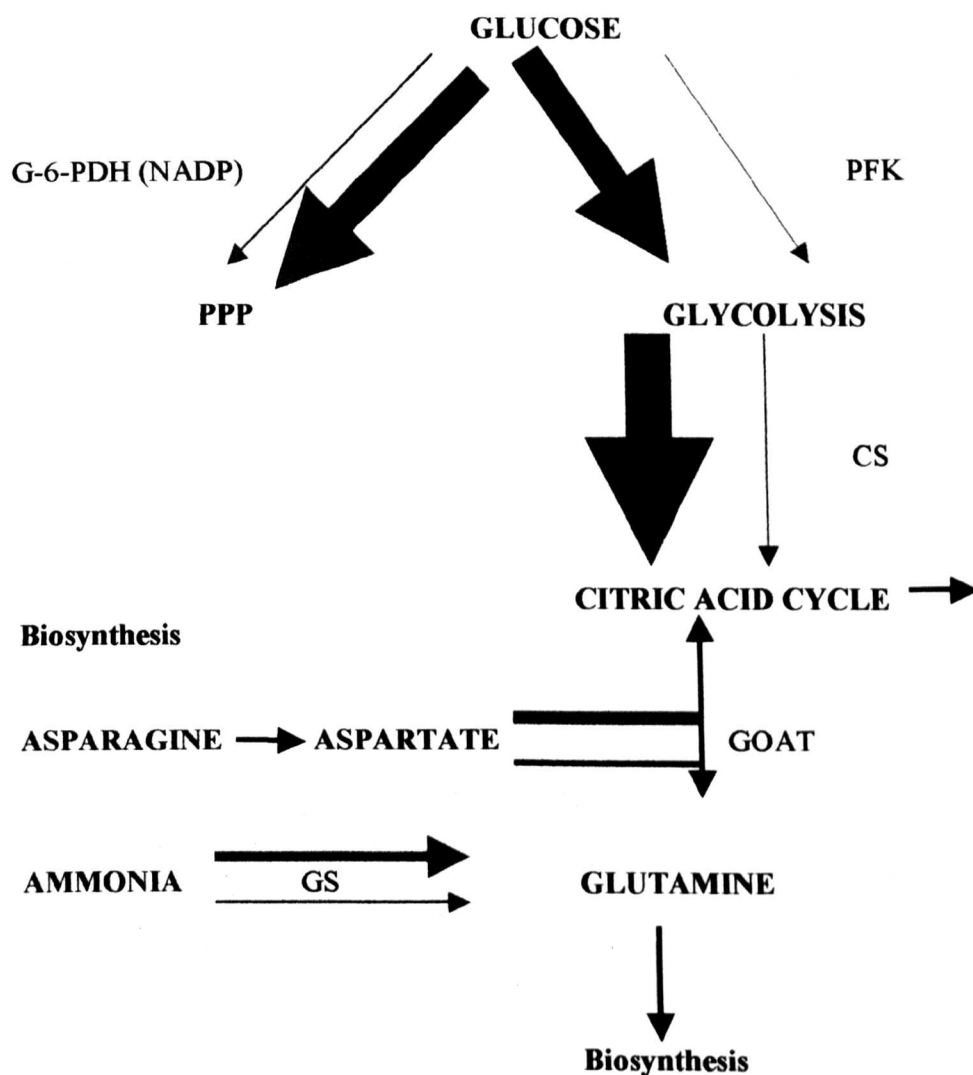
Figure 94. Diagrammatic representation of primary metabolic flux model for nitrogen-limited growth of *M. echinospora*. Line weight is relative to pathway activity. Black lines represent common pathways or hypothetical reactions. Green lines represent slow growth rate (0.034 h^{-1}). Red lines represent fast growth rate (0.093 h^{-1}). Blue text represents starting substrates.

3.3.12 Proteolytic enzymes of *Micromonospora echinospora* and differentiation

The previous section demonstrated the importance of amino acids as major carbon sources for *M. echinospora*. In many fermentation media and in the environment free amino acids are seldom encountered. The main organic nitrogen source in both these situations are proteins and peptides, which are only accessible following degradation by proteolytic enzymes, yielding small peptides and amino acids. Proteases in prokaryotic cells perform many functions, including cleavage of signal peptides during protein export, inactivation of regulatory proteins and removal of aberrant proteins (Gottesman, 1999) in addition to their nutritional role. It has been demonstrated in streptomycetes that proteases play an integral role in development with production correlating with sporulation (Ginter, 1978). A similar phenomenon was described for *Bacillus subtilis* where a number of intracellular and extracellular proteases are known to be associated with the initiation of sporulation (Ochi, 1985). These experiments aimed to investigate and characterize the activities of the extracellular proteases of *M. echinospora*, identifying both constitutive and temporally controlled species that are expressed during the growth cycle of the organism, and correlate these activities with sporulation.

3.3.12.1 Dynamics of protease production

The changes in extracellular proteolytic activity were studied in *M. echinospora* during batch culture in mYEME. Biphasic production of extracellular proteolytic activity was observed, with maximum levels measured during the stationary phase of growth (Fig. 95). A rapid increase in extracellular proteolytic activity was observed from 68 hours of growth, coinciding with a rapid decrease in ninhydrin-positive

amino compounds in the medium (Fig. 34). A second phase of increased extracellular proteolytic activity was observed with the onset of sporulation at 120 hours (*Fig. 95*).

To assess whether the extracellular proteolytic activity of *M. echinospora* was inducible by extracellular proteins and peptides supplied in the medium, batch culture was performed in minimal medium under specific nutrient limiting conditions, containing asparagine as the sole nitrogen source. The production of extracellular proteolytic enzymes during nitrogen-limited culture resulted in a single phase of protease production (Fig. 97), coinciding with the onset of sporulation, and the depletion of asparagine from the medium (Fig. 56). Extracellular proteolytic activity during culture under carbon-limitation revealed a single phase of protease production that was found to coincide with the onset of sporulation (*Fig. 96*). Furthermore nitrogen-limitation resulted in a two-fold higher level of extracellular proteolytic activity. This suggests that proteolytic enzymes are produced in response to depletion of readily accessible nitrogen from the medium. Additionally the mYEME experiments indicate that the proteolytic enzymes are induced in response to the presence of complex nitrogen sources, as the enzyme activities are up to ten-fold higher than those observed in minimal medium.

M. echinospora was grown in continuous culture to assess the effect of growth rate on protease production under carbon- and nitrogen-limited conditions. The level of proteolytic activity was higher under nitrogen-limiting conditions, than carbon-limiting conditions. Under carbon and nitrogen-limiting conditions proteolytic activity was not markedly influenced by growth rate (*Fig.98*). This latter result indicates that it is nutrient limitation, rather than decreasing growth rate that influences the activity of extracellular proteolytic enzymes.
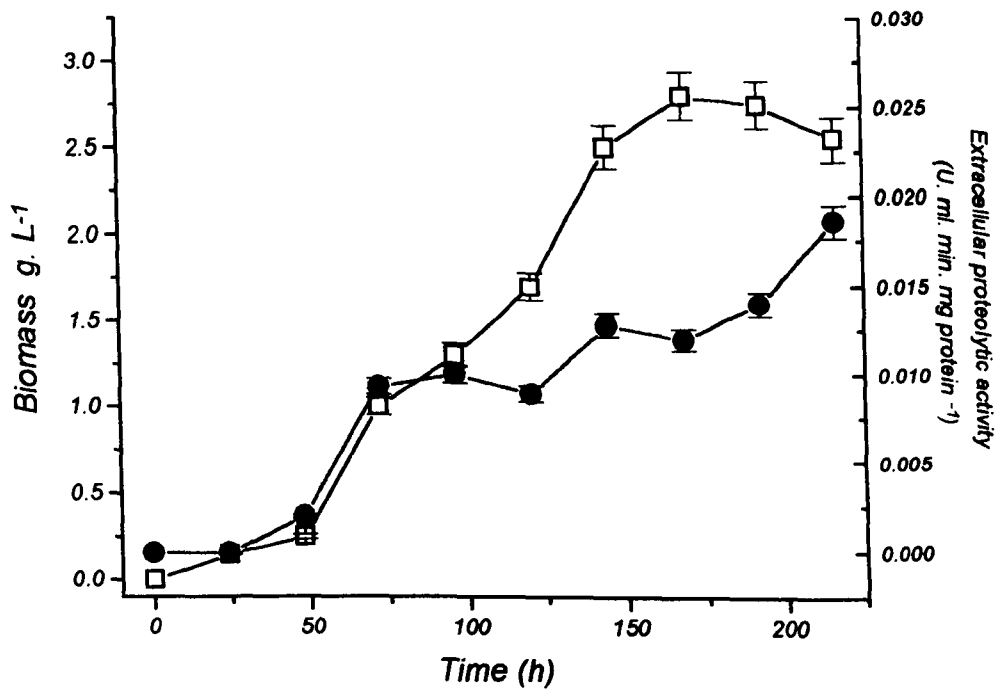
Figure 95. **Growth kinetics during batch culture in liquid YEME.** Biomass dry weight (-□-), and extracellular proteolytic activity (-●-). Values are the mean of at least three determinations. Error bars represent the standard deviation of the data.
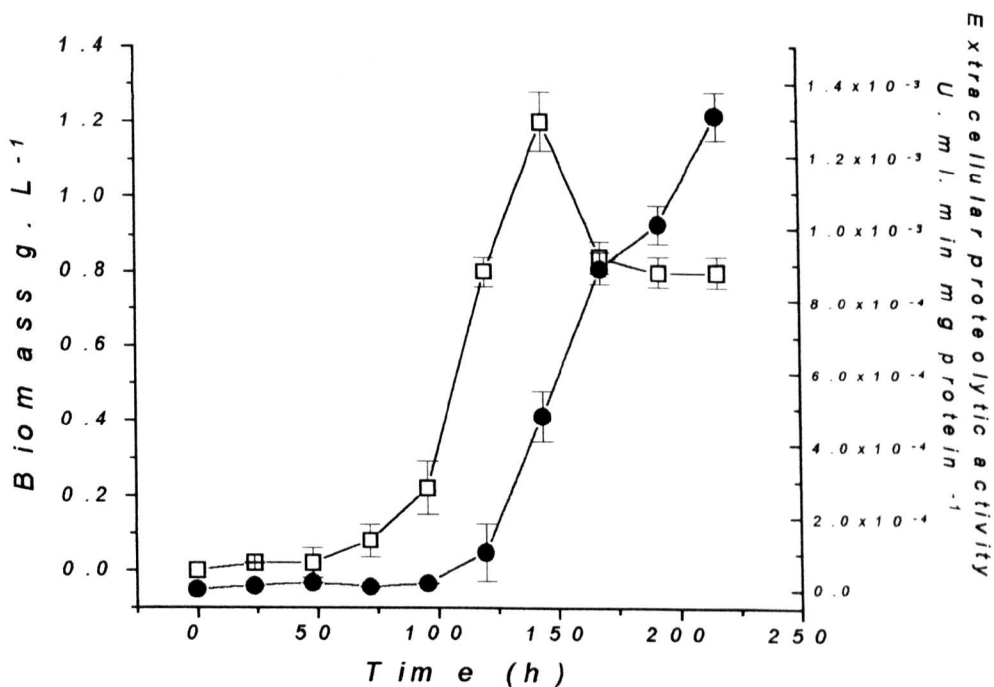
Figure 96. **Growth kinetics during batch culture in Carbon-limited minimal medium.** Biomass dry weight (-□-), and extracellular proteolytic activity (-●-). Values are the mean of at least three determinations. Error bars represent the standard deviation of the data.
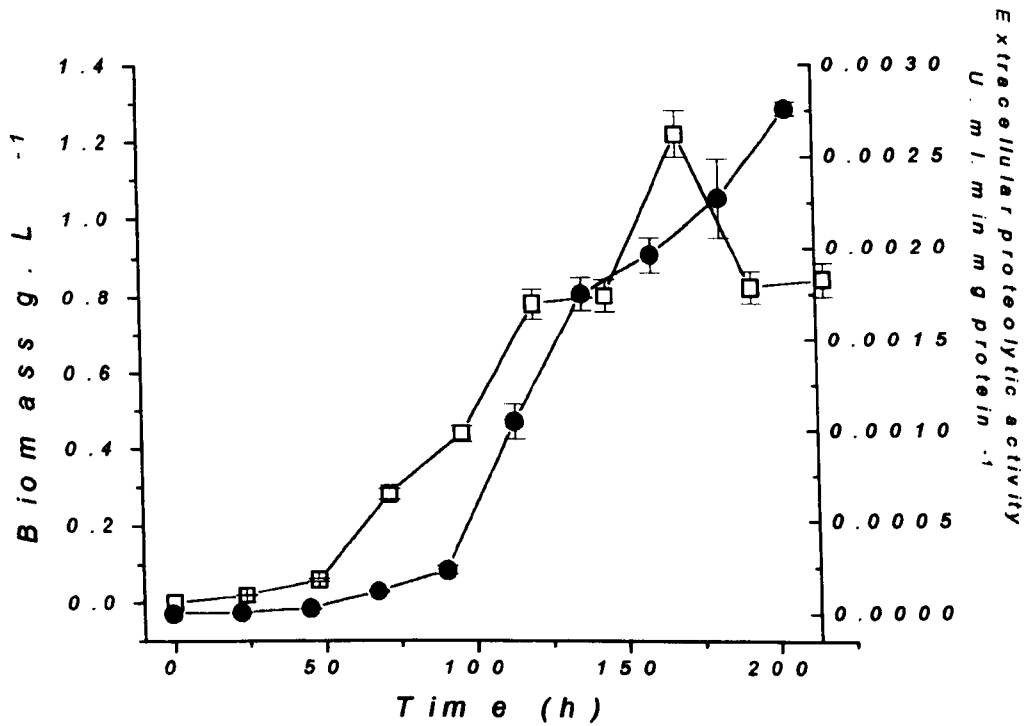
Figure 97. **Growth kinetics during batch culture in Nitrogen-limited minimal medium.** Biomass dry weight (-□-), and extracellular proteolytic activity (-●-). Values are the mean of at least three determinations. Error bars represent the standard deviation of the data.
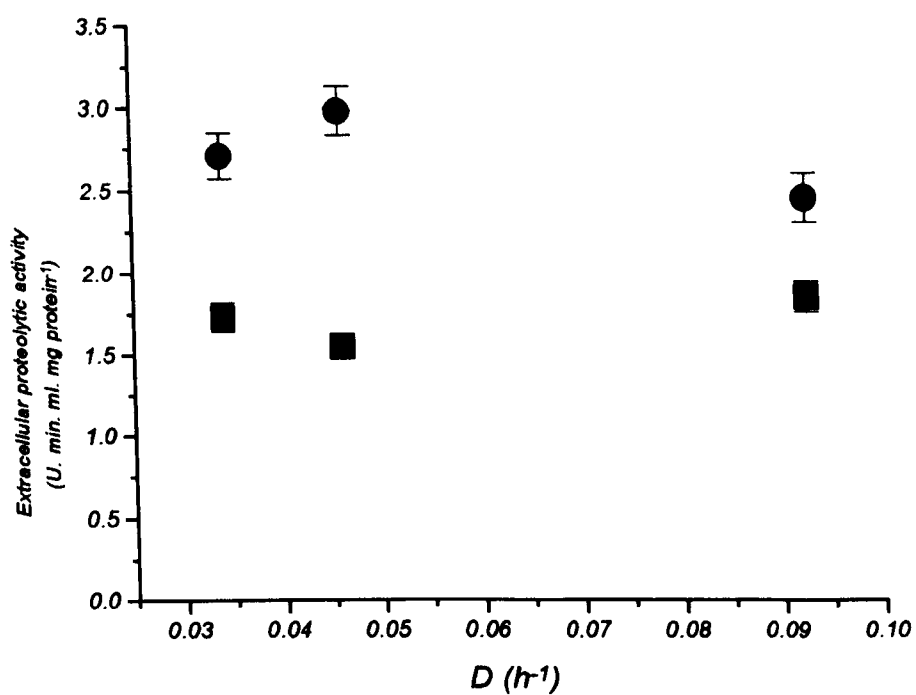
Figure 98. **Growth kinetics during continuous culture.** Extracellular proteolytic activity from nitrogen-limited chemostat (-●-), and from carbon-limited chemostat (-■-). Values are the mean of at least three determinations. Error bars represent the standard deviation of the data.

3.3.12.2 Response surface examination of extracellular proteolytic enzyme production.

To examine the effects and interactions of glucose and asparagine at various initial concentrations on the production of extracellular proteolytic enzymes a response surface was constructed . The x, y and z data are shown in table 16. The data was analysed and interpolated using Stanford graphics software (Stanford University). The response surface for protease production is presented in Figure 99. It was found that low concentrations of nitrogen (< 6 mM) and high levels of carbon (>400 mM) resulted in the highest levels of proteolytic activities within the cultures. These data also suggest that proteolysis is a response to nutrient limitation.

Table 16. Two-variable surface response design and biomass formation by shake flask cultures.

mM of Carbon	mM of Nitrogen	Extracellular protease
43.5	7.6	1.22×10^{-7}
87	7.6	1.43×10^{-7}
174	7.6	8.11×10^{-8}
261	7.6	6.48×10^{-8}
348	0.945	1.27×10^{-7}
348	1.89	1.04×10^{-7}
348	3.79	1.57×10^{-7}
348	5.68	4.91×10^{-8}
348	9.47	6.76×10^{-8}
348	11.4	6.8×10^{-8}
348	13.25	6.24×10^{-8}
435	7.6	6.6×10^{-8}
522	7.6	5.88×10^{-8}
609	7.6	4.94×10^{-8}
43.5	0.945	1.06×10^{-7}
609	1.89	3.67×10^{-7}
174	3.79	1.24×10^{-7}
261	5.68	8.00×10^{-8}
43.5	13.26	2.95×10^{-7}
174	11.4	3.14×10^{-8}
522	3.79	4.30×10^{-7}
435	9.47	2.67×10^{-7}
522	11.4	7.41×10^{-8}
609	13.26	2.74×10^{-7}
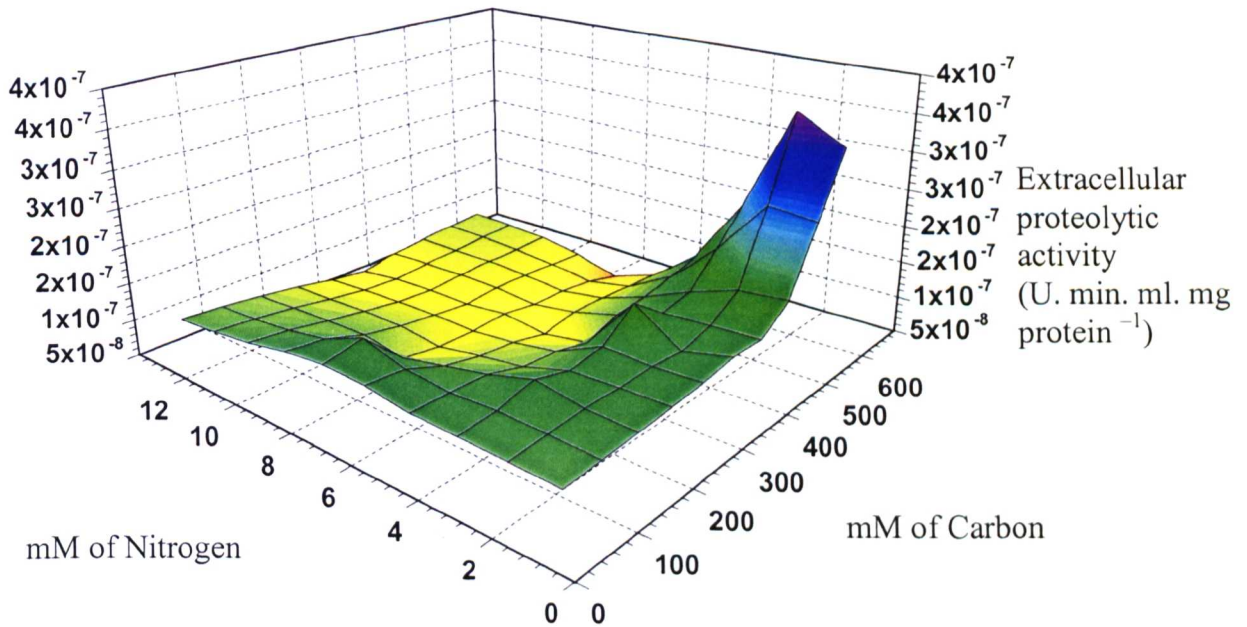
Figure 99. **3-D response surface plot of protease production.** Plot of protease (z) using initial medium concentrations of carbon (x) and nitrogen (y) as variables. Data was recalculated from initial data (Table x) using Stanford graphics (Stanford Software).

3.3.12.3 Properties of *M. echinospora* proteases

The activity of *M. echinospora* proteases was investigated over a broad pH range (pH 5-9.5). Protease activity was found to have an optimum of pH 7 throughout growth (Fig. 100). No differences were observed in the pH optima of the proteases from different media, or culture conditions.

An indication of proteolytic class was determined by incubation of culture supernatants with a range of protease inhibitors. Protease inhibitor studies were conducted on samples taken from the stationary phase of mYEME grown cultures. The extracellular proteases of *M. echinospora* were subject to significant inhibition of proteolytic activity with TLCK (an inhibitor of Trypsin-like serine proteases) and EDTA (an inhibitor of Metalloproteases) indicating the presence of trypsin-like proteases and metalloproteases (Table 17).

To examine the possible physiological role of the proteolytic enzymes, protease inhibitors were applied on to the surface of growing cultures (on filter papers (8 mm) impregnated with the inhibitor). Sporulation was inhibited by the application of TLCK and TPCK, established by the absence of the culture darkening and confirmed by impression mounts of the culture surface (Plate. 6). This latter result suggests a possible link between protease activity and morphological differentiation in *M. echinospora*. This coordination of protease production and development has previously been shown in *S. lactamdurans* with an asporulant mutant unable to produce proteases or antibiotics (Ginther 1978). It has also been demonstrated that the addition of inhibitors to growing submerged cultures of *S. albidoflavus* resulted in the inhibition of sporulation, suggesting an essential role for proteases in the development of these organisms (Kang *et al.*, 1995).

Table 17. The effect of various inhibitors on the activity of extracellular proteases. Values are means of at least three determinations (Standard deviations are shown in parentheses).

Inhibitor	Inhibition (%)
Control (no inhibitor)	100
TLCK (100 mM)	30 (± 4)
TPCK (100 mM)	15 (± 2)
EDTA (100 mM)	88 (± 5)
E-64 (1 mM)	15 (± 1)
Pepstatin (1 mM)	12 (± 3)

Plate 6. The effect of direct application of protease inhibitors to the surface of growing cultures. Clockwise from the top right inhibitors are as follows:- TLCK; TPCK; EDTA; E64; Pepstatin.
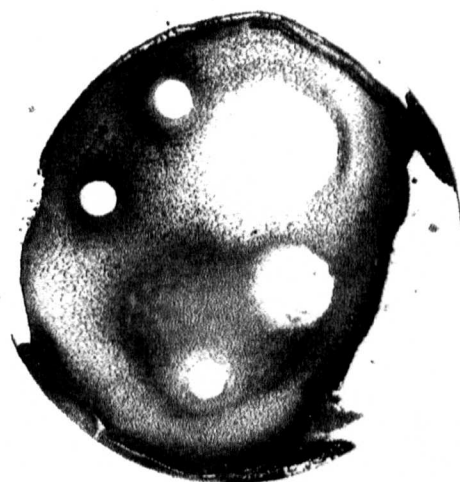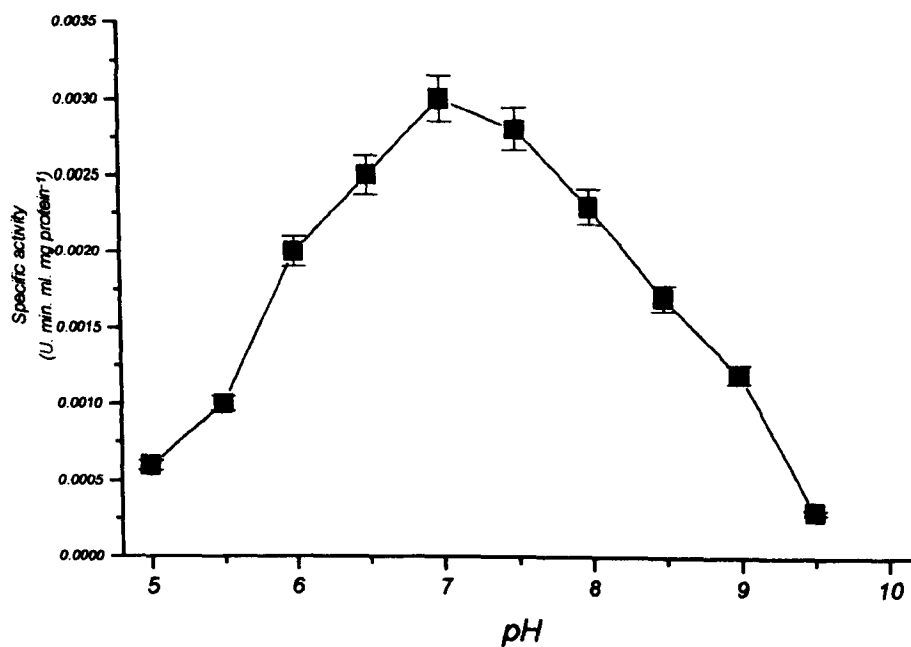
Figure 100. **The establishment of the pH optima for *M. echinospora* proteolytic enzymes.** Extracellular proteolytic activity was determined by the azocasein assay (Brock *et al.*, 1982). Values are the mean of at least three determinations. Error bars represent the standard deviation of the data.

3.3.12.4 Purification of proteases by affinity chromatography

Analysis of proteases produced by *M. echinospora* was achieved by a single step purification utilizing the peptide antibiotic, bacitracin, coupled to sepharose 4B (van Noort *et al.*, 1989). An initial set of experiments determined the ability of this method to purify proteolytic enzymes of *Micromonospora* (*Fig.* 101). The passage of culture supernatant over bacitracin-sepharose resulted in complete binding of the proteolytic component, with two passages. The proteolytic component of the supernatant remained bound following washing with 25 mM sodium acetate/acetic acid buffer (pH 4), and allowed the removal of unbound material, retained within the column matrix. The proteolytic enzymes were successfully eluted, (with retention of their activity, Fig. 101), with 25 % (v/v) Iso-propanol/1M NaCl in 200 mM Tris-HCl, (pH 7.8), and collected in 1 ml fractions. This allowed the detection of fractions containing the majority of the extracellular proteolytic activity, and the subsequent visualization of the proteins by SDS-PAGE. The visualization of intense protein bands following SDS-PAGE and increasing proteolytic activity, detected by the azocaesin assay, indicated a correlation between the fraction activity and the proteolytic protein bands obtained by SDS-PAGE (Fig. 102). This rationale allowed the prediction of fractions containing bands for all the purified proteases from the culture supernatants.
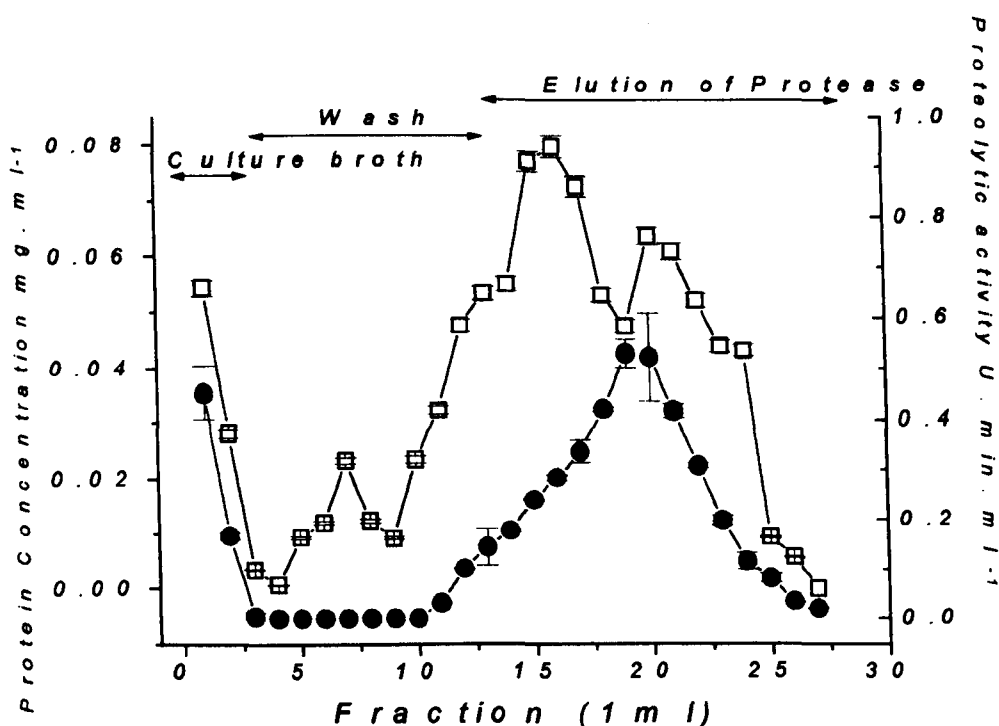
Figure 101. **The optimisation of bacitracin-sepherose affinity chromatography column for the purification of** *M. echinospora* **proteolytic enzymes.** Proteolytic activity of affinity column fractions (-●-) was determined by the azocasein assay (Brock *et al.*, 1982). The protein content (-□-) of the fractions was determined by the method of Bradford (1976). Fractions 1 & 2 were obtained after the culture supernatant was passed through the column. Fractions 3 to 10 were obtained during the wash stage. Fractions 11 to 27 were obtained during the elution of the column. Values are the mean of at least three determinations. Error bars represent the standard deviation of the data.

Figure 102. SDS-PAGE of purified proteolytic enzymes from YEME liquid culture demonstrating the binding capabilities of the bacitracin-sepherose affinity chromatography column.
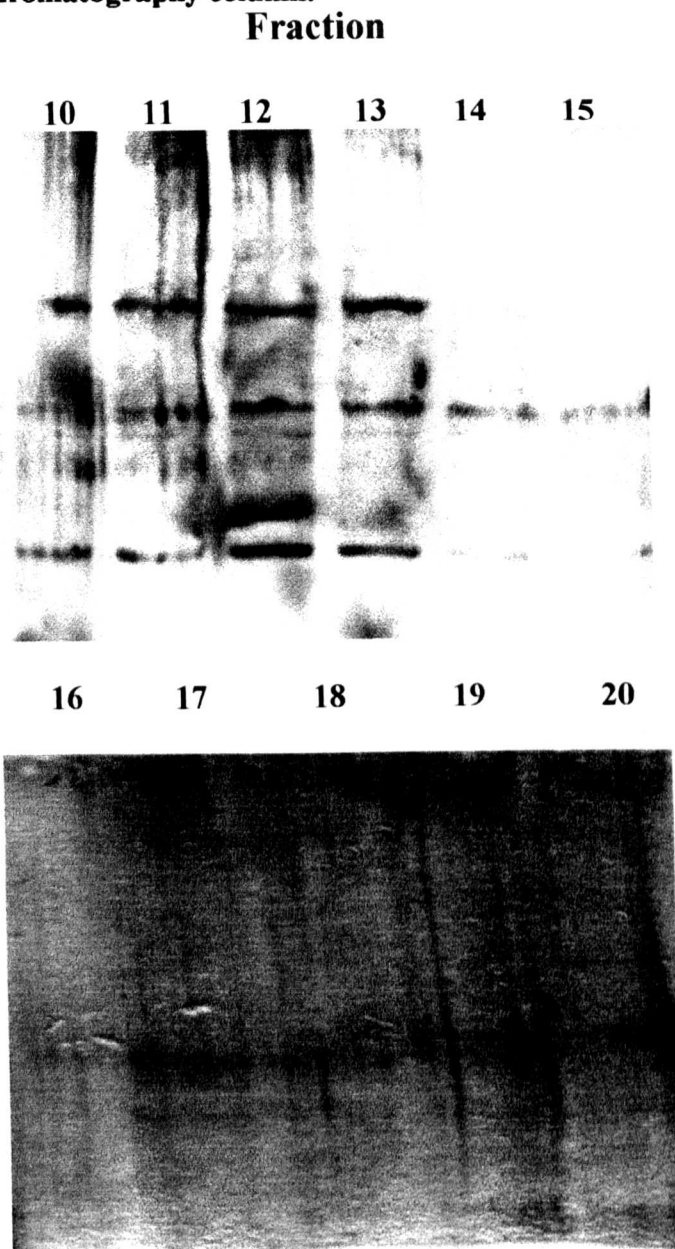
Fraction

Fractions collected from the bacitracin-sepharose affinity chromatography column were run on 10 % SDS-PAGE gels, with a 7.5 % stacking gel.

The proteolytic enzymes from carbon-limited batch culture were purified and revealed that three proteolytic enzymes were produced during growth and differentiation. A protease of 97.4 kDa appears throughout growth. At the onset of sporulation (120 h) a second protease (50 kDa) is produced transiently in the culture supernatant, and during stationary phase (192 h), a protease of 29 kDa is detected (Fig. 104).

The growth of *M. echinospora* in nitrogen-limited minimal medium revealed a complex profile of proteolytic proteins. Proteolytic enzymes appear in the culture supernatants following depletion of asparagine from the medium. Proteolytic proteins of masses, 32, 33, 50, and 90 kDa are present at 96 h, prior to the onset of differentiation. Following the onset of sporulation, additional proteases appear in the medium with approximate masses of 61.5 and 29 kDa (*Fig. 103*).

The growth of *M. echinospora* in continuous culture revealed proteolytic enzymes of 29 and 32 kDa expressed at all the studied growth rates under carbon and nitrogen-limiting conditions. As demonstrated by batch culture, the majority of proteolytic enzymes were produced under nitrogen-limiting conditions (Fig. 105).

The visualisation of the 50.2 kDa putative proteolytic protein at the onset of sporulation during carbon and nitrogen-limited batch culture, and at the slow growth rate under nitrogen-limitation may implicate a role in differentiation.

Figure 103. **SDS-PAGE of purified proteolytic enzymes from nitrogen-limiting batch culture**.
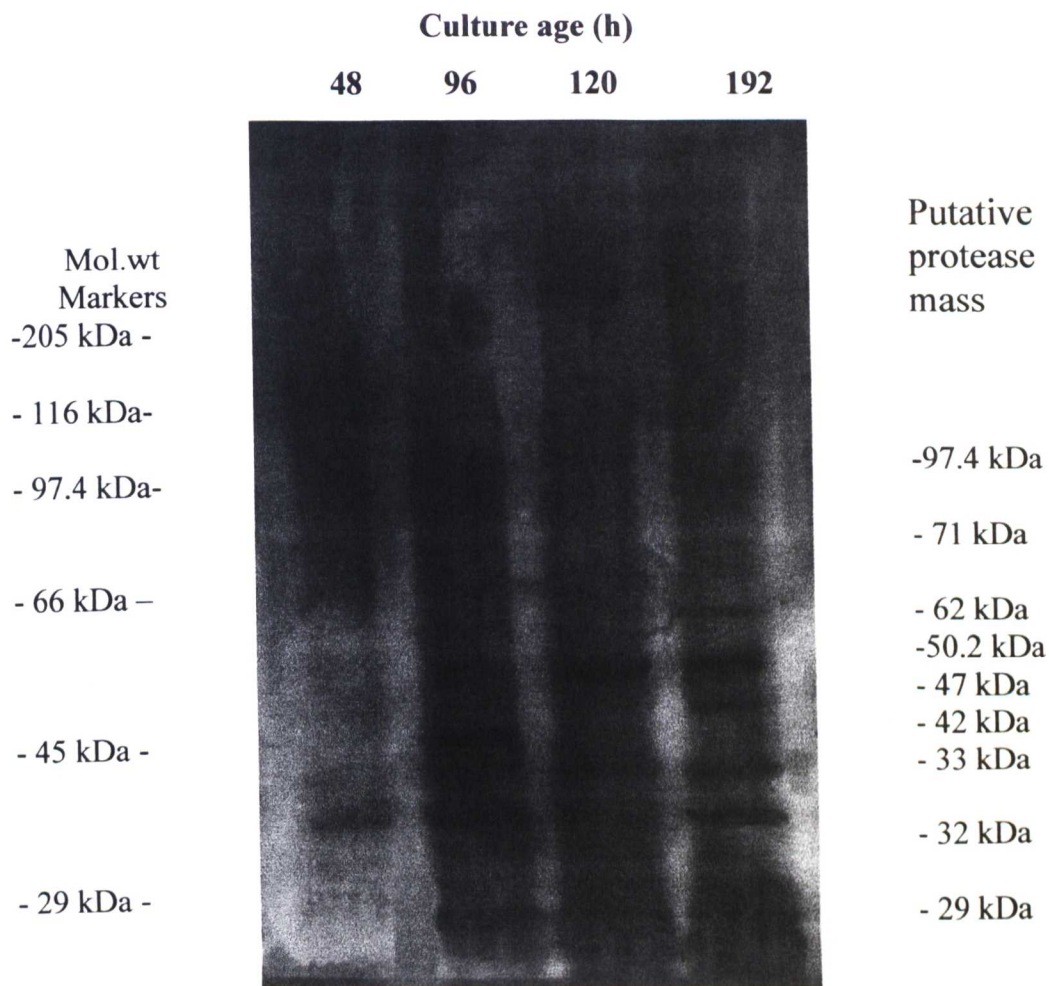
Culture age (h) is indicated on the top edge of the gel. Purified protein apparent masses are indicated on the right. Molecular mass markers are given on the left (Mol. Wt. Markers 30-200 kDa, Sigma).

Figure 104. SDS-PAGE of purified proteolytic enzymes from carbon-limiting batch culture.
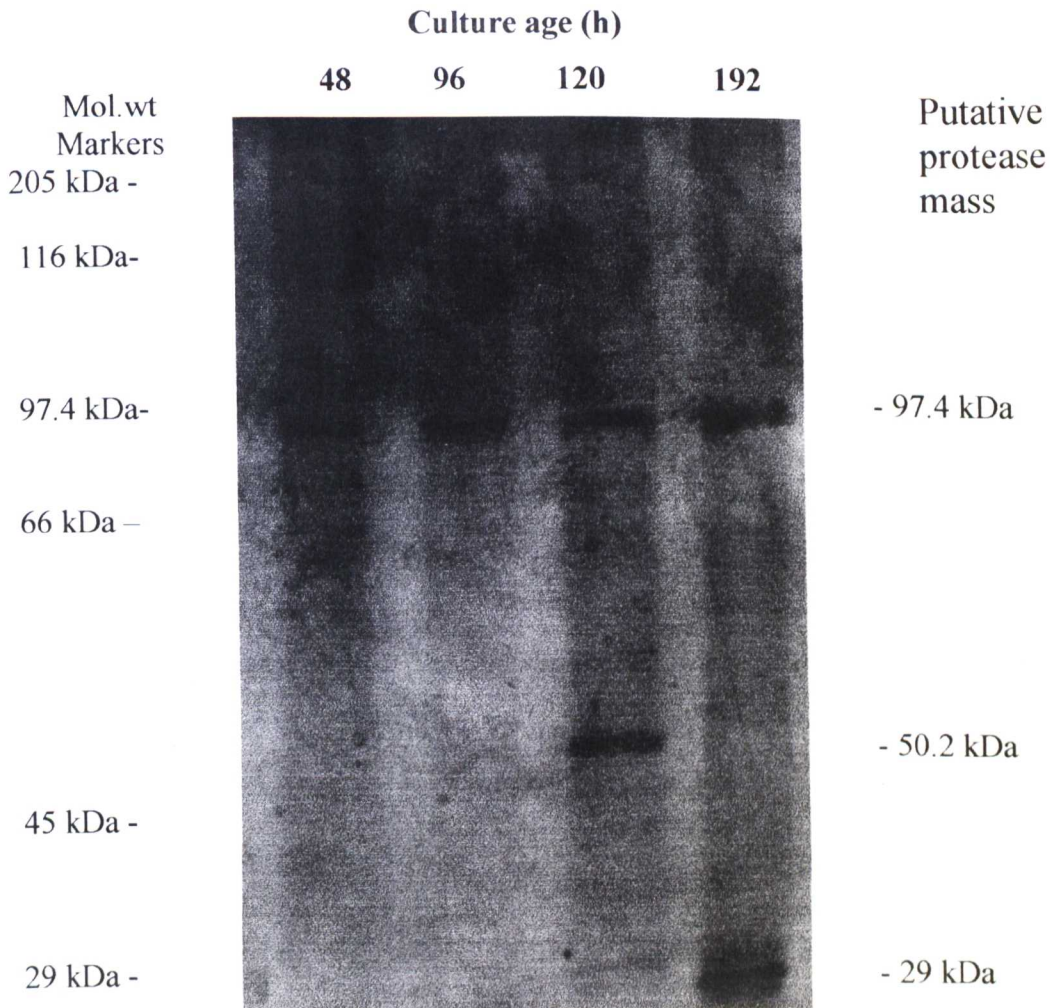
Culture age (h) is indicated on the top edge of the gel. Purified protein apparent masses are indicated on the right. Molecular mass markers are given on the left (Mol. Wt. Markers 30-200 kDa, Sigma).

Figure 105. SDS-PAGE of purified proteolytic enzymes from nutrient limiting chemostat.
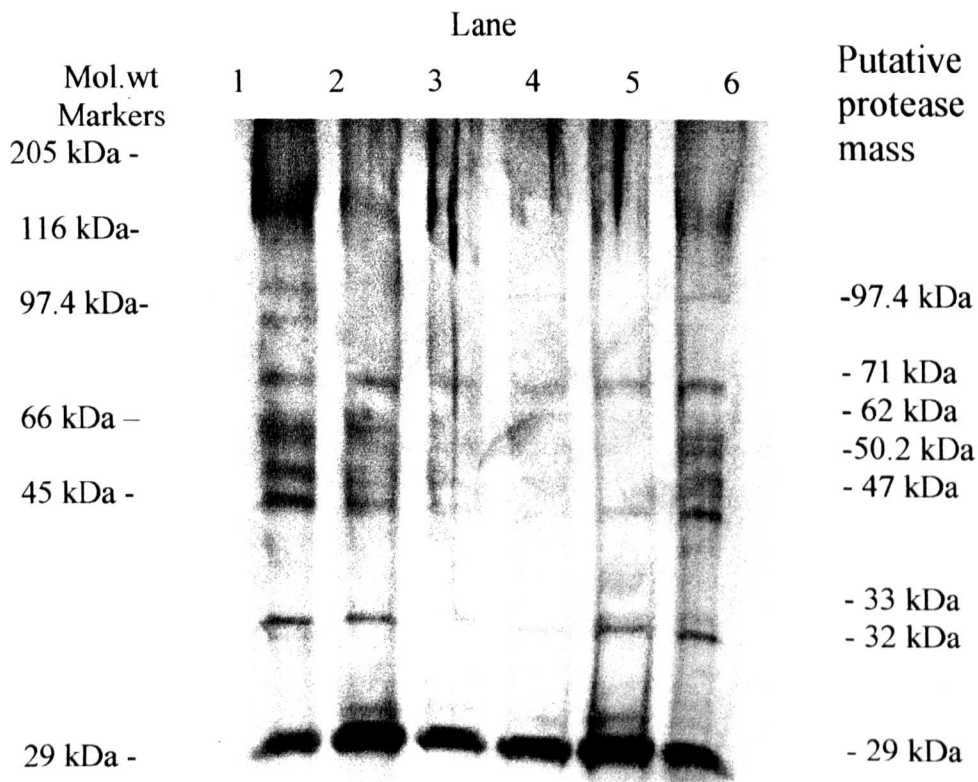
Lane 1: Nitrogen-limited chemostat (D=0.034 h^{-1}). **Lane 2**: Nitrogen-limited chemostat (D=0.046 h^{-1}). **Lane 3**: Nitrogen-limited chemostat (D= 0.092 h^{-1}). **Lane 4**: Carbon-limited chemostat (D=0.034 h^{-1}). **Lane 5**: Carbon-limited chemostat (D=0.046 h^{-1}). **Lane 6**: Carbon-limited chemostat (D= 0.092 h^{-1}). Purified protein apparent masses are indicated on the right. Molecular mass markers are given on the left (Mol. Wt. Markers 30-200 kDa, Sigma).

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3.3.12.5 Summary of studies on the proteolytic activity of *M.echinospora*

The production of proteolytic enzymes was studied during batch and continuous culture of *M. echinospora* in complex and nutrient-limited minimal medium. The production of extracellular proteases was found to correlate with the appearance of spores, and the depletion of nitrogen sources. The proteases were purified and visualised by a bacitracin-sepharose affinity chromatography and SDS-PAGE. Nitrogen-limited culture resulted in the production of up to nine proteolytic enzymes at different stages of growth. Carbon-limitation resulted in the production of up to three proteolytic enzymes. A continuous culture approach to the production of proteases was found to be regulated by the nutrient limitation, and by growth rate. Characterisation of the proteases revealed a pH optima of 7, and inhibition studies indicated the presence of trypsin-like serine proteases and metalloprotease.

3.3.13 Investigation of developmental gene expression in *M.echinospora*

The genetically best-characterised filamentous actinomycete, *Streptomyces coelicolor* has been shown to contain key developmental genes that exhibit homology to genes found in other actinomycete genera (Soliveri *et al.*, 2000). A PCR approach was used to identify genes within the genome of *M. echinospora* demonstrating similarity to those previously identified and sequenced in *S. coelicolor* (http://www.sanger.ac.uk/projects/S_coelicolor/). Genes that had previously been shown to be developmentally regulated in *S. coelicolor*, were identified and their expression investigated in *M. echinospora*.

3.3.13.1 Establishing the presence of *Streptomyces coelicolor* homologous gene sequences in *M. echinospora*.

The importance of glycogen accumulation in this organism has been demonstrated in previous sections. The biosynthesis of glycogen involves three enzymes: ADP glucose pyrophosphorylase, glycogen synthase, and the branching enzyme (1,4-α-D-glucan: 1,4-α-D-glucan 6-glycosyltransferase), which are products of the *glgC*, *glgA*, and *glgB* genes respectively (Homerova *et al.*, 1996; Preiss & Romeo, 1989). In *Streptomyces coelicolor*, glycogen is accumulated in two distinct phases, each associated with duplicated operons, containing a developmentally specific glycogen branching gene *glgB* (Schneider *et al.*, 2000). The correct structure of glycogen requires the activity of unlinked genes involved in glycogen biosynthesis such as *glgC*, which is only essential for the first phase of accumulation in *S. coelicolor*. Primers designed for *S. coelicolor* (Martin *et al.*, 1997) were used to amplify a

product of 450 bp, corresponding to the approximate mass of PCR products obtained for *glgC* in *S. coelicolor (Fig. 106)*.

Primers based on a highly conserved region of the *glgB* gene (Bruton *et al.*, 1995) were used to amplify a PCR product of approximately 500 bp, similar in mass to the product obtained in *S. coelicolor* using the same primers (Fig. 106).

The presence of a stringent response, and formation of ppGpp was demonstrated in *M. echinospora* in a previous section. The formation of ppGpp is dependant upon the product of the *relA* gene, ppGpp synthetase. The *relA* gene of *S. coelicolor* has previously been cloned (Chakraburtty *et al.*, 1996). Primers designed during this study were used to amplify a PCR product of 550 bp, in *M. echinospora*, this product being larger than that obtained in *S. coelicolor* using the same primers (Fig. 107).

The *bldD* gene encodes a transcription factor in *S. coelicolor* (Elliot *et al.*, 2001), and has been shown to act on developmental genes, repressing their activity, during vegetative growth (Keleman *et al.*, 2001). Primers designed for *S. coelicolor* (Keleman *et al.*, 2001) were used to amplify a PCR product in *M. echinospora* with an apparent mass of 450 bp *(Fig. 107)*.

The sporulation gene cascade in *S. coelicolor* requires the expression of the early sporulation gene *whiB*. The morphology of *whiB* deletion mutants exhibit coiled, undifferentiated aerial hyphae lacking septa, indicating the involvement of *whiB* in the delimitation of spores (Flardh *et al.*, 1999). The presence of *whiB* like genes have previously been demonstrated in morphologically diverse actinomycetes, including *Micromonospora chalcea* (Soliveri *et al.*, 1993; & 2000). The use of previously published primers (Soliveri *et al.*, 2000) allowed the amplification of a PCR product from genomic DNA with an apparent mass of 300 bp *(Fig. 108)*. The expected product in *S. coelicolor* was 309 bp.

The bldA gene of *S. coelicolor* encodes a leucyl tRNA that recognises the rare UUA

codon in this organism. The observation that this codon is present in genes that are

expressed only late in growth, such as sporulation and antibiotic genes, has led to the

suggestion that *bldA* may represent a translational regulatory mechanism for

developmental genes in *Streptomyces*. The presence of this gene has been

established in several *Streptomyces* species (Leskiw *et al.*, 1991). A PCR approach

using previously designed primers for this gene (Lekiw & Mah, 1995), allowed the

detection of a product 297 bp in length corresponding to the previously detected

product in *S. coelicolor* (fig, 108).

A PCR product for each gene amplified was sequenced. The sequence data was

analysed in a basic local alignment search tool (BLAST) program (NCBI & EMBL).

Each sequence was found to demonstrate similarity to the intended gene sequence in

each search.

3.3.13.2 Monitoring of differential gene expression

The presence of genes in *M. echinospora* demonstrating similarity to developmentally regulated genes in *S. coelicolor* enabled the use of reverse transcriptase PCR (RT-PCR) to monitor transcription of genes expressed in response to environmental and physiological conditions. RT-PCR has previously been used to investigate differences in the relative expression level of genes involved in the sporulation cascade of *Bacillus subtilis* (Hernandez *et al.*, 2000).Initial experiments were conducted to discover the boundaries of the RT-PCR kit used; that is, do different amounts of RNA incorporated in to the reaction result in different levels of PCR product following amplification? Figure 109. demonstrates that increasing the amount of template RNA per reaction results in detectable differences in the amount of product produced during the PCR. This enabled the relative differences in the level of transcripts of each gene to be assessed in a temporal profile of gene expression in *M. echinospora*.

Figure 106. **Agarose gel electrophoresis of PCR products formed using primers for *glgC* and *glgB*, amplified from genomic DNA (Bruton *et al.*, 1997; Martin *et al.*, 1997).**
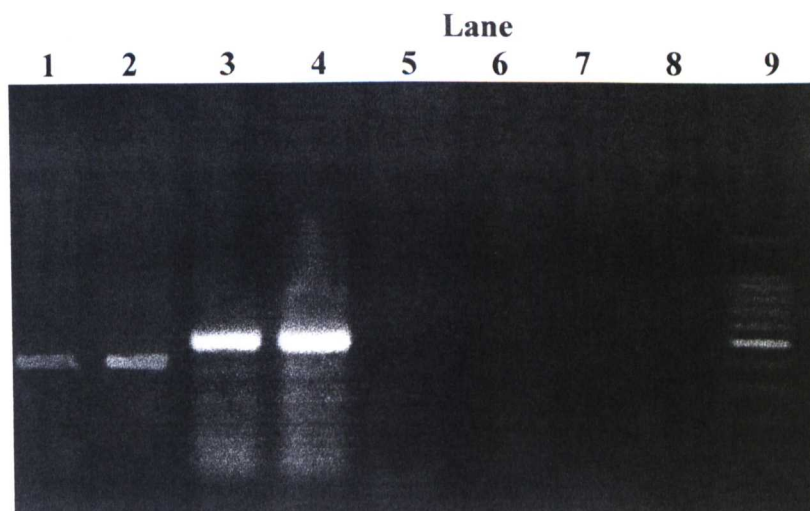
Lane 1: PCR using *glgC* primers. Apparent mass of product is 450 bp. **Lane 2**: PCR using *glgC* primers. Apparent mass of product is 450 bp. **Lane 3**: PCR using *glgB* primers. Apparent mass of product is 500 bp. **Lane 4**: PCR using *glgB* primers. Apparent mass of product is 500 bp. **Lane 5**: Control *glgC* primers only (No genomic DNA). **Lane 6**: Control genomic DNA only (No primers). **Lane 7**: Control *glgB* primers only (No genomic DNA). **Lane 8**: Control genomic DNA only (No primers). **Lane 9:** Markers 100 - 1500 bp, with 500 bp marker at triple intensity (Promega).

Figure 107. **Agarose gel electrophoresis of PCR products formed using primers for *relA* and *BldD* amplified from genomic DNA (Chakraburtty *et al.*, 1996; Kelemen *et al.*,2001).**
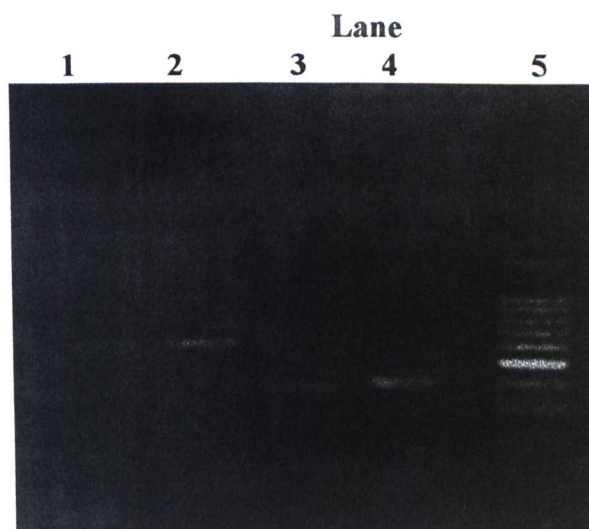
Lane 1: PCR using *relA* primers. Apparent mass of product is 550 bp. **Lane 2**: PCR using *relA* primers. Apparent mass of product is 550 bp. **Lane 3**: PCR using *BldD* primers. Apparent mass of product is 450 bp. **Lane 4**: PCR using *BldD* primers. Apparent mass of product is 450 bp. **Lane 5**: Markers 100 - 1500 bp, with 500 bp marker at triple intensity (Promega).

Figure 108. Agarose gel electrophoresis of PCR products formed using primers for *whiB*, *BldA* amplified from genomic DNA (Soliveri *et al.*, 2000 & Leskiw *et al.*, 1993).
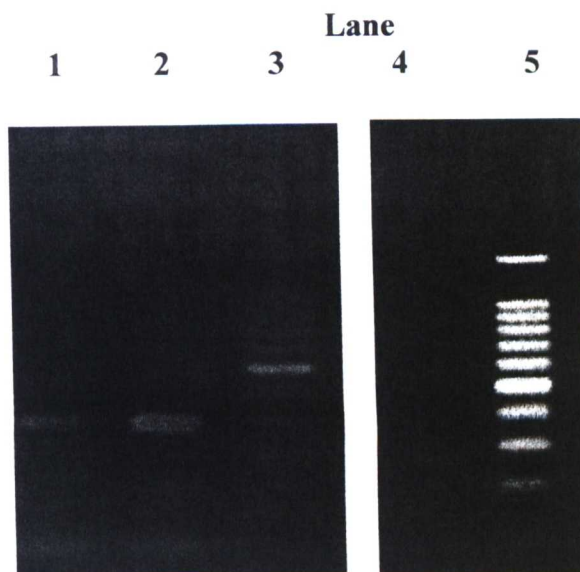
Lane
1   2   3   4   5

Lane 1: PCR using *whiB* primers. Apparent mass of product is 300 bp. **Lane 2**: PCR using *whiB* primers. Apparent mass of product is 300 bp. **Lane 3**: Markers 100 - 1500 bp, with 500 bp marker at triple intensity (Promega).**Lane 4**: PCR using *BldA* primers. Apparent mass of product is 297 bp. **Lane 5**: Markers 100 - 1500 bp, with 500 bp marker at triple intensity (Promega).

NB: Control lanes omitted from figure for clarity.

Figure 109. **Agarose gel electrophoresis of RT-PCR products formed using primers for *glgB* (Bruton *et al.*, 1997), amplified from total RNA to indicate the relationship between RNA template added to reaction and product formed.**
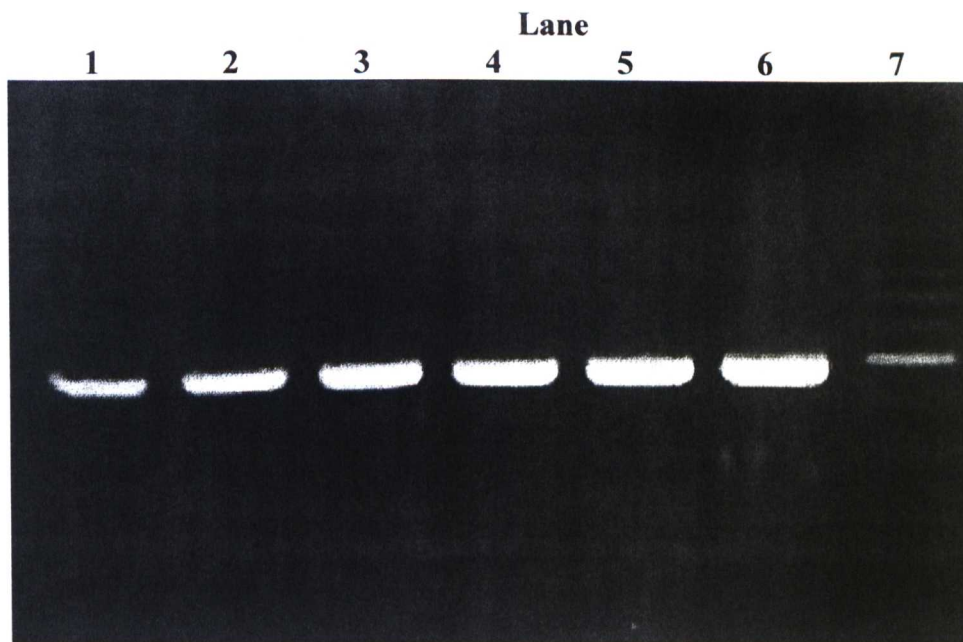
Lane 1: RT-PCR using *glgB* primers. RNA loaded = 25 ng μl^{-1} **Lane 2**: RNA loaded = 50 ng μl^{-1} **Lane 3**: RNA loaded = 100 ng μl^{-1} **Lane 4**: RNA loaded = 150 ng μl^{-1} **Lane 5**: RNA loaded = 200 ng μl^{-1} **Lane 6**: RNA loaded = 400 ng μl^{-1} **Lane 7**: Markers 100 - 1500 bp, with 500 bp marker at triple intensity (Promega).

Previous experiments have demonstrated that differentiation is regulated by the availability of nitrogen in *M. echinospora*. It was decided to investigate the transcription of the homologous genes identified by PCR, during batch culture under nitrogen-limited conditions. The swift manner in which *M. echinospora* responds to nutrient shiftdown, such as the initiation of the stringent response, indicated a profile of gene expression following nitrogen shiftdown would provide an insight into the manner in which differentiation is modulated in this organism.

3.3.13.3 Gene expression following nitrogen shiftdown.

To elicit nitrogen starved conditions *M. echinospora* mycelia grown for 72 hours in YEME medium were transferred to minimal medium, containing sodium nitrate (0.5 g. l^{-1}) as the sole nitrogen source and glucose as the sole carbon source. RNA was harvested at time intervals following shiftdown, and RT-PCR performed. Each RT-PCR assay contained 100 ng of RNA per reaction.

The transcripts of genes involved in glycogen biosynthesis (*glgB* & *glgC*) appear to be at greater abundance following shiftdown to nitrogen-limited medium (Fig. 110). These data correspond with the physiological data obtained in earlier experiments where glycogen is accumulated in response to nitrogen-limitation. Additionally, in many bacteria, intracellular storage materials have been shown to be produced in response to nitrogen starvation (Dawes, 1992).

The abundance of transcripts for the early sporulation gene, *whiB*, was increased following the depletion of nitrogen in the medium (Fig. 110). The abundance of these transcripts appears to diminish fours hours post-shiftdown. In surface grown cultures of *S. coelicolor* the transcript for *whiB* is transiently expressed during

normal growth, and interacts with other developmental genes resulting in the formation of sporulation septa (Soliveri *et al.*, 1992).

The transcripts of both *relA*, and *bldD* were undetectable under the conditions studied (Fig. 111). The products of both genes are known to be transient and this may explain why the transcripts were not detected during RT-PCR. Experiments were repeated with RNA extraction at two-minute intervals, along with the addition of further PCR cycles, and increased gel loading. Again, PCR-products were not detected for either of these genes. It is possible that the Qiagen RNeasy RNA extraction kit is not suitable for such transient transcripts, due to the length of the initial centrifugation stage. The use of different methods for the extraction of RNA, such as those detailed by Kieser *et al.*, (2000), may provide a way forward in the future.

The detection of *bldA* transcripts occurred within 30 minutes of nitrogen downshift, and were found to persist over the following two hours (Fig. 112). This is consistent with reports in *S. coelicolor*, where *bldA* transcripts are present prior to sporulation and antibiotic biosynthesis and persist into stationary phase (Leskiw & Chater 1991; Chater 1989).

Figure 110. Agarose gel electrophoresis of RT-PCR products formed using primers for *glgB*, *glgC*, and *whiB* (Bruton *et al.*, 1997; Martin *et al.*, 1997; Soliveri *et al.*, 2000), amplified from total RNA, extracted following nitrogen shiftdown.

Lane
1   2   3   4   5   6   7   8   9   10   11   12   13   14   15   16
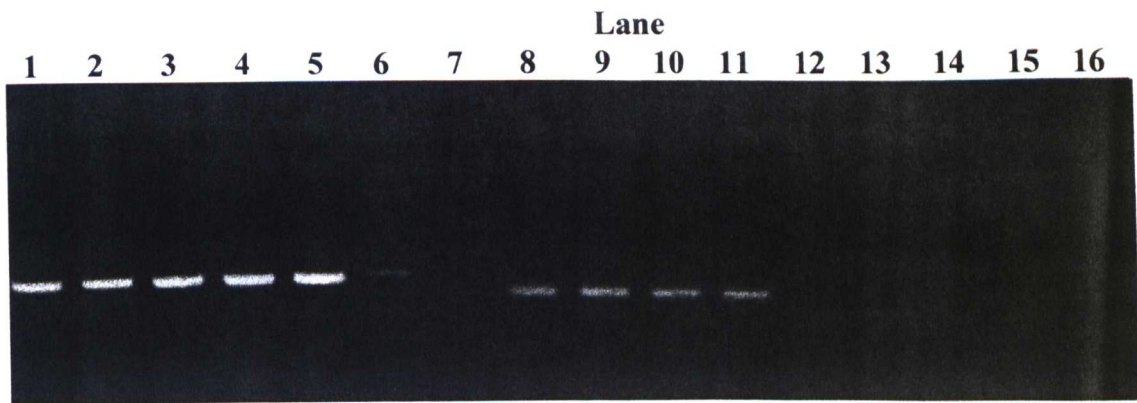
Lane 1: *glgB* , Time = 0; **Lane 2:** *glgB* , Time = 30; **Lane 3:** *glgB* , Time = 60; **Lane 4:** *glgB* , Time = 120; **Lane 5:** *glgB* , Time = 240; **Lane 6**: Markers 100 - 1500 bp, with 500 bp marker at triple intensity (Promega). **Lane 7:** *glgC* , Time = 0; **Lane 8:** *glgC* , Time = 30; **Lane 9:** *glgC* , Time = 60; **Lane 10:** *glgC* , Time = 120; **Lane 11:** *glgC* , Time = 240;   **Lane 12:** *whiB* , Time = 0; **Lane 13:** *whiB* , Time = 30; **Lane 14:** *whiB* , Time = 60; **Lane 15:** *whiB* , Time = 120; **Lane 16:** *whiB*, Time = 240;

NB: Control lanes omitted from figure for clarity.

Figure 111. **Agarose gel electrophoresis of RT-PCR products formed using primers for *relA*, and *bldD* (Chakraburtty *et al.*, 1996; Kelemen *et al.*,2001), amplified from total RNA, extracted following nitrogen shiftdown.**

Lane

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
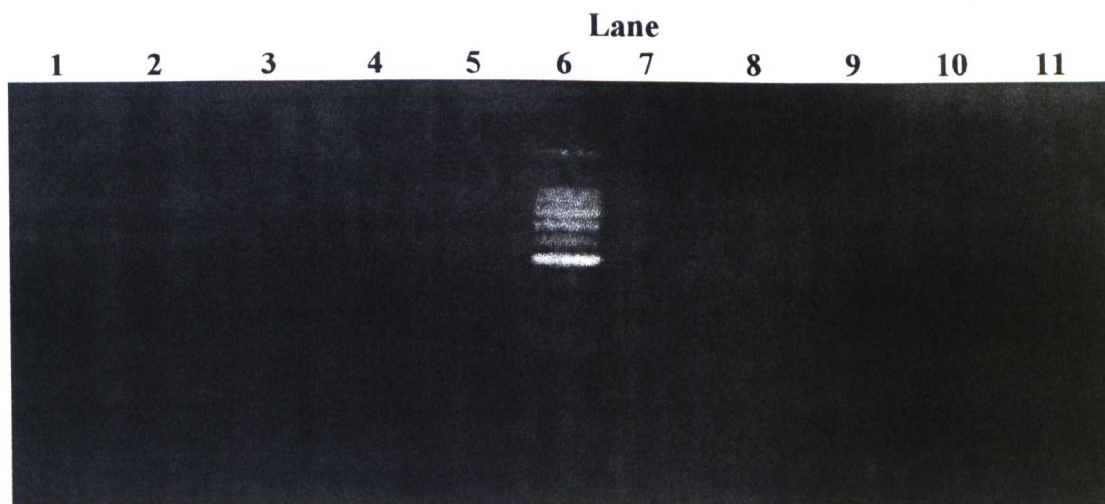
Lane 1: *relA* , Time = 0; **Lane 2:** *relA* , Time = 30; **Lane 3:** *relA* , Time = 60; **Lane 4:** *relA*, Time = 120; **Lane 5:** *relA*, Time = 240; **Lane 6**: Markers 100 - 1500 bp, with 500 bp marker at triple intensity (Promega). **Lane 7:** *bldD* , Time = 0; **Lane 8:** *bldD* , Time = 30; **Lane 9:** *bldD*, Time = 60; **Lane 10:** *bldD*, Time = 120; **Lane 11:** *bldD*, Time = 240;

NB: Control lanes omitted from figure for clarity.

Figure 112. **Agarose** gel **electrophoresis of RT-PCR products formed using primers for** *bldA* **(Leskiw** *et al.,* **1993), amplified from total RNA, extracted following nitrogen shiftdown.**
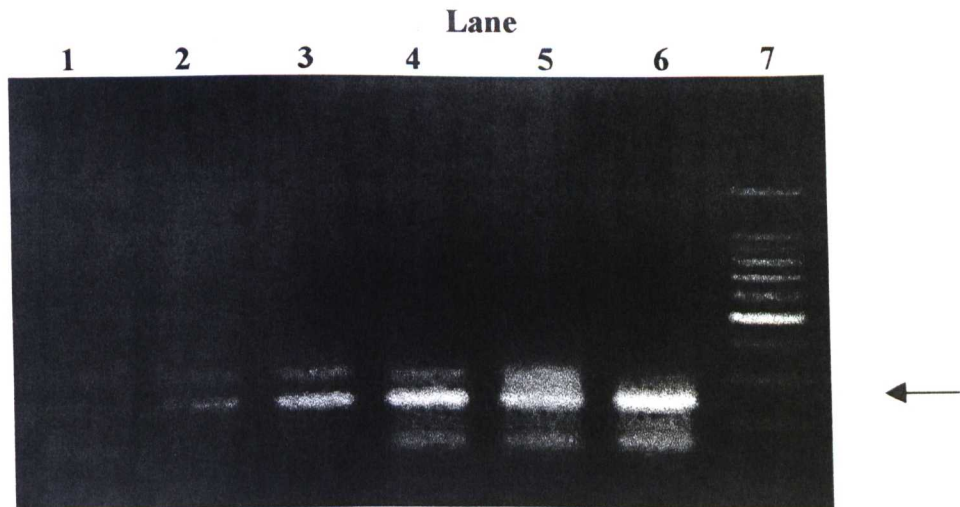
Lane 1: *bldA*, **Time = 0; Lane 2:** *bldA*, **Time = 30; Lane 3:** *bldA*, **Time = 60; Lane 4:** *bldA*, **Time = 120; Lane 5:** *bldA*, **Time = 240; Lane 6**: Markers 100 - 1500 bp, with 500 bp marker at triple intensity (Promega).

3.3.13.4 Temporal profile of gene expression during nitrogen-limited batch culture.

M. echinospora was grown in nitrogen-limited batch culture and RNA was extracted throughout growth. The RNA time courses obtained was subjected to RT-PCR and a temporal profile of gene expression was obtained. The appearance of spores, observed by phase contrast microscopy, was from 120 hour onwards under the conditions studied.

The glycogen biosynthetic genes exhibited a complex profile of expression throughout growth (Fig. 113). The branching enzyme (*glgB*) was only active following 72 hours of incubation, and continued to be transcribed throughout the remainder of the growth period. The presence of the *glgB* transcripts following 48 hours growth correlates with the physiological data provided in earlier sections. There is no previous temporal transcriptional data available for this gene. The *glgC* gene exhibited a profile that was found to change throughout growth. The detection of transcripts at 48 hours post-inoculation, and again at low levels at 96 hours, with increasing PCR product detected during the remainder of the fermentation. These data correlate temporally with the physiological data provide in earlier sections, for the biphasic accumulation of glycogen, the first phase peaking at 48 hours, and the second phase at 144 hours. Biosynthesis of glycogen requires the interplay of *glgB* and *glgC* gene products (amongst others). The operons present in *S. coelicolor* indicate that *glgC* is widely separated from the polycistronic operon containing the *glgB* gene (Alderson. Per. Comm.). This would be concurrent with the differing expression profiles of both genes in *M. echinospora*.

The transcripts of *relA* were not detected during nitrogen limited batch culture (Fig. 114). The transient nature of the transcripts would explain the inability to detect these transcripts under these conditions.

The RT-PCR product generated from the use of *bldA* primers was detected, with levels increasing throughout growth (Fig. 114). The presence of this transcript early in growth, despite the lack of morphological differentiation is consistent with the early role in development of this gene, the presence of *bldA* transcripts during exponential growth has previously been demonstrated in *S. coelicolor* (Leskiw *et al.*, 1993).

The presence of *whiB* transcripts was detected during late growth stages during nitrogen-limited batch culture, with PCR product detected at 144 and 168 hours (Fig. 115). The presence of *whiB* transcripts at this stage of growth correlates with the putative role in the formation of sporulation septa in *S. coelicolor* (Soliveri *et al.*, 1992).

Transcripts corresponding to that of *bldD* were not detected during batch culture (Fig. 115). One product was detected following 48 h of growth, however, this product is not homologous to *bldD*.

3.3.13.5 Summary of RT-PCR studies of *M. echinospora* genes.

The use of RT-PCR enabled the monitoring of gene experession in *M. echinospora*. This approach has previously been shown to provide a reliable and sensitive method of gene expression analysis, correlating well with northern blot analysis of *Bacillus subtilis* genes (Hernandez *et al.*, 2000). The shiftdown of *M. echinospora* mycelium to nitrogen-limited medium resulted in the appearance of, or increased, PCR product formation of *glgB*, *glgC*, *whiB*, and *bldA*. The transcripts of *bldD* and *relA* were not detected.

The investigation of temporal profiles in nitrogen-limited batch culture also resulted in changing transcript detection throughout growth. It was found that the expression of glycogen biosynthetic genes (*glgB* and *glgC*) correlate well with the physiological data provided in previous sections regarding biphasic accumulation of this polyglucan. The expression profiles of the developmental gene homologues (*whiB* and *bldA*) were found to coincide with the appearance of spores in the culture vessel. However, the transcripts of *relA* were undetectable under the studied conditions, reflecting the transient nature of ppGpp within the mycelium.

Figure 113. **Agarose gel electrophoresis of RT-PCR products formed using primers for *glgB* and *glgC* (Bruton *et al.*, 1997; Cruz Martin *et al.*, 1997), amplified from total RNA, extracted during nitrogen-limited batch culture.**
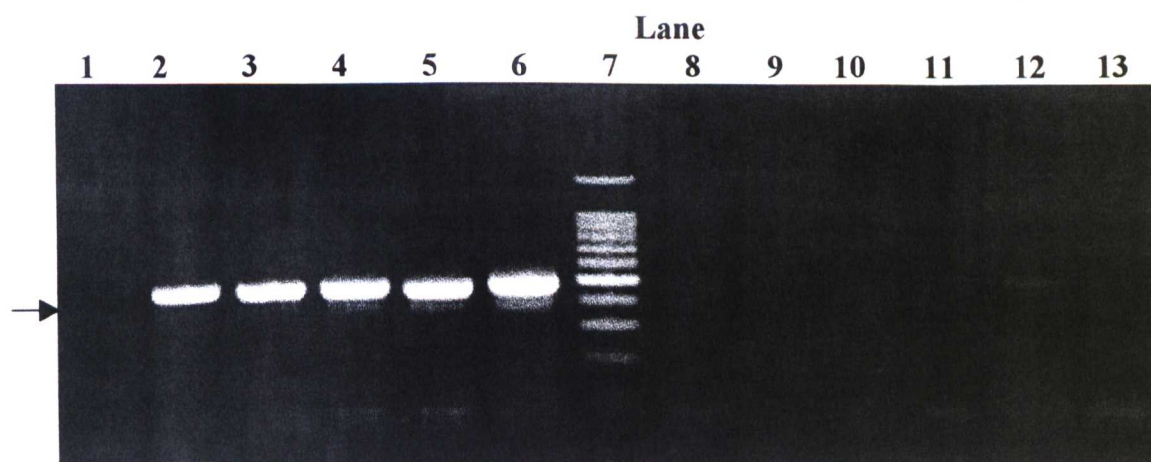
Lane 1: *glgB* , Time = 48 h; **Lane 2:** *glgB*, Time = 72 h; **Lane 3:** *glgB*, Time = 96 h;

Lane 4: *glgB*, Time = 120; **Lane 5:** *glgB*, Time = 144; **Lane 6:** *glgB*, Time = 168 h;

Lane 7: Markers 100 - 1500 bp, with 500 bp marker at triple intensity (Promega).

Lane 8: *glgC*, Time = 48 h; **Lane 9:** *glgC*, Time = 72 h; **Lane 10:** *glgC*, Time = 96

h; **Lane 11:** *glgC*, Time = 120 h; **Lane 12:** *glgC*, Time = 144 h; **Lane 13:** *glgC*,

Time = 168 h;

Arrows indicate the product of interest.

NB: Control lanes omitted from figure for clarity.

Figure 114. **Agarose gel electrophoresis of RT-PCR products formed using primers for *relA* and *bldA* (Chakraburtty *et al.*, 1996; Leskiw *et al.*, 1993), amplified from total RNA, extracted during nitrogen-limited batch culture.**
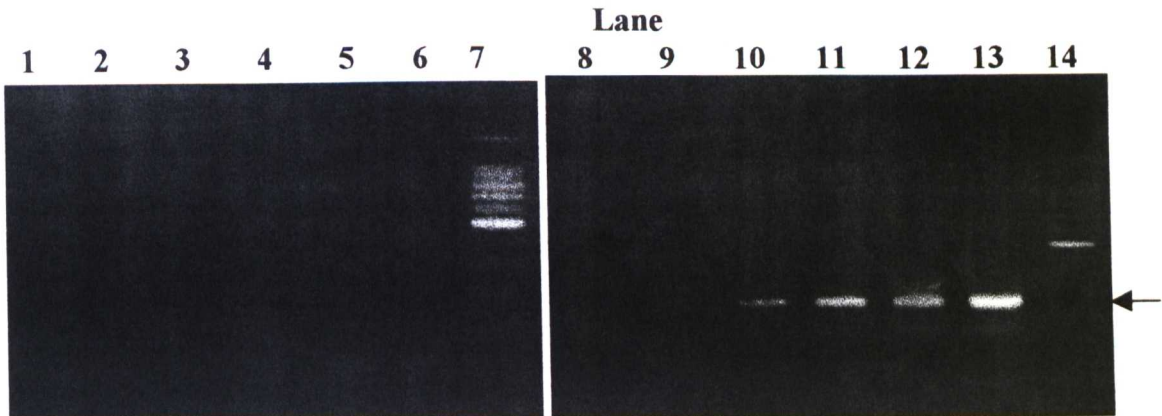
Lane 1: *relA* , Time = 48 h; **Lane 2:** *relA* , Time = 72 h; **Lane 3:** *relA*, Time = 96 h;

Lane 4: *relA*, Time = 120; **Lane 5:** *relA*, Time = 144; **Lane 6:** *relA*, Time = 168 h;

Lane 7: Markers 100 - 1500 bp, with 500 bp marker at triple intensity (Promega).

Lane 8: *bldA* , Time = 48 h; **Lane 9:** *bldA*, Time = 72 h; **Lane 10:** *bldA*, Time = 96

h; **Lane 11:** *bldA*, Time = 120 h; **Lane 12:** *bldA*, Time = 144 h; **Lane 13:** *bldA*,

Time = 168 h; **Lane 14:** Markers 100 - 1500 bp, with 500 bp marker at triple

intensity (Promega).

Arrows indicate the product of interest.

NB: Control lanes omitted from figure for clarity.

Figure 115. **Agarose gel electrophoresis of RT-PCR products formed using primers for *whiB* and *bldD* (Soliveri *et al.*, 2000; Keleman *et al.*, 2001), amplified from total RNA, extracted during nitrogen-limited batch culture.**
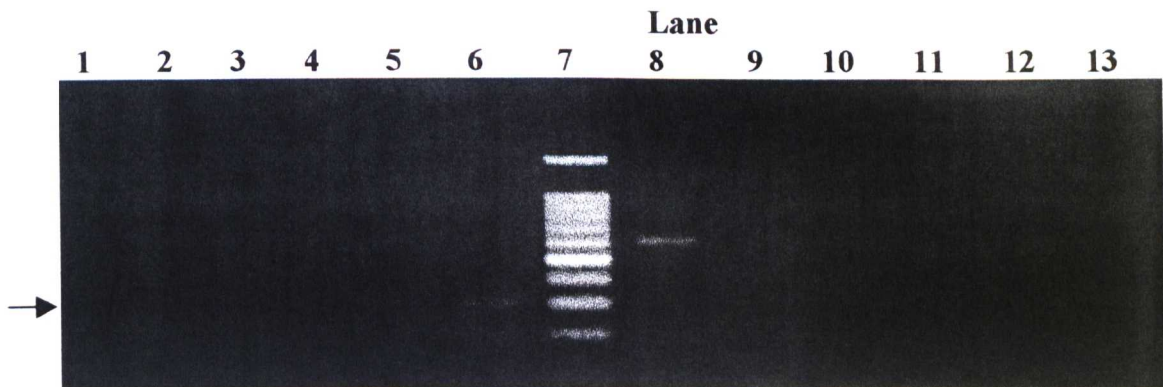
Lane 1: *whiB* , Time = 48 h; **Lane 2:** *whiB* , Time = 72 h; **Lane 3:** *whiB* , Time = 96 h; **Lane 4:** *whiB*, Time = 120; **Lane 5:** *whiB*, Time = 144; **Lane 6:** *whiB*, Time = 168 h; **Lane 7:** Markers 100 - 1500 bp, with 500 bp marker at triple intensity (Promega). **Lane 8:** *bldD* , Time = 48 h; **Lane 9:** *bldD*, Time = 72 h; **Lane 10:** *bldD* , Time = 96 h; **Lane 11:** *bldD*, Time = 120 h; **Lane 12:** *bldD* , Time = 144 h; **Lane 13:** *bldD*, Time = 168 h;

Arrows indicate the product of interest.

NB: Control lanes omitted from figure for clarity.

4.0 Discussion

Growth of *Micromonospora echinospora* was studied in solid medium and in submerged culture. The general characteristics of growth were examined on solid medium, allowing features of the developmental cycle to be elucidated, prior to a detailed physiological examination of growth and development in stoichiometrically carbon-limiting and nitrogen-limiting liquid minimal media.

The development of a convenient method of assessing the properties of spores produced by *M. echinospora* revealed an interesting phenomenon regarding the biology of this organism. Heat treatment increased the level of culturability of a spore population of *M. echinospora*. This indicates that spores are constitutively dormant and therefore require an activation treatment to initiate germination. The only other documented example of constitutive dormancy and heat activation in actinomycetes is that demonstrated by Hirsch and Ensign (1976) for aged (4 week-old) spores of *Streptomyces viridochromogenes*.

There have been two other studies to date detailing the effects of heat activation on spores of *Micromonospora* species. Suarez *et al* (1980) noted that spore germination in *M. chalcea* was unaffected by mild heat treatments. In contrast, Ensign (1982) working with *M. echinospora*, demonstrated an increase in the rate of spore germination, but not culturability, when cells were exposed to mild heating regimes. This has important implications for the design of isolation methods for rare actinomycetes. The low level of culturability measured in untreated spore suspensions compared with heat-treated suspensions implies that many potentially

viable cells may evade the culturable state. Recovery of micromonosporas from natural habitats is known to be difficult (Hayakawa *et al.*, 1991). Judicial heat treatment in the light of this study may well provide a way ahead in the search for new species of this genus.

The mechanism of heat activation is unknown but the experiments examining the respiration of the treated spores indicate that the response to mild heat treatment involves a rapid metabolic upshift, which may represent the start of a cascade of events leading to the breaking of dormancy. In fungi where heat activation has been described respiration is known to respond in a similar manner (Hill *et al.*, 1992).

The data presented on the heat response of *M. echinospora* spores to higher temperatures indicate that the spores of this organism are only mildly resistant to heat when compared with the reported resistance of typical bacterial endospores such as *Bacillus* and *Clostridium* (Russell 1999). *M. echinospora* can, however, be considered heat resistant when compared with vegetative bacteria (Tomlins and Ordal, 1976). In terms of other micromonosporas, the decimal reduction values (D-value) obtained indicate that this strain of *M. echinospora* is less resistant to heat ($D_{80°C}$ = 2.67 min) than *M. chalcea* ($D_{80°C}$ = 12.5 min, as reported by Suarez *et al.*, 1980), but exhibits similar D-values to *M. sagamiensis* and *M. olivoasterospora* (Kawamoto *et al.*, 1982).

Previous studies of differentiation on actinomycete differentiation in surface culture have primarily examined the influence of nitrogen source on sporulation (Ahronowitz & Demain, 1979: *Streptomyces clavuligerus*; Coleman & Ensign, 1982: *S. viridochromogenes*; Karandikar, 1998: *S. coelicolor*). In each of these studies, a complex interaction between the nature of the nitrogen source and the formation of spores was demonstrated. Assessment of the effect of various nitrogen sources has revealed different responses from a variety of organisms. In streptomycetes it has been reported that ammonium exerts a suppressive effect upon sporulation (Ahronowitz & Demain, 1979) and antibiotic production (Brana *et al.*, 1985). Coleman & Ensign (1982) reported that amino acids served as poor sources of nitrogen for supporting differentiation, although the nature of the suppression was not indicated. There is a general lack of information concerning nitrogen metabolism in actinomycetes. In the present study, it has been demonstrated that *M. echinospora* undergoes full differentiation on solid and in liquid YEME medium. The major nitrogenous components of this medium are yeast extract and bacteriological peptone. Quantification of these components indicated that their rapid depletion resulted in the formation of spores. The presence of high levels of residual glucose within the medium indicated that the cultures were nitrogen-limited and that glucose was not a readily utilised source of carbon. Sporulation of streptomycetes is subject to glucose repression (Chater 1993). The carbon requirements of developmental (*bld*) mutants of *S. coelicolor* have been shown to conditionally affect the formation of spores. It would appear from the present study that development of *M. echinospora* is not subject to regulation by glucose.

Ammonia was found to appear within the cultures during growth on YEME medium, indicating that deamination of amino-containing compounds was occurring. The

utilisation of carbon skeletons from amino compounds may explain the low rate of glucose utilisation by this organism.

The results drawn from studies on cultures containing complex nitrogen sources need to be interpreted with caution. A defined minimal medium was therefore developed to investigate processes of differentiation in cultures stoichiometrically limiting for both carbon and nitrogen.

The development of a minimal medium for studying the development of *M. echinospora* was initiated by investigating the suitability of various carbon and nitrogen sources for growth and sporulation. It would appear that the suitability of amino acids as nitrogen sources for *M. echinospora*, contrasts with the work of Ahronowitz & Demain (1979) and Brana *et al.*, (1985), who showed that the presence of amino acids in the medium resulted in poor sporulation in streptomycetes. The importance of selecting carbon and nitrogen sources that complement each other, resulting increased sporulation has previously been demonstrated in *S. coelicolor* (Karandikar, 1998). The molar ratio of carbon to nitrogen also provided a further example of this interdependency of major nutrient sources. The finding that stoichiometrically, nitrogen-limited conditions, promote abundant sporulation in *M. echinospora* is consistent with previous reports in the literature. Such reports describe nitrogen-limitation as a prerequisite for sporulation in a range of diverse microorganisms such as filamentous fungi (*Penicillium griseofulvum*: Morton, 1961; *Neurospora crassa*: Turian & Bianchi, 1972; *Geotrichum candidum*: Trinci & Collinge, 1974), and *Bacillus* spp. (*B. brevis*: Ristow & Paulus, 1982; *B. thuringiensis*: Starzak & Bajpai, 1991).

Promotion of differentiation (i.e. sporulation) under carbon-limiting conditions has been reported in a number of microorganisms such as *Mucor racemosus* (Trinci & Collinge, 1974), *Paecilomyces fumsoroseus* (Inch *et al.*, 1986), and *Bacillus thuringiensis* (Starzak & Bajpai, 1991). In actinomycetes, Karandikar (1998) demonstrated that carbon-limiting conditions did not promote sporulation in *S. coelicolor*. The physiological impact of carbon-limited growth is poorly studied in actinomycetes. *M. echinospora* exhibited delayed and reduced sporulation under carbon-limiting conditions.

It has been shown that extracellular proteolytic enzymes in bacteria may be synthesised in response to the presence of an inducing substrate within the medium, or when growth conditions are sub-optimal. In the genus *Bacillus*, protease production and sporulation have been linked (Ochi, 1985). The presence of genes having pleiotropic effects on both processes has also been demonstrated (Piggot & Coote, 1976). In this study, an affinity chromatography method was developed, based on the selective binding of proteases to the peptide antibiotic, bacitracin (van Noort *et al.*, 1991). Elution of proteolytic enzymes from the affinity column and subsequent visualisation by SDS-PAGE, allowed a temporal profile of proteases to be formed. Up to nine extracellular proteases were present in nitrogen-limited conditions, and were found to correlate with the exhaustion of nitrogen in the medium. The control of protease synthesis by nitrogen availability has been demonstrated in *Streptomyces clavuligerus* (Bascaran *et al.*, 1990). The growth of *M. echinospora* in carbon-limiting conditions, resulted in the suppression of the nitrogen-starvation induced proteases. Under these conditions, only three proteolytic enzymes were detected. The appearance of two of these proteases (50.2 & 29 kDa) correlated with morphological differentiation under both carbon and nitrogen-limited

conditions, suggesting a role for these in the process of sporulation. Proteolytic enzymes of similar masses to those isolated from *M. echinospora* have been shown to be present in some *Streptomyces* species. A 32 kDa, trypsin-like protease has been implicated in morphological differentiation in *S. exfoliatus* SMF13 (Kim & Lee, 1996). Additionally, a 50 kDa serine protease has been discovered in *S. lividans*, although no role in the life cycle of the organism was specified (Butler *et al.*, 1996). Serine proteases have been implicated in a number of organisms as regulators of differentiation (Ochi, 1995; Gibb & Strohl, 1987; Kim & Lee, 1996; Kang *et al.*, 1995). The application of chymotrypsin-like and trypsin-like protease inhibitors to the surface of growing cultures, prior to sporulation, inhibited sporulation, which suggests a role for serine proteases in development of *M. echinospora*.

It would appear that proteases play an integral role in the development of actinomycetes (Ginter, 1978), the sequencing of the *S. coelicolor* genome has revealed the presence of twenty or more putative proteolytic enzyme genes (http://www.sanger.ac.uk/projects/S_coelicolor/).

In *M. echinospora* nothing is known about the molecular level of control of differentiation, but parallels can be drawn between differentiation in streptomycetes and micromonosporas. In an attempt to elucidate common control elements in actinomycetes a number of known developmental genes were chosen from the literature on *Streptomyces coelicolor*. In the present study homologues of *whiB*, *bldA* and *bldD* were identified from *M. echinospora* genomic DNA. This would suggest that at least certain elements of the molecular architecture supporting differentiation in *M. echinospora* and perhaps other actinomycetes are common. Further evidence

has been demonstrated from transcriptional studies of *whiB* in a wide range of actinomycetes (Soliveri *et al.*, 2000).

The early sporulation gene, *whiB*, was found to be transcribed within 30 minutes following nitrogen-shiftdown, which indicates that *Micromonospora* responds rapidly to unfavourable nutritional conditions. Investigations of transcription during nitrogen-limited batch culture, allowed the correlation of *whiB* transcription with the appearance of spores. The *bldA* gene has been shown to represent a developmental regulatory mechanism (Leskiw *et al.*, 1993). The transcription of the *bldA* homologue was also shown to be induced rapidly on the depletion of nitrogen during batch growth, correlating with the appearance of spores and gentamicin. Transcription of *bldA* was also demonstrated following downshift to nitrogen depleted media. This is consistent with its speculated role as a regulator of secondary metabolism and morphogenesis (Leskiw *et al.*,1991). Although these genes are well understood functionally (Soliveri *et al.*, 1993 & 2000; Leskiw *et al.*, 1991 & 1993; Pope *et al.*, 1996; Keleman & Buttner, 1998), studies of their expression under defined nutritional conditions are few. The presence of a *bldA* homolgue is interesting in itself and it remains to be seen as to how common the *bldA*-type control mechanism is amongst the actinomycetes in general.

The excretion of ammonium and higher than expected residual glucose levels in minimal media indicated that preferential use of amino acids as a carbon source was occurring. The repression of glucose uptake is probably not mediated by the amino acid itself but by the carbon skeleton remaining following deamination. Chatterjee and Vining (1981) demonstrated that acetate and citrate acted as repressors of the

maltase operon in *S. venezuelae*, which is an inducible carbohydrate catabolic pathway. Additionally the repression of glucose uptake by citrate has been shown in *S. niveus* (Hodgson, 2000).

From early work in the present study, it was apparent that the metabolism of *M. echinospora* did not follow the central dogma, which would predict that simple sugars would be used as the primary carbon source. Clearly organic nitrogenous compounds figured highly in the nutrient hierarchy of this organism. How does the metabolism of *M. echinospora* differ from other bacteria?

The uptake of simple sugars appeared to occured slow rates. Studies on glucose uptake using radioactive substrates indicated the presence of a low affinity, high capacity glucose uptake system. K_m values for glucose previously reported for streptomycetes range from 0.05-6.2mM (Hodgson, 2000), indicating that *M. echinospora*, with a Km of 53 mM, has a poor affinity for glucose. The presence of a low affinity, high capacity system, may in some way explain the relatively slow growth rates observed. It has previously been shown that glucose is not a preferred carbon source for *S. coelicolor* (Karandikar, 1998). Poor glucose usage is also confirmed by the low growth yields observed from glucose, which are consistently lower than those of asparagine, even under carbon-limiting conditions.

The growth of *Streptomyces spp.* on glucose as a sole carbon source often results in acidification of the medium (Hobbs *et al.*, 1992; Dekleva & Strohl, 1987; Surowitz & Pfister, 1985). This acidification is the result of excretion of citric acid cycle intermediates, suggesting that glucose usage is poorly regulated in many actinomycetes. Studies on the major primary metabolic pathways during continuous

culture of *M. echinospora* also provided further evidence that this organism preferred amino acids to glucose as a carbon source. During carbon-limited culture, a linear response was observed for the activity of Glutamate:oxaloacetate aminotransferase (GOAT). This enzyme allows the formation of glutamine from aspartate, with the concomitant formation of the citric acid cycle intermediate oxaloacetate (Hodgson, 2000). Such increases in pathway activity in response to increased supply of asparagine in the medium, and the observation that GS activity was low, suggest that the GOAT activity was not a response to a requirement for glutamine but a mechanism for generating citric acid cycle intermediates.

Species of *Micromonospora* living in soils are dependant on a supply of energy-yielding substrates. The main supply of nutrients are insoluble polymers, requiring the action of hydrolytic enzymes to release smaller oligosaccharides, sugars, amino acids and peptides. Hodgson (2000) reviewed the catabolic pathways of streptomycetes and indicated that the majority of carbon pathways are inducible, whereas nitrogen catabolic pathways are constitutive. Additionally the constitutive uptake mechanisms for carbohydrate transport appear to be low affinity, high capacity systems. Extracellular hydrolytic enzyme activity would result in transient and localised, high concentrations of nutrients, requiring the action of such transport systems.

Systematic studies on the formation of antibiotics from the genus *Micromonospora* are few. The majority of these studies relate to production of gentamicin in the non-sporulating species, *M. purpurea* (Escalante *et al.*, 1992; Obregon *et al.*, 1994; Gonzalez *et al.*, 1995). In the present study, the production of gentamicin has been investigated in a strain capable of sporulation, enabling a temporal profile of

gentamicin production to be obtained and related to the formation of spores. Many studies have shown that the production of antibiotics is influenced by the state of nitrogen in the culture (Ahronowitz, 1980). It appears that amino acids are the most favourable nitrogen sources for antibiotic production in *S. clavuligerus* (Ahronowitz & Demain, 1979), with asparagine and glutamine giving rise to the highest titres. The stimulation of aminoglycoside antibiotic production by certain amino acids, such as glutamine and alanine, can be explained by their role as direct nitrogen donors (Pipersberg, 1997). The mechanisms underlying the positive effects of asparagine are at present not understood (Neumann *et al.,*1996). In the present study *M. echinospora* was able to form gentamicin in media containing either complex nitrogen sources or asparagine as the sole nitrogen source. Antibiotic production and sporulation have often been intimately linked (Kondo *et al.*1988) and common controlling elements such as phosphorylated guanosine nucleotides have been identified (Hodgson 1992). Both processes appear to be mutually exclusive with antibiotic production ceasing at the onset of sporulation. This may reflect the fact that antibiotic production is spatially constrained to mycelial forms of the organism and sporulation precludes antibiotic production. Spatial localisation of antibiotic biosynthetic capacity has been elegantly demonstrated to occur in *Streptomyces coelicolor.* Sun *et al.* (1999) have demonstrated the transcription of an undecylprodigiosin regulatory gene to be confined to aged mycelia with no transcription in spores. Further evidence for antibiotic being confined to the mycelium of *M. echinospora* is provided from the chemostat studies, where gentamicin was produced at low growth rates under nitrogen-limiting conditions. Traditionally, the formation of antibiotics has been associated with the slow growth rateof an organism prior to entry into the stationary phase of growth (Bibb, 1996). It

is considered that in *E. coli* the highly phosphorylated guanosine nucleotide ppGpp plays a central role in the control of growth rate (Marr, 1991). In streptomycetes much attention has been focussed upon correlating antibiotic production with the formation of ppGpp. Ochi (1986) demonstrated that the accumulation of ppGpp following nutrient shiftdown was accompanied by an eight-fold increase in formycin production by *S. lavendulae*. The transcription of pathway specific activators of undecylprodigiosin and actinorhodin has also been correlated with accumulation of ppGpp (Strauch *et al.*, 1991; Takano *et al.*, 1992).

The present study allowed the correlation of gentamicin and ppGpp detection in cultures of *M. echinospora*, although the formation of gentamicin during continuous culture occurred in the absence of detectable ppGpp. It was noted that ppGpp accumulation also occurred prior to morphological differentiation. Morphological differentiation in streptomycetes has been correlated with a fall in intracellular GTP levels (Glazebrook *et al.*, 1990; Ochi, 1988). This pattern was also observed in *M. echinospora*. Morphological differentiation and antibiotic production have since been shown to not be solely dependent upon decreasing GTP and ppGpp accumulation. Ochi (1990), has shown that relaxed mutants (*rel*) in *S. coelicolor*, which were unable to form ppGpp, still exhibited a fall in GTP concentration prior to aerial mycelium formation. Recent work by Sun *et al.*, (2001) has demonstrated that a ppGpp independent pathway exists for the formation of actinorhodin in *S. coelicolor*. This antibiotic has previously been shown to be repressed by nitrogen and phosphorus (Hobbs *et al.*, 1990; Bystryth *et al.*, 1996). The limitation of nitrogen elicits the ppGpp dependant mechanism of transcription. The limitation of

phosphate, however, allows the ppGpp-independent mechanism mediated by the *rshA* gene product, to be initiated.

Early studies of carbon catabolic pathways in actinomycetes have indicated that glycolysis dominated the early stages of growth with the second, slower phases of growth being characterised by increased activity through the pentose phosphate pathway (Cochrane, 1961). Primary metabolism and antibiotic formation have been correlated in a number of organisms (Dekleva & Strohl, 1988; Obanye *et al.*, 1996; Pipersberg, 1997; Padilla *et al.*, 1991; Lee & Lee, 1993). Such associations have been made in batch culture systems, which are intrinsically transient, and, therefore, enzymatic flux and antibiotic formation cannot be correlated with confidence. In the present study, steady state biomass was analysed for flux through major carbon and nitrogen metabolic pathways with respect to gentamicin formation under both nitrogen and carbon-limited conditions. In *S. griseus* the production of the aminoglycoside antibiotic, streptomycin, has been correlated with increased activity of the pentose phosphate pathway (PPP). Increased activity of the PPP during the formation of gentamicin, in continuous culture at low dilution rates (0.034 h^{-1}), under nitrogen-limitation was observed in the present study. Ikeda *et al.*, (1988) correlated the activity of the PPP with avermectin production in *S. avermitilis*, emphasising the importance of this pathway in providing NADPH for antibiotic biosynthesis. Carbon-limitation or higher dilution rates did not permit the formation of gentamicin. Limitation of nitrogen was a prerequisite for the formation of gentamicin in *M. echinospora*. The limitation of nitrogen also increased the activity of GS. Gonzalez *et al.*,(1995) reported that glutamine exerted a stimulatory effect upon the formation of gentamicin, with the GS inhibitor, methionine sulphoximine,

preventing antibiotic formation. Caution, however, is required in the interpretation of these results as methionine has been shown to be a direct precursor of gentamicin (Rinehart & Stroshane, 1976). This suggests that antibiotic formation was inhibited by the blocking of the methylation reactions from methionine to gentamicin rather than by inhibition of GS.

Bacteria have been shown to accumulate storage compounds in conditions of carbon excess and nitrogen limitation (Dawes, 1992). The ability of streptomycetes to accumulate glycogen has been reported previously (Brana *et al.*, 1982, 1986; Plaskitt & Chater, 1995). It has been demonstrated that this polyglucan is accumulated in two, temporally and spatially distinct phases (Brana *et al.*, 1986; & Plaskitt & Chater, 1995). The first phase, which is in the substrate mycelium, prior to the emergence of aerial hyphae, is believed to be supported by the mobilisation of phase I deposits, due to the physical distance between the aerial hyphae and the environmental nutrients (Schneider *et al.*, 2000). The second phase of accumulation is believed to fuel spore maturation (Chater, 1989; & Plaskitt & Chater, 1995). On solid medium, *M. echinospora* accumulated glycogen in two temporally distinct phases, prior to sporulation and again after the onset of sporulation. The first phase of glycogen accumulation accounted for a similar dry weight percentage of cells (6 % w/w) as shown in *Streptomyces coelicolor* (Karandikar *et al.*, 1997).

The second phase of accumulation accounted for up to 15.2 % (w/w) of cellular mass, which was two-fold higher than in *S. coelicolor*. The absence of aerial mycelium in *M. echinospora* (Kawamoto, 1989) may indicate that the first phase of glycogen accumulation serves a different physiological role than that previously suggested for streptomycetes. The large amount of glycogen in the second phase of

accumulation in *M. echinospora* was degraded when glucose ceased to be utilised and may be involved in providing energy for spore maturation. In submerged culture glycogen was again accumulated in a biphasic manner. Under these conditions, however, the phase I deposits were higher than those in the second phase of accumulation.

The study of glycogen biosynthetic genes during nitrogen-limited batch growth revealed that *glgB* was transcribed after 72 hours of growth. Analysis of *glgC*, however, indicated a complex pattern of transcription. This pattern of *glgC* transcription correlated well with the biphasic glycogen accumulation model. It is tempting to speculate that *glgC* plays a controlling role in glycogen accumulation. The presence of large storage metabolism operons, controlling the branching and de-branching of glycogen and trehalose metabolism (Schnieder *et al.*, 2000) would suggest a complex controlling element which would be required to regulate glycogen biosynthesis. The appearance of a complex transcription profile, correlating with glycogen accumulation for *glgC*, may suggest such a role for the product of this gene, ADPglucose pyrophosporylase. Additional evidence is provided by the distance of the *glgC* gene from the other glycogen biosynthetic genes on the chromosome of *S. coelicolor* (Schneider *et al.*, 2000).

Lipids represent a significant portion of the cell mass in *M. echinospora*, with accumulation being mainly towards the end of the primary growth phase and during the secondary growth phase. Total lipid and TAG accumulation coincided with rapid medium glucose usage, as previously observed in *Streptomyces coelicolor* and *S. lividans* (Olukoshi & Packter 1994). It was speculated that excess sugar was converted to TAG, in nitrogen-limiting conditions, as observed with many storage

polymers. Schauner *et al.*,(1999) demonstrated that *S. ambofaciens* accumulated lipid throughout growth, but primarily during the stationary phase, of which a significant proportion was TAG.

It appears that Micromonosporas displays several features likely to enhance survival of this organism under conditions of nutrient flux. Accumulation of reserve carbonaceous compounds is significant to their feast or famine existence in soils providing cellular energy reserves. Antibiotic production is transient and may reflect the spatial confinement of antibiotic biosynthesis to mycelial forms of the organism, while abundant sporulation ensures survival in conditions unfavourable to growth.

In the present study, defined conditions for the examination of growth and physiology of *Micromonospora echinospora* were developed. This enabled the basic physiological processes invoked in response to nutrient-limitation to be investigated. The foundation of physiological knowledge presented here will hopefully lead to increased interest in Micromonosporas.

The sequencing of the *Streptomyces coelicolor* genome has allowed the detection of homologous developmental genes in *Micromonospora*. Further use of the genome could advance our understanding of micromonosporas very rapidly, by identification of further homologues. A rapid way ahead would be via the use of DNA microarray technology being developed at the University of Manchester Institute of Science and Technology (UMIST) with *S. coelicolor* to rapidly identify homologues and identify targets for further study. It would also be interesting from a taxonomic point of view to compare these organisms utilising such a rapid technique.

Recently there have been significant advances in the development of cloning vectors for use in *Micromonospora* (Alexander *et al.,* 2001). This area has lagged significantly behind that of *Streptomyces* and as such has restricted studies of *Micromonospora*. Such systems will enable the manipulation of strains to increase the ease of study of sporulation and antibiotic formation, for example, by the use of reporter genes. The creation of 'knock-out' mutants will allow the physiological role of certain genes to be elucidated. It would be interesting to use this approach to identify the protease genes and study the impact of deletions on morphogenesis.

The sequencing of the gentamicin biosynthetic cluster has recently been completed (J. Unwin, personal communication). The publication of this sequence will allow studies of transcription and regulation of gentamicin formation. The model of streptomycin biosynthesis will provide a useful basis with which to study gentamicin production. Preliminary data on the gentamicin cluster has revealed the similarity of the biosynthetic cluster to that of streptomycin (Unwin *et al.,* 2001).

5.0 References

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