A Quantitative Study of the Relationships between Morphology, Physiology and Geldanamycin Synthesis in Submerged Cultures of *Streptomyces hygroscopicus* var. *geldanus*

A thesis submitted for the qualification of PhD by

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Writing these acknowledgements has proved to be almost as difficult as compiling this entire thesis. To use the words of one author, 'the most important things in life are often the hardest things to say; words can diminish and shrink what otherwise seems limitless in your mind'. As I try to put my thoughts on paper I know that there are a number of people to whom I will be forever indebted and there are no appropriate words to describe my endless gratitude for their advice, support, friendship and love over the last number of years.

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To Arthur and Noelle Dobson

Amongst the metabolic activities of micro-organisms,

those usually classed as *secondary* are far from trivial……

Published peer-reviewed articles related to the work in this thesis

Dobson, L.F., O'Cleirigh C.C., and O'Shea D.G. (2008). The influence of morphology on geldanamycin production in submerged fermentations of *Streptomyces hygroscopicus* var. *geldanus. Applied Microbiology and Biotechnology*, 79, 859-866.

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Proposed control of pathway metabolics for geldanamycin synthesis. *International Workshop on Systems Biology 2006*. Hamilton Institute, NUI Maynooth, Ireland, 17-19 July 2006.

The development of an optimised production strategy for geldanamycin synthesis. *National Symposium of the Irish Research Council for Science Engineering and Technology*. Dublin, Ireland, 3rd November 2005.

The development of an optimised fermentation medium for geldanamycin production. *12th European Congress on Biotechnology*. Copenhagen, Denmark, 21-24 August 2005.

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ABSTRACT

Microbially produced secondary metabolites such as antibiotics have tremendous economic importance. However, most are produced by filamentous organisms which exhibit diverse growth patterns presenting challenges for industrial fermentation. There are many factors affecting secondary metabolite production which concomitantly impact on morphology, thus it is difficult to distinguish the key driver for productivity. *Streptomyces* spp. is a genus of filamentous organisms that together synthesise over 4000 bioactive compounds. *Streptomyces hygroscopicus* var. *geldanus* produces the secondary metabolite geldanamycin, a novel chemotherapeutic compound, in submerged fermentation. This organism represents an ideal system for experimentation in order to elucidate the relationships between morphology, physiology and secondary metabolite production.

The effects of a variety of microbiological (inoculum size), physical (glass beads) and chemical (surfactants, calcium ions, magnesium ions) factors on morphological development were examined as part of this study. Inclusion of the divalent cations magnesium or calcium was demonstrated to alter the cell surface hydrophobicity of the organism, provoking dispersion or aggregation of cells respectively, and stimulating great disparity in geldanamycin yields. Indeed, in all instances, morphology was found to impact considerably on secondary metabolite formation, with smaller pellet sizes optimal for geldanamycin synthesis. Investigation of the respiration rate of *Streptomyces hygroscopicus* var. *geldanus* revealed that a linear relationship existed between this parameter and geldanamycin production. Submerged cultures consisting primarily of small pellets, less than 0.5mm in diameter, were more metabolically active and concomitantly produced more geldanamycin. Nonetheless, it was also demonstrated that other explicit factors exist which do not affect morphology or respiration but regulate geldanamycin synthesis through feedback inhibition of the direct metabolic pathway.

This study has demonstrated that, in *Streptomyces hygroscopicus* var. *geldanus*, the bulk of factors that affect morphology impact significantly on respiration, and it is this parameter that is the key driver of secondary metabolite production. This case study provides new insights into the regulation of geldanamycin production in *Streptomyces hygroscopicus* var. *geldanus* and provides a basis for elucidation of the relationships between morphology, physiology and secondary metabolism in other filamentous micro-organisms.

NOMENCLATURE AND ABBREVIATIONS

°C	degrees celsius
17-AAG	17-allylamino-17-demethoxygeldanamycin
17-DMAG	17-dimethylaminoethylamino-demethoxygeldanamycin
2DGE	2D gel electrophoresis
µl	microlitre
µm	micrometre
γ	shear rate
μ	viscosity
μ_{app}	apparent viscosity
τ	shear stress
Ø	degree of aggregation
$Ø_1$	degree of dispersion
v/v	volume per volume
w/v	weight per volume
au	absorbance units
AcCoA	acetyl coenzyme A
ACN	acetonitrile
ADE2	adenosine2
ADP	adenosine di-phosphate
AHBA	3-amino-5-hydroxybenzoic acid
AMP	adenosine mono-phosphate
ANN	artificial neural network
ATP	adenosine tri-phosphate
BATH	bacterial adherence to hydrocarbons
Ca^{2+}	calcium ion
CFU	colony forming unit
CO_2	carbon dioxide

CSH	cell surface hydrophobicity
CTC	5-cyano-2,3-ditolyl tetrazolium chloride
DAHP	D-arabino-heptulosonate 7-phosphate
DCW	dry cell weight
DHS	5-deoxy-5-amino-3-dehydroshikimic acid
$DiOC_6$	3,3-dihexyloxocarbocyanine
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dpi	dots per inch
E4P	erythrose-4-phosphate
EDTA	ethylenediaminetetraacetic acid
EF2	elongation factor-2 kinase
FFD	fractional factorial design
Fru6P	fructose-6-phosphate
GA	geldanamycin
Glu6P	glucose-6-phosphate
GOD-PAP	glucose oxidase-phenol 4-aminophenazone peroxidase
HER2	human epidermal growth factor receptor 2
HIC	hydrophobic interaction chromatography
HPLC	high performance liquid chromatography
Hsp90	heat shock protein 90
IEF	iso-electric focusing
ISPR	*in-situ* product recovery
k	fluid consistency index
MEN	menadione
Mg^{2+}	magnesium ion
mm	millimetre
mM	micro molar
Mn^{2+}	manganese ion

n	flow behaviour index
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NCCLS	National committee for clinical laboratory standards
Nm	newton metre
NPF	normalised particle frequency
NVF	normalised volume frequency
O_2	oxygen
OAA	oxaloacetic acid
OD	optical density
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PEP	phosphoenylpyruvate
Phe	phenylalanine
psi	pounds per square inch
RNA	ribonucleic acid
rpm	rotations per minute
RR_g	global respiration rate
RR_s	specific respiration rate
RSM	response surface methodology
RT-PCR	reverse transcriptase polymerase chain reaction
SELDI	surface enhanced laser desorption ionisation
spp	species (plural)
TCA	tricarboxylic acid
tiff	tagged image file format
TOF-MS	time of flight mass spectrometry
Trp	tryptophan
TW	tween80
TX	tritonX100

Tyr	tyrosine
uv	ultra-violet
VBNC	viable but non-culturable
XTT	3-(1-[(phelylamino)-carbonyl]-3,4-tetrazolium)-bis(4-methoxy-6-nitro) benzenesulfonic acid hydrate
Zn^{2+}	zinc ion

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CHAPTER 1: INTRODUCTION

SECTION 1A: Factors affecting secondary metabolite production in filamentous micro-organisms

1.1 The commercial significance of filamentous organisms

It is a remarkable paradox that to most academic researchers filamentous organisms represent an unconventional class, and yet these micro-organisms are extensively studied by the industrial researcher, particularly within the pharmaceutical industry. Filamentous micro-organisms account for the majority of industrial fermentations, in terms of bulk as well as the diversity of metabolites produced (Braun and Vecht-Lifshitz, 1991, Makagiansar et al., 1993, Tamura et al., 1997). They produce a plethora of compounds of great value to society. These may be large molecules such as proteins, nucleic acids, carbohydrate polymers or even the cells themselves; or they can be smaller metabolite molecules such as amino acids, vitamins or organic acids (Demain, 2000). However any discussion pertaining to the commercial significance of filamentous organisms, and in particular the Actinomycetes, would be wholly incomplete without mention of the vast array of clinically important secondary metabolites, particularly antibiotics, produced by these organisms.

Microbial metabolites may be classified into two major categories, growth or non-growth associated (Figure 1.1). Primary metabolism (growth associated) is essentially identical for all living things and involves an interconnected series of enzyme mediated catabolic and anabolic pathways providing energy and essential macromolecules such as deoxyribonucleic acid (DNA) ribonucleic acid (RNA), proteins, lipids and polysaccharides (Martin and Demain, 1980).

Secondary metabolites (non-growth associated) are compounds produced following the main stage of growth or primary metabolism. Often they are only produced by some particular species of a genus, and usually as families of closely related components (Martin and Demain, 1980). They have no role in the growth of the producing cultures but are important for the organisms that produce them, with many varied functions including competitive weapons against other bacteria, fungi, amoeba, insects and plants (Demain, 2000).

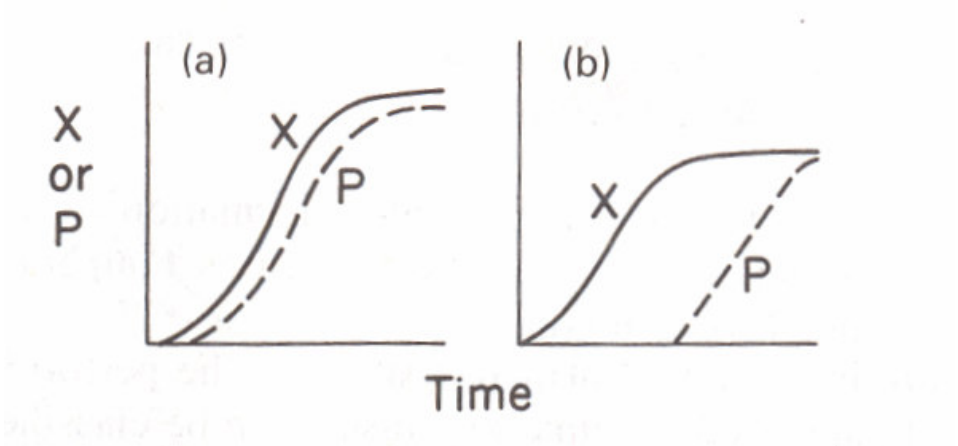


Figure 1.1: Kinetic patterns of growth and product formation in batch fermentations: (a) growth associated; (b) non-growth associated product formation (Schuler and Kargi, 1992).

The best known of the secondary metabolites are the biologically active compounds known as antibiotics. Antibiotics are defined as low molecular weight, natural products made by micro-organisms which are active against other organisms at low concentrations (Demain, 1999). They attack virtually every type of microbial activity such as DNA; RNA; protein synthesis; membrane function; electron transport; sporulation; germination; and many others (Demain, 2000).

Over 60 years ago the golden age of antibiotics dawned with considerable achievements in the discovery and development of penicillin and the sulfonamides produced by filamentous fungi (Overbye and Barrett, 2005). However the Actinomycetes have since provided the vast majority of antibacterial compounds (Overbye and Barrett, 2005). Actinomycin, the first antibiotic from a streptomycete, was discovered by Waksman and Woodruff in 1940, and this was followed closely by the discovery of the first anti-tuberculosis drug streptomycin in 1944 (Omura, 1986). These microbially produced antibiotics are extremely important to human and animal health and nutrition, and have tremendous economic importance. Recent figures have

indicated that the antibiotic market amounts to almost US$30 billion (Demain, 2000).

Following the discovery of the first antibiotics, the quest for understanding and regulating biological systems for the production of valuable products led to a dramatic increase in microbiological and bioengineering research (Hecker and Mullner, 2003). Chemical and pharmaceutical companies quickly realised the enormous commercial potential of these bio-products, and huge investment was made in research and development projects, as well as on a build-up of production facilities (Hecker and Mullner, 2003).

In the mid-1970's the term 'Biotechnology' was brought into popular usage as a result of the increased potential for the application of biological organisms, systems or processes to both the chemical and pharmaceutical industries (Trevan et al., 1987, Hecker and Mullner, 2003). Biotechnology is very much an interdisciplinary field comprising of microbiology, biochemistry and process engineering. All of these disciplines are vital to optimisation of systems that inherently exhibit low product yields, in particular antibiotic fermentations. Several approaches are available for intensification of bioprocesss, with most centred on enhancement of yield at the bioreaction stage (Chisti and MooYoung, 1996). The methods of strain improvement, media composition or feeding strategies, and the optimisation of fermentation conditions, either individually or synergistically, have a proven record of achieving spectacular improvements in bioprocess productivities.

1.2 Factors affecting secondary metabolite production

1.2.1 Metabolic engineering

For the overproduction of industrial products, improved microbial strains with enhanced productivity or other desired attributes are often employed (Li et al., 2001). The generation of an improved strain requires alteration of the informational content of DNA in such a way as to increase the levels of enzymes involved in biosynthesis; eliminate or reduce the effects of control mechanisms; eliminate detrimental characteristics of the organism; or generate a novel and more efficient route to the desired product (Normansell, 1986).

Strain improvement for antibiotic production has traditionally been based on random mutagenesis methods (Borodina et al., 2005). Indeed, in an attempt to improve the yield of penicillin, *Penicillium chrysogenum* was exposed to a variety of mutants such as nitrogen mustard, ultraviolet radiation and X-radiation. In contrast metabolic engineering enables the introduction of rational changes to the central metabolism in order to achieve a specific phenotype (Borodina et al., 2005, Vemuri and Aristidou, 2005, Tyo et al., 2007). In technical terms, metabolic pathway engineering encompasses directed modification of cellular physiology through the introduction, deletion, and/or modification of metabolic pathways or regulatory functions of a cell (Sandford et al., 2004).

The first step is establishing the best pathway from a substrate to a given product (Sandford et al., 2004). Cloning the genes of interest into the production host, fine-tuning the expression, and eliminating transcriptional and/or allosteric regulatory mechanisms will optimise production (Sandford et al., 2004). Such metabolic engineering techniques have been demonstrated to improve the yield of secondary metabolites. For example, Butler et al., (2002), engineered primary carbon metabolism in *Streptomyces lividans*, making deletions of two genes encoding isoenzymes for the first step in the pentose

phosphate pathway, allowing more efficient glucose utilisation by the glycolytic pathway. Combined with transformations of multicopy plasmids containing transcriptional activator genes, the yield of antibiotics in the mutant strain was greatly improved (Butler et al., 2002). To improve doxorubicin productivity in a strain of *Streptomyces peucetius*, a doxorubicin pathway-specific regulatory gene was cloned into a high-copy-number plasmid containing a promoter system and approximately 9.5-fold higher doxorubicin productivity was exhibited compared with the wild-type (Park et al., 2005). Overexpression of a rate-limiting enzyme in a recombinant strain of *Streptomyces clavuligerus* also yielded 60% higher production of cephamycin C (Malmberg et al., 1995).

Critical to successful metabolic engineering is the identification of rate-limiting steps, however this requires detailed analyses of specific metabolic controls in the biosynthetic pathways (Malmberg et al., 1995). In general, primary and secondary metabolite pathways are differentially expressed, are tightly controlled by enzymatic steps, and the complexity of these interactions represents the challenging task of identifying which enzymes control the relevant metabolic processes (Malmberg et al., 1995). Even the concept of rate-limiting steps is transient, as control is ultimately distributed across a pathway: as one bottleneck is relieved, another step becomes limiting (Tyo et al., 2007). Thus, the central paradox of contemporary biology has been defined as the dissonance between our understanding of the parts and the whole in complex biological systems (Hellerstein, 2003). Nonetheless, the increased capacity to measure and catalogue both cellular components and their interactions through advances in 'omics' technologies have provided an expanded view of the cell, and offer metabolic engineers a more complete understanding of the cell in various environments, which can be exploited for strain improvement (Tyo et al., 2007).

1.2.2 Medium composition and culture conditions

Fermentation media and cultural conditions are the most important factors in a production process, providing the main environment of the organism. All micro-organisms require water; oxygen (if aerobic); sources of energy; carbon; nitrogen; mineral elements; and possibly vitamins. In the particular case of secondary metabolites, the interaction between growth metabolism and product secretion is often critically influenced by growth limiting nutrient concentrations (Sircar et al., 1998). A successful media should meet the following criteria: produce the maximum yield of product or biomass; result in minimal yield of undesired products; be cheap and of a consistent quality; and cause minimal problems in other aspects of the production process particularly aeration and agitation, extraction, purification and waste treatment.

1.2.2.1 Carbon source regulation

Most antibiotic-producing micro-organisms are heterotrophic, requiring organic compounds as a source of carbon and energy. Carbon sources such as corn starch; glucose; fructose; sucrose; and molasses which are rapidly taken up are commonly used as growth substrates to produce secondary metabolites by fermentation (Sanchez and Demain, 2002, Papagianni, 2004). However glucose is generally the best carbon and energy source for growth of many antibiotic-producing micro-organisms (Park et al., 1994). Nonetheless, rapid catabolism of glucose can cause a decrease in the rate of biosynthesis of many antibiotics, termed carbon catabolite repression (Choi et al., 1996, Sanchez and Demain, 2002). Carbon catabolite repression is widely distributed among microbial systems and functions primarily to assure an organised and sequential utilisation of carbon sources when more than one is present in the environment (Sanchez and Demain, 2002). Limiting the concentration of the carbon source which causes repression by using fed-batch mode can help to maintain substrate levels at low concentrations, thus avoiding the repressive effects (Sanchez and Demain, 2002).

Alternatively, the use of carbon sources with low solubility such as vegetable oils is a practical means of preventing catabolite repression (Choi et al., 1996). Lipids and oils were traditionally added to bioprocess media because of their natural antifoam properties, but their ability to increase secondary metabolite titres has since been noted (Large et al., 1998). However, adequate dispersal and mixing are integral to facilitate maximal contact of oil droplets with the cell surface (Peacock et al., 2003). For example, Choi et al., (1996), and Tamura et al., (1997), noted that oil consumption by *Streptomyces fradiae* increased in conjunction with agitation rate. Residual oil levels may also lead to problems associated with increased medium viscosity and warrant additional downstream processing (Large et al., 1998).

1.2.2.2 Nitrogen source regulation

Nitrogen is a major component of living materials with an ability to govern cellular growth and the formation of cellular enzymes and products (Choi et al., 2000). It is thus one of the most important macronutrients required by micro-organisms. Most industrially used micro-organisms can utilise both inorganic (e.g. ammonium salts) and organic (e.g. amino acids, proteins) sources of nitrogen. Nonetheless, the production of some antibiotics may be inhibited by the presence of ammonium ions and rapidly utilised nitrogen sources. Although the exact biochemical basis of the inhibition has not yet been determined, the phenomenon is called nitrogen catabolite repression by analogy with carbon catabolite repression (Omura et al., 1980). In such instances slowly assimilated organic nitrogen sources are used to encourage high production of secondary metabolites (Sanchez and Demain, 2002).

1.2.2.3 Minerals

In many media minerals such as magnesium, phosphorous, potassium, sulphur, calcium, copper, iron, manganese and zinc are essential components and may be distinctly added or are present as impurities in other constituents. Numerous enzymes require certain metal ions as cofactors, and although needed in very low concentrations, they may be essential in achieving high production rates

(Bader, 1986). The requirement for metals varies with the type of organism as well as with the nature of the basal medium used (Basak and Majumdar, 1975).

1.2.2.4 Precursor inclusion

Other components of fermentation media help to regulate the synthesis of a desired product rather than support the growth of the organism. This compound, the inducer, is able to 'turn on' production in cells in such a way that the enzymes or metabolites are produced only when needed (Sanchez and Demain, 2002). However, it is sometimes difficult to determine whether the stimulatory effect is a true induction effect or merely a precursor effect (Martin and Demain, 1980). A precursor is a compound that may be directly incorporated into the product, for example chloride in the synthesis of chlorotetracycline by *Streptomyces aureofaciens*. Addition of phenylacetic acid, a side chain precursor for penicillin production, is probably the earliest example of precursor feeding, with a three-fold increase in antibiotic observed following its inclusion. It is unsurprising that precursor feeding often simply consists of the supplementation of primary metabolites, as the availability of these molecules will dictate the rate of secondary metabolism and product formation.

1.2.2.5 Inoculum type, age and size

The performance of a microbial culture can be strongly influenced by the choice of the inoculum, with optimisation of type, age and size used to improve the performance of the biological process (Elibol et al., 1995). Filamentous organisms in liquid cultures are normally grown from spore inocula, however vegetative inocula may be advantageous by reducing the lag phase in batch culture (Elibol et al., 1995). In addition, a low inoculum density may give insufficient biomass causing reduced product formation, whereas a higher inoculum concentration may produce too much biomass and deplete the substrate of nutrients necessary for product formation (Ellaiah et al., 2004).

1.2.2.6 Agitation and aeration

In order to perform a given bioconversion in a bioreactor appropriate mass transfer, heat transfer, gas dispersion, and a certain homogenisation are required, and these are normally achieved by agitation with an impeller (Cui et al., 1997). Agitation thus serves two primary purposes in fermentation processes: to provide a uniform environment for the organism and to transfer oxygen from air to the liquid phase for microbial uptake (Bader, 1986).

Aeration in industrially important antibiotic fermentations is a critical factor since growth and production can seriously be affected by the dissolved oxygen concentration (Elibol, 2002). The problem arises mainly because of the low solubility of oxygen in aqueous media (Tolosa et al., 2002, Berry, 1975); poor mass transfer capacities of bioreactors; and the highly viscous nature of the dense mycelial cultures of filamentous organisms. Oxygen limitation can lead to a slow-down of metabolism or in some cases a complete change in metabolism (Buchs, 2001). Recently, Elibol, (2002), reported on the applicability of perfluorocarbons, chemically inert petroleum based compounds that exhibit oxygen solubility 10-20 times higher than that in water, as oxygen carriers for biotechnological processes.

1.2.3 Process mode

Most commercial bioprocesses are based on batch processing, which is better for non-growth associated product formation and genetic stability, and has a lower risk of contamination (Wang et al., 2005). Indeed, antibiotic production has traditionally been a batch-process based industry (Bader, 1986). Batch growth may typically be divided into a number of phases: lag phase, exponential phase, and stationary phase. The lag phase is generally thought to be a period of adaptation to the conditions imposed by a change of environment (Righeato, 1975). The length of this phase is dependent not only on the physiological state of the organism, but also on the morphology and level of inoculum; for example spore inoculum will often require a germination period (Papagianni, 2004). The exponential phase is characterised by a

significant increase in cell mass and the organism will continue to grow, consuming nutrients and producing metabolites and additional cells until the development of an adverse pH, the accumulation of inhibitory end products of metabolism or, most commonly, the depletion of some nutrient causes a reduction in the specific growth rate signifying the lag or stationary phase (Papagianni, 2004, Bader, 1986). It is usually at this point that secondary metabolism begins and the desired antibiotic is produced. Production can occur for a considerable time period before the rate declines and the batch is harvested (Bader, 1986).

During fed-batch cultivation, one or more nutrients are supplied to the bioreactor, while cells and products remain in the vessel until the end of the operation (Wang et al., 2005). Thus, as fresh nutrients are added, the volume of the culture is continually increasing (Papagianni, 2004). Fed-batch cultivation is generally superior to batch processing when changing nutrient concentrations effect the productivity and yield of the desired product, as is the case in substrate inhibition (Wang et al., 2005). For example, Cruz et al., (1999), reported that fed-batch fermentation favoured maintenance of higher production of cephalosporin C by promoting minimisation of catabolite repression. Substrate is converted immediately on entry into the bioreactor by high levels of active biomass, thus metabolism is directed towards product formation rather than growth (Papagianni, 2004). However, whilst fed-batch cultures ensure that essential nutrients are supplied at optimal concentrations or in excess for the duration of the culture, they do not allow for the removal of undesirable by-products of metabolism which may be inhibitory (Moran et al., 2000).

Continuous processes generally result in high productivity with many advantages including the facilitation of long fermentation runs with little downtime, steady-state conditions resulting in easier process control, and consistency of product quality (Wang et al., 2005). Indeed, the maximum specific production rate of tylosin in continuous cultures of *Streptomyces tendae* was 35% higher than that of a batch culture (Veelken and Pape, 1982).

Continuous cultures are usually preceded by growth of the organism in batch culture to stationary phase (Papagianni, 2004). However genetic instability can pose a serious problem in continuous cultures (Fazeli et al., 1995). Also, the growth of filamentous organisms by means of continuous cultures is problematic with increased wall growth and morphological heterogeneity making maintenance of steady-state difficult to achieve (Papagianni, 2004). Often immobilisation of filamentous organisms is required for successful continuous culture. For example, the production of actinomycin D by *Streptomyces parvullus* in immobilised continuous cultures was more advantageous than batch or fed-batch cultures (Dalili and Chau, 1988).

1.3 The morphological diversity of filamentous organisms

Micro-organisms have been characterised by their shape ever since the microscope became a standard instrument in microbiology (Scheper 1998). For example, bacteria are frequently characterised morphologically as rods (*Bacillus cereus*), cocci (*Staphylococcus aureus*), or spirals (*Helicobactor pylori*). In contrast, filamentous organisms are irregularly-shaped and grow as filaments or hyphae rather than as single cells. The most identifiable of the filamentous organisms are the filamentous fungi, for instance *Aspergillus* spp. and *Penicillium* spp.. The order Actinomycetales provides many examples of filamentous bacteria such as the genera *Streptomyces* spp. and *Nocardia* spp., although these were originally studied and accepted as fungi. Dimorphic yeasts such as *Saccharomyces cerevisae* and *Kluyveromyces marxianus*, although unicellular organisms, can also exhibit a filamentous growth phenotype.

The growth and reproduction of filamentous organisms is widely different from that of their unicellular counterparts. While binary fission and budding generally serve to create new bacterial or yeast cells respectively, in filamentous organisms cells do not separate, instead mycelial growth oscillates between hyphal extension and branch formation, resulting in a completely different macroscopic morphology. Although filamentous fungi and Actinomycetes, as eukaryotes and prokaryotes respectively, exhibit differences at the cellular level, the two groups display similar growth patterns (Prosser and Tough, 1991). The duplication cycle for filamentous organisms as represented in Figure 1.2 considers spore formation and germination; tip extension; and septation and branching.

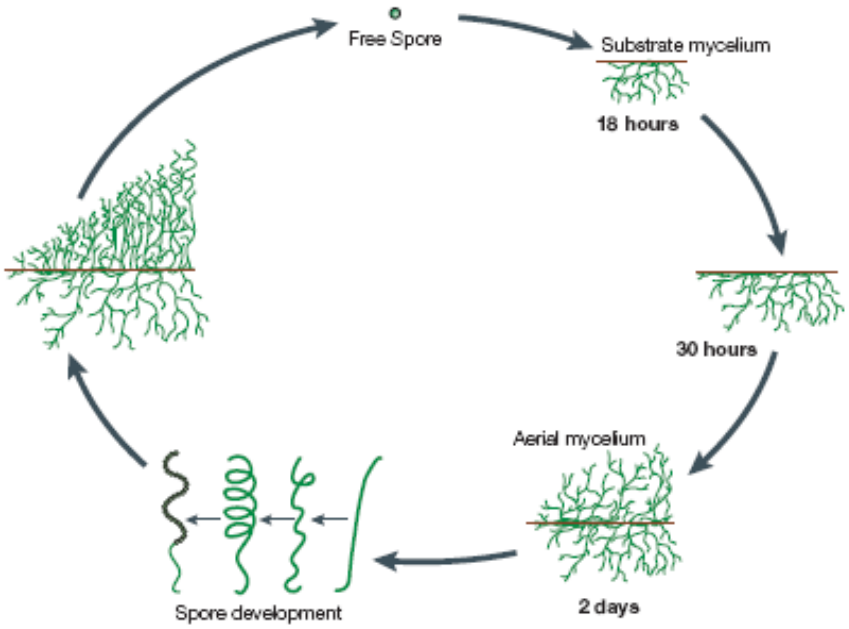


Figure 1.2: The life cycle of *Streptomyces coelicolor*, a filamentous micro-organism (Angert, 2005).

1.3.1 Growth kinetics

1.3.1.1 Spore formation and germination

The life cycle of filamentous organisms starts and ends in the form of spores (Znidarsic and Pavko, 2001). In biology, a spore is a reproductive structure that is adapted for dispersion and survival for extended periods of time in unfavourable conditions. Indeed, there have been some reports that dry aerial spores of Actinomycetes have survived for greater than 14 years, although the germinating fraction decreased significantly (Kalakoutskii and Pouzharitskaja, 1973). Improvement in environmental conditions is generally deemed to induce most spores to transition from dormancy to the filamentous vegetative state i.e. germinate. During the germination process swelling and the production of germ tubes occur, with streptomycetes capable of producing 1-4 germ tubes per spore depending on nutritional conditions of the growth medium (Kalakoutskii

and Pouzharitskaja, 1973). Vegetative cells emerge from this phase of outgrowth.

1.3.1.2 Tip extension

The basic vegetative structure of growth of filamentous organisms consists of tubular filaments known as hyphae which make up a so-called mycelium, with a hyphal element arising from a single spore (Nielsen, 1996, Papagianni, 2004). Elongation of vegetative hyphae occurs by apical tip growth and extension, supported by the sub apical regions. This hyphal growth appears to be driven by internal hydrostatic pressure acting on an extension zone (Prosser and Tough, 1991). The hyphal extension rate will depend on the amount of material supplied to the tip, and eventually reaches a constant value, generally when transport of materials from regions distant from the tip is limited (Papagianni, 2004). The hyphal diameter of filamentous fungi can reach up to 10 μm whereas streptomycete hyphae are generally 1 to 5 μm in diameter and only achieve lengths of several millimetres (Bader, 1986).

1.3.1.3 Septation and branching

Some fungi and all Actinomycetes are septate, having cross-walls which divide the hyphae into a series of compartments (Paul and Thomas, 1998). Once the hyphal apex has extended, the compartment is bisected by septum formation, with successive rounds of septation equivalent to the doubling time of the organism (Prosser and Tough, 1991). Thus as growth occurs, these compartments vary greatly in age and in physiological state, and can show various forms of structural and biochemical differentiation (Paul and Thomas, 1998). However, growth in filamentous organisms is not solely restricted to hyphal tip extension. If all growth takes place in the apical segment of the hyphae and the individual hypha extend at a constant linear rate, then exponential growth will require that new branches are produced at a rate proportional to the rate of increase of cell mass (Papagianni, 2004). Thus, the formation of branches enables an exponential growth phase, with the increase in total mycelium length with increasing number of actively growing tips by branching (Znidarsic and Pavko, 2001).

1.3.2 Solid state cultivation

Mycelial micro-organisms are well adapted to growth on solid substrates where growth is not self-limiting and large spreading colonies can form (Tough et al., 1995). Hyphal extension provides a means of exploring new regions for fresh nutrients, while branch formation enables full utilisation of medium already colonised (Prosser and Tough, 1991). Solid state fermentation has been known for centuries and used successfully for the production of oriental foods, enzymes, fine chemicals and antibiotics from obligate aerobic organisms (Ellaiah et al., 2004). It is generally a simple process, with low manufacturing costs due to utilisation of unprocessed or moderately processed raw materials as substrates, and a low requirement for pre-processing energy (Ellaiah et al., 2004).

Historically, there are numerous examples of the use of solid substrate fermentations including citric acid synthesis by *Aspergillus oryzae*, blue cheese production by *Pencillium roqueforti*, and the fermentation of koji by *Aspergillus oryzae* for the manufacture of sake. Indeed the industrial synthesis of penicillin by *Penicillium chrysogenum* was pioneered using solid state fermentation. However, solid state cultivations are often slow processes because of the diffusion barriers imposed by the solid nature of the fermented mass (Ellaiah et al., 2004); are labour-intensive; require a large surface area; and have a high risk of cross-contamination. Certainly it was the requirement for aseptic or sterile operations that led to the development of the stirred-tank reactor and the cultivation of organisms by way of submerged fermentations.

1.3.3 Submerged fermentation

Submerged fermentation is generally preferred to solid state cultivation in industrial processes, allowing greater process control and homogeneity. Indeed most commercial antibiotics are produced by the submerged culture of filamentous micro-organisms (Tamura et al., 1997). In submerged liquid

cultures nutrients, when present, are readily available to the organism by diffusion, and a relatively homogeneous environment can be maintained by adequate agitation (Berry, 1975).

When grown in submerged culture filamentous organisms exhibit different morphological forms ranging from dispersed individual mycelial elements to densely interwoven mycelial masses referred to as pellets (Figure 1.3) (Papagianni, 2004, Nielsen, 1996). Such variation is due in part to the great number of parameters that influence morphology, and also to the ability of each species to exhibit diverse growth forms. For example, Lawton et al., (1989), examined 49 streptomycete strains and found that each strain could produce one or more morphological forms including compact pellets; spiky or fluffy pellets; oblong pellets; flakes; hydrophobic rafts; aggregates; dispersed mycelium and fragmented mycelium. However, generally on a macroscopic level one can distinguish between the filamentous growth form where the hyphae are freely dispersed in the medium, and the pellet form, where the mycelium develops spherical aggregates consisting of highly entangled networks of hyphae (Znidarsic and Pavko, 2001).

Dispersed cultures ideally consist of a homogeneous suspension of branched hyphae, and in this respect are often equivalent to the cultures of unicellular organisms. Thus, in submerged cultures, adequate dispersal of the mycelium is an important factor in maintaining uniform environmental conditions (Glazebrook et al., 1992). However, in reality, dispersed mycelia frequently results in the formation of highly viscous solutions exhibiting non-Newtonian pseudoplastic rheological properties. The occurrence of pseudoplastic behaviour in mycelial suspensions is due to the alignment of the hyphae, which brings about a decrease in the apparent viscosity when high shear rates are applied (Sousa et al., 2002, Nienow, 1990). Highly viscous solutions reduce the mass transfer of nutrients, oxygen, and heat within cultures.

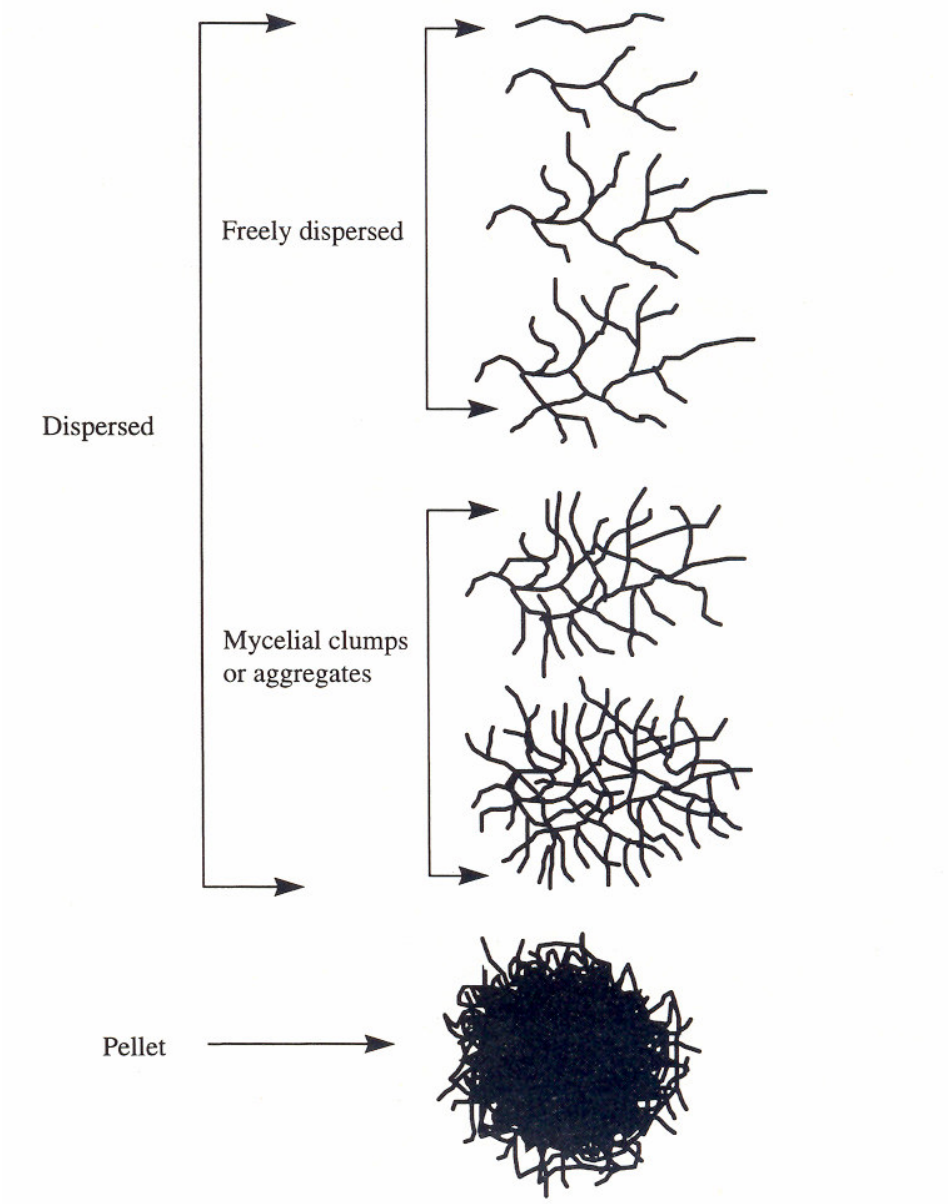


Figure 1.3: Diverse forms of morphology found in typical submerged cultures of filamentous fungi and Actinomycetes (Paul and Thomas, 1998).

Under conditions of rapid growth and high branching frequency, filamentous organisms may produce pellets (Bader, 1986). Pellet formation in submerged fermentations is often preferred, but is in itself not without the inherent disadvantage of increased heterogeneity. While broth rheology returns to Newtonian behaviour, the formation of tightly packed mycelial pellets can result in diffusional limitation of nutrients and oxygen through to the dense

core. The mycelial clumps also compose of entangled hyphae of different ages, states of nutrition and stages of development (Glazebrook et al., 1992). Often growth will only occur in a peripheral zone where a good supply of oxygen is available (Braun and Vecht-Lifshitz, 1991). Cell lysis is likely to occur in the centre of the pellet leading to a hollow interior surrounded by cells in differing metabolic states due to solute gradients, resulting in a suspension of pellets containing a heterogeneous complement of cells. Accordingly, small pellets as opposed to large ones are generally considered desirable in developing filamentous fermentations (Papagianni, 2004).

1.3.4 Characterisation of morphology by image analysis

Compared to many unicellular organisms, filamentous fermentation processes present special challenges in optimisation and scale-up because of the varying morphological forms (Wang et al., 2005). Therefore adequate monitoring of microbial aggregation is of great importance to many biochemical processes such as brewing; activated sludge; biomass production; and the synthesis of commercially important metabolites in submerged fermentations.

In the 17th century Antoine van Leuwoenhoek made possible the first observation of micro-organisms and for at least two centuries, until the arrival of photography, microbiologists relied exclusively on drawings to report their microscopic observations (Pons et al., 1998). Investigations of mycelial morphology then depended upon manual measurements from photographs of the objects assessed using a ruler or micrometer gauge (Papagianni, 2004, Paul and Thomas, 1998). However such conventional microscopic studies necessitated the use of multiple images in order to obtain statistically valid outcomes (O'Shea and Walsh, 1996). Other restrictions include the limited size of the field of view of a microscope at a given magnification, and the time consuming nature of the sequential image capture and analysis technique (O'Cleirigh et al., 2003).

The use of automated image analysis systems during the last decade has proved to be a fast and accurate method for quantitatively characterising complex mycelial morphologies, physiological states, and relationships between morphology and productivity (Papagianni, 2004, Znidarsic and Pavko, 2001). Image analysis systems have been developed to detect and quantify both pelleted and dispersed morphology. For example, O'Cleirigh et al., (2003), developed a high-throughput method for quantification of *Streptomyces hygroscopicus* var. *geldanus* pelleted morphology using a flatbed scanner and high performance desktop computer.

Quantified morphological information may be used to build morphologically structured models of predictive value, with mathematical modelling of growth and process performance leading to improved design and operation of mycelial fermentations (Papagianni, 2004). Structured models for fungal fermentations are available that consider hyphal growth, differentiation and metabolite production (Papagianni, 2004). Indeed, Aynsley et al., (1990), developed a mathematical model for the growth of mycelial fungi in submerged culture which was successfully applied to describe fed-batch penicillin fermentations. Subsequently Tough et al., (1995), and Cui et al., (1998a), published mathematical models for the growth of fungal pellet populations. A comparison of simulation versus experimentation for the model developed by Cui et al., (1998a), showed that it could suitably describe the time course of fungal growth (such as biomass and carbohydrate concentrations) and fungal morphology (such as pellet size and the fraction of pellets in the total biomass).

Nonetheless, it is sometimes difficult to define a single mechanism for pellet formation from reported results, as often more than one parameter is adjusted by changing only one variable (Vecht-Lifshitz et al., 1989). Thus generalised models are frequently disregarded due to poor reproducibility and inherent differences between strains, and it is recommended that the factors influencing morphology be examined for each particular organism.

1.4 Factors affecting morphological development

There are a great number of microbiological and physiochemical factors that can affect the morphology of filamentous organisms in submerged cultures.

1.4.1 Microbiological factors

1.4.1.1 Inoculum size

An inoculum typically consists of either a suspension of spores or a solution of vegetative biomass. There is much evidence to suggest that the morphological form of a number of filamentous organisms is influenced by the concentration of viable spores in the inoculum, with high concentrations producing a dispersed form of growth, and low concentrations resulting in pellet formation (Whitaker, 1992). Indeed, Tucker and Thomas, (1995); Nielsen et al., (1995); Kim and Hancock, (2000); Vecht-Lifshitz et al., (1990); and Vecht-Lifshitz et al., (1989), observed that pellet sizes of *Streptomyces* spp. and *Penicillium* spp. decreased progressively with increasing inoculum size, with small hyphal elements increasingly evident leading to partially dispersed growth.

Two distinct mechanisms of pellet formation can be recognised depending on whether or not the organism is itself coagulating or non-coagulating. Pellet formation in coagulating systems e.g. *Aspergillus* spp. involves the initial clumping of spores that then germinate to produce hyphae which gradually become entangled and develop into pellets (Whitaker, 1992, Nielsen, 1996). In contrast, in non-coagulating cultures, such as *Streptomyces tendae* and *Streptomyces griseus*, a single spore develops into a pellet, with further branching leading to a dense core (Nielsen, 1996).

1.4.1.2 Medium composition

Media may be chemically defined (synthetic) or complex and many common media constituents have been observed to impact significantly on morphology of cultures. Dispersed growth is more likely in rich, complex media, and pellets

are more likely to occur in chemically defined media (Whitaker, 1992). However the effects of media can be extremely varied, and individual components may only change the morphological state of a few strains (Whitaker, 1992).

1.4.1.2.1 Carbon source

Certain specific sugars have been demonstrated to disperse flocculent cells of *Saccharomyces cerevisae*, with glucose and sucrose being the most effective (Mill, 1964). Jonsbu et al., (2002), observed that cultures of *Streptomyces noursei* grown on fructose as the sole carbon source exhibited smaller 'clump' sizes throughout the fermentation compared with cultivation on glucose. However, Glazebrook et al., (1992), reported that there was no consistent relationship between the size of mycelial aggregates and carbon source in cultures of *Streptomyces akiyoshiensis*. In many instances where the choice of carbon source may appear to directly influence mycelial morphology, disparity may be related to the growth rate of the organism, which is itself controlled by the concentration of substrate in the fermentation media.

1.4.1.3 Nitrogen source

There is much evidence to suggest that nitrogen source greatly influences morphological type. Ammonium nitrate was found to exert a profound effect on the morphology of *Aspergillus niger* in submerged culture (Joung and Blaskovitz, 1985). Depending on the ammonium nitrate concentration, three different types of morphology were possible: (1) vegetative mycelia at low concentrations; (2) natural morphology with mycelia and fruiting bodies at intermediate concentrations; and (3) short branched mycelia with bulbous masses at high concentrations (Joung and Blaskovitz, 1985). Choi et al., (2000), illustrated the difference in morphology of *Streptomyces fradiae* cultivated on separate nitrogen sources. In cultures containing ammonium ions, the pellet area was about 8-fold larger than cultures grown on an amino acid mixture, although large entangled filamentous mycelia were observed. The morphology of *Mortierella alpina* was examined by Park et al., (1999), using various different nitrogen sources and two morphological types were detected.

Pelleted growth was obtained in the case of yeast extract, gluten meal and corn steep liquor; but pellets with radial morphology 2-3 times greater in area were produced on soybean meal and fish meal (Park et al., 1999). Du et al., (2003), also reported differences in the length of hyphae and degree of branching of *Rhizopus chinesis*, with dispersed and filamentous morphology evident on peptone and yeast extract, and pelleted growth on corn steep liquor and ammonium sulphate.

1.4.1.3.1 Minerals

Certain essential minerals and metals are required for growth such as phosphate, magnesium, calcium, manganese and zinc. Extensive information exists on the effects of metal and other ions on growth (Papagianni, 2004), but to a lesser extent, their impact on morphology. Morphology in cultures of *Streptomyces akiyoshiensis* was observed to be dependent on phosphate concentration in the media, with a gradual increase in mycelial aggregation and pellet size with a corresponding decrease in phosphate concentration (Glazebrook et al., 1992). Okba et al., (1998), investigated the effect of divalent cations Mg^{2+}, Ca^{2+}, Mn^{2+} and Zn^{2+} on pellet formation in submerged cultures of *Streptomyces azureus*. Mn^{2+}, Mg^{2+} and Ca^{2+} were observed to exert an effect on morphology, inducing pellet formation. The authors suggest that cell surfaces bound to these divalent cations may become electrostatically unstable, aggregated and entangled, leading to pelleting (Okba et al., 1998). These results confirm the findings of Braun and Vecht-Lifshitz, (1991), who concluded that addition of polycations usually induces aggregation, whereas polyanions suppress it. In contrast, Glazebrook et al., (1992), suggested that mineral nutrition was not a critical factor in mycelial aggregation in cultures of *Streptomyces akiyoshiensis*. The inclusion or omission of inorganic media constituents, along with varying Mg^{2+} concentrations yielded consistently large pellets of >0.5mm in diameter.

1.4.1.4 Cell wall composition

Many studies have indicated that physiochemical properties such as charge and, in particular, cell surface hydrophobicity can influence agglomeration of

cells. Dynesen and Nielsen, (2003), studied morphology in mutants of *Aspergillus nidulans* by disruption of the genes *dewA* and *rodA* which encode hydrophobic proteins located in the cell wall of the organism. Their study observed that absence of the hydrophobins reduced the surface hydrophobicity of conidiospores and caused a concomitant increase in the percentage of free mycelia. Analogously, flocculence of *Saccharomyces cerevisae* was found to be induced by an increase in cell surface hydrophobicity (Smit et al., 1992), indicating that the influence of surface hydrophobicity is not confined to spore-spore interactions.

Atkinson and Daoud, (1976), suggested that aggregation can result from the interactions of extracellular polymers that accumulate at the microbial surface during growth. These polymers combine either electrostatically or physically and bridge the cells in suspension. Indeed, Vecht-Lifshitz et al., (1989), observed a recurring phenomenon in cultures of *Streptomyces tendae*, whereby conditions yielding smaller pellets reduced the cell wall hydrophobicity, thus demonstrating that larger pellets were more hydrophobic. They postulated that hyphae are either brought together by adhesive forces determined by their surface properties, or are repulsed, and hence form free mycelia, but that the exact nature of surface interactions may differ in distinct biological systems.

1.4.2 Physiochemical factors

1.4.2.1 Incubation temperature

As most organisms have an optimal temperature for growth, temperature is frequently a disregarded parameter in fermentation development. However, Mill, (1964), noted that flocculence of *Saccharomyces cerevisae* was dependent on temperature, with complete dispersion achieved at high temperatures of 60°C. As the temperature was lowered the flocculence reappeared (Mill, 1964). The morphology of *Streptomyces* spp. has also been found to be somewhat dependent on temperature of incubation. An increase in temperature from 30 to 37°C caused a decrease in pellet size by one third in cultures of *Streptomyces griseus* (Kim and Hancock, 2000). Vecht-Lifshitz et

al., (1989), also reported that high incubation temperatures (31-32°C) induced pulpy growth of *Streptomyces tendae*, in contrast to lower temperatures where pellet growth was prominent (26-30°C).

1.4.2.2 Dissolved oxygen concentration

The effect of dissolved oxygen on filamentous morphology has often been observed, but as it is generally coupled with studies on agitation and mechanical shear it becomes unclear as to whether any impact can be attributed to the mechanical forces or the dissolved oxygen tension (Cui et al., 1998b). Braun and Vecht-Lifshitz, (1991), noted that pelleted morphology predominates in the early life of a culture where oxygen supply is sufficient, while dense, older cultures tend to be filamentous. Vecht-Lifshitz et al., (1990), also observed a correlation between the reduction of the oxygen transfer rate in cultures and a decrease in the pellet size of *Streptomyces tendae*. In addition, increasing the growth rate by enriching the culture medium, or by raising the temperature or pH, depleted the oxygen supply and prevented pellet formation (Braun and Vecht-Lifshitz, 1991). Cui et al., (1998b), studied the effects of dissolved oxygen and mechanical forces independently in cultures of *Aspergillus awamori*. The authors concluded that, from the data obtained, pellet size is independent of dissolved oxygen tension and is determined by mechanical forces which are related to the agitation intensity. However higher dissolved oxygen tensions did lead to the formation of denser pellets (Cui et al., 1998b).

1.4.2.3 Shear forces

The effects of mechanical stress due to agitation on morphology have been studied fairly extensively, though not always with rigorous control of other parameters (Nienow, 1990). For coagulating cells, high turbulence or agitation is often required for effective collision of particles for aggregation. Indeed Lu et al., (1998), found that the effective collision was directly proportional to the agitation intensity, however this is only true to a maximum agitation point, beyond which fragmentation of aggregates occurs and coagulation decreases. The extent of shear forces is highly dependent on the choice of bioreactor.

1.4.2.3.1 Stirred tank reactors

For aerobic bioprocessing the stirred tank reactor is the most popular choice of bioreactor (Ohta et al., 1995). Pellets do not normally form in bioreactors because of the high aeration and vigorous agitation, and dispersed mycelial growth is usually the predominant form (Whitaker, 1992). However shear stresses arising in these reactors can cause undesired effects on mycelial morphology and product formation (Ohta et al., 1995). The use of non-mechanically agitated bioreactors may provide a solution and contribute to a reduction in power costs, because they are considered to have a lower power requirement for comparable values of oxygen mass transfer than mechanically agitated vessels (Adinarayana et al., 2004). Indeed, air-lift reactors contain a draft tube which increases mixing and reduces bubble coalescence, thereby ensuring smaller bubbles with increased surface area are available for oxygen transfer (Adinarayana et al., 2004). Shear stress has also been reported to be considerably reduced compared to that in stirred-tank reactors (Ohta et al., 1995).

Agitation conditions and shear stress in stirred tank bioreactors are strongly associated with the type of impellers employed (Znidarsic and Pavko, 2001). While rushton turbines are generally the most frequently utilised agitators in industrial fermentations, other configurations may be used in order to generate an optimal morphological composition. Justen et al., (1996), demonstrated the dependency of morphology of *Penicillium chrysogenum* on impeller geometry, using radial flow impellers (rushton turbines, paddles); axial flow impellers (pitched blades); and counterflow impellers (Intermig). Damage to mycelia was characterised by image analysis and, at the same power input, paddle impellers and rushton turbines were construed to produce no significant damage. In contrast, pitch blade impellers were observed to cause the most damage to mycelia (Justen et al., 1996). Nonetheless, low power number impellers such as pitched or hydrofoil impellers are often used to replace rushton turbines for mycelial fermentations (Nienow, 1990). They can be of larger diameter than the rushton turbines and thus inherently give better bulk flow mixing and homogenisation, and the profile of the blades eliminates cavities from behind them (Nienow, 1990).

In general, increasing the agitation rate improves the overall homogeneity of filamentous cultures, although this also raises the power consumption and often damages the cells due to high shearing (Braun and Vecht-Lifshitz, 1991). Cui et al., (1997), postulated that three damaging mechanisms may exist for mycelial cultures in stirred vessels: the interaction between particles and eddies; the impact between particles and impellers or baffles; and the collision between particles and particles. This may result in damage to cell structure, morphological changes, or variations in growth rate (Papagianni, 2004). However, fragmentation of hyphal elements and pellets due to shear forces by agitation may also result in growth renewal since fragments may act as centres for new growth (Papagianni, 2004).

1.4.2.3.2 Shake-flasks

Shaking bioreactors (shake-flasks) have been used for decades in research and development laboratories and have the advantages of reduced overhead and operating costs, allowing one to set up large numbers of simultaneous experiments with relative ease. Indeed it has been estimated that probably much more than 90% of all culture experiments in biotechnology are performed in shaking bioreactors (Buchs, 2001). They are an essential tool in the screening processes for fermentation technology as well as the first step in scale-up studies for most bioprocessing applications (Webb and Furusaki, 2001). In addition, shake-flasks are usually retained in the commercial bioprocess as the first step in inoculum development (Webb and Furusaki, 2001). At the laboratory scale, typical shake-flask fermentations are conducted on rotary shakers and the rate of oxygen transfer to the liquid is highly dependent on the shaker speed; the type of sterile enclosure on the flask; the size and design of the flask; the quantity of medium; and the viscosity of the broth (Bader, 1986).

In shake-flask cultures shear is generally quite low, promoting the development of pelleted morphology. Baffled flasks have the ability to increase shear and oxygen transfer in shake-flask cultures, and are often used to obtain a dispersed mycelial morphology. Indeed, Vecht-Lifshitz et al., (1989), reported that the

upper limit for pellet formation in *Streptomyces tendae* was markedly decreased from 10^{11} spores/m^3 in an unbaffled flask to 10^9 spores/m^3 in baffled flasks.

1.4.2.3.3 Micro-titre plates

The use of micro-titre plates for the growth and maintenance of microbial strains reduces the demand for incubation space and medium, and allows the parallel screening of large numbers of strains (Duetz et al., 2000). Minas et al., (2000), cultivated mycelium forming streptomycete strains in 96-well format micro-cultures and found that extensive wall growth of the organism occurred, although biomass and antibiotic production did compare well with shake-flask or bioreactor cultivations. Such a small scale format was amenable for the screening of many different streptomycete strains, media formulations, and secondary metabolites. It has been postulated that the greatest benefits of micro-scale processing techniques will arise from the automated operation of whole bioprocess sequences, which has yet to come about (Micheletti and Lye, 2006).

Apart from hyphal fragmentation, release of intracellular material into the fermentation broth due to changes in membrane structure and permeability has also been ascribed to mechanical forces (Papagianni, 2004). Therefore agitation and shear forces may indirectly affect morphology by prompting the release of intracellular products such as biosurfactants, surface active substances synthesised by the organism in question.

1.4.2.4 Polymers and surfactants

The inclusion of polymers and surfactants is believed to induce electrostatic repulsion among cells or spores, preventing the initial aggregation of spores or cells in the inocula and the subsequent clumping of mycelia in the growing culture (Okba et al., 1998). The inclusion of the charged polymers Carbopol and Junlon, and surfactants such as Pluronic F68, Brij 58 and Triton X100, have all been demonstrated to enhance dispersion of filamentous morphology (Hobbs et al., 1989, Vecht-Lifshitz et al., 1989, Morrin and Ward, 1990).

1.4.2.5 pH

The pH of a culture is an important environmental factor that can profoundly affect any activity being studied (Papagianni, 2004). At pH values above 5.5, the cell walls of most organisms are negatively charged, tending to cause separation of the aggregating cells by electrostatic repulsion (Braun and Vecht-Lifshitz, 1991). However, many conflicting results on the influence of pH on morphology have been reported. Carlsen et al., (1996), found that at acidic pH values the morphology of *Aspergillus oryzae* was freely dispersed, whereas for pH values higher than 6 pellets were the only growth form observed, with pellet radius increasing with pH. Vecht-Lifshitz et al., (1989), reported that at pH optimal for growth (pH 7-8), the pellet size of *Streptomyces tendae* was significantly smaller (0.1-0.2mm) than that of cells grown at lower pH (1.2–1.5mm at pH 4). Kim and Hancock, (2000), demonstrated the ability of Tris / HCl buffer to disperse preformed pellets of *Streptomyces griseus* at neutral pH values. However, at a lower pH the dispersing activity of the reagent was abolished. In cultures of *Streptomyces akiyoshiensis*, the maximum size of aggregates was 4- to 5- fold smaller in cultures at pH 5.5 than at higher pH values (Glazebrook et al., 1992).

Dynesen and Nielsen, (2003), observed a dependency between pH and morphology in cultures of *Aspergillus nidulan*s. At low or acidic pH values, the cells carried a sufficiently positive charge to inhibit coagulation, and free mycelia were formed. When the pH was increased toward the isoelectric point, surface charge decreased, allowing the spores to agglomerate and form pellets. However, at a pH above the isoelectric point of approximately 4.5, the cells attained a negative surface charge and repellence should be re-established among the cells. Yet, in this example, the percentage of free mycelium was seen to progressively decrease, leading the author to the conclusion that electrostatic interactions alone cannot explain the pH dependency of the agglomeration process.

1.4.2.6 Viscosity and rheology

Rheological behaviour is closely related to mycelial morphology, and can determine the transport phenomenon in bioreactors which may be the key to improving the product yield (Sinha et al., 2001). Viscosity is an important factor to consider in *Streptomyces* spp. cultures: the larger the pellet size and ratio of the pellet area, the lower the viscosity in the culture broth (Tamura et al., 1997). In contrast, dispersed mycelial broths are generally highly viscous. An increase in the apparent viscosity during the culture of filamentous fungi or Actinomycetes can result in a decrease in oxygen transfer and in the production rate (Choi et al., 1998). Most mycelial fermentations will be shear-thinning or pseudoplastic because increasing shear rate pulls the intertwined mycelial structure apart and aligns the hyphae, thereby reducing broth viscosity (Nienow, 1990, Sousa et al., 2002). Shear thinning fluids such as mycelial broths have the characteristic of being of low viscosity at high shear rates, and of high viscosity at low shear rates (Nienow, 1990).

O'Cleirigh et al., (2005), artificially regulated broth viscosity by the inclusion of varying concentrations of the non-Newtonian agent xanthan gum. An increase in the apparent viscosity of any liquid should result in a decrease of turbulence within the system, and thus a reduction in the collision of particles, a common prerequisite for pellet formation. Indeed, an increase in viscosity resulted in a four-fold increase in the pellet count of *Streptomyces hygroscopicus* var. *geldanus*, and a concurrent decrease in pellet volume (O'Cleirigh et al., 2005).

1.4.2.7 Suspended solids

The attachment of growing hyphae to solid substrates may act as a nucleus for forming pellets (Braun and Vecht-Lifshitz, 1991). Kim and Kim, (2004), postulated that there should be substances responsible for the strong cohesion of hyphae in pellet formation. They showed that the addition of an insoluble calcium salt to the fermentation acted as a physical support in the formation of pellets of *Streptomyces coelicolor* A3(2). The inclusion of kaolin particles, the

main constituent of clay, yielded similar findings with a mycelium-kaolin complex observed (Kim and Kim, 2004).

1.5 Relationship between morphology, physiology and secondary metabolite production

It is apparent that there are a myriad of factors affecting secondary metabolite production (Section 1.2). However, many of these parameters have also been independently demonstrated to affect the morphological development of filamentous organisms (Section 1.4). Thus it is often difficult to elucidate the true effect of a parameter and identify the key driver of secondary metabolite production.

Although many models have been developed to describe filamentous morphology, only a few have simultaneously dealt with the influence of morphology on metabolite formation (Wang et al., 2005). Table 1.1 details the morphological type preferred for metabolite production in a number of commercially important filamentous organisms.

Table 1.1: Mycelial morphology and metabolite production.

Organism	Antibiotic	Morphology	Reference
Streptomyces tendae	Nikkomycin	Pelleted	Vecht-Lifshitz et al., 1992
Streptomyces noursei	Nystatin	Pelleted	Jonsbu et al., 2002
Rhizopus chinesis	Antibiotic	Pelleted	Du et al., 2003
Penicillium chrysogenum	Penicillin	Pelleted Dispersed	Hotop et al., 1993; Makagiansar et al., 2003
Streptomyces akiyoshiensis	5-hydroxy-4-oxonorvaline	Dispersed	Glazebrook et al., 1992
Rhizopus arrhizus	Fumaric acid	Dispersed	Morrin and Ward, 1990

Reports on the preferred morphology can often be contradictory since each one of the two extreme forms, pellets or filaments, has its own characteristics concerning cell physiology, growth kinetics, nutrient consumption and broth rheology, which can be regarded as either advantages or drawbacks (Papagianni and Mattey, 2006). Indeed even for the most studied, industrially important *Aspergillus* and *Penicillium* species, reports of the dependency of productivity on morphology can be conflicting. For example whilst Hotop et al., (1993), observed a doubling in yields of penicillin as pellet size decreased; Makagiansar et al., (1993), determined that decreasing hyphal lengths of *Penicillium chrysogenum* suppressed productivity; and, indeed, Nielsen et al., (1995), found that no relationship at all existed between the two parameters!

Vecht-Lifshitz et al., (1990), postulated that variations in morphology may cause disparity in oxygen transfer rates between cultures of *Streptomyces tendae*. The macroscopic (gross) morphology affects the micro-environment of the hyphae by determining the culture rheology, mixing, and mass transfer capabilities of the culture (McIntyre et al., 2001). For example, the presence of the organism in a dispersed form would afford proximity to the liquid medium for mass transfer purposes such as nutrient uptake and excretion of toxic compounds and metabolites (Prosser and Tough, 1991). At the microscopic level hyphal dimensions, segregation, and differentiation may influence metabolic pathway activity (McIntyre et al., 2001). Thus, while the production of metabolites may depend on the morphological type of the synthesising organism, it is most probable that this represents an indirect relationship induced by alterations in cell physiology (Figure 1.4).

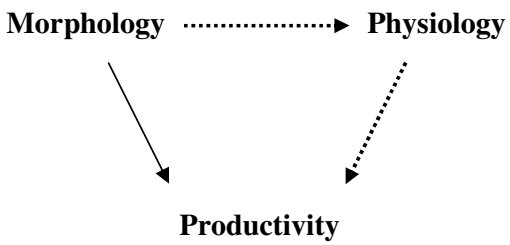


Figure 1.4: Proposed relationship between morphology, physiology and secondary metabolite production.

As the growth of filamentous organisms occurs, hyphal compartments can vary greatly in age and in physiological state and can show various forms of structural and biochemical differentiation (Paul and Thomas, 1998). In dispersed mycelial cultures an increase in vacuoles or empty zones in conjunction with mycelium age is commonplace, and their appearance has been used as an indicator of the physiological state of *Penicillium chrysogenum* (Lendenfeld et al., 1993) and *Streptomyces ambofaciens* (Drouin et al., 1997). Indeed, Lendenfeld et al., (1993), identified the vacuole as the cellular compartment containing the precursor amino acids for B-lactam biosynthesis in *Penicillium chrysogenum*, establishing a solid link between physiological differentiation and secondary metabolite production.

When cells are present in pelleted form, intra-particle resistance is likely to be significant as oxygen has to diffuse through the solid pellet to reach the interior cells with the magnitude of this resistance depending on the size of the clumps (Doran, 1995). Wittler et al., (1986), proposed the existence of four different layers in a c1.7mm diameter pellet of *Penicillium chrysogenum*, ranging from L1 consisting of viable hyphae to L4 in the centre of the pellet where no hyphae could be distinguished. Pons and Vivier, (1998), also classified the physiology of *Penicillium chrysogenum* into a number of different states, although no clear link between the physiological stages of biomass and penicillin production was determined. Jonsbu et al., (2002), demonstrated that the main core of pellets of *Streptomyces noursei* appeared to lack metabolic

activity with the areas of high activity primarily located at the loosely packed mycelia around the pellet surfaces. However, the relationship between morphology, physiology and nystatin yield was not comprehensively explored.

Many authors have previously highlighted the need for further investigation of the relationships between morphology, physiology and productivity (Wang et al., 2005, Papagianni, 2004, Tamura et al., 1997). Clearly there is a deficiency of knowledge in this area as previous studies have concentrated solely on the effects of one parameter. Thus, conclusive evidence to establish whether factors affecting morphology have a direct or indirect effect on secondary metabolite production remains to be demonstrated. In order to elucidate between these parameters, an ideal model system for experimentation must be selected. The model system must meet the following criteria:

- Be a filamentous organism with the ability to exhibit diverse morphological forms
- Produce a secondary metabolite of interest that is derived from a tightly regulated complex metabolic pathway

SECTION 1B: Choice of Model System and Experimental Aims and Objectives

1.6 *Streptomyces hygroscopicus* var. *geldanus* and geldanamycin synthesis

The bacterial genus *Streptomyces* spp. belongs to Actinomycetales, a bacterial order of more than thirty genera of gram-positive bacteria that show branching, filamentous or irregularly rod-shaped cell morphology (Omura, 1986). The most outstanding characteristic of the Actinomycetales, and in particular *Streptomyces* spp., is their ability to produce a huge number of secondary metabolites which are of significant clinical importance. Indeed streptomycetes are remarkably versatile chemists, and are reported to be the largest antibiotic-producing genera discovered in the microbial world to date (Watve et al., 2001, Crandall and Hamill, 1986).

Of the 12,000 antibiotics known in 1995, 55% were produced by filamentous bacteria of the genus *Streptomyces* spp. (Demain, 1999). Some *Streptomyces griseus* strains produce over 40 different antibiotics, while strains of *Streptomyces hygroscopicus* make almost 200 antibiotics (Demain, 2000). The fundamental process of secondary metabolism is a key and highly developed attribute of streptomycetes, and the vast array of chemically diverse products have ensured its position as the object of intense research in university and industrial laboratories (Queener and Day, 1986). Table 1.2 summarises the major structural classes of antibiotics produced by *Streptomyces* spp..

1.6.1 Geldanamycin, a new antibiotic

Geldanamycin (GA) was discovered in 1970 by De Boer et al. during a screening process for compounds which inhibit growth and multiplication of protozoa. The substance was produced under submerged fermentation by an Actinomycete originally isolated from a Kalamazoo soil sample and subsequently identified as *Streptomyces hygroscopicus* var. *geldanus* (De Boer et al., 1970).

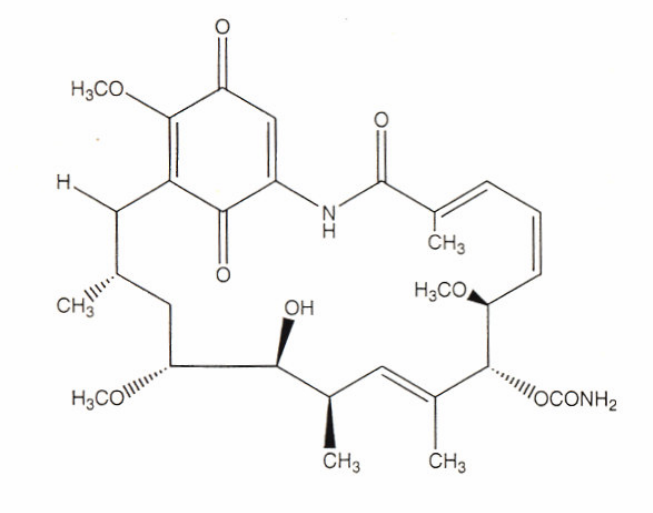
Table 1.2: Major structural classes of antibiotics produced by Streptomycetes

Classification	Example	Mode of action	Clinical Use
Aminoglycosides	Streptomycin (*S. griseus*)	Inhibition of protein synthesis, disruption bacterial cell membranes	Gram-negative antibiotics
Anthracyclines	Daunorubicin (*S. peucetius*)	Inhibition DNA replication, disruption cell membranes	Chemotherapeutic compounds
Ansamycins	Rifamycin (*S. mediterranei*)	Inhibition of DNA dependent RNA synthesis	Broad spectrum antibiotics
β-Lactamase Inhibitors	Clavulanic Acid (*S. clavuligerus*)	Inhibition of β-lactamase enzymes	In conjunction with penicillins for treatment of gram-positive bacterial infections
Macrolides	Tylosin (*S. fradiae*)	Inhibition protein synthesis	Broad spectrum antibiotics, effective against fungal infections
Polypeptides	Actinomycin (*S. antibioticus*)	Inhibition DNA replication	Chemotherapeutic compounds
Polyenes	Nystatin (*S. noursei*)	Disruption fungal cell membranes	Antifungal agents
Tetracyclines	Tetracycline (*S. aureofaciens*)	Inhibition protein synthesis	Broad spectrum antibiotics

Geldanamycin is classified, structurally, as a polyketide ansamycin antibiotic and a profile of the compound is presented in Table 1.3. Polyketide natural products are among the most important microbial metabolites in human medicine, with polyketide derived pharmaceuticals comprising 20% of the top-selling drugs and worldwide revenues of over UK£10 billion per year (Weissman and Leadlay, 2005). Polyketides make good drugs: of the 7,000 known polyketide structures, more than 20 (0.3%) have been commercialised, which is considerably better than the typical <0.001% 'hit rate' from standard pharmaceutical screens (Weissman and Leadlay, 2005). Polyketide ansamycin antibiotics, including geldanamycin, are hybrids of polyketides and peptides (Kim et al., 1998). The ansamycins are characterised by an aliphatic-ansa bridge which connects two nonadjacent portions of an aromatic nucleus (Crandall and Hamill, 1986). The ansamycins can be further subdivided depending on the nature of the aromatic group, in the case of geldanamycin, a benzoquinone (Janin, 2005).

Geldanamycin possesses both *in-vitro* and *in-vivo* antimicrobial activity. Using an agar diffusion bioactivity assay, geldanamycin was originally shown to be a potent inhibitor of protozoa such as such as *Crithidia* spp. and fungal plant pathogens, including *Penicillium* spp. (De Boer et al., 1970). In contrast, the compound was found to be inactive against viral test strains Parainfluenza and Herpes simplex, and exhibits limited anti-bacterial activity against organisms such as *Bacillus* spp. and *Salmonella* spp. (De Boer et al., 1970). However, recently many ansamycin compounds such as radicicol, herbimycin A, rifampicin and geldanamycin have received considerable attention, primarily focused on their potential as therapeutic lead compounds for new anti-cancer agents (Andrus et al., 2003).

Table 1.3: Profile of geldanamycin

Physio-chemical properties	
Formula	$C_{29}H_{40}N_2O_9$
Structure	 (Agnew et al., 2001)
Molecular Weight	560.6
Melting Point	265-268°C
Appearance	Yellow powder
Solubility	Soluble in DMSO, Dichloromethane, Acetonitrile; very low solubility in H_2O
Classification	
CAS	30562-34-6
Sources	
Natural	Isolated from *Streptomyces hygroscopicus* var. *geldanus*

1.6.2 Geldanamycin, a novel chemotherapeutic agent

Geldanamycin has been demonstrated to be a potent cytotoxic compound, active in tumour cell lines and reducing the size of solid tumours in mouse models. Initially it was thought that its cytotoxic properties were due to its activity as a non-specific protein kinase inhibitor (Sittler et al., 2001). A protein kinase is an enzyme that modifies other proteins, resulting in changes in enzyme activity, cellular location or interactions with other proteins. Deregulated kinase activity is known to be a frequent cause of disease, particularly cancer, where kinases regulate many aspects that control cell growth, movement and death. However the method of action of some benzoquinone ansamycins, including geldanamycin, has now been re-evaluated following their demonstrated ability to bind to and antagonize the function of the chaperone protein heat shock protein 90 (Hsp90) (Neckers et al., 1999).

Chaperone proteins mediate the correct assembly of other polypeptides but are not themselves part of the assembled structure (Neckers et al., 1999). The molecular chaperone Hsp90 is a 90kDa protein and is one of the most abundant proteins expressed in cells. In the mammalian system, Hsp90 is involved in the folding and maturation of key regulatory proteins like hormone receptors, transcription factors and kinases, an expanding subset of medically relevant signalling molecules important in cancer. Of interest to cancer researchers, Hsp90 is constitutively expressed at 2-10 fold higher levels in tumour cells than in their normal counterparts, suggesting that it may play a critical role in tumour cell growth and/or survival (Neckers et al., 1999).

The remarkable sensitivity of cancer cells to pharmacologic disruption of Hsp90 suggests that this family of proteins may be a novel target for anti-cancer drug development (Neckers et al., 1999). Indeed, cancer cells appear to be especially sensitive to interference with chaperone activity because so many growth and survival-regulating proteins in tumour cells depend upon stable or transient interactions with Hsp90 for their activities (Neckers et al., 1999). Clinical and preclinical trials of Hsp90 inhibitors are currently underway, with

radicicol and geldanamycin among the most studied (Janin, 2005). Stebbins et al., (1997), and Neckers et al., (1999), demonstrated that geldanamycin binds to Hsp90 via its essential ATP binding site in a C-shaped conformation (Figure 1.5). This inhibits Hsp90 chaperone function, resulting in dissociation of complexes between Hsp90 and its partners and accelerating the degradation of these proteins (Suttitanamongkol et al., 2000). In the National Cancer Institute (US) *in-vitro* screening of drug sensitivity in 60 tumour cell lines, geldanamycin achieved 50% growth inhibition in highly responsive cell lines (Supko et al., 1995).

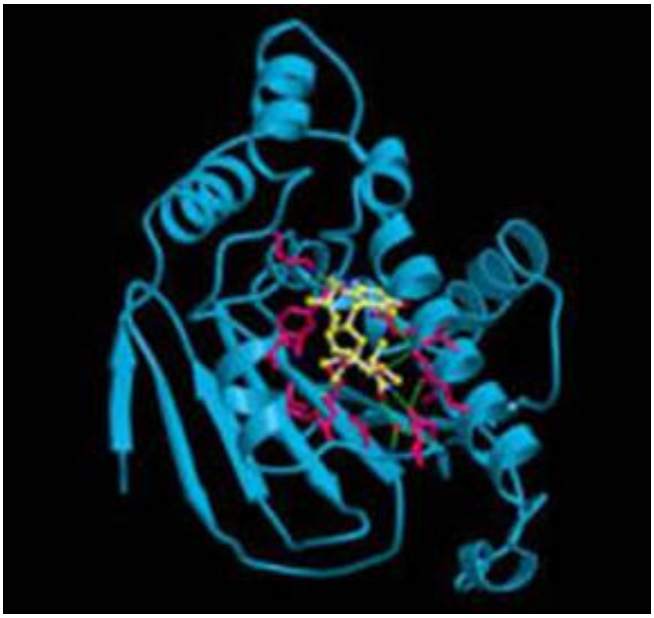


Figure 1.5: The interaction of geldanamycin (yellow) with Hsp90 side chains (pink) in a well-defined deep pocket lined by residues conserved across different species expressing Hsp90 (Stebbins et al., 1997). Geldanamycin occupies the ATP-binding site on the protein.

There are four main classes of signalling proteins that depend on chaperone action:

- *Receptor and non-receptor protein kinases*

Elongation factor-2 kinase (EF-2), is a unique kinase overexpressed in glioblastoma multiforma, a highly resistant lethal malignancy (Yang et al., 2001). Following exposure to geldanamycin or its analogues, a 70% decrease in EF-2 kinase was reported in a glioma cell line (Yang et al., 2001). HER-2 is a membrane receptor whose overexpression is strongly associated with poor prognosis in breast carcinomas (Mandler et al., 2000). By coupling geldanamycin to an anti-HER-2 monoclonal antibody to form an immunoconjugate, HER-2 levels were reduced by 86% (Mendelsohn, 2000).

- *Serine/threonine kinases*

Raf-1 is a central component of the signal transduction pathway utilised by many growth factors involved in various malignancies (An et al., 1997). Dissociation and rapid degradation of this kinase follows treatment with geldanamycin.

- *Proteins that regulate cell cycle and apoptosis*

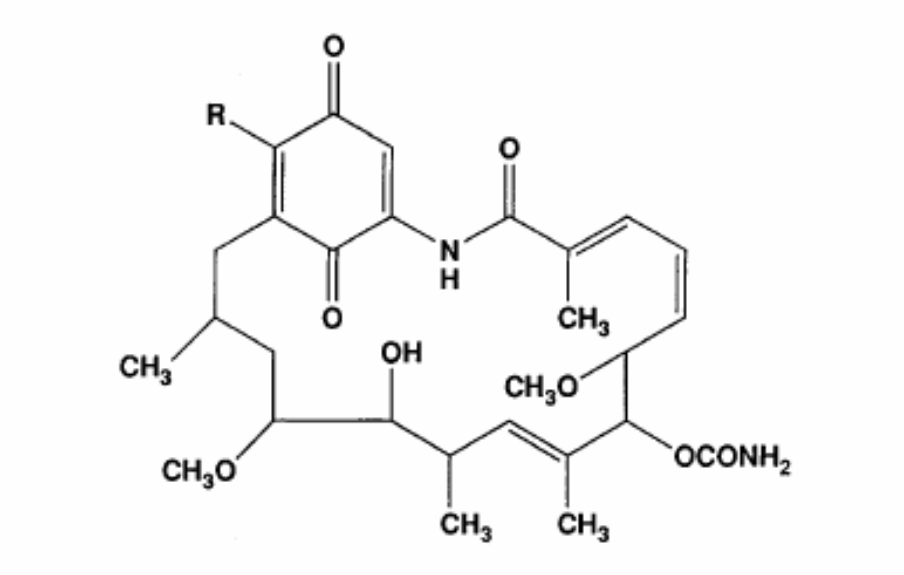
The wild type p53 gene product is a tumour suppressor protein, and is one of the most commonly mutated proteins found in cancer cells (An et al., 1997). Targeting of Hsp90 by geldanamycin results in a conformational change in mutant p53, with subsequent reduction in its stability (An et al., 1997).

- *Steroid hormone receptors*

Androgen and oestrogen are known to play pivotal roles in the progression of prostate and breast cancer respectively. Cellular receptors for these hormones also function in an Hsp90 dependent manner, thus, Hsp90 inhibitors may effectively inhibit the progression of these tumours (Miyata, 2005).

Unfortunately the application of geldanamycin as a clinical agent has been limited by its severe hepatic toxicity. Nonetheless, because of the promising anti-tumour properties of the compound, the development of biologically active, low toxicity derivatives has become an interesting and important endeavour (Schulte and Neckers, 1998). To try and understand the cause of such severe toxicity, proteome mining technology was employed. This confirmed that Hsp90 is a target for geldanamycin, but also demonstrated that the compound has a high affinity for the purine synthetase adenosine 2 (ADE2) (Dishman, 2002). Interestingly, exposure of the mouse proteome to a number of geldanamycin derivatives indicated that the group which elute only ADE2 had significant toxicity, demonstrating that the major side-effects associated with geldanamycin were likely caused by its ability to inhibit this purine synthetase (Dishman, 2002).

It has been established that a single side chain substitution can increase biological activity and stability, resulting in the derivative 17-allylamino-17-demethoxygeldanamycin (17-AAG), the structure of which is shown in Figure 1.6. 17-AAG and geldanamycin show almost equivalent Hsp90 inhibiting activity in cultured cells, while toxicity of 17-AAG in experimental animals and humans is significantly lower (Miyata, 2005). The geldanamycin derivative 17-AAG has been selected for further clinical development and Phase 1 clinical trials of intravenously administered 17-AAG were recently initiated (Agnew et al., 2001). Since then, the geldanamycin analogue 17-DMAG has also been found to be active on patient-derived tumour explants and is less metabolised (Janin, 2005). The results of initial Phase I and Phase II trials of these inhibitors should be available in the near future (Neckers and Neckers, 2005).



GA: R= CH_3O

17-AG: R= $H_2C=CHCH_2NH$

Figure 1.6: Comparison of wild-type geldanamycin with the synthetic derivative 17-AAG (Schulte and Neckers, 1998).

Janin, (2005), reported that close to 500 compounds related to geldanamycin have been developed, most being 17-aminated derivatives. Thus, it appears that the clinical success of geldanamycin will be dependent on chemically altered derivatives, with large quantities of natural geldanamycin required for chemical modification. Accordingly, process optimisation for the yield of high titres of geldanamycin from submerged fermentations of *Streptomyces hygroscopicus* var. *geldanus* remains an important focus of biotechnology research.

1.6.3 The Shikimate Pathway

In any discussion of secondary metabolism, one is mainly interested in the progress of carbon along the various pathways (Turner, 1973). Carbohydrate metabolism is traditionally divided into 3 phases: glycolysis, pyruvate metabolism and the tricarboxylic acid (TCA) cycle, and oxidative

phosphorylation (Berry, 1975). However, the catabolism of glucose does not only lead to the production of energy in the form of ATP, as the pathways involved also provide many precursor molecules for secondary metabolite production. The shikimate pathway, ubiquitous in micro-organisms and plants, represents a reaction sequence that proceeds from two precursors of carbohydrate biosynthesis, D-erythrose-4-phosphate (E4P) and phosphoenolpyruvate (PEP), through shikimate to aromatic compounds such as the aromatic amino acids, vitamin K, folic acid and ubiquitone (Wilson et al., 1998). A schematic representation of the shikimate pathway and the pathways of central metabolism is shown in Figure 1.7.

Aromatic metabolites such as tryptophan, phenylalanine, and tyrosine are essential amino acids for humans and animals (Patnaik and Liao, 1994). Their biosynthesis is generally very well regulated, as they are the most energetically expensive amino acids to synthesise (Euverink, 1995). Moreover, an interesting group of secondary metabolites containing aromatic rings are derived from intermediates of the aromatic amino acid biosynthesis pathway or indeed the aromatic amino acids themselves (Euverink, 1995). The shikimate pathway and variations therefore play a critical role in providing precursors such as amino acids and other aromatic acids and moieties for the biosynthesis of secondary metabolite products of medicinal importance, particularly in the bacterial genus *Streptomyces* spp. (Moore and Hertweck, 2002, Wilson et al., 1998, Stuart and Hunter, 1993).

Ansamycin antibiotics including geldanamycin contain a novel structural mC_7N unit, 3-amino-5-hydroxybenzoic acid (AHBA), which is biosynthetically derived from the common aromatic amino acid pathway (Moore and Hertweck, 2002). The AHBA pathway parallels the first three steps of the shikimate pathway, but is modified by introduction of glutamine-derived nitrogen at an early stage to give 3,4-dideoxy-4-amino-D-*arabino*-heptulosonic acid 7-phosphate (aminoDAHP) instead of the normal shikimate pathway intermediate, 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP) (Watanabe et al., 2003). It is speculated that AroAII type DAHP

synthases catalyse a critical step for the production of the precursor molecule AHBA (Gosset et al., 2001). These types apparently possess an altered substrate specificity in which either an aminated derivation of E4P is recognised or additional overall aminating ability exists. There is also evidence to suggest that a single isoenzyme exists significantly feedback inhibited by tryptophan (Stuart and Hunter, 1993).

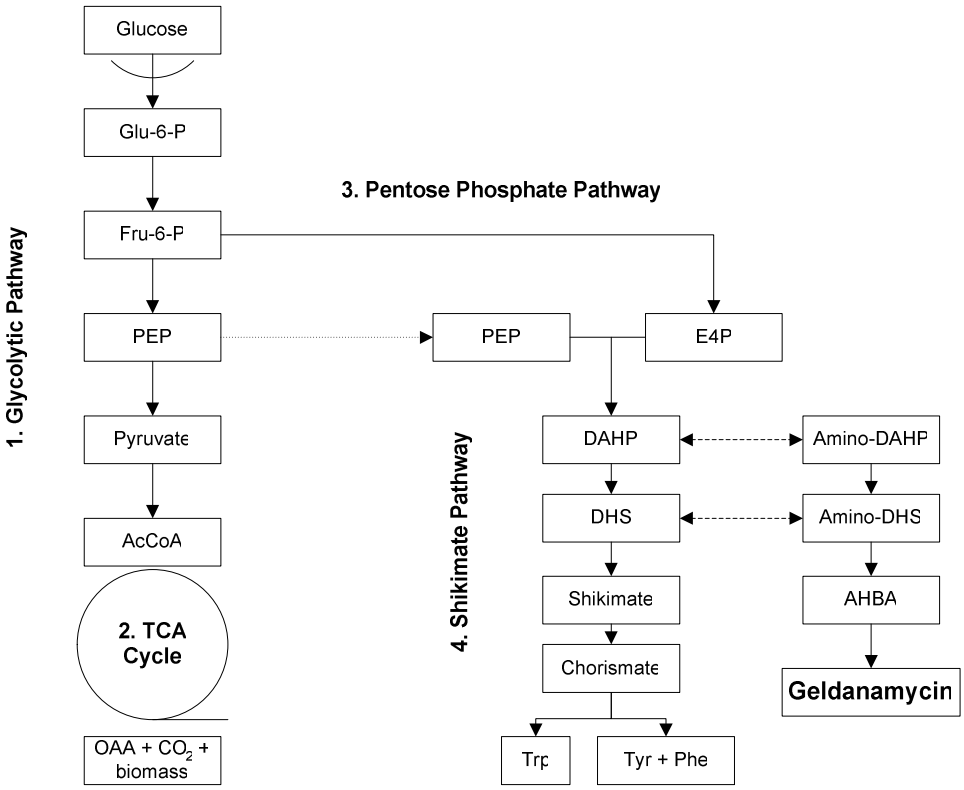


Figure 1.7: Schematic representation of the Glycolytic, TCA, Pentose Phosphate and Shikimate Pathways. Abbreviations: Glucose-6-phosphate (Glu-6-P); Fructose-6-phosphate (Fru-6-P); Phosphoenolpyruvate (PEP); Erythrose-4-phosphate (E4P); Acetyl Co-Enzyme A (AcCoA); Oxalo-Acetic Acid (OAA); D-arabino-heptulosonate 7-phosphate (DAHP); 5-deoxy-5-amino-3-dehydroshikimic acid (DHS); 3-amino-5-hydroxybenzoic acid (AHBA); Tyrosine (Tyr); Tryptophan (Trp); Phenylalanine (Phe).

1.7 Aims and Objectives

Streptomyces hygroscopicus var. *geldanus* represents an ideal system for investigation of the relationships between morphology, physiology and secondary metabolite production. It is a filamentous organism that may be induced to form diverse morphological types ranging from dispersed mycelial fragments to pelleted biomass. It produces a secondary metabolite, geldanamycin, which is of interest to clinical researchers as a novel chemotherapeutic agent. This compound is derived from the Shikimate pathway, a highly complex metabolic pathway whose regulation is of significant relevance for biosynthesis of geldanamycin.

The aim of this research is to provide a greater understanding of the mechanisms which control the synthesis of geldanamycin in *Streptomyces hygroscopicus* var. *geldanus*. In order to comprehensively and conclusively accomplish this objective, the following studies will be completed:

- **The optimisation of a quantitative assay for antibiotic determination**

High Performance Liquid Chromatography (HPLC) is one of the most widely used analytical techniques for identification and quantification of components within a sample. However, samples are rarely in a form that can be analysed directly and a number of treatment stages are often required. Indeed, as geldanamycin is only sparingly soluble in water-based liquid medium, an extraction protocol is currently utilised prior to HPLC analysis. Nonetheless, the impact of this procedure on sample stability must be examined in order to demonstrate that a reproducible measurement of geldanamycin in fermentation broth can be made.

- **The development of an optimised medium for antibiotic production**

The successful production of any secondary metabolite requires a detailed knowledge of the growth characteristics of the organism in question and the design of an appropriate production medium (Papagianni, 2004, Elibol, 2004).

Medium optimisation has been proven to increase the yields of actinorhodin by *Streptomyces coelicolor* almost 32% (Elibol, 2004); natamycin by *Streptomyces natalensis* (Farid et al., 2000); meilingmycin production by *Streptomyces nanchangensis* by 4-fold (Zhuang et al., 2006); penicillin G acylase by *Bacillus* spp. by 2-fold (Rajendhran et al., 2002); and bacteriocin production by *Lactococcus lactis*, also by 2-fold (Li et al., 2002). Despite this, there have been no references in literature on the effects of media formulation on geldanamycin synthesis since the initial studies in the 1970's (De Boer and Peterson, 1971, De Boer and Dietz, 1976).

Geldanamycin is currently produced commercially by Pfizer using the complex medium, PK1 (Short et al., 2003). PK1 medium contains a significant concentration of potato starch ($87.5gl^{-1}$), and thus requires time-consuming hydrolytic stages by bacterial amylases prior to fermentation. The medium also exhibits high viscosity which has reportedly led to problems with downstream processing and product recovery (Short et al., 2003). The initial objective of this study is to develop reproducible growth conditions using an optimised medium which eliminates the disadvantages of PK1.

- **The application of a suitable method for estimation of cell physiology**

In the quest for understanding the relationship between mycelial morphology and metabolite production the contribution from physiologists has increased gradually. Biochemical staining has allowed visualisation of cell lysis in the central core of pellets of *Streptomyces tendae* (Lawton et al., 1989) and *Streptomyces noursei* (Jonsbu et al., 2002); and in the empty parts or vacuoles of hyphae of *Penicillium chrysogenum* (Pons and Vivier, 1998) and *Streptomyces ambofaciens* (Pons et al., 1998). However, no quantitative studies of the metabolic activity of mycelial pellets in *Streptomyces* spp. have been made to date. The application of a quantitative method for the measurement of metabolic activity of mycelial pellets of *Streptomyces hygroscopicus* var. *geldanus* will provide a feasible tool for elucidation of the relationship between morphology, physiology and secondary metabolite production.

- **The impact of morphology on secondary metabolite production**

A comprehensive study of the factors influencing morphological development of *Streptomyces hygroscopicus* var. *geldanus* will be undertaken. The plethora of factors influencing the morphological development of filamentous organisms was discussed in Section 1.4 and a variety of microbiological and physiochemical factors will be exploited for this purpose. Whereas information on morphology is often obtained by visual inspection and microscopy, an image analysis method previously developed by O'Cleirigh et al., (2003), will be employed in this study allowing quantitative investigations to be made. The existence of a relationship or dependency between morphology and geldanamycin production in the organism *Streptomyces hygroscopicus* var. *geldanus* may then be established.

- **The impact of physiology on secondary metabolite production**

A quantitative assessment of metabolic activity in morphologically diverse cultures of *Streptomyces hygroscopicus* var. *geldanus* will be made using an appropriate assay. It will then be possible to ascertain the impact of morphology on physiology, and the implications of this with regard to production of the secondary metabolite geldanamycin.

Collectively, these studies will enable elucidation of the relationships between morphology, physiology and secondary metabolism. It is envisaged that, upon completion, the key driver of geldanamycin production in the filamentous organism *Streptomyces hygroscopicus* var. *geldanus* will be determined.

CHAPTER 2: MATERIALS AND METHODS

2.1 Bacterial strain

The organism used for all studies was *Streptomyces hygroscopicus* var. *geldanus* NRRL 3602 (ARS Patent Culture Collection, Peoria, IL, USA).

2.2 Media preparation

Recipes for media are shown below. In each case, glucose was always autoclaved separately and added later.

2.2.1 Bennett's medium

Bennett's medium contained the components listed in Table 2.1, resuspended in distilled water prior to autoclaving.

Table 2.1: Bennett's medium formulation.

Constituent	Manufacturer	Concentration (gl^{-1})
Yeast Extract	Oxoid	1
'Lab-Lemco' Beef Extract	Oxoid	1
NZ-Amine A	Sigma-Aldrich	2
Glucose Monohydrate	BDH	10

2.2.2 Bennett's medium (solid)

This medium was as listed in Table 2.1, along with the addition of $20gl^{-1}$ Oxoid Technical Agar No.3 (Basingstoke, England).

2.2.3 DeBoer and Dietz medium (D & D)

The DeBoer and Dietz medium contained the components listed in Table 2.2, resuspended in distilled water prior to autoclaving (De Boer and Dietz, 1976).

Table 2.2: DeBoer and Dietz medium formulation.

Constituent	Manufacturer	Concentration (gl^{-1})
Yeast Extract	Oxoid	2.5
Bacteriological Peptone	Oxoid	2.5
Tryptone	Oxoid	2.5
Oatmeal	Odlums	5
Molasses	Unknown	10
Glucose Monohydrate	BDH	40

2.2.4 GYM medium

GYM medium contained the components listed in Table 2.3, resuspended in distilled water prior to autoclaving.

Table 2.3: GYM medium formulation.

Constituent	Manufacturer	Concentration (gl^{-1})
Yeast Extract	Oxoid	2.5
Malt Extract	Oxoid	10
Calcium Carbonate	BDH	2
Glucose Monohydrate	BDH	4

2.2.5 GYM medium (solid)

This medium was as listed in Table 2.3, along with the addition of $20gl^{-1}$ Oxoid Technical Agar No.3 (Basingstoke, England).

2.2.6 YEPD medium

YEPD medium contained the components listed in Table 2.4, resuspended in distilled water prior to autoclaving.

Table 2.4: YEPD medium formulation.

Constituent	Manufacturer	Concentration (gl^{-1})
Yeast Extract	Oxoid	10
Bacteriological Peptone	Oxoid	20
Glucose Monohydrate	BDH	20

2.2.7 M2M medium

M2M medium contained the components listed in Table 2.5, resuspended in distilled water prior to autoclaving (Trejo-Estrada et al., 1998).

Table 2.5: M2M medium formulation.

Constituent	Manufacturer	Concentration (gl^{-1})
Casamino acids	Difco	5
$MgSO_4.7H_2O$	BDH	10
NaCl	BDH	5
KH_2PO_4	BDH	4
$CaCl_2.6H_2O$	BDH	0.05
$FeSO_4.7H_2O$	BDH	0.01
$MnSO_4.H_2O$	BDH	0.003
$ZnSO_4.7H_2O$	BDH	0.0044
$CuSO_4$	BDH	0.0025
$NaMoO_4.2H_2O$	BDH	0.00025
Glucose Monohydrate	BDH	20

2.2.8 YEME medium

YEME medium contained the components listed in Table 2.6, resuspended in distilled water prior to autoclaving.

Table 2.6: YEME medium formulation.

Constituent	Manufacturer	Concentration (gl^{-1})
Yeast Extract	Oxoid	3
Malt Extract	Oxoid	3
Bacteriological Peptone	Oxoid	5
Glucose Monohydrate	BDH	10

2.2.9 Resuspension solution

The resuspension solution contained the components listed in Table 2.7, resuspended in distilled water prior to autoclaving.

Table 2.7: Resuspension solution formulation.

Constituent	Manufacturer	Concentration (gl^{-1})
Yeast Extract	Oxoid	3
Bacteriological Peptone	Oxoid	5
$MgSO_4.7H_2O$	BDH	1

2.2.10 Ca^{2+}, Mg^{2+}, surfactants and glass beads

Ca^{2+} and Mg^{2+} were supplemented as sulphate ions (BDH Laboratory Supplies, Merck Chemicals Ltd.). The non-ionic surfactants Dow Corning 1510 silicone antifoam, Tween80 and TritonX100 were supplied by BDH Laboratory Supplies. The glass beads utilised were 5mm in diameter and supplied by Sigma Aldrich.

2.2.11 Autoclaving procedure

All media, both solid and liquid, were sterilised by autoclaving at 121^{o}C (15 psi) for 15 minutes in a Tomy SS-325 autoclave (Tomy Seiko Co., Ltd., Japan).

2.3 Spore generation

Streptomyces hygroscopicus var. *geldanus* was grown on GYM solid medium for spore generation. Sterile incubation of the organism on 100g of solid media in a 5L Erlenmeyer flask for a period of 21 days at 28^{o}C resulted in the production of aerial spores. A spore suspension was prepared by washing the surface of the solid media at 100rpm on an orbital shaker for 1 hour at 4^{o}C using 100ml of sterile resuspension solution. The resulting spore containing solution was enumerated following the procedure outlined in Section 2.4, aliquoted, and stored at –20^{o}C for use in subsequent experimental procedures.

2.4 Colony Forming Unit (CFU) test

The following procedure was employed to determine the amount of colony forming units (CFU) present in a given sample. The sample of interest was serially diluted 1 in 10 from neat to $1*10^{-6}$ in sequential steps in sterile distilled water to bring it within range for testing. Following this, 0.1ml of each of the diluted samples was spread on Bennett's solid medium in petri dishes in duplicate, and incubated at 28^{o}C for five days prior to enumeration.

2.5 Glucose assay

Residual glucose levels in the cultures were determined using a GOD-PAP glucose assay kit (Randox Laboratories Ltd., UK), modified for high throughput analysis using 96 microwell plates (Sarstedt, Ireland). Samples were diluted with distilled water to obtain a glucose concentration in the range 0–1gl^{-1}. Standard glucose concentrations in the same range were also prepared to generate a standard curve. Aliquots of 20µl diluted fermentation broth and standard curve samples were loaded onto a 96-well plate and 200µl of GOD-PAP reagent was added. Residual glucose was determined after enzymatic

oxidation in the presence of glucose oxidase, the GOD-PAP reagent. The hydrogen peroxide formed reacts to form a red-violet dye as an indicator, the intensity of which was measured after 25 minutes at 492nm using a 96-well Plate Reader (Tecan, Switzerland). The glucose concentration was then extrapolated using a glucose standard curve (Appendix A).

2.6 Harvesting and Dry Cell Weight (DCW) determination

Biomass accumulation was estimated using dry weight analysis determined by the following method. Samples of fermentation broth (20ml) were placed in a plastic universal and subjected to two centrifugation cycles at 3500rpm. After the first centrifugation step, the supernatant was removed and the pellet was resuspended in distilled water to a final volume of 20ml. After the second centrifugation step the supernatant was again removed and the pellet was transferred to a pre-weighed glass universal. The glass universal was then dried to constant weight in a 104°C oven for 24 hours, removed and allowed to cool in a desiccator to ensure it was free from additional moisture derived weight. The universal was then re-weighed and the biomass concentration was determined using the following equation:

Biomass Concentration (gl^{-1}) = [(Final weight- Initial weight)/20] * 1000

2.7 Viscosity determination

The determination of the apparent viscosity of fermentation broths was achieved using a Brookfield Rotational DV-I+ Cone and Plate Viscometer (Brookfield, USA) at 20°C. The viscometer facilitated the calculation of the apparent viscosity over a wide range of shear rates. According to flow behaviour, fluids may be divided into:

1. Newtonian fluids which obey Newton's law of viscosity:

$$\tau = \mu\gamma \qquad \text{Equation 2.1}$$

where μ is the dynamic viscosity (Nsm^{-2}), τ is the shear stress (Nm^{-2}) and γ is the shear rate (s^{-1}).

2. Non-Newtonian fluids, the most common of which obey the power law:

$$\tau = k\,\gamma^{n} \qquad \text{Equation 2.2}$$

where k is the fluid consistency index ($Ns^{n}m^{-2}$) and n the flow behaviour index (no units). An apparent viscosity at any shear rate can be calculated by

$$\mu_{app} = \frac{\tau}{\gamma} \qquad \text{Equation 2.3}$$

Figure 2.1 examines the typical flow curves for Newtonian and non-Newtonian time independent fluids.

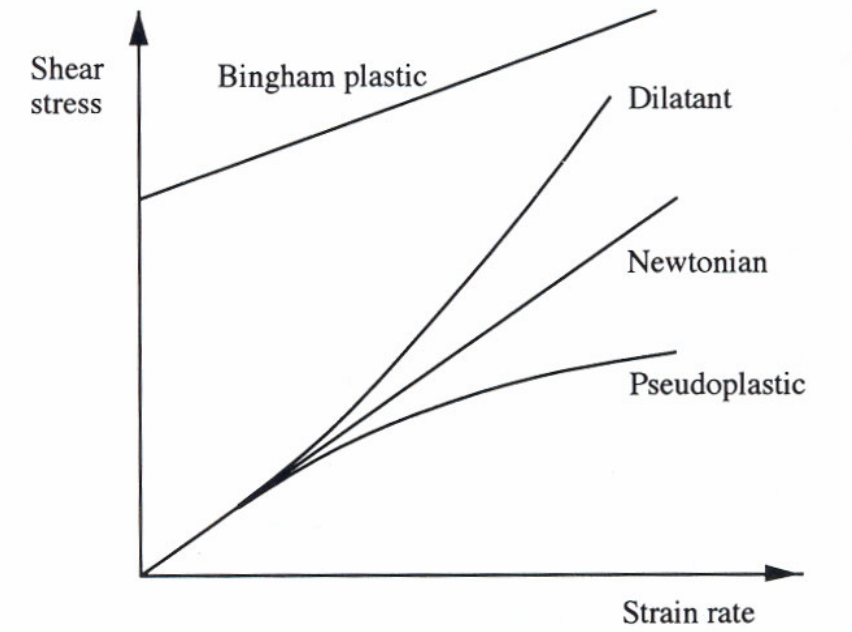


Figure 2.1: Stress/strain behaviour of various fluids (Wilkes, 1999).

2.8 Surface tension measurement

Broth surface tension was determined using a "Model OS" Tensiometer and the Du Nouy ring method (White Electrical Instrument Co., UK). The tensiometer incorporates a precision microbalance connected to a platinum ring with defined geometry and a clock glass on a stage that can be precisely vertically manoeuvred. The platinum ring hanging from the balance hook is first immersed into 0.5ml liquid and then carefully pulled up, by lowering the clock glass, through the surface of the liquid until the two detach. The surface tension is the maximum force (N/m) needed to detach the ring from the liquid surface and may be read directly from the gauge.

2.9 The development of a quantitative assay for geldanamycin detection

2.9.1 High Performance Liquid Chromatography (HPLC)

De Boer et al., (1970), determined geldanamycin concentration using a standard disc plate agar diffusion assay. Antibiotic activity was expressed in bio-units, where one bio-unit equalled the amount of antibiotic necessary to produce a 20mm zone of inhibition under standard conditions. However, calculation of inhibition zones can be subjective, particularly where in-growth of the organism occurs; time consuming; material intensive; and, indeed, such methods are often more qualitative than quantitative. Casey et al., (2004), endeavoured to evade these limitations by the development of a high-throughput micro-titre plate-based assay for detection of geldanamycin bioactivity. Nonetheless, growth retardation of test organisms could not be exclusively attributed to the bioactivity of geldanamycin, as the method was not compound-specific.

Casey et al., (2006), subsequently developed a specific method for the analysis and quantification of geldanamycin, based on the High Performance Liquid Chromatography (HPLC) method described by Agnew et al., (2001), for detection of the geldanamycin derivative 17-AAG. HPLC is unquestionably one of the most widely used analytical techniques for identification and quantification of components within a sample. The separation relies on the use of two different phases, one of which is stationary (the column) and the other which moves over it (the mobile phase). Separation occurs because, under an optimal set of conditions, each component in a mixture will interact with the two phases differently relative to the other components in the mixture. The most popular classification scheme stems from the manner in which the analyte reacts with the stationary phase i.e. the mechanism of retention. The HPLC system employed for sample assessment was a Merck-Hitachi LACHROM 7000-series HPLC system, comprised of a D-7000 interface device, L-7200

auto-sampler, L-7400 U.V. detector and a L-7100 pump system (Hitachi Ltd., Japan). The HPLC column utilised was a reverse phase C18 Kingsorb 5µm column (Phenomenex, UK) with mobile phase comprised of acetonitrile and ultrafiltered H_2O (50:50 v/v). Samples were delivered at a flowrate of 1ml/min for a run time of 20 minutes, with the geldanamycin peak eluted at approximately 10 minutes. Geldanamycin U.V. detection was achieved at 308nm and its concentration was determined using a standard curve prepared with commercially available preparations of the antibiotic (Fluorochem, UK) (Appendix A).

2.9.2 Geldanamycin extraction protocol

When analysing samples by HPLC, they are rarely in a form that can be injected directly onto the column, and some form of preparation is usually required. Geldanamycin is only slightly soluble in water but may be extracted from filtered broth using water-immiscible solvents in which it is soluble, for example butanol (De Boer and Peterson, 1971). Pre-processing steps such as liquid–liquid extraction also serve to reduce the amount of non-specific contaminant material within the sample and prevent column fouling. Column fouling is characterised by increases in column pressure and reduced performance due to deposition of particulate material or non-elution of compounds from the column and can result in reduced life-span of the column.

Following HPLC analysis of v/v butanol contacted and non-extracted broth supernatant, it was observed that the liquid-liquid extraction step removed a significant amount of poorly retained non-specific material from the samples (Figure 2.2). Such less-hydrophobic contaminant material refers to all compounds other than the one of interest, geldanamycin, and may include fermentation by-products, proteins, minerals, vitamins and carbohydrates. The extraction process also served to increase the sample purity from 34% to 84% geldanamycin, reducing the amount of contaminant matter entering the column.

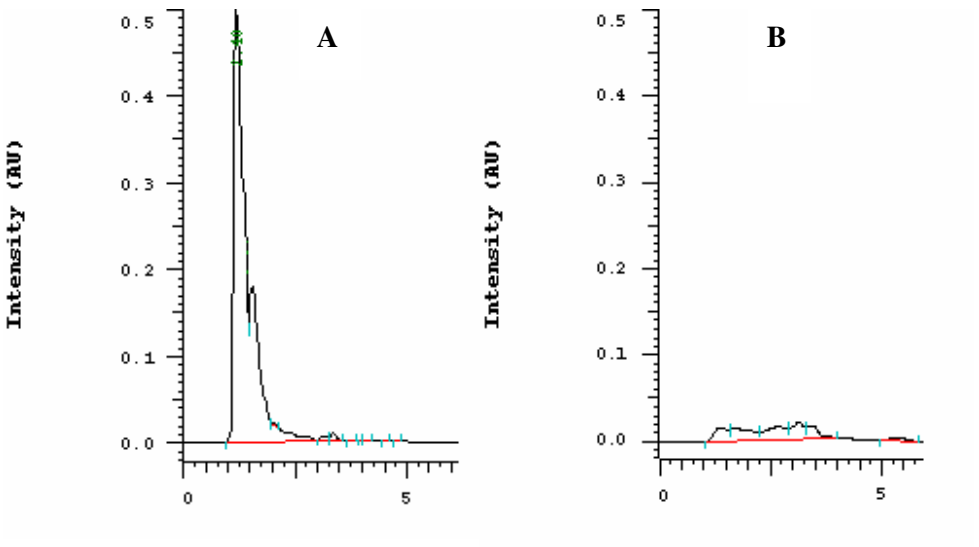


Figure 2.2: Chromatograms of non-extracted (A) versus extracted (B) crude broth samples, highlighting the removal of non-specific material from the samples.

Nevertheless, butanol was found to be incompatible with the HPLC system, leading to poorly resolved peaks (Casey, 2006). Accordingly, a low pressure evaporation step was required to remove the butanol from samples prior to HPLC assessment. Butanol evaporation was effected by heating the sample in a pressurised silicone oil bath at 40°C for 40 minutes. The dried samples were then resuspended in acetonitrile (Lennox Chemicals Ltd., Ireland), filtered using a 0.2µm nylon filter (VWR International, U.K.) into a vial and analysed by HPLC. However, geldanamycin is a heat-labile compound, known to readily decompose with exposure to high temperature (De Boer et al., 1970). Some degradation of the compound was anticipated due to its exposure to temperatures of over 40˚C for a period of 40 minutes. In order to determine any losses incurred due to the heating step in the extraction process, three samples containing different concentrations of geldanamycin were prepared and analysed both prior and subsequent to evaporation (Figure 2.3). A substantial loss of the compound of interest was observed over the concentration range, averaging at 21% which was deemed unacceptable.

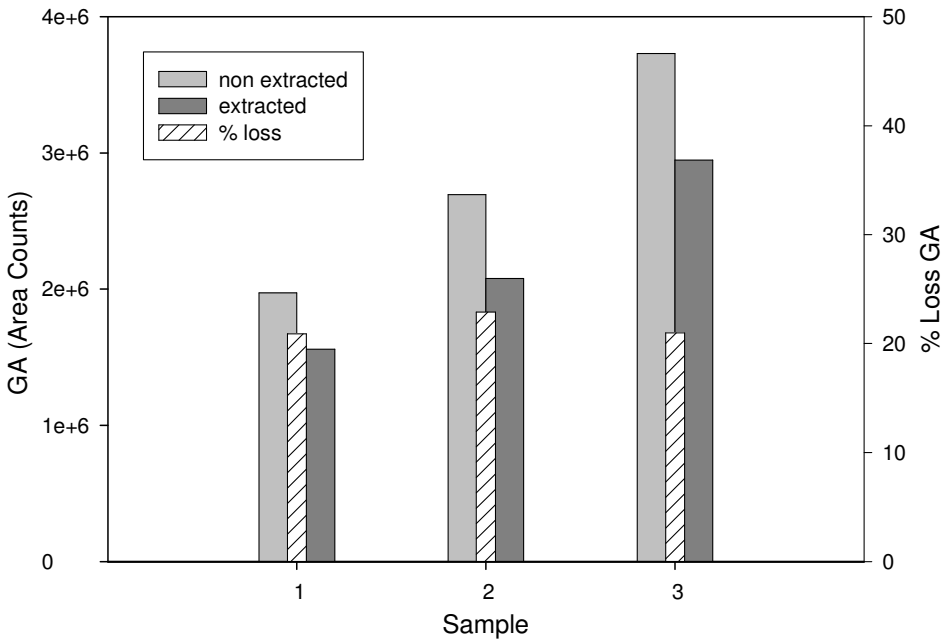


Figure 2.3: Degradation of geldanamycin in three separate samples following liquid-liquid extraction.

There have been many reports documenting the poor solubility of geldanamycin and its derivatives in water and water based media. As butanol extraction was no longer deemed suitable, the identification of a similar solvent which could simultaneously be utilised as a diluent and aid in the extraction of geldanamycin from the fermentation broth was required. DeBoer and Peterson, (1971), and DeBoer et al., (1970), had previously identified that geldanamycin is soluble in lower-alkalones such as acetonitrile, which is less-polar than butanol but miscible in water and therefore highly compatible with the HPLC protocol. The effects of broth supernatant dilution in acetonitrile (100%) are shown in Figure 2.4.

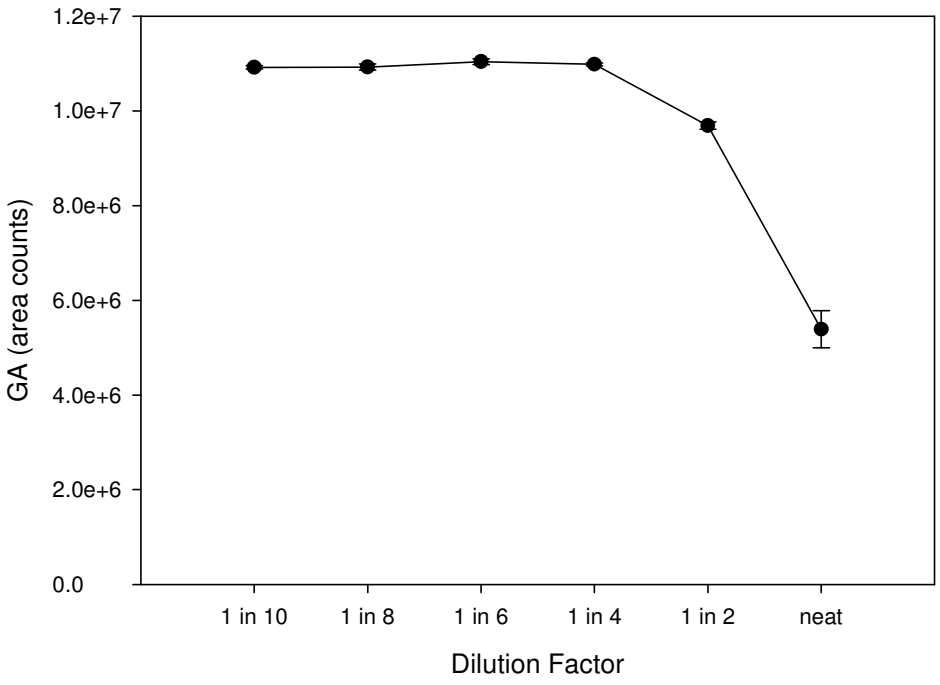


Figure 2.4: Effect of broth supernatant dilution in acetonitrile on geldanamycin signal linearity and reproducibility.

Figure 2.4 confirms that geldanamycin is only slightly soluble in water, and analysis of neat broth samples will underestimate the amount of the compound present by approximately 52%. However, solubility is increased by the inclusion of acetonitrile with a linear signal return observed. Diluting fermentation broth in acetonitrile thus increases the reproducibility of the detection method, ensuring a high degree of consistency when compared to the analysis of neat samples.

Subsequent to dilution in acetonitrile, samples were filtered using a 0.2µm nylon filter prior to HPLC analysis as an additional aid to remove particulate matter and prevent column fouling. However the poor solubility of geldanamycin raised the possibility that a quantity may be retained by the filter. It was determined that such losses were also limited by increasing the amount of acetonitrile present in each sample (Figure 2.5).

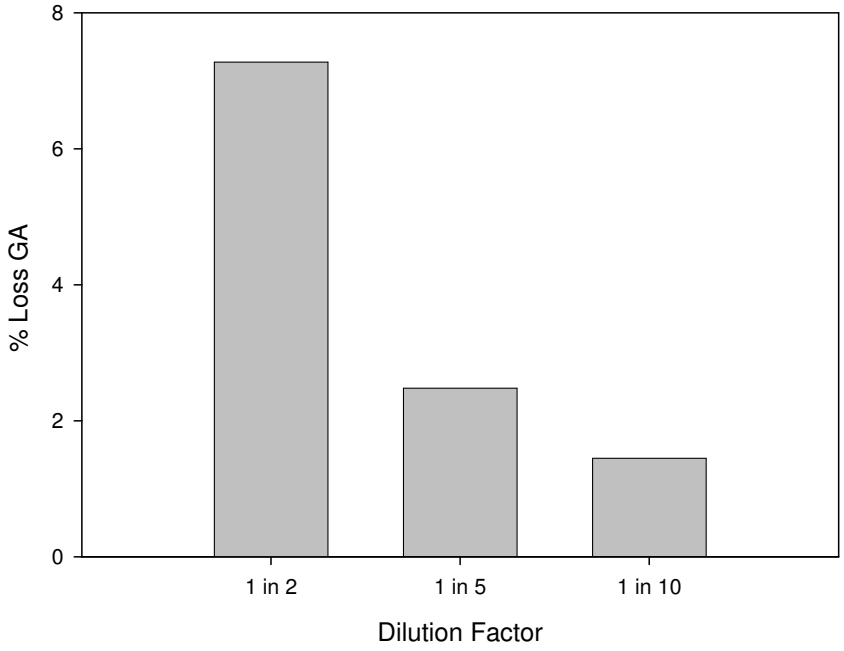


Figure 2.5: Effect of acetonitrile dilution on the loss of geldanamycin by filtration.

In general, secondary metabolites enter the extracellular milieu as they are formed, although this depends on the secretory capacity of the organism. Despite the fact that usually more than 85% of the total yield accumulates in culture supernatants (Weinberg, 1970) the liquid stream is more commonly than not the only phase examined in the detection of fermentation products. However in some instances product may be entrapped or immobilised; retained intracellularly by association with cell walls and membranes; or may be insoluble in the fermentation broth, a phenomenon exhibited by geldanamycin. Thus the benefits of treating the solid phase on the extraction of geldanamycin were examined (Table 2.8).

Table 2.8: Effect of sample treatment on the extraction of geldanamycin from cultures of *Streptomyces hygroscopicus* var. *geldanus*.

Sample Treatment	GA (gl^{-1})	% Increase
Supernatant	0.1504	------
Crude Broth	0.1564	3.99
Crude Broth + Sonication	0.1722	14.49

Sonication is often used to disrupt cell membranes by exposure to high frequency sound waves supporting the release of intracellular material. Indeed, Lindemann, (2000), found that sonication was advantageous in the extraction of heavy metals from different cell types. Sonication was determined to be of great benefit in the extraction of geldanamycin from crude broth, with an increase in yield of almost 15% observed (Table 2.8). This figure correlates well with the estimations of cell-associated product made by Weinberg, (1970).

Following the identification of appropriate sample preparation parameters, the stability of geldanamycin with respect to time was determined. Signal stability was monitored in a sample containing $0.1gl^{-1}$ geldanamycin at room temperature by periodic sampling. Negligible losses were recorded over the first 20 hours; however a 27% loss of geldanamycin was detected at 72 hours (Figure 2.6).

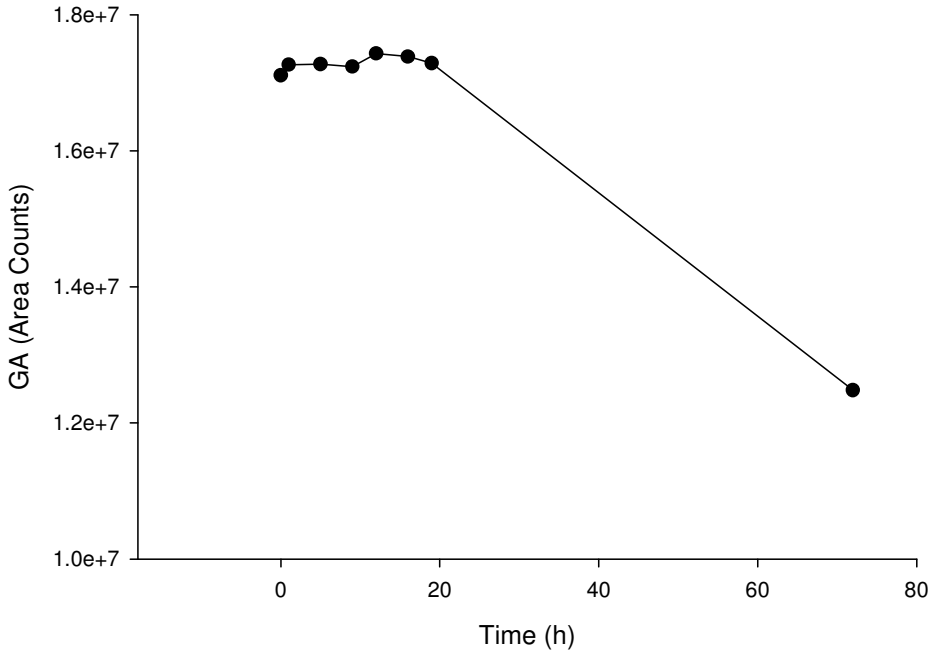


Figure 2.6: The stability of geldanamycin over time following sample preparation.

The observations made regarding the extraction, solubility and stability of geldanamycin dictated an amendment of the quantification protocol. The revised procedure eliminates the butanol extraction step and introduces the following stages prior to filtration:

- Dilution of crude fermentation samples with acetonitrile 100% v/v 1:9 to ensure solubility of geldanamycin
- Vortex and sonication of samples for a period of 15 minutes to include cell associated product

Subsequent to preparation, the samples should remain stable for a period of 20 hours, although it is recommended that analysis be performed as soon as possible.

2.10 Image analysis technique

The morphology of cultures was quantified using an image analysis method adapted from that developed by O'Cleirigh et al., (2003), and described below.

2.10.1 Image analysis hardware/ software

The image analysis system used was a Dell Inspiron 6400 with an Intel Centrino Duo processor (Dell Computer Corporation). A HP Scanjet 5590 flatbed scanner (Hewlett Packard, U.S.A.) was used for image capture. The image analysis software used was Optimas 6.5® (Media Cybernetics Inc., U.S.A.). The code for the image analysis algorithm was developed by O'Cleirigh et al., (2003), and is given in Appendix B. The statistical analysis software utilised was SigmaPlot 8.0 (SPSS Inc., U.S.A.).

2.10.2 Fermentation sample preparation

For the purpose of staining, 60µl of Safranin O (1% w/v) (BDH Laboratory Supplies), an organic stain, was used to stain 10ml samples of fermentation broth. The mixture was gently rotated for 20 minutes to ensure homogeneous dye uptake. Excess stain was removed by three sequential centrifugation steps at 2000rpm for 10 minutes, followed by removal of supernatant and replacement with distilled water. Samples were subsequently homogenised by gentle vortexing and diluted with distilled water as required. Aliquots of 10ml were then transferred into petri-dishes for image capture and analysis.

2.10.3 Image capture and analysis

The petri-dish containing the 10ml aliquot of the stained sample was placed on the glass surface of the flatbed scanner and gently swirled to ensure even distribution of biomass. The dish was covered with a white plastic tray to

exclude external light and also to provide contrast for the sample. Images were then captured using the flatbed scanner at a high resolution of 1200dpi. The resulting image was saved as a tagged image file format (tiff) for subsequent image analysis. The images were processed and parameters such as mean pellet volume and count, frequency distributions and mean pellet area equivalent diameter were extracted. The pellet area equivalent diameter is a derived measurement, based on the measured cross sectional area of individual particles. This measurement, as calculated by Optimas, is taken to be a good representation of the pellet diameter as both the microbial pellets and the calibration particles (see below) were approximately spherical.

2.10.4 Assay validation

The assay was validated on the Scanjet 5590 flatbed scanner with 109μm ± 5μm and 644μm ± 13μm diameter red spherical particles (Duke Scientific Corporation, U.S.A.) used for calibration of the image analysis system. Pre-weighed samples containing a mixture of the two were used for this procedure and the data from each particle set were separated and subjected to separate histogram analysis. Figure 2.7 contains the sample histograms, overlaid with Gaussian distributions of the calibration particles derived from the manufacturers mean and standard deviation. The combined sample consisted of defined masses of both sizes, which in conjunction with the total measured particle volume facilitated the estimation of the material density. Table 2.9 demonstrates that the deviation of the experimentally measured density from the known density of polystyrene divinylbenzene ($1.05 gcm^{-3}$) is 4.5%.

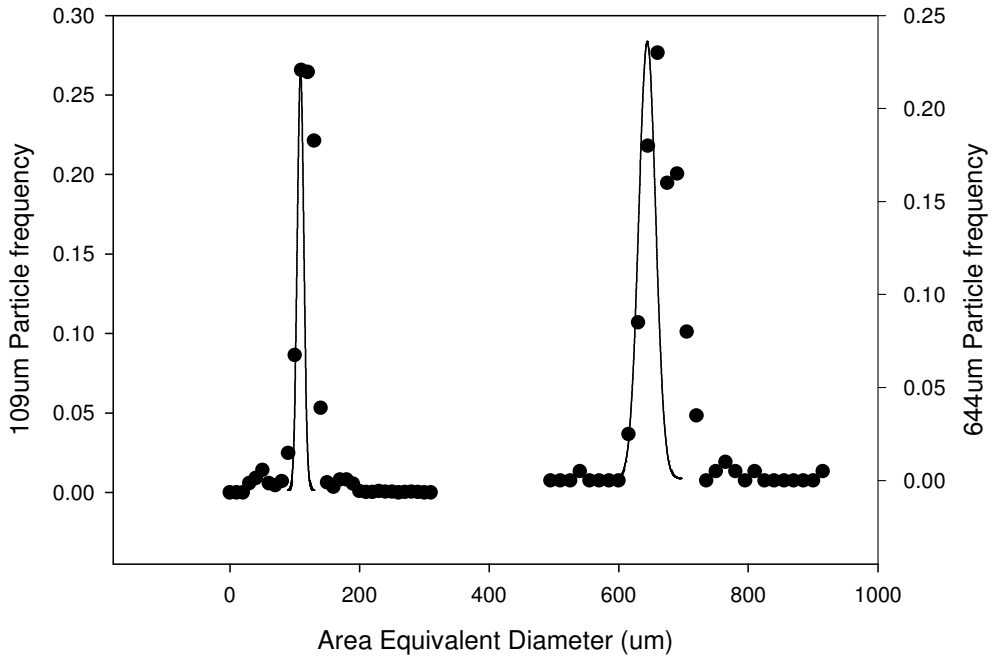


Figure 2.7: Measured area equivalent histogram for 109µm and 644µm combined sample overlaid with Gaussian distribution.

Table 2.9: Morphological characteristics of 109µm and 644µm size calibration particles.

Particle count (ml^{-1})	Measured volume of particles (cm^3)	Mass of particles (g)	Experimentally determined density (g/cm^3)	Polystyrene density (g/cm^3)	Density deviation (%)
3289	0.0368	0.0369	1.003	1.05	-4.5

2.11 pH measurement

pH was measured using a WTW pH 583 meter.

2.12 Cell Surface Hydrophobicity (CSH) measurement

Microbial cell surface hydrophobicity (CSH) is one of the most studied properties of the cell surface, playing a major role in the manner in which the cell interacts with other cells or with solid surfaces (Kiely et al., 1997, van der Mei et al., 1998). Measurement of bacterial hydrophobicity has been of great importance in many research areas, including: the association and stability of bacteria in granulated sludge reactors (Thaveesri et al., 1995); the formation of microbial biofilms (Briandet et al., 2001); the interaction of pathogenic bacteria with mammalian cells in immunocompromised patients (Miorner et al., 1982, Glee et al., 1995); the adhesion of probiotic lactic acid bacteria to the gastrointestinal epithelium (Schar-Zammaretti et al., 2005, Vadillo-Rodriguez et al., 2004); and the interaction of oral bacteria suspended in saliva with human enamel in dentistry (Busscher et al., 1984).

Adhesion-based CSH assays such as bacterial adhesion to hydrocarbons (BATH) and hydrophobic interaction chromatography (HIC) essentially probe an interplay of all physio-chemical and structural factors involved in microbial adhesion rather than one single factor, the CSH (van der Mei et al., 1998). Indeed, Zita and Hermansson, (1997), postulated that inconsistencies between the two methods used to evaluate a specific bacterial strain were due to the fact that BATH evaluates the overall surface charge, whereas the HIC method more than likely measures localised hydrophobicity. Both assays are also susceptible to gravitational forces, and entrapment of bacterial cells in chromatographic gels can produce erroneous results. Ahimou et al., (2001), assessed the hydrophobicity of *Bacillus subtilis* by water contact angle measurement, HIC, and BATH. Their results confirmed on a statistical basis that both HIC and BATH are affected by electrostatic interactions but that water contact angle revealed surface hydrophobicity without such interference, providing a better estimate of CSH (Ahimou et al., 2001). Contact angle measurements have thus been nominated as the standard assay for determination of microbial cell surface hydrophobicity (Ahimou et al., 2001, van der Mei et al., 1998, van Loosdrecht et al., 1987).

Contact angle measurement has been utilised to determine the CSH of many different bacteria, including: *Streptococcus* spp. (Busscher et al., 1984); *Lactobacillus* spp. (Vadillo-Rodriguez et al., 2004); *Bacillus subtilis* (Ahimou et al., 2001); *Pseudomonas* spp. (van Loosdrecht et al., 1987); *Escherichia coli* (van Loosdrecht et al., 1987) and *Streptomyces tendae* (Vecht-Lifshitz et al., 1989). The measurement is typically carried out by depositing a layer of bacterial cells on a membrane filter, and measuring the contact angle of a drop of a diagnostic liquid on the bacterial filter cake with a goniometer (Pembrey et al., 1999).

In this study, following batch fermentation, 60ml culture samples were centrifuged at 4000rpm for 10 minutes and the supernatant was removed. The biomass pellet was recovered, washed in distilled water and subsequently re-centrifuged. Using a dead-end filtration apparatus at 2psi, bacterial filter cakes were prepared on 1.2µm filter paper and allowed to dry overnight to ensure only surface-bound water was present. The apparatus utilised to measure the CSH was a FTA200 goniometer which consisted of specimen stage, syringe pump, camera and associated data retrieval software (First Ten Angstroms, US). The contact angle of a 200µl drop of distilled water was then measured at a minimum of five different locations on the bacterial filter cake. Contact angles thus represent mean values of 10 measurements, equally distributed over 2 bacterial lawns prepared from duplicate bacterial cultures. According to the water contact angle, cell hydrophobicity may be roughly classified into two categories: hydrophobic or hydrophilic. If water molecules have a greater tendency to surround each other than to contact to a bacterial cell surface, the surface appears hydrophobic and water droplets do not spread; if water molecules favour a microbial cell surface rather than each other, the surface appears hydrophilic (Vadillo-Rodriguez et al., 2004). Thus, the greater the contact angle, the greater the hydrophobicity of the cell surface. A contact angle of 45° has been arbitrarily assumed as the cut-off between hydrophilicity and hydrophobicity (Thaveesri et al., 1995).

2.13 The application of a quantitative assay for the estimation of metabolic activity

The application of inexpensive, reliable and quantitative methods to detect the viable fraction of micro-organisms in complex environments is of great interest in a variety of research domains, ranging from the assessment of microbial diversity to monitoring pathogen presence (Keer and Birch, 2003); and from microbial ecology (Nielsen *et al.*, 2003b) to public health microbiology (Chaiyanan et al., 2001). The viability of micro-organisms can be influenced by a great number of factors. Strain dependency (Kuhn et al., 2003); substrate limitation (Rodriguez et al., 1992); heat (Yaqub et al., 2004); culture age (Mauss et al., 1997); salinity (Caro et al., 1999); and morphology (Kuhn et al., 2002, Jonsbu et al., 2002) have all been demonstrated to affect metabolic activity.

Classical methods for the determination of bacterial viability rely on the ability of cells to actively grow and form visible colonies on solid media (Keer and Birch, 2003). However the enumeration of colony forming units (CFU) incurs significant disadvantages. At the outset the assay is time consuming (because of lengthy incubation periods) and moreover the bacteria are removed from native samples and are no longer subject to inhibitory substances or situations occurring *in-situ* (Rodriguez et al., 1992). In addition, under some circumstances, the number of viable organisms may be severely underrepresented due to the presence of viable but non-culturable (VBNC) cells.

Measurements based on capacitance, microcalorimetry, oxygen (O_2) consumption rates and carbon dioxide (CO_2) production may also be used to estimate the metabolic activity of micro-organisms. Capacitance is a measure of the electrical charge for a given potential and can be used to estimate viable microbial cells due to the presence of the plasma membrane which behaves as a capacitor (Mas et al., 2001). Capacitance readings have been shown to give

an accurate description of the biomass profile when compared to determinations by standard techniques e.g. dry cell weight (Neves et al., 2001). Microcalorimetry monitors the metabolic heat produced by cells during respiration and can generally be estimated to within 10% from oxygen uptake data (Bader, 1986). Indeed, O_2 consumption rates are a good measure of catabolism since the bulk of the oxygen consumed is respired (Braeckman et al., 2002). It is less clear what CO_2 production rates measure as the amount of CO_2 produced by respiration will depend on the nature of the metabolised substrate (Braeckman et al., 2002). However, a precise evaluation of each of these parameters *in-situ* requires the use of expensive and sophisticated probes and is therefore not amenable for the simultaneous derivation of physiological data in multiple fermentations.

2.13.1 Fluorochrome $DiOC_6$

Metabolic activity and proliferation depend on an intact cytoplasmic membrane which separates the cell from its environment (Nebe-von-Caron et al., 2000). This plays a critical role in the transportation of ions and metabolites both into and from the cell. Cells without an intact membrane cannot maintain the electrochemical gradient which generates membrane potential and can be classified as dead cells: as their internal structures are freely exposed to the environment they will eventually decompose (Nebe-von-Caron et al., 2000). One of the major classes of membrane potential dyes are the carbocyanines, with 3,3-dihexyloxocarbocyanine ($DiOC_6$) the most commonly used in physiology assays (Laflamme et al., 2005, Penyige et al., 2002, Agger et al., 1998).

Laflamme et al., (2005), previously demonstrated that $DiOC_6$ could rapidly predict the germination of *Bacillus* spores. Penyige et al., (2002), also utilised $DiOC_6$ to establish the mechanism of action of β-lactam antibiotics such as penicillin G and gramicidin D on cultures of *Streptomyces griseus*. In addition, Agger et al., (1998), successfully used $DiOC_6$ to stain the cell wall and organelles of *Aspergillus oryzae* in order to detect the active region of hyphae.

This facilitated the development of a morphologically structured model to describe growth and product formation of the organism. Thus initial attempts were made to quantify the physiology of cultures of *Streptomyces hygroscopicus* var. *geldanus* on the basis of membrane integrity using the fluorochrome $DiOC_6$.

A stock solution of 3,3-dihexyloxocarbocyanine ($DiOC_6$) (Sigma-Aldrich) was prepared by dissolving in dimethyl sulphoxide (DMSO) (Lennox, Ireland) to the appropriate concentration. Cell suspensions (1ml) were then incubated with the dye (100µl) at 28°C and 150rpm. A 10 minute incubation time was required for $DiOC_6$, and samples were then placed on microscopic slides and visualised using fluorescence microscopy at wavelength excitation:emission 483:501nm.

The metabolically active region of cultures of *Streptomyces hygroscopicus* var. *geldanus* was clearly visible following fluorescent staining with $DiOC_6$ (Figure 2.8 A).

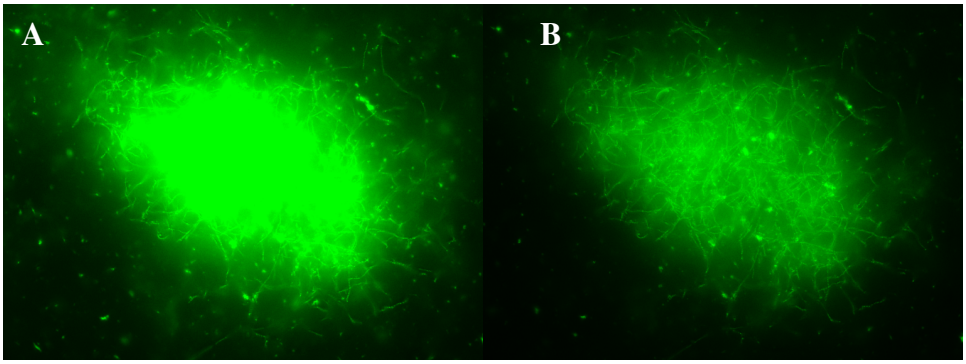


Figure 2.8: Analysis of metabolic activity using fluorochrome $DiOC_6$ (10µM) and the effects of photobleaching on fluorescence intensity A: Initial image capture; B: Image capture following one minute exposure to excitation light source (200x magnification).

However, while observing the interaction of light and dye, the fluorochrome was found to readily photobleach during microscopy, due to exposure to high

energy excitation light (Figure 2.8 B). Such photodynamic damage has previously been reported (Terasaki, 1989). This characteristic could present a serious problem in the evaluation of viable cells and could lead to the phenomenon of false negative scoring if the lack of fluorescence was incorrectly attributed to the presence of non-viable cells rather than the destruction of the fluorochrome. Accordingly, the application of a more suitable biochemical test to measure metabolic activity was required.

In recent times molecular methods have been utilised for the assessment of bacterial viability. These rely on the analysis of DNA and RNA by PCR and RT-PCR respectively. Nonetheless, due to the variable persistence of nucleic acids in cells post death the correlation between the presence of DNA, RNA and viability is not clear–cut (Keer and Birch, 2003). Another marker used for the estimation of cellular activity is bioluminescence, emitted in a reaction with adenosine triphosphate (ATP), catalysed by luciferase enzymes. ATP is commonly referred to as the universal energy currency of cells, providing energy for biosynthesis reactions. Thus luciferase activity is linked with cellular physiology and viability as the reaction depends on the ATP concentration in the cell (Billard and DuBow, 1998). However, the measurement of intracellular ATP requires a preceding extraction step, for example with tris-EDTA buffer (Gikas and Livingston, 1993). Moreover, energy can also be stored in the form of ADP and AMP therefore the quantification of ATP alone is said to be unrepresentative of the energy state of the cell (Rakotonirainy et al., 2003).

Aerobic organisms utilise a series of oxidation-reduction (redox) reactions known as the electron transport chain to convert energy into ATP. Redox reactions are chemical reactions in which electrons are transferred from a high energy molecule (donor) to a lower energy molecule (acceptor) producing energy in the form of a proton gradient which, in the electron transport chain, is used to generate ATP. In chemotrophic organisms NADH and succinate are the electron donors, entering the chain via NADH dehydrogenase or succinate dehydrogenase, and in aerobic environments the terminal electron acceptor is

O_2. The estimation of electron transport activity using artificial electron acceptors such as redox dyes that can successfully compete with oxygen for electrons is regarded as a suitable measure of metabolic activity (McCluskey et al., 2005).

2.13.2 Tetrazolium salt CTC

Tetrazolium salts are widely utilised to measure the redox potential of cells based on their reduction by dehydrogenase enzymes in the electron transport chain of metabolically active cells to coloured formazan derivatives (Braeckman et al., 2002, Roehm et al., 1991). Tetrazolium salts are heterocyclic organic compounds that substitute the natural final acceptor (oxygen) in the biological redox process (Meletiadis et al., 2001). Thus they are not stains *per se* but are transformed by reduction into red, water-insoluble crystals (formazan crystals). So, while tetrazolium salts are water-soluble due to the ionisation of the tetrazolium ring, when the ring is opened by reduction this charge is lost leading to an uncharged water-insoluble formazan salt (Berridge et al., 2005). Healthy cells respiring via the electron transport chain will absorb and reduce tetrazolium salts into coloured formazan products. Cells not respiring or respiring at slower rates will reduce less compound, giving a quantitative estimate of metabolic activity.

Tetrazolium salts have become some of the most widely used tools in cell biology for measuring the metabolic activity of cells ranging from mammalian to microbial origin (Berridge et al., 2005). Indeed, it is not uncommon for over 350 reports quoting reduction of tetrazolium salts to be published annually, for example 373 in 1995 and 354 in 1999 (medline) (Bernas and Dobrucki, 2000). 5-Cyano-2,3-ditolyl tetrazolium chloride (CTC) is an example of a tetrazolium salt that has been used by many scientists to evaluate the respiratory activity of numerous bacterial populations (Chaveerach et al., 2003, Rodriguez et al., 1992, Schwartz et al., 2003). The large crystals which are formed are relatively insensitive to light but are easily observed by microscopy (Mauss et al., 1997).

The reduction of CTC by *Streptomyces hygroscopicus* var. *geldanus* as a method to characterise the physiology of the cells was investigated.

A stock solution of 5-Cyano-2,3-ditolyl tetrazolium chloride (CTC) (Sigma-Aldrich) to the appropriate concentration was made in distilled water. Cell suspensions (1ml) were then incubated with the dye (100µl) at 28°C and 150rpm. A 3 hour incubation time was required for CTC, and samples were then placed on microscopic slides and visualised using fluorescence microscopy at wavelength excitation:emission 430:600nm.

It was observed that *Streptomyces hygroscopicus* var. *geldanus* reduced CTC into an insoluble fluorescent formazan salt (Figure 2.9). The deposition of the insoluble formazan product intracellularly enabled identification of the site of reduction and illustrated that a peripheral area of biomass at the pellet surface exhibited high metabolic activity.

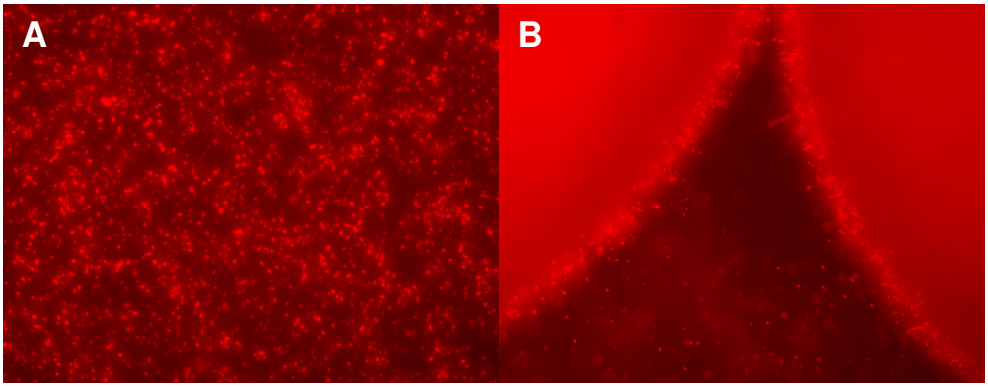


Figure 2.9: Determination of metabolic activity using the tetrazolium salt CTC (0.5mM). A: Dispersed culture; B: Pelleted culture (200x magnification).

However, while the insolubility of the formazan salt proved advantageous for the differentiation of active and inactive regions of cells, it complicated any quantitative measure of metabolic activity. Indeed, Mauss et al., (1997), studied respiration activity in *Streptomyces ambofaciens* spiramycin producing strains using a similar insoluble tetrazolium salt. In order to develop a

quantitative assay they were required to utilise the ratio of the total crystal projected surface to the filament surface, the crystal density, and the distribution of inter-crystal distances. The extraction of the formazan product often requires the use of hazardous solvents and can significantly lengthen the assay. Thus XTT, a tetrazolium salt that reduces to a soluble formazan product, has become a more applicable reagent for quantitative assessments.

2.13.3 Tetrazolium salt XTT

Paull et al., (1988), synthesised a new tetrazolium salt, 3-(1-[(phenylamino)-carbonyl]-3,4-tetrazolium)-bis(4-methoxy-6-nitro) benzenesulfonic acid hydrate (XTT). This new compound, a light yellowish tetrazolium salt, is metabolically reduced by viable cells to a water-soluble, reddish-brown coloured formazan product (Zhao et al., 2002) (Figure 2.10).

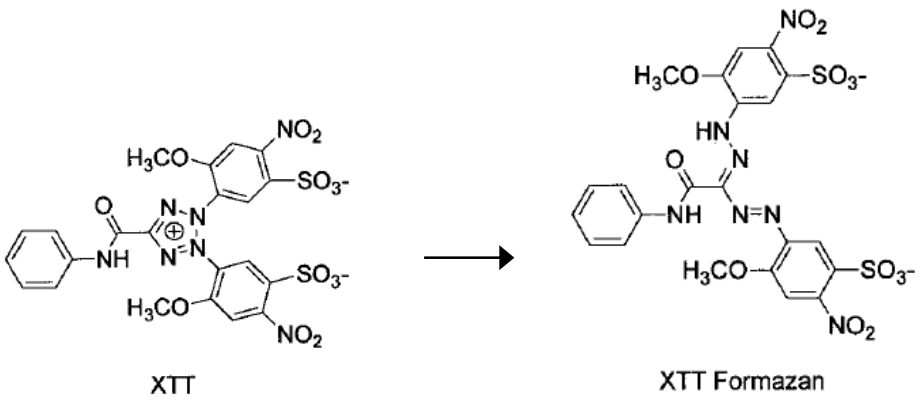


Figure 2.10: Chemical structure of XTT and its formazan salt (Zhao et al., 2002).

In contrast to other tetrazolium salts, XTT offers the advantage of eliminating the need for formazan crystal solubilisation prior to scanning spectrophotometric measurements, thereby reducing both labour and assay time (Roehm et al., 1991). Since the formazan product is water soluble it is easily measured in cellular supernatants, allowing the study of intact cells and pellets (Kuhn et al., 2003). However, Berridge et al., (2005), demonstrated that the increased negative charge on tetrazolium molecules which allows the formation of soluble formazans may have reduced their ability to move across cell membranes, raising the possibility that reduction of XTT may be extracellular. Nonetheless, the use of plasma membrane permeable electron carriers can facilitate a transfer of electrons from intracellular donors to extracellular tetrazolium, thus accelerating the reduction process (Bernas and Dobrucki, 2000). Indeed, Berridge et al., (2005), demonstrated that reduction of such tetrazolium salts in the absence of an electron carrier was negligible but that reduction was strongly promoted by its inclusion. Berridge et al., (2005), postulated that the electron carrier mediates tetrazolium salt reduction by picking up electrons at the cell surface, or at a site in the plasma membrane to form a radical intermediate that then reduces the dye by two single electron reduction events. Berridge et al., (1996, 2005), and McCluskey et al., (2005), have demonstrated that XTT is efficiently reduced by NADH, NADPH and succinate dehydrogenase, with NADH produced in the TCA cycle the primary reductant for extracellular tetrazolium reduction via transplasma membrane electron transport in the presence of an electron carrier.

Many previous studies have evaluated the use of XTT and demonstrated its ability to quantitatively measure metabolic activity. Assays for the activity of *Aspergillus* spp. (Meletiadis et al., 2001); T-cells (Roehm et al., 1991); *Candida* spp. (Kuhn et al., 2002, Kuhn et al., 2003); and *Mycobacterium bovis* BCG (Kairo et al., 1999) have all been developed using XTT indicating that it may also be a suitable assay for the physiological assessment of *Streptomyces hygroscopicus* var. *geldanus.*

2.13.3.1 Sample preparation

XTT (Sigma-Aldrich) was prepared by dissolving in 1M phosphate buffer saline (PBS) pH7 to the required concentration. The electron carrier menadione (MEN) (Sigma-Aldrich) was prepared as a solution in dimethyl sulphoxide (DMSO). Both mixtures were used immediately. Samples of homogeneous cultures (2ml) at appropriate concentrations of biomass were aliquoted to sterile universals. Universals were then incubated with 100µl of XTT and 25µl MEN at appropriate concentrations in a rotary shaker at 28°C and 150rpm. Apposite controls or 'blanks' were prepared similarly but with cell-free supernatants of cultures. Subsequently, after a suitable time period, samples were centrifuged at 4000rpm for 10 minutes to remove cells. Aliquots of 200µl of each sample supernatant were placed in the wells of a 96-well plate. In contrast to CTC, the soluble formazan product of XTT does not fluoresce, and instead may be measured colourimetrically using spectrophotometric analysis. Therefore the optical density of each well was measured at 492nm using a Tecan Sunrise plate reader (Tecan, Mannedorff, Switzerland) and associated XFlour4 data retrieval software.

2.13.3.2 Absorption spectrum

There has been much discussion about the requirement of an electron carrier for efficient reduction of XTT to its formazan salt. Menadione has previously been selected as an effective electron carrier (Meletiadis et al., 2001, Roehm et al., 1991). Figure 2.11 depicts the absorption spectra characterised using a Varian Cary 50 Scan UV Spectrometer and associated CaryWinUV data retrieval software of XTT/MEN substrate in culture medium in comparison to that of the reduced soluble formazan derivative.

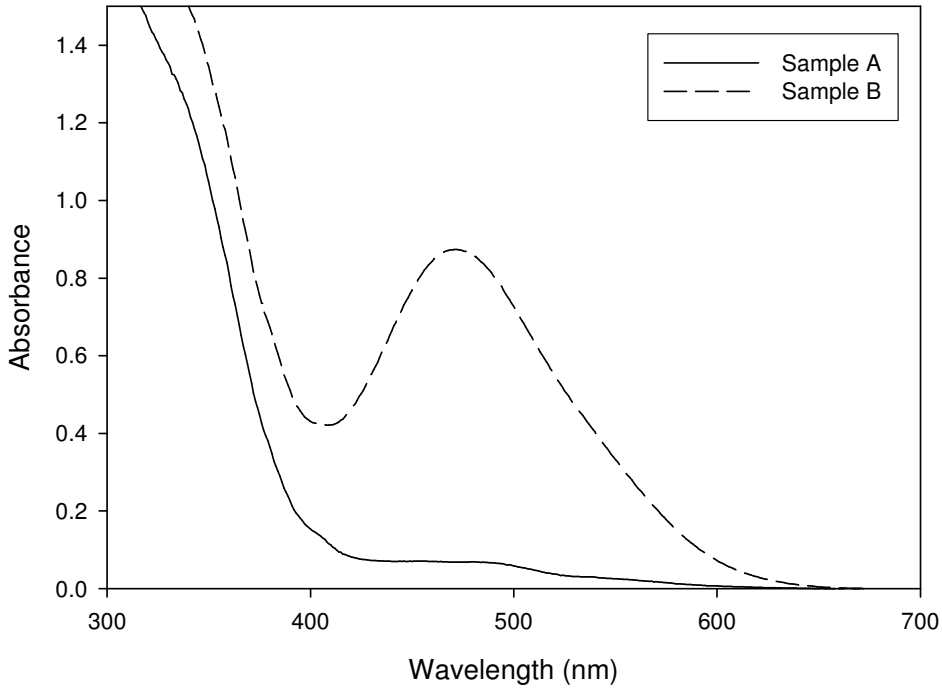


Figure 2.11: Absorbance spectrum of XTT/MEN substrate mixture in culture medium (Sample A) in comparison to that of the formazan product (Sample B) released into the supernatant by respiring *Streptomyces hygroscopicus* var. *geldanus* cells cultured with XTT/MEN.

The XTT formazan product exhibited maximal absorbance at wavelengths between 450 and 500nm. In contrast the medium and XTT/MEN substrate mixture had a low absorbance in this wavelength range. Accordingly a test wavelength of 492nm was chosen for the detection of formazan production in respiring cultures of *Streptomyces hygroscopicus* var. *geldanus*.

2.13.3.3 Concentration dependency

When developing an assay based on XTT reduction for *Aspergillus* spp., Meletiadis et al., (2001) found that the concentration of the electron carrier was critical in order to obtain a good correlation between the formazan product and the number of viable cells. Consequently, a range of concentrations (0.0625-2mM) of menadione (MEN), an electron carrier, were assessed for their ability

to potentate the conversion of XTT (0.075-0.3mM) for the detection of metabolic activity in *Streptomyces hygroscopicus* var. *geldanus* (Figure 2.12).

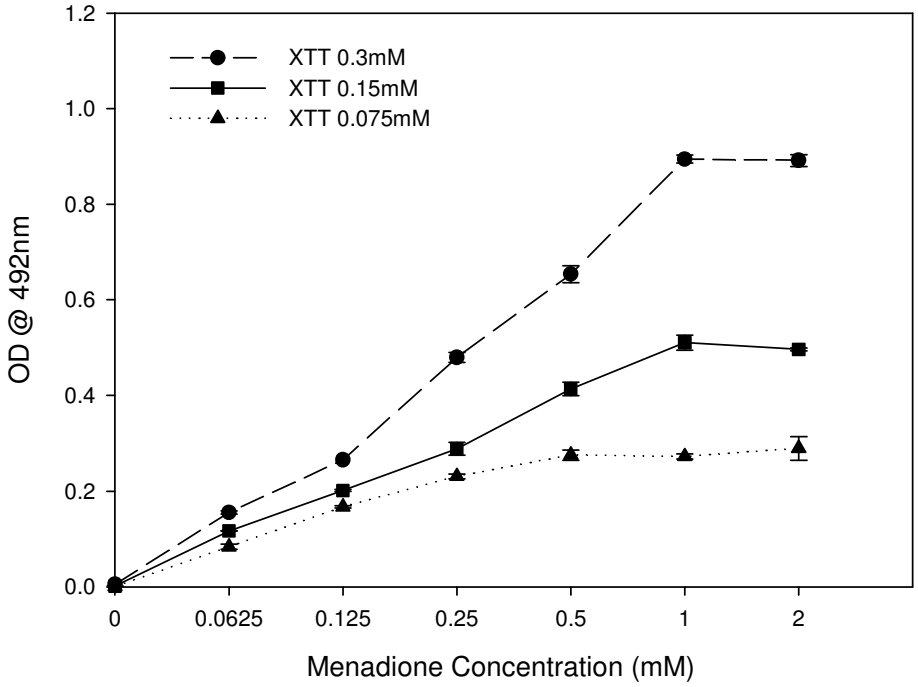


Figure 2.12: Analysis of optimum XTT and MEN concentrations for the measurement of metabolic activity in *Streptomyces hygroscopicus* var. *geldanus* following a 2 hour incubation period.

Analogous with many other reports, the tetrazolium salt XTT was only metabolised by *Streptomyces hygroscopicus* var. *geldanus* in the presence of an electron carrier, menadione. Menadione had previously been selected as an effective electron coupling agent for susceptibility testing of *Aspergillus* spp. (Meletiadis et al., 2001), and in the development of a proliferation assay for T-cells (Roehm et al., 1991). Formazan production depended on the concentration of XTT as well as the electron carrier and its concentration, with the inclusion of MEN leading to a concentration dependent increase in absorbance values. Indeed, it was observed that XTT reduction reached a maximum when high concentrations of MEN (1 and 2mM) were present, which is indicative of substrate saturation. Accordingly, in order to ensure that

the reaction is not substrate limited, a concentration of 0.3mM XTT and 2mM MEN were utilised in all subsequent assays.

2.13.3.4 Kinetics of bioreduction

Having established the optimum working concentration of XTT and MEN, an appropriate incubation time was determined. Figure 2.13 illustrates the kinetics of bioreduction of XTT / MEN over a time period of 260 minutes.

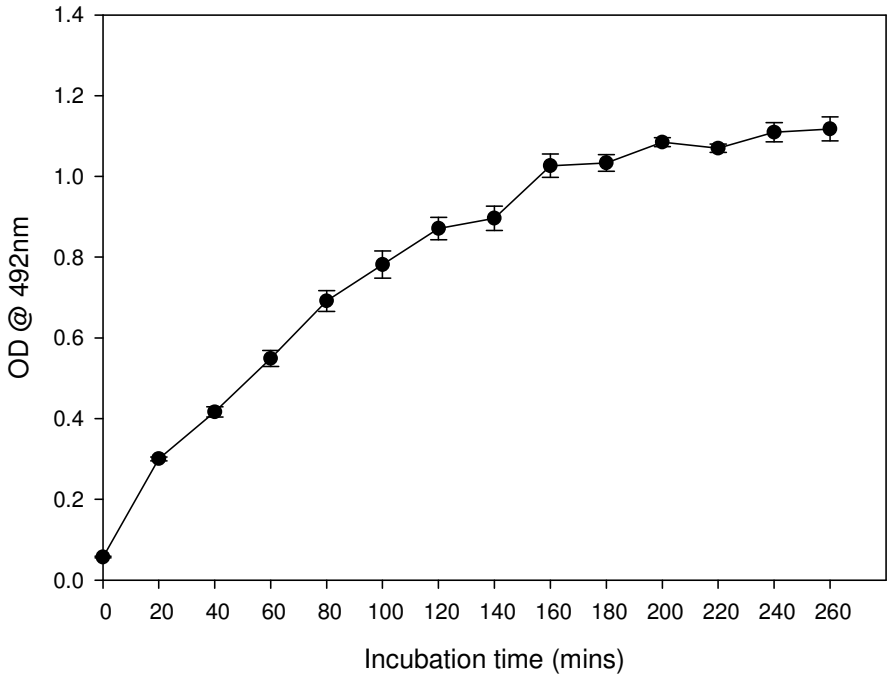


Figure 2.13: Progress curve for the reduction of XTT (0.3mM) and MEN (2mM) over a time period of 260 minutes.

Figure 2.13 confirms that XTT reduction occurs at a linear rate initially, with the rate slowing down as substrate becomes limiting. The initial kinetics of bioreduction of XTT to its formazan salt were found to give a good linear correlation with an R^2 value of 0.97 observed up to 120 minutes of incubation. Approximately 50% of the maximum signal was reduced within 60 minutes;

accordingly, an incubation time of 60 minutes was employed in all further experimentation.

2.13.3.5 Linearity and reproducibility

To make the assay practically useful, responses should be proportional to the number of cells. There have been many reports using XTT to measure metabolic activity showing a direct relationship between colorimetric signal and cell number (Kuhn et al., 2003). Indeed, formazan production was found to correlate with dry cell weight of *Streptomyces hygroscopicus* var. *geldanus*, indicating that there is a linear relationship between tetrazolium reduction and the mass of respiring bacteria (Figure 2.14). Similar effects have been documented by McCluskey et al., (2005), and Roslev and King (1993).

Frequently, within identical sets of flasks a degree of flask to flask variability can occur. Consequently, in order to accurately represent the metabolic state of the organism it is necessary to sample a sufficient number of flasks to obtain accurate results. The impact of flask-to-flask variability is also represented in Figure 2.14. Deviation from the average in this data set where triplicate flasks were assayed for XTT reduction was determined to be ±6.75%. The analysis of duplicate samples from triplicate flasks is thus considered a sufficient sample size to accomplish significant confidence in results.

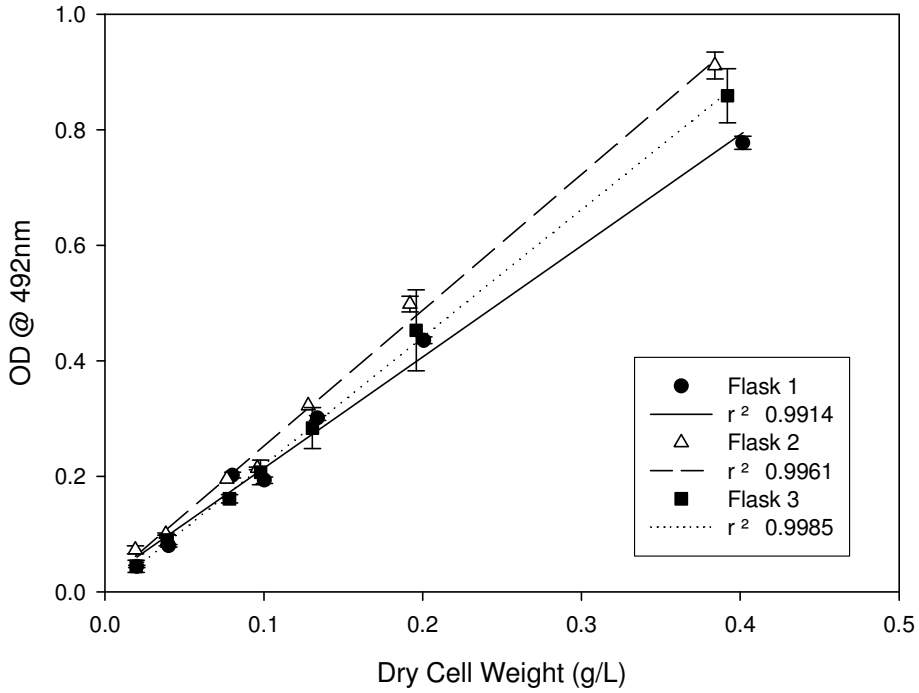


Figure 2.14: Linearity and reproducibility of XTT assay from multiple shake-flask fermentations.

2.13.3.6 Assay validation

The assay was validated by correlation of XTT reduction with colony forming unit (CFU) enumeration of *Streptomyces hygroscopicus* var. *geldanus* as the reference method. As shown in Figure 2.15, a good correlation was observed ($R^2 = 0.96$). Stentelaire et al., (2001), also observed good agreement between tetrazolium reduction and CFU enumeration during the development of an assay to detect spore viability of *Penicillium digitarium*, *Aspergillus niger* and *Metarhizium flavoviride.*

When evaluated alongside other assays utilised for physiological investigations, those based on tetrazolium reduction compare favourably. For example, McCluskey et al, (2005), determined that oxygen uptake rate of activated sludge was directly proportional to formazan production with a correlation coefficient of 0.973. Indeed, Meletiadis et al., (2000), compared

tetrazolium reduction with a method recommended by the National Committee for Clinical Laboratory Standards (NCCLS) for susceptibility testing in six species of filamentous fungi and high levels of agreement (94%) were found.

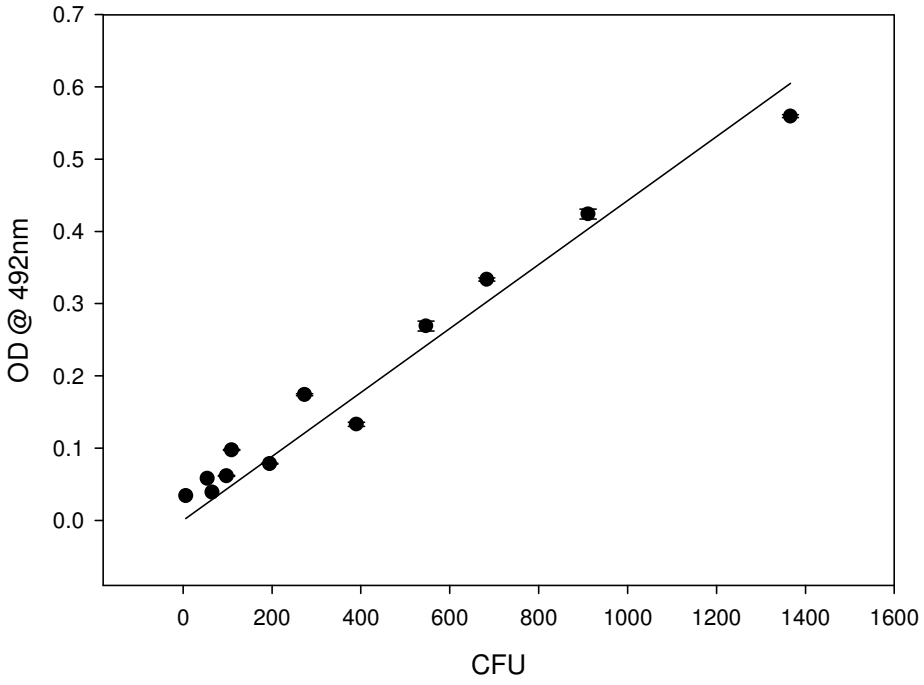


Figure 2.15: Relationship between formazan production and colony forming units (CFU) of *Streptomyces hygroscopicus* var. *geldanus* ($R^2 = 0.96$).

In this study the configuration of the assay using 96-well micro-titre plates facilitated high-throughput analysis of samples, and the assay gave reproducible results with maximum 7% deviation from the average detected during analysis of triplicate data sets. The tetrazolium-based assay compared well with the reference method of CFU enumeration, and has the significant advantage of far shorter incubation times. Accordingly, the quantification of metabolic activity in cultures of *Streptomyces hygroscopicus* var. *geldanus* may be achieved by the application of a rapid, high-throughput, accurate and reproducible assay based on tetrazolium reduction.

2.14 Submerged culture conditions

Submerged cultures were grown in Bennett's liquid medium in 250ml Erlenmeyer flasks containing 100ml medium inoculated with 1% (v/v) of a stock suspension of spores at a concentration of $1*10^7$ spores per ml. All fermentations were carried out in duplicate and incubated at 28°C on an orbital shaker at an agitation rate of 150rpm for a maximum time-course of 21 days, unless otherwise stated.

Having generated an improved standard operating procedure for the quantification of geldanamycin, the effect of medium formulation on growth and product formation could be accurately assessed. Fermentation medium comprises the environment in which the organism must grow and produce. Interactions between an antibiotic producing strain and its chemical environment have long been known to play an important role in determining the rates of production and yields of the antibiotic (Young et al., 1985). Thus, medium formulation is an essential stage in the design of a successful process.

2.14.1 Selection of basal medium

A selection of defined media were analysed for their ability to support both growth of the organism and biosynthesis of geldanamycin, as depicted in Figure 2.16.

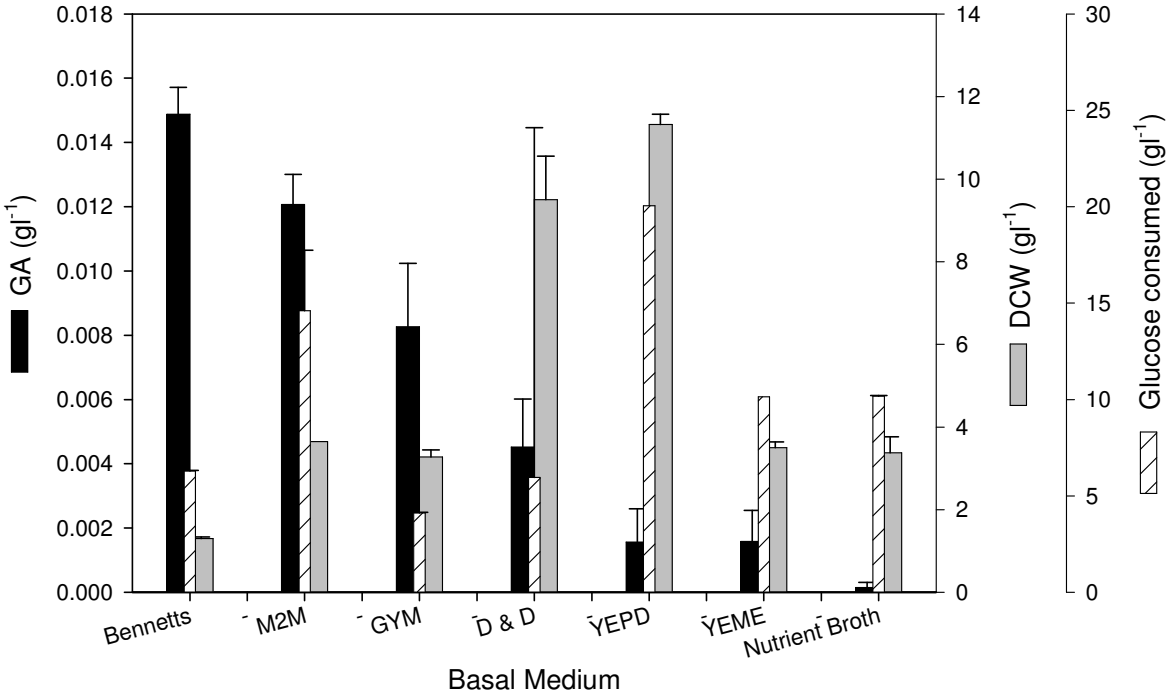


Figure 2.16: Effects of basal medium on geldanamycin (GA) and dry cell weight (DCW) production by *Streptomyces hygroscopicus* var. *geldanus* following 10 day fermentation.

All of the media investigated were found to support growth, with YEPD and De Boer and Dietz (D&D) medium achieving the greatest concentration, although it must be noted that the determination of biomass in D&D medium may be inaccurate due to the presence of unconsumed residual oatmeal. However, similar to the findings of Glazebrook et al., (1992), the optimal medium for growth is often not the same as that for production, as both YEPD and D&D media failed to support any considerable concentrations of geldanamycin. Bennett's medium, as recommended by De Boer and Peterson, (1971), was determined to be the optimal medium for the fermentation process in question, sustaining low levels of dry cell weight but, more importantly, significant amounts of geldanamycin. Figure 2.17 describes the growth of the organism and production of geldanamycin throughout a 20 day fermentation in Bennett's medium.

Dry cell weight increased until stationary phase was reached at day 14 due to the exhaustion of glucose from the medium. Maximal concentrations of geldanamycin were detected at day 12 which coincided with the transition to stationary phase of growth as denoted by stability in biomass accumulation levels. The end of the production phase corresponded with substrate depletion. These results confirm that geldanamycin is a secondary metabolite produced by *Streptomyces hygroscopicus* var. *geldanus* following the main phase of exponential growth.

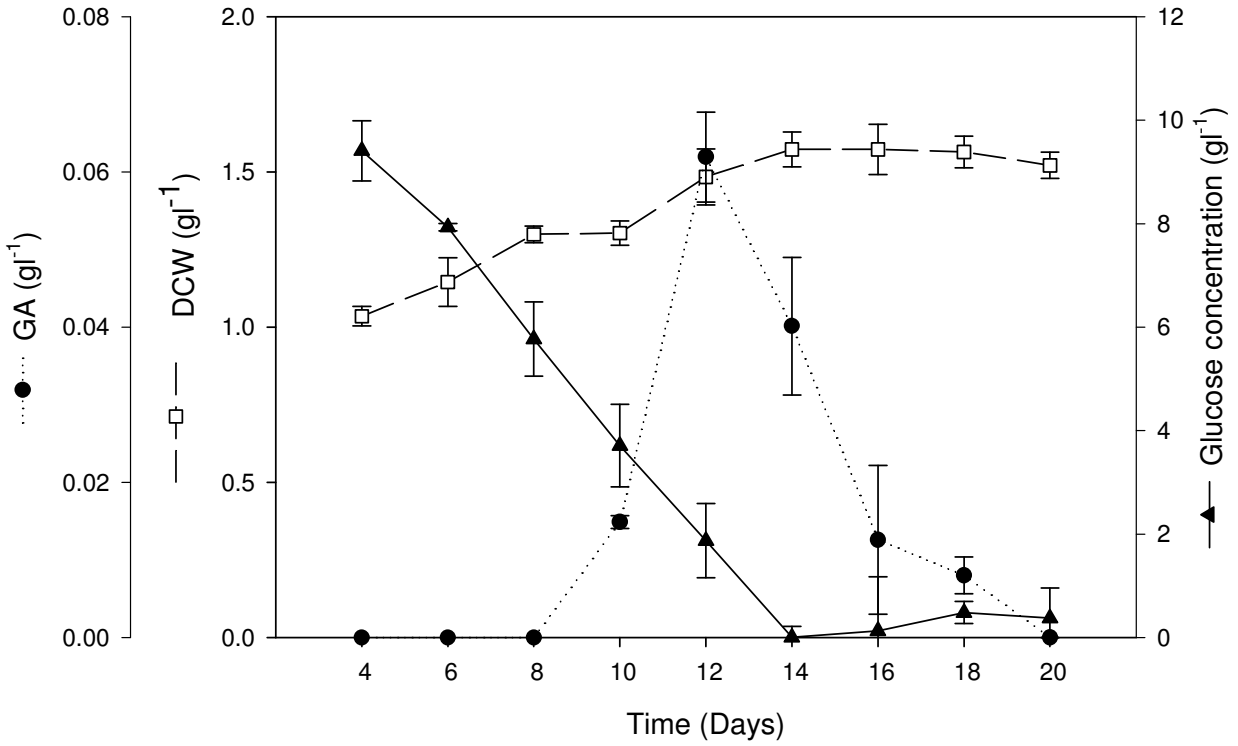


Figure 2.17: Fermentation profile of *Streptomyces hygroscopicus* var. *geldanus* in standard Bennett's medium.

2.14.2 Selection of optimal carbon source

Standard Bennett's medium contains the carbon substrate glucose at a concentration of $10gl^{-1}$ which was found to be completely utilised by the organism by day 14, initiating the onset of a decline phase (Figure 2.17). Thus, the inclusion of a more efficient, or a greater concentration, of carbon source was investigated. Various monosaccharides and disaccharides at concentrations of $20gl^{-1}$ were supplemented to Bennett's medium and examined for their ability to support both growth and production of geldanamycin by *Streptomyces hygroscopicus* var. *geldanus* (Figure 2.18). The other constituents of the medium were present at constant amounts as detailed in Table 2.1.

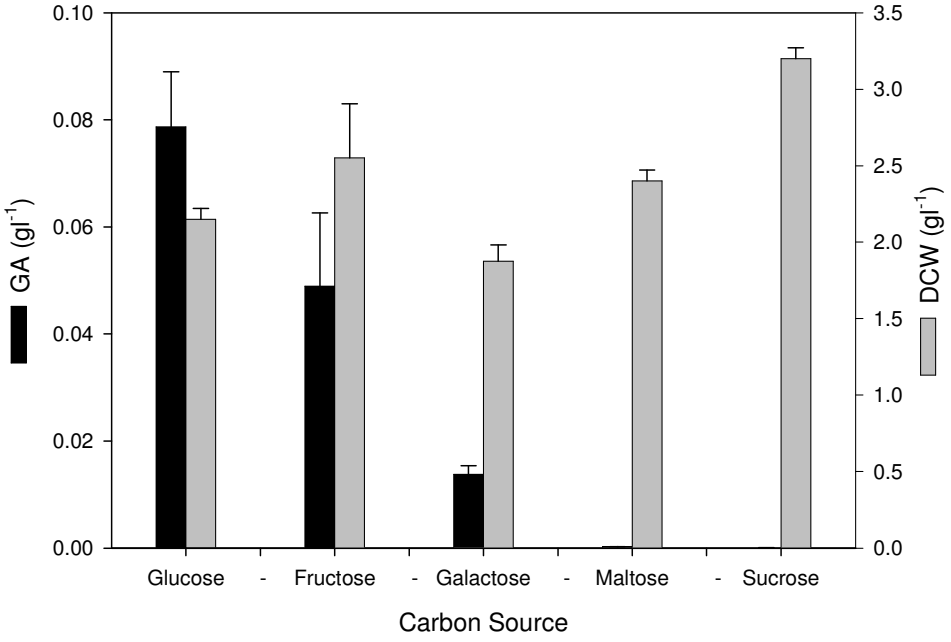


Figure 2.18: Suitability of different carbon sources ($20gl^{-1}$) in Bennett's medium for the production of geldanamycin analysed at day 12.

It was observed that while monosaccharides had the ability to support both growth of the micro-organism and antibiotic synthesis, both disaccharides tested supported only biomass production. Of those carbon sources assessed,

glucose was observed to be the optimal carbon and energy source for geldanamycin production. Figure 2.19 illustrates that, from the range of concentrations examined, the most favourable concentration of glucose for antibiotic production was found to be $50gl^{-1}$, with a 42% increase over Bennett's standard medium containing $10gl^{-1}$ observed. It was noted that the differences in specific yield of geldanamycin on biomass ($Y_{p/x}$) between those cultures supplemented with 40 and $50gl^{-1}$ was only 2%; therefore the inclusion of higher concentrations of glucose was deemed to be economically unviable.

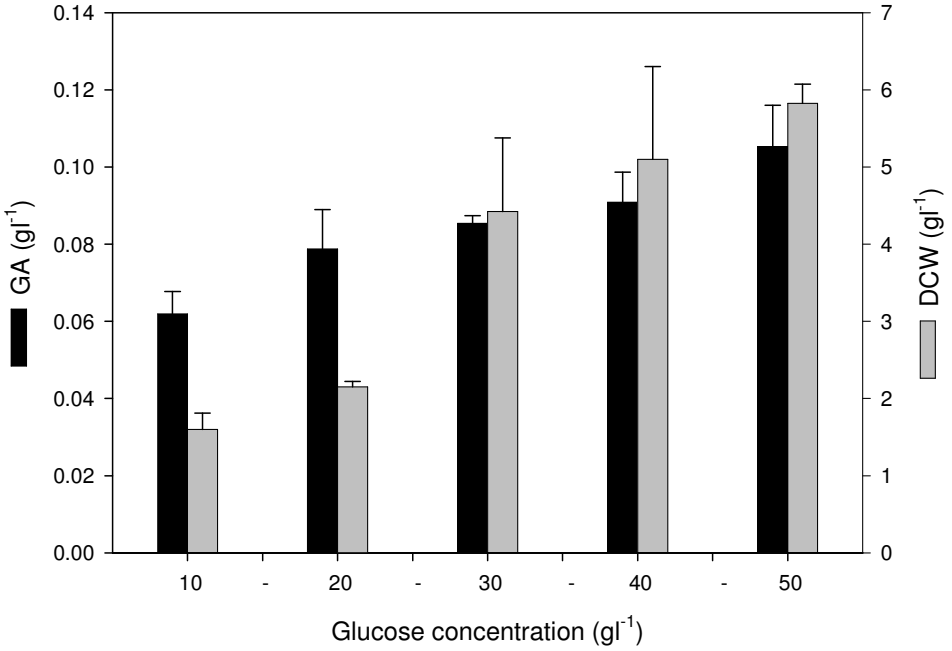


Figure 2.19: Effect of glucose concentration on DCW accumulation and geldanamycin production on fermentation parameters at day 12 in Bennett's medium.

These findings also suggest that geldanamycin production by *Streptomyces hygroscopicus* var. *geldanus* is not affected by carbon catabolite regulation, whereby high concentrations of rapidly utilisable carbon sources such as glucose repress the synthesis of enzymes that are required for antibiotic production (Young et al., 1985). In contrast, the production of both doxorubicin by *Streptomyces peucetius* var. *caessius* (Sanchez and Demain,

2002) and actinorhodin by *Streptomyces coelicolor* A3(2) (Fazeli et al., 1995) are reported to be highest under carbon-limited growth conditions.

2.14.3 Analysis of Bennett's medium constituents

Fermentation media may be either complex or defined depending on the nutritional composition. While defined media are made up of exact quantities of pure compounds and allow greater regulation of cellular metabolism and product formation, they are typically more expensive to formulate. Complex media such as Bennett's often consist of crude supplements, in this instance Beef and Yeast extract and NZ-Amine-A, and the exact elemental composition is not known although an approximation can be made. The optimal concentrations of the organic constituents of Bennett's medium were determined and are presented in Figure 2.20. Of those tested, concentrations of Yeast and Beef extract at $3gl^{-1}$ and NZ-Amine-A at $2gl^{-1}$ were deemed most advantageous for geldanamycin production.

Table 2.10 provides a summary of the nitrogen composition of these constituents. In general, they provide complex nitrogen consisting of a proportion of amino nitrogen (amino acids and peptides), although they also contain water soluble vitamins, carbohydrates and adequate amounts of trace elements (Farid et al., 2000). Most micro-organisms cannot fix atmospheric nitrogen and thus require a readily utilisable source. Figure 2.21 demonstrates that the stimulation of geldanamycin production observed when these compounds are included in Bennett's medium may be attributed to the increasing concentration of amino nitrogen.

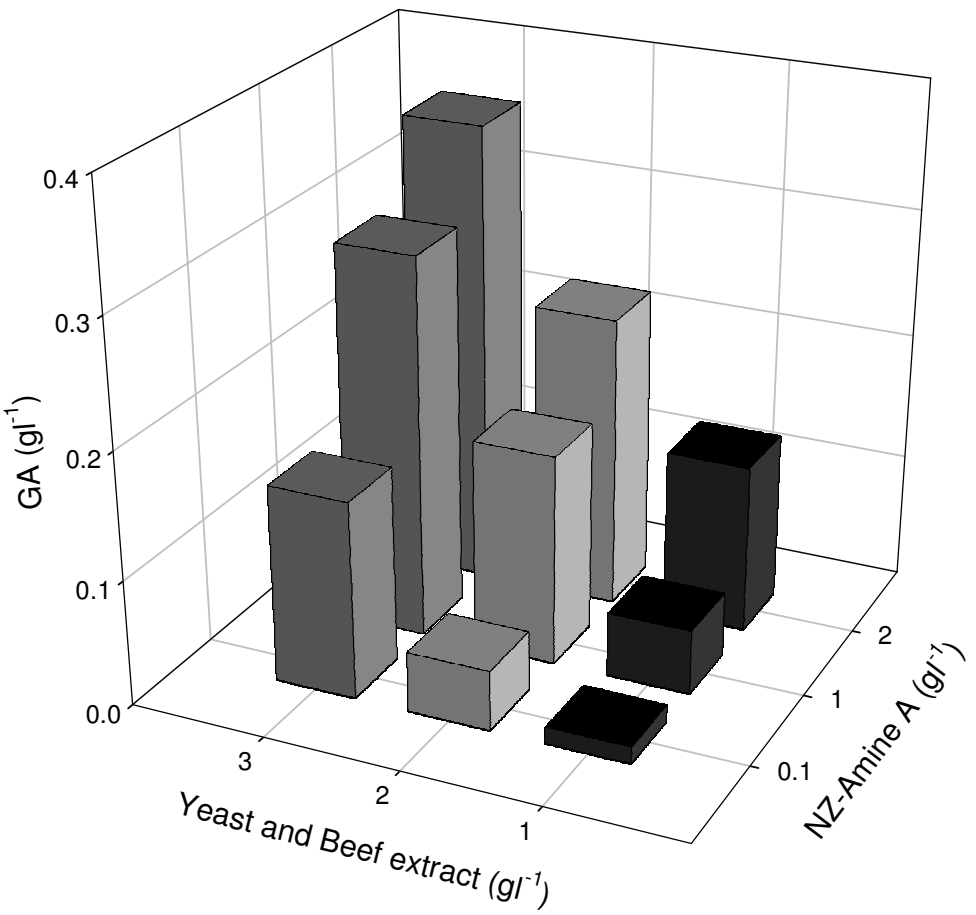


Figure 2.20: Concentration effect of the organic constituents Yeast extract, Beef extract and NZ-Amine-A on geldanamycin synthesis at day 14 in Bennett's medium supplemented with 50gl^{-1} glucose.

Table 2.10: Nitrogen composition of Bennett's medium constituents.

Constituent	Total Nitrogen %	Amino Nitrogen %
NZ-Amine-A	13.1	6.5
Yeast Extract	10.9	5.3
Beef Extract	13.3	2.5

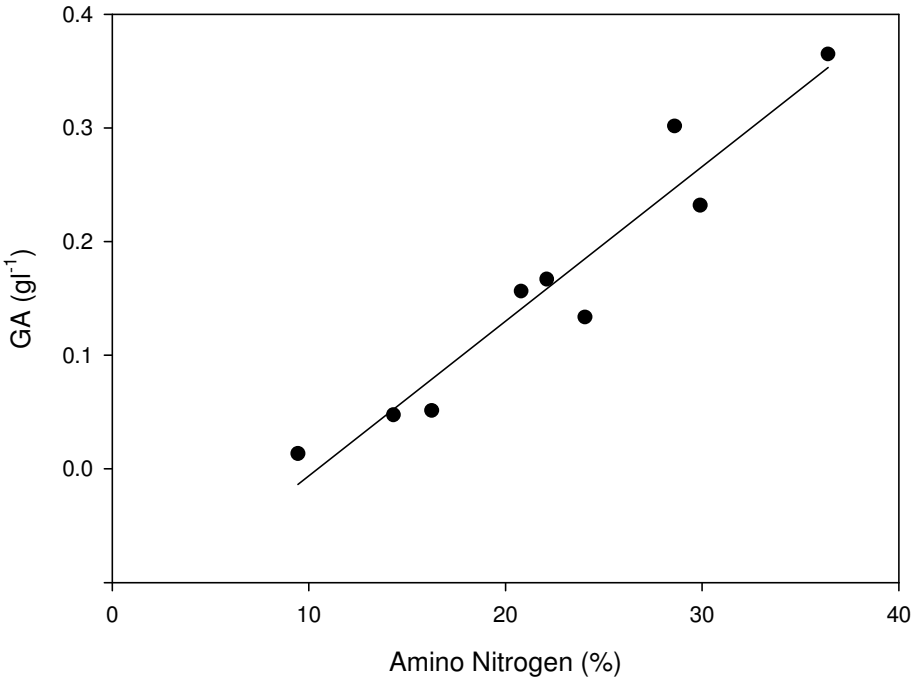


Figure 2.21: Stimulation of geldanamycin production following supplementation of Bennett's medium with amino nitrogen, $R^2 = 0.92$.

2.14.4 Specific respiration rate RR_s and fermentation profile of *Streptomyces hygroscopicus* var. *geldanus* cultivated on optimised Bennett's medium

Having optimised the concentrations of Bennett's medium constituents, it was decided to investigate whether the fermentation profile had altered from that cultured in standard Bennett's medium depicted in Figure 2.17. A comparison between the two media is given in Table 2.11.

Table 2.11: Comparison of standard and optimised Bennett's medium formulations.

	Standard Bennett's Medium	Optimised Bennett's Medium
Glucose (gl^{-1})	10	50
Yeast extract (gl^{-1})	1	3
Beef extract (gl^{-1})	1	3
NZ Amine A (gl^{-1})	2	2

It was also of interest to investigate whether the tetrazolium assay for cell physiology investigated in Section 2.13.3 would correlate with the factors more traditionally measured during the fermentation of micro-organisms, namely dry cell weight accumulation, glucose consumption and secondary metabolite production. The vast majority of assays measure cell proliferation where it is assumed that tetrazolium reduction is proportional to the number of viable cells (Berridge et al., 2005). Nevertheless, although this is usually a good approximation, reduction can vary widely within and between cell populations depending on the cell growth conditions, whether the cells are in exponential growth phase and with the stage of the cell cycle (Berridge et al., 2005). Thus it has been recommended by Braeckman et al., (2002), that experimental data be normalised appropriately for quantitative comparisons between groups e.g. normalising with dry weight data. By normalising the formazan signal with dry cell weight, it is possible to contrast different systems by assessment of metabolic activity on a specific basis, equivalent to the determinations of Mauss et al., (1997). Following an incubation period of 1 hour, the absorbance at 492nm of sample (A_{assay}) was measured against its blank (A_{blank}) in order to calculate the net absorbance (A_{net}).

The global respiration rate RR_g (au/L/h) is thus given by:

$$RR_g = \frac{A_{net}}{2} * 1000 \qquad \text{Equation 2.4}$$

The specific respiration rate RR_s (au/g DCW/h) is then given by:

$$RR_s = \frac{RR_g}{[X]} \qquad \text{Equation 2.5}$$

where [X] is the biomass concentration in gl^{-1}.

Figure 2.22 depicts the fermentation profile of *Streptomyces hygroscopicus* var. *geldanus* and reveals that the specific respiration rate RR_S accurately describes the fermentation kinetics. Metabolic activity remains constant and at a high level during the exponential phase of growth and dry cell weight accumulation from Days 4-12. From Day 14 onwards, RR_s drops significantly, signalling the initiation of the stationary and decline phase of growth with a decrease in glucose consumption also observed during this period.

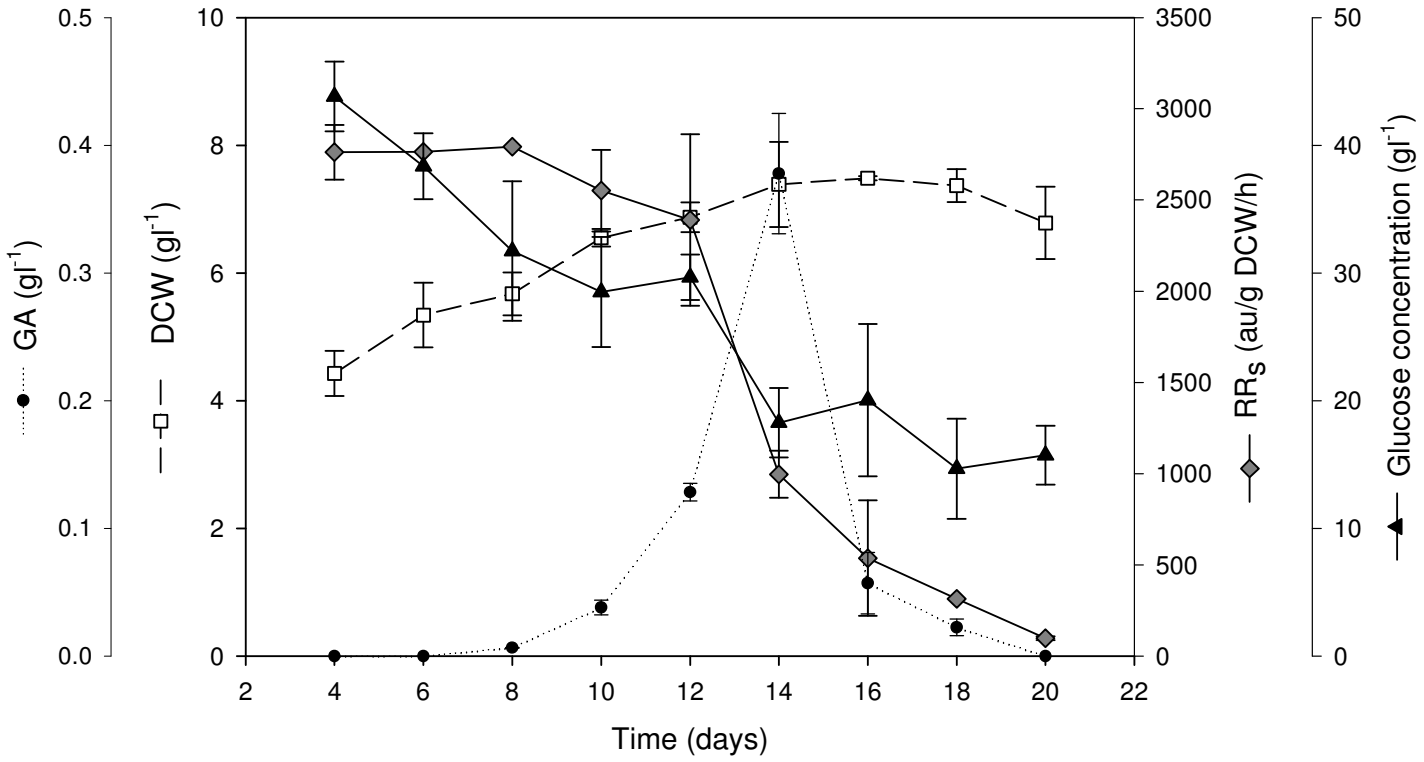


Figure 2.22: Fermentation profile of *Streptomyces hygroscopicus* var. *geldanus* including the determination of specific respiration rate (RR_s) as calculated using an assay based on reduction of the tetrazolium salt XTT.

Figure 2.22 demonstrates that the fermentation kinetics remain similar when the organism is cultivated on optimised Bennett's medium. The onset of geldanamycin synthesis was found to follow the phase of exponential growth and the rate of antibiotic synthesis was greatest during a 48 hour period between days 12 and 14. In Actinomycetes and fungi it is not uncommon for the phase of antibiotic synthesis to be short, in some instances lasting only 20 hours (Martin and Demain, 1980).

The cellular yield coefficient and geldanamycin synthesis were significantly increased when the organism was cultured on optimised Bennett's medium (Table 2.12). Accordingly, this medium was utilised in all further fermentations.

Table 2.12: Suitability of Bennett's standard and optimised media for biomass and antibiotic production.

	Standard Bennett's Media	**Optimised Bennett's Media**	**% Increase**
Cellular yield coefficient ($Y_{x/s}$)	0.057	0.108	89
Maximum Volumetric GA (gl^{-1})	0.062 (day 12)	0.378 (day 14)	509

Following the period of geldanamycin synthesis, the organism entered a decline phase between days 14 and 16 despite high residual concentrations of glucose, indicating the presence of another limiting substrate(s). Concomitantly, a sharp decline in antibiotic concentration was observed. The cessation of antibiotic synthesis is often attributed to feedback inhibition whereby the product triggers enzymatic repression of the synthesising pathway, as is evident in the production of cycloheximide and thienamycin (Dykstra and Wang, 1990, Wang et al., 1989). Generally the synthesis inhibiting level of antibiotic in a particular organism is similar to the production level of that

strain (Martin and Demain, 1980). However the steep and rapid decline of geldanamycin in fermentation indicates that either chemical or enzymatic mechanisms are responsible for product degradation.

For instance, the main losses of cephamycin C and clavulanic acid in fermentation broths have been attributed to chemical breakdown due to their instability (Usher et al., 1988, Bersanetti et al., 2005). In contrast, a cell-associated enzyme is responsible for the break-down of pristinamycin in the culture broth following the production phase (Paquet et al., 1994). Enzymatic degradation of geldanamycin as the organism enters a decline or death phase may occur in order to replace the limiting substrate. Nonetheless, regardless of the mechanism, it is paramount that batch fermentations be ceased before reaching the later phases of cultivation where geldanamycin degradation surmounts production. The 48 hour interval up to day 14 therefore represents the optimum sampling time for geldanamycin in batch fermentations cultured on optimised Bennett's medium.

CHAPTER 3: RESULTS AND DISCUSSION

3.1 Impact of surfactants on morphology and geldanamycin synthesis

Surfactant compounds consist of both hydrophobic and hydrophilic regions on the same molecule, and act to reduce the surface tension of foams leading to their collapse. In many microbiological processes, excessive foaming can become a significant problem causing overflow in bioreactors, providing an entry for contamination, blockage of outlet gas lines, a loss of bioreactor volume and damage to cells (Doran, 1995). A high degree of aeration and agitation, combined with the production of proteins and polysaccharides can thus lead to a requirement for foam control, most commonly by the addition of surfactant agents.

However, a reduction in broth surface tension can also decrease the potential for interaction and aggregation of biomass, resulting in the dispersion of pellets. For this reason, surfactants have been demonstrated by many authors to influence the morphological development of filamentous micro-organisms in submerged fermentations (Davoust and Hansson, 1992, Okba et al., 1998, Hobbs et al., 1989, Morrin and Ward, 1990, Vecht-Lifshitz et al., 1989).

Figure 3.1 details the effect of silicone antifoam concentration on the surface tension of cell-free optimised Bennett's medium with the subsequent effect on morphology and geldanamycin production depicted in Figures 3.2 and 3.3.

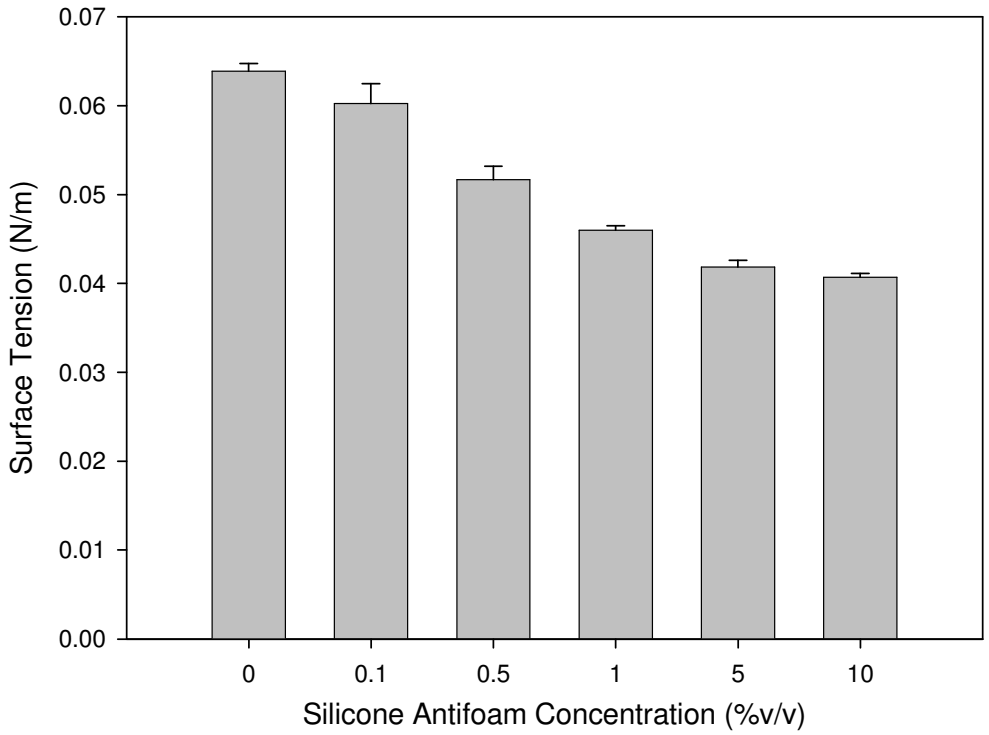


Figure 3.1: Effect of silicone antifoam concentration on surface tension of cell-free optimised Bennett's medium

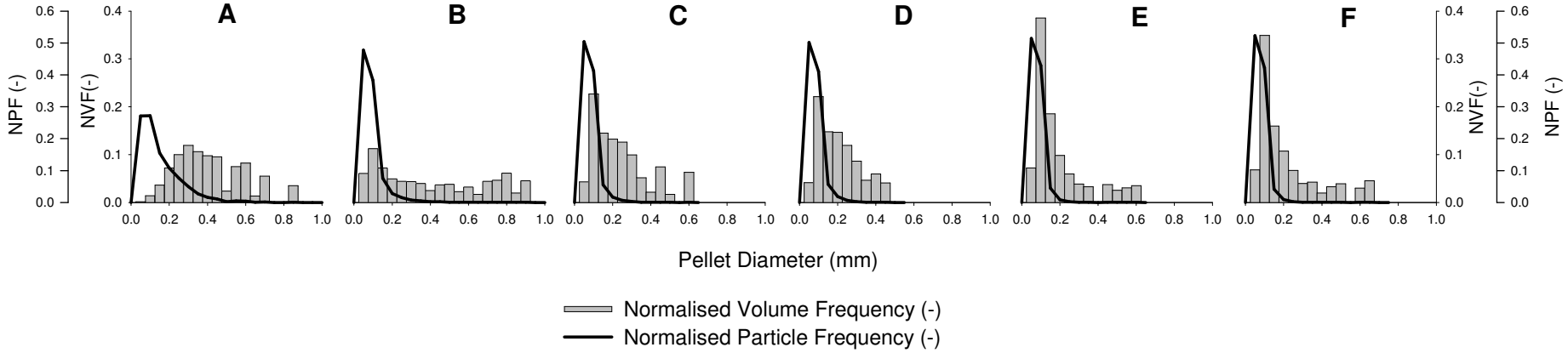


Figure 3.2: Impact of varying concentrations of silicone antifoam on particle distributions at day 12: A = 0; B = 0.1; C = 0.5; D = 1; E = 5; F = 10 %v/v.

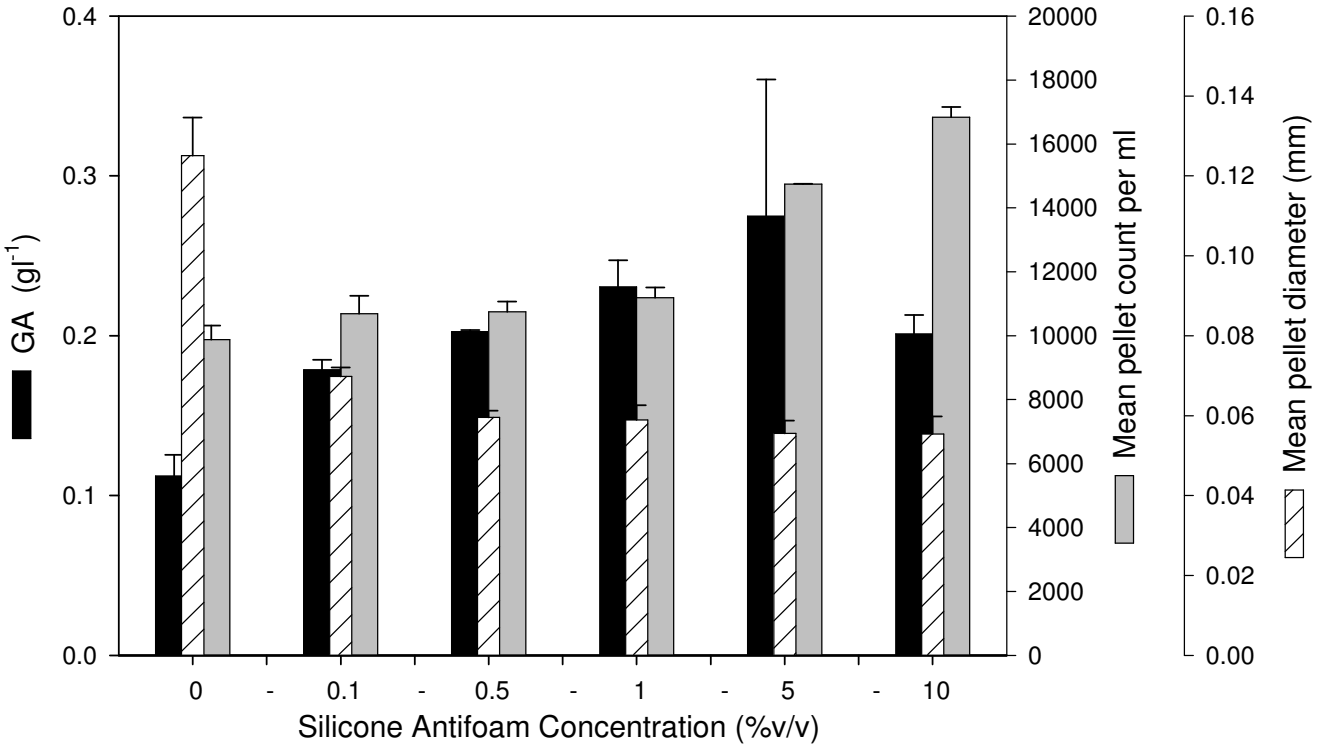


Figure 3.3: Effect of silicone antifoam concentration on geldanamycin production and morphological characteristics at day 12.

Certainly, an increase in the amount of antifoam present in optimised Bennett's medium prompted an increase in the dispersion of pellets, with a greater distribution of smaller pellets observed. The increase in pellet count and simultaneous decrease in pellet diameter illustrates the diversity in morphology within these cultures. It transpires from Figure 3.3 and Table 3.1 that the production of geldanamycin is significantly influenced by the morphological characteristics of the organism. Volumetric concentration of the antibiotic increased by 145% to a maximum of $0.275gl^{-1}$ as the pellet count increased by 70% and the mean pellet size decreased by over 50% (Table 3.1).

Table 3.1: Comparison of geldanamycin production in control and morphologically regulated cultures at day 12.

Surfactant concentration	Mean pellet count per ml	Mean pellet diameter (mm)	GA concentration (gl^{-1})
0%	9875.78	0.1251	0.112
0.1%	10692.94	0.0698	0.178
0.5%	10751.38	0.0596	0.203
1%	11186.06	0.0590	0.231
5%	16836.62	0.0556	0.275

The inclusion of various concentrations of the non-ionic surfactants TritonX100 and Tween80 also affected the morphology of *Streptomyces hygroscopicus var. geldanus* (Figure 3.4).

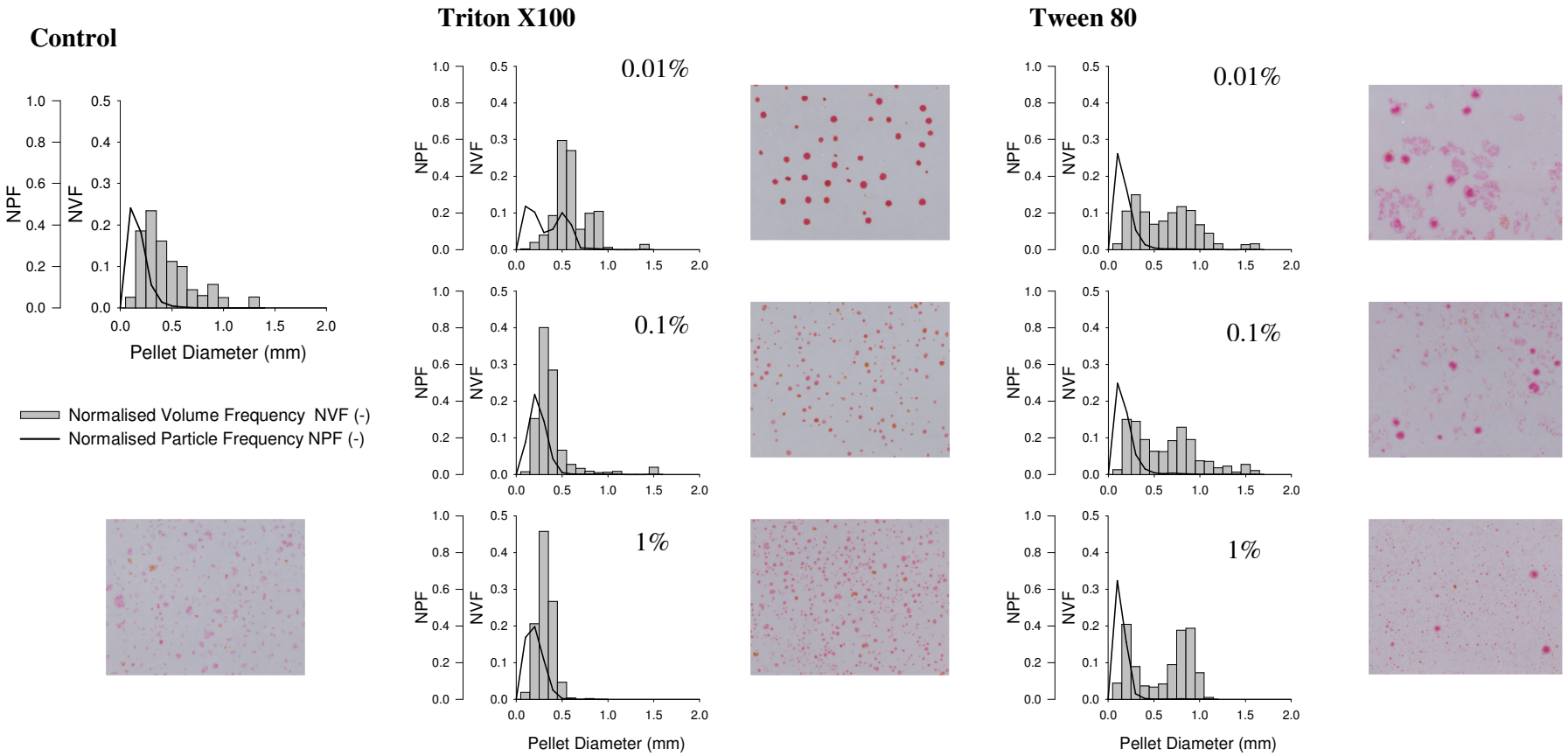


Figure 3.4: The disparity in morphological profiles in cultures supplemented with varying concentrations of surfactants (%v/v) analysed at day 12.

Both TritonX100 and Tween80 were determined to act as binding or flocculent agents at low concentrations (0.01%) inducing pellet formation; however at high concentrations (1%) the culture morphology regressed to control-like behaviour. Vecht-Lifshitz et al., (1989), also reported that pellet sizes increased with surfactant addition to a certain point, above which an inhibition of pellet formation was observed.

It was apparent that both the type and concentration of surfactant could influence morphology, and furthermore geldanamycin synthesis. For both surfactants an increase in concentration (%v/v) resulted in a greater degree of dispersion of biomass with an increase in pellet count per ml (Figure 3.5 A) and a concomitant decrease in the mean pellet size observed (Figure 3.5 B). Volumetric titres of geldanamycin could again be correlated with morphology, and antibiotic synthesis appeared to be repressed by the formation of large pellets, implying that cultures with smaller pellet sizes are optimal for geldanamycin production (Figure 3.5 C). However, it was noted that while the addition of 1% (v/v) TritonX100 yielded control-like morphology, these cultures failed to produce similar concentrations of geldanamycin. It was concluded that TritonX100 exerted a toxic effect on cultures of *Streptomyces hygroscopicus* var. *geldanus*, with a decrease of 26% in dry cell weight analyses observed for these samples. Davoust and Hanson, (1992), and Vecht-Lifshitz et al., (1989), have also reported that high concentrations of certain surfactants were detrimental to cell physiology and antibiotic synthesis in cultures of *Absida* spp. and *Streptomyces tendae* respectively.

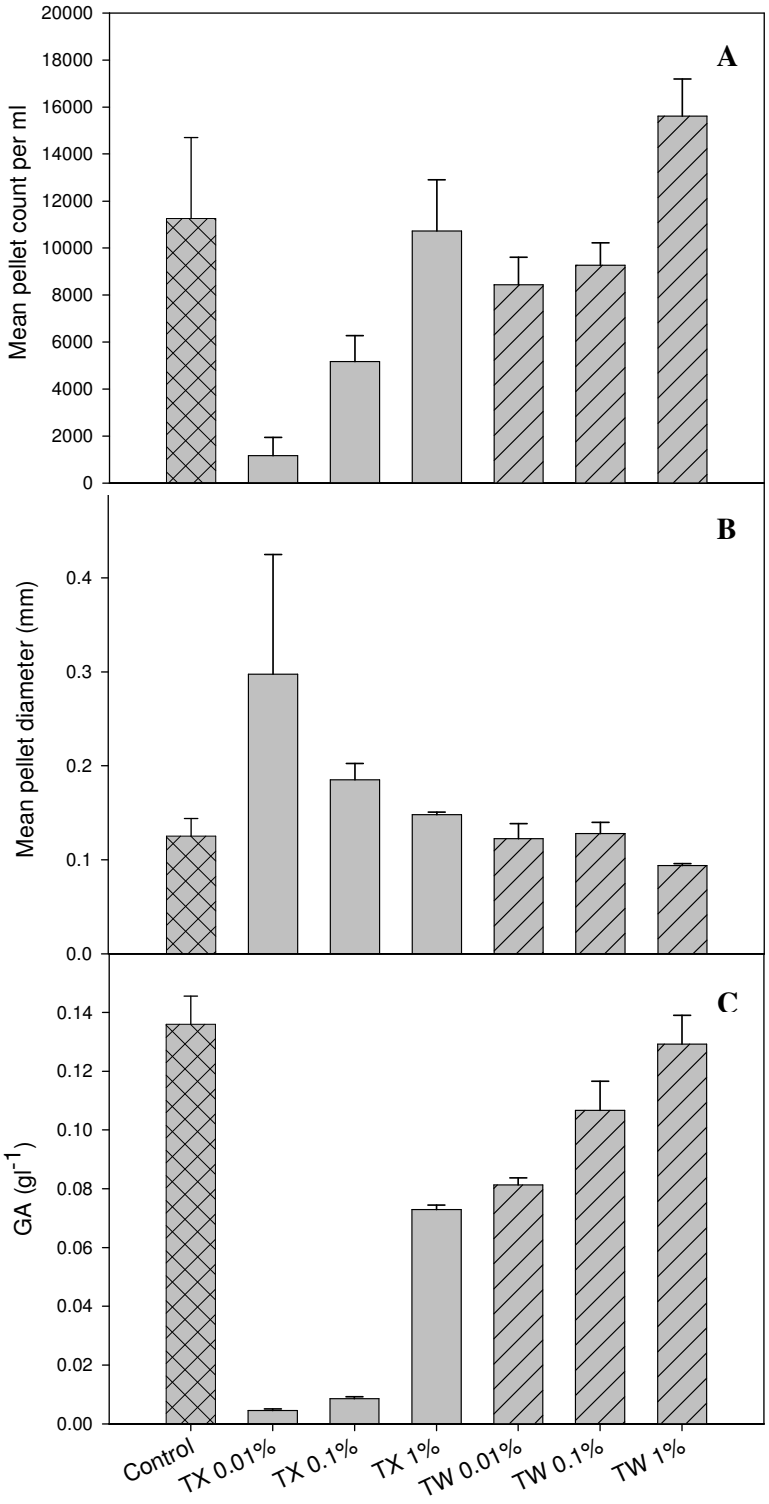


Figure 3.5: Effect of surfactant (%v/v) on morphological characteristics and geldanamycin production at day 12. TX = Triton X100; TW = Tween 80.

Whilst the addition of surfactants or antifoams to nutrient media has traditionally been used to prevent both foaming and/or wall growth in submerged fermentations, the inclusion of silicone antifoam, Tween80, and TritonX100 was found to have great implications on morphological development in cultures of *Streptomyces hygroscopicus* var. *geldanus*. The experimental results presented here support the use of silicone antifoam to effectively control cell aggregation. Morrin and Ward, (1990), and Hobbs et al., (1989), also demonstrated that surfactant addition resulted in an increase in the degree of dispersion of *Rhizopus arrhizus* and *Streptomyces lividians* respectively, with the latter demonstrating a decrease in mean pellet diameter from 300μm to 65μm.

3.2 Impact of spore inoculum level on morphology and geldanamycin synthesis

Thus far, a spore inoculum had been utilised in all fermentations. However, the use of vegetative inocula has often been recommended for the production of secondary metabolites by reducing the lag phase of growth required for spore germination (Elibol et al., 1995, Ashy et al., 1982). Consequently, the impact of employing a vegetative inoculum of *Streptomyces hygroscopicus* var. *geldanus* for geldanamycin fermentation was examined. To provide vegetative inoculum 1ml of a spore suspension containing 10^7spores/ml was used to inoculate 100ml of standard Bennett's medium in 250ml Erlenmyer flasks and incubated on an orbital shaker (150rpm) for a period of 3, 4 or 5 days before inoculation in optimised Bennett's medium.

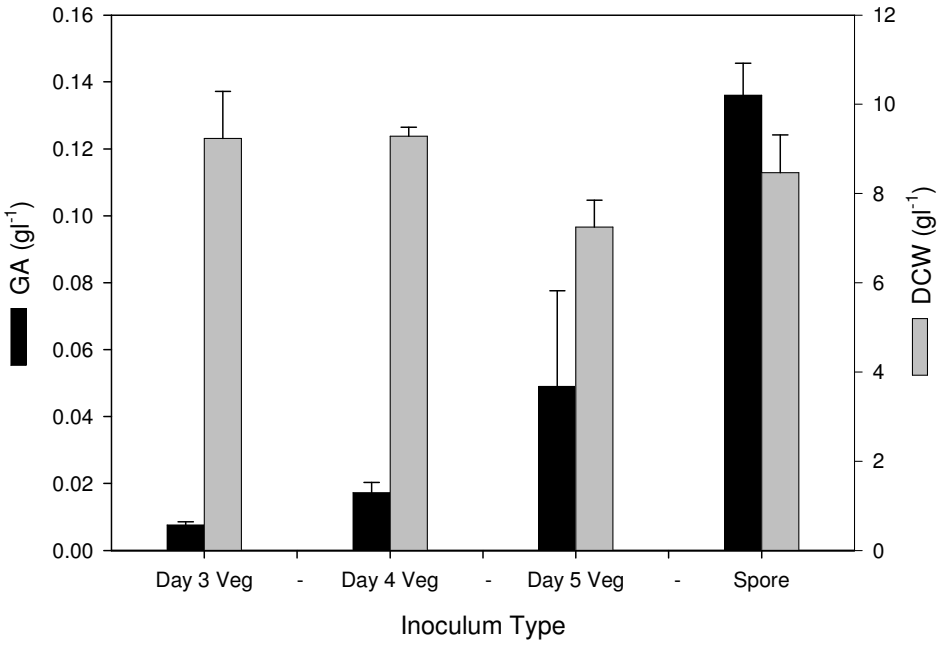


Figure 3.6: Impact of inoculum type on fermentation parameters at day 12. All cultures contained a final in-flask spore inoculum concentration of 10^5spores/ml, and vegetative inocula were produced by culturing in standard Bennett's medium for the appropriate time before introduction to optimised Bennett's medium.

Figure 3.6 established that while both inoculum types supported equivalent levels of growth, volumetric geldanamycin production was considerably increased in cultures that were inoculated with spores. The spore is regarded as a dormant stage in the streptomycete life cycle with a low metabolic turnover. Normally two types of dormancy are recognised: constitutional dormancy where spores need some kind of activation, and exogeneous dormancy, where germination occurs when spores are placed in a suitable medium (Krabben and Nielsen, 1998). It would appear that spores of *Streptomyces hygroscopicus* var. *geldanus* used for the inoculation of submerged cultivation were in exogeneous dormancy, germinating readily when placed in growth medium.

Once the optimum inoculum type had been established, it was then important to provide an optimum inoculum level for the fermentation process. According to Ellaiah et al., (2004), a low inoculum density may give insufficient biomass causing reduced product formation whereas a higher inoculum concentration may produce too much biomass and deplete the substrate of nutrients necessary for product formation. Previous reports have also detailed the influence of spore inoculum level on morphology. Tucker and Thomas, (1992); Nielsen et al., (1995); Kim and Hancock (2000); and Vecht-Lifshitz et al., (1990), have all illustrated that a transition from pelleted to dispersed morphology occurred as inoculum levels were increased in cultures of *Penicillium chrysogenum*, *Streptomyces tendae* and *Streptomyces griseus* respectively. In agreement with these observations, this study established that pellet formation in *Streptomyces hygroscopicus* var. *geldanus* occurred at low spore inoculum levels, and pellet sizes decreased progressively with increasing spore concentration as represented by Figure 3.7. Volumetric concentrations of geldanamycin were found to be minimal at large pellet sizes, but increased concurrently with a reduction in the mean pellet diameter (Figure 3.8).

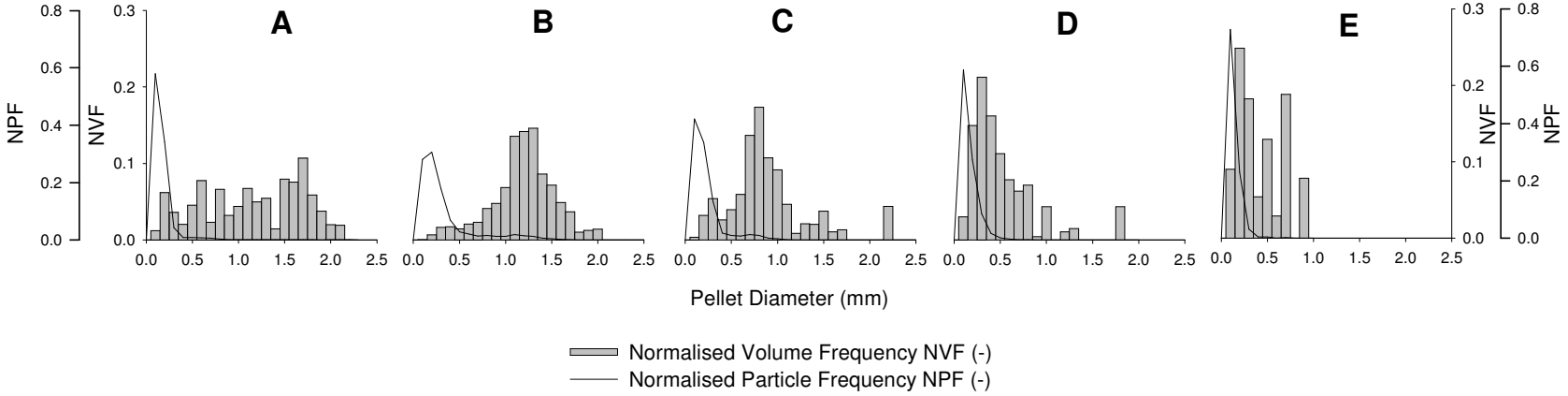


Figure 3.7: Impact of initial in-flask spore inoculum concentration on the morphological profile of *Streptomyces hygroscopicus* var. *geldanus* cultures at day 12, A = 10^2; B = 10^3; C = 10^4; D = 10^5; E = 10^6 spores/ml.

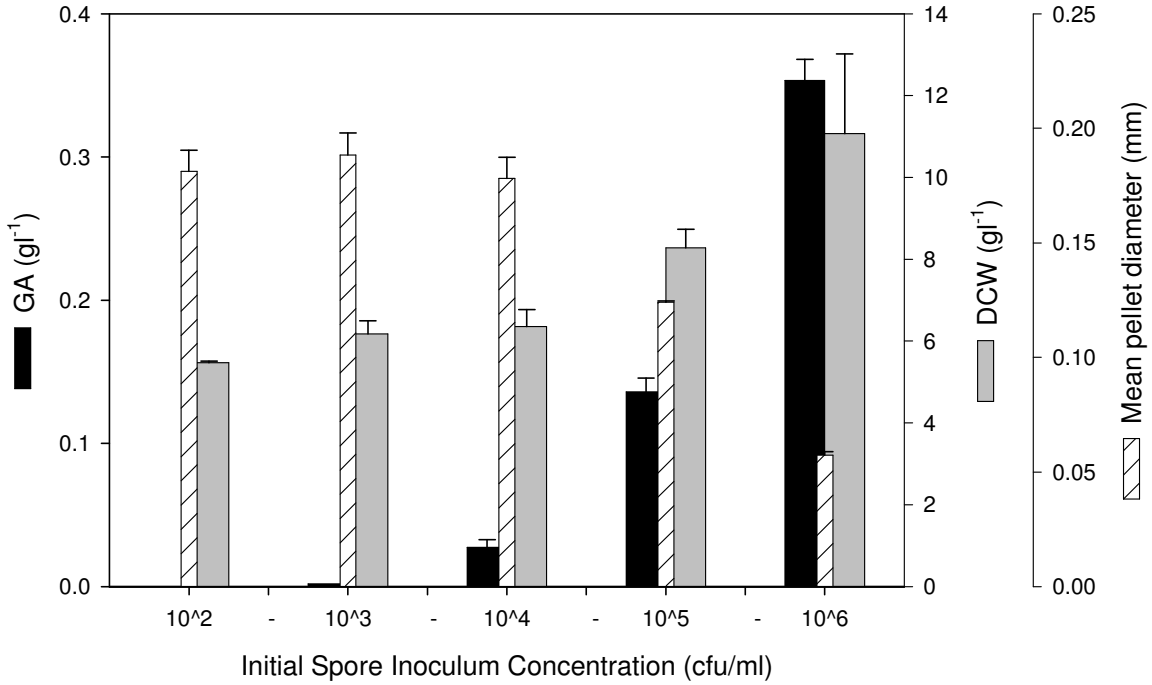


Figure 3.8: The impact of spore inoculum level on pellet diameter (mm) and geldanamycin production at day 12.

3.3 Impact of shear on morphology and geldanamycin synthesis

Pellet size is strongly influenced by shear forces: high energy input supports small, smooth and compact pellets, while under low shear stress pellets are larger and more hairy (Bellgardt, 1998). In addition, the dependency of mycelial morphology on agitation intensity and various shear conditions has been documented for the filamentous organisms *Streptomyces fradiae* (Tamura et al., 1997); *Aspergillus niger* (Fujita et al., 1994); and *Pencillium chrysogenum* (Justen et al., 1996). Generally, effective collision of particles is required for aggregation and this may be proportional to the agitation intensity up to a maximum point (Lu et al., 1998). However, shearing of pellets via collisions or interactions with impellers can cause fragmentation of aggregates, culminating in pellet break-up and dispersion.

An environment of high mechanical shear may be simulated in shake-flask fermentations by the introduction of glass beads, with collisions between particles and beads inducing fragmentation of cells and thus morphological change. Figure 3.9 demonstrates the effect of various quantities of glass beads on pellet dispersion in cultures with an initial in-flask spore inoculum level of 10^4spores/ml. The degree of dispersion was calculated using equation 3.1.

$$\text{Degree of dispersion } \phi_1 = \frac{pellet\ count\ per\ ml}{number\ of\ spores\ inoculated\ per\ ml} \quad \text{Equation 3.1}$$

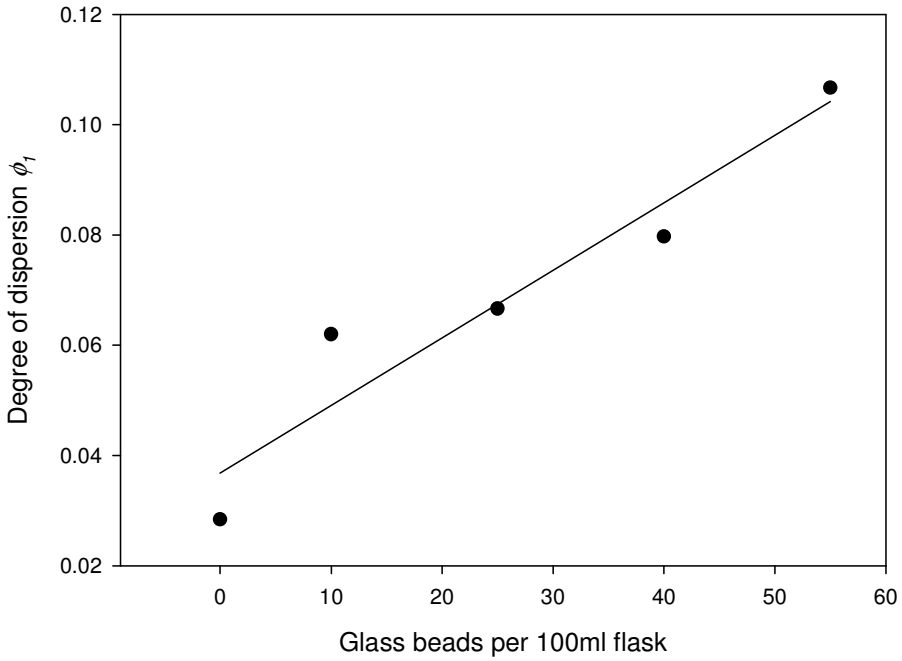


Figure 3.9: The impact of varying concentrations of glass beads on pellet break-up and dispersion at day 12, $R^2 = 0.91$.

Shear, as affected by the inclusion of glass beads, greatly increased the dispersion of pellets of *Streptomyces hygroscopicus* var. *geldanus* (Figure 3.9). As shear was intensified by the inclusion of incrementing amounts of glass beads, the degree of dispersion of pellets increased linearly. Hotop et al., (1993), also reported that the use of glass spheres in cultures of *Penicillium chrysogenum* resulted in considerably smaller pellets than in flasks without. This trend is also evident in Figure 3.10.

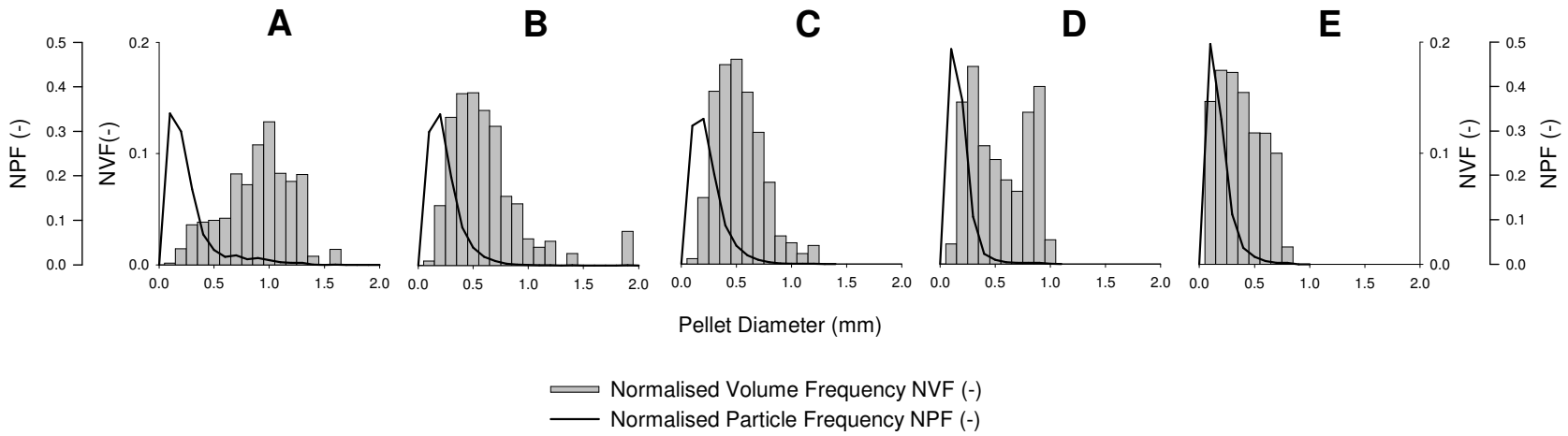


Figure 3.10: Image analysis of day 12 fermentations supplemented with A: 0; B: 10; C: 25; D: 40; and E: 55 glass beads per 100ml culture flask.

Figure 3.11 demonstrates the increase in pellet count and reduction in pellet diameter with heightening shear. Similar to previous results geldanamycin synthesis was again found to be dependent on decreasing pellet size, substantiating the relationship between morphology and geldanamycin production.

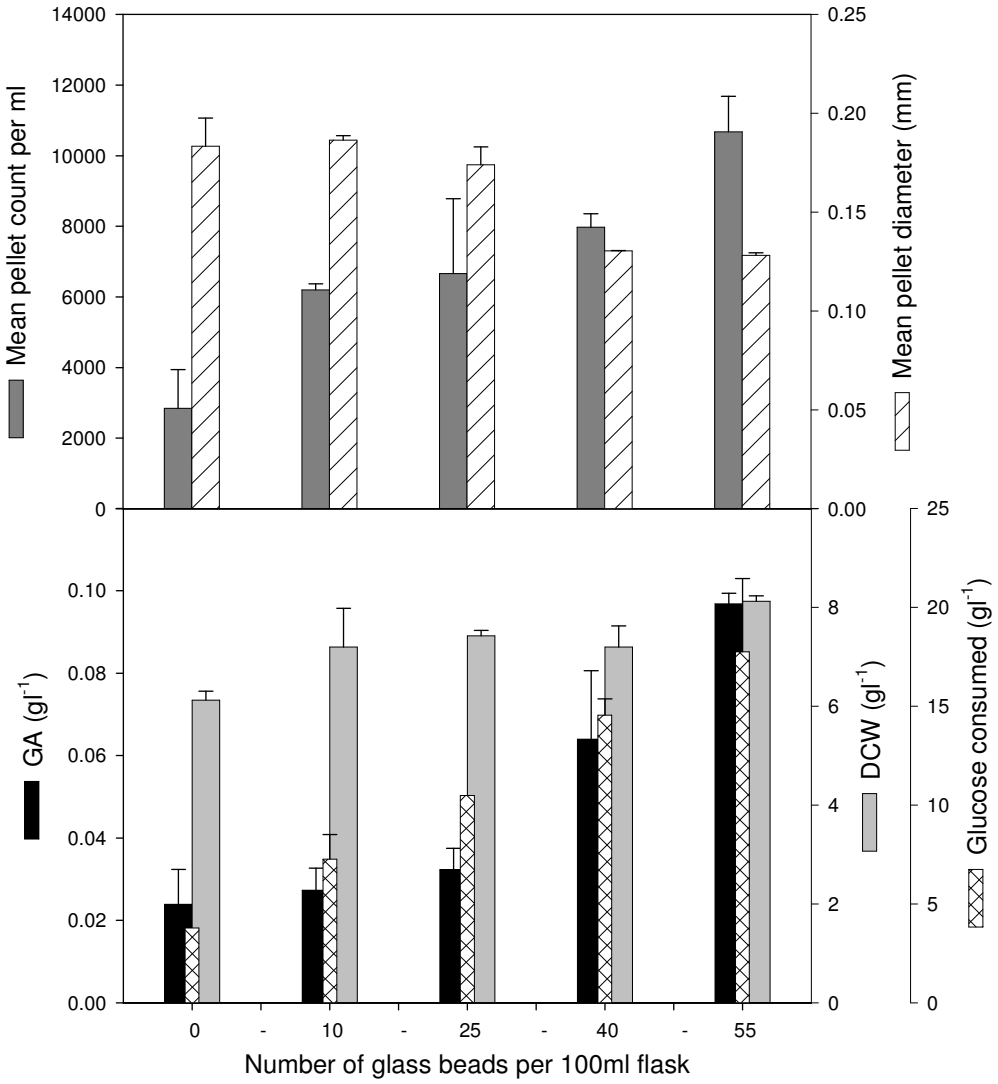


Figure 3.11: Dependency of antibiotic synthesis on morphological characteristics of cultures supplemented with varying amounts of 0.5mm diameter glass beads and analysed at day 12.

3.4 Antagonistic effect of divalent cations Ca^{2+} and Mg^{2+} on the morphological development of *Streptomyces hygroscopicus* var. *geldanus*.

Minerals such as calcium, magnesium and iron are essential constituents of any fermentation medium. They may be added as distinct components or may be present as impurities in other major ingredients. Many of these minerals are critical in secondary metabolism, with the yield of product varying linearly with the log concentration of the 'key' metal, excluding concentrations which are either insufficient or toxic to cell growth. Calcium and magnesium are examples of physiologically active divalent cations and are more often associated with growth and metabolism (Abbas and Edwards, 1990). It is almost impossible to over-emphasise the role of calcium in biological systems, with calcium dependent steps controlling a number of metabolic pathways (Frausto da Silva and Williams, 2001). Magnesium is also an essential ion for all life forms with all known DNA polymerases having active sites requiring two magnesium ions (Frausto da Silva and Williams, 2001).

A number of publications have examined the impact of magnesium and calcium on the physiology of cells in terms of growth yields and secondary metabolite formation (Abbas and Edwards, 1990, Cheng et al., 1995, Young et al., 1985, Basak and Majumdar, 1975, Danova et al., 1997). For instance, calcium and magnesium were both seen to be particularly effective in reducing antibiotic titres of actinorhodin by *Streptomyces coelicolor* (Abbas and Edwards, 1990). Cheng et al., (1995), and Basak and Majumdar, (1975), observed similar findings, with rapamycin production by *Streptomyces hygroscopicus* and kanamycin synthesis by *Streptomyces kanamyceticus* under specific negative control by magnesium and calcium salts respectively. In contrast, Young et al., (1985), and Byrne and Greenstein, (1986), observed that Mg^{2+} exerted a stimulatory effect on lincomycin production by *Streptomyces lincolnensis* and gilvocarcin V production by *Streptomyces arenae* that was independent of cell growth. There is speculation that the apparent stimulatory

activity of magnesium ions reflects its role as an ammonium ion-trapping agent, thereby eliminating nitrogen catabolite repression in fermentations (Byrne and Greenstein, 1986, Omura et al., 1980). In addition, magnesium has been demonstrated to reduce the degradation rate of clavulanic acid in cultures of *Streptomyces clavuligerus* (Roubos et al., 2002).

Previous reports have also detailed that cations may be involved in the aggregation and flocculation of micro-organisms. The ability of Ca^{2+} ions to induce aggregation has previously been demonstrated in cultures of *Chlamydia trachomatis* (Majeed et al., 1993), *Escherichia coli* (Onoda et al., 2000), *Streptococcus downei* (Rose, 2000), and *Saccharomyces cerevisae* (Mill, 1964). There have been few previous reports on the effects of divalent cations on the morphological development of *Streptomyces* spp., although Okba et al., (1998), demonstrated that the divalent cations Mg^{2+} and Ca^{2+} stimulated pellet formation in submerged cultures of *Streptomyces azureus*. The present study aims to determine whether inclusion of the divalent cations magnesium and calcium to optimised Bennett's medium exerts any influence on morphological development or geldanamycin synthesis in submerged cultures of *Streptomyces hygroscopicus* var. *geldanus*.

Optimised Bennett's fermentation medium was supplemented with various concentrations of Mg^{2+} and Ca^{2+} ions ranging from 0.025-0.5 gl^{-1}. Evidence of a concentration dependent effect on geldanamycin production was apparent as indicated in Figure 3.12.

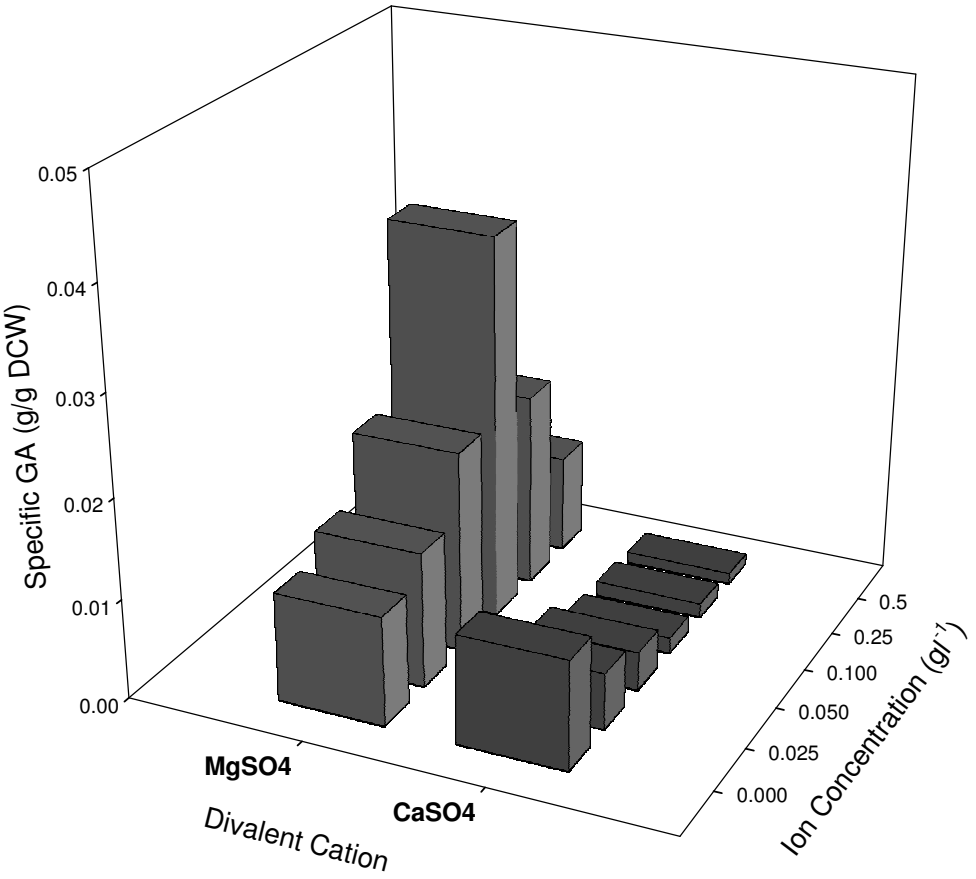


Figure 3.12: The effect of calcium and magnesium ion concentration on geldanamycin production at day 12.

Increasing concentrations of calcium were observed to have a detrimental effect on geldanamycin production. Conversely, cultures supplemented with magnesium ions exhibited improved antibiotic synthesis, with the yield of geldanamycin on biomass enhanced almost 3.5 fold in cultures containing $0.1 gl^{-1}$ magnesium compared to the control.

The time of addition of the metal ions to fermenting cultures was found to be significant for both antibiotic production and morphological development. Figure 3.13 depicts how supplementation of magnesium in the early phase of growth is beneficial to antibiotic production, but later additions resulted in lesser production, approximating that of the control if added on the 7^{th} day.

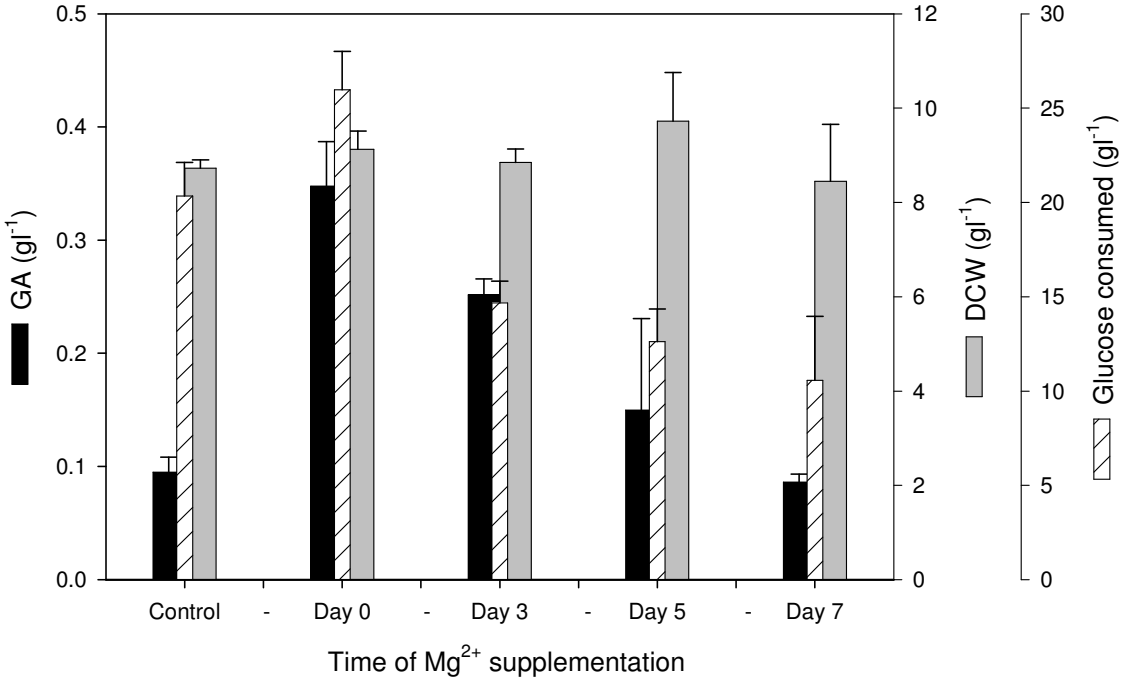
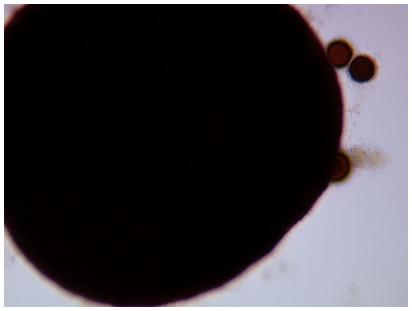


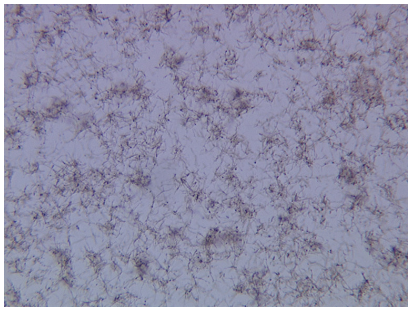
Figure 3.13: Impact of time of addition of $MgSO_4$ ($0.1gl^{-1}$) to cultures analysed at day 12. Cultures were supplemented at days 0, 3, 5, and 7.

Such time dependent effects have been referred to previously. Omura et al., (1980), investigating the impact of magnesium phosphate on leucomycin production by *Streptomyces kitasatoensis*, found that significant stimulation occurred when added at early to middle logarithmic phase of growth but the addition at later stages gave smaller or no effect on antibiotic synthesis. These findings imply that the reaction(s) mediated by ions during the growth period may be a pre-requisite either directly or indirectly for the operation of the biosynthetic pathways by which secondary metabolites are produced. Indeed, magnesium has previously been associated with the activation of enzymes involved in the primary glycolytic pathway (Gaikwad et al., 1992). Such physiological effects may also occur in *Streptomyces hygroscopicus* var. *geldanus* as glucose consumption was observed to be over 2.5 times greater when Mg^{2+} was added at culture initiation rather than at day 7, although biomass levels were comparable.

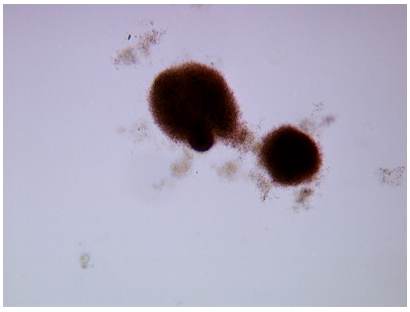
Microscopic examination of safranin stained biomass indicated that the cations also affected the morphological development of the filamentous organism (Figure 3.14). The inclusion of calcium ions was found to promote aggregation whereas magnesium supplemented cultures yielded predominantly dispersed growth with shorter, more branched hyphae visible. It was confirmed that the ability of the metal ions to induce morphological variation was also reduced when added at progressively later stages of growth, until cultures regressed to control-like behaviour. This suggests that these ions are affecting the germination and development of mycelial pellets of *Streptomyces hygroscopicus* var. *geldanus*. When the ions are added after germination has occurred, the effect is gradually reduced. The initial fermentation period has previously been reported to be extremely important for the overall morphological development of *Aspergillus niger*; permanent aggregates were detected at 18 hours while by 24 hours the pelleted morphological profile was established (Papagianni and Mattey, 2006). Generally, within the first five days of growth the subsequent morphological profile is determined after which it is difficult to engineer superficially, as proven in this instance with the inclusion of divalent cations to cultures of *Streptomyces hygroscopicus* var. *geldanus*.



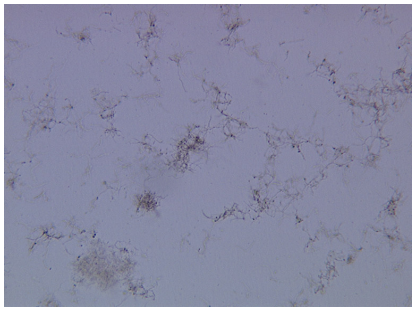
A: Ca^{2+} added at day 0 (40x)



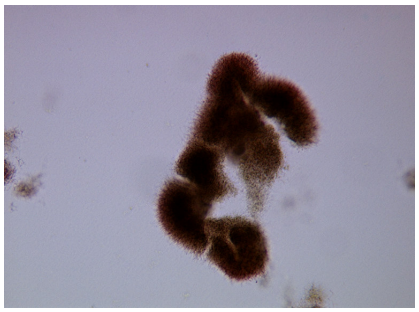
E: Mg^{2+} added at day 0 (100x)



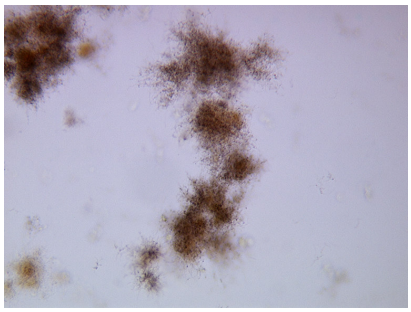
B: Ca^{2+} added at day 3 (40x)



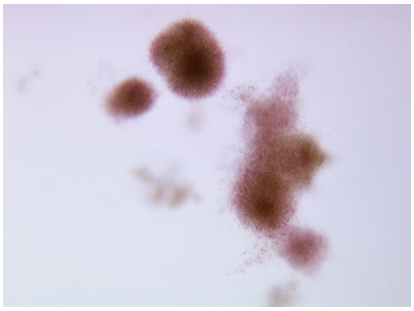
F: Mg^{2+} added at day 3 (100x)



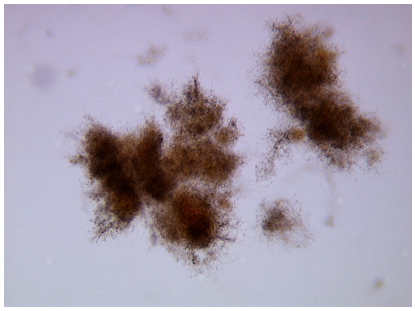
C: Ca^{2+} added at day 5 (40x)



G: Mg^{2+} added at day 5 (40x)



D: Ca^{2+} added at day 7 (40x)



H: Mg^{2+} added at day 7 (40x)

Figure 3.14: Time dependent effects of the addition of divalent cations ($0.1gl^{-1}$) on morphology as analysed at day 12 at 40 or 100 times magnification.

The microscopic analysis of cultures detected substantial divergence in the morphology of the organism when grown in the presence of different cations from the initiation of fermentation. Certainly, quantification of morphology indicated that cultures grown in a calcium rich environment exhibited increased aggregation of cells, with pelleted growth observed. Figure 3.15 revealed the occurrence of a concentration dependent shift in the morphological profiles of cultures supplemented with calcium.

Figure 3.16 clearly illustrates that calcium ions exerted a powerful mechanism of aggregation within the fermentation. Incrementing amounts of Ca^{2+} initiated a 40% increase in pellet diameter across the concentration range. Significant decreases in the pellet count, up to 70%, were also determined, reflecting the extent of the aggregation. Volumetric concentrations of geldanamycin were found to be dramatically decreased by 85% in cultures exhibiting larger pellet sizes, providing substantial evidence that morphological behaviour affects antibiotic synthesis in *Streptomyces hygroscopicus* var. *geldanus*.

Morphological quantification of the magnesium supplemented cultures analogous to that carried out in Figure 3.15 and Figure 3.16 was only feasible up to a concentration of $0.05gl^{-1}$. Cultures supplemented with Mg^{2+} concentration in excess of this exhibited dispersed, free filamentous mycelia and the image analysis system was developed for the detection and quantification of pelleted morphology only. Nonetheless, microscopic analysis illustrates the extreme divergence between the morphologies of the two cultures (Figure 3.17).

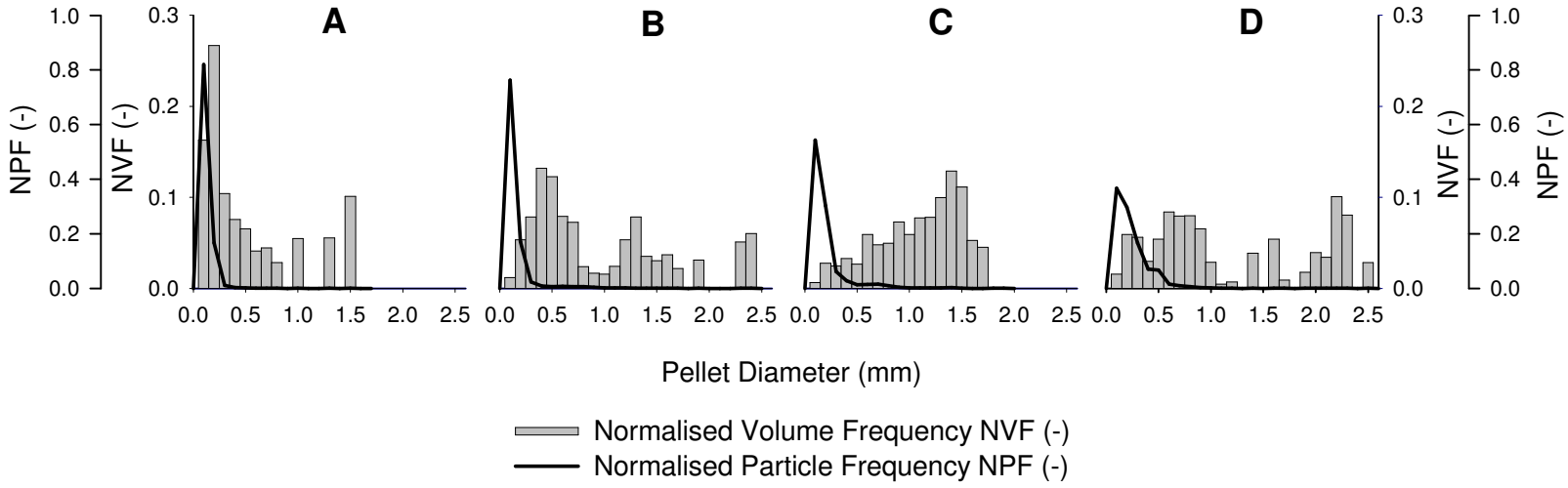


Figure 3.15: Day 12 morphological profiles of cultures grown in media with varying concentrations of $CaSO_4$, A: 0.01 gl^{-1}; B: 0.05 gl^{-1}; C: 0.1 gl^{-1}; D: 0.5gl^{-1}.

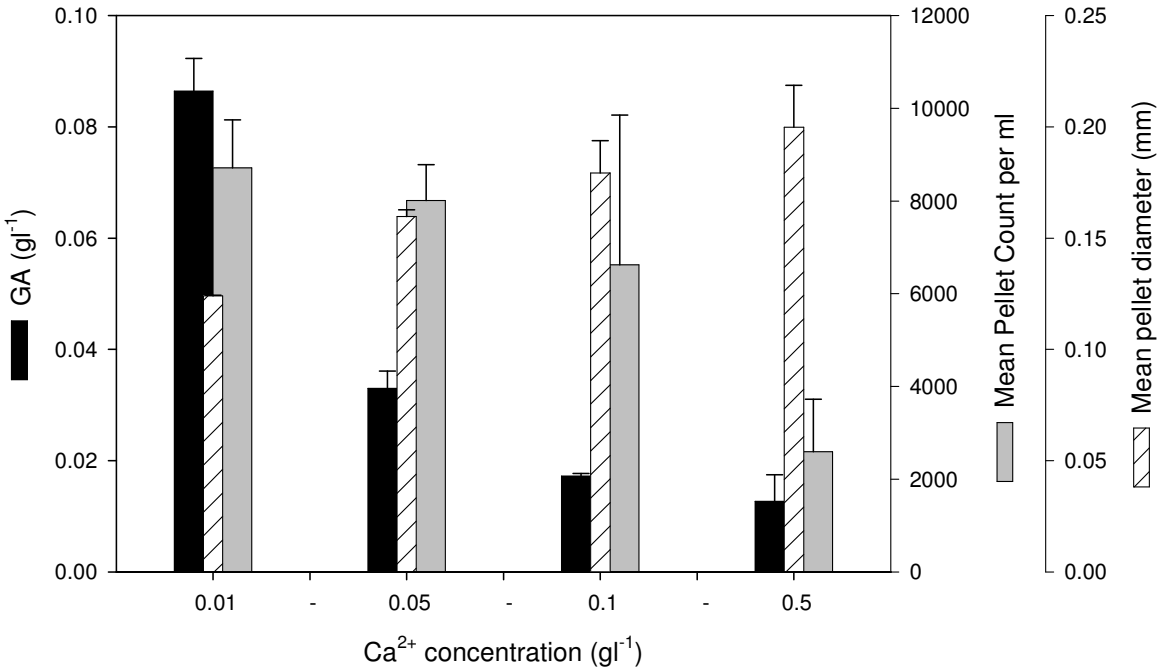


Figure 3.16: Influence of $CaSO_4$ on antibiotic synthesis, pellet size and pellet count per ml in submerged fermentations analysed at day 12.

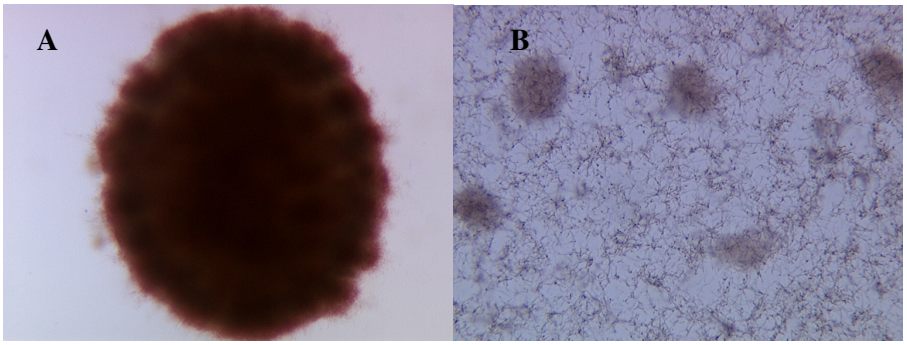


Figure 3.17: Diverse morphology of cultures supplemented with cations and examined at day 12. A: $CaSO_4$ ($0.1gl^{-1}$), 4 times magnification; B: $MgSO_4$ ($0.1gl^{-1}$), 10 times magnification.

An analysis of the rheological properties of the fermentation broths revealed that while both the control and calcium supplemented cultures conformed to Newtonian behaviour, the dispersed filamentous magnesium population exhibited non-Newtonian flow characteristics (Figure 3.18).

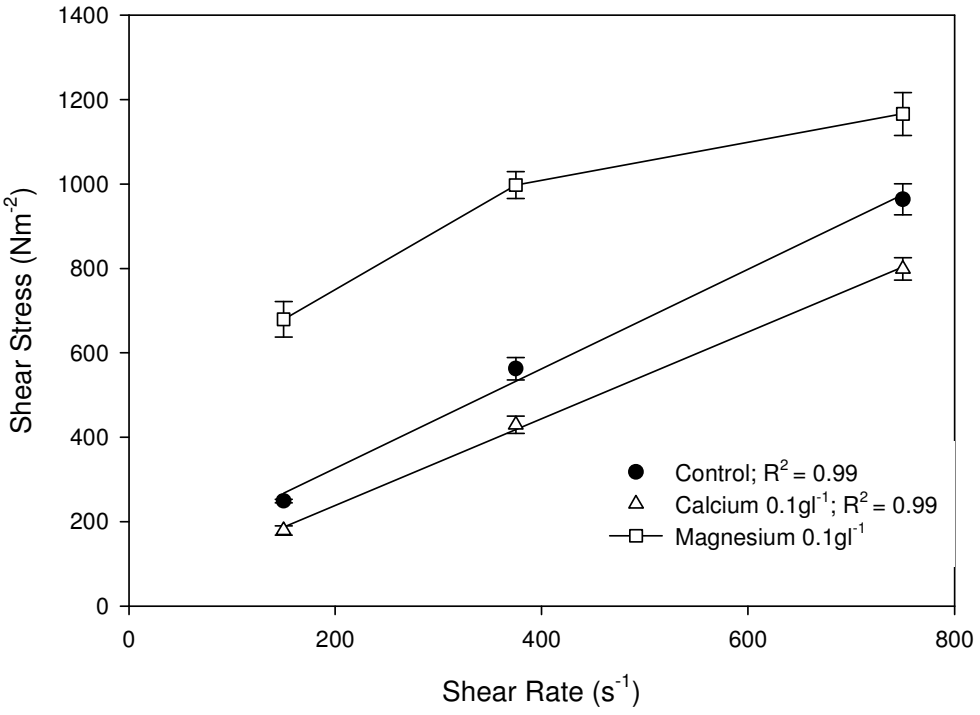


Figure 3.18: Relationship between shear rate and shear stress in fermentations with respect to cation inclusion at day 12.

Calculation of the flow behaviour index, n, according to Equation 2.2 confirmed that the rheological behaviour of magnesium cultures was consistent with pseudoplastic power law conduct, with n=0.34 (Table 3.2). Pseudoplastic fluids are also described as shear-thinning because the apparent viscosity of the fluid decreases with increasing shear rate. Many biological fluids are classified as pseudoplastic, and in particular free filamentous fermentation broths, where increasing shear rate pulls the intertwined mycelial structure apart and aligns the hyphae, thereby reducing broth viscosity (Nienow, 1990, Sousa et al., 2002). Indeed, Warren et al., (1995), noted that the rheology of three Actinomycetes that also synthesise secondary metabolites obeyed pseudoplastic behaviour.

Fujita et al., (1994), calculated that the shear rate for 100ml media in a 300ml shake flask rotating at 150rpm, similar to the fermentation conditions employed in these experiments, was $28s^{-1}$. Thus, using Equation 2.3, the apparent viscosity of the three fermentation broths was estimated. As expected, and in agreement with the findings of Tamura et al., (1997), morphological properties have a significant impact on the viscous nature of streptomycete cultures: the larger the pellet size, the lower the viscosity of the broth.

Table 3.2: Rheological characteristics of fermentation broths containing divalent cations.

Sample	Flow Behaviour Index (n)	Fluid Consistency Index (K) (Ns^nm^{-2})	Apparent Viscosity (μ_a) @ $28s^{-1}$ (Nsm^{-2})
Control	0.85	3.50	2.14
Ca^{2+} $0.1gl^{-1}$	0.93	1.71	1.35
Mg^{2+} $0.1gl^{-1}$	0.34	126.40	14.02

Many previous reports have agreed on a general consensus: that the addition of polyvalent cations induces aggregation, whereas polyvalent anions suppress it (Braun and Vecht-Lifshitz, 1991, Okba et al., 1998). Nonetheless, in this

instance two divalent cations, magnesium and calcium, caused antagonistic effects in cultures of *Streptomyces hygroscopicus* var. *geldanus*. Addition of calcium encouraged mycelial clumping, as predicted by the studies previously mentioned, whereas magnesium addition instigated the dispersion of mycelial fragments, resulting in free filamentous growth.

A number of mechanisms have been proposed which pertain to explain how cations stimulate aggregation. For example, Kim and Kim, (2004), proposed that the insoluble form of calcium salts functions as a physical support in the formation of pellets in *Streptomyces coelicolor* A3 (2). However, this hypothesis does not explain aggregation in this system, as a soluble solution of calcium ions was utilised. Yeast flocculation based on Ca^{2+} ions acting as 'cationic bridges' between cells is widely accepted (Domingues et al., 2000, Smit et al., 1992, Mill, 1964). Indeed, many potential anionic sites in the walls of gram-positive bacteria such as *Streptomyces* spp. would be available to sequester cations. For example, carboxyl, phosphoryl groups and secondary polymers such as teichoic acids constitute a proportion of gram-positive cell walls and would be candidates for cation binding (Archibald, 1974, Beveridge, 1989). In addition, several cell surface proteins that specifically bind calcium ions have previously been characterised. Yousef and Espinsa-Urgel, (2007), analysed the bacterial genomes of 351 prokaryotes and defined seven families of large cell surface proteins, three of which are associated with cell surface interactions and cell-cell adhesion. Interestingly, many of these proteins contained calcium binding domains suggesting that this cation plays a significant role in their functioning. Indeed, aggregation of *Kluyveromyces bulgaricus* and *Rhizobium leguminosarum* are mediated by Ca^{2+} dependent cell surface proteins (Smit et al., 1989, Thiebault and Coulon, 2005). In eukaryotes, 'EF hand' calcium binding proteins such as calmodulin are ubiquitous (Yonekawa et al., 2005). Calcium binding to calmodulin causes a small change in the geometry of the β-sheet which rotates the helices revealing hydrophobic sites (Figure 3.19).

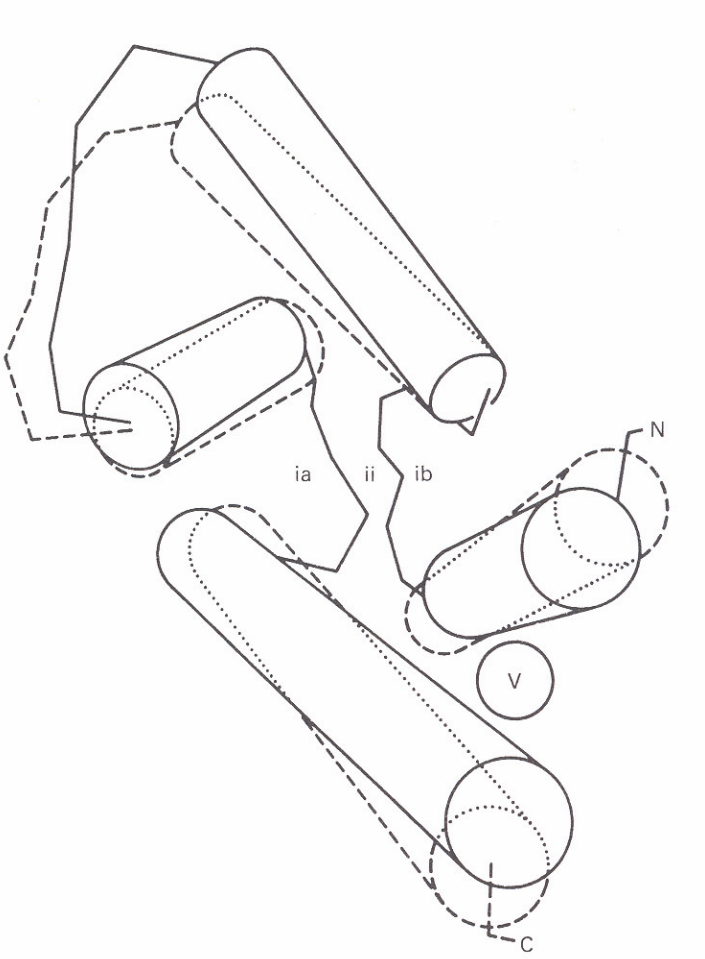


Figure 3.19: The proposed helix/helix motion in the calmodulin family of proteins on binding calcium. Calcium ions bind at EF hands (ia) and (ib) and the sites are connected by a short β-sheet (ii). The rolling of the helices reveals hydrophobic sites (V) (Frausto da Silva and Williams, 2001).

The 14-3-3 proteins are a family of conserved regulatory molecules expressed in all eukaryotic cells. Like calmodulin they bind to a variety of cellular proteins and modulate their function (Ishii and Kurachi, 2002). Analogous to calmodulin, common cellular divalent cations such as calcium have been shown to interact with 14-3-3 proteins causing a conformational change which is manifested as an increase in hydrophobicity (Manak and Ferl, 2007). Another calmodulin like protein, CabB, with two 'EF' hand motifs binding two calcium ions has recently been isolated from *Streptomyces coelicolor* A3(2)

and following the binding of calcium a conformational change in the protein was observed (Yonekawa et al., 2005).

Thus, as a certain degree of conservation and homology is often demonstrated between the Actinomycetes, it is feasible to suggest that the strain utilised in this study, *Streptomyces hygroscopicus* var. *geldanus* could possess a similar Ca^{2+} binding cell surface protein. It is postulated that in a similar fashion, Ca^{2+} binding induces a conformational change in this surface protein which is manifested as an increase in cell hydrophobicity and ultimately the attraction of cells causing pellet formation and aggregation. Certainly cell surface hydrophobicity has previously been associated with the flocculence of *Saccharomyces cerevisae* (Smit et al., 1992), *Aspergillus niger* (Ryoo and Choi, 1999), and *Streptomyces tendae* (Vecht-Lifshitz et al., 1989), suggesting that it may be the driving force between hyphal interaction and pellet formation.

Supporting the theory that cell surface hydrophobicity, as affected by divalent ions, was controlling the morphology of *Streptomyces hygroscopicus* var. *geldanus*, was the visual observation that dispersion of the Ca^{2+} supplemented pelleted cultures was unachievable, even in high shear environments containing large amounts of glass beads. Lu et al., (1998), also reported that shear stress could not disrupt hydrophobic aggregates due to their much greater strength. Indeed, even the presence of the strong calcium chelator EDTA at 1M concentrations could not disperse the aggregates in this study, similar to the findings of Kelstrup and Funder-Nielsen, (1972). Thus, in order to elucidate whether the antagonistic influences of divalent cations on morphology were as a result of differences in cell surface hydrophobicity, it was decided to thoroughly investigate this parameter.

Analysis of the CSH of *Streptomyces hygroscopicus* var. *geldanus* by application of the contact angle method determined that the cell wall of a control culture was hydrophilic with a contact angle of 37.65 ± 4.1. This is in agreement with the general consensus that the cell walls of gram-positive

bacteria are typically hydrophilic making the surfaces good interfaces for binding of surrounding metal ions (Beveridge, 1989). However, a great disparity was detected between the surface hydrophobicity of cells cultured with high concentrations of divalent cations. Figure 3.20 illustrates that pelleted cultures containing Ca^{2+} ions were strongly hydrophobic. In contrast, Mg^{2+} supplemented cultures exhibiting dispersed morphology were classed as hydrophilic, with contact angles of less than 45° detected.

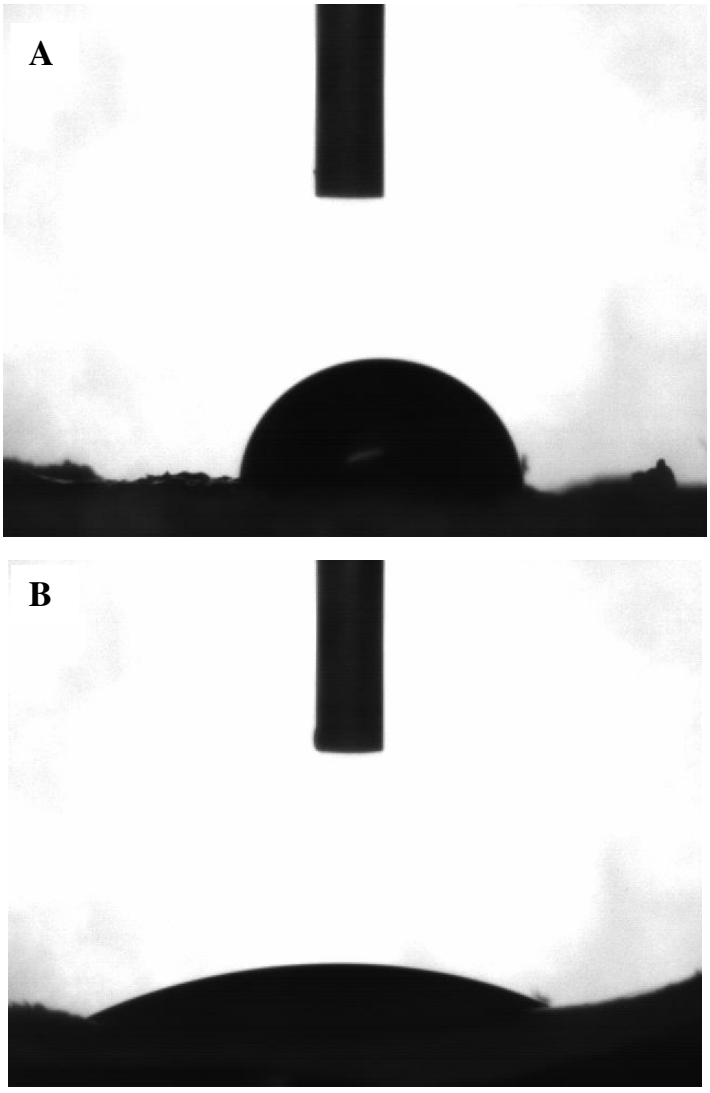


Figure 3.20: Cell surface hydrophobicity (CSH) measurement of day 12 *Streptomyces hygroscopicus* var. *geldanus* cultures supplemented with divalent cations calcium and magnesium at concentrations of $0.5gl^{-1}$. A: Ca^{2+} Contact angle $\theta = 76.64 \pm 5.5$; B: Mg^{2+} Contact angle $\theta = 22.89 \pm 4.9$.

This result confirms that the disparity in morphology induced by divalent cations is likely to be caused by a change in the CSH of these cultures. According to the DVLO theory the potential energy of particle-particle interaction comprises of the van der Waals attractive energy and the electrical double bond layer energy (Lu et al., 1998). However, for particles with a hydrophobic surface, hydrophobic interaction energy is the dominant factor being one or two magnitudes larger than that of the others (Lu et al., 1998). The relationship between pellet size and CSH demonstrated in Figure 3.21 verifies the theory that this parameter significantly affects morphological development, with increasing hydrophobicity instigating aggregation and pellet formation.

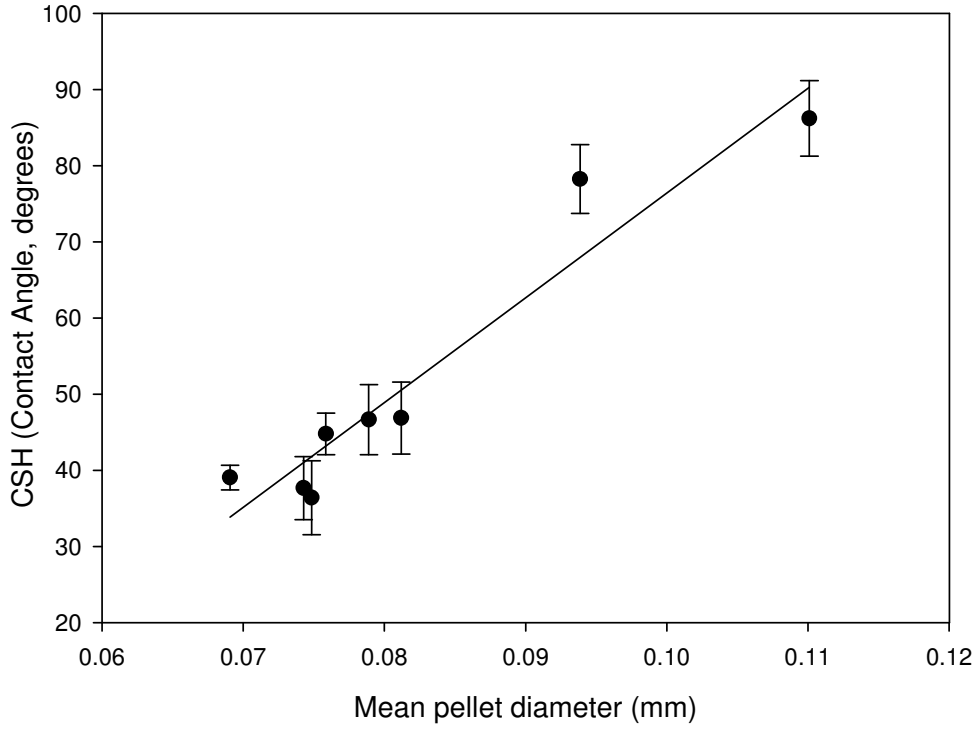


Figure 3.21: Relationship between pellet size and CSH in cultures with varying Ca^{2+} concentrations at day 12 ($R^2 = 0.92$).

The inclusion of the divalent cations calcium and magnesium to optimised Bennett's fermentation medium impacted significantly on the growth, morphological development and antibiotic production of *Streptomyces hygroscopicus* var. *geldanus*. These cations elicited distinct responses, with concentration dependent aggregation and repression of geldanamycin production characteristic of calcium addition. In contrast, magnesium induced dispersion of cells and yielded high geldanamycin synthesis. The findings presented in this study have identified the mechanism behind such morphological disparity and the results strongly suggest that physiochemical interactions due to cell surface hydrophobicity are involved in the flocculation process of *Streptomyces hygroscopicus* var. *geldanus.*

Many reports have indicated that physiochemical properties such as cell hydrophobicity can influence agglomeration of cells. Dynesen and Nielsen, (2003), studied morphology in mutants of *Aspergillus nidulans* by disruption of the genes *dewA* and *rodA* which encode hydrophobic proteins located in the cell wall of the organism. Their study observed that absence of the hydrophobins reduced the surface hydrophobicity of conidiospores and caused a concomitant increase in the percentage of free mycelia. Analogously, flocculence of *Saccharomyces cerevisae* was found to be induced by an increase in cell surface hydrophobicity (Smit et al., 1992), demonstrating that flocculent yeast cells are highly hydrophobic.

Whereas it was initially thought that each microbial strain possessed a fixed degree of hydrophobicity, it is now clear that environmental factors can modulate cell surface hydrophobicity, for example the concentration of available carbon (Vecht-Lifshitz et al., 1990, Thiebault and Coulon, 2005); surface or submerged fermentation (Castellanos et al., 1997); temperature (Castellanos et al., 1997, Singleton et al., 2001); ionic strength (Vadillo-Rodriguez et al., 2004); and indeed metal ions (Smit et al., 1992). In this study, conditions yielding larger pellet sizes were found to be strongly hydrophobic, with the presence of Ca^{2+} ions mediating this change in hydrophobicity.

However Mg^{2+} ions were incapable of replacing the Ca^{2+} ions indicating that calcium was specifically required for aggregation.

Accordingly, it was hypothesised that both Mg^{2+} and Ca^{2+} cations become bound at anionic sites on cell surface proteins, but induce different conformational changes which are manifested by an alteration in cell surface hydrophobicity. Specific calcium binding proteins have been isolated from a number of organisms, including the calmodulin-like CabB from *Streptomyces coelicolor A3(2)*. It is probable that *Streptomyces hygroscopicus* var. *geldanus* expresses a similar cell surface protein, and, in a Ca^{2+} free state, such binding regions would exhibit considerable electrostatic repulsion between their large numbers of negatively charged centres, thus explaining why in the absence of Ca^{2+} ions tightly packed mycelial pellets were not observed. Indeed, the cell surface hydrophobicity of dispersed mycelia formed in the presence of Mg^{2+} ions was determined to be hydrophilic. Such antagonism between Ca^{2+} and Mg^{2+} may be justified by comparing the main properties of these divalent cations (Table 3.3).

Table 3.3: Comparison of the properties of magnesium and calcium ions (Frausto da Silva and Williams, 2001).

Property	**Mg^{2+}**	**Ca^{2+}**
Size (Å)	0.6	0.95
Bond lengths	Regular	Irregular
Bond angles	Regular	Irregular
H_2O exchange (s)	10^{-6}	10^{-9}

Frausto de Silva and Williams, (2001), clearly detailed the differences between Mg^{2+} and Ca^{2+} ions. They explained that differences in structure could not be greater: Mg^{2+} has a simple regular symmetry with Ca^{2+} having irregular bond lengths and angles, and there are significant differences in the radius of the ions with Mg^{2+} over 33% smaller. Kinetic differences between Mg^{2+} and Ca^{2+} have

also been documented with the exchange of water approximately 10^3 times slower from Mg^{2+}; thus Ca^{2+} constitutes a fast trigger ion in biology using strong binding in rapid exchange (Frausto da Silva and Williams, 2001). Calcium has an ability to bind to a large number of centres at once, a function not shared by magnesium or smaller cations giving it a selective binding chemistry based on its size to charge ratio. It has previously been demonstrated that although Mg^{2+} may bind weakly to at least 10% of some of the Ca^{2+} 'EF' binding motifs present on calmodulin, Mg^{2+} will not act to produce the same response since the conformational change produced in the protein would not be the same (Frausto da Silva and Williams, 2001). These findings may serve to explain why Mg^{2+} supplemented cultures did not display the same hydrophobic phenotype as those with similar concentrations of Ca^{2+}.

This study has established that divalent cations induce morphological divergence by altering cell surface hydrophobicity, with Mg^{2+} encouraging the dispersion of mycelia, and Ca^{2+} promoting aggregation and pellet formation. This variation in mycelial dispersion accounts for the observed difference in geldanamycin production, indicating that geldanamycin production by *Streptomyces hygroscopicus var. geldanus* is strongly dependent on the morphological type and is negatively affected by mycelial aggregation. It was concluded that in this instance, filamentous or dispersed growth behaviour, as represented by the magnesium cultures, was optimal for geldanamycin synthesis.

3.5 Relationship between morphology and geldanamycin synthesis

Chapter 1 detailed a range of microbiological and physiochemical factors that impact on the morphology of filamentous organisms. A number of these were examined in Sections 3.1 - 3.4 as suitable methods to stimulate morphological diversity. Cultures were supplemented with the surface active agents Tween80 (0.01; 0.1; 1% v/v), Triton X100 (0.01; 0.1% v/v) and silicone antifoam (0; 0.1; 0.5; 1; 5; 10 % v/v); varying inoculum sizes (10^2; 10^3; 10^4; 10^5; 10^6 spores/ml); numbers of glass beads (0; 10; 25; 40; 55 per 100ml flask); and concentrations of calcium and magnesium ions (0.01; 0.05; 0.1; 0.5 gl^{-1}). However, morphological data was unavailable for those fermentations supplemented with high concentrations of magnesium ions (0.1 and 0.5 gl^{-1}) as these cultures were observed to be completely dispersed and of free filamentous behaviour.

Collating the data obtained, and expressing geldanamycin production as a specific yield per gram of dry cell weight, it was apparent that a linear relationship between antibiotic production and the mean pellet diameter existed (Figure 3.22). This confirms that morphology plays a significant role in the regulation of geldanamycin production by *Streptomyces hygroscopicus* var. *geldanus*.

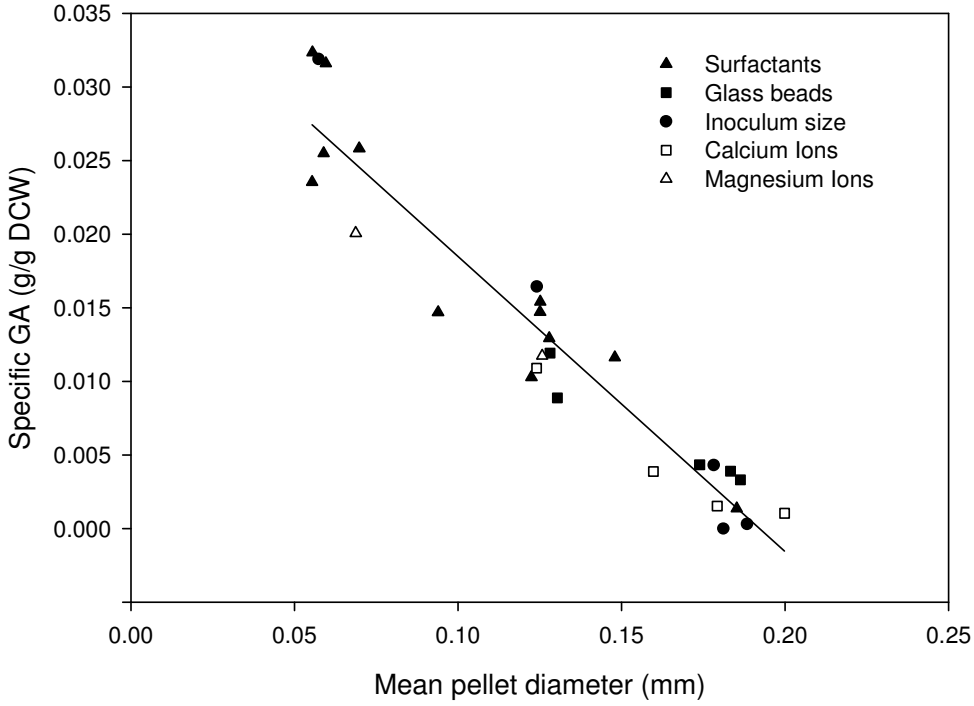


Figure 3.22: Relationship between mean pellet size and specific geldanamycin yield in morphologically diverse cultures at day 12, $R^2 = 0.93$.

Nonetheless, when comparing morphology with metabolite production it is important that the morphological parameter selected provides a true representation of the pellet population. Statistically, the mean pellet diameter may not describe the data accurately. For example, whilst smaller particles may be numerically in the majority, they may not contribute significantly to the overall biomass volume. Consider Figure 3.23 (A). In this instance 74% of the particles are between 0.05-0.2mm in diameter and pellets within this range also make up 98% of the biomass volume. However, in Figure 3.23 (B) whilst 76% of the pellets have a diameter between 0.1 and 0.4mm, these particles constitute less than 3% of the biomass volume. Evidently a depiction of the pellet population in Figure 3.23 (B) by measure of the mean pellet diameter would be inaccurate as it is unlikely that the low volume of biomass at this value contributes greatly to geldanamycin production.

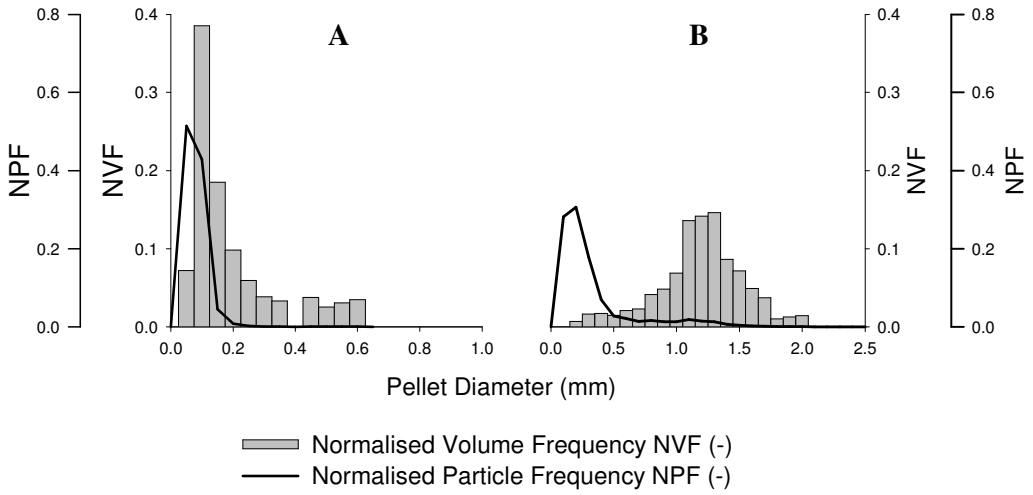


Figure 3.23: Selection of an appropriate morphological parameter to accurately describe the morphology in differentially distributed pellet populations.

Thus, it was concluded that the pellet diameter at which the majority of the biomass volume was located i.e. the mode would provide a more statistically accurate representation of the pellet population in cultures of *Streptomyces hygroscopicus* var. *geldanus*. Indeed, a correlation between geldanamycin synthesis and the pellet diameter at which the mode of the biomass was located was obtained (Figure 3.24). The relationship was observed to be non-linear, indicating that significant geldanamycin production occurred only in cultures where the majority of the biomass volume consisted of small particles, generally less than 0.5mm in diameter.

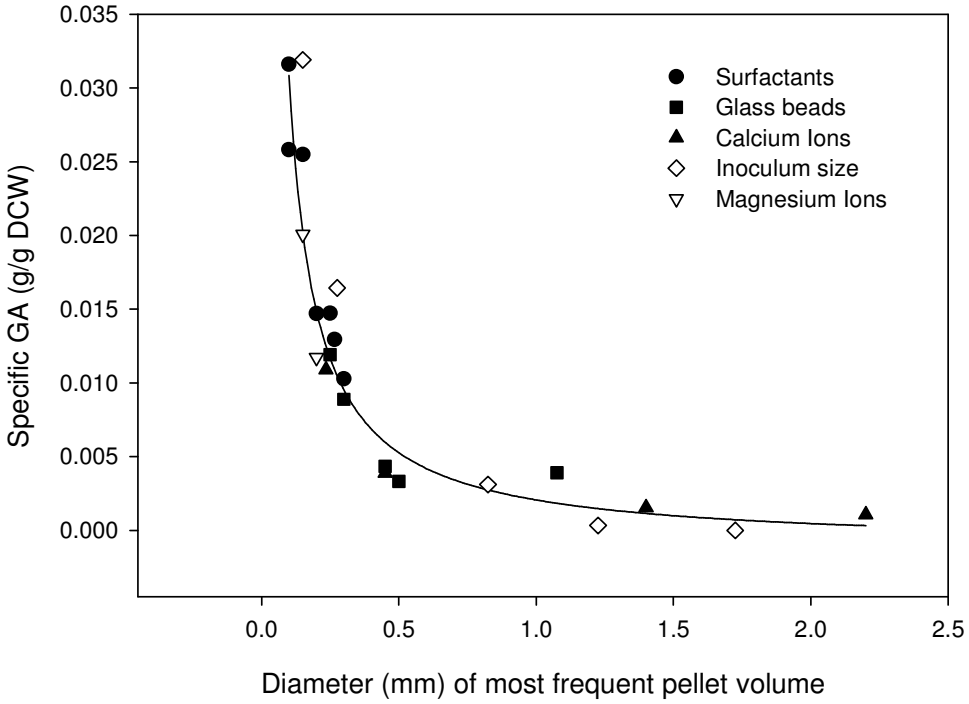


Figure 3.24: Relationship between the pellet diameter (mm) of most frequent pellet volume and geldanamycin synthesis analysed at day 12.

This study confirms that geldanamycin synthesis within *Streptomyces hygroscopicus* var. *geldanus* is highly dependent on the morphological profile of the culture. In order to maximise geldanamycin production it is necessary to limit pellet formation to an appropriate size, and it has been demonstrated that chemical, microbiological or physical factors may be exploited for this purpose.

On the contrary, the production of Nikkomycin Z by *Streptomyces tendae* was observed to increase steadily with pellet size, with larger pellets (2mm) twofold more productive than smaller pellets (0.35mm) (Vecht-Lifshitz et al., 1992). They postulated that diffusion barriers in aggregates prevented the release of immature secondary metabolites, which either stimulate secondary metabolism or act as substrates for the 'mature' products. However, more often than not a decrease in pellet size is associated with increased product formation, in

agreement with the findings presented here. Indeed, Tamura et al., (1997), demonstrated that small mycelial sizes were more effective for tylosin production by *Streptomyces fradiae*. Hotop et al., (1993), also established that a decrease in the diameter of *Penicillium chrysogenum* pellets from 1.0 to 0.6mm resulted in a doubling of concentration of penicillin G.

Section 1.5 introduced the hypothesis that the relationship between morphology and secondary metabolite production is caused by alterations in cell physiology (Figure 3.25).

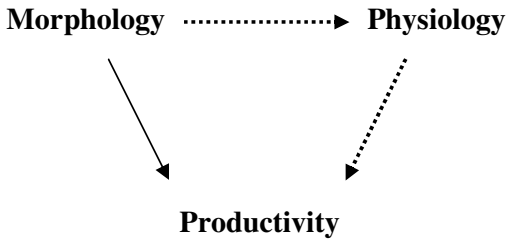


Figure 3.25: Proposed relationship between morphology, physiology and geldanamycin synthesis in *Streptomyces hygroscopicus* var. *geldanus*.

Such relationships are logical considering that pellets may suffer from oxygen limitation if they are large than a critical size, due in part both to mass transfer limitations within pellets and the low solubility of oxygen in water which ensures it is frequently a limiting substrate in submerged fermentations. The observation that significant concentrations of geldanamycin were only produced in pellet populations where the modal biomass volume consisted of pellets no greater than 0.5mm in diameter is particularly interesting, given that the critical radius for oxygen penetration in mycelial pellets has been estimated to be between 0.13 and 0.2mm (Wittler et al., 1986).

Additionally, in this study, improved glucose consumption rates were observed in pellets of a smaller size. Figure 3.26 demonstrates that for those cultures supplemented with varying concentrations of glass beads, the amount of

glucose consumed increased concomitantly with the degree of dispersion of biomass. This evidence suggests that smaller pellet sizes may be more physiologically active than larger pellet populations.

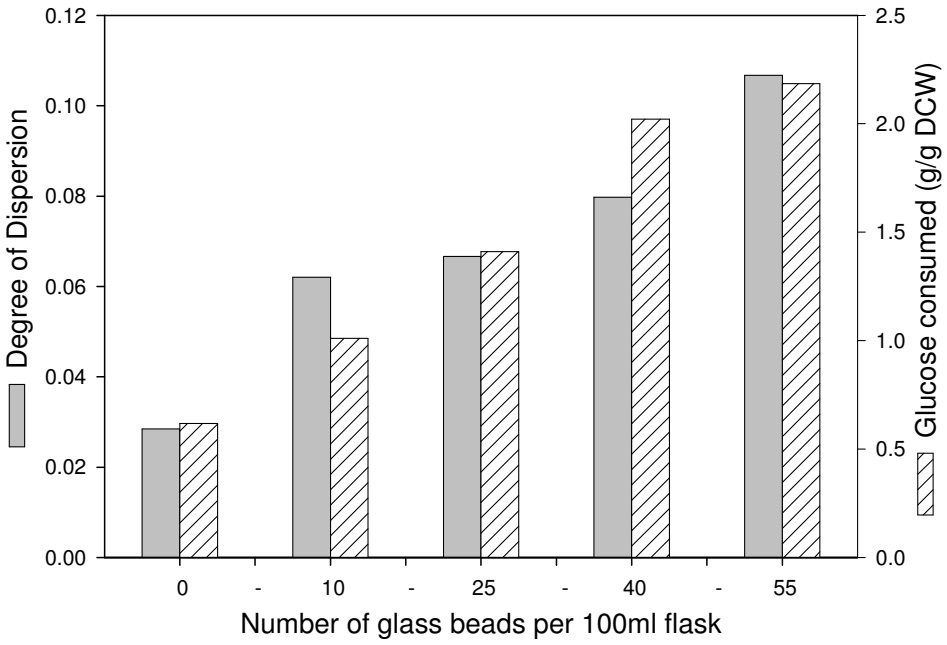


Figure 3.26: The effect of morphology on glucose consumption in cultures supplemented with varying concentrations of glass beads.

3.6 Relationships between morphology, physiology and geldanamycin synthesis

In many instances mycelial aggregates or pellet populations are advantageous for secondary metabolite synthesis and there have been some reports on the relationship between the structure and size of pellets and their activity (Znidarsic and Pavko, 2001). On these occasions, the major cause of physiological heterogeneity occurs due to diffusional limitation of oxygen into the pellets. When cells are present in clumps or pellets, intra-particle resistance is likely to be significant as oxygen has to diffuse through the solid pellet to reach the interior cells with the magnitude of this resistance depending on the size of the clumps (Doran, 1995). Thus it is generally accepted that at some point during the fermentation the internal sections of pellets will start to degenerate due to substrate and oxygen limitation, and the proportion of metabolically active biomass is often restricted to the outer zone of less dense mycelium (Bader, 1986, Papagianni, 2004).

Using microprobe measurements, Wittler et al., (1986), demonstrated that the dissolved oxygen tension in mycelial pellets of *Penicillium chrysogenum* decreased until a penetration depth of approximately 130-200µm where the signal reached zero. Such observations have led to the phenomenon of the pellet 'critical radius' above which oxygen limitation is a significant problem. The critical radius has also been estimated for pellets of *Aspergillus niger* (150µm) (Huang and Bunay, 1973); *Aspergillus awamori* (185µm) (Cui et al., 1997); and *Aspergillus oryzae* (145µm) (Carlsen et al., 1996). Pellets larger than the critical size are often hollow in the centre as a result of autolysis caused by strong limitation of oxygen into the inner zones (Bellgardt, 1998).

In this study the effect of morphology on the synthesis of geldanamycin in cultures of *Streptomyces hygroscopicus* var. *geldanus* was examined and production was consistently greater in those pellet populations where the mode of the biomass volume was composed of pellets which were equal to or less

than 0.5mm in diameter (Section 3.5). It is postulated that, as a consequence of the diffusional limitations of oxygen within pellets, large pellets (≥0.5mm in diameter) are physiologically different to small pellets (<0.5mm in diameter), thus contributing to a disparity in geldanamycin production in morphologically diverse cultures. In order to conclusively prove this hypothesis and determine the relationship between the three parameters, a means of measuring cell physiology must be combined with image analysis and productivity data.

Assays using tetrazolium salts are widely employed to measure the redox potential of metabolising cells and have been demonstrated to give good estimates of cell physiology and respiration rates in a variety of organisms (Roehm et al., 1991, Kairo et al., 1999, Kuhn et al., 2003, Meletiadis et al., 2001, Roslev and King, 1993, Mauss et al., 1997). In Chapter 2 the specific respiration rate (RR_s) of *Streptomyces hygroscopicus* var. *geldanus* was quantitatively measured using the tetrazolium salt XTT. Nonetheless, in Chapter 2 the tetrazolium assay for metabolic activity was applied to cultures conforming to 'control' morphology. The hypothesis in question infers that metabolic activity is influenced by pellet size; therefore it is prudent to demonstrate that the assay maintains reproducibility across a diverse range of morphological types (Figure 3.27).

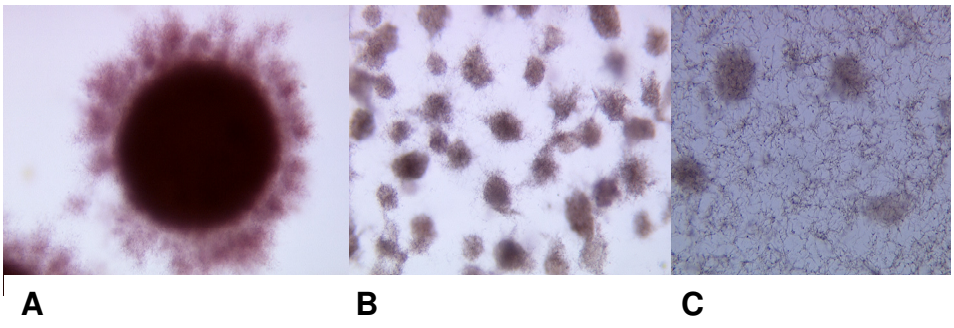


Figure 3.27: Morphological diversity in safranin stained cultures of *Streptomyces hygroscopicus* var. *geldanus.* A: Pelleted biomass (40x magnification); B: 'Control' loosely clumped morphology (40x magnification); C: Dispersed growth (100x magnification).

The rate of any reaction in a solid phase catalyst such as a microbial pellet can be considerably influenced by mass transfer processes (Doran, 1995). For effective diffusion to occur within a pellet, a concentration gradient is required and the concentration of a substrate inside a pellet must be smaller than at the surface for any mass transfer to take place (Nielsen et al., 2003a). Figure 3.28 demonstrates the typical substrate concentration profile for a spherical pellet. The concentration of substrate in the particle C_{As} is lower than that of the bulk liquid C_{Ab}; it is this concentration gradient that drives mass transfer of substrate to reaction sites in the pellet interior (Doran, 1995). In this study, if the rate of reduction of XTT was much faster than the rate of mass transfer, cells near the centre of the pellet would be starved of XTT substrate and C_{As} would reach zero. Thus the measurement of formazan product would not truly reflect the physiological status of the culture.

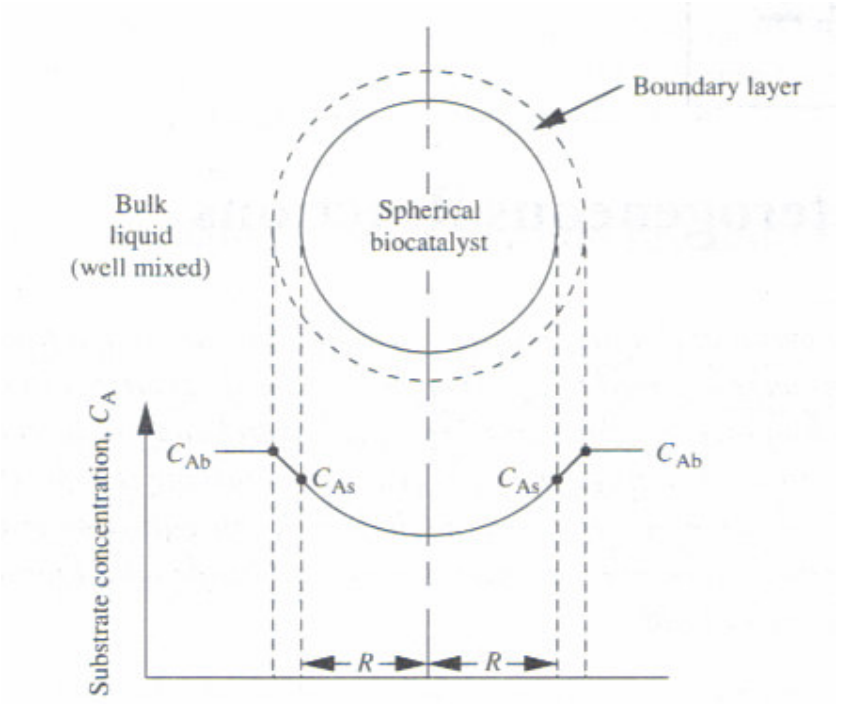


Figure 3.28: Typical substrate concentration profile for a spherical biocatalyst (Doran, 1995).

Nonetheless, in aerobic reactions the mass transfer of oxygen is much more likely to limit the rate of reaction than the mass transfer of any other substrate

due to its poor solubility in aqueous solutions (Doran, 1995). Indeed, most other substrates give rise to an approximately uniform behaviour inside a pellet (King, 1998). Therefore if the concentration of XTT/MEN in the pellet core falls to a low level or even zero, it is more than likely that these cells are already starved of oxygen and are metabolically inactive. For example, Carlsen et al., (1996) determined the critical radius for glucose ($1.8gl^{-1}$) uptake by pellets of *Aspergillus oryzae* to be 1255µm compared to 145µm for air containing 21% oxygen at 1atm (Carlsen et al., 1996).

Figure 3.29 demonstrates that the XTT assay was not subject to substrate limitation within the appropriate biomass concentration range regardless of the morphological growth pattern. The preliminary results in Figures 3.27 and 3.29 also endorse the theory that dispersed morphology is more metabolically active than pelleted growth behaviour.

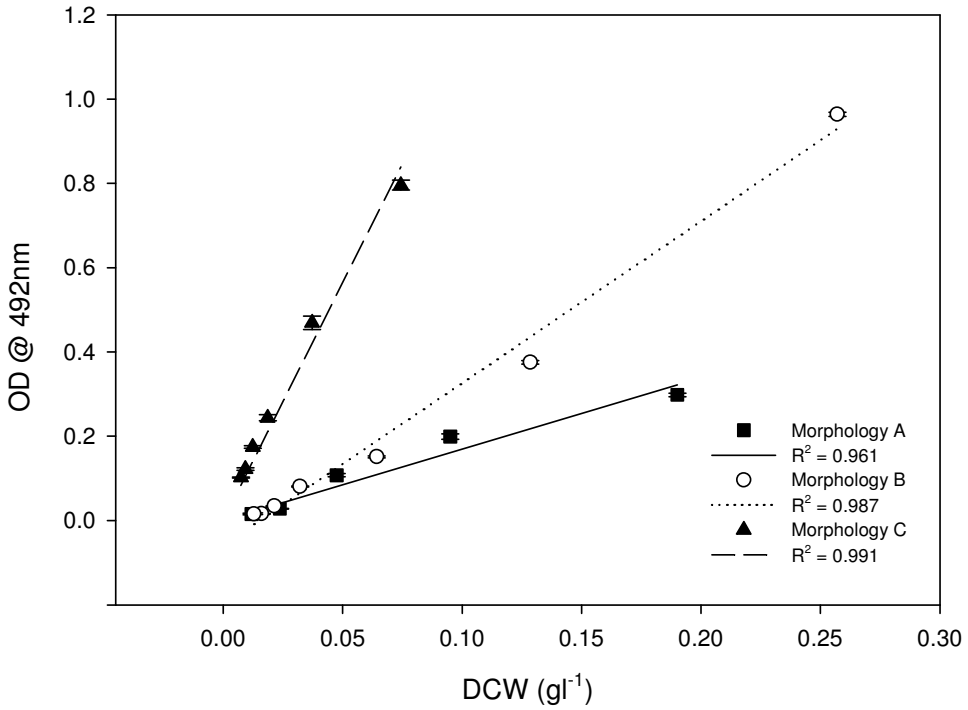


Figure 3.29: Linearity and reproducibility of XTT assay for metabolic activity in morphologically diverse cultures. The morphology of cultures A, B and C may be visualised in Figure 3.27.

In order to stimulate morphological diversity cultures were once again supplemented with the surface active agents Tween80 (0.01; 0.1; 1% v/v) and silicone antifoam (0; 0.1; 0.5; 1 % v/v); varying inoculum sizes (10^2; 10^3; 10^4; 10^5; 10^6 spores/ml); numbers of glass beads (0; 10; 25; 40; 55 per 100ml flask); and concentrations of calcium and magnesium ions (0.01; 0.05; 0.1; 0.5 gl^{-1}). The opaque nature of those cultures containing high concentrations of silicone antifoam (5 and 10 % v/v) prevented accurate determinations of the specific respiration rate. Morphological data was unavailable for those fermentations supplemented with high concentrations of magnesium ions (0.1 and 0.5 gl^{-1}) as these cultures were observed to be completely dispersed and of free filamentous behaviour.

Figure 3.30 demonstrates that geldanamycin production is intimately regulated by the respiration rate of the organism with a linear relationship existing between the two parameters. The exhibited morphological profile of the culture significantly affected the respiration rate of the organism (Figure 3.31). The most productive fermentations were found to be the most metabolically active and the mode of the biomass in those cultures consisted of pellets with a diameter of equal to or less than 0.5mm. Thus, factors which induce morphological diversity have a direct impact on the respiration rate of the organism and this in turn is the key driver of geldanamycin synthesis.

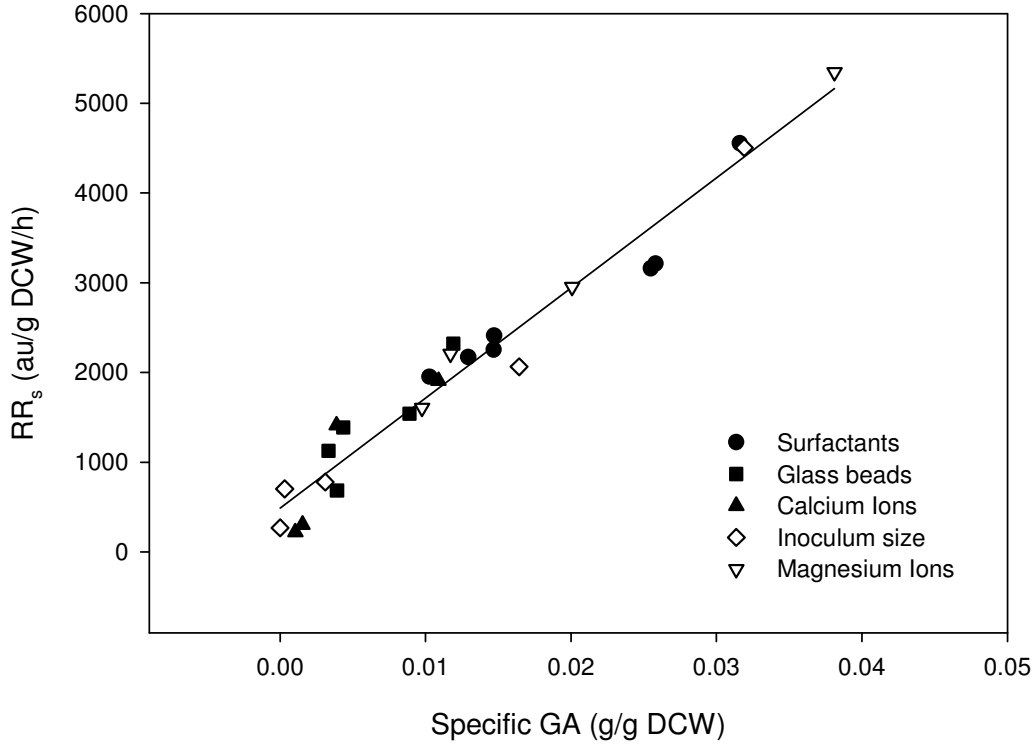


Figure 3.30: Linear relationship between geldanamycin synthesis and respiration rate at day 12, $R^2 = 0.96$.

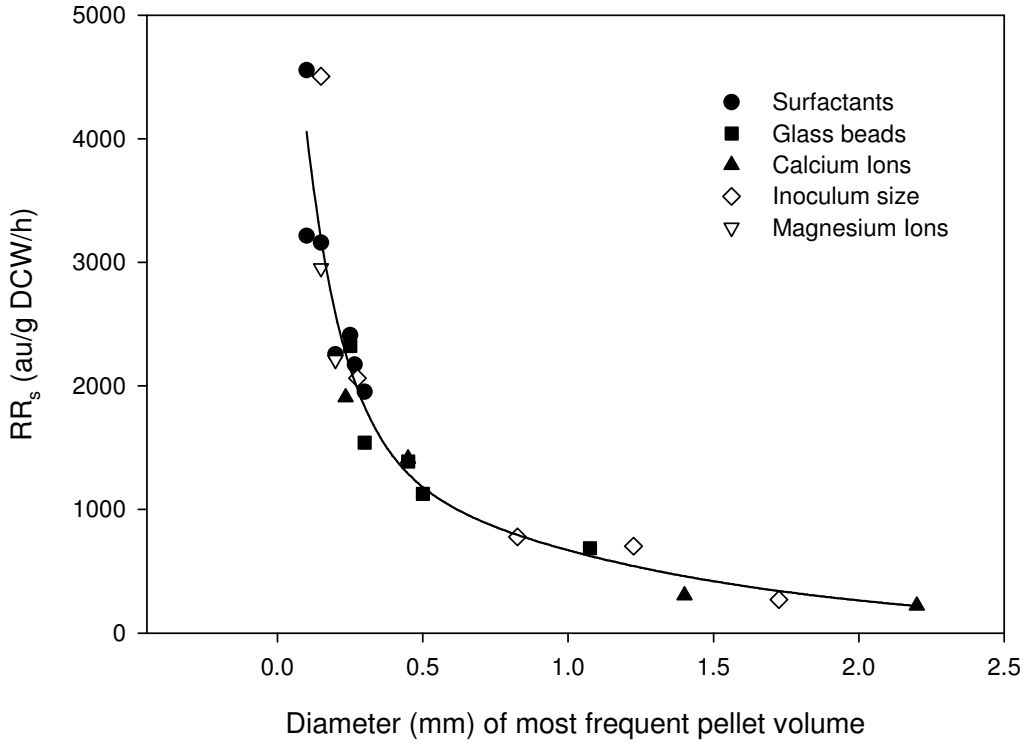


Figure 3.31: Evaluation of the relationship between morphology and the specific respiration rate RR_s at day 12.

Many previous reports have estimated the critical radius of fungal pellets to be between 130 and 200μm; however streptomycete fermentations do not produce tight pellets like many fungi (Bader, 1986). Thus, it may be interpreted from Figure 3.31 that pellets of *Streptomyces hygroscopicus* var. *geldanus* greater than 0.5mm in diameter are subject to oxygen limitation, which would estimate that the critical radius for this organism is approximately 250μm. At larger pellet sizes an increase in catabolic repression would be expected and indeed a decrease in the respiration rate was observed.

Nonetheless it is widely acknowledged that low specific growth rates and the onset of stationary phase initiate secondary metabolite synthesis. On that basis, it could well be assumed that those cultures with lower respiration rates would produce more geldanamycin. However, the aromatic amino acids and moieties which provide the building blocks for geldanamycin are amongst the most energetically expensive compounds to synthesise (Euverink, 1995). Indeed, Craig and Weber, (1998), quantified the energetic costs of the synthesis of amino acids in *Escherichia coli* on the basis of ATP equivalents and the aromatic amino acids tryptophan, phenylalanine, and tyrosine required 78.5, 63 and 56.5 ATP equivalents respectively, substantially greater than the next closest at 33 ATP equivalents. Thus, *Streptomyces hygroscopicus* var. *geldanus* must be in a sufficiently active metabolic state in order to synthesise considerable concentrations of geldanamycin.

3.7 Metabolic regulation of geldanamycin synthesis

Collectively, the findings in Section 3.6 have endorsed the theory that respiration rate is dictated by the pellet size distribution and this in turn affects secondary metabolism. However, it was decided to investigate whether, in a culture consisting of optimal size pellets, a parameter could be exploited which could exclusively regulate antibiotic production (Figure 3.32).

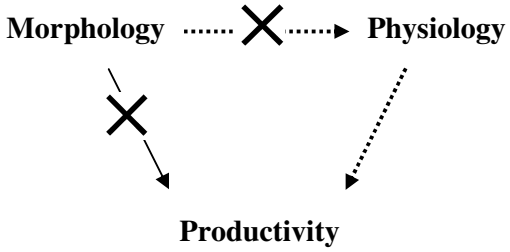


Figure 3.32: Proposed metabolic regulation of geldanamycin production.

The Shikimate Pathway was introduced in Chapter 1 and is illustrated in Figure 3.33. This pathway represents a reaction sequence which provides aromatic amino acids and moieties that constitute as metabolic precursors for many aromatic compounds, including geldanamycin. Up- or down- regulation of productivity in such metabolic pathways may be achieved by supplementation with metabolic intermediates or end-products. For example, rapamycin, produced by *Streptomyces hygroscopicus*, is an antifungal and antitumour macrolide whose cyclohexane moiety is derived from shikimic acid, an intermediate of the shikimate pathway (Fang and Demain, 1995). The inclusion of exogeneous shikimate to fermentation media strongly stimulated the production of rapamycin, with an increase of 125% observed (Fang and Demain, 1995). The α-aminoadipic acid (AAA) moiety of Cephamycin C produced by *Streptomyces clavuligerus* has been shown to be derived from lysine (Mendelovitz and Aharonowitz, 1982). Addition of lysine and diaminopimelate, an intermediate of the lysine production pathway, stimulated

specific antibiotic production by 75%, or by 4-fold with simultaneous addition (Mendelovitz and Aharonowitz, 1982).

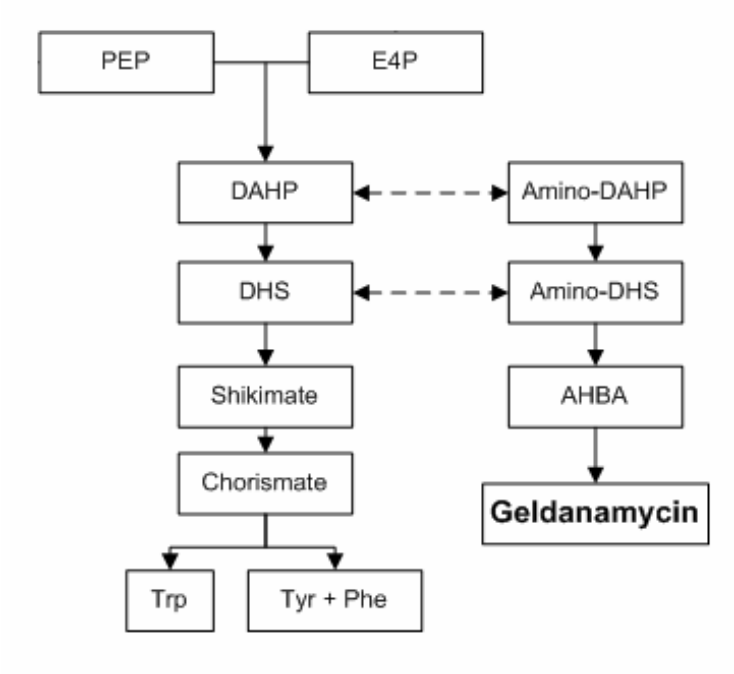


Figure 3.33: Schematic representation of the shikimate pathway.

In a number of pathways, amino acids also turn on the production of secondary metabolites and therefore function as inducers (Sanchez and Demain, 2002). However in this instance the aromatic amino acids represent end-products of the shikimate pathway and it was postulated that their supplementation could stimulate feedback repression of geldanamycin. The inclusion of shikimate intermediate molecules and aromatic amino acids to *Streptomyces hygroscopicus* var. *geldanus* fermentation broths and their effect on geldanamycin synthesis is represented in Figure 3.34.

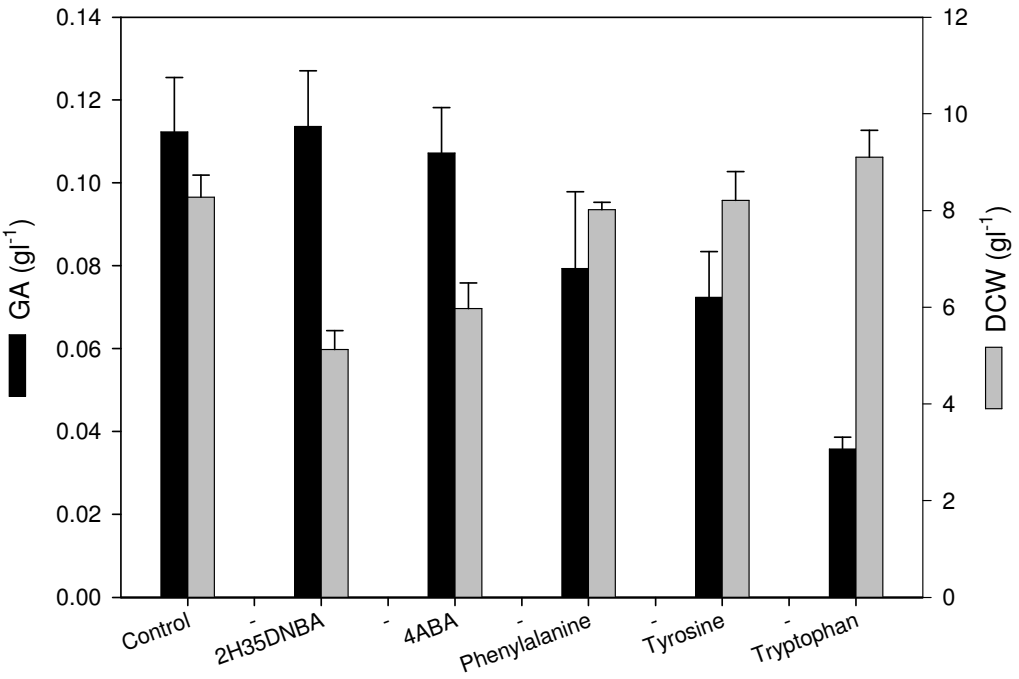


Figure 3.34: Impact of the inclusion of shikimate intermediate structural analogues 2-hydroxy-3,5-dinitro-aminobenzoic acid (2H35DNBA) and 4-aminobenzoic acid (4ABA) ($0.1gl^{-1}$); and aromatic amino acids tyrosine, phenylalanine and tryptophan (5mM) on geldanamycin synthesis at day 12.

The supplementation of amino-benzoates was found to exert negligible effects on geldanamycin production. In contrast, antibiotic synthesis was significantly affected by the inclusion of aromatic amino acids. These compounds are found in sources of complex nitrogen such as Yeast extract, Beef extract and NZ-Amine-A; thus they are inherently present in optimised Bennett's medium (Table 3.4). Whilst the supplementation of 5mM tyrosine and phenylalanine was observed to repress geldanamycin production by approximately 30% respectively, tryptophan inhibited geldanamycin synthesis by almost 70% compared to the control value while maintaining similar biomass production, behaviour indicative of feedback repression.

Table 3.4: Quantity of aromatic amino acids in Bennett's medium

	Tyrosine	**Tryptophan**	**Phenylalanine**
Yeast Extract (g/g)	0.0495	0.0085	0.0378
Beef Extract (g/g)	0.0066	0.0034	0.0234
NZ Amine A (g/g)	0.0280	0.0100	0.0400
Total Bennett's Optimised Medium (gl^{-1})	0.2243	0.0557	0.2636

The committed step and most tightly regulated reaction in the shikimate pathway is the condensation of phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P) to D-arabino-heptulosonate 7-phosphate (DAHP) by DAHP synthase (Bongaerts et al., 2001). As the first enzyme in the pathway, DAHP synthase therefore dictates the amount of cellular carbon flux directed down the cascade. DAHP synthases have been characterised in a few Actinomycetes only and in a few species of *Streptomyces spp*. (Euverink, 1995) It is speculated that AroAII type DAHP synthases in Actinomycetes have a specialised role in antibiotic synthesis, catalysing a critical step for the production of the precursor molecule AHBA which acts as a starter unit for polyketide assembly of ansamycin antibiotics (Gosset et al., 2001). These types apparently possess an altered substrate specificity in which either an aminated derivation of E4P is recognised or additional overall aminating ability exists. There is evidence to suggest that a single isoenzyme exists significantly feedback inhibited by tryptophan alone. For instance, Stuart and Hunter, (1993), found that DAHP synthase purified from *Streptomyces rimosus* was significantly inhibited by tryptophan.

A comparison of the morphological profiles of *Streptomyces hygroscopicus* var. *geldanus* cultures supplemented with varying concentrations of tryptophan in Figure 3.35 revealed that these fermentations do not conform to the relationships derived in Section 3.6. For example, the morphological profile and biomass volume mode was similar in the control, 1mM, and 5mM supplemented flasks and the respiration rates were comparable yet great

disparity regarding secondary metabolite production was observed (Figure 3.36).

Accordingly, this study has demonstrated that factors exist which do not appear to influence morphology or respiration but nonetheless affect geldanamycin synthesis in *Streptomyces hygroscopicus* var. *geldanus*. Despite the fact that the pellet population conformed to the optimum distribution for geldanamycin production, and that respiration rates remained constant between the cultures, the aromatic amino acid tryptophan exerted a negative control over antibiotic synthesis. This observed effect by an end-product of the shikimate pathway is consistent with feedback inhibition. Thus, this study has determined that whilst in many instances respiration rate is the key parameter affecting geldanamycin production, it is also possible to repress geldanamycin antibiotic synthesis in *Streptomyces hygroscopicus* var. *geldanus* by the inclusion of tryptophan and this represents an alternative means of controlling secondary metabolite production.

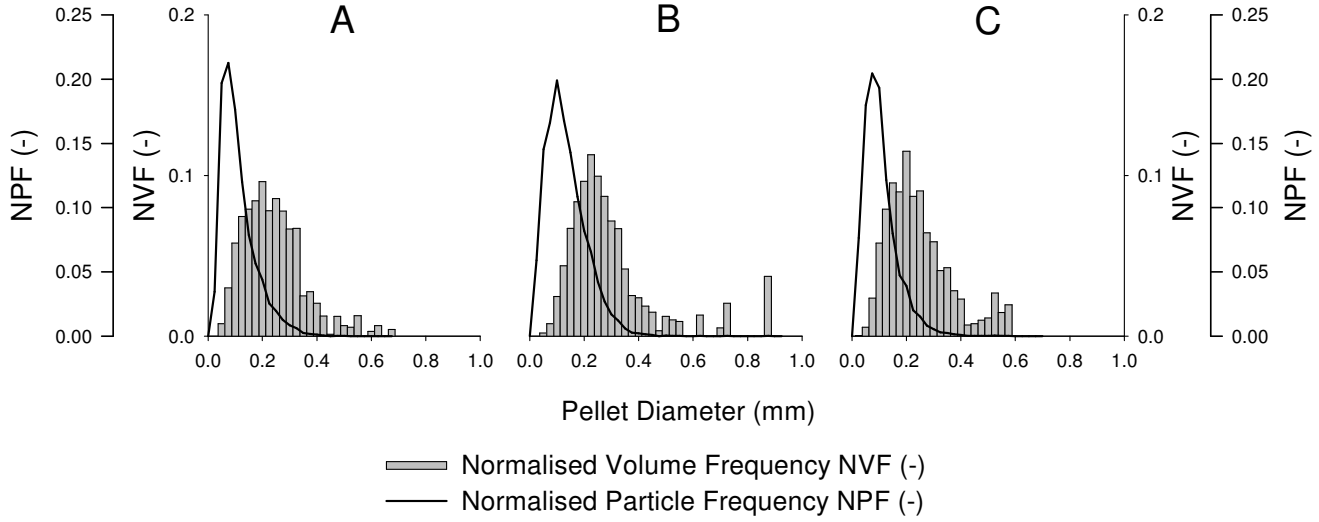


Figure 3.35: Morphological distributions of cultures at day 12 supplemented with varying concentrations of tryptophan, A: 0; B: 1; C: 5mM.

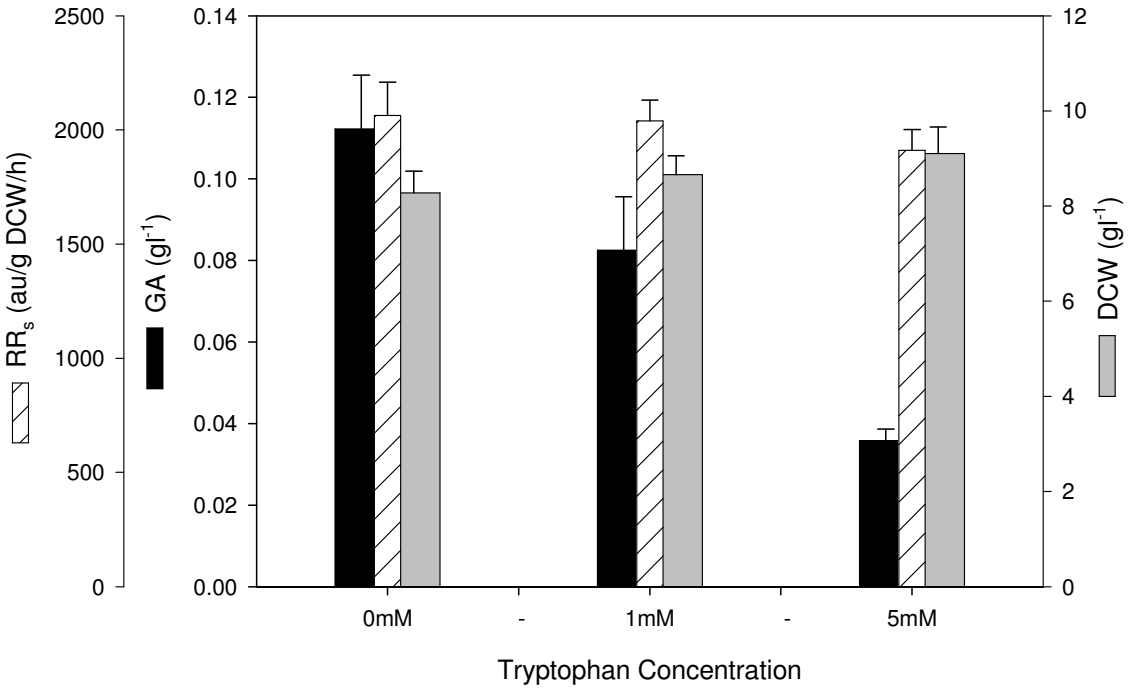


Figure 3.36: Inhibition of geldanamycin synthesis in cultures supplemented with varying concentrations of the aromatic amino acid tryptophan and analysed at day 12.

CHAPTER 4: CONCLUSIONS AND FUTURE RECOMMENDATIONS

4.1 Conclusions

Microbially produced secondary metabolites such as antibiotics are of tremendous economic importance. However, the majority of these are produced by filamentous organisms that exhibit diverse growth patterns in submerged cultures presenting challenges for industrial fermentations. Indeed, although the relationship between morphology and metabolite production has been the subject of some previous reviews (Braun and Vecht-Lifshitz, 1991, Papagianni, 2004, Whitaker, 1992), with a myriad of factors impacting on morphology, physiology and productivity there is discontinuity of thought regarding which parameter is the key driver of secondary metabolite synthesis. This study aimed to elucidate between these parameters using an appropriate model system.

Streptomyces spp. has long been identified as a comprehensive source of antibiotics, synthesising over 4000 bioactive compounds. Geldanamycin is a polyketide ansamycin antibiotic derived from the Shikimate pathway and produced by *Streptomyces hygroscopicus* var. *geldanus* in submerged fermentation. This compound has been shown to bind to and inhibit the function of heat shock protein 90 (Hsp90), a chaperone protein constitutively expressed at 2-10 fold higher levels in tumour cells compared to their normal counterparts. This suggests that geldanamycin may play a critical regulatory role in tumour cell growth and/or survival and the compound is currently being investigated as a novel chemotherapeutic agent.

Initial studies focused on the optimisation of a suitable method for geldanamycin recovery and detection from fermentation broth. The measurement of geldanamycin has traditionally been carried out using plate diffusion bioactivity assays; however this method is more qualitative than quantitative and high performance liquid chromatography represents a more suitable quantitative technique. Nonetheless, the low solubility of the compound in water based medium created a requirement for pre-processing steps and dilution of sample in the solvent acetonitrile. A significant amount of

cell associated product could also be recovered by inclusion of a sonication step prior to analysis.

To achieve high product yields, it is a prerequisite to design a proper production medium in an efficient fermentation process (Elibol, 2004). In this study Bennett's medium was selected as the most efficient for geldanamycin synthesis as previously recommended by DeBoer and Peterson, (1971). However Bennett's medium is nutritionally poor and consequently was found to be unable to sustain high levels of growth or antibiotic production. The production process was positively affected by the inclusion of higher glucose concentrations ($50gl^{-1}$), suggesting that this streptomycete strain is neither sensitive to carbon catabolite repression, nor is geldanamycin synthesis initiated by carbon source depletion. Antibiotic production was further stimulated by the supplementation of higher amino nitrogen concentrations in the form of Yeast extract, Beef extract and NZ-Amine A. The development of an optimised Bennett's medium significantly exceeded all previous shake-flask yields of geldanamycin. Indeed, a 6-fold increase in volumetric yield was observed. A fermentation time course characterised biomass accumulation, product formation and morphological development in optimised Bennett's medium over a 20 day period. The fermentation of *Streptomyces hygroscopicus* var. *geldanus* followed standard kinetics with characteristic exponential, stationary and decline phases of growth observed. The yield of the secondary metabolite geldanamycin reached a maximum as the organism entered stationary phase. However, the instability of geldanamycin represented a significant problem in the sampling of batch cultivations, and recovery of the antibiotic must be made before the degradation rate surmounts production. Thus the 48 hour interval up to day 14 represented the optimum sampling time for geldanamycin in batch fermentations.

The effects of chemical (surfactant inclusion), microbiological (inoculum size) and physical (shear) factors on the morphological development of *Streptomyces hygroscopicus* var. *geldanus* were examined. Surface active agents such as silicone antifoam, TritonX100 and Tween80 reduced the surface

tension of fermentation broth and it was apparent that both the type and concentration of surfactant could influence morphology, and, furthermore, geldanamycin synthesis. For all surfactants an increase in concentration (%v/v) resulted in a greater degree of dispersion of biomass with an increase in pellet count and a simultaneous decrease in the mean pellet size observed. Volumetric titres of geldanamycin could be correlated with morphology, and antibiotic synthesis appeared to be repressed by the formation of large pellets. Previous reports have detailed the influence of spore inoculum level on morphology. In agreement with these observations, this study established that pellet formation in *Streptomyces hygroscopicus* var. *geldanus* occurred at low spore inoculum levels, and pellet sizes decreased progressively with increasing concentration. A concomitant increase in geldanamycin synthesis was observed as the mean pellet diameter was reduced by 68%. An environment of high mechanical shear was stimulated by the introduction of glass beads to shake-flask fermentations, with collisions between particles and beads inducing fragmentation of cells and thus morphological change. Geldanamycin synthesis was found to be dependent on decreasing pellet size, substantiating the relationship between morphology and geldanamycin production.

In this study, inclusion of the divalent cations magnesium and calcium was also demonstrated to alter the morphology of *Streptomyces hygroscopicus* var. *geldanus*, provoking dispersion or aggregation of cells respectively, and stimulating great disparity in geldanamycin yields. Cell surface hydrophobicity assessment using the contact angle method revealed a great divergence between the cells cultured with high concentrations of either cation, suggesting that physiochemical interactions due to cell surface hydrophobicity are involved in the flocculation process of *Streptomyces hygroscopicus* var. *geldanus.* Whilst magnesium supplemented cultures were hydrophilic, the presence of calcium ions mediated significant increases in hydrophobicity and cultures exhibiting larger pellet sizes were found to be strongly hydrophobic. It is postulated that Ca^{2+} binding induced a conformational change in a surface protein which manifested as an increase in cell hydrophobicity and ultimately the attraction of cells causing pellet formation and aggregation.

Whereas information on morphology is often obtained by visual inspection and microscopy, an image analysis method was employed in this study allowing quantitative investigations to be made. Five methods which had previously been determined as suitable (surfactant inclusion; inoculum size; glass beads; calcium and magnesium supplementation) were utilised to stimulate morphological diversity in *Streptomyces hygroscopicus* var. *geldanus*. Geldanamycin production was consistently greater in those pellet populations where the mode of the biomass volume was composed of pellets which were equal to or less than 0.5mm in diameter. It has previously been noted that the deduction of general relationships between process variables, product formation, and morphology is inherently difficult since so many parameters influence these interrelationships (Papagianni, 2002). Nonetheless, in this instance, a number of different parameters were investigated for their ability to stimulate morphological diversity, and, persistently, the findings indicated that morphology regulates geldanamycin synthesis in *Streptomyces hygroscopicus* var. *geldanus*.

It was postulated that, as a consequence of the diffusional limitations of oxygen within pellets, large pellets (≥0.5mm in diameter) were physiologically different to small pellets (<0.5mm in diameter), contributing to a disparity in geldanamycin production in morphologically diverse cultures. Indeed, improved glucose consumption rates were observed in pellets of a smaller size, indicating that they may be more physiologically active. A number of authors have previously highlighted the need for further investigation of the relationships between morphology, physiology and productivity. Thus, in this study, image analysis was utilised in conjunction with an applicable quantitative assay for metabolic activity in order to elucidate any variations in physiology within these morphologically diverse cultures.

As a high fraction of dead or dormant cells present during any part of a bio-process can be detrimental; it is important to have accurate information on the physiological states of individual cells within a population (Nebe-von-Caron et al., 2000). Tetrazolium salt reduction represented a rapid, high-throughput, and

reproducible assay for the quantitative estimation of cell physiology in respiring cultures of *Streptomyces hygroscopicus* var. *geldanus*. Analogous with the determinations of Mauss et al., (1997), metabolic activity was characterised on the basis of a specific respiration rate (RR_s). It transpired that the most productive fermentations were also the most metabolically active, and the mode of the biomass in these cultures consisted of pellets with a diameter of less than 0.5mm. It was postulated that pellets equal to or larger than 0.5mm had exceeded the critical radius for oxygen diffusion and limiting concentrations of this vital nutrient resulted in a decrease in respiration rate.

Oxygen is often the limiting substrate in fermentations because of its low solubility, and as a result pellets greater than a critical size more than likely have mass transfer limitations (Wang et al., 2005, Cui et al., 1998b). The critical radius may be defined as the point at which diffusion of nutrients to the core is limited and the nutrient which is likely to limit growth first is oxygen. The profile of the dissolved oxygen tension in pellets has been measured with a microprobe by a number of authors, and the critical radius for oxygen transfer is often estimated to be between 130 and 200μm. Oxygen supply to the culture can greatly affect productivity because it sets bounds on the maximum attainable growth rate and even short oxygen limitations can have lasting negative effects on the product synthesis (Bellgardt, 1998). However pellets of streptomycete species are inherently less compact than fungal pellets and thus the critical radius may be somewhat larger. Indeed, in this study oxygen limitation occurred in pellet populations of *Streptomyces hygroscopicus* var. *geldanus* where a substantial fraction of the biomass volume consisted of pellets greater than 0.5mm in diameter, as low respiration rates were observed in those cultures. In these instances it is likely that growth only occurred in a peripheral zone where a good supply of oxygen was available (Braun and Vecht-Lifshitz, 1991). Such heterogeneity is unfavourable for secondary metabolite synthesis.

In this study, only pellet populations of *Streptomyces hygroscopicus* var. *geldanus* which were sufficiently metabolically active were found to support

the production of geldanamycin. These results quantitatively confirmed the hypothesis that cultures exhibiting small pellet sizes are more metabolically active and produce more geldanamycin than those populations with a higher proportion of larger diameter pellets. Indeed, a linear relationship between the specific respiration rate and antibiotic synthesis was observed, confirming that respiration is the key driver of geldanamycin synthesis in *Streptomyces hygroscopicus* var. *geldanus.*

Nevertheless, whilst morphology has a direct effect on respiration rate, and this in turn impacts on geldanamycin production, an alternative means of regulating antibiotic production was identified in this study. The shikimate pathway by which geldanamycin is synthesised may be feedback inhibited by the supplementation of the end-point aromatic amino acid tryptophan. Indeed, whilst there is always a certain level of variability in size distribution, the control and tryptophan supplemented cultures were quite similar in their morphological profiles. However, despite being deemed 'optimal' for geldanamycin synthesis, great discrepancies in volumetric production between these fermentations were observed. This result confirmed that in the presence of tryptophan, neither morphology nor physiology is the controlling factor in the production of geldanamycin. Instead the shikimate pathway was determined to be feedback inhibited up to 70% compared to control levels by the inclusion of the aromatic amino acid at a concentration of 5mM. In effect, it was possible to regulate or 'turn off' geldanamycin synthesis by the inclusion or omission of tryptophan to optimised Bennett's medium, regardless of the morphology or physiological state of the culture. It is speculated that DAHP synthase in *Streptomyces hygroscopicus* var. *geldanus*, the first enzyme in the shikimate pathway, is feedback inhibited by tryptophan, analogous to the findings of Stuart and Hunter (1993).

This study has demonstrated that, in *Streptomyces hygroscopicus* var. *geldanus*, the bulk of factors that affect morphology impact significantly on respiration, and it is this parameter that is the key driver of secondary metabolite production. In order to maximise geldanamycin production it is

necessary to limit pellet formation to an appropriate size, and it has been conclusively demonstrated that chemical (surfactant inclusion, magnesium and calcium supplementation), microbiological (inoculum size), or physical (shear) factors may be exploited for this purpose. Previous reports have documented the dependency of antibiotic production on pellet size (Tamura et al., 1997, Vecht-Lifshitz et al., 1992, Jonsbu et al., 2002), however there have been no prior attempts to correlate these two parameters with physiology. From this study, it is evident that the importance of culture heterogeneity on growth and metabolite formation cannot be underestimated. Certainly the frequency distribution of pellet sizes had a significant affect on both cellular metabolism and product synthesis; consequently small pellet populations (<0.5 mm diameter) as opposed to large ones (≥0.5mm diameter) are considered optimal for geldanamycin synthesis. A reduction in the particle size resulted in an increase in respiration rate, and by virtue high geldanamycin yields were concomitantly observed.

Nonetheless, it was also demonstrated that other factors exist which do not affect morphology or respiration but appear to regulate geldanamycin synthesis through repression of the direct metabolic pathway. The inclusion of an end-product of the shikimate pathway, the pathway from which the antibiotic is derived, significantly inhibited geldanamycin production. This behaviour is consistent with feedback inhibition and represents an alternative means of controlling geldanamycin production.

This case study is the first to provide a comprehensive understanding of the regulation of geldanamycin production in *Streptomyces hygroscopicus* var. *geldanus* and provides a basis for elucidation of the relationships between morphology, physiology and secondary metabolism in other filamentous micro-organisms. One general conclusion is inevitable: careful examination of size phenomenon and its effect on physiology is necessary whenever pellet formation and secondary metabolite production are considered.

4.2 Future Recommendations

4.2.1 In-situ product recovery (ISPR)

Many antibiotic fermentations are sensitive to high concentrations of their own product due to toxicity or feedback regulation mechanisms, limiting the attainable product yield. For example, the production of thienamycin by *Streptomyces cattelya* and cycloheximide by *Streptomyces griseus* are sensitive to high concentrations of the antibiotic (Wang et al., 1989, Dykstra and Wang, 1990). In addition, simultaneous production and decomposition of antibiotics can occur in laboratory batch fermentations. Indeed, the degradation of clavulanic acid and cycloheximide in cultures of *Streptomyces clavuligerus* and *Streptomyces griseus* has previously been demonstrated (Roubos et al., 2002, Dykstra and Wang, 1990). Generally chemical or enzymatic mechanisms are active in the degradation process. For instance, Usher et al., (1988), demonstrated that the main loss of cephamycin C in fermentation broth was chemical breakdown and Bersanetti et al., (2005), showed that stability of clavulanic acid is dependent on temperature and pH.

However, in other examples the antibiotic degradation rate increases upon reaching substrate limitation and the decline phase of growth. As cell lysis occurs, materials such as protein, nucleic acid and in particular enzymes may leak from cell mass and be the major cause of antibiotic degradation. A cell-associated enzyme is responsible for the break-down of pristinamycin in the culture broth following the production phase (Paquet et al., 1994) and it is likely that a similar phenomenon is affecting geldanamycin degradation in batch fermentations of *Streptomyces hygroscopicus* var. *geldanus*. Nonetheless, regardless of the mechanisms, product degradation has a major effect on product titres in fermentations (Roubos et al., 2002) and optimisation of culture media and environmental conditions will yield only nominal increases in volumetric productivity if product limitation and instability are significant (Wang et al., 1989).

One approach for circumventing product limitation and product instability is to simultaneously remove the product from the fermentation broth as it is formed (Wang et al., 1989). Indeed, such methods can also reduce the amount of downstream processing required which may be troublesome when dealing with mycelial media. The feasibility of whole cells (Wang et al., 1989), adsorbent resins (Lam et al., 1995, Casey et al., 2007), solvent extraction and micro-encapsulation as methods for in-situ product recovery have previously been examined.

Adsorbent resins are characterised by their surface properties and polarities and possess large surface areas for mass transfer; however their selectivity may often be non-specific. Resin addition has been demonstrated to significantly improve the titres of dynemicin A (Lam et al., 1995), kirromycin (Gastaldo et al., 1996), and teicoplanin (Lee et al., 2003) by relieving inhibition and preventing product degradation. Indeed, a preliminary study carried out by Casey et al., (2007), described that such resins were capable of adsorbing geldanamycin, thus stabilising the product by removing it from the producing organism and other environmental or metabolic hazards. However, both Lam et al., (1995), and Casey, (2006), observed that the production and growth rates of the respective organisms were inhibited by the addition of high concentrations of resin at fermentation initiation. They postulated that the non-specificity of these resins resulted in the removal of essential nutrients or metabolites from the culture medium, which presents significant disadvantages to their *in-situ* application.

Solvent extraction involves the use of suitable solvents into which the product of interest may be preferentially recovered. The application of organic solvent extraction has been successful in the recovery of penicillins, however the formation of stable emulsions at broth-solvent interfaces limit its wider application (Yang et al., 1994). In addition, the extractive solvent phase may often be toxic to the producing organism.

Nonetheless, the encapsulation of extractive solvents in polymeric membranes can relieve these problems, protecting the cells from the solvent and preventing the formation of stable emulsions (Stark et al., 2003). Such microcapsules also offer the advantage of having a large interfacial contact area that promotes rapid extraction (Wyss et al., 2004). Stark et al., (2003), demonstrated that microcapsules represent a novel ISPR method, relieving the inhibitory effects of 2-phenylethanol on yeast cells by continuous product extraction, although optimisation of the process was required. Indeed, Wyss et al., (2004), established the importance of microcapsule optimisation for the removal of pesticides and herbicides from aqueous environments. Different liquid core solvents; the size of capsules; agitation rate; and treatment with complexing agents were all found to affect the extraction rate, revealing that the correct microcapsule properties are critical for effective ISPR.

It is envisaged that ISPR of geldanamycin from fermentation broths using micro-encapsulation could provide a successful means to prevent the significant degradation of the antibiotic which has previously been observed. The selection of an optimal solvent for encapsulation, and optimisation of the process similar to Wyss et al., (2004), could enable the recovery of much greater yields of geldanamycin from *Streptomyces hygroscopicus* var. *geldanus* fermentations.

4.2.2 Process optimisation using statistical methodology

Process optimisation has traditionally been managed empirically, using a 'one factor at a time' approach, which is often laborious and time-consuming. Hence, the problem is to find, in the least number of experiments, the factor levels at which the response is said to be maximised (Bloor and England, 1991). Experimental design techniques can be a very useful tool for this purpose, as they can provide statistical models with a relatively small number of experiments (Sircar et al., 1998).

Factorial design offers a solution by reducing the number of factor combinations of a design and, therefore, runs or experiments required (Landsheer and van den Wittenboer, 2002). For example, in a two-level full factorial design, the experiments involved are 2^n where n is the number of ingredients (Sircar et al., 1998). So if there were 5 ingredients in a medium, and each was to be set as a factor for optimisation studies, one would need to carry out 2^5 or 32 experiments. However, the number of experiments can be reduced by using only part of the factorial designs (*fractional* factorial design, FFD), without loss of information about the main effects (Li et al., 2002). Therefore, for a 2^{5-2} fractional factorial design with 5 factors at 2 levels, only 8 experimental runs are required. However, two level factorial design only involves simultaneous adjustment of experimental factors at two levels: high and low (Anderson and Whitcomb, 1997). While it indicates the importance of each constituent and how it tends to affect, for example, growth and product formation, it cannot determine the exact quantity of each constituent required for the medium to generate an optimal response (Rajendhran et al., 2002). Thus, this first step in the process of seeking optimum conditions is only to identify the input variables that have the greatest influence on the experimental response (Elibol, 2002).

Response surface methodology (RSM) is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors, and searching optimum conditions of factors for desirable response (Li et al.,

2002). Following experimentation, one can generate a response surface map and move the process to the optimum location (Anderson and Whitcomb, 1997). Therefore, in addition to analysing the effects of independent variables, this experimental methodology generates mathematical models that should accurately describe the overall process (He et al., 2004). From these models, the relative influence of the various factors studied can be determined and their optimal concentration calculated for maximal metabolite production (Sircar et al., 1998). A prior knowledge and understanding of the process and the variables under investigation are necessary for achieving a more realistic model (Elibol, 2004). Thus, a response surface design is only appropriate once the key parameters and their ranges have been established.

Optimisation through factorial design and response surface analysis is a common practice in biotechnology (Kalil et al., 2000). Fractional factorial design and response surface methodology have frequently been utilised in media design for optimisation of product yield, for example: meilingmycin production by *Streptomyces nanchangensis* (Zhuang et al., 2006); doxorubicin productivity of *Streptomyces peucetius* (Park et al., 2005); actinorhodin production by *Streptomyces coelicolor* A3(2) (Elibol, 2004); neomycin synthesis by *Streptomyces marinensis* (Adinarayana et al., 2003); nisin production by *Lactococcus lactis* (Li et al., 2002); ethanol production by *Zymomonas mobilis* (Sreekumar et al., 1999); and penicillin G acylase from *Bacillus spp.* (Rajendhran et al., 2002). This method of optimisation has been found to be efficient, relatively simple and material saving (Elibol, 2004), with satisfactory model accuracy (Adinarayana et al., 2003).

Artificial neural networks (ANN) also lead to the development of process models but differ in their ability to provide an adaptive system which can be trained. Such learning abilities are attributed to the fact that neural networks, possessing many simple parallel processing units (called neurons or neurodes) crudely resemble the architecture of the human brain (Himmell et al., 1992). The network is formed by a series of connected neurons: each neuron in the first layer being associated with a variable or characteristic of the problem e.g.

pH, temperature, salt; each neuron in the hidden layer carries out a double transformation or function that gives the models their non-linearity; and the neurons in the output layer are the values or 'targets' given (Garcia-Gimeno et al., 2002). ANN are commonly used as 'black box' models of key variables whose relationship to other process entities are neither formally described nor mathematically established, but assumed to occur (Franco-Lara et al., 2006). Once the best model has been obtained by the training process, a validation step is carried out by testing with a data set not used for training to evaluate the system performance (Garcia-Gimeno et al., 2002, Franco-Lara et al., 2006). An ANN was successfully used by Kase, (1996), for the modelling of cephalosporin C production. Comparisons of ANN models with response surface models have indicated that neural network models exhibit superior accuracy, with lower percentage errors of prediction (Himmell et al., 1992, Garcia-Gimeno et al., 2002).

It is proposed that both RSM and ANN techniques could be utilised to build predictive models of the effects of multiple parameters on the synthesis of geldanamycin by *Streptomyces hygroscopicus* var. *geldanus*. Following the development of the models by both methods, a comparison by means of the percentage standard error of prediction could be made. Accordingly, this would determine whether the predictive capabilities of neural networks are superior to that of their statistical counterparts for the same experimental data.

4.2.3 Proteomics technology

Now that the sequences of several bacterial genomes have been or are about to be completed, researchers are aware that they represent just the start of understanding how organisms develop and function (Abbott, 1999b). The next stage encompasses an understanding of gene function and hence characterisation of every protein that may be deduced from the coding sequence of a genome, the proteome. Thus, proteomics is one of the most important of the so-called 'post-genomic' approaches to understanding gene function because it is the proteins expressed by all genes that are ultimately responsible for all processes that take place within the cell (Abbott, 1999b).

No known organism needs and uses all gene products at the same time and in the same concentration (Mullner, 2003). When a bacterium encounters a harmful environment, or just experiences changes in its growth conditions, it responds by rearranging its metabolism to accommodate the changes. Knowledge of up- and down- regulation of proteins following stress response is important for prediction of bacterial performance. Since cell metabolism is influenced in many ways by the fermentation conditions, e.g., aeration; pH; media; bioreactor volume; temperature; mechanical stress; cell density; feedback effects of the product etc., proteomics is presently the method of choice for identifying functional co-regulated and cooperating protein networks (Hecker and Mullner, 2003).

By the identification of key metabolic enzymes and regulatory proteins, proteomics should allow researchers to build a complex map of cell function (Abbott, 1999b). It will enable one to determine the individual proteins involved in a biochemical pathway, including those that have not yet been characterised; to follow the quantitative aspects of the process; and to study the effects of overproduction of certain proteins (Li et al., 2001). Elucidation of the mechanisms of action and regulation would provide fundamental knowledge for improvement of biotechnological processes via metabolic pathway engineering.

However, while proteins may yield the most important clues to cellular function, they are also the most difficult cell components to detect and identify on a large scale (Abbott, 1999b). Successful proteomic analysis using 2DGE is hampered by many limitations, not least of which is poor reproducibility and sensitivity. In addition, 2DGE can often fail to detect low abundance proteins such as regulatory proteins. While these tend to do the most interesting jobs in a cell their detection is drowned out by high-abundance 'housekeeping proteins', which can be present at up to 10,000 times the concentration (Abbott, 1999a).

A potential alternative to the 2D approach for the multidimensional separation of proteins is the high-throughput, array-based novel analytical technique referred to as surface enhanced laser desorption ionisation (SELDI) technology (Seibert et al., 2004). SELDI technology is an affinity based mass spectrometry method in which proteins are selectively adsorbed and captured on chromatographic array surfaces (protein chips) (White et al., 2004), thus offering a single unified platform for protein separation and identification. Analysis by SELDI time of flight mass spectrometry (SELDI TOF-MS) produces spectra of complex protein mixtures based on the mass-to-charge ratio of proteins and their binding affinity to the chip surface, facilitating the detection of differentially expressed proteins by comparing peak intensities (Seibert et al., 2004).

The understanding of metabolic (protein) networks within an industrially used cell is of clear importance for productivity of the organism and the whole process (Hecker and Mullner, 2003). Indeed, companies like BASF, Bayer, Degussa, Novartis, and others involved in larger scale biotechnological production processes, e.g., vitamins, amino acids, organic acids, antibiotics, have started in-house proteomics projects to optimise their respective fermentation processes (Mullner, 2003). Proteomics technology could be similarly utilised to gain insight into the physiology, differentiation and antibiotic production in *Streptomyces hygroscopicus var. geldanus*.

For example, Li et al., (2001), was able to identify a subset of proteins that were related to the formation of chlortetracycline by *Streptomyces aureofaciens*. Consistently, these proteins exhibited a remarkably increased intensity when the cells were grown in the presence of a known stimulatory compound, benzyl thiocynate (BT), with expression levels stimulated up to 15-fold (Li et al., 2001). Analogously, proteomics may help to identify the major proteins involved in geldanamycin synthesis and indeed, the effect of tryptophan as a demonstrated inhibitor of geldanamycin on their expression in cultures of *Streptomyces hygroscopicus* var. *geldanus*. Indeed, while studying the intracellular protein profile of *Streptomyces griseus,* four proteins were found to be significantly and specifically repressed by addition of cycloheximide to a producing fermentation (Dykstra and Wang, 1990). As such, proteomics would provide a platform for the identification of enzymatic targets for metabolic pathway engineering for improved geldanamycin synthesis by *Streptomyces hygroscopicus* var. *geldanus*.

4.2.4 Metabolic engineering

Strain development programs in industry have markedly increased the production capabilities of many antibiotic–producing cultures (Martin and Demain, 1980). While most of these efforts were traditionally performed by random mutagenesis, designed mutants are now being developed with decreases in regulatory controls. The Shikimate and related pathways (Figures 1.5 and 3.32) play a critical role in providing aromatic precursors for microbial biosynthesis of many antibiotics, including geldanamycin.

To improve the production of geldanamycin both the availability of precursors from central carbon metabolism and the optimisation of enzyme expression within the shikimate pathway need to be addressed. One of the first limiting steps of the pathway is the availability of the precursor molecule PEP derived from the glycolytic pathway of central metabolism. The major PEP consumer is the phosphotransferase system (PTS) which is responsible for the uptake of glucose into cells and its subsequent phosphorylation and conversion to pyruvate (Bongaerts et al., 2001), with the reconversion to PEP non-energetically favourable. This stoichiometric limitation greatly reduces the yield of aromatic metabolites (Patnaik and Liao, 1994). An approach to avoid PEP consumption during substrate uptake is to use a non-PTS carbon source such as xylose (Patnaik et al., 1995). Amplified expression of PEP synthase which catalyses the conversion of pyruvic acid back to PEP has been observed to double the final yield of 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) (Patnaik and Liao, 1994).

Derived from the Pentose Phosphate Pathway, the shikimate precursor E4P is prone to di- tri- and poly- merisation, and dissociation to monomeric forms is quite slow (Williams et al., 1980). Thus there is generally a low steady state concentration in the cell. Transketolase and transaldolase are the main enzymes that convert fructose-6-phosphate (F6P) and glyceraldehyde-3P to E4P in the glycolytic and pentose phosphate pathways respectively (Bongaerts et al., 2001). Ikeda et al., (1999), demonstrated that the overexpression of

transketolase in *Corynebacterium glutamicum* resulted in an increase in aromatic amino acid production by 5-20%. They concluded that overexpression of the enzyme contributed to an increased availability of E4P, and consequently the engineered producing strain was able to commit more carbon to the aromatic amino acid pathway (Ikeda et al., 1999). Bongaerts et al., (2001), reported the impact of transaldolase on the shikimate pathway. Overexpression of the enzyme was found to increase the flux into the aromatic pathway of an *Escherichia coli* phenylalanine production strain.

The large family of ansamycin antibiotics contains a novel structural element, an mC_7N unit which is biosynthetically derived from 3-amino5-hydroxybenzoic acid (AHBA). AHBA is generated by a newly discovered biosynthetic reaction sequence, the amino-shikimate pathway, which parallels the shikimate pathway but is optimised by the introduction of nitrogen to give 3,4-dideoxy-4-amino-D-arabino-heptulosonic acid 7-phosphate (aminoDAHP) instead of the normal shikimate intermediate 3-deoxy-D-arabino-heptulosonic acid 7-phosphate (DAHP) (Kim et al., 1998, Watanabe et al., 2003, Yu et al., 2001). Cyclisation and dehydration leads to amino-DHS which is then aromatised by the enzyme AHBA synthase (Kim et al., 1998, Watanabe et al., 2003).

Rifamycin is an ansamycin antibiotic containing a C_7N unit derived from the shikimate pathway. Inactivation of the gene encoding AHBA synthase in *Amycolatopsis mediterranei* resulted in a loss of rifamycin formation, but production of the antibiotic was restored when the mutant was supplemented with AHBA (Kim et al., 1998). Watanabe et al., (2003), expressed the entire AHBA biosynthetic pathway in *Escherichia coli* in order to provide the primer unit for biosynthesis of a variety of ansamycin derivatives.

Analogous to the findings of Bongaerts et al., (2001); Ikeda et al., (1999); Kim et al., (1998); and Patnaik and Liao, (1994), up-regulation of the shikimate pathway for improved geldanamycin synthesis may be achieved by the

overexpression of PEP synthase, transketolase, transaldolase and AHBA synthase either individually or synergistically.

However, aside from engineering the wild-type strain to produce greater yields of geldanamycin, genetic manipulation could also be utilised to produce novel derivatives of geldanamycin with more desirable attributes. Despite its potent activity as a chemotherapeutic agent, clinical use of geldanamycin has been limited by its severe hepatic toxicity. Whilst chemical modifications have yielded some success due in particular to the development of the low-toxicity analogues 17-AAG and 17-DMAG, the identification and characterisation of the gene cluster for geldanamycin (Rascher et al., 2003) should lead to greater opportunities for the development of a wider range of geldanamycin analogues.

For example, amphotericin B is an important antibiotic used to treat serious systemic fungal infections in humans, and similarly to geldanamycin, it can cause many severe side effects including nephrotoxicity, cardiotoxicity and neurotoxicity (Caffrey et al., 2001, Byrne et al., 2003). Caffrey et al., (2001), proposed that analogues with improved properties could be generated by manipulating the amphotericin biosynthetic genes in *Streptomyces nodosus*. Indeed, Carmody et al., (2005), demonstrated that mutants of *Streptomyces nodosus* produced a novel polyene antibiotic which retained comparable antifungal activity to amphotericin B but was 10 times less haemolytic. It is thus postulated that similarly to amphotericin B, novel geldanamycin analogues could be produced by genetic manipulation of *Streptomyces hygroscopicus* var. *geldanus*.

CHAPTER 5: REFERENCES

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CHAPTER 6: APPENDICES

6.1 APPENDIX A: STANDARD CURVES

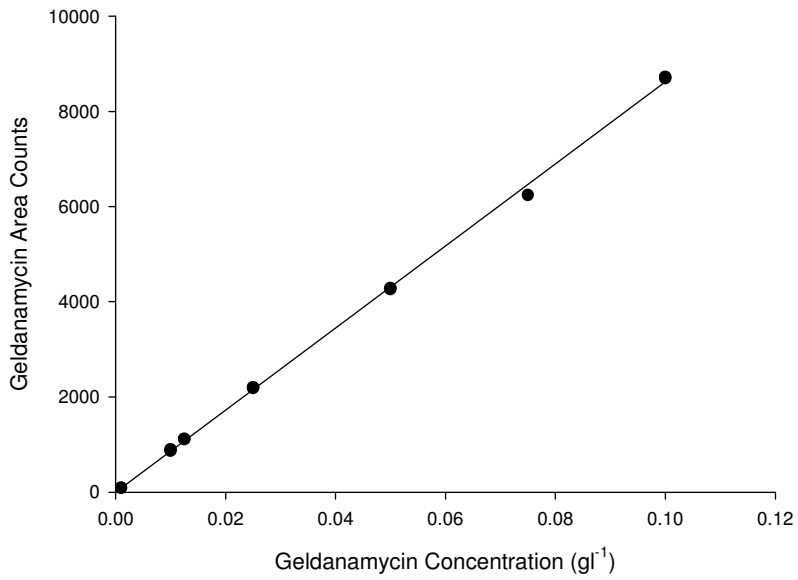

Figure 6.1: Standard Curve for Geldanamycin; y=86226x; R^2=0.9993.

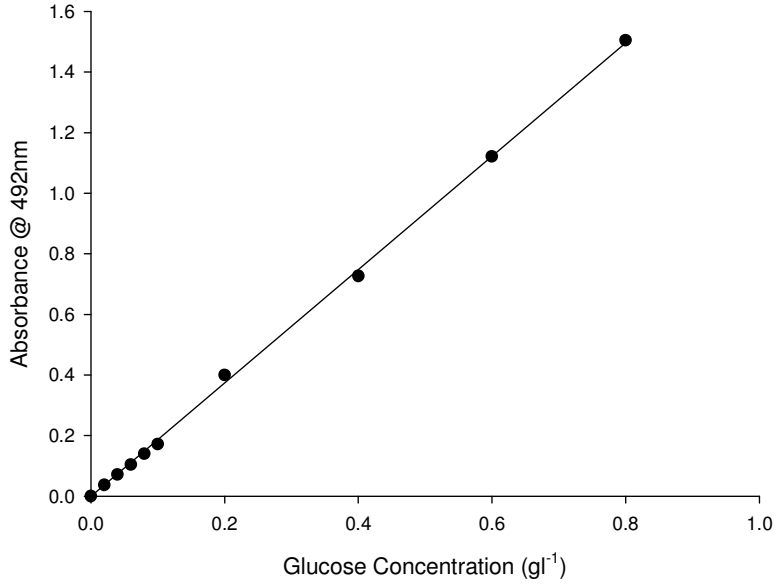

Figure 6.2: Standard curve for glucose; y=1.8704x; R^2=0.9994.

6.2 APPENDIX B: IMAGE ANALYSIS ALGORITHM

The following is the code for the image analysis algorithm developed by O'Cleirigh et al., (2003), and used in conjunction with Optimas 6.5® for the morphological quantification of pellets in *Streptomyces hygroscopicus* var. *geldanus* using a flatbed scanner.

```
OpenConfiguration("C:\\ProgramFiles\\Optimas6.5\\Config\\cormac
.cfg");
Calibrate (Calibration5);
ScalarToList (255.0);
ScalarToList (0.0);

RunMacro ("C:/Documents and Settings/lynne\My Documents/Optimas
Stuff/low frequency filter.mac");
RunMacro ("C:/Documents and Settings/lynne\My Documents/Optimas
Stuff/low frequency filter.mac");

Filters ( Median3x3 );

Calibrate (Calibration5);

BandOfInterest = 2;
BandToView = 2;

AreaCNVFactors[6]=1;
AreaCNVFactors[7]=-2.0;
AreaCNVFactors[8]=2.0;

AreaCNVFactors[4:5] = 0.0:1.0;
AreaCNVFactors[12]=2.0;

Threshold(0:169);
GrayToBinary ();
InvertFilter();

ScrollPosXView = 0;
ScrollPosYView = 0;
ZoomFactor = -10;
PositionWindow ("Image2", 0, 0, 570, 589);

show("Manual ROI Selection Required");

SelectFullScreen (optSelectFullImageFrame);

hid=CreateArea (, FALSE, , , 1);
SetExport (ArArea,1, TRUE);
SetExport(ArAreaEquivDiameter,1,TRUE);
Extract();

real forcounting=ArArea;

ImageMask (1,hid);

ZoomFactor = 1;
CreateArea (, , TRUE);
```

```
SetExport (mArArea, , TRUE);
SetExport (mArCircularity, , TRUE);
SetExport (mArGV, , TRUE);
SetExport (mArBreadth, , TRUE);
SetExport (mArMajorAxisLength, , TRUE);
SetExport (mArAreaEquivDiameter, , TRUE);
SetExport (mArCircularity, , TRUE);

MultipleExtract (TRUE);
ImageMask(1,mArHandle);
mmararea=mararea;
mmmararea=mararea;
Imagemask(4096);
ArithmeticOp ("Copy", "#0", , , "Clip", FALSE, FALSE);
Imagemask(8192);
ArithmeticOp ("Copy", "#255", , , "Clip", FALSE, FALSE);
ImageMask (8);
ZoomFactor = -10;
SelectFullScreen (optSelectFullViewedArea);
ZoomFactor =1;
BINB_iIterations = 1;
RunMacro("dialogs/brkapart.mac");
Threshold ( 127.5:255.0 );
BRK_nHoleDilates = 0;
BRK_nRawErodes = 0;
BRK_nErodesToPoint = 5;
BRK_bAutoAreas = TRUE;
BRK_bAutoPoints = FALSE;
BRK_bAutoNone = FALSE;
BRK_bShowWork = FALSE;
BRK_BreakApartBlobs ();

InvertFilter();

MultipleExtract (TRUE);

rResult = Prompt("Enter a value for dilution (e.g. 1 in 5 =
0.2)", "REAL");

mmararea=mararea;
real volume;

cpm=594.46*vectorlength(mmararea)/forcounting/rResult;

//show("the value for pellet count per ml is...");
//show(cpm);

volume=pow(mArAreaEquivDiameter,3)*3.1417/6.0;
//show("the pellet volume per ml is...");
//show(moments(volume)[0]*cpm);

delete(volume);

if (ChanID = DDEInitiate ("Excel", "Sheet1"))
{
      statval=Moments(mmararea);

      output="R1C1";
      DDEPoke (ChanID,output,"Area Equivalent Diameter");
//       output="R1C2";
```

```
//    DDEPoke (ChanID,output,"Circularity");
//       output="R1C3";
//    DDEPoke (ChanID,output,"Minor Axis Length");
//       output="R1C4";
//    DDEPoke (ChanID,output,"Major Axis Length");
//    output="R1C1";
//    DDEPoke (ChanID,output,"Pellet Area");


      for(i=0;i<vectorlength(mmararea);i++)
      {
         output="R":totext(i+2):"C1";
         DDEPoke (ChanID,output, marareaequivdiameter[i]);
//       output="R":totext(i+2):"C2";
//       DDEPoke (ChanID,output, marcircularity[i]);
//       output="R":totext(i+2):"C3";
//       DDEPoke (ChanID,output, marbreadth[i]);
//       output="R":totext(i+2):"C4";
//       DDEPoke (ChanID,output, marmajoraxislength[i]);
//       output="R":totext(i+2):"C5";
//       DDEPoke (ChanID,output, mmararea[i]);
      }

      output="R2C3";
      DDEPoke (ChanID,output,"Sample Size");
      output="R2C4";
      DDEPoke (ChanID,output,vectorlength(mmararea));
      output="R3C3";
      DDEPoke (ChanID,output,"Mean");
      output="R3C4";
      DDEPoke (ChanID,output,statval[0]);
      output="R4C3";
      DDEPoke (ChanID,output,"Standard Deviation");
      output="R4C4";
      DDEPoke (ChanID,output,statval[1]);
      output="R6C3";
      DDEPoke (ChanID,output,"Pellet Count/ml");
      output="R6C4";
      DDEPoke (ChanID,output,cpm);
      output="R7C3";
      DDEPoke (ChanID,output,"Pellet Volume/ml");
      volume=pow(mArAreaEquivDiameter,3)*3.1417/6.0;
      output="R7C4";
      DDEPoke (ChanID,output,moments(volume)[0]*cpm);

      output="R8C3";
      DDEPoke (ChanID,output,"Mean Area eq dia");
      volume=moments(mArAreaEquivDiameter)[0];
      output="R8C4";
      DDEPoke (ChanID,output,volume);

      output="R9C3";
      DDEPoke (ChanID,output,"St.dev. Area eq dia");
      volume=moments(mArAreaEquivDiameter)[1];
      output="R9C4";
      DDEPoke (ChanID,output,volume)

      statvals=Moments(mararea);
DDETerminate (ChanID);
}
```