QUANTIFICATION AND REGULATION OF PELLET MORPHOLOGY IN *STREPTOMYCES HYGROSCOPICUS* VAR. *GELDANUS* CULTURES

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At the School of Biotechnology,

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I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Ph.D. (Biotechnology) is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

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ABSTRACT

Quantification and Regulation of Pellet Morphology in *Streptomyces Hygroscopicus* var. *Geldanus* Cultures

Streptomyces hygroscopicus var. *geldanus*, an actinomycete, produces a range of antibiotic compounds, one of which, Geldanamycin, is currently under investigation for its potential anticancer properties. Filamentous microbes, both bacterial and fungal, possess the ability to grow in a range of morphological forms in submerged fermentations. Secondary metabolite production by filamentous microorganisms is often dependent on the morphology of biomass aggregates within the culture and therefore morphological regulation in such fermentations is desirable in order to produce specific compounds of interest.

Morphological assessment of pellet formation and growth necessitated the development of an image analysis technique to allow high throughput morphological characterisation of microbial fermentation broths. An assessment of environmental and nutritional conditions for culturing *S. hygroscopicus*, concentrating specifically on the impact of nutrient broth composition, spore loading density and shear rate on the morphological and physiological profiles of the organism, was undertaken.

The formation of pelleted biomass in submerged fermentations occurs as a result of spore aggregation, hyphal aggregation or entanglement; each of which was identified as a potential morphological control strategy. The microbial polysaccharide xanthan gum was used to artificially regulate the apparent viscosity and hence the rheological characteristics of *S. hygroscopicus* fermentations broths and succeeded in controlling particle collision, aggregation and hence pellet formation. Control of broth surface tension through the introduction of surfactants, facilitates regulation of hydrophobic particle interaction, thus enabling regulation of hyphal aggregation and hence pellet formation in *S. hygroscopicus* cultures. Furthermore, we have identified the production of a previously unreported biosurfactant which appears to have significant effect on the morphology of the organism. Preliminary purification and characterisation of this molecules and an assessment of its usefulness as a morphological control agent in Streptomyces fermentations has been conducted.

NOMENCLATURE

Symbol	Definition	Units
Abs	Absorbance	nm
CFU	Colony forming unit	ml^{-1}
C_{AL1}	Dissolved oxygen concentration at time t_1	gl^{-1}
C_{AL2}	Dissolved oxygen concentration at time t_2	gl^{-1}
C_{ss}	Final steady dissolved oxygen concentration	gl^{-1}
CCD	Charge coupled device	
dpi	Dots per inch	
ε	Energy dissipation rate	s^{-1}
H-bonding	Hydrogen bonding	
k	Constant	
K	Fluid consistency index	Ns^nm^{-2}
K_La	Gas-liquid mass transfer coefficient	s^{-1}
K_r	Colony radial growth rate	s^{-1}
M	Biomass concentration at time t	gl^{-1}
M_0	Biomass concentration at time 0	gl^{-1}
n	Flow behaviour index	(-)
η	Kolmogorov length	µm
NIST	National institute of standards and technology	
NPF	Normalised particle frequency	(-)
NVF	Normalised volume frequency	(-)
PC	Pellet count	ml^{-1}
RAM	Random access memory	
RGB	Red green blue	
t	Time	s
τ	Shear stress	Nm^{-2}
τ_E	Eulerian integral time scale	
tiff	Tagged image file format	
u	Mean turbulent velocity	$m\ s^{-1}$
μ_a	Apparent viscosity	Nsm^{-2}

υ	Kinematic viscosity	$m^2 s^{-1}$
VF	Volume fraction	$mm^3 ml^{-1}$
XG	xanthan gum	
γ	Shear rate	s^{-1}

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Figure 5.24 The impact of artificially regulating broth surface tension on *S. hygroscopicus* dry weight of biomass concentration after 7 days.

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CHAPTER 1 – THE INFLUENCE OF ENVIRONMENTAL CONDITIONS ON FILAMENTOUS MORPHOLOGY

1.1. GROWTH AND MORPHOLOGICAL DEVELOPMENT OF FILAMENTOUS MICROORGANISMS

1.1.1. Introduction

Unicellular bacteria are thought to have been one of the earliest forms of life to appear on earth. These organisms usually take one of three shapes, rod shaped (bacilli), spherical shaped (cocci) and spiral or helical shaped (Prescott, 1993). The process of reproduction within these organisms can take one of two forms, either sexual, in the form of conjugation, or asexual, in the form of binary fission. When grown in liquid cultures these unicellular bacteria do not typically attach to one another but remain dispersed in suspension. However, when aggregation does occur it is generally by means of flocculation, a process of loose aggregate or floc formation based on temporary cell interactions. The attractive forces that hold the cells together are typically either cell surface interactions or the presence of an extracellular binding agent. Flocs are only loose aggregates and are prone to disruption given the weak temporary nature of the bonds formed.

Filamentous bacteria on the other hand are more complex in structure and in their mechanisms of growth than their unicellular counterparts. The basic units of growth in a filamentous organism are long multicellular string-like structures called hyphae (Prosser & Tough, 1991). Reproduction within these organisms is usually by means of hyphal tip extension, followed by septation and branching in the sub-apical regions. Filamentous bacteria experience the same attractive forces as unicellular bacteria except that once they have attached they begin to intertwine with one another giving rise to permanent aggregates. The morphology of these organisms may vary from a free

filamentous suspension to pellets, depending on the degree of aggregation and the nature of apical and branched growth. Filamentous bacteria possess the ability to grow in a range of morphological forms in submerged fermentations depending on process conditions. They are an important group of microbes due to their ability to produce commercially valuable secondary metabolites. Product formation within these filamentous microbes, in submerged fermentations, is often dependent not only on the level of biomass present but also on the morphological profile of the culture. Given the comparative complexity of filamentous bacteria, careful consideration must be given to the organisms' growth mechanisms in both solid and liquid culture in order to understand the processes of colony or pellet formation, cellular differentiation and product formation.

1.1.2. Growth on Solid Medium

1.1.2.1. Spore Germination

Filamentous bacteria, such as the actinomycetes, share certain common characteristics with fungi, one of which is the ability to produce endospores. These spores are not fragments of vegetative biomass, but a distinct stage in the lifecycle of the producing organism. Vegetative growth occurs when favourable environmental and nutritional conditions exist. Once these conditions become adverse the organism can no longer sustain growth and must make provision for survival in the form of spore formation. The spore is the simplest inert form of the organism and can remain dormant for extended periods of time. Once favourable environmental and nutritional conditions prevail the spore will germinate, giving rise to vegetative biomass.

Germination may be defined as the first irreversible change in the formation of vegetative biomass from a spore, the first visual evidence of which is the formation of a germ tube. Optimum spore germination occurs in rich complex media with high oxygen levels, whereas the presence of carbon dioxide and poor mineral based media are inhibitory. In less favourable conditions

Streptomyces spores was seen to be capable of auto-regulating germination, whereby the proportion of germinating spores decreased with increasing initial population density (Triger *et al.*, 1991). This auto-regulatory mechanism has been found to be intrapopulational and dependent on the proximity of one spore to another. The existence of favourable environmental and nutritional conditions causes the spore to swell, become metabolically active and it begins to synthesise additional intra-cytoplasmic membranous structures. This is subsequently followed by a disruption in the spore wall leading to the emergence of a germ tube. The emergence of the first germ tube occurs somewhere between 2.5 and 8.0 hours after inoculation and is dependent on the species of the organism and the prevailing process conditions. Streptomycetes are capable of producing 1 – 4 germ tubes per spore, depending on the nutritional conditions of the growth medium (Kalakoutskii & Pouzharitskaja, 1973). Continued growth and extension of germ tubes leads to the formation of hyphae.

1.1.2.2. Filamentous Growth Mechanisms

There are two major types of microorganism that develop by means of filamentous growth, the eukaryotic filamentous fungi and the prokaryotic actinomycetes. Within the filamentous fungi, there is a further sub-division in the form of true filamentous fungi and the dimorphic forms; the latter can be either filamentous or unicellular, depending on process conditions. The actinomycetes can range from true filamentous forms such as the streptomycetes to those that form mycelia that easily fragment to give filamentous growth or even those that grow as single cells based on process conditions. An example of such microorganisms are the *Nocardiae* (Prosser & Tough, 1991). Unicellular and filamentous bacteria differ primarily in the mechanism of cellular reproduction, the former by binary fission while the latter by hyphal tip growth, septation and branching. Although the filamentous fungi and the actinomycetes share common morphological forms and growth mechanisms the similarity is mostly physical and internal structure of the organisms differ. Microscopic examination has revealed that the hyphal diameter of filamentous fungi (~ 10 μm) is typically an order of magnitude

greater than that of the actinomycetes (~ 1 - 2 µm). Furthermore, the filamentous fungi, as eukaryotes, have membrane-bound organelles and a cytoskeleton, structures which are not present in the prokaryotic actinomycetes.

Microscopic examination of germinating *Streptomyces* spores indicates that the process begins with the formation of germ tubes from a central node in any direction (Kalakoutskii & Pouzharitskaja, 1973). Once these filaments have formed they proceed to grow and develop into strands known as hyphae. As the hyphal elements develop the process of elongation becomes autocatalytic as the extended filament contributes to sustained biosynthesis by the uptake and metabolism of nutrients from the medium. Hyphal extension in filamentous organisms can be by tip growth or intercalary growth, the latter does not occur in vegetative mycelia of filamentous bacteria. Intercalary growth involves extension from within the hyphae itself, a process more prevalent in the development of aerial than vegetative hyphae, due to reduced resistance to distal motion in air. Hyphal tip growth involves the biosynthesis of extension material in the distal regions of the hyphae followed by transportation to the point of extension, the tip, where it is incorporated into the wall. Furthermore, the hypha forms branches in the regions behind the tip that will themselves extend through tip growth. The hypha effectively 'rolls out' over the growth media. The combination of tip growth and branch formation in hyphae enables the organism to colonise multi-directionally and efficiently utilise the available space and nutrients.

1.1.2.3.1. Hyphal Tip Growth

Hyphal elongation within the filamentous actinomycetes is by means of the addition of new material in the apical extension zone. The driving force behind hyphal growth appears to be hydrostatic pressure within the hypha itself (Prosser & Tough, 1991). The sub-apical regions within the hyphal element, although not actively growing, are continuously biosynthesising material for inclusion in the cell wall. The component material is then transported, most likely by diffusion, from the sub-apical zones to the apical extension zone where it fuses with the tip wall and expands the hyphal wall

outwards. Under favourable growth conditions, the apical zone has a constant diameter, length and shape. The newly added wall material in the apical extension zone is quite flexible and not at all rigid like the wall in the sub-apical regions. However, this flexibility coupled with the thinness in this region makes it susceptible to lysis. The continual assimilation of additional material into the apical wall ensures that the tip is constantly moving forward. Within the apical extension zone the further from the tip the assimilated material is the more rigid it becomes until it, too, becomes fully rigid and part of the sub-apical zone.

The rate of tip extension in hyphae is dependent on two criteria. Firstly, the rate at which the new material is supplied to the tip, which is dependent on both the rate of synthesis in the sub-apical regions and the rate of transport from this region to the apical extension zone and secondly, the rate at which the newly supplied material is absorbed into the tip wall, which is determined by the dimensions of the apical extension zone (Prosser & Tough, 1991). Filamentous growth in this manner provides for the elongation of hyphae in a single dimension, an effective but inefficient use of space and nutrients.

1.1.2.3.2. Nuclear Division, Branching & Septation

The nuclear material present in actinomycetes is distributed evenly throughout filamentous hyphae in the form of a fibrillar matrix, except in the apical 2 – 5 µm. In much the same way as hyphal extension only occurs in the apical extension zone, the majority of nuclear replication occurs in the 15 – 25 µm behind the apical non-nuclear material containing zone. The nuclear material can also form independent nuclear bodies called nucleoids, which are thought to contain copies of the organisms genome. Branch formation in filamentous bacteria is not dependent on septation to the same degree as in filamentous fungi but is thought to be more dependent on the presence of nucleoid aggregates at particular locations within the hyphae (Prosser & Tough, 1991).

As mentioned previously, filamentous growth by hyphal elongation provides for colonisation in a single dimension, which is an inefficient use of space and

nutrients. To counteract this, the organism produces branches that extend out from the parent hyphae at various angles, which elongate through subsequent apical and branched growth. This mechanism allows the organism to effectively fill in the gaps left by the apical growth of the parent hyphae and colonise the growth medium. Apical growth in hyphae does not give rise to large single cell filaments. A process known as septation is responsible for dividing the filamentous hyphae into component cells. Kretschmer (1982) proposed that septation within *S. granaticolor* occurred in the following manner. Hyphal tip growth extends the apical compartment to approximately 28 μm before a septum is formed, dividing it into two. The apical component enlarges through tip growth and the process repeats itself. The formation of branches in sub-apical cells is also accompanied by the migration of nuclear material into the new cell and the septation of the new from the old. The process of septation in filamentous organisms serves a useful purpose as it allows for the generation of discrete cellular units within a filamentous superstructure. These septae impart greater shear tolerance, isolate the growing tip from distal regions where conditions may be less favourable and shorten the distance across which nutrients have to be transported to the tip.

Given the importance of early stage filamentous growth to the morphological and physiological development of these organisms, attempts have been made to measure the major processes involved such as tip growth, branching and septation and to formulate a mathematical model to describe them. Yang *et al.* (1992b) examined the morphological development of *Streptomyces tendae* cultures and discovered that the angles at which both the tip and branches grow from the parent hyphae are normally distributed. The tip growth was found to be in a straight line and the formation of branches found to be at right angles to the parent. A novel aspect, considered in this model, was the three dimensional nature of filamentous growth. Following on from the experimental determination of filamentous growth in *S. tendae,* Yang *et al.* (1992a) formulated a mathematical model to describe the apical growth, septation and branching of mycelial organisms. Mathematical models have also been postulated for filamentous fungi

(Nielsen, 1993; Nielsen & Krabben, 1995; Viniegragonzalez *et al.*, 1993) that may also be applicable to hyphal development in the actinomycetes.

1.1.2.3. Colony Growth and Differentiation

One of the major competitive advantages that filamentous microorganisms possess over unicellular counterparts, is that they are adapted for growth on solid substrates. Filamentous extension, through apical and branched growth, not only enables the organism to explore new ground for fresh nutrients but also to fully utilise the area already colonised, a process not possible in simpler systems. Unlike unicellular microorganisms, whose growth is at an exponential rate, filamentous microorganisms extend apically at a constant rate due to the limiting rate of wall synthesis, a problem solved through the ability of these organisms to generate hyphal branches at an exponential rate (Trinci, 1974).

One of the most important factors governing colony development is the spatial organisation of hyphae relative to one another. Hyphae of the filamentous actinomycetes avoid coming into contact with one another and rarely if ever crossover, when in this situation hypha often form branches and grow in the opposite direction. Neighbouring hyphae are thought to be able to sense proximity to one another, most likely as a result of nutrient limitation in the solid medium. Just as for unicellular microorganisms the growth of filamentous microbes on solid medium results in the creation of circular colonies formed as a consequence of apical and branched hyphal growth mechanisms. The measurement of colony expansion is usually expressed in terms of the colony radius in the form of the colony radial growth rate (K_r) (Pirt, 1967). In the early phase of colony formation this radial growth rate is exponential, however as the colony ages, and steady state conditions develop, this rate becomes constant. Expansion in mature colonies only occurs at the perimeter, the peripheral growth zone, which is responsible for maintaining the radial growth rate (Trinci, 1971).

Colony expansion is driven by apical growth with the branching mechanisms essentially performing a space-filling role. As mentioned previously, one of the major advantages of filamentous growth is the efficiency of medium colonisation and hence nutrient utilisation provided by both apical and branched growth. Colony aging also brings about differentiation in the growth kinetics and morphology of parent and branch hyphae, a process of cell specialisation. Branched growth in the central region of a colony results in the formation of a dense mycelium that over time utilizes all the available nutrients in that region. Once growth conditions become less favourable in the central region the formation of many secondary metabolites is triggered, which in itself stimulates the formation of aerial hyphae and the production of spores. It is not uncommon for individual colonies of filamentous microorganisms to be actively growing in one region and making preparations for death in another. Mature colonies consist of a viable vegetative peripheral growth zone and a dying sporulation zone in the centre of the colony (Allan & Prosser, 1983).

1.1.2.4. Spore Formation

The initiation of spore formation in actinomycetes is triggered by the onset of adverse environmental or nutritional conditions. The spore is an inert reproductive cell capable of remaining dormant until favourable conditions prevail, at which time it will germinate and generate fresh vegetative biomass. The growth of filamentous bacteria, such as the actinomycetes, on solid medium results in a very densely packed mycelium of vegetative hyphae. When conditions become less favourable for continued growth in this zone the organism will generate aerial mycelia, at the top of which spores will be formed, the major advantage of which is the ease of spore dispersal. The formation of spores in actinomycetes can be broadly categorised into two classes. Firstly hyphal fragmentation is the process whereby an existing hypha fragments to give rise to multiple smaller subunits, i.e. spores. Secondly, endogenous spore production results from the reorganisation of the cytoplasm and the formation of a spore completely contained within the hypha. Spore production by hyphal fragmentation may

also be accompanied by the presence or absence of an outer sheath, which enables spore retention until dispersal is initiated (Williams *et al.*, 1973).

Given that spore formation is a response to the onset of adverse growth conditions, the concentration and composition of the nutritional medium will influence sporulation. Nabais and da Fonseca (1995), working with *Streptomyces clavuligerus,* noted that a high carbon to nitrogen ratio and the addition of calcium carbonate to the growth medium stimulated increased sporulation. Furthermore, Dionigi *et al.* (1992) cultured *S. tendae* on four different nutrient media and examined the incidence of spore formation and the production of the secondary metabolite, Geosmin, in each case. Geosmin production occurred on all four media whereas sporulation occurred on only two. However, geosmin levels were higher in the two media where spore formation had taken place. This highlighted a possible link between secondary metabolite production and the onset of sporulation on solid media. The process of spore production, although normally confined to solid media systems, has also been observed in submerged cultures such as those of *Streptomyces antibioticus* (Novella *et al.*, 1992), where it was also observed to be dependent on medium composition.

1.1.3. Growth in liquid medium

Filamentous microorganisms are uniquely adapted for growth on a solid medium, in a way that unicellular organisms are not, through the colonising capabilities of apical and branched growth (Allan & Prosser, 1983; Prosser & Tough, 1991). However, cultivation of these microorganisms on an industrial scale is rarely if ever conducted on a solid medium and is generally performed in liquid media in the form of a submerged fermentation. One of the major differences experienced by the microorganism when growing in liquid media is the mobility of particles in suspension; the filamentous microbe is submerged in a moving liquid and not rooted to a solid medium. Furthermore, growth on solid media results in a flat, circular, two-dimensional colony, whereas growth in liquid media maximises the potential of branched growth by enabling the organism to develop in three dimensions.

The cultivation of filamentous microorganisms in liquid culture presents the fermentation engineer with a far more complex process than those containing unicellular organisms. The root of this complexity is the nature of filamentous growth and the resulting heterogeneity, whereby the culture morphology can range from dispersed mycelia to large compact pellets within the same vessel. The heterogeneity experienced in submerged fermentations is not only within the population as a whole but also within individual pellets. Pelleted growth in liquid medium results in many different internal regions in much the same way as colony growth on solid medium does. Hyphal growth in liquid medium is similar to that observed on solid medium, i.e. apical and branched growth. Culture morphology may range from dispersed mycelia to large compact pellets, as illustrated in Figure 1.1, depending on the organism itself and the processing conditions experienced in the liquid medium (Prosser & Tough, 1991).

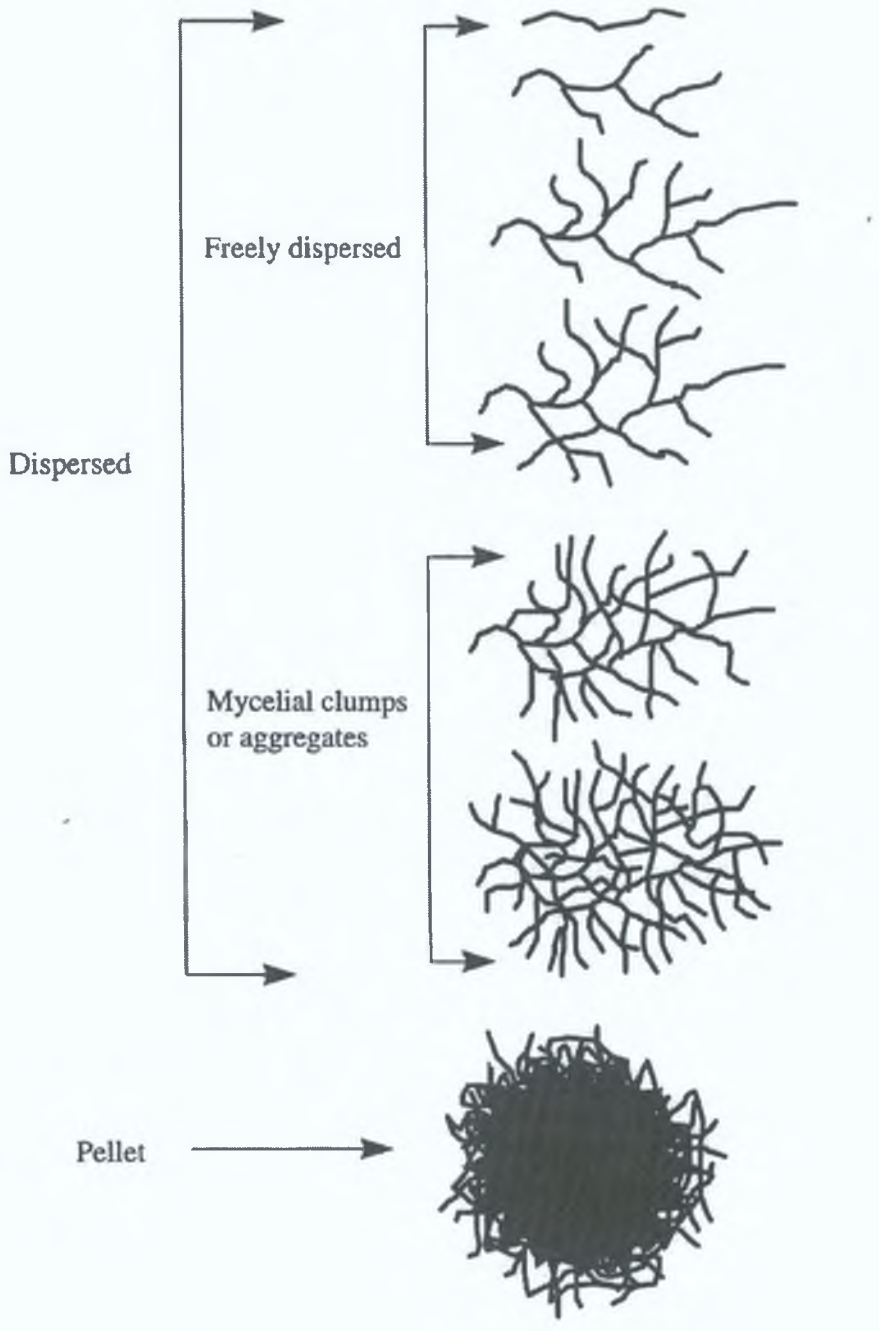


Figure 1.1. Classification of mycelial morphology from (Paul & Thomas (1998)).

1.1.3.1. Dispersed Mycelia

The morphological profile of filamentous microorganisms in liquid culture may vary from dispersed mycelia to pellets depending on both the organism itself and the process conditions experienced during the fermentation. In its simplest morphological form a culture can contain dispersed mycelia, a

homogeneous suspension of relatively short, branched hyphae. This free filamentous behaviour is the closest possible approximation to unicellular microorganisms. The development of a dispersed mycelial culture under optimum nutritional and environmental conditions enables the organism to achieve an exponential growth rate. Furthermore, the presence of the organism in a dispersed form affords proximity to the liquid medium for mass transfer purposes such as nutrient uptake and excretion of toxic compounds and metabolites (Prosser & Tough, 1991). In submerged fermentations, mycelia tend towards pellet formation either through continued growth or aggregation. The generation of a culture of dispersed mycelia is only possible through the careful regulation of process parameters to prevent pellet development and maintain a tight morphological distribution (Znidarsic & Pavko, 2001).

1.1.3.2. Pelleted Growth

Pellets are spherical or ellipsoidal agglomerates made up of a stable intertwined network of filamentous hyphae. Pellet formation can be either coagulative or non-coagulative, depending on the ability of spores to agglomerate and form pellets. Pellet formation and development within the filamentous bacteria is variable as observed by Tresner *et al.* (1967) who examined 145 *Streptomyces* species and found a continuum of morphological types, from large pellets several millimetres in diameter to free filamentous cultures resembling those of unicellular microorganisms. Filamentous growth mechanisms within pellets are responsible for enlargement and consolidation; apical growth is responsible for pellet expansion whereas branched growth is responsible for hyphal intertwining. The branching frequency of hyphal elements determines pellet density, which can range from loosely packed fluffy pellets to tightly packed compact pellets (Papagianni, 2004).

Pellet intrastructure varies greatly with respect to time. Colony enlargement on solid medium occurs as a result of the continual expansion of the peripheral growth zone surrounding a non-growing region. A similar

phenomenon occurs in liquid cultures where pellets are made up of a number of different layers. The simplest form is that of a young pellet where the structure consists of two layers, an outer shell of actively growing hyphae surrounding a core of metabolically active, slow growing, densely packed hyphae. Mature pellets consist of a more complex structure; as well as the two, previously mentioned layers, these pellets can also consist of a layer of dead cells showing signs of lysis and a hollow core (Prosser & Tough, 1991). The intrastructure of *Streptomyces fradiae* pellets was elucidated by Park *et al.* (1997). Using the fluorescent dyes flourescein isothiocyanate and propidium iodide, in conjunction with confocal scanning laser microscopy, it was shown that mycelial density increases and biomass viability decreases from the periphery to the core of the pellet.

The density or compactness of mycelial pellets is of particular interest for the mass transfer of oxygen and nutrients from the fermentation broth to the internal regions of the pellet. Cell metabolism depends heavily on mass transfer within the pellets for the provision of essential compounds and the removal of toxins. Limitations in the supply or removal of these compounds can trigger differentiation within pellets leading to the modification of cell metabolism, which can in turn lead to the formation of certain secondary metabolites of interest. The morphological distribution of biomass from dispersed mycelia to pellets may influence product formation in filamentous organisms, an example being maximal Nikkomycin production by *S. tendae* pellets of approximately 1.4 mm in diameter (Braun & Vechtlifshitz, 1991; Vechtlifshitz *et al.*, 1992).

1.1.3.2.1. Pellet Formation

Pellet formation in filamentous microorganisms has been traditionally classified as coagulative or non-coagulative, depending on the ability of individual spores to aggregate and form pellets (Nielsen *et al.*, 1995). However, subsequent aggregation may still occur within systems traditionally designated non-coagulative. For example, *Penicillium chrysogenum* was originally thought by Takahashi and Yamada (1959) to be non-coagulating but Packer & Thomas (1990) identified that pellet formation was as a result of

hyphal element aggregation. Another example is *S. tendae*, which is non-coagulative in the early stages of growth but subsequently capable of hyphal aggregation as observed by Vechtlifshitz *et al.* (1990). Pellet formation in submerged fermentations of filamentous microorganisms is a complex, multi-parameter process initially involving of particle collision, facilitates interaction leading to subsequent aggregation or entanglement.

In submerged fermentation systems, fluid mixing through agitation, brings about particle collision. Lu *et al.* (1998) examined the kinetics of fine particle aggregation using quartz (SiO_2) and rhodochrosite ($MnCO_3$) in a stirred tank reactor. This investigation showed that the kinetics of particle aggregation often depend on the existence of an energy barrier between colliding particles; if the collision energy is not sufficient then the particles will not aggregate. Collision energy is primarily a function of fluid turbulence, which is, in turn, influenced by broth rheology, control of which enables regulation of particle interaction. Viscosity affects the motion of particles within a fermentation broth through its impact on viscous drag forces and fluid flow characteristics. An increase in broth apparent viscosity results in an increase in viscous drag forces and a decrease in the Reynolds number. Consequently the turbulence within the system decreases, which in turn reduces the rate of particle collision.

Once a collision has occurred and sufficient energy is present the particles will aggregate by one of the following mechanisms. The composition, structure, hydrophobicity or charge of the cell wall, as well as the presence of extracellular polymeric substances may influence flocculent behaviour. In coagulative systems, multiple spores aggregate at a very early stage in the fermentation and give rise to pelleted biomass. One of the primary forces regulating this process is the presence of charged groups on the cell surface, an example being carboxyl groups in proteins (Atkinson & Daoud, 1976). The regulation of broth pH may be responsible for altering the electrical charge on the cell surface, thereby influencing cell-cell interaction and aggregation. Carlsen *et al.* (1996) altered the pH in submerged fermentations of *Aspergillus oryzae*, a coagulative organism, and observed the impact on both

cell growth and morphology. The morphological profile of the culture was found to vary significantly with broth pH. At pH 3 – 3.5 the culture consisted entirely of dispersed mycelia; at pH 4 – 5, a mixed population of both pellets and dispersed mycelia prevailed; at pH values of 6 and higher the culture consisted entirely of pellets. The influence of pH on pellet formation in this coagulative system was shown to be specific to spores. In a similar experiment, performed using filamentous vegetative inocula, it was shown that pH does not influence culture morphology (Carlsen *et al.*, 1996). Pellet formation in *A. oryzae* is of the coagulating type and the extent to which it occurs is pH dependent, possibly as a result of altered cell surface charges.

Pellet formation within coagulating systems is not just dependent on the cell surface charge but has also been shown to depend on hydrophobicity. In a study undertaken by Dynesen & Nielsen (2003) it was demonstrated that the surface hydrophobicity of *Aspergillus nidulans* conidiospores influences pellet formation. This study investigated the importance of two surface hydrophobic proteins, DewA and RodA, in hydrophobic interaction, aggregation and pellet formation. Disruption of the corresponding genes *dewA* and *rodA* led to the generation of mutants deficient in either or both of these proteins. The absence of either of these hydrophobic proteins led to a decrease in the surface hydrophobicity of *Aspergillus nidulans* conidiospores. In cultures inoculated with DewA- and RodA-deficient conidiospores it was observed that decreasing hydrophobicity increased the percentage of dispersed mycelia compared to the control. The authors concluded that both the electrical charge and the hydrophobicity of the conidiospores influenced pellet formation but that this process could not be ascribed to these factors alone.

The influence of surface hydrophobicity is not confined to spore-spore interactions but is also prevalent in vegetative biomass. The nature of filamentous growth through apical extension ensures that the cell wall composition, structure and hence hydrophobicity are constantly changing. Ryoo and Choi (1999) investigated the influence of surface properties on pellet formation in *Aspergillus niger*, using a thermodynamic parameter, the

Gibbs free energy of pellet formation, which is derived from the hydrophobicity of the biomass and the surface tension of the cell free broth. A negative Gibbs free energy is indicative of aggregation, the more negative the number the greater the extent of pellet formation. Experimental analysis, using cultures pitched with varying strength inocula, indicated that increasing spore concentration increased the Gibbs free energy and hence reduced the extent of pellet formation. Pellet formation in submerged fermentations of filamentous microorganisms is partly dependent on the cell surface hydrophobicity and broth surface tension.

Cell surface properties such as the electrical charge and hydrophobicity are partly responsible for regulating spore and hyphal aggregation leading to pellet formation. For example, Vechtlifshitz *et al.* (1990), proposed that the primary forces inducing cellular aggregation in cultures of *S. tendae* are the hydrophobic interactions of the vegetative cell walls. Once entanglement and/or aggregation has occurred and the disruptive forces are not prohibitive the entwining nature of filamentous growth ensures irreversible binding of the constituent parts to form a pellet.

Thus, pellet formation in submerged fermentations is a function of cell surface properties and bioprocessing parameters within the fermentation vessel. Papagianni (2004) states that the factors that influence mycelial aggregation and hence pellet formation in filamentous organisms are;

- Inoculum size, type and age
- Genetic factors and the ability to produce bioflocculants
- Medium composition
- Biosynthesis or addition of polymers, surfactants or chelators
- Shear forces
- Temperature and pressure
- Medium Viscosity

Mycelial biomass is subject to many adhesive and disruptive forces within a submerged fermentation and the morphological profile of any filamentous microorganism may be said to represent the final result of these competing forces (Braun & Vechtlifshitz, 1991). Pellet formation in submerged fermentations of filamentous microorganisms is a function of particle collision, interaction and aggregation/entanglement.

1.1.3.2.2. Pellet Growth

Mycelial pellets consist of a complex structure of filamentous biomass. The typical structure of a pellet consists of a number of different layers; an outer shell of actively growing hyphae surrounds a core of metabolically active yet non-growing densely packed hyphae, which in turn surrounds a layer of dead cells beginning to show signs of lysis and finally a hollow core (Braun & Vechtlifshitz, 1991; Prosser & Tough, 1991). Pellet growth is restricted to the outermost layer where there is unrestricted access to nutrients. Filamentous expansion through apical and branched growth is a continual process whereby actively growing biomass in the peripheral zone will eventually become part of the second and third layers.

The densely intertwining nature of filamentous growth in the inner regions of mycelial pellets presents problems for nutrient influx and metabolites and toxin efflux. Assessment of whether there are mass transfer limitations within a pellet may be possible by determining the critical radius, which has been defined as the pellet radius where the substrate reaches zero. For a typical pellet, the critical radius is found to be in the range of 50-200 μm when oxygen is the limiting substrate. Consequently, for pellets with a radius larger than the critical radius there will only be growth in the outer shell of the pellet (Nielsen & Carlsen, 1996). Mycelial density increases and biomass viability decreases from the periphery to the core of the pellet (Park *et al.*, 1997). Mass transfer limitations cause the differentiation of viable distal biomass and the concomitant alteration of cell biochemistry from general to secondary metabolism (Papagianni, 2004). When mass transfer limitations in the inner regions of the pellet become critical the cells lyse and disintegrate to form a hollow core.

During the early phase of colony formation on a solid medium the radial growth rate is exponential, however as the colony ages and steady state conditions develop this rate becomes constant. Similarly, for growth in liquid medium, early stage growth is exponential but as the pellet enlarges mass transfer limitations alter biomass production and enlargement is assumed to follow cube root kinetics.

$$M^{1/3} = M_0^{1/3} + kt \qquad (1.1)$$

where M_0 represents biomass concentration at time *0*, M represents biomass concentration at time t and k is a constant. Emerson (1950) demonstrated the existence of a linear relationship between the cube-root of the biomass concentration and the culture age for the filamentous fungi *Neurospora crassa*. This relationship has been shown to apply to a variety of filamentous fungi and bacteria (Marshall & Alexander, 1960). Although useful, cube-root kinetics have limitations as they do not account for impact of mass transfer and substrate concentration on growth within heterogeneous pellets (Tough *et al.*, 1995). Mass transfer limitations arise as a result of densely packed hyphae within the pellet, which in turn causes cellular differentiation and heterogeneity. If mass transfer limitations persist this will result in cell autolysis and the formation of a hollow core at which point cube-root kinetics will no longer apply (Papagianni, 2004).

1.1.3.2.3. Pellet Fragmentation and Disruption

Pellet formation and growth within filamentous fungal and bacterial systems does not proceed unchecked. Two of the most important aspects of pellet development in submerged culture are the destructive forces of fragmentation and disruption. Pellet fragmentation occurs almost exclusively as a result of shear forces causing hyphal breakage from the pellet surface (Papagianni, 2004). Furthermore, Paul *et al.* (1994), working with *P. chrysogenum,* also noted that physiological effects, a greater incidence of vacuolisation for this particular filamentous fungi, in conjunction with shear

may enhance fragmentation. This would seem to support the idea that fragmentation only occurs when an internal decay process has weakened the hyphae. Nielsen & Krabben (1995) showed that a linear relationship existed between the rate of fragmentation and the energy input into the bioreactor. The fragmentation of viable hyphae often results in growth renewal, as the newly liberated hyphal fragments can generate new pellets, a useful method of reseeding the pellet population. The processes of hyphal fragmentation and new pellet generation enable the organism to fully utilise the available resources.

Pellet disruption is generally a two-stage process consisting initially of the loss of structural stability through lysis of the pellet core followed by pellet break-up as a result of mechanical forces. The nature of apical and branched growth in filamentous fungi and bacteria ensures the formation of a dense network of intertwining hyphae within the inner regions of a pellet, the exact density and compactness of which varies with respect to processing conditions over the course of the fermentation. The densely woven hyphae impart a rigidity and structural stability to the pellet that allows it to withstand many disruptive forces. The formation and development of a mycelial pellet brings about hyphal differentiation as a result of limitations in the mass transfer of nutrients into and metabolites and toxins out of densely packed hyphae (Papagianni, 2004). Should mass transfer limitations become critical, cell lysis, brought about by material imbalances in the hyphae, will begin. Factors that can trigger cell lysis include accumulation of toxic compounds, intracellular disturbances, lack of nutrients or the presence of lytic enzymes. Cell lysis in the innermost region of the pellet, results in the formation of a hollow core, which just like the peripheral growth zone is continuously enlarging. A reduction in pellet density and a loss of structural stability as a result of the formation of the hollow core increases the susceptibility of the pellet to disruption by mechanical forces (Nielsen *et al.*, 1995). Pellet disruption in this manner completes the pellet lifecycle and any viable hyphal fragments that survive can reseed the culture for further growth.

1.1.3.2.4. Advantages of Pelleted Growth

The growth of filamentous organisms in pellets is preferable to that of dispersed mycelia, from a bioprocessing point of view, as it reduces the tendency of the microorganism to grow on fermenter walls, wrap around impellers and foul gas distribution apparatus thereby improving operating conditions (Papagianni, 2004). Furthermore one of the major advantages of pellet growth over dispersed mycelia is the reduction in broth viscosity (Sinha *et al.*, 2001a), which ensures more efficient power consumption for mass transfer processes within the system.

1.1.4. Influence of culture morphology on product formation

The morphological profile of submerged fermentations of filamentous microorganisms can vary from dispersed mycelia to pellets depending on process conditions. The close link between culture morphology and physiology has lead to the hypothesis that product formation may be morphologically regulated. Macroscopic morphology influences the rheological characteristics of the fermentation broth as pelleted cultures tend to behave like Newtonian fluids while dispersed mycelial cultures tend to exhibit non-Newtonian characteristics. Alteration of broth rheology influences the heat and mass transfer properties of the fluid and potentially alters the metabolism of the biomass within the system. The development of mycelial pellets through apical and branched growth generates a tightly packed hyphal network within the non-growing regions. Diffusional limitations within the mycelial pellet may be responsible for cellular differentiation in the inner regions and thereby trigger biochemical specialisation. Differentiation and associated biochemical specialisation result in cells switching from general metabolism to secondary metabolism. Given the influence of pellet morphology on cellular differentiation and the associated shift to secondary metabolism then the influence of mycelial morphology on product formation is to be expected (Znidarsic & Pavko, 2001).

Mycelial biomass is subject to many adhesive and disruptive forces within a submerged fermentation and the morphological profile of any filamentous

microorganism may be said to represent the final result of these competing forces (Braun & Vechtlifshitz, 1991). Many authors have suggested the dependence of product formation on a particular morphological profile in submerged fermentations. However this is not always the case as morphological dependence, if any exists at all, varies from one compound to another and one organism to another. Virginiamycin production by *Streptomyces virginiae* (Yang *et al.*, 1996), clavulanic acid production by *Streptomyces clavuligerus* (Belmarbeiny & Thomas, 1991) and penicillin production by *P. chrysogenum* (Nielsen *et al.*, 1995) were all shown to be independent of culture morphology. On the other hand Morrin & Ward (1990) linked the increased production of fumaric acid in *Rhizopus arrhizus* to the switch from clumped pellets to dispersed mycelial growth. Furthermore the production of tylosin by *S. fradiae* (Tamura *et al.*, 1997), striatals by *Cyanthus striatus* (Gehrig *et al.*, 1998) and lovastatin by *Aspergillus terreus* (Casas Lopez *et al.*, 2005) was influenced by the morphological distribution of the pellet population. Jonsbu *et al.* (2002) noted that product formation is also a function of pellet size; once pellets of *Streptomyces noursei* exceeded a certain size limit, nystatin production ceased. This was observed to be more than likely as a result of diffusional limitations leading to core death. Although a particular culture morphology is in some instances a pre-requisite for product formation this is not always the case. The relationship between morphology and productivity is system specific. The influence of pellet morphology on the formation and composition of secondary metabolites is still largely unexplored (Braun & Vechtlifshitz, 1991).

1.2. INFLUENCE OF FERMENTATION CONDITIONS ON CULTURE MORPHOLOGY

1.2.1. Introduction

The morphological development of filamentous microorganisms in submerged fermentations is a complex process of apical and branched growth, and particle aggregation and entanglement. The impact of culture morphology and specifically the size and density of mycelial pellets has been shown in many instances to influence product formation in submerged fermentations of filamentous microorganisms (Casas Lopez *et al.*, 2005; Gehrig *et al.*, 1998; Tamura *et al.*, 1997). Given that culture morphology influences product formation in many cases; the regulation of morphological development in order to control compound production is the next logical step. An understanding of the mechanisms of pellet formation and development and the processing conditions that influence them, is essential when trying to regulate the morphological profile of a culture. The growth of filamentous microbes in submerged fermentations is highly dependent on the environmental and nutritional conditions experienced. Some of the parameters that influence culture morphology in these systems are nutrient media composition, inoculum type, concentration and age, agitation and fluid characteristics such as viscosity and surface tension. Alterations in these parameters will influence particle aggregation and pellet formation. This leads to the hypothesis that the morphological regulation of pellet formation and development may be achieved through the control of processing parameters.

1.2.2. Nutrient Medium Composition

The composition of nutrient media must be sufficient to support growth and product formation in submerged fermentations. The basic requirements of filamentous microorganisms in submerged fermentations include water, molecular oxygen, an energy source, organic carbon, nitrogen other than in a molecular form and several other elements. There are approximately thirteen elements essential to life, five of which are required in large quantities

(macronutrients); carbon, hydrogen, oxygen, nitrogen and phosphorous; the remaining eight are required in small amounts (micronutrients): potassium, sulphur, magnesium, manganese, iron, zinc, copper and molybdenum (Papagianni, 2004). Filamentous microorganisms depend heavily on the presence of the requisite nutritional compounds at optimal concentrations to ensure maximal growth and product formation. One point of note in these systems is that optimal nutrition for growth is not necessarily the same for production (Glazebrook *et al.*, 1992; Kojima *et al.*, 1995; Schrader & Blevins, 2001).

Fermentation media may be either complex or defined depending on the nutritional composition. Complex media are typically made up crude supplements such as beef or yeast extract, which contain a mixture of nutritional compounds. The exact elemental composition of complex media is not known, although a rough approximation can be made. On the other hand, defined media are made up of exact quantities of all the required nutrients in the most elemental form possible – sugars, salts etc. Complex media generally provide good growth and product formation, but often cause bioprocessing problems. Choi *et al.* (1998) using two complex nitrogenous compounds, pharmamedia and gluten meal, for the production of tylosin from *S. fradiae* encountered non-Newtonian behaviour in the fermentation broth, a problem not usually associated with defined media. Defined media are often preferable in production systems as they allow greater regulation of cellular metabolism and hence compound formation. However, defined media containing exact quantities of pure compounds are typically more expensive to formulate, given the associated purification costs, than complex media containing unknown quantities of crude compounds.

The type and concentration of the organic carbon source is of the utmost importance to the nutritional welfare of microorganisms, both as a supply of carbon and energy. Microorganisms may utilise carbohydrates, proteins, lipids or hydrocarbons as their carbon and energy source. Compound and concentration specificity varies from one organism to the next. Glazebrook *et al.* (1992), investigating the influence of a number of carbohydrates on the

growth and morphology of *Streptomyces akiyoshiensis* in submerged cultures, noted that maximal biomass growth was achieved with starch which also produced the smallest mycelial aggregates, compared to lactose which produced the largest. Papagianni *et al.* (1999) investigated the impact of glucose concentration on the morphology and citric acid production by *Aspergillus niger* in both batch and chemostat cultures. The morphology of the organism seemed to be related to the growth rate of the organism which, in turn, depended on the glucose levels; low glucose levels resulted in smaller mycelial clumps. Optimal carbon source for product formation varies from one organism to another, examples being a combination of fructose and mannose for rapamycin biosynthesis by *Streptomyces hygroscopicus* (Kojima *et al.*, 1995), mannitol for geosmin biosynthesis by *Streptomyces halstedii* (Schrader & Blevins, 2001) and glycerol for the production of a broad spectrum antibiotic by *Streptomyces antibioticus* $sr_{15.4}$ (Haque *et al.*, 1995).

Two of the most important macronutrients needed for growth and product formation in submerged fermentations of filamentous microorganisms are nitrogen and phosphorous. Most microbes cannot fix atmospheric nitrogen so they need a readily utilisable source, usually in the form of ammonia or urea, nitrates or organic compounds such as amino acids and proteins. Similarly, phosphorous can be obtained from phosphates or organic forms. Both nitrogen and phosphorous are essential for cell growth and regulate or participate in cell metabolism, thereby influencing culture growth, morphology and product formation. The inclusion of yeast extract, gluten meal and corn steep liquor has been shown to result in the formation of circular pellets, whereas pharmamedia, soybean meal or fish meal resulted in the formation of radial filamentous mycelia, for an arachidonic acid-producing strain of *Mortierella alpina* (Park *et al.*, 1999). Lee *et al.* (1997) found that media including the nitrogen-containing salt ammonium sulphate produced comparable levels of biomass and increased rapamycin biosynthesis by *Streptomyces hygroscopicus* compared to media containing a mixture of three organic nitrogen sources. It was observed in cultures of *S. akiyoshiensis* that increasing phosphate concentration resulted in a

corresponding decrease in mycelial aggregation in cultures while not affecting growth (Glazebrook *et al.*, 1992). The level of phosphorous can also influence compound formation, as demonstrated for geosmin production by *S. halstedii* where increasing phosphorous concentration increased product formation (Schrader & Blevins, 2001).

As well as the macronutrients, microorganisms also require smaller amounts of potassium, sulphur, magnesium, manganese, iron, zinc, copper and molybdenum to maintain life. These micronutrients are primarily involved in the regulation of cellular metabolism and, consequently, in product formation. The specific requirements for and utilisation of micronutrients varies from one organism to another. Schrader & Blevins (2001) investigated the impact of several micronutrients on geosmin production by *S. halstedii* and showed that increased zinc levels increased growth but decreased geosmin production whereas increasing iron and copper concentrations decreased growth and production.

A submerged fermentation of filamentous microorganisms is a complex process with many different variables impacting on growth and product formation. In aerobic systems, one of the most important is the supply of oxygen to the organism via gas-liquid mass transfer from sterile air contacted with the fermentation media. The nature of filamentous growth varies from dispersed mycelia to pellets depending on the degree of hyphal aggregation, with the rheological characteristics of fermentation broths depending heavily on culture morphology, especially dispersed mycelia. Pelleted cultures may become oxygen deficient due to the mass transfer limitations experienced as a result of the prohibitive hyphal density in the inner regions of the pellet. In unicellular fermentations, when oxygen limitation occurs, counteractive measures in the form of increased aeration and agitation are put in place. However, in filamentous systems increased agitation influences pellet formation, which may in turn alter culture morphology, broth rheology and gas-liquid mass transfer (Cui *et al.*, 1998).

The dissolved oxygen tension and the rate of gas-liquid mass transfer can have a profound effect on the growth and product formation in submerged filamentous fermentation systems. Vechtlifshitz *et al.* (1990) working with *S. tendae* examined the impact of oxygen transfer on pellet morphology by varying culture volume in agitated shake flasks. It was observed that an increase in shake flask volume at an approximately constant shear rate decreased the oxygen transfer rate and resulted in a larger population of smaller pellets while maintaining comparable biomass levels. The authors noted that, although agitation was one of the major parameters controlling pellet development, it was oxygen supply that regulated the maximal size of the pellet. The rate of gas-liquid mass transfer is largely regulated by three process parameters. Firstly agitation, which is controlled by the impeller type and speed in the case of a bioreactor or the shaker table speed for shake flasks, regulates gas-liquid mass transfer by controlling the interfacial surface area to volume ratio. Secondly aeration, which is controlled by the air flowrate for a bioreactor and the liquid-to-headspace ratio in a shake flask, regulates the supply and hence gas-liquid mass transfer. Thirdly the broth, the presence of dispersed mycelia or extracellular polysaccharides may cause non-Newtonian behaviour and thus influence gas-liquid mass transfer (Banerjee, 1993; El-Enshasy *et al.*, 2000; Garcia-Ochoa *et al.*, 2000). Furthermore, the biomass concentration within the broth may regulate gas-liquid mass transfer through oxygen consumption for metabolic purposes. The oxygen transfer rate influences product formation in submerged fermentations of filamentous microorganisms whereby sensitivity to the dissolved oxygen concentration in the nutrient broth regulates productivity (Banerjee, 1993; El-Enshasy *et al.*, 2000). For example Vechtlifshitz *et al.* (1992) states that the formation of Nikkomycin greatly depends on the availability of oxygen, with increasing oxygen transfer typically increasing productivity.

1.2.3. Inoculum Type, Concentration and Age

As mentioned previously, Lu *et al.* (1998) proposed the existence of an energy barrier between colliding particles whereby once collision energy is

sufficient, the particles will interact and aggregate. Furthermore, the rate of collision is a function of the number of particles present in the system. Therefore, an increased concentration of spores or hyphae should result in an increased incidence of collision, which should, in turn, result in increased aggregation and/or entanglement and hence pellet formation.

Pellet formation in submerged fermentations is a function of inoculum type, concentration and age (Papagianni, 2004). An inoculum typically consists of either a suspension of inert spores or a solution of vegetative biomass. In a multi-stage industrial fermentation process, the former is normally used in the initial scale-up stages with the latter in the final stages. The major advantage of using vegetative inocula is the reduced lag time associated with germination and initial biomass development. Given that vegetative cell wall properties are often responsible for aggregation, it seems logical that the type of inoculum used will influence the final outcome. El-Enshasy *et al.* (2000) observed that both inoculum concentration and type were responsible for regulating growth and productivity in *Streptomyces natalensis* cultures. On comparison of both vegetative and spore inocula, it was observed that although comparable biomass levels were present in all cultures, those inoculated with spores produced the most natamycin. van Suijdam *et al.* (1980), working with three different fungal strains, inoculated a bubble column fermenter with a spore suspension, a pelleted preculture and a dispersed mycelial preculture to investigate the impact of inoculum type on culture morphology. Inoculation with the spore suspension led to the gradual development of a uniformly sized pellet population, while the pelleted preculture led to the development of a wide size distribution of pellets and the dispersed mycelial preculture lead to the initial development of hyphal flocs and eventually pellets.

Vechtlifshitz *et al.* (1990) investigated the impact of spore inoculum concentration on pellet formation for *S. tendae.* Firstly it was noted that an inverse relationship existed between inoculum concentration and pellet size whereby increasing the order of magnitude of spores in the system produced smaller pellets. The morphological profile of the culture varied from free

filamentous growth at 10^6 spores ml^{-1}, through a mixed pellet population at 10^3 spores ml^{-1} to a few large pellets at 1 spore ml^{-1} (in-flask concentration). The authors classify *S. tendae* as a non-coagulative organism given that one spore yielded one pellet as indicated by the spore-to-pellet ratio. Only at spore loading densities of greater than 10^3 spores ml^{-1} did the organism deviate from this behaviour and begin to aggregate. A similar situation was observed by Nielsen *et al.* (1995) for *P. chrysogenum*, another non-coagulating organism where aggregation occurs between hyphal elements and not spores. The regulation of culture morphology through inoculum concentration has been observed by a number of other researchers (Domingues *et al.*, 2000; Kim & Hancock, 2000; Papagianni & Moo-Young, 2002; Tucker & Thomas, 1992) whereby increasing inoculum concentration increased particle count and decreased particle size.

As well as concentration and type, the age of spore and vegetative inocula has been demonstrated to influence culture morphology and product formation. Given that spore or vegetative cell wall properties are responsible for aggregation it seems logical that the age influences the final outcome. The age of *Aspergillus niger* spores was determined to be a factor in citric acid production (Vergano *et al.*, 1996). The authors observed that spores harvested between 3 and 7 days after incubation and subsequently used as inocula produced five and a half times as much citric acid as those that were inoculated with 9 to 11 day old spores. Similarly the age of vegetative inocula was shown to have an impact on product formation in *S. natalensis* cultures (El-Enshasy *et al.*, 2000). The length of the preculture step was demonstrated to have a marked affect on natamycin production. Increasing the preculture step from 24 to 48 hours increased production while increasing it from 48 to 96 hours decreased production.

1.2.4. Agitation and Shear

The cultivation of filamentous microorganisms in submerged fermentations relies heavily on sufficient agitation to promote mixing within the system. Adequate mixing is a prerequisite in such fermentation processes in order to

achieve uniform distribution of temperature and concentration of nutrients and oxygen, should it be required, within the vessel. Agitation is responsible for sufficient heat and mass transfer by promoting mixing, which in turn results in the blending of substrates and the dispersal of oxygen and heat. These processes are essential to the growth of the organism and the production of compounds of interest. Furthermore, agitation plays a vital role in the morphological development of filamentous fermentations by regulating particle-to-particle collision and thus subsequent interaction and aggregation. Both the incidence of collision and the energy that particles possess when they do collide are a function of agitation. Once particles collide and the collision energy is sufficient spore and/or hyphal aggregation can take place and pellets may form, thus agitation plays a vital role in pellet formation.

Once pellets have formed, agitation is responsible for regulating culture morphology through fragmentation and disruption. Hyphal fragmentation can occur as a result of shear forces on the particle by the fluid, by collision with another particle or by collision with process equipment. Nielsen & Krabben (1995) demonstrated that the incidence of hyphal fragmentation in submerged fermentations of *P. chrysogenum* was a function of the effective hyphal length, which in turn was linearly related to the power input to the vessel. However, Paul *et al.* (1994) demonstrated that agitation alone was not responsible for fragmentation and that physiological effects, increased vacuolization for the particular filamentous fungus studied, in conjunction with agitation enhanced fragmentation. Similarly, agitation influences pellet disruption through the regulation of incidence and energy of collision. A decrease in pellet density and a loss of structural stability as a result of the formation of the hollow core increases the susceptibility of the pellet to disruption by mechanical forces (Nielsen *et al.*, 1995). Both fragmentation and disruption provide new centres of growth for future mycelial development (Cui *et al.*, 1997).

One phenomenon commonly caused by fragmentation and/or disruption in fermentations of this type, is the development of cultures with a high degree of filamentous growth, resulting in non-Newtonian behaviour, which

negatively impacts on heat and mass transfer within the broth. Counteractive measures to restore heat and mass transfer to the levels desired usually involve increased agitation, which starts the cycle all over again, consumes considerable power input and possibly even causes shear damage to the cells (Vechtlifshitz *et al.*, 1992). Tamura *et al.* (1997) working with *S. fradiae* in a 3L fermenter noted that at an agitation speed below 400 rpm, the majority of the biomass was pelleted, whereas above 400 rpm the majority was filamentous. The authors state that the optimal mycelial area for maximum tylosin production was approximately 4×10^3 μm^2. Furthermore, the authors recommend regulation of pellet development through the control of the agitation speed as a means of ensuring maximal productivity. In filamentous fermentation systems agitation speed and the corresponding hydrodynamic forces influence pellet formation, growth, fragmentation and disruption. The common trend is that increasing agitation results in decreasing particle size (Amanullah *et al.*, 2001; Casas Lopez *et al.*, 2005; Cui *et al.*, 1997; Hotop *et al.*, 1993).

The influence of agitation on culture morphology and the optimum conditions for pellet size regulation and compound formation vary from one microorganism to another (Papagianni, 2004). With this in mind, a few general statements can be made regarding the influence of agitation on culture morphology. Low agitation levels lead to the formation of a small population of large fluffy clumps. Intermediate agitation levels lead to the formation of a mixed population made up predominantly of medium sized compact pellets. High agitation levels lead to the formation of a mixed population containing a large number of small compact pellets and an increasing proportion of filamentous growth. Very high agitation speeds lead to predominantly free filamentous growth.

1.2.5. Fermentation Fluid Properties

In order to fully understand the impact of the fermentation broth on culture morphology we must first understand the properties of the fluid itself. Fluid mechanics involves the study of forces and motions in fluids. A fluid may be

defined as a substance which when exposed to the required shear force starts to move and continues as long as the force is applied. Fluids are typically liquids and gases although solid-liquid intermediates exist and exhibit either solid or liquid characteristics depending on the shear force applied. A shear force is one that is applied to a system that makes one surface slide parallel to another and results in the fluid experiencing a shear stress i.e. shear force per unit area. The primary difference between liquids and gases is the proximity of the molecules to one another due to varying attractive forces; molecules in liquids are close together whereas gases are far apart. The forces of attraction and hence the proximity of the molecules to one another are dependent on the temperature and pressure of the system. Fermentation broths are liquids and from this point on we will confine the discussion to these alone. Fluid properties such as density, specific gravity, viscosity and surface tension greatly influence fluid mechanics (de Nevers, 1991). The latter two are of particular interest in the context of the project work undertaken.

1.2.5.1. Viscosity

Viscosity is the resistance of a fluid to deformation under shear stress and is related to the resistance to motion in a fluid, a measure of drag force. With increasing viscosity comes an increased resistance to flow. Fluids are typically divided into one of two categories depending on their rheological characteristics, Newtonian and non-Newtonian. For Newtonian systems the viscosity remains constant with respect to alterations in the shear rate. An example of a typical Newtonian liquid would be a simple solution containing inorganic salts, nitrogenous compounds and sugars, i.e. a fermentation broth. However many fluids do not exhibit Newtonian behaviour, these are generally complex mixtures usually in the form of slurries, gels or pastes in which the dissolved molecules, especially polymers, are much larger than water.

The flow behaviour of non-Newtonian fluids depends on the type of particles or molecules that are present in solution, an example being pseudoplastic fluids. Pseudoplastic, or *shear-thinning* fluids have a lower apparent viscosity

at higher shear rates, and typically have dissolved long-chain molecules randomly oriented within the fluid at rest. Once shear is applied to the system the orientation becomes progressively less random as the particles line up with one another and begin to flow in a linear fashion, hence the shear thinning nature of this system (de Nevers, 1991). From a bioprocessing point of view the rheology of a fermentation broth is of interest given that it impacts on fluid pumping and mixing, which in turn affect the rates of heat and mass transfer within the system (Doran, 1995).

The viscosity of a fermentation broth influences culture morphology in two ways. Firstly it influences the motion of particles within the fluid and hence the rate of collision, interaction and aggregation and secondly it influences the rates of heat and mass transfer to and from the filamentous biomass. The viscosity of a fermentation broth is in turn influenced by the culture morphology.

1.2.5.1.1. Viscosity of Fermentation Broths

Most fermentation fluids are non-Newtonian primarily due to the presence of dissolved molecules or suspended solids with many exhibiting pseudoplastic behaviour (Doran, 1995), the causes of which may be threefold. Firstly, the initial fermentation medium may contain nutritional compounds capable of inducing non-Newtonian behaviour, for example, complex polysaccharides such as starch or simpler disaccharides such as sucrose (Cho *et al.*, 2002; Sinha *et al.*, 2001b) or nitrogenous compounds such as pharmamedia or gluten meal (Choi *et al.*, 1998). Secondly, the biomass may be capable of producing compounds during the fermentation, such as the production of xanthan gum by *Xanthamonas campestris*, which cause non-Newtonian behaviour (Garcia-Ochoa *et al.*, 2000). Thirdly, in the case of filamentous microbes the organism itself may be responsible for the viscosity of the solution (Choi *et al.*, 2000; Riley *et al.*, 2000). The rheology of the fermentation broth is heavily dependent both on the biomass concentration and the morphological profile of the organism (Allen & Robinson, 1990); pelleted fermentation broths may behave in a Newtonian fashion whereas free filaments can exhibit pseudoplastic behaviour (Sinha *et al.*, 2001a).

Warren *et al.* (1995) examined the relationship between morphology and broth rheology in three actinomycetes (*Saccharopolyspora erythraea, Actinomadura roseorufa and Streptomyces rimosus*). The authors noted that the generation of biomass in each of the three cultures induced pseudoplastic behaviour in the broth. The advent of significant growth caused a decrease in the flow behaviour index, *n*, to between 0.20 and 0.25 and the fluid consistency index, *K*, increased with respect to biomass concentration.

Alteration of the rheological characteristics of a fermentation broth through either the addition of nutrient components, synthesis of extracellular polymers or the altered morphological profile of the biomass can cause significant mixing and aeration problems. Counteractive measures to restore heat and mass transfer to the levels desired usually involve increased mixing, which may further aggravate the situation, consume considerable power input and possibly even causes shear damage to the cells (Vechtlifshitz *et al.*, 1992). Both heat and mass transfer are vital to the metabolism of filamentous microorganisms. An alteration in metabolism will influence cellular growth, differentiation and product formation as discussed in previous sections. The rheological characteristics of a fermentation fluid influence heat and mass transfer to and from the biomass and hence regulate compound formation as is the case of tylosin production by *S. fradiae* (Choi *et al.*, 1998).

1.2.5.1.2. Manipulation of Broth Viscosity

Viscosity affects the motion of particles within a fermentation broth through its impact on drag forces within the fluid and also the Reynolds number, a measure of fluid turbulence. Büchs *et al.* (2000) defined the Reynolds number in shake flasks as:

$$\mathrm{Re} = \frac{\rho n d^2}{\mu} \qquad (1.2)$$

where *Re* is the Reynolds number, ρ is the fluid density, *n* is the rotational speed of the shaker, *d* is the maximum inner diameter of the flask and μ is

the fluid viscosity. The Reynolds number is the ratio of inertial forces to viscous forces and is typically used for determining whether flow will be laminar or turbulent. Turbulent flow occurs at high Reynolds numbers and is driven by inertial forces, producing random eddies, vortices and other flow fluctuations whereas, laminar flow occurs at low Reynolds numbers and is driven by viscous forces, and is characterized by smooth, constant fluid motion (de Nevers, 1991; Douglas *et al.*, 1985). Büchs *et al.* (2000) calculated that at a shaking frequency of approximately 80 to 120 rpm, the Reynolds number in shake flasks ranges from 5,000 to 50,000. Furthermore, the authors hypothesize that, within this range, the transition from laminar to turbulent flow can be found. The Reynolds number is inversely proportional to the fluid viscosity and consequently an increase in broth apparent viscosity through compound addition decreases the Reynolds number, i.e. decreases fluid turbulence and causes flow to become more laminar. As mentioned previously particle aggregation relies upon collision frequency and collision energy, both of which are reduced by laminar flow (Lu *et al.*, 1998). Alteration of fluid flow within the system through the control of broth rheology enables the regulation of particle collision, interaction and subsequent aggregation.

Fermentation broth rheology can be influenced by a number of broth components or culture morphology. Only the composition of the nutrient medium can regulate broth rheology from the beginning of the fermentation, the most important phase in the morphological development of the organism. Choi *et al.* (1998) observed that decreasing the concentration of pharmamedia from 15 to 10 gl^{-1} and gluten meal from 25 to 10 gl^{-1} resulted in a shift from free filamentous to pelleted cultures and a reduction in the apparent viscosity of the cultures at day 10 from 0.19 to 0.050 Nsm^{-2}. The addition of polymeric compounds, such as polyethylene-glycol, to nutrient media has been reported to influence the morphological development of *Streptomyces coelicolor* A3(2), whereby the introduction of this agent into the solution favours the generation of a population of dispersed mycelia as opposed to pellets (Hodgson, 1982). Hobbs *et al.* (1989) observed a similar phenomenon when culturing *Streptomyces lividans* in the presence of polyethylene-glycol, whereby increasing polymer concentration in the region

of 10 - 50 gl^{-1} resulted in a concomitant increase in dispersed growth, which was attributed to increased medium viscosity. The addition of nutrient thickening agents such as various carbohydrates and proteinaceous substances have been used to used increase the viscosity of *Blakeslea* and *Choanephora* cultures to prevent clumping and the resultant pellet formation (Corman, 1959). The author noted that varying the type and concentration of compounds added enabled the generation of nutrient broths with (apparent) viscosity levels in the range of 1 to 30 Nsm^{-2} and regulated the morphological development of the cultures resulting in an increased proportion of dispersed growth.

1.2.5.2. Surface Tension

The surface tension of a liquid greatly influences fluid mechanics, especially in multi-phase systems such as filamentous fermentations containing air, nutrient broth and biomass particles. The inter-molecular attractive forces within liquids cause surface tension. Within the bulk of the liquid all molecules attract one another equally, for example hydrogen bonding between adjacent water molecules (Zubay, 1998). However, at the liquid surface the molecules are drawn towards the bulk of the liquid because there are no other molecules to draw them outwards (Claesson, 1998). This constant inward pressure caused by molecular attraction, results in the liquid adopting a shape with the least surface area to volume ratio, the sphere (de Nevers, 1991). Not only does the sphere occur for liquids in gases, i.e. water droplets, but also for gases in liquids, i.e. air bubbles. In the case of two or more immiscible liquids a similar phenomenon known as interfacial tension arises. In much the same way as with surface tension the attractive forces within each of the liquids is stronger than the attractive forces for each other and molecules at the interface are drawn towards the bulk liquid. Thus an interfacial tension exists between the liquids, an example being oil and water (Rosen, 1989).

The surface tension of a fermentation broth can influence bioprocessing parameters such as gas-liquid mass transfer. In a stirred tank reactor

sparged with air, the surface tension of the liquid affects the surface chemistry of bubbles and their tendency to coalesce, both of which will influence the gas-liquid mass transfer rate (k_La) (Doran, 1995). For example, a decrease in surface tension reduces the average bubble diameter and therefore increases the interfacial area (a), meanwhile reducing the mobility of the gas-liquid interface and therefore decreasing the liquid-phase mass transfer coefficient (k_L). With the introduction of a surface tension reducing compound such as a surfactant into the fermentation system the decrease in k_L is typically larger than the increase in a so that, overall k_La is reduced (Doran, 1995; Kawase & Mooyoung, 1990). Both the surface tension and the interfacial tension have units of Nm^{-1} and can be measured using the Du Nouy ring method whereby a platinum ring is placed on the surface or at the interface of the liquid(s) and the force required to separate the ring from the liquid is recorded (de Nevers, 1991).

The surface tension of a liquid will also influence its interaction with a solid in a process known as wetting. Placing a water droplet on a glass slide will result in the dispersal of the liquid over the solid surface, the degree of which is dependent on the surface tension of the liquid and the hydrophobicity of the solid. Pure water wets a clean glass slide well but a dirty one poorly. The measurement of the wetting of a solid by a liquid is performed by placing a drop of liquid on a solid surface and measuring the contact angle that it makes, the lower the contact angle the greater the liquid has spread on the solid and the greater degree of wetting (de Nevers, 1991; Rosen, 1989). Surface tension may be controlled by the addition of specialised molecules known as surface active agents or surfactants, which alter the surface properties of the liquid and regulate molecular interaction. Surface tension influences the interaction of liquids with solids, gases and other liquids and thus influences fluid mechanics.

1.2.5.2.1. Hydrophobicity and Surfactants

In aqueous systems polar water molecules interact and form hydrogen bonds with one another. It is thought that water consists of both structured and non-structured regions undergoing constant re-orientation (Attwood & Florence,

1983). The introduction of a non-polar compound such as a hydrocarbon into an aqueous system initially disrupts the ordered structure of the H-bonded water molecules. Hydrophobic molecules such as non-polar hydrocarbons cannot form H-bonds and cannot interact with polar water molecules. The system quickly re-establishes equilibrium and H-bonds are once again formed between the polar water molecules, effectively repelling the non-polar hydrophobic molecules (Attwood & Florence, 1983). Density issues aside, the presence of hydrophobic compounds in an aqueous phase will result in encapsulation and the formation of an aqueous 'shell' around a hydrophobic core (Zubay, 1998). In such systems hydrophobic molecules will not mix with the aqueous phase, an example being oil and water. Should two or more hydrophobic molecules come into contact in such a system they will effectively interact due to the repulsive forces of the aqueous phase, which is termed hydrophobic interaction (Claesson, 1998; Norde, 1998). The polar aqueous phase and the non-polar hydrocarbon are made miscible by the inclusion of a bridging molecule, a surfactant.

Surface-active agents or surfactants, as they are better known, adsorb at the surface or interface of two immiscible phases, an example being an oil and water system. Ionic surfactants, for example, posses both polar (hydrophilic) and non-polar (hydrophobic) regions on the same molecule. The hydrophilic region of the molecule interacts with the aqueous and the hydrophobic region with the organic, thus forming a link between the two previously immiscible phases (Attwood & Florence, 1983; Claesson, 1998). These molecules are typically made up of a hydrophilic 'head' and a hydrophobic 'tail', the length of which regulates surface activity. As mentioned previously, hydrophobic molecules are essentially encapsulated by water due to H-bonding within the aqueous phase. The introduction of a surfactant into such a system, results in the formation of a cluster of surfactant molecules around the hydrophobic particle in the form of a micelle, vesicle or bi-layer. The hydrophobic surfactant tail interacts with the encapsulated hydrophobic particle and the hydrophilic head interacts with the aqueous phase, thereby solubilising the hydrophobic particle in the aqueous phase (Claesson, 1998; McMurray, 1996). Surfactants decrease the surface tension of a liquid by interacting with

the molecules at a surface and reducing the strong attractive forces that they have for one another. The regulation of broth surface tension through the addition of a surfactant controls the solubility of hydrophobic molecules in solution.

1.2.5.2.2. Surface Tension of Fermentation Broths

The addition of polymeric substances such as surfactants or antifoams to nutrient media has traditionally been used as a counteractive measure to prevent foaming and/or wall growth in submerged fermentations. However it has been observed that the inclusion of such compounds also influences the morphological and physiological development of filamentous microorganisms. These compounds may affect the aggregation of spores and/or biomass, the biochemical functioning of the cell or even the availability and/or solubility of nutrient constituents. Vechtlifshitz *et al.* (1990) proposed that pellet formation in *S. tendae* was as a result of hydrophobic interactions of cell walls. Furthermore, Dynesen & Nielsen (2003) demonstrated that the presence of two hydrophobic proteins on the surface of *Aspergillus nidulans* conidiospores played a role in pellet formation. The absence of either of these proteins led to a decrease in the surface hydrophobicity and a decrease in pellet formation. The interaction of hydrophobic biomass in aqueous fermentation media is partly responsible for particle aggregation and pellet formation.

Surfactants are capable of reducing broth surface tension and regulating hydrophobic interactions in submerged fermentations. The addition of a wide variety of non-ionic surfactants to fermentation broths has been investigated and the potential morphological impact assessed. Compounds such as Span 20 (Jeong *et al.*, 2001) and Tween 40 (Lucatero *et al.*, 2004) have been shown to influence culture morphology for *Blakeslea trispora* and *Trichoderma harzianum,* respectively, by reducing pellet formation and increasing the dispersed mycelial content in the culture. Some conflicting reports exist in the literature as to the exact influence of Tween 80 on culture morphology. For example, Znidarsic *et al.* (2000) working with *Rhizopus nigricans,* observed a slightly larger pellet size and increased growth rate in

the presence of 0.5 to 2.0 gl^{-1} of Tween 80, whereas Domingues *et al.* (2000) working with *Trichoderma reesei,* observed the prevention of pellet formation and the induction of pulpy growth in the presence of 0.5 gl^{-1}. It is highly likely that the mode of action and consequently the morphological impact of this and other surfactants are dependent on both the concentration of the compound and the organism itself. The impact of surfactants on culture morphology was also observed in solid media where the inclusion of Tween 80 in nutrient agar was observed to convert the colony morphology of *Mycobacterium avium* from rough to smooth (Wright *et al.*, 1992).

Vechtlifshitz *et al.* (1989) investigated the impact of three surfactants on pellet formation in cultures of *S. tendae.* The surfactants used were Pluronic F68, Brij 58 and Triton X-100, all of which are non-ionic. Triton X-100 differed from the other two compounds in that, although it enhanced pellet formation, it was observed to decrease both the growth rate and the cellular yield with increasing concentration. Both Pluronic F68 and Brij 58 in the range of 0.0001 – 0.01 % w/w brought about increased pellet formation and resulted in a morphological distribution containing larger pellets with increasing surfactant concentration. Increasing the concentration beyond this point resulted in a concomitant decrease in pellet formation until it ceased completely and dispersed mycelia were obtained. It would appear from the experimental data that comparable levels of biomass were obtained throughout. Measurement of the contact angles indicated that inclusion of surfactant at low concentrations increased biomass hydrophobicity whereas higher concentrations decreased the hydrophobicity, which would explain the dependence of pellet formation on surfactant concentration. *S. tendae* is non-coagulative and the impact of these compounds was demonstrated to be on the vegetative mycelia, for example, the introduction of surfactant into control cultures grown as dispersed mycelia resulted in flocculation.

The dependence of pellet formation on surfactant concentration in submerged fermentations of *S. tendae,* suggests that the mode of action of Pluronic F68, Brij 58 and Triton X-100 differs accordingly. At low concentrations the surfactant adheres to the cell wall and increases the

overall hydrophobicity, which in turn increases the attractive forces between molecules and results in increased agitation. Once a critical concentration is reached, the hydrophobicity of the cell wall decreases as the adhered surfactant reduces the surface tension and increases the particle solubility in the aqueous phase. With reduced hydrophobicity comes reduced particle interaction and aggregation. Surfactant molecules are capable of regulating pellet formation in submerged fermentations of filamentous microorganisms.

1.3. SUMMARY

Filamentous bacteria are more complex in structure and in their mechanisms of growth than their unicellular counterparts. The lifecycle of a filamentous bacteria begins with the germination of the inert spore, the first visual evidence of which is the formation of a germ tube, which will subsequently develop into a long multicellular string-like structure called a hypha. Reproduction within these organisms is usually by means of hyphal tip extension, followed by septation and branching in the sub-apical regions. Filamentous microorganisms have adapted for growth on solid substrates through apical and branched growth, which not only enables the organism to explore new areas for fresh nutrients but also to fully utilise the area already colonised, a process not possible to the same extent in simpler unicellular systems. However, cultivation of these microorganisms on an industrial scale is rarely conducted on a solid medium and is generally performed in a liquid medium as a submerged fermentation. When grown in liquid medium, filamentous growth, through tip extension and branch formation, leads to the development of hyphae in a three-dimensional fashion, typically resulting in the development of a pellet.

The morphological profile of filamentous microorganisms in submerged fermentations may vary from dispersed mycelial suspensions to entirely pelleted cultures. Pellet formation and development in these vegetative fermentation systems depends on particle collision frequency and energy, aggregation due to cell surface properties and entanglement/intertwining due to apical and branched growth. The impact of culture morphology and

specifically the size and density of mycelial pellets has been shown in many instances to influence product formation in submerged fermentations of filamentous microorganisms. The growth of filamentous microbes in submerged fermentations is highly dependent on the environmental and nutritional process conditions experienced. Some of the parameters that influence culture morphology in these systems are nutrient media composition, inoculum type, concentration and age, agitation and fluid characteristics such as viscosity and surface tension. Thus leading to the hypothesis that the morphological engineering of culture development may be achieved through the control of processing parameters.

1.4. AIMS AND OBJECTIVES

The actinomycete *Streptomyces hygroscopicus* var. *geldanus* produces a range of antibiotic compounds, one of which, Geldanamycin, a benzoquinone ansamycin, was first examined for its broad-spectrum antibiotic activity against protozoa, bacteria and fungi (Deboer *et al.*, 1970; Deboer & Dietz, 1976). Although initially isolated and studied for its antibiotic properties, recent work with Geldanamycin has concentrated on its potential anti-cancer properties.

Filamentous bacteria are capable of growing in a range of morphological forms, with compound production often linked to the morphological profile of the culture (Casas Lopez *et al.*, 2005; Tamura *et al.*, 1997). Pellet formation in submerged fermentations of filamentous microorganisms is a complex process dependent on the organism in question and the bioprocessing conditions experienced (Kojima *et al.*, 1995; Tamura *et al.*, 1997; Vechtlifshitz *et al.*, 1990).

The primary focus of the experimental work will be to gain an overview of the mechanisms involved in the morphological regulation of pellet development of *S. hygroscopicus* cultures and to propose strategies for the morphological engineering of the organism that can subsequently be exploited by other researchers working on productivity improvement.

The first objective to be achieved is the sourcing of *Streptomyces hygroscopicus* var. *geldanus* from a culture collection. Once obtained, it will be necessary to establish the organism in the laboratory, become proficient in the microbiological techniques associated with culturing it and generate sterile spore stocks for subsequent experimental procedures.

In order to investigate the process of pellet development, it will first be necessary to develop a method to quantify *S. hygroscopicus* cultures, which will facilitate high throughput enumeration and morphological quantification of pellets in fermentation broths.

Once such a technique has been developed it will enable an investigation into the influence of bioprocessing parameters, specifically different nutrient broth formulations, varying inoculum spore concentrations and varying shear rates, on the morphological and physiological development of the culture.

The formation of pelleted biomass in submerged fermentations is thought to be as a result of particle collision, interaction and aggregation, each of which has been identified as a potential point of morphological control. Therefore, it will be necessary to assess the influence of the fluid properties of viscosity and surface tension on pellet development in *S. hygroscopicus* cultures.

Once the impact of altering bioprocessing conditions and fluid attributes on culture morphology is known this will provide a means to propose new morphologically engineering strategies for *S. hygroscopicus* cultures.

CHAPTER 2 – MATERIALS AND EXPERIMENTAL METHODS

2.1. ORGANISM

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

2.2. MEDIA PREPARATION

The nutrient media, both solid and liquid, were prepared as described in the following sections.

2.2.1. Bennett's Medium

The 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
N-Z-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 20 gl^{-1} Oxoid Technical Agar No.3 (Basingstoke, England).

2.2.3. DeBoer and Dietz Medium

The DeBoer and Dietz medium contained the components listed in Table 2.2, resuspended in distilled water prior to autoclaving (Deboer & 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	Odiums	5
Molasses	Unknown	10
Glucose Monohydrate	BDH	40

2.2.4. GYM Medium

The 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 20 gl^{-1} Oxoid Technical Agar No.3 (Basingstoke, England).

2.2.6. M2M Medium

The M2M medium contained the components listed in Table 2.4, resuspended in distilled water prior to autoclaving (Trejo-Estrada *et al.*, 1998).

Table 2.4. M2M medium formulation

Constituent	Manufacturer	Concentration (gl^{-1})
Casaminoacids	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

2.2.7. Mueller-Hinton Medium

The Mueller-Hinton medium was ready-mixed by Oxoid and contained the components listed in Table 2.5, which were resuspended in 1 litre of distilled water prior to autoclaving.

Table 2.5. Mueller-Hinton medium formulation

Constituent	Manufacturer	Concentration (gl^{-1})
Meat Infusion	Oxoid	2
Casein Hydrosylate	Oxoid	17.5
Starch	Oxoid	1.5

2.2.8. YEPD Medium

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

Table 2.6. YEPD medium formulation

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

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. Autoclaving Procedure

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

2.3. ANALYTICAL TECHNIQUES

2.3.1. Spore Enumeration

The following procedure was employed to determine the number of colony forming units (CFU) present in a given sample. The sample of interest was serially diluted (1 in 10) with the stock concentration reduced by a factor of 1 x 10^6 in sequential steps. Subsequently, 0.1 ml of each diluted samples was spread on Bennett's solid medium in a petri dish and incubated at 28°C for six days prior to enumeration. The spread plating of the individual samples was performed in duplicate.

Microscopic examination of spore inocula revealed the presence of spore clusters and rings as illustrated in Figure 2.1. Disruption of these clusters and rings was attempted using vigorous agitation and the introduction of a chemical dispersal agent. Spore suspensions were variously vortexed for 5, 30 and 120 seconds and for 120 seconds including 1% (v/v) Silcorel AFP 20 Silicone Antifoam (BDH Laboratory Supplies, UK). None of these approaches disrupted the spore clusters, suggesting that these aggregates are potentially stable under normal fermentation conditions. As a result, it was proposed that each cluster or ring effectively behaves as one colony forming unit when germinating and gives rise to one colony or pellet.

Figure 2.1. Microscopic image of a ring of spores (100X magnification).

The spore inocula used for all experimental procedures was taken from a frozen spore suspension stock stored at -20 °C. The material was thawed and diluted with sterile water, as needed, for use in subsequent experimentation. An examination of the reproducibility and consistency of using this material was undertaken by examining the spore concentration in the inoculum for four separate experimental procedures conducted over a four month time period. As illustrated in Figure 2.2 the spore inocula used for these procedures was consistent, thereby confirming the reproducibility of using the frozen spore suspension stock for inoculation purposes.

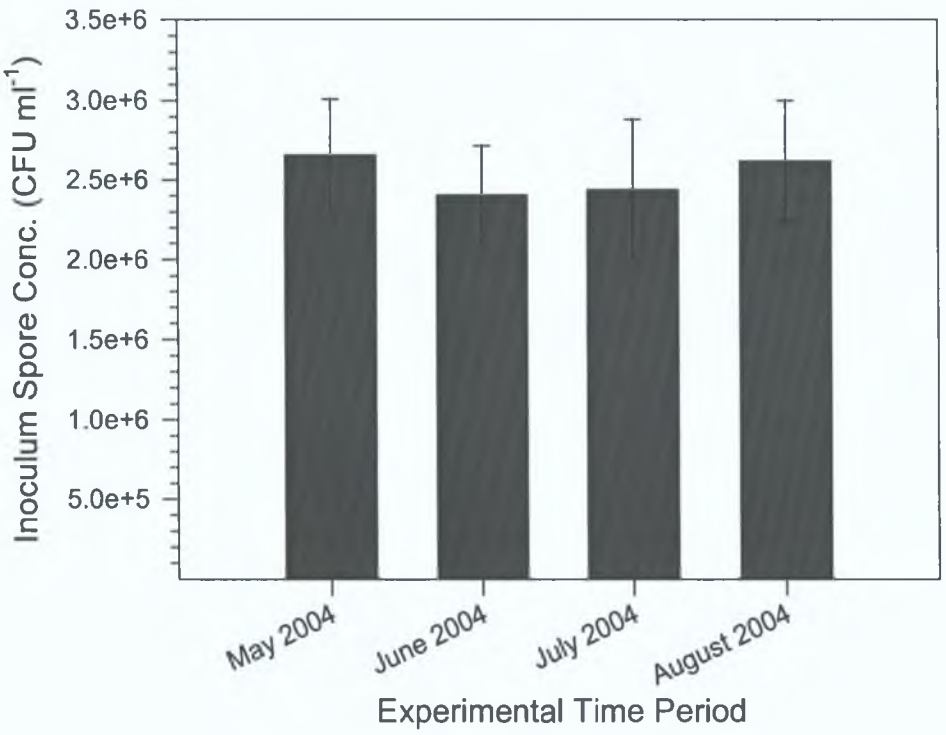


Figure 2.2. The concentration of spore suspension inoculum used for four separate experimental procedures performed during May - August 2004.

2.3.2. Biomass Concentration

The dry weight of biomass in fermentation broth was determined using the following procedure. A 20 ml sample of the fermentation broth was placed in a plastic universal container and subjected to two centrifugation cycles at 3500 rpm for 10 mins. After the first centrifugation step, the supernatant was removed and the pellet was resuspended with distilled water, to a final

volume of 20 ml. After the second centrifugation step the supernatant was again removed and the pellet was resuspended in 2 ml ethanol, a volatile liquid with a boiling point of 78°C and transferred to a pre-weighed glass universal. The glass universal was placed in an oven at 105°C, where the ethanol evaporated leaving only the dry biomass behind. After 24 hours the glass universal was removed from the oven, allowed to cool and weighed to determine the dry weight of biomass in the sample.

2.3.3. Glucose Concentration

The procedure for determination of glucose concentration within fermentation broths is a version of the GOD-PAP method, adapted for high sample throughput using 96-well microtitre plates. Samples were prepared by performing two successive 1-in-10 dilutions with distilled water in order to obtain a glucose concentration in the range 0 – 0.1 gl^{-1}. A standard curve consisting of standards with concentrations in the same range was also prepared. 20 μl of the diluted fermentation broth and standard curve samples were loaded onto a 96-well plate and 200 μl of GOD-PAP (Randox Laboratories Ltd., UK) reagent was added. The reaction was incubated at 25 °C for 35 mins and then the absorbance was read at 492 nM using a 96-well plate reader (Tecan, Mannedorff, Switzerland).

2.3.4. Viscosity Determination

The determination of the cell-free fermentation broth apparent viscosity was achieved using 0.5 ml sample in a Brookfield Rotational DV-I+ Cone and Plate Viscometer (Brookfield, Middleboro, MA, USA) at 20°C. Solutions of xanthan gum, as used in Chapter 5, are non-Newtonian power law fluids, whose apparent viscosity is dependent on shear rate (Doran, 1995). The viscometer facilitated the calculation of the apparent viscosity of the solution over a wide range of shear rates. The experimentally determined relationship between shear rate and shear stress for cell-free Bennett's broth containing a range of xanthan gum concentrations is shown in Figure 2.3.

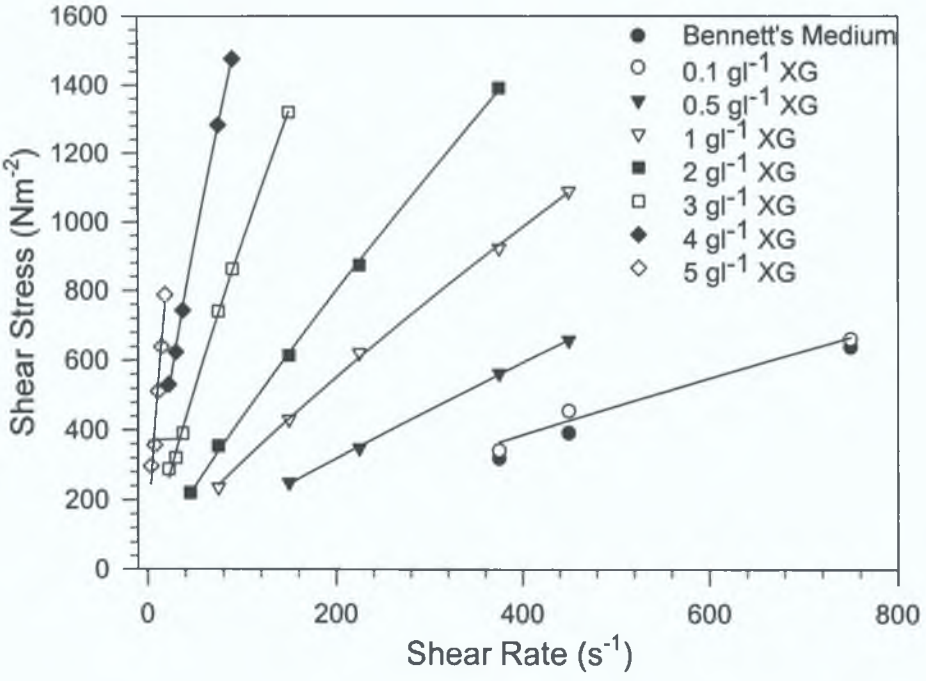


Figure 2.3. Relationship between shear rate and shear stress with respect to varying xanthan gum (XG) concentration.

For non-Newtonian fluids the relationship between apparent viscosity (μ_a), shear stress (τ) and shear rate (γ) may be represented as follows:

$$\mu_a = \frac{\tau}{\gamma} \qquad (2.1)$$

Using Equation 2.1 and Figure 2.3 it is possible to calculate the apparent viscosity of a solution at a given shear rate and corresponding shear stress. Fujita *et al.* (1994) calculated the shear rate experienced by 100 ml of nutrient media in a 300 ml shake flask rotating on a shaker table at 150 rpm to be 28 s^{-1}. The author estimated this shear rate value based on the power required for rotational shaking and the volume of liquid in the shake flask. Taking a shear rate value of 28 s^{-1}, it was possible to calculate the corresponding apparent viscosity for each of the different concentration xanthan gum solutions. The results of which are presented in Table 5.1.

The most common types of non-Newtonian fluids are power law fluids. These fluids can be further sub-divided into pseudoplastic and dilatant where the shear stress is a function of the fluid consistency index (K), shear rate and the flow behaviour index (n). If $n < 1$ then the fluid is pseudoplastic; if $n > 1$ the fluid is dilatant. The behaviour of power law fluids may be described as follows:

$$\tau = K\gamma^n \tag{2.2}$$

Equation 2.2 may be rearranged to give:

$$\ln(\tau) = \ln(K) + n(\ln(\gamma)) \tag{2.3}$$

The relationship between $\ln(\tau)$ and $\ln(\gamma)$ is represented in Figure 2.4, from which n and K were calculated for each of the different concentration xanthan gum solutions. The results of which are presented in Table 5.1.

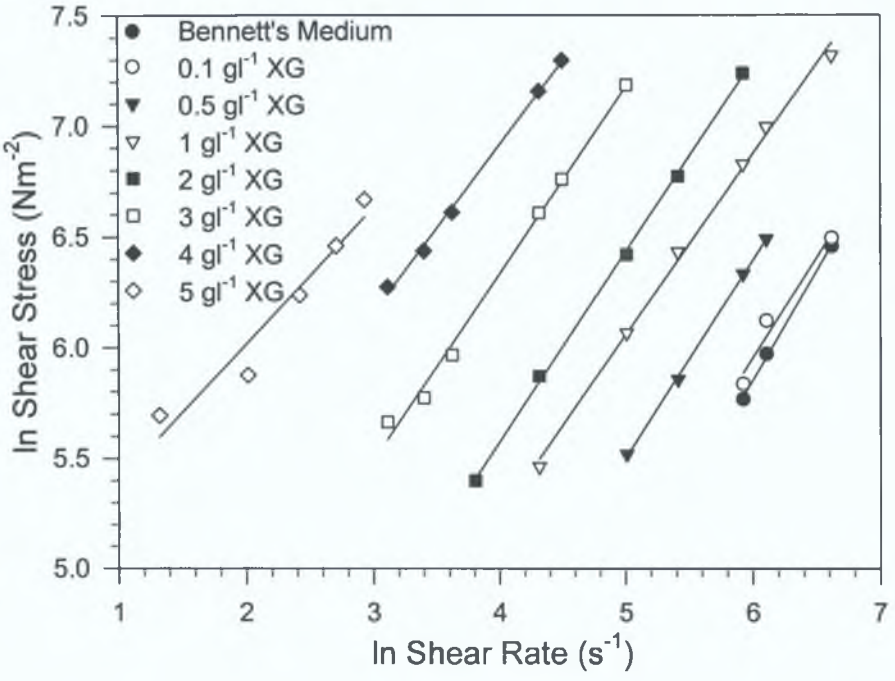


Figure 2.4. Relationship between $\ln\tau$ and $\ln\gamma$ with respect to varying xanthan gum (XG) concentration.

2.3.5. Gas-liquid mass transfer determination

The gas-liquid mass transfer coefficient K_La was determined using the dynamic gassing-out method (Doran, 1995). 100 ml of the relevant cell-free solution at 20^0C was aliquoted into a 250 ml Erylenmeyer flask and de-oxygenated by sparging pure Nitrogen through the liquid phase. The headspace in the flask was then sparged with air before the dissolved oxygen probe was positioned in the centre of the flask. The flask was placed on an orbital shaker and agitated at 150 rpm while manually recording the % saturation at 20 second intervals from a dissolved oxygen meter (WTW, Weilheim, Germany). A diagrammatic representation of the apparatus is given in Figure 2.5.

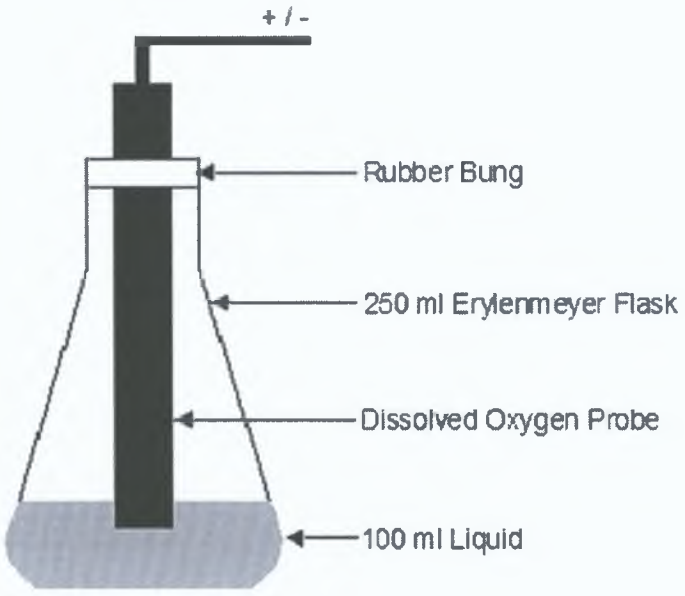


Figure 2.5. Diagram of a 250 ml Erlenmeyer shake flask containing 100 ml of liquid and a dissolved oxygen probe used in the determination of the gas-liquid mass transfer coefficient.

The gas-liquid mass transfer coefficient (k_La) was determined using the following equation, where C_{SS} is the final steady dissolved oxygen concentration and C_{AL1} and C_{AL2} are the dissolved oxygen concentrations at times t_1 and t_2 respectively.

$$k_L a = \frac{\ln\left(\frac{C_{SS} - C_{AL1}}{C_{SS} - C_{AL2}}\right)}{t_2 - t_1} \qquad (2.4)$$

The relationship between $ln[(C_{SS}-C_{AL1})/(C_{SS}-C_{AL2})]$ and $(t_2 - t_1)$ is represented in Figure 2.6, from which k_La was calculated for each of the different concentration xanthan gum solutions. The results of which are presented in Figure 5.4.

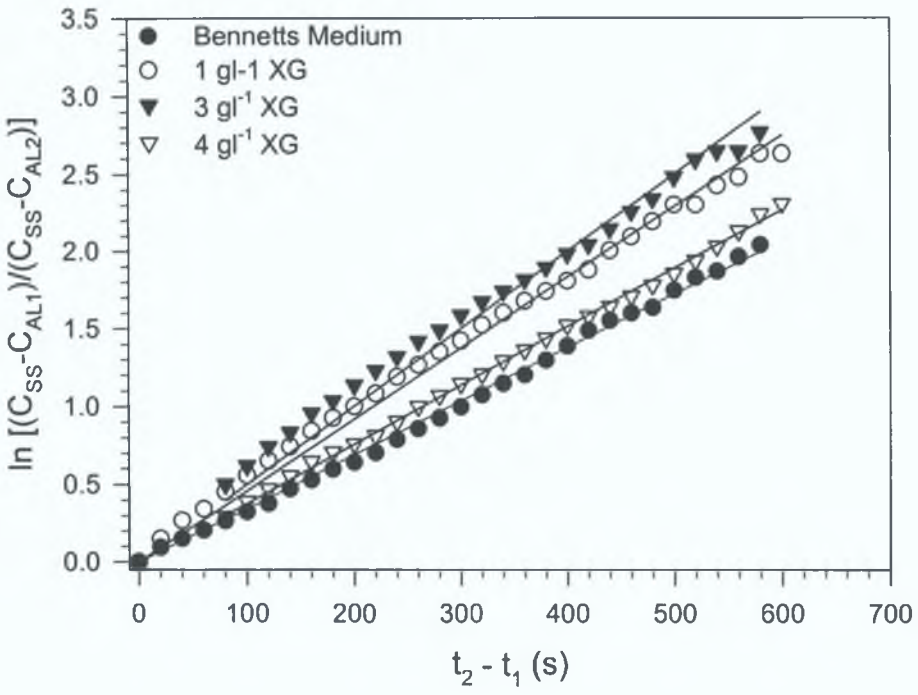


Figure 2.6. The relationship between $ln[(C_{SS}-C_{AL1})/(C_{SS}-C_{AL2})]$ and $t_2 - t_1$ with respect to varying xanthan gum (XG) concentration.

2.3.6. Surface Tension Measurement

The broth surface tension was determined using a "Model OS" Tensiometer (White Electrical Instrument Co., Malvern, WR14 1BL, UK), shown in Figure 2.7. The Du Nouy ring method was used for surface tension determination (de Nevers, 1991). A 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 0.5 ml 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 needed to detach the ring from the liquid surface and may be read directly from the gauge.

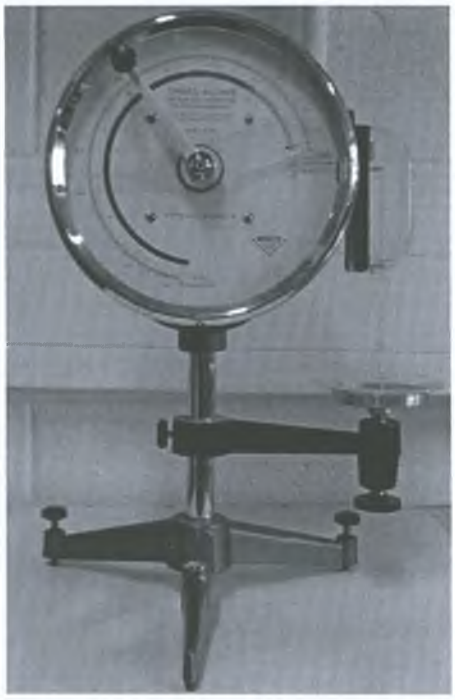


Figure 2.7. White Electrical Instruments "Model OS" Tensiometer.

2.4. SEPARATION AND PURIFICATION TECHNIQUES

The following chromatographic techniques were used in the purification of fermentation products of interest from the crude Bennett's fermentation broth.

2.4.1. Gel Filtration Chromatography

A Bio-Rad Glass Econo-Column (internal diameter x length, 1.5 x 75 cm) was used for gel filtration chromatography. Two different packing materials were used: Sephadex G-10 and G-25 with molecular weight fractionation ranges of <700 and 100-5000, respectively. The sample application procedure involved manually loading 1 ml of the desired sample onto the top of the column. Distilled water was used as the mobile phase, the flowrate of which was controlled using a Bio-Rad Econo Pump EP-1 at 0.25 ml min^{-1}.

2.4.2. Hydrophobic Interaction Chromatography

A Bio-Rad Glass Econo-Column (internal diameter x length, 2.5 x 5 cm) was used for hydrophobic interaction chromatography. The packing material used

was Phenyl Sepharose CL-4B. Sample application was performed using a Bio-Rad Econo Pump EP-1 at flowrates in the range of 0.25 – 2.5 ml min^{-1}. The mobile phase in this procedure was either distilled water or 4 M NaCl aqueous solutions.

2.5. SOLID CULTURING (SPORE PRODUCTION)

S. hygroscopicus was grown on GYM solid medium for spore production. Sterile incubation of the organism on 14 g of solid medium in a 250 ml Erlenmeyer flask, for a period of 21 days at 28°C, resulted in the production of aerial spores (Figure 2.8). A spore suspension was prepared by washing the surface of the solid medium at 100 rpm on an orbital shaker for 1 hour at 4°C using 20 ml of sterile resuspension solution. The resulting spore suspension was enumerated, as per section 2.3.1, and stored at –20°C for use in subsequent experimental procedures.

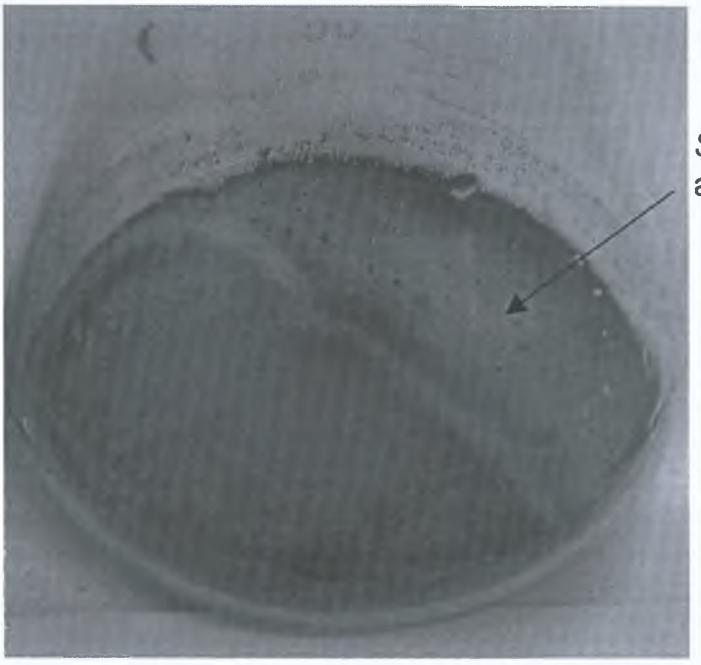


Figure 2.8. A 250 ml Erlenmeyer flask containing solid nutrient medium supporting a sporulating culture of *S. hygroscopicus*. The grey colour observed is indicative of aerial spore production, otherwise the medium is a light brown colour.

2.6. LIQUID CULTURING (SHAKE FLASKS)

The standard operating conditions for the cultivation of the organism in shake flasks involved inoculating 100 ml of Bennett's nutrient broth in 250 ml Erlenmeyer flasks with 1% (v/v) of a 2 x 10^6 ml^{-1} spore suspension and incubating the cultures on an orbital shaker at 150 rpm and 28°C. A set of five flasks was prepared for each timepoint. These conditions form the basis for all subsequent shake flask culturing experiments involving *S. hygroscopicus* unless otherwise explicitly stated.

CHAPTER 3 – MORPHOLOGICAL QUANTIFICATION OF PELLETS IN *S. HYGROSCOPICUS* BROTHS USING A FLATBED SCANNER

3.1. INTRODUCTION

Filamentous microorganisms, which include the actinomycetes, are an important group of microbes due to their ability to produce commercially valuable secondary metabolites (Okami & Hotta, 1988). Many authors have suggested the dependence of product formation on a particular morphological profile in submerged fermentations. However, the morphological dependence of product formation varies for different compounds and organisms and although an appropriate culture morphology is, in some instances a pre-requisite for product formation, this is not always the case. For example, the production of virginiamycin (Yang *et al.*, 1996), clavulanic acid (Belmarbeiny & Thomas, 1991) and penicillin (Nielsen *et al.*, 1995) have all been shown to be independent of culture morphology. Whereas, the production of tylosin (Tamura *et al.*, 1997), striatals (Gehrig *et al.*, 1998) and lovastatin (Casas Lopez *et al.*, 2005) have been shown to be dependent on pelleted growth. Braun and Vechtlifshitz (1991) have suggested that the influence of pellet morphology on the formation and composition of secondary metabolites is still largely unexplored.

The morphological profile of submerged fermentations of filamentous microorganisms can consist of a continuum of morphological forms from dispersed mycelia to pellets depending on process conditions. Characterisation of the morphology of filamentous microorganisms facilitates the elucidation of the impact of environmental and nutritional conditions on pellet formation and, in turn, on productivity. The predominant methodology used for the determination of pellet morphology is microscopic image analysis (Cox *et al.*, 1998), which generally involves sample preparation, microscopic examination and multiple image capture for subsequent image analysis. Traditionally, samples for microscopic examination are placed on a

glass slide and covered with a glass slip. However, several authors have reported other methods better suited to the three dimensional nature of filamentous organisms such as immobilization on cellophane (Reichl *et al.*, 1990), use of a measurement chamber (Reichl *et al.*, 1992) and containment within a flow-through cell (Spohr *et al.*, 1998) or in-situ measurement using focused beam reflectance measurements (Pearson *et al.*, 2003). Traditionally, microscopic examination is performed manually but may also be programmed to allow for automated light correction, focusing and stage movement. Image capture is normally by means of a charge coupled device (CCD) camera mounted on a microscope, which is in turn linked to a frame grabber within a personal computer. The CCD camera outputs a video signal that the frame grabber converts to a digital format, which the computer can save for further analysis.

The subsequent image analysis procedure typically involves two steps. The first step involves pre-processing the image to remove any unwanted distortions such as low and high frequency background noise, caused by lighting inconsistencies, by applying a series of filters. The second step involves processing the resulting image to identify, isolate and measure the particles of interest (Paul & Thomas, 1998). Manipulation of the resulting data yields a morphological assessment of the culture of interest. Many authors have used microscopic image analysis methods to determine various morphological parameters. Reichl *et al.* (1992) used microscopic image analysis to describe the characterisation of pellet morphology of *S. tendae*. Morphological quantification of fermentation broths facilitated the construction of a model describing hyphal aggregation and thus pellet formation (Nielsen, 1996).

Conventional microscopic image analysis necessitates the acquisition of multiple images, in order to obtain a statistically valid outcome (O'Shea & Walsh, 1996). This process does not typically allow for high sample throughput. Furthermore, there are limitations to the applicability of microscopic image analysis in describing the macroscopic morphological profile of fermentation broths. For example, 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 procedure. Some headway has been made in the macroscopic study of pellet morphology by Ryoo (1999), which incorporated the use of a CCD camera in 'macro' mode, i.e. without being attached to a microscope, for image capture of *A. niger* fermentation broths. However, when using a conventional CCD array in this manner, the user must compromise between image resolution and field of view size. To enable high sample throughput the number of image capture and analysis steps must be minimised by increasing the field of view while maintaining resolution. This may be achieved by capturing a single image with a large field of view as opposed to numerous images with small fields of view.

The use of a flatbed scanner is especially applicable when a single image of moderate (>10μm per pixel) resolution and a large field of view is required. The potential of flatbed scanners has been recognised by many authors and utilised for image capture and in conjunction with image analysis in many diverse fields. One such application of this approach is the automated counting of microbial colonies on a Petri dish during routine CFU analysis (Marotz *et al.*, 2001). Furthermore, Gabrielson *et al.* (2002) demonstrated the advantages of using a flatbed scanner instead of a conventional microplate reader for the quantification of microbial growth in 96-well microplates. This approach has been used in fields as diverse as determining the size distribution and the proportion of broken kernels in rice (van Dalen, 2004), automated counting of mammalian cell colonies in petri dishes (Dahle *et al.*, 2004) and quantification of barley root morphology (Simojoki, 2000).

The use of a scanner instead of conventional microscopic techniques negates the requirement for a microscope, CCD camera and frame grabber, thus potentially providing a cost effective and easily implemented procedure applicable in the macroscopic image analysis of pelleted microbial fermentation broths. This chapter describes the development, validation and

application of an image analysis technique for the morphological quantification of *S. hygroscopicus* fermentation samples.

3.2. IMAGE ANALYSIS TECHNIQUE

3.2.1. Image Analysis Hardware

The image analysis system used was a Dell OptiPlex GX150 SMT with a 900 MHz Intel Celeron processor and 256Mb of SDRAM (Dell Computer Corporation, Round Rock, TX, United States). A HP Scanjet 5400c flatbed scanner (Hewlett-Packard, Palo Alto, CA, U.S.A.) was used for image capture.

3.2.2. Image Analysis Software

The image analysis software used was Optimas 6.5 ® (Media Cybernetics Inc., Silver Spring, MD, USA). This software can be used for any life science research or industrial application where the analysis of an image can help the user to understand processes, make comparisons, or identify objects. The code for the image analysis algorithm is given in Appendix A. The statistical analysis software used was SigmaPlot 8.0 (SPSS Inc., Chicago, IL, USA).

3.2.3. Size Calibration Particles

109µm and 644µm diameter red spherical particles (Duke Scientific Corporation, 2463 Faber Place, Palo Alto, U.S.A.) were used for calibration of the image analysis system. The manufacturer provided NIST traceable calibration certificates for the particles. Pre-weighed samples (0.0025g of the 109µm particles and 0.0346g of the 644µm particles) were used for the calibration procedure.

3.2.4. Fermentation Sample Preparation

The cultivation of the fermentation sample was as described in section 2.6. For the purposes of image analysis a homogeneous fermentation sample was required. This was achieved by withdrawing the required 10ml sample using a pipette, while gently stirring the culture using a magnetic follower. The microbial biomass required staining in order to provide sufficient contrast for image analysis purposes. For the purpose of staining 60 µl of Safranin O (1% w/v) (BDH Laboratory Supplies), an organic dye, was used to stain 10 ml of microbial culture. The mixture was gently mixed for 20 minutes to ensure dye uptake. Excess stain was removed via three sequential steps, each involving centrifugation at 2000 rpm for 10 minutes, followed by removal of the supernatant and replacement with distilled water. Following excess stain removal the 10 ml sample was placed in a plastic Petri dish for imaging purposes.

3.2.5. Image Analysis Procedure

The 10 ml aliquot of the stained calibration particles or microbial biomass was transferred to a plastic Petri dish and gently swirled to ensure an even distribution of biomass. The Petri dish was then placed on the glass surface of the flatbed scanner. The dish was covered with a white plastic tray to exclude external light and also to provide contrast for the sample. An image was obtained by scanning the Petri dish using the following standard conditions: resolution 21µm per pixel (1200 dpi); "medium sharpen"; "exposure highlights" level 179 (both "medium sharpen" and "exposure highlights" are in-built functionalities within the HP Scanjet 5400c software). The resulting image, of area 93.98 x 93.98 mm, was then saved as a 24 bit RGB, tagged image file format (tiff) for subsequent image analysis. The average file size under these standard conditions is in the region of 58 Mb and takes approximately 2.5 minutes to capture, per image.

The acquired, 24-bit colour image, requires pre-processing in order to remove unwanted distortions such as low and high frequency background noise (caused by lighting inconsistencies). The first part of image pre-

processing is the isolation and removal of low frequency background noise from the image in two sequential steps. The image of interest is opened in an Optimas window and scaled to 1/100th of its original size in a separate image window. The rescaled image is subjected to 20 sequential passes of an Average 5x5 filter, The purpose of this step is to blur the high frequency foreground signal (particles), while retaining the low frequency background signal, resulting in the formation of a mask image. Resizing the image to 1/100th of it original size reduces the processing overhead and serves to bring the size of the pellets in the image into the working range of the Average 5x5 filter. The mask image is subsequently rescaled to its original size and divided into the original image. Division of the original image by the mask image serves to remove unwanted shading distortions (low frequency noise). The resultant image is then multiplied by 200 to bring the signal back into the visible range of a 24-bit colour digital image.

The second part of the image pre-processing is the application of a Median 3x3 filter, which reduces high frequency noise present as single pixel speckles. Figure 3.1 indicates the impact of pre-processing on the original image, predominantly highlighting the removal of low frequency noise, such as the shadowing present in Figure 3.1 (a).

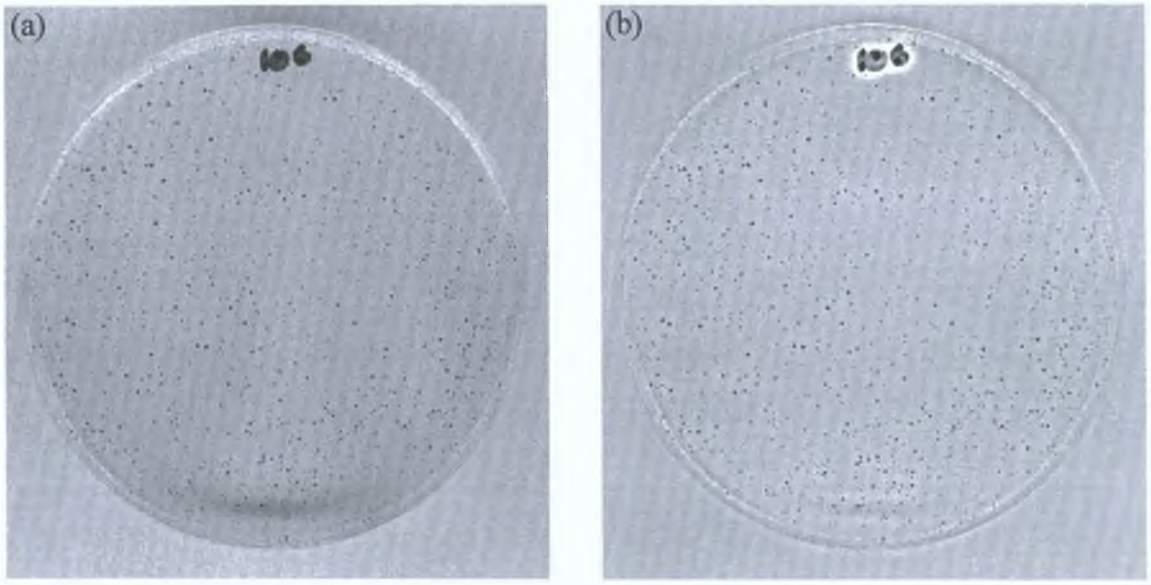


Figure 3.1. The result of image processing on the acquired image. (a) original image (b) processed image (image size 4380 x 4552 pixels).

The 24-bit RGB image is made up of a red, a green and a blue band. Upon closer inspection, the green band was judged to have the strongest signal and was subsequently chosen for the image processing steps. A threshold value (0:169) was manually chosen, and applied to the green band, to distinguish the particles from the background. Following the designation of a threshold value, the monochrome image was binarised and inverted in order to differentiate between background and foreground (particles). A region of interest within the pre-processed, binarised image was automatically selected for further processing. Due to the hydrophobic nature of the particles and pellets, there was a degree of overlapping and/or touching between neighbouring particles. Touching particles were separated using the "break apart touching blobs" function available in Optimas (a combination of binary erosions to point and dilations within image masks). The following parameters were then extracted from the image and exported automatically to a Microsoft Excel ™ spreadsheet.

- Area Equivalent Diameter for each particle.
- Number of Particles in Analysed Region of Interest
- Particle Count/ml of Solution
- Volume of Particles/ml of Solution (assuming spherical particles)
- Mean & Standard Deviation of the Area Equivalent Diameter

The 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 particle diameter, as both the calibration particles and the microbial pellets were approximately spherical. For example, Figure 3.2 contains an image of stained microbial biomass demonstrating the approximate circularity of *S. hygroscopicus* pellets. The image analysis procedure takes approximately 3 minutes per image with a further 1 minute for the export of data to an Excel spreadsheet. Table 3.1 contains timing data for the analysis of a specific sample, containing 1940 particles.

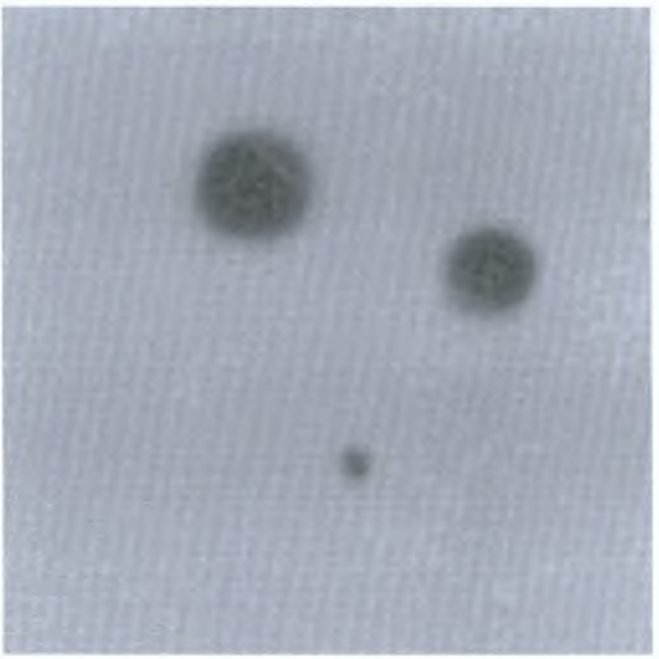


Figure 3.2. An image of stained microbial biomass demonstrating the approximate circularity of *S. hygroscopicus* pellets (image size 480 x 480 pixels, 21 μm per pixel).

Table 3.1. Breakdown of times for each step in the image analysis procedure performed on a 10^6 spore ml^{-1} inoculum culture.

Step	Time (s)
Image Acquisition	157
Image Processing	149
Data Output	30

3.2.6. Data Manipulation and Interpretation

Of the parameters calculated by the image analysis algorithm and exported to an Excel spreadsheet, the area equivalent diameters for each particle, pellet count and mean pellet volume per ml of solution were transferred to SigmaPlot. The area equivalent diameter for each particle was used to estimate its corresponding volume. The particle volume data was sorted on the basis of size and assessed, resulting in an incremental increase in both the corresponding numerical particle count and the cumulative volume fraction size ranges. This data manipulation facilitates the quantitative analysis of the morphological state of pelleted fermentation systems as illustrated later in Figures 3.5. A diagrammatic representation of the sample preparation and image analysis procedures are given in Figure 3.3.

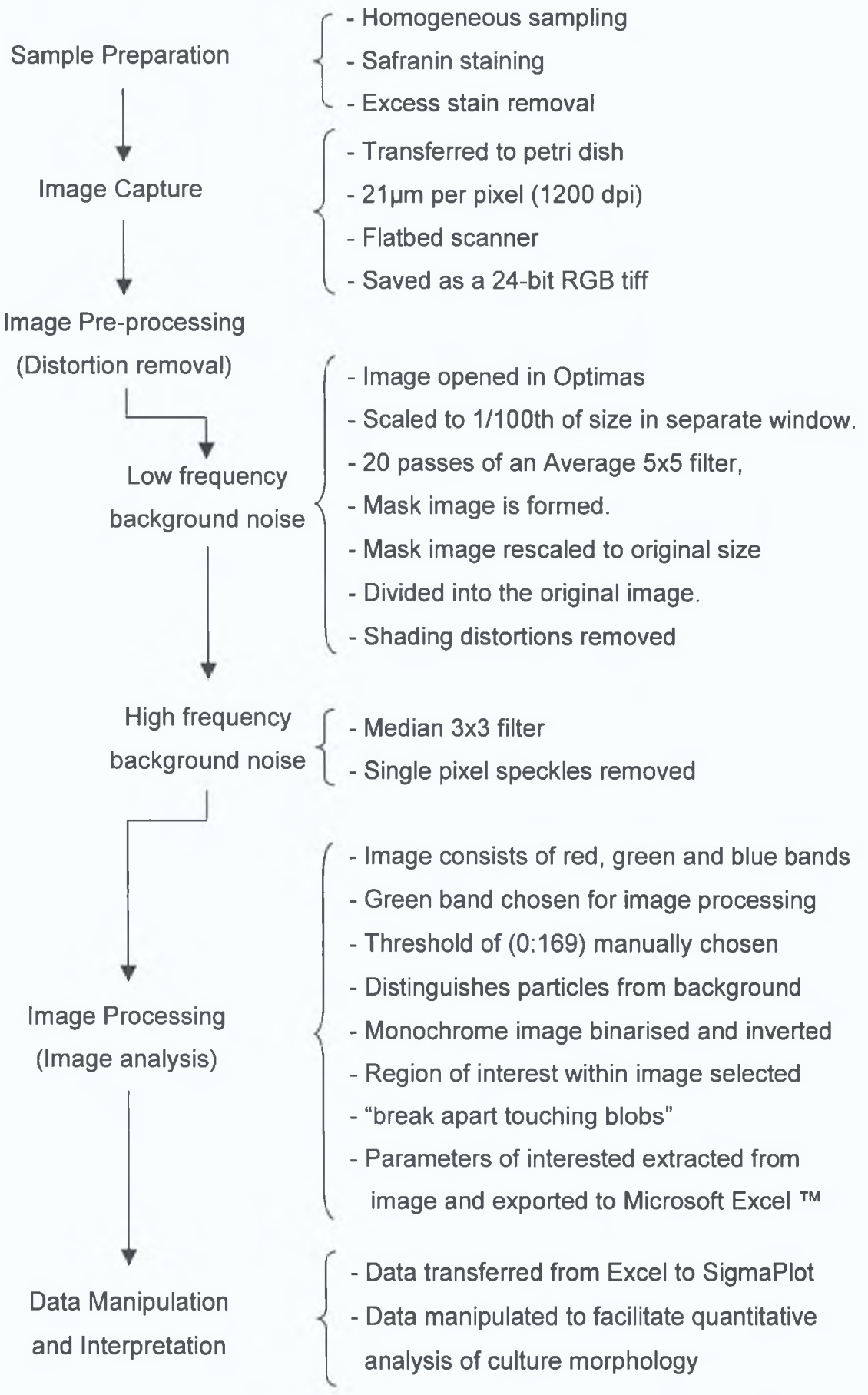


Figure 3.3. Diagrammatic representation of the sample preparation and image analysis procedures for *S. hygroscopicus* cultures.

3.3. VALIDATION OF THE IMAGE ANALYSIS SYSTEM

The image analysis procedure was applied to a sample containing a mixture of the 109 μm and 644 μm calibration particles. The data for each particle set were separated and subjected to separate histogram analysis. Figure 3.4 contains the sample histograms, overlaid with Gaussian distributions prepared using the manufacturers specified mean and standard deviation for both particle sets. Both the histogram analysis and the Gaussian distributions of the 109 μm and 644 μm data sets were prepared using bin ranges of 10 μm and 15 μm, and particle counts of 3102 and 225, respectively. The manufacturers certified mean diameters for each particle population were 109 μm ± 5 μm and 644 μm ± 13 μm. The 109 μm and 644 μm combined sample was made up of defined masses of both sizes, which in conjunction with the total measured particle volume facilitated the estimation of the material density. The manufacturing specifications of the particles indicate that the density of polystyrene divinylbenzene is 1.05 g cm^{-3}. Table 3.2 indicates that the deviation of the experimentally measured density from the known density is 3.2%.

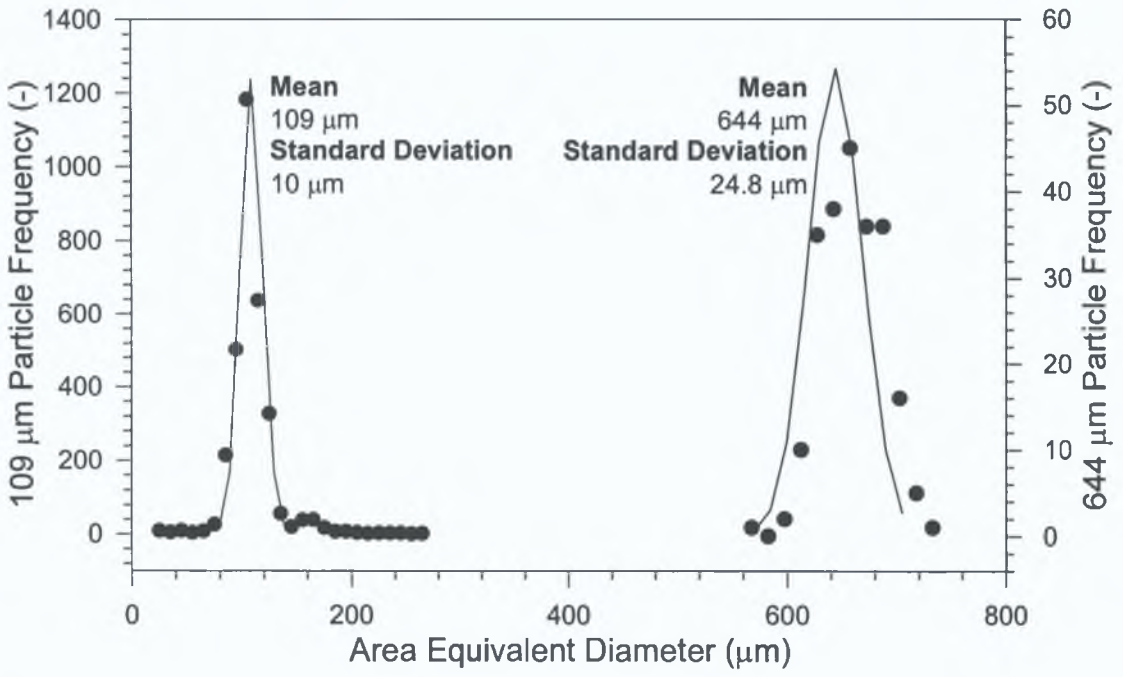


Figure 3.4. Measured area equivalent diameter histogram for 109 µm and 644 µm combined sample with overlaid Gaussian distributions derived from particle calibration certificates (NIST traceable).

Table 3.2. Morphological characteristics of 109 µm & 644 µm polystyrene divinylbenzene 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 (%)
3327	0.0365	0.0371	1.02	1.05	-3

Error analysis was performed on both the image analysis method and the dry weight determination procedure in order to ascertain the reproducibility of the data and derived measurements. Table 3.3 contains the mean value as well as the percentage standard deviation from the mean for each of the experimental and derived measurements. Both the image analysis method and the dry weight determination procedure were found to be reproducible to within 5%.

Table 3.3. Error analysis on measurements taken from five identical *S. hygroscopicus* var. *geldanus* fermentation samples.

	Mean	% Standard Deviation
Particle Count (ml^{-1})	853	3.87
Volume of Particles (mm^3 ml^{-1})	7.7	4.93
Dry Weight of Biomass (g l^{-1})	0.357	2.39
Mean Pellet Volume (mm^3)	9.0E-03	4.68
Dry Weight Equivalent Density (g l^{-1})	4.7E-05	4.36

3.4. APPLICATION OF THE TECHNIQUE TO THE STUDY OF *S. HYGROSCOPICUS* MORPHOLOGY

The assessment of *S. hygroscopicus* morphology was aided by the quantitative analysis described in section 3.2.6, whereby each individual particle is analysed and its contribution to both the numerical count and volume fraction is considered. The presentation of this data in the format of Figure 3.5 facilitates the direct comparison of both the pellet counts and volume fractions with respect to pellet diameter for *S. hygroscopicus* cultures. Figure 3.5 represents the estimated volume (based on measured area equivalent diameter) distribution with respect to pellet diameter for three separate *S. hygroscopicus* cultures; each one was pitched with a different concentration of spore inoculum. Figure 3.5 contains the day 7 normalised volume and particle frequency distributions for cultures inoculated with the 10^4, 10^5 and 10^6 spore ml^{-1} suspensions and a pictorial representation of the corresponding stained samples. This figure clearly illustrates the disparity in the morphological profiles of cultures inoculated with increasing concentration spore suspensions. The narrow size distribution in the culture pitched with a 10^6 ml^{-1} inoculum is due primarily to the aggregative nature of higher density cultures resulting in a single phase of growth from germination and vice versa for the culture pitched with a 10^4 ml^{-1} inoculum. An overlay of volume fraction distribution with particle frequency distribution indicates that particles with a diameter of less than 0.5 mm in diameter, although

numerically in the majority, do not contribute significantly to the overall biomass volume.

The image analysis procedure is also capable of determining a wide range of particle measurements, including particle count per ml of solution and volume of particles per ml of solution as seen in Table 3.4. Biomass concentration is determined by dry weight analysis and the volume of pellets per ml of solution is calculated using image analysis, both of which allow the calculation of the biomass (dry weight) equivalent density. This measurement provides an indication of the compactness of internal pellet structure. Utilisation of the biomass dry weight equivalent density value along with the pellet count per ml and the volume fraction distribution allow for a more in-depth view of microbial pellet development. Upon closer examination of the pellet count per ml of solution, volume fraction distribution and mean pellet volume for the 10^6 and 10^4 spore ml^{-1} inoculum cultures it is observed that the 10^4 spore ml^{-1} inoculum culture consists of a fewer number of particles, most of which are in the 0.75 to 1.5 mm size range, indicating the development of a single phase of growth from germination. A more in-depth analysis of the influence of inoculum spore concentration on the morphological development of *S. hygroscopicus* cultures is conducted in section 4.3.

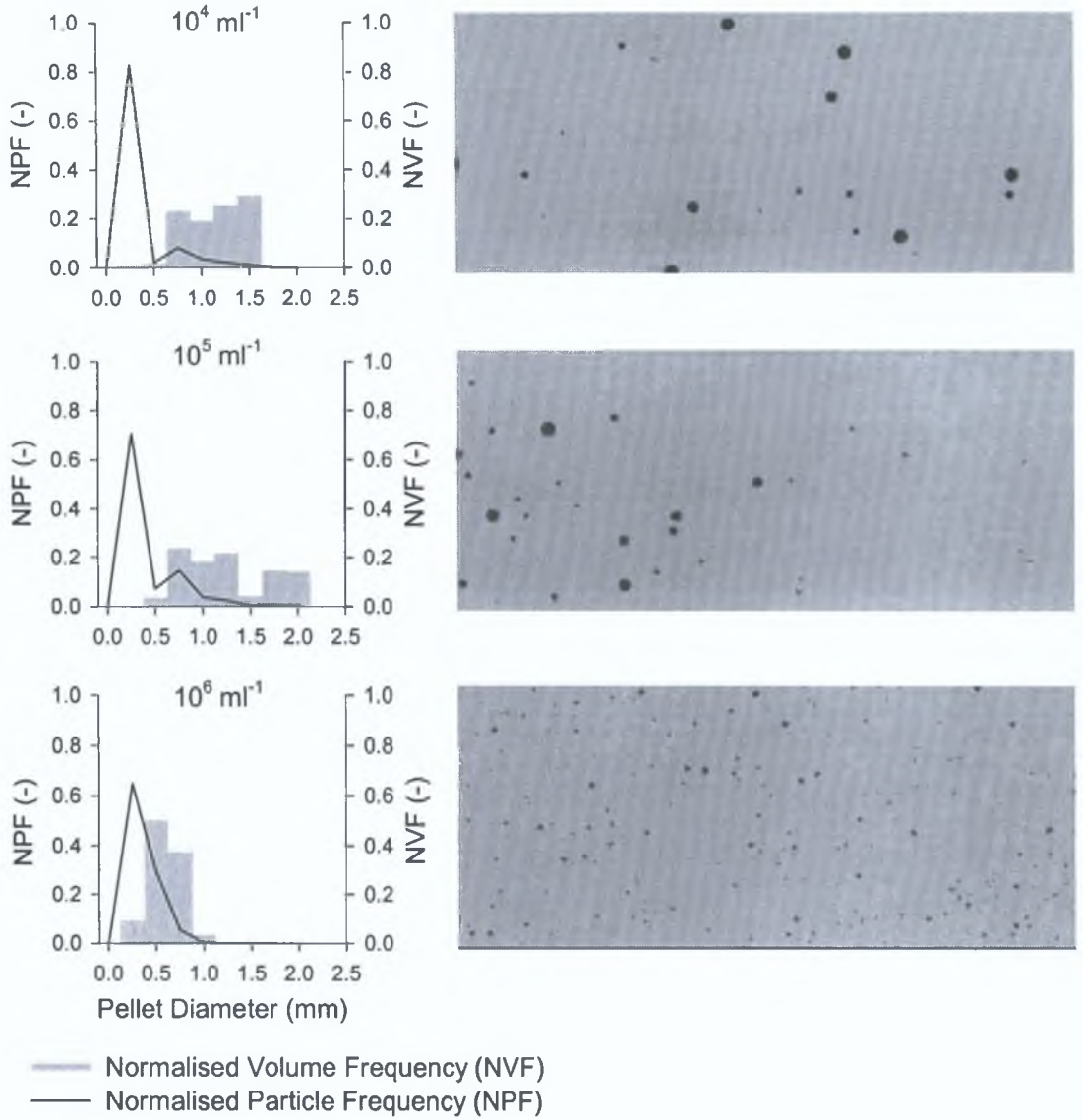


Figure 3.5. Stained microbial biomass and normalised volume and particle frequency distributions for 10^4 spores ml^{-1} (1 in 2 Dilution), 10^5 spores ml^{-1} (1 in 5 Dilution) and 10^6 spores ml^{-1} (1 in 10 Dilution) day 7 fermentation samples.

Table 3.4. Morphological characteristics of *Streptomyces hygroscopicus* var. *geldanus* fermentation samples.

Inoculum Conc. (ml^{-1})	Pellet Count (ml^{-1})	Volume of Pellets ($mm^3\ ml^{-1}$)	Dry Weight of Biomass $(g\ l)^{-1}$	Mean Pellet Volume (mm^3)	Biomass Dry Weight Equivalent Density ($g\ mm^{-3}$)
1×10^6	2650	37	1.39	0.014	3.8e-5
1×10^5	230	18	0.77	0.081	4.2e-5
1×10^4	190	10	0.73	0.056	6.8e-5

The technique developed applies not only to the study of pelleted fermentation broths but may apply to any procedure that necessitates the macroscopic image analysis of non-translucent spherical particles of diameter greater than 100 µm.

3.5. FLASK-TO-FLASK VARIABILITY

During the investigation into the morphological and physiological characteristics of *S. hygroscopicus* fermentation cultures it was observed that there was a significant degree of flask-to-flask variability within supposedly identical sets of flasks. These variations were observed across all measured parameters and suggested an apparent randomness in culture development. The physical manifestation of this randomness, in the form of flask-to-flask variability, provided cause for considerable concern regarding the statistical accuracy of experimental outcomes.

It is thought that flask-to-flask variability may be as a result of variability in the mechanisms of particle collision, interaction and subsequent aggregation leading to pellet formation within the system. The standard experimental procedure described in section 2.6 uses quintuplicate flasks per time point. However, in order to assess the degree of flask-to-flask variability within this system it was decided to scale up from five to fifty flasks to facilitate the

generation of a larger set of experimental data for subsequent analysis. The remainder of the operating conditions were as described in section 2.6 and the analysis was performed 72 hours after inoculation, where variability within the system was found to be at its highest.

The degree of variability inherent in the system was assessed in terms of the pellet count, mean pellet volume and dry weight of biomass. The variability in pellet count, as illustrated in Figure 3.6 (a), appears to be considerable, ranging from a minimum of 651 to a maximum of 2659 ml^{-1}, a four-fold difference. The mean pellet volume, as shown in Figure 3.6 (b), varies from a minimum of 5.3×10^{-4} to a maximum of 5×10^{-3} mm^3, an approximately nine and a half-fold difference, with the majority of the cultures in the range of 5×10^{-4} to 15×10^{-4} mm^3. The variability observed in the dry weight of biomass data as shown in Figure 3.6 (c) randomly varies from a minimum of 0.1 up to a maximum of 0.455 gl^{-1}, a four and a half-fold difference, with the majority of flasks in the region of 0.3 to 0.45 gl^{-1}.

Each of the fifty flasks used in this experimental procedure, including the nutrient broth, were prepared in an identical manner. The spore inoculum utilised was prepared from a frozen spore stock, the stability and reproducibility of which is examined in section 2.3.1 and verified in Figure 2.2. The fifty flasks were placed on two identical shaker tables in a 28°c warm room for the duration of the experimental time period. The image analysis procedure, as demonstrated in section 3.3, has been validated to within a 5% deviation on biological samples. Given the identical nature of the flask preparation, inoculation and incubation and image analysis procedures, it is surprising to see such a degree of morphological and physiological variability within this set of flasks.

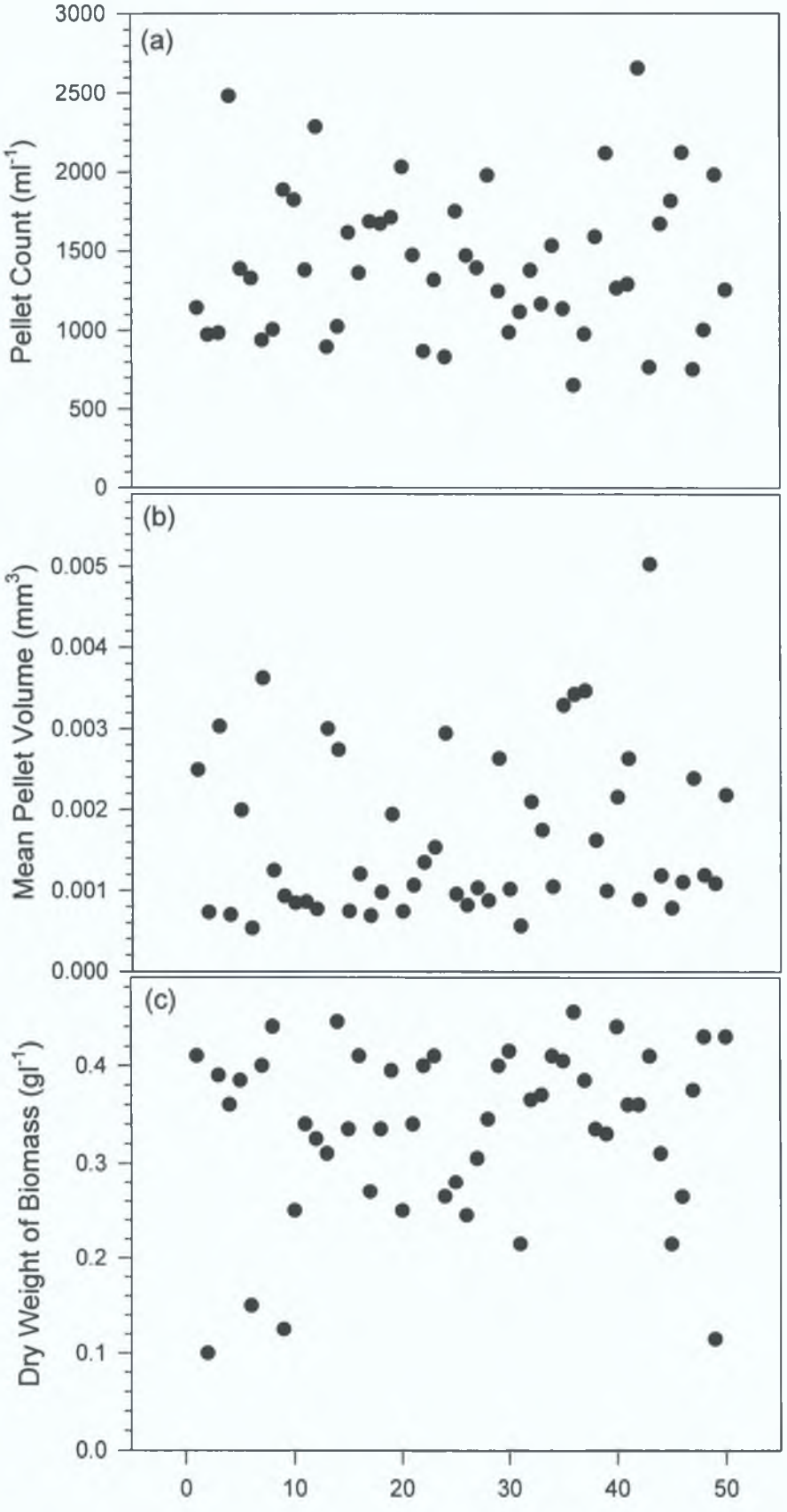


Figure 3.6. Flask-to-flask variability in the morphological and physiological parameters (a) pellet count, (b) mean pellet volume and (c) dry weight of biomass after 72 hours.

The variable nature of culture development in the first 72 hours is further illustrated in Figure 3.7. This figure shows the particle count and volume fraction distributions of five randomly chosen flasks from the total population. The particle counts vary in two respects. Firstly, the numerical particle count varies, as shown earlier in Figure 3.6 (a), with the greatest disparity amongst the five being between flasks 4 and 8 with 2480 and 1004 pellets ml^{-1}, respectively. Secondly, the range of the particle count distribution varies, with the greatest disparity here being between flasks 4 and 19. The wider size distribution in flask 19 indicates that this particular culture contained more small to mid-sized particles than any of the other selected flasks. Just as is the case with the particle count the volume fraction also varies in two respects. Firstly, the cumulative volume fraction varies from one flask to another; this is a measure of the total volume of biomass present. The greatest disparity observed is between flasks 25 and 19 with approximately 4 and 9 mm^3ml^{-1} respectively. Secondly, the different morphological distributions (i.e pellet count and volume fraction distributions) present in the cultures illustrate that they are fundamentally dissimilar. In flasks 19 and 25, not only is the volume fraction different but also the distribution of pellet volumes. The biomass in flask 25 appears to a certain extent bi-modal, with two pellet populations, one containing a small number of large particles and the other containing a large number of small particles. Flask 19 however contains a uni-modal distribution of small to mid-sized pellets present in a single phase.

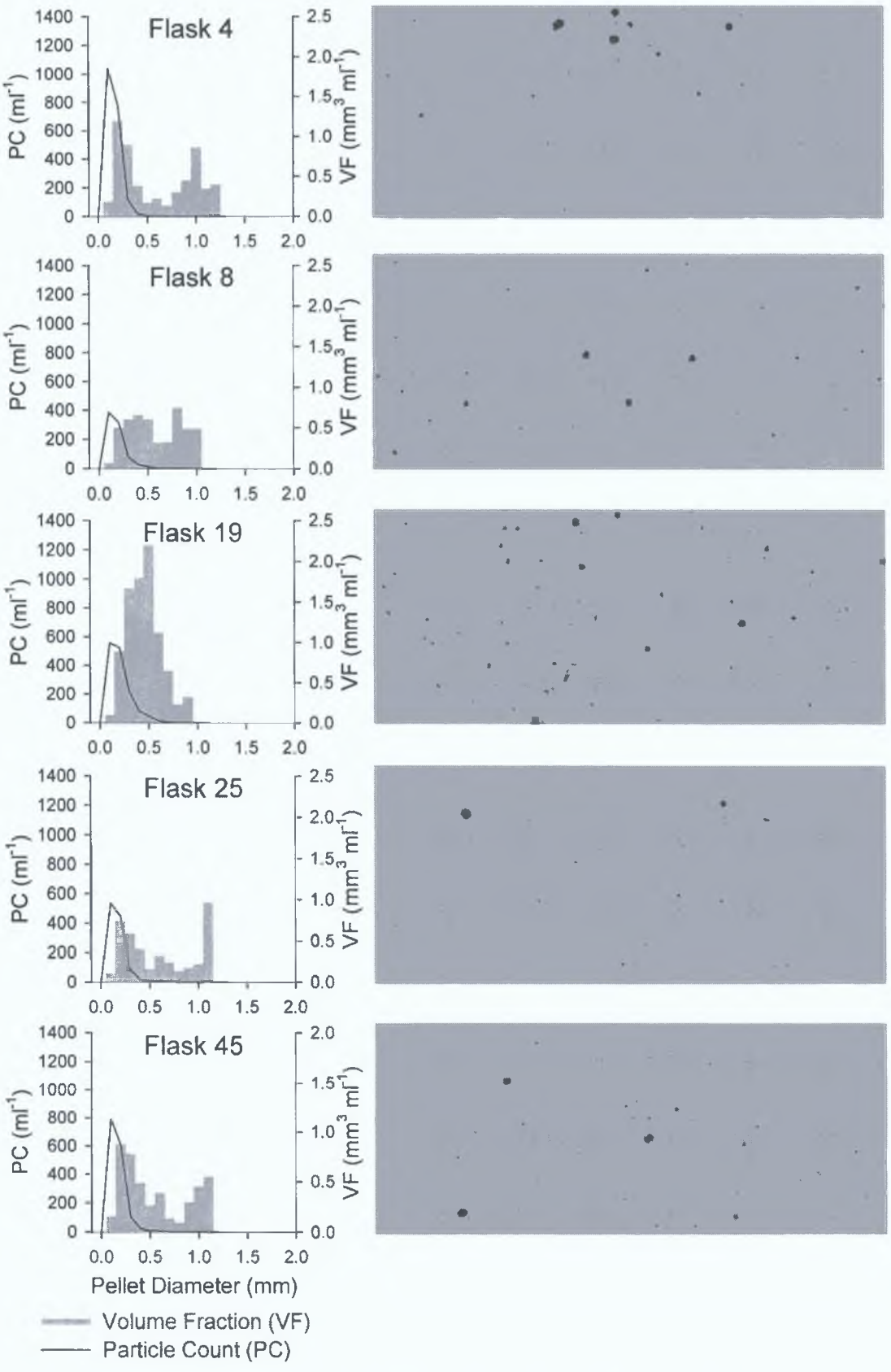


Figure 3.7. Stained microbial biomass and volume fraction and particle count distributions for flasks 4, 8, 19, 25 and 45 (1 in 5 Dilution), 72-hour fermentation samples.

The volume fraction and particle count distributions shown in Figure 3.7 illustrate the degree of morphological variability in a randomly chosen sub-set of the larger flask population. Within these five flasks, the differences in the cumulative particle counts and volume fractions and their respective distributions are evident. The cultures can randomly vary from single normally distributed populations with a relatively large volume fraction and a mode of approximately 0.4 – 0.5 mm (as demonstrated by flask 19) to bi-modal distributions of relatively low volume fraction with one mode at approximately 0.2 – 0.3 mm and the other > 1.0 mm (as demonstrated by flask 25). This variation in culture morphology is observed in the complete set of fifty flasks and not just for the sub-set shown.

It is proposed that the morphological and physiological variability observed from flask-to-flask is as a result of variability within the process of particle aggregation, which in turn is a function of collision and interaction. The occurrence of variability to the extent demonstrated highlights a major issue associated with culturing filamentous microorganisms, the statistical accuracy of experimental data. Given the variability of data shown within the flask set, the requirement to analyse sufficient flasks, to obtain an accurate statistical representation, becomes apparent.

3.6. EXPERIMENTAL SAMPLE SIZE

The occurrence of flask-to-flask variability as discussed in the previous section underlines the necessity of running sufficient flasks to obtain an accurate statistical representation, while remaining within experimental limitations. By utilising a larger data set, it is proposed to determine the optimum number of flasks per experimental run, to accurately describe the morphological and physiological condition of the organism, without exceeding experimental limitations.

The set of fifty data points was randomised ten times to yield ten randomly ordered data sets for both pellet counts and biomass concentrations. To

investigate the statistical accuracy of duplicate sampling, the first two points from each of the ten data sets were chosen, for triplicate, the first three, quadruplicate, the first four and quintuplicate, the first five. Figure 3.8 illustrates the statistical variability in dry weight of biomass analysis for duplicate, triplicate, quadruplicate and quintuplicate flasks in randomly chosen *S. hygroscopicus* cultures, in comparison to the mean of fifty flasks. Figure 3.9 shows a comparative analysis for the pellet count per ml of fermentation broth in the same flasks. As illustrated in both Figures 3.8 and 3.9, increasing sample size increases the statistical accuracy, for example, when quintuplicate sampling is used there is a maximum deviation from the mean of approximately ± 12%.

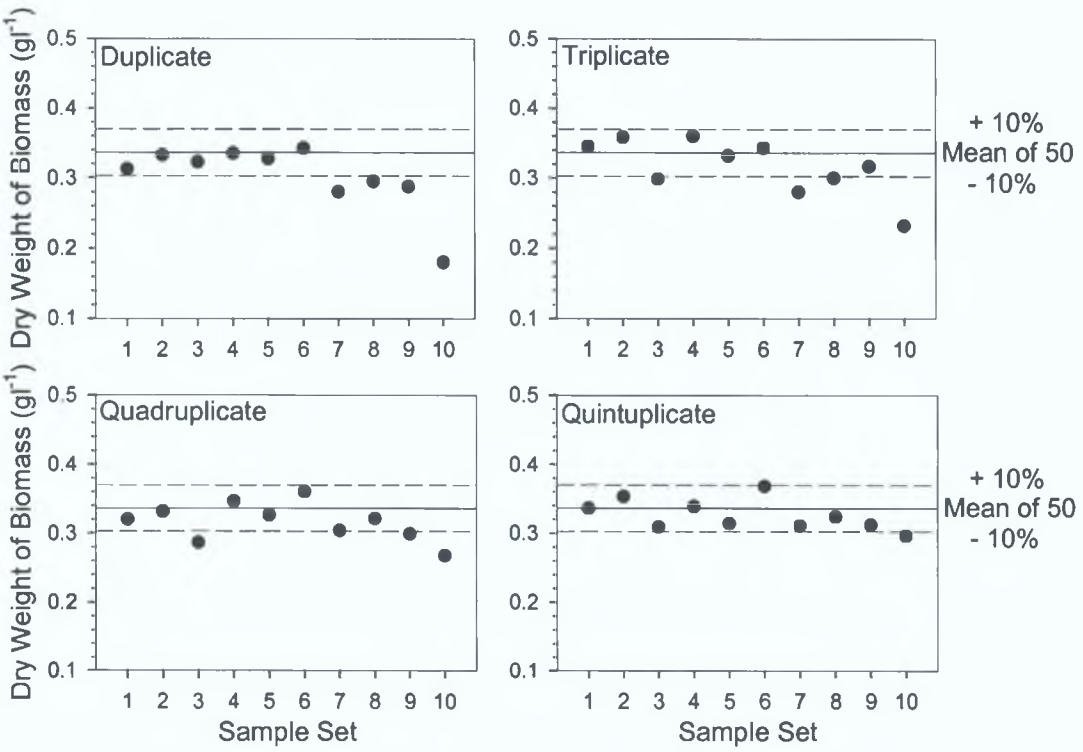


Figure 3.8. The impact of varying sample size on the measurement of dry weight of biomass within ten randomly chosen sample sets from a population of fifty flasks.

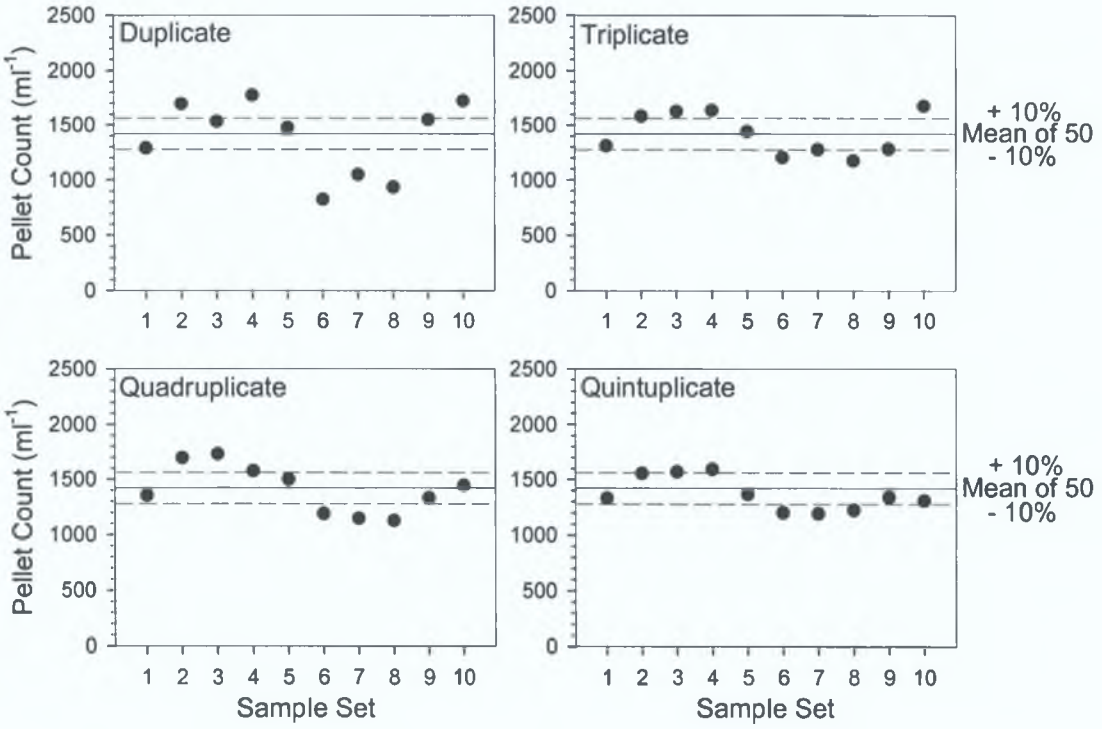


Figure 3.9. The impact of varying sample size on the measurement of pellet count per ml of broth within ten randomly chosen sample sets from a population of fifty flasks.

Figure 3.10 illustrates the morphological mean of the entire set of fifty flasks and the accuracy of randomly choosing a sub-set of the population for analysis. Once again it is shown that increasing sample size increases accuracy and results in a distribution closer to the population mean. The most statistically accurate yet still experimentally feasible set of data for the dry weight of biomass, pellet count and morphological profile is obtained by sampling five flasks. In theory, increasing the sample size will provide an ever-closer approximation of the entire population. However, there are experimental limitations associated with running more than five flasks per set. Firstly, each flask can only be sampled once, given the invasive and destructive nature of the sampling procedures, hence one flask per sample per time point is required. Secondly, equipment limitations, such as shaker table space and glassware availability, limit the number of flasks that it is physically possible to use. Thirdly, the manpower requirements of performing analysis for each individual sample is excessive.

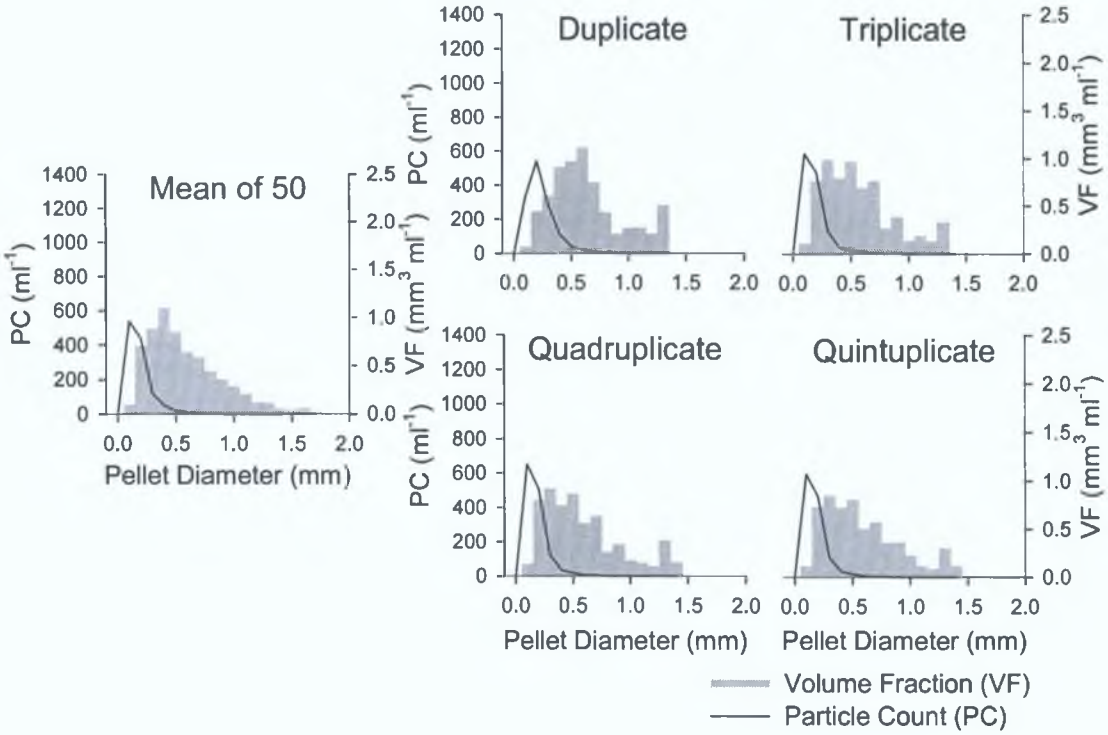


Figure 3.10. Volume fraction and particle count distributions for varying sample size analysis from a randomly chosen sample set of 72-hour fermentation cultures.

3.7. CONCLUSIONS

Microscopic image analysis is a powerful tool for the morphological quantification of filamentous fermentation broths. However it does have its limitations, most notably the small field of view and the concomitant need for multiple image capture. The applicability of such a system to pelleted fermentation systems is questionable given the sample preparation techniques and the size of the particles to be captured. The adaptation of these traditional image analysis methods to facilitate the morphological quantification of pelleted systems in an efficient and cost effective manner is desirable.

The technique developed allows high throughput morphological characterisation of *S. hygroscopicus* pelleted fermentation samples, at a rate hitherto difficult using conventional microscopic imaging techniques. The

advent of high performance desktop scanners has allowed for a cost effective image acquisition system for images of moderate resolution. This, combined with high performance desktop computing, has allowed for the handling and image processing of large field of view images. The technique developed applies not only to the study of pelleted fermentation broths but may apply to any procedure that necessitates the macroscopic image analysis of non-translucent spherical particles of diameter greater than 100 μm. This technique will form the cornerstone for all subsequent investigations into the quantification, regulation and morphological engineering of pellet development in *S. hygroscopicus* fermentations.

Morphological and physiological variability, as observed in the investigation into the characteristics of *S. hygroscopicus* cultures, appears to be due to inconsistent culture development within the shake flasks and not as a result of procedural error in the analytical techniques, which have been demonstrated to be accurate to less than 5% of the mean. Flasks prepared, inoculated and incubated in an identical manner should have given rise to morphologically and physiologically similar cultures. Yet, there was significant variability between flasks with respect to pellet counts, mean pellet volumes, biomass concentrations and morphological distributions. The overall outcome is the generation of cultures inherently different from one another. Indeed the pellet size distributions of the cultures vary in continuum from uni-modal to bi-modal pellet size distributions. The degree of morphological and physiological variation observed from flask-to-flask is thought to be as a result of random variation in the kinetics of particle collision, interaction, aggregation and thus pellet formation.

Theoretically, increasing sample size will provide an ever-closer approximation of the attributes of an entire population. However, the experimental overheads associated with running more than five flasks per sample are prohibitive. In this case a finite selection of samples from the population must be used to determine as accurate a representation of the distribution for the parameters of interest as possible. When using a selection of specimens it will not be possible to gain a completely accurate

representation of the whole population, however the correct sampling procedure will allow the approximation of an accurate relationship between the sample set and the overall population (Mandel, 1984). Taking into consideration the degree of flask-to-flask variability and the procedural limitations, the optimum sample size is thought to be five flasks per point.

The investigation into the extent of flask-to-flask variability in submerged fermentations of *S. hygroscopicus* has provided a very useful insight into the apparently random nature of particle aggregation and pellet formation. The extent to which this phenomenon occurs and its influence on the morphological and physiological development of these cultures illustrates the potential for morphological engineering of pellet formation and development.

CHAPTER 4 – THE INFLUENCE OF BIOPROCESSING PARAMETERS ON THE MORPHOLOGICAL AND PHYSIOLOGICAL DEVELOPMENT OF *S. HYGROSCOPICUS* CULTURES

4.1. INTRODUCTION

Filamentous microorganisms are specially adapted for surface colonisation of solid substrates, through the foraging nature of apical and branched growth in a way that simpler single celled microorganisms are not. The growth of these microbes in a submerged liquid fermentation, which is not the organism's natural habitat, forces the organism to alter its morphological and physiological developmental patterns. Bioprocessing parameters greatly influence culture development within such systems (Papagianni, 2004; Prosser & Tough, 1991), chiefly the medium composition, spore inoculum concentration and shake flask agitation speed.

The nutritional composition of fermentation media must be sufficient to enable growth and/or product formation in submerged cultures. Fermentation media may be either complex or defined depending on the nutritional composition. Complex media generally provide good growth and product formation, which are not mutually exclusive. Nutrient rich fermentation media may lead to bioprocessing problems; the presence of complex substrates may increase culture viscosity, by altering the rheological characteristics of the fluid, and thus affecting the mass transfer of both nutrients and oxygen (Cho *et al.*, 2002; Choi *et al.*, 1998; Sinha *et al.*, 2001). The type and concentration of the carbon, nitrogen and phosphorous sources are important to the nutritional welfare of filamentous microorganisms and affect biomass growth, morphological development and product formation (Glazebrook *et al.*, 1992; Kojima *et al.*, 1995; Lee *et al.*, 1997; Park *et al.*, 1999; Schrader & Blevins, 2001). Micronutrients are primarily involved in the regulation of cellular metabolism and consequently in product formation.

The morphological development of an organism in submerged fermentation is a function of inoculum type, concentration and age (Papagianni, 2004). The inoculum may be either spores or vegetative cells, the choice of which depends on the application. Vegetative inocula are typically used when the lag time associated with germination and initial biomass development is not desirable. Spore inocula on the other hand consist of inert single spores or clusters of spores, typically used in the initial preparatory stages of a process. As well as the type of inocula used, the concentration of the spores or biomass, depending on which type is used, has also been shown to influence culture development. Vechtlifshitz *et al.* (1990) investigated the impact of spore inoculum concentration on pellet formation for *S. tendae*. An inverse relationship was shown to exist between inoculum concentration and pellet size whereby increasing the order of magnitude of spores in the system produced larger numbers of smaller pellets, with the cultures varying from free filamentous, through a mixed pellet population to a few large pellets.

The cultivation of microorganisms in submerged fermentations relies heavily on sufficient agitation to promote mixing within the system, which is a prerequisite to achieve uniform distribution of temperature and concentration of nutrients and oxygen. Furthermore, not only is agitation responsible for maintaining favourable growth conditions but it is also responsible for regulating the morphological development of the cultures (Amanullah *et al.*, 2001; Casas Lopez *et al.*, 2005; Cui *et al.*, 1997; Hotop *et al.*, 1993). Within this developmental process, agitation is thought to play a role in pellet formation and break-up, the former being as a result of particle aggregation and/or entanglement while the latter occurs through hyphal fragmentation and pellet disintegration. The relationship that has been shown to exist between agitation and morphology, suggests increasing agitation speed typically results in decreasing particle size and increasing particle number.

Of the environmental and nutritional conditions experienced by the organism in submerged fermentation, perhaps the most important are nutrient medium composition, inoculum concentration and agitation speed. The impact of

these three bioprocess parameters on the growth and morphological development of *S. hygroscopicus* cultures was explored. The morphological and physiological development of the organism was assessed in a variety of different nutrient media, in cultures pitched with varying concentration inocula and in cultures agitated at different rates. Characterisation of the effect of bioprocess conditions on culture development enabled the designation of a standard set of operating conditions under which to further study the morphological and physiological development of *S. hygroscopicus*.

4.2. NUTRIENT MEDIA COMPOSITION

The cultivation of *S. hygroscopicus* was attempted in six different nutrient broths, as outlined in Table 4.1, in order to assess the morphological and physiological impact of each on the lifecycle of the organism. The experimental procedure performed involved culturing 100 ml of the relevant nutrient media in a 250 ml Erlenmeyer flasks with 1% (v/v) of a 2×10^6 ml^{-1} *S. hygroscopicus* spore suspension and incubating on an orbital shaker at 150 rpm and 28°C. The duration of the experimental procedure was 7 days, with sampling for image analysis purposes occurring on the third, fifth and seventh day and sampling for dry weight of biomass determination occurring on the seventh day. A single set of five flasks was prepared for each of the nutrient media and was used throughout the experimental time period for the aforementioned sampling. All data shown is based on that obtained from the set of five identical flasks.

Of the six broths tested one, M2M, a defined medium that may have been lacking in certain germination-dependent nutrients, did not support growth. The remaining five supported growth, however the determination of biomass concentration in two of these broths was complicated by the presence of suspended solids: DeBoer & Dietz medium contained oatmeal and GYM contained calcium carbonate. Morphological assessment of the Mueller-Hinton samples was complicated by the fact that the majority of the biomass was in one of two forms, wall growth or free filaments, neither of which is morphologically quantifiable with the current image analysis system. The

phenomenon of wall growth occurs when biomass adheres to the wall of the vessel above the liquid. Some of the biomass adhered to the glass surface and continued to grow, forming a ring around the inside of the flask at the air-liquid interface, where it continued to be provided with nutrient material. This material periodically became detached and fell back in to the liquid. Growth in this manner did not conform to conventional pellet development and so could not be assessed in the same way. Figure 4.1 contains a pictorial representation of biomass wall growth both in the flask and prepared for image capture in a petri dish, as described in section 3.2.4.

Table 4.1. Capability of different nutrient media to support growth and the applicability of dry weight of biomass determination and image analysis techniques to the resulting broth.

Nutrient Medium	Growth	Dry Weight Determination	Image Analysis
Bennett's	✓	✓	✓
DeBoer & Dietz	✓	×	×
GYM	✓	×	×
M2M	×	×	×
Mueller-Hinton	✓	✓	×
YEPD	✓	✓	✓

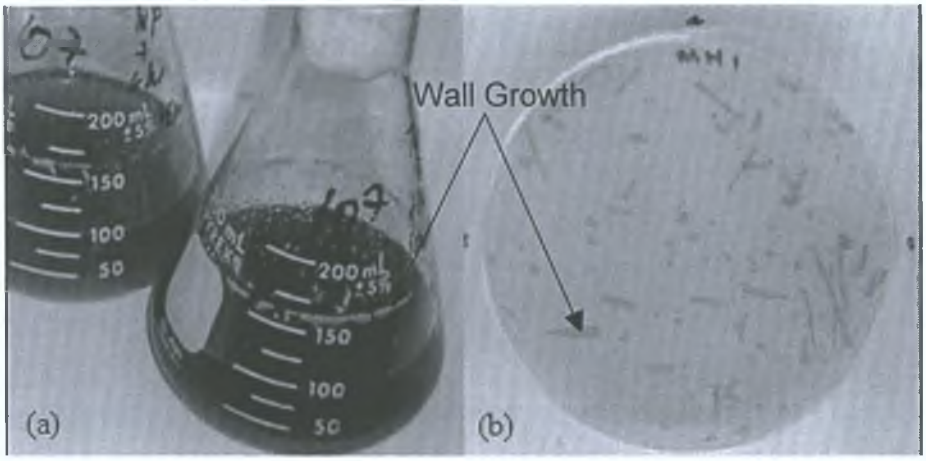


Figure 4.1. Pictorial representation of biomass wall growth in Mueller-Hinton broth (a) immobilised on the shake flask wall and (b) stained and presented for scanning in a petri dish.

Morphological profiling of the pellet population was possible in Bennett's and YEPD cultures as shown in Table 4.1 and Figure 4.2.

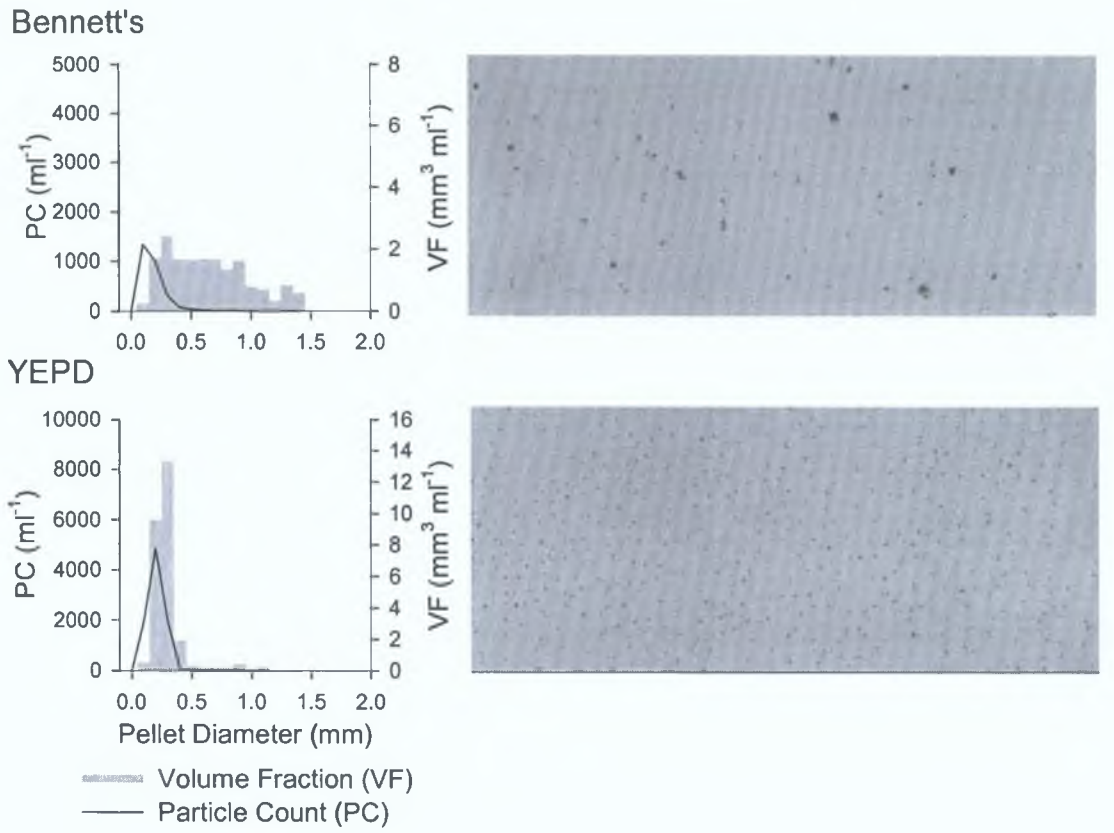


Figure 4.2. Morphological distributions and stained microbial biomass for Bennett's (1 in 10 Dilution) and YEPD (1 in 20 Dilution) Day 7 fermentation samples.

A direct comparison of the impact of broth composition on the morphological profiles of the organism was undertaken using both these nutrient media, the results of which are given in Figures 4.3 and 4.4. Both cultures were inoculated with the same density of spores yet exhibit significantly different morphological profiles. As illustrated in Figure 4.3 (a) the pellet count in YEPD cultures was shown to be 4 – 7 fold higher than that present in Bennett's over the experimental time period. In Bennett's, the pellet count remains approximately constant at ~2500 ml^{-1} from day 3 – 5 and then increases to ~3500 ml^{-1} by day 7, whereas in YEPD cultures, the pellet count increases day 3 – 5 from ~8500 to ~13500 ml^{-1} and decreases again to ~11000 ml^{-1} by day 7. The mean pellet volume in Bennett's cultures is 0.0010 mm^3 at day 3 and increases slightly to 0.0013 mm^3 by day 7, whereas the value is 0.0005 mm^3 in YEPD at day 3 and increases significantly day 3 – 7

finally reaching a value of 0.0022 mm^3. Figure 4.3 (c) illustrates the ratio of pellets formed to the concentration of CFUs added in both Bennett's and YEPD cultures. At day 3, the ratio is 1:10 in Bennett's and 1:2 in YEPD indicating that there may be less aggregation in YEPD cultures, leading to a higher numerical frequency of smaller pellets.

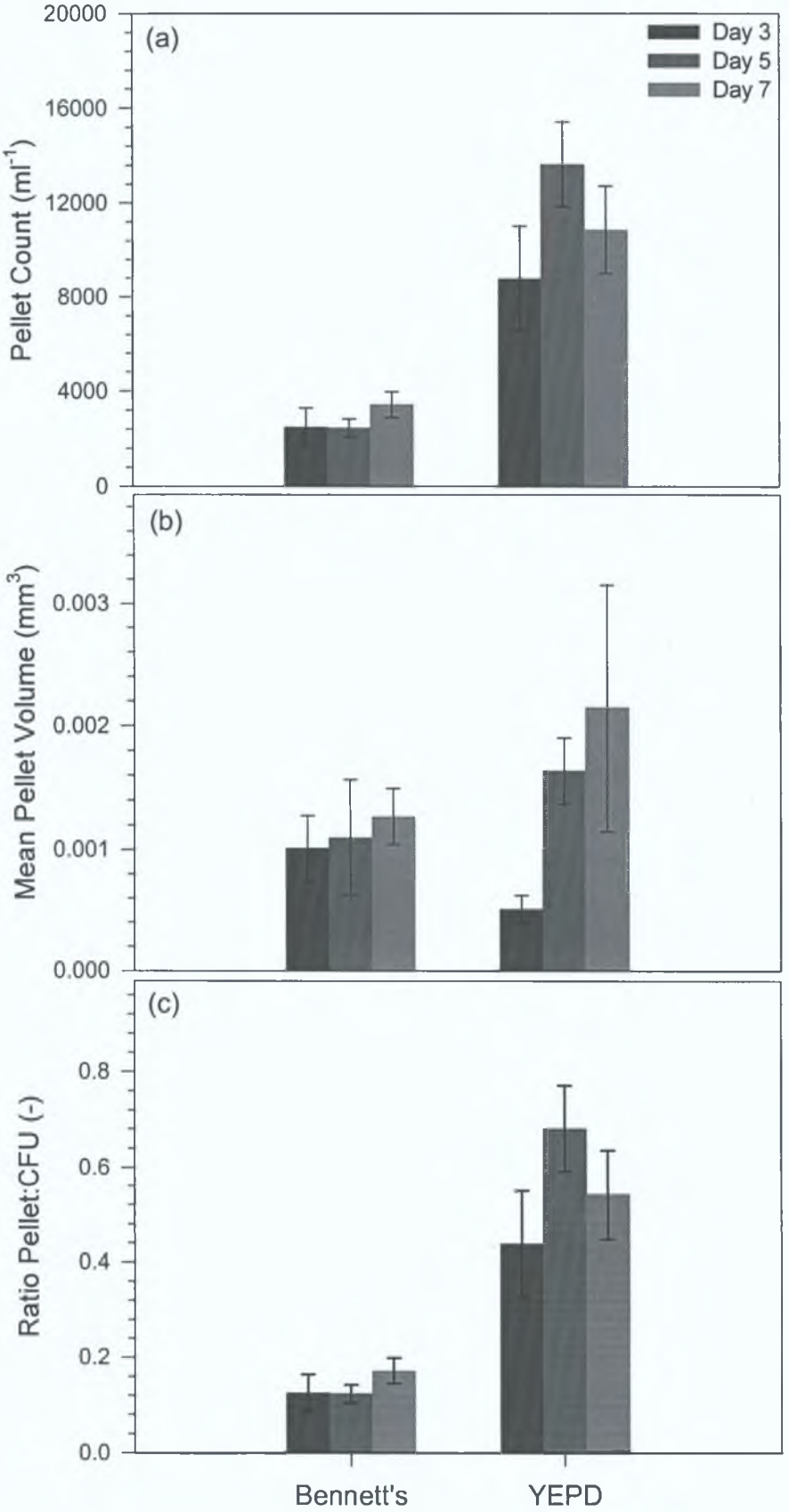


Figure 4.3. Influence of nutrient broth on (a) pellet count, (b) mean pellet volume and (c) pellet to CFU ratio at days 3, 5 and 7. Error bars represent the +/- standard deviation of the mean obtained from a set of five flasks.

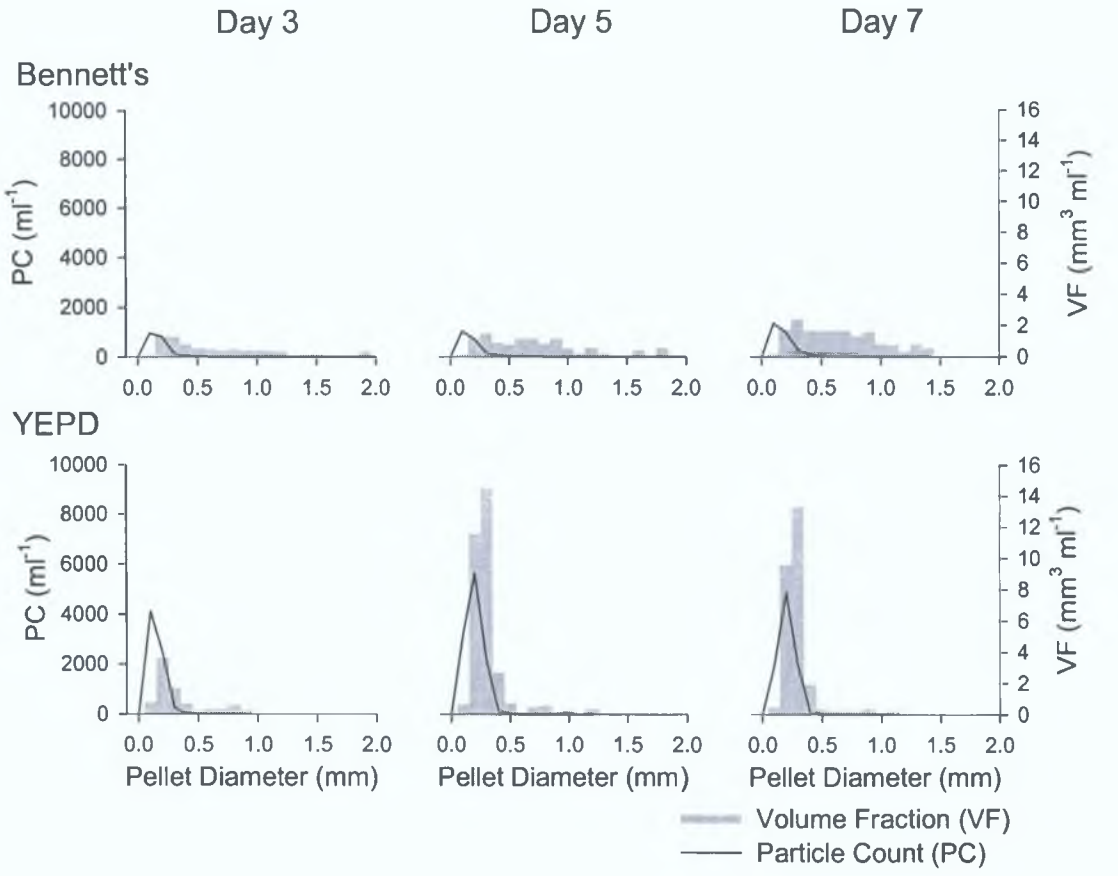


Figure 4.4. Volume fraction and particle count distributions for Bennett's and YEPD fermentation cultures at days 3, 5 and 7.

As illustrated in Figures 4.3 and 4.4, by day 3, the biomass within Bennett's cultures is made up of ~2500 pellets ml^{-1} of varying size, with approximately 12% of the numerical fraction contributing in excess of 80% of the cumulative volume fraction i.e. pellets with a diameter in the range 0.3 - 2.0 mm. There is a high degree of morphological heterogeneity within these cultures as demonstrated by the differences in the particle count and volume fraction distributions. In contrast, biomass within YEPD cultures is made up of ~8500 small pellets ml^{-1}, which account for the majority of the cumulative volume fraction. The particle count and the volume fraction size distribution overlap, indicating a high degree of morphological homogeneity in these cultures.

As demonstrated in Figure 4.4, by day 3 the morphological fate of both cultures is set. All subsequent culture development is governed by the morphological profile at this point in time. This subsequent development of Bennett's cultures involves a broadening of the morphological distribution, pellet count remaining static or increasing slightly and mean pellet volume and cumulative volume fraction increasing. Pellet enlargement is not simply a function of hyphal growth alone but also of particle aggregation. Within the system it is suspected that the larger pellets, as well as growing themselves, are responsible for actively binding smaller pellets. By day 7 these cultures consist of a mixed pellet population with a widely distributed heterogeneous population. Similarly in YEPD cultures the subsequent development involves a slight broadening of the morphological distribution. Pellet count increases day 3 - 5 and then decreases day 5 - 7 and mean pellet volume and cumulative pellet volume increase significantly, as illustrated in Figures 4.3, 4.4 and 4.5. This indicates that the existing pellet population is continuing to grow as new particles emerge and that pellet development is primarily through hyphal growth and not particle aggregation. By day 7 these cultures consist of a single homogeneous population of pellets, as illustrated by the overlapping particle count and volume fraction distributions in Figure 4.4.

Figure 4.5 illustrates the differential levels of biomass production over 7 days, with YEPD cultures producing approximately 4 times as much biomass. Upon comparison of the cumulative pellet volume data in the same figure it becomes apparent that although there is disparity from Bennett's to YEPD it is not of the same magnitude as the dry weight of biomass. The cumulative pellet volume of YEPD cultures is one and a half times that of Bennett's, which would suggest that YEPD cultures contain pellets that are more compact with a higher density than their Bennett's counterparts.

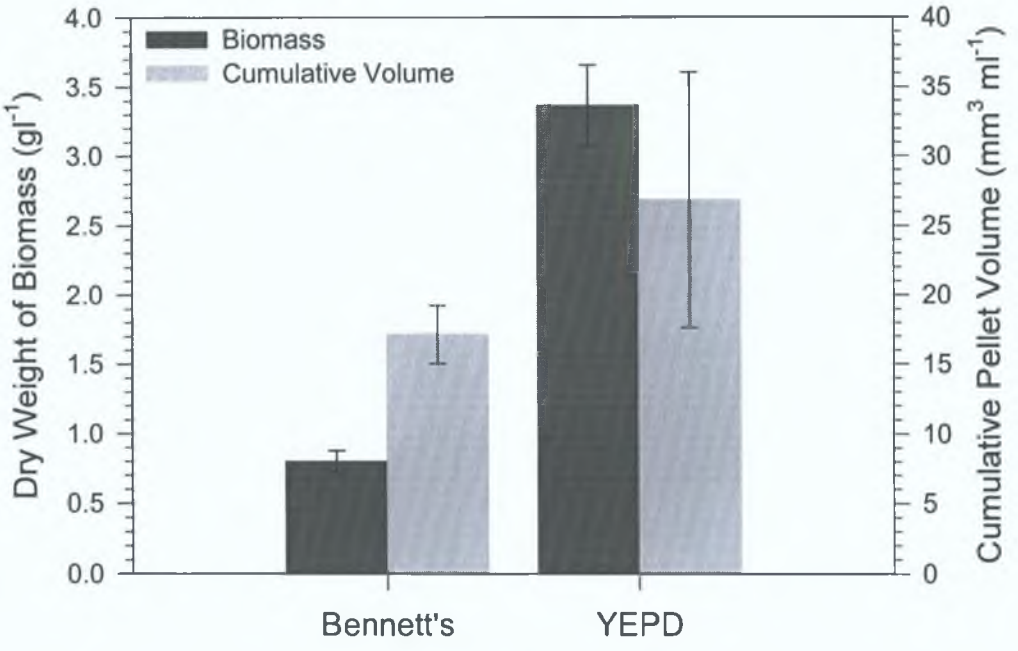


Figure 4.5. Day 7 dry weight of biomass and cumulative pellet volume for Bennett's and YEPD cultures. Error bars represent the +/- standard deviation of the mean obtained from a set of five flasks.

The nutritional composition of the fermentation medium, in particular the type and concentration of the carbon and nitrogen sources, can greatly influence culture morphology and physiology. Papagianni *et al.* (1999), investigating the influence of glucose concentration on *A. niger* morphology, concluded that glucose concentration (60 - 150 gl^{-1}) altered the growth rate (0.01 - 0.03 h^{-1}), which in turn affected the morphological profile of the organism, with higher initial glucose levels resulting in the formation of smaller pellets. The type and concentration of nitrogenous compounds used in the preparation of fermentation media has also been shown to influence the morphology of *Mortierella alpina* (Park *et al.*, 1999). Culture morphology varied from pelleted in the presence of yeast extract, gluten meal and corn steep liquor to dispersed mycelia in the presence of pharmamedia, fish meal or soybean meal.

The major nutritional differences between Bennett's and YEPD media are the concentration of glucose and the type and concentration of the nitrogenous

compounds used. YEPD contains 7.5 times the nitrogenous compounds and double the glucose of Bennett's. Consequently, growth in YEPD (3.36g at day 7) is greater than in Bennett's (0.80g at day 7) with an approximate four-fold increase in the amount of biomass produced. The morphological development of the cultures in the first 72 hours is most significant, with the resulting profiles forming the basis for all subsequent growth and enlargement. YEPD cultures produce high biomass levels containing a large number of small pellets, with lower sample-to-sample variability and a narrow size distribution that varies little with respect to time. Bennett's cultures produces significantly lower biomass levels containing a mixed pellet population with a wider size distribution and a greater degree of sample-to-sample variability.

4.3. INOCULUM CONCENTRATION

The nutritional composition of the fermentation medium has been shown to influence the morphological development of *S. hygroscopicus* cultures. Another bioprocessing parameter that influences culture morphology is inoculum concentration. The morphological development of the organism in submerged fermentations is a function of inoculum type, concentration and age (Papagianni, 2004). Spore inocula are used throughout the experimental work reported in this thesis as they are inert and their morphological development from germination can be investigated in a manner not possible with vegetative inocula, where the morphological distribution of the culture is already formed. Inoculum concentration has been shown to influence the morphological development of filamentous microorganisms such as *S. tendae* (Vechtlifshitz *et al.*, 1990). However, no information is currently available as to the influence of varying inoculum concentration on the morphological and physiological profiles of *S. hygroscopicus* cultures. An investigation into the influence of inoculum concentration on the morphological and physiological development of *S. hygroscopicus* cultures is reported in this section.

The experimental procedure performed involved culturing 100 ml of Bennett's nutrient medium in a 250 ml Erlenmeyer flasks with 1% (v/v) of *S. hygroscopicus* spore suspensions varying in concentration from 2×10^3 to 2×10^7 ml^{-1} in orders of magnitude. The flasks were subsequently incubated on an orbital shaker at 150 rpm and 28°C. The duration of the experimental procedure was 7 days, with sampling for image analysis purposes occurring on the third, fifth and seventh day and sampling for dry weight of biomass determination occurring on the seventh day. A single set of five flasks was prepared for each inoculum concentration and was used throughout the experimental time period for the aforementioned sampling. All data shown is based on that obtained from the set of five identical flasks.

Figure 4.6 contains the day 7 volume fraction and particle count distributions for cultures inoculated with the 10^3 – 10^7 spores ml^{-1} suspensions and a pictorial representation of the corresponding stained samples. This figure clearly illustrates the disparity in the morphological profiles of cultures inoculated with spore suspensions of increasing concentration. Further examination of the influence of spore suspension concentration is presented in Figure 4.8.

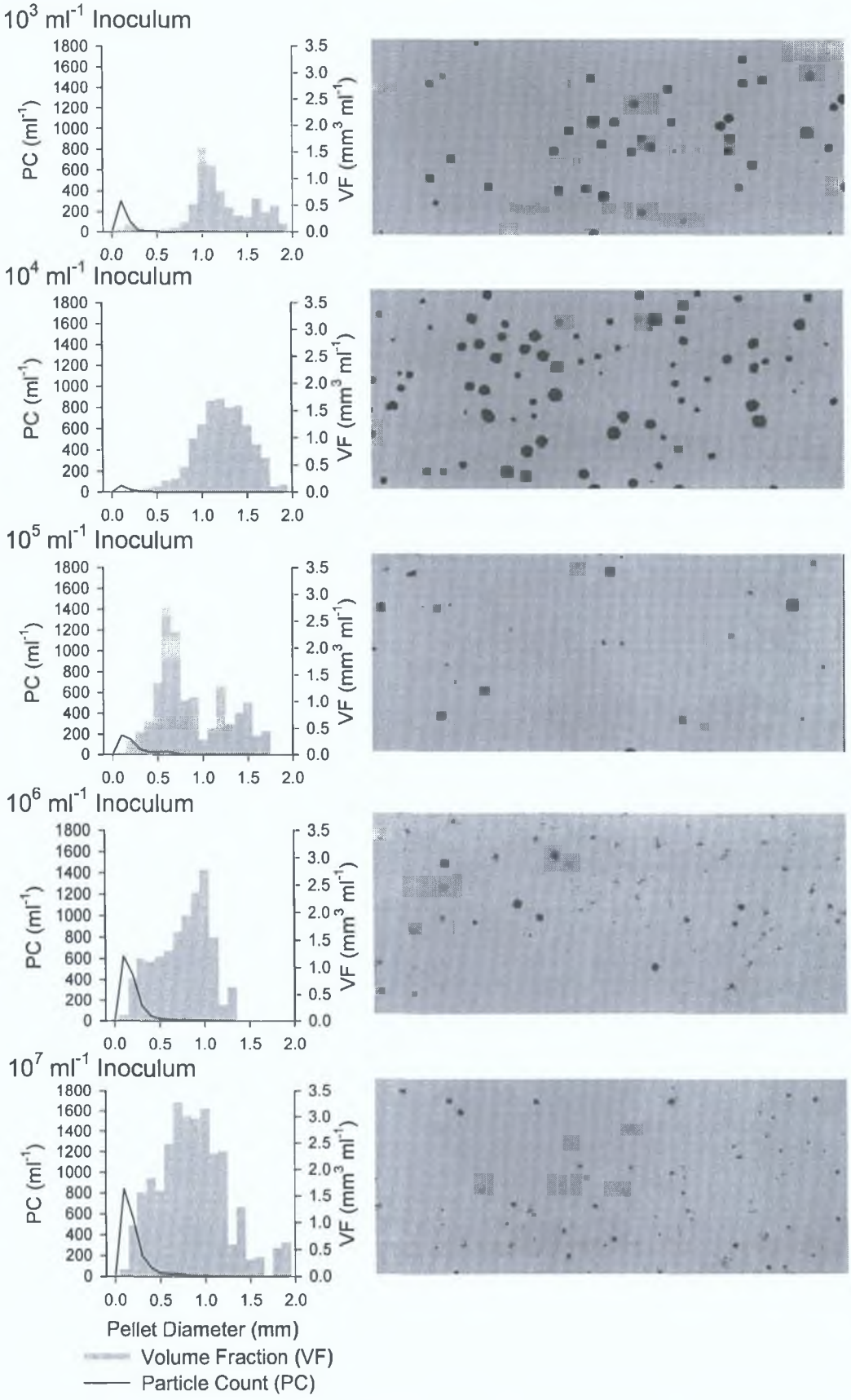


Figure 4.6. Morphological distributions and stained microbial biomass for 10^3 and 10^4 spores ml^{-1} (Neat) and 10^5, 10^6 and 10^7 spores ml^{-1} (1 in 10 Dilution) day 7 fermentation samples.

As illustrated in Figure 4.7 (a), increasing CFU loading density in orders of magnitude does not increase the number of pellets formed by the same extent. Indeed as shown in Figure 4.7 (c) the ratio of pellets formed to CFUs pitched indicates that increased inoculum concentration results in a reduced CFU to pellet ratio and suggests that a greater degree of aggregation is occurring in these cultures. Vechtlifshitz *et al.* (1990) encountered a similar phenomenon with *S. tendae.* The more CFUs present in the system, the more aggregation was found to occur and the lower the CFU to pellet ratio. Aggregation of this kind can be between individual spores or hyphal elements and is thought to be influenced by the composition, structure, hydrophobicity or charge of the cell wall, as well as by the presence of extracellular polymeric substances (Atkinson & Daoud, 1976). In order for spores and/or hyphae in solution to aggregate, they must first come into contact with one another in the form of a collision. An increase in the inoculum concentration provides for a greater probability of collision and hence aggregation. Thus, by increasing the concentration of CFUs in the fermentation broth, the rate of pellet formation will also be increased. Upon closer examination of Figures 4.7 (a) and (b) from day 3 - 5 the pellet count decreases in all but the 10^3 spores ml^{-1} culture and the mean pellet volume increases indicating that aggregation is occurring within these cultures. Subsequently, from day 5 - 7, the pellet counts increase in all cultures and the mean pellet volumes decrease or remain static indicating that aggregation is occurring to a lesser extent.

In the first 72 hours an increased rate of aggregation as a result of increased inoculum concentration appears to be advantageous to the morphological development of biomass populations as illustrated in Figure 4.8. Increased inoculum concentration and hence particle aggregation enables pellets to form more quickly, generate a greater volume fraction and yield a broader morphological distribution within this time period. The generation of larger pellets in a shorter period of time is advantageous as the Kolmogorov microscale for a shake flask system similar to the one under investigation was experimentally determined by Kaku et al. (2003) to be approximately 300 μm. For particles in the size range below this point there is a greater

probability of disaggregation and disruption, whereas for particles above 300 μm in diameter, this probability is reduced, therefore aggregation is advantageous for pellet formation as it enables pellets to exceed this size limit in a shorter period of time. In the 10^3 spores ml^{-1} cultures, the frequency of aggregation is lower, than in those pitched with higher concentration inocula. The ratio of CFUs pitched to pellets formed is 1:4 after 72 hours (Figure 4.7 (c)), which is likely to be as a result of fragmentation, leading to new centres of growth and eventually pellet formation. This inoculum density results in the formation of cultures with a small number of particles, a reduced volume fraction and a normally distributed pellet population. In the 10^4, 10^5, 10^6 and 10^7 spores ml^{-1} cultures particle aggregation occurs and increases with increasing inoculum concentration. All of these factors give rise to an increasing volume fraction of particles after 72 hours as shown in Figure 4.8.

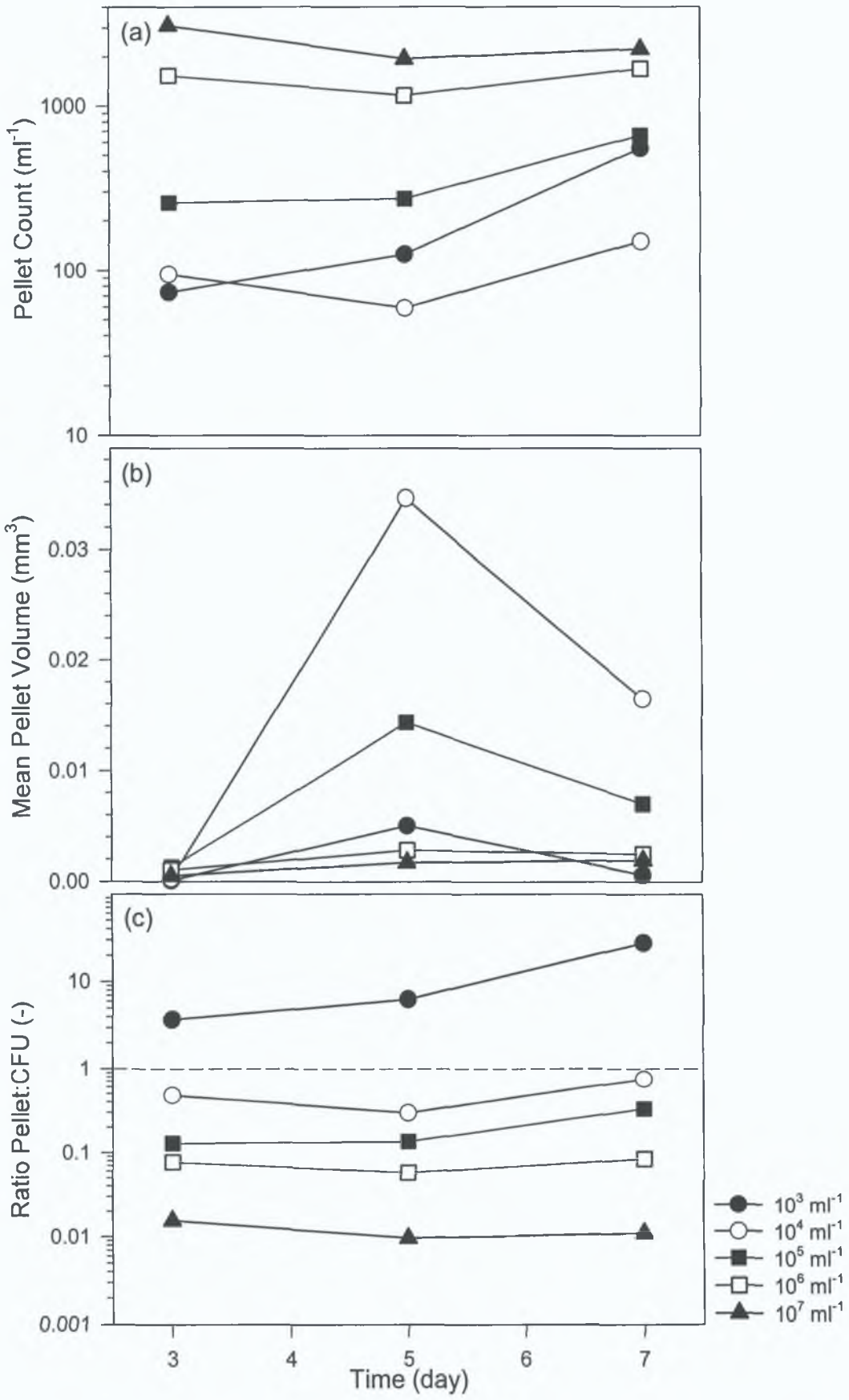


Figure 4.7. Influence of spore loading density on (a) pellet count, (b) mean pellet volume and (c) pellet to CFU ratio, at days 3, 5 and 7.

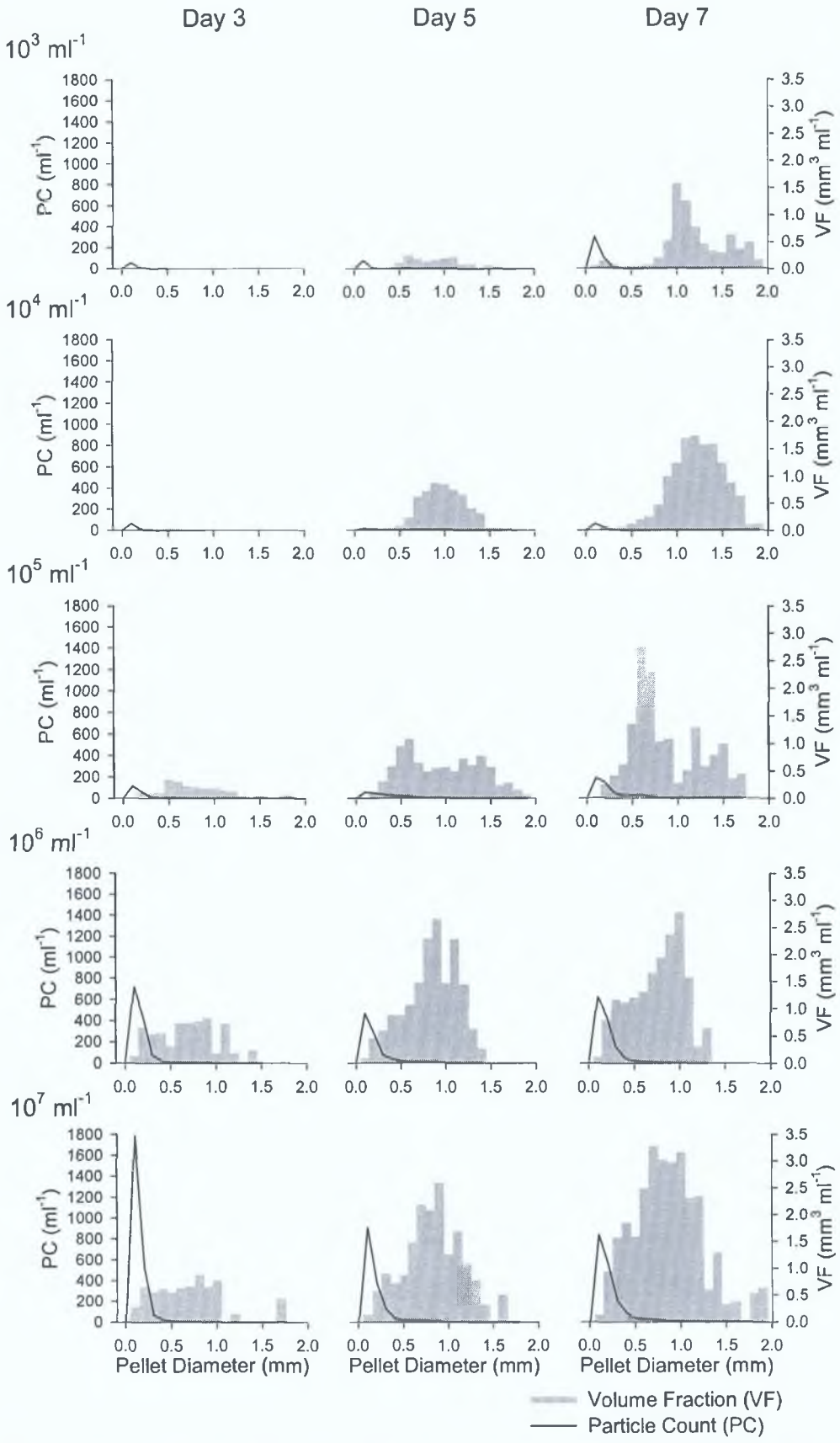


Figure 4.8. Volume fraction and particle count distributions for cultures inoculated with increasing concentration spore suspension at days 3, 5 and 7.

The development of the 10^3 spores ml^{-1} cultures between day 3 and 7 is thought to be through both hyphal growth and fragmentation, given that the cultures exhibit a CFU to pellet ratio that is greater than one and which increases with respect to time. Aggregation is thought to occur to a lesser extent in these cultures. A single phase of spores germinates, develops slowly and once large enough, begins to fragment leading to the generation of new pellets. By day 7 the size distribution appears to contain two populations of particles, the larger one (~1.75 mm in diameter) most likely being those derived from spores and the smaller one (~1.0 mm in diameter) being derived from fragments. The subsequent development of the 10^4 spores ml^{-1} culture is thought to be through both growth and aggregation. Unlike the 10^3 spores ml^{-1} culture it would appear that there are sufficient particles in this system for aggregation to occur. Although fragmentation is also thought to be occurring it would appear that whatever fragments are formed are subsequently aggregating with larger particles. In both the 10^3 and 10^4 spores ml^{-1} cultures approximately 98% of biomass by volume is contained within less than 18% of the particles, in the size range 0.3 - 2.0 mm diameter.

By day 3 a broad size distribution is achieved in the 10^5 spores ml^{-1} cultures, which continues to enlarge through growth and aggregation. The subsequent emergence of bi-modality in the distribution indicates that the larger pellets are fragmenting and giving rise to the development of a second population of smaller pellets. It is thought that the difference between the 10^4 and the 10^5 spores ml^{-1} cultures is that fragmentation is outstripping aggregation and resulting in the generation of new centres of growth that go on to form a second phase of pellets. As in the 10^5 spores ml^{-1} cultures, the increased inoculum concentration in the 10^6 and 10^7 spores ml^{-1} cultures ensures that by day 3 there is significant particle breakthrough giving rise to a mixed population containing a broad distribution of particle sizes. The subsequent development of these cultures is dependent on hyphal growth, fragmentation and aggregation. A complex inter-relationship exists between these three

processes and results in the formation of mixed populations predominantly containing a large number of small to medium-sized pellets.

Figure 4.9 shows the dry weight of biomass concentrations for each of the cultures. This figure clearly illustrates that even though the inoculum spore loading densities differed by several orders of magnitude, the cultures had similar levels of biomass (0.55 - 0.62 gl^{-1}) by day 7. Increasing loading density resulted in only marginally increased biomass levels indicating that biomass production is weakly dependent on the initial seeding density. The apparent disparity between the day 7 cumulative volume fractions and the biomass concentrations as illustrated in Figure 4.9, suggest that volume is not proportional to mass and that pellet density is variable. For example, the ratio of dry weight of biomass to cumulative pellet volume increases from approximately 1:13 in the 10^3 spores ml^{-1} culture up to approximately 1:48 in the 10^7 spores ml^{-1} cultures. Upon comparison of Figures 4.7, 4.8 and 4.9 it would appear that the mechanisms of pellet formation and development vary with increasing inoculum concentration, resulting in the formation of an increasing number of smaller particles with lower density.

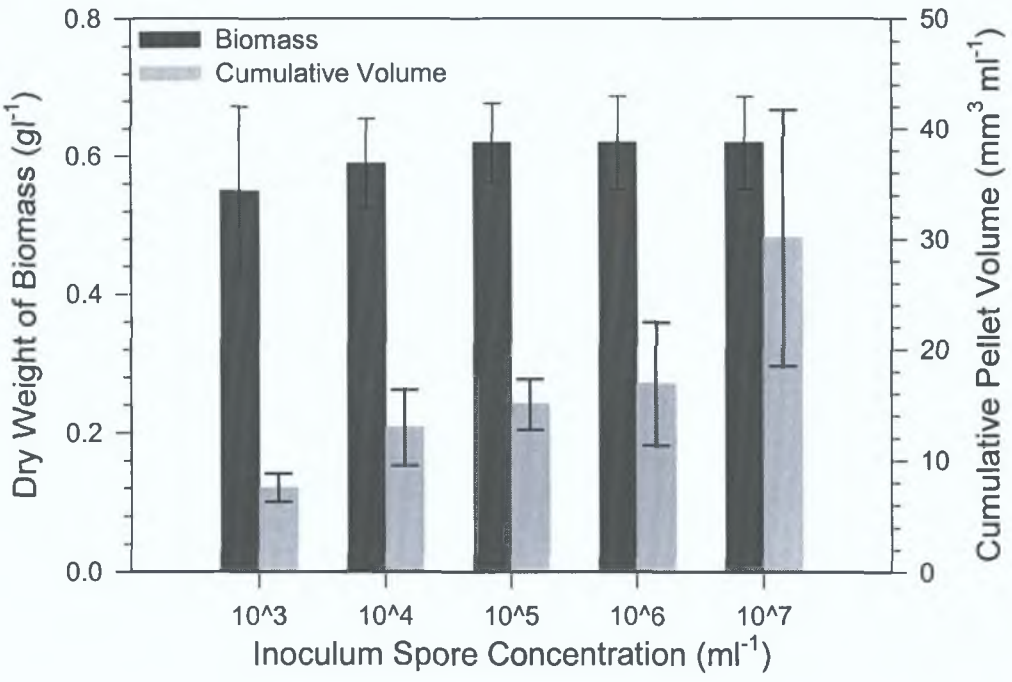


Figure 4.9. Influence of inoculum CFU loading density on dry weight of biomass and cumulative pellet volume at day 7. Error bars represent the +/- standard deviation of the mean obtained from a set of five flasks.

The experimental observations are broadly in line with those of Vechtlifshitz *et. al.* (1990) on growth of *S. tendae* under similar operating conditions, where the average pellet size seemed to be inversely proportional to the inoculum concentration. The morphological profile of the cultures varied from a uni-modal population of large pellets (1.5 - 2.5 mm diameter) at low inoculum concentrations, through bi-modal distributions of both large and small pellets (0.4 - 0.7 mm diameter) at intermediate inoculum concentrations to uni-modal distributions of small particles at high inoculum concentrations.

Inoculum spore loading density heavily influences culture morphology in shake flask cultures. In the work reported here cultures inoculated with different concentration spore suspensions produced comparable levels of biomass yet significantly different morphological profiles indicating that the loading density has no significant impact on biomass production by day 7. Given that the morphological distributions and pellet to CFU ratios vary with respect to inoculum concentration it is proposed that different degrees of

particle aggregation are occurring in each of the cultures. Furthermore, given that aggregation rates vary with respect to the inoculum concentration, as does the pellet density it is proposed that the overall mechanism of pellet formation varies with increasing aggregation as a result of increased inoculum concentration.

4.4. AGITATION SPEED

The cultivation of filamentous microorganisms in submerged fermentations relies heavily on sufficient agitation to promote mixing within the system. Adequate mixing is a prerequisite in such fermentation processes in order to achieve uniform distribution of temperature and concentration of nutrients and oxygen, within the vessel. The provision of these services is essential to the growth of the organism. Furthermore, agitation is thought to play a vital role in the morphological development of filamentous fermentations by regulating particle-to-particle collision and aggregation. The collision and aggregation of spores and/or hyphae is generally thought to depend on cell surface properties, as in the case of cellular hydrophobicity, for *S. tendae* (Vechtlifshitz *et al.*, 1990). Both the incidence of collision and the energy that particles possess when they collide are a function of agitation. Not only is agitation thought to be responsible for aggregation but also conversely for hyphal fragmentation. Disruption can occur as a result of shear forces on the particle by the fluid, by collision with another particle, or by collision with process equipment. Several researchers have noted the agitation dependence of pellet formation and development with increasing agitation typically resulting in decreasing particle size (Amanullah *et al.*, 2001; Casas Lopez *et al.*, 2005; Cui *et al.*, 1997; Hotop *et al.*, 1993). However, no information is currently available as to the influence of varying agitation speed on the morphological and physiological profiles of *S. hygroscopicus* cultures.

The experimental procedure performed involved culturing 100 ml of Bennett's nutrient medium in a 250 ml Erlenmeyer flasks with 1% (v/v) of 2 x 10^6 spores ml^{-1} *S. hygroscopicus* spore suspension and incubating on an orbital

shaker at 110, 150 and 200 rpm and 28°C. The orbital shaker agitation speeds of 110, 150 and 200 rpm correspond to estimated in-flask shear rates of 18, 28 and 39 s^{-1} respectively, as reported by Fujita *et al.* (1994). The authors calculated the shear rate experienced by 100 ml of nutrient media in a 300 ml shake flask rotating on the orbital shaker based on the power required for rotational shaking and the volume of liquid in the flask. The duration of the experimental procedure was 7 days, with sampling for image analysis purposes occurring on the third, fifth and seventh day and sampling for dry weight of biomass determination occurring on the seventh day. A single set of five flasks was prepared for each agitation speed and was used throughout the experimental time period for the aforementioned sampling. All data shown is based on that obtained from the set of five replicate flasks.

Figure 4.10 shows the day 7 volume fraction and particle count distributions for cultures agitated at 110, 150 and 200 rpm and an image of the corresponding stained samples. This figure illustrates the different morphological profiles of cultures agitated at varying shaker table rotational speed. Variations in agitation speed will alter fluid turbulence within the fermentation broth, which in turn is thought to alter the rate of spore and/or hyphal aggregation. The aggregation of Silicone Carbide (SiC) particles was studied in a water-based system by Johansen and Taniguchi (1998), where a theoretical model was derived to describe the combined processes of aggregation and break-up and applied to the metallurgical system of SiC particles in liquid aluminium. The authors noted that the equilibrium particle size, regulated by the antagonistic processes of aggregation and fragmentation, increased with a decrease in the agitation in a stirred tank reactor. Therefore, increased agitation resulted in smaller particles. As mentioned in Chapter 1 the kinetics of particle aggregation often depend on the existence of an energy barrier between colliding particles; if the collision energy is not sufficient then the particles will not aggregate. Collision energy is primarily a function of fluid turbulence, which is, in turn, influenced by fluid mixing (Lu *et al.*, 1998). Several methods of aggregate fragmentation have been proposed including fluctuating pressure difference occurring at opposite sides of the aggregate, aggregate-aggregate collision and the collision of

aggregates with the vessel walls or equipment. Lu *et al.* (1998) also note that the maximum aggregate size is correlated with the intensity of the turbulence field and consequently the Kolmogorov microscale length, which is, as already mentioned, a function of fluid mixing.

The Kolmogorov microscale, one of the three standard turbulence length scales, characterises the smallest dissipitation-scale eddies. At the smallest scales, the rate at which energy is supplied must equal the rate at which it is dissipated. The Kolmogorov microscale for a shake flask system similar to the one under investigation was experimentally determined by Kaku et al. (2003) to be approximately 300 µm. For particles in the size range below this point there is a greater probability of disaggregation and disruption, whereas for particles above 300 µm in diameter this probability is reduced. The Kolmogorov length, η, is a function of the energy dissipation rate, ε and the kinematic viscosity, υ.

$$\eta = \left(\frac{\upsilon^3}{\varepsilon}\right)^{1/4} \tag{4.1}$$

where the energy dissipation rate, ε, is in turn a function of constant, A, the mean turbulent velocity, u and Eulerian integral time scale, τ_E.

$$\varepsilon = A\left(\frac{u^2}{\tau_E}\right) \tag{4.2}$$

Therefore, an increase in the mean turbulent velocity, u, through an increase in the shake flask agitation speed, will increase the energy dissipation rate, ε and in turn decrease the Kolmogorov microscale length (Doran, 1995; Kaku *et al.*, 2003). Consequently, an increase in the agitation speed of the shaker table will decrease the scale of the smallest turbulent eddies and therefore decrease the size of aggregates formed in the system (Lu *et al.*, 1998).

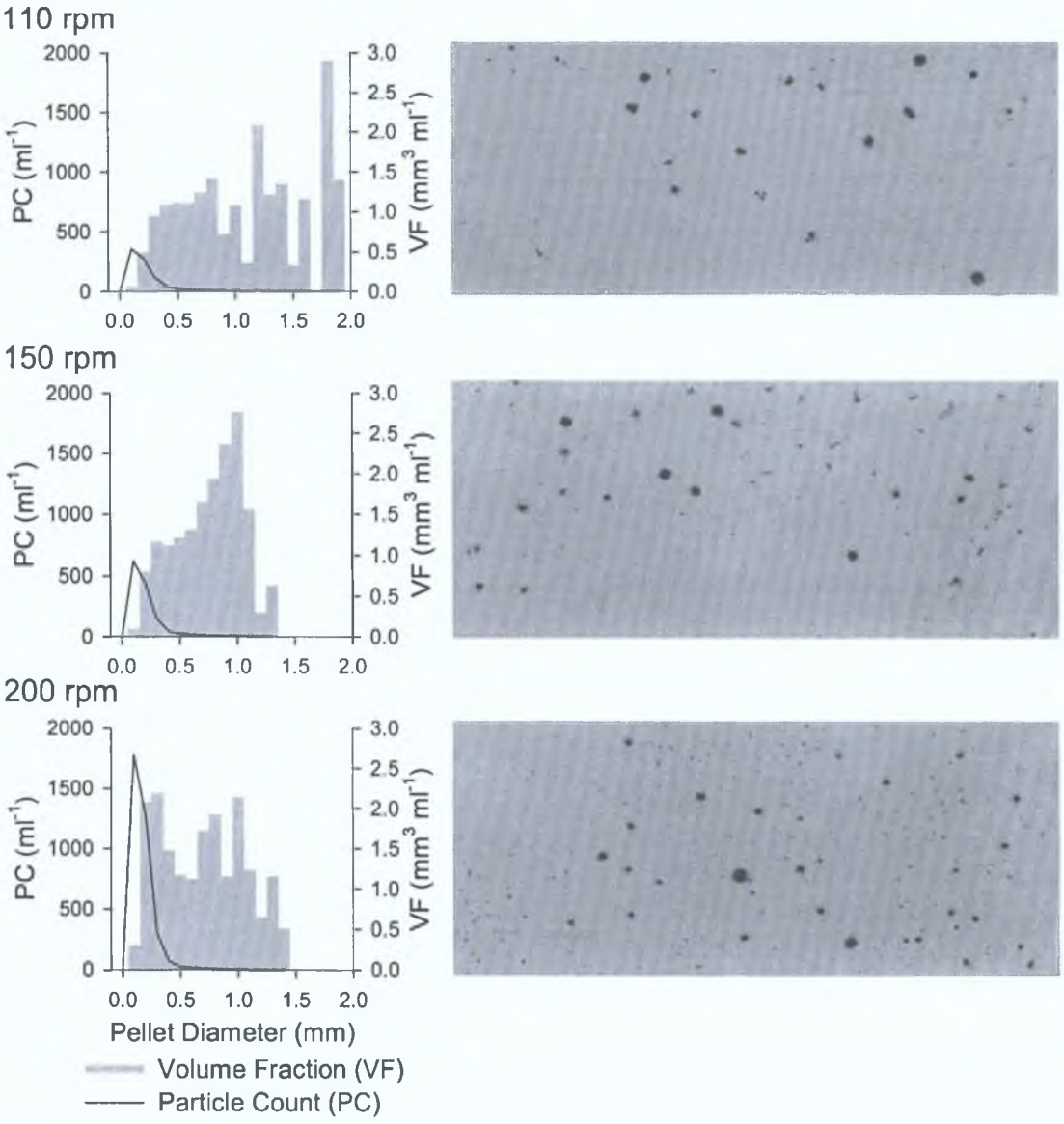


Figure 4.10. Morphological distributions and stained microbial biomass for 110, 150 and 200 rpm (1 in 10 Dilution) day 7 fermentation samples.

As illustrated in Figure 4.11 (c), by day 3, three and a half-fold more spores are aggregating to form one pellet in the 110 rpm than the 200 rpm cultures. The increased particle aggregation in the 110 rpm compared to the 200 rpm cultures facilitated the generation of a smaller population of pellets, 597 as opposed to 2083 ml^{-1} as shown in Figure 4.11 (a), forming more quickly, producing a slightly larger cumulative pellet volume of 7.16 in comparison to 6.62 mm^3 ml^{-1} and yielding a broader morphological distribution of 1.1 as opposed to 0.8 mm by 72 hours, as illustrated in Figure 4.12. Since particle

aggregation occurred quite extensively in the 110 rpm cultures in the first 72 hours (approximately 33 CFUs aggregate to form one pellet as shown by Figure 4.11 (c)) it is reasonable to assume that sufficient collision energy is present in the system. As the agitation speed is increased so does particle collision, which in turn is thought to affect the disaggregation of temporary aggregates and fragmentation, both of which are thought to be responsible for an increased population of smaller particles (Lu *et al.*, 1998). For example, 597 pellets ml^{-1} in with a volume fraction modal point of 1.1 mm in the 110 rpm cultures as opposed to 2083 pellets ml^{-1} with a volume fraction modal point of 0.8 mm in the 200 rpm cultures at day 3, as illustrated in Figures 4.11 (a) and 4.12. Therefore it would appear that as the agitation speed increases fragmentation and disaggregation are exceeding aggregation, which is evident in the disparate morphological behaviour of the 110 and the 150 with respect to the 200 rpm cultures in Figures 4.11 and 4.12. The morphological profile of the cultures is thought to be dependent on the rate of particle aggregation and fragmentation, both of which are regulated by shake flask agitation speed (Johansen & Taniguchi, 1998; Lu *et al.*, 1998).

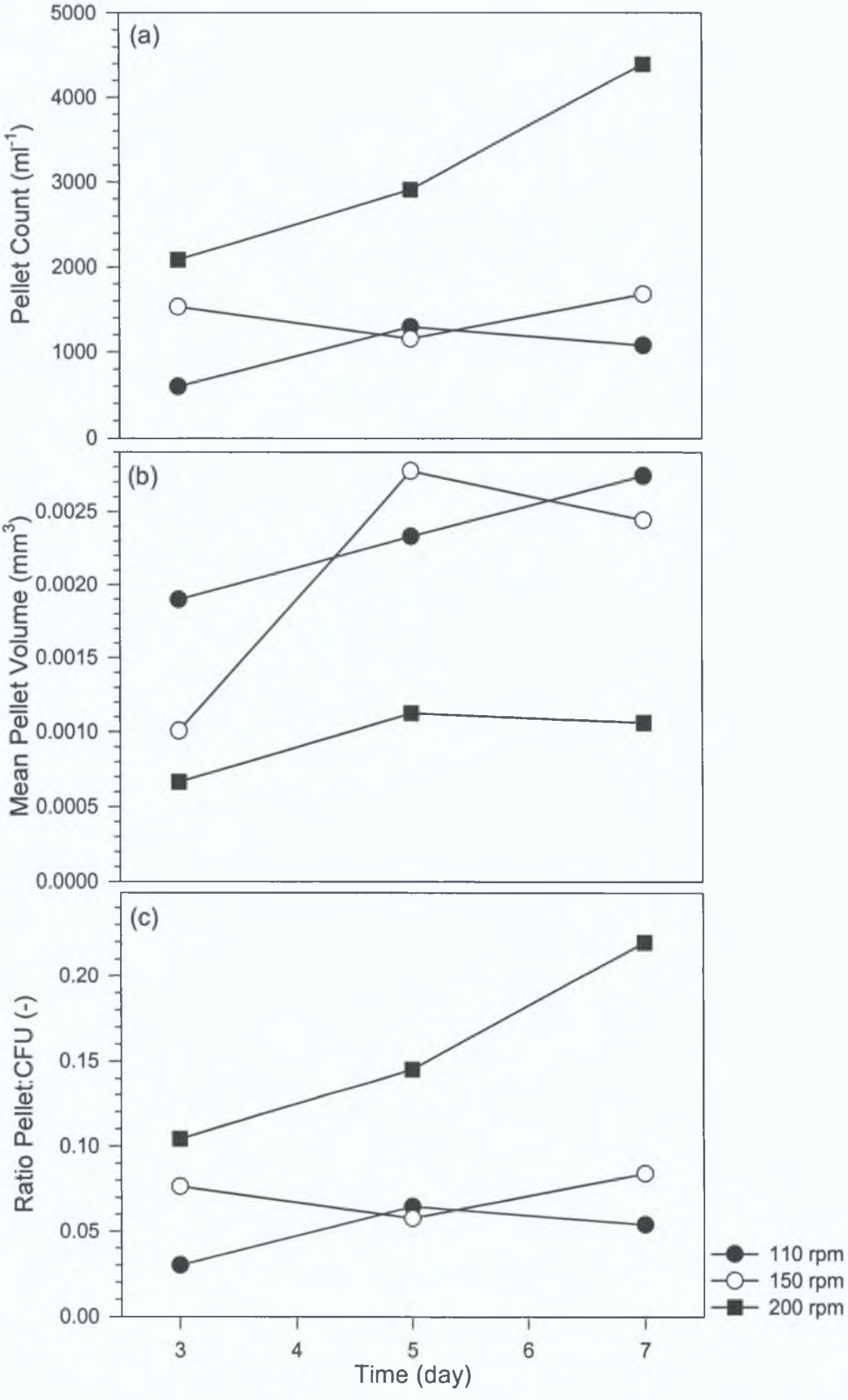


Figure 4.11. Influence of shake flask agitation on (a) pellet count, (b) mean pellet volume and (c) pellet to CFU ratio at days 3, 5 and 7.

The impact of shake flask agitation on the morphological profile of the cultures after the first 72 hours is illustrated in Figure 4.12. Each of the systems contains a comparable cumulative pellet volume yet the morphological profile differs. Increasing agitation results in a tighter distribution with the majority of the cumulative pellet volume, almost 80% for both the 150 and 200 rpm cultures compared to only 58% for the 110 rpm cultures, residing in the small to medium size range (0 - 1 mm diameter).

The subsequent development of the 110 rpm cultures involves enlargement as a single population with an increasing cumulative pellet volume, 7.16 to 20.04 mm^3 ml^{-1}, and a broadening morphological distribution. This coupled with an increasing pellet count, 597 to 1293 pellets ml^{-1}, and then decreasing, 1293 to 1077 pellets ml^{-1}, and an increasing mean pellet volume, 0.0019 to 0.0027 mm^3, indicates that at low agitation speeds aggregation is occurring to a greater extent than fragmentation. The subsequent development of the 150 rpm cultures involves an increasing cumulative pellet volume, 6.51 to 16.89 mm^3 ml^{-1}, and an initially broadening (day 3 - 5) but then narrowing (day 5 - 7) morphological distribution. The initial decrease, 1529 to 1153 pellets ml^{-1}, and subsequent increase, 1153 to 1681 pellets ml^{-1}, coupled with the advent of a greater proportion of smaller particles in the volume fraction distribution indicates that a greater degree of fragmentation is occurring later in the cultures. In the case of the 200 rpm cultures the subsequent development involves an increasing cumulative pellet volume, from 6.62 to 18.27 mm^3 ml^{-1}, and an initially broadening but then narrowing morphological distribution containing an increased proportion of smaller particles. Furthermore, the increasing pellet count, 2083 to 4394 pellets ml^{-1}, and the advent of a greater proportion of smaller particles in the volume fraction distribution indicates that fragmentation is occurring to a greater degree in these cultures than in either the 110 or the 150 rpm cultures.

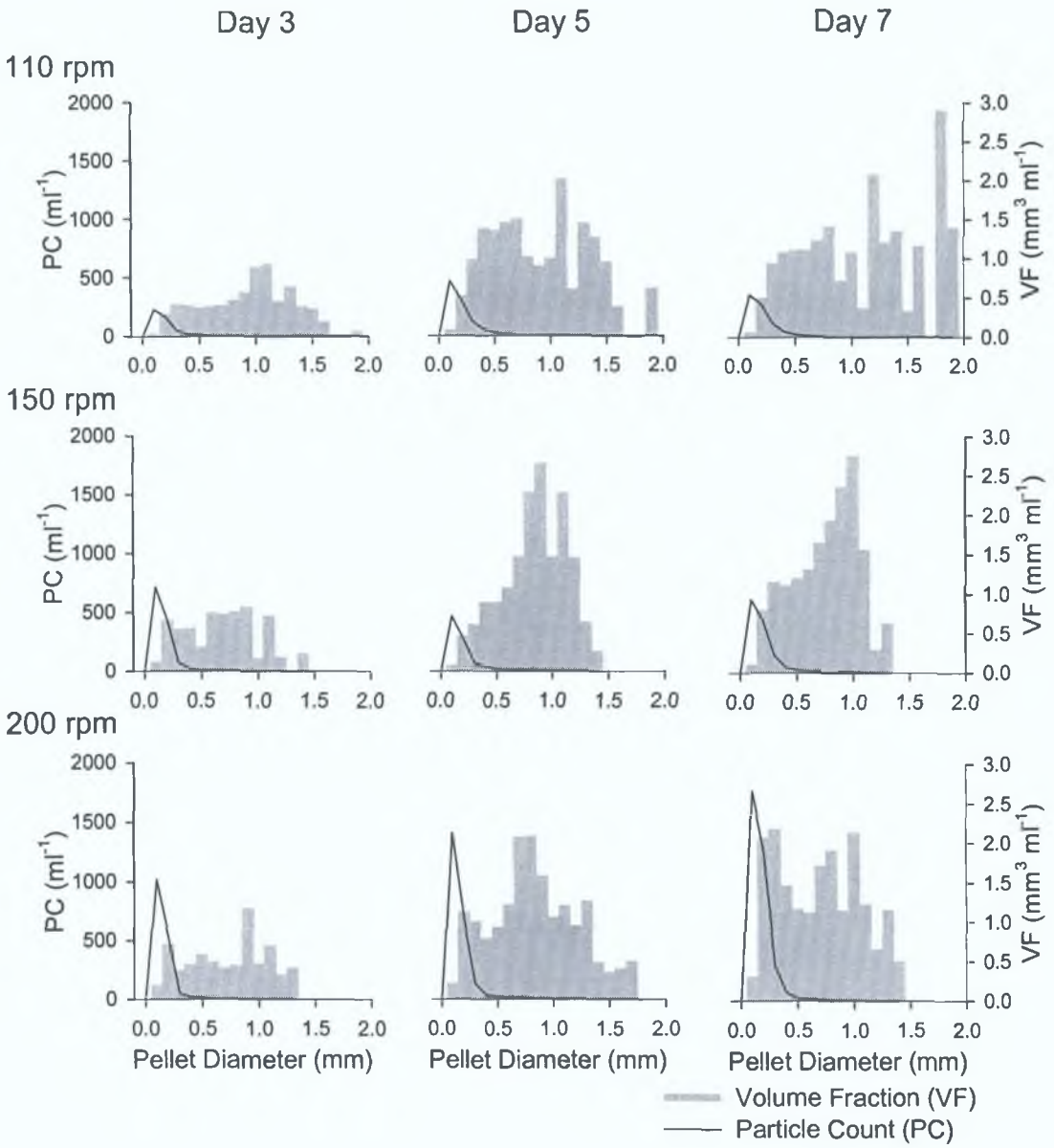


Figure 4.12. Volume fraction and particle count distributions for shake flask cultures agitated at varying speeds after 3, 5 and 7 days.

The morphological distributions as illustrated in Figure 4.12 indicate that shake flask agitation impacts on the pellet population distribution and results in cultures of varying morphology. Increasing agitation speed controls particle aggregation and fragmentation, thereby regulating pellet formation, development and disruption. An increase in the agitation speed is thought to increase the rate of fragmentation. Indeed as indicated in Figure 4.13, comparable biomass levels are produced in each of the cultures, yet it is evident that there is a significant impact on the morphological development of

the culture given the narrow experimental agitation range. A comparison of the biomass concentrations with the corresponding cumulative pellet volumes indicates that there is a slight, but not significant, difference in density. However, it is worth noting the decreasing ratio of biomass concentration to cumulative pellet volume with respect to increasing agitation, 1:29, 1:27 and 1:25 at 110, 150 and 200 rpm respectively, suggests that higher agitation speeds may result in denser pellets.

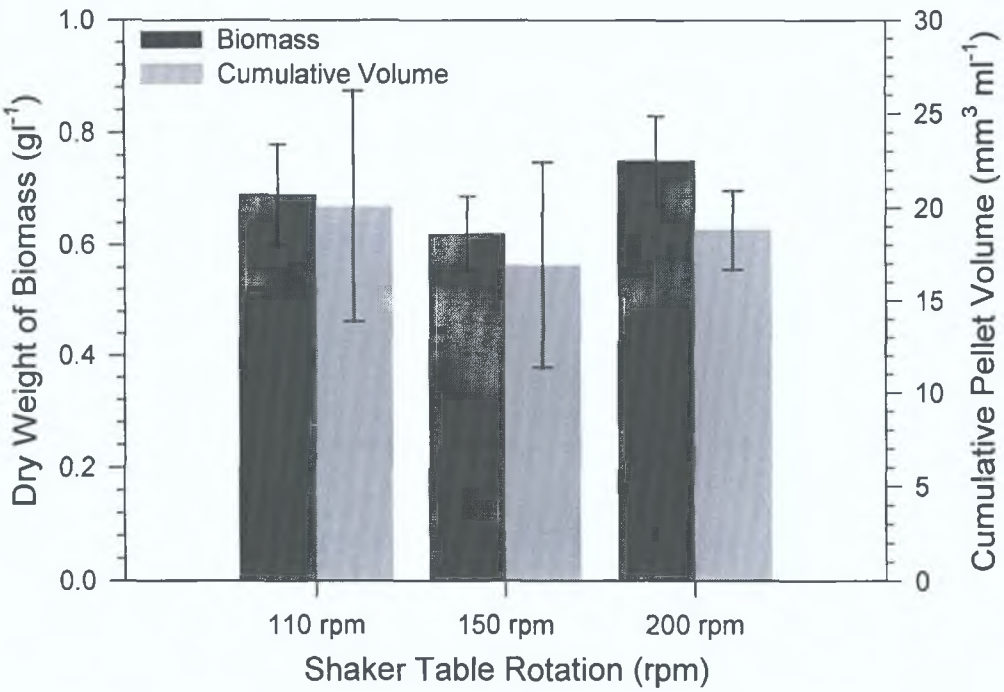


Figure 4.13. Influence of shake flask agitation on *S. hygroscopicus* dry weight of biomass concentrations and cumulative pellet volume at day 7. Error bars represent the +/- standard deviation of the mean obtained from a set of five flasks.

The influence of agitation on the development of *A. oryzae* cultures was demonstrated by Amanullah *et al.* (2001). A step decrease in agitation speed within a 5.3 L bioreactor from 1000 to 550 rpm resulted in an increase in pellet size as a result of aggregation. In the case of fragmentation, Tamura *et al.* (1997) working with *S. fradiae* in a 3 L jar fermenter observed that increasing agitation rates altered culture morphology. At agitation speeds below 400 rpm, pelleted growth predominated but increasing agitation above

this point resulted in the formation of a greater portion of dispersed and entangled filaments. Casas Lopez et al. (2005) working with *A. terreus* noted that although increased agitation speed, in the range of 300 to 800 rpm in a 5 L bioreactor, reduced pellet size it did not affect biomass production profiles. The experimental results suggest that pellet formation and subsequent development in *S. hygroscopicus* cultures is a combination of the competing forces of aggregation and fragmentation. The shear rate experienced by the organism in the shake flasks rotating at 110, 150 and 200 rpm was between approximately 19 and 39 s^{-1}, in order to assess the morphological impact of a wider range of shear rates it would be necessary to culture the organism in a bioreactor.

4.5. MORPHOLOGICAL AND PHYSIOLOGICAL CHARACTERISTICS OF *S. HYGROSCOPICUS* FERMENTATION CULTURES

Previous sections focused on the impact of bioprocessing parameters such as broth composition, spore loading density and shake flask agitation speed on the morphology and physiology of the organism. Arising out of this investigative work was a need to design a defined set of operating conditions under which the organism was to be substantially studied. Of the bioprocess conditions examined, Bennett's nutrient broth, 10^6 spore ml^{-1} inoculum and 150 rpm agitation speed provided sufficient biomass growth and development to generate a wide range of morphological behaviour through which the mechanisms of pellet formation and its impact on culture morphology were studied. This model system was to form the basis for all subsequent work.

An in-depth study of the lifecycle of the organism that involved culturing 100 ml of Bennett's nutrient medium in a 250 ml Erlenmeyer flasks with 1% (v/v) of 2 x 10^6 spores ml^{-1} *S. hygroscopicus* spore suspension and incubating on an orbital shaker at 150 rpm and 28°C was performed. The duration of the experimental procedure was 21 days, with sampling for image analysis

purposes and the determination of dry weight of biomass and glucose concentration occurring on day 3, 4, 5, 6, 7, 8, 9, 10, 14 and 21. A single set of five flasks was prepared for each time point for the aforementioned sampling. All data shown is based on that obtained from sets of five replicate flasks.

Bennett's nutrient media was inoculated with 2 x 10^6 CFU ml^{-1} inoculum giving an in-flask concentration of 2 x 10^4 CFU ml^{-1} which, by day 3, have formed approximately 2 x 10^3 pellets ml^{-1} as shown in Figure 4.14 (a). In the first 72 hours after inoculation, the growing hyphal elements are thought to aggregate randomly to form pellets, resulting in a mixed population containing a wide distribution of particle sizes as illustrated in Figure 4.15. As illustrated in Figures 4.14 (a) and (b), 4.15 and 4.16 (a), between day 3 and 8, the pellet count fluctuates between approximately 800 and 2000 ml^{-1} while the mean pellet volume increases from 0.0013 to 0.0126 mm^3, biomass increases from 0.38 to 01.05 gl^{-1} and the modal point of the size distribution increase from 0.3 to 1.2 mm indicating that the biomass population is collectively enlarging as a single phase through both hyphal growth and aggregation. Smaller particles arising out of late-germinating spores and shear fragments are aggregating with bigger pellets. It appears that there is a pellet diameter size limit of ~ 2.0 mm, which is probably a function of shear with particle collision resulting in fragmentation beyond this point. Other researchers have observed the upper size limits of Streptomycetes to be in the same range, most notably Vechtlifshitz *et al.* (1992) working with *S. tendae* (~2.0 mm) and Tamura *et al.* (1997) working with *S. fradiae* (~1.7 mm).

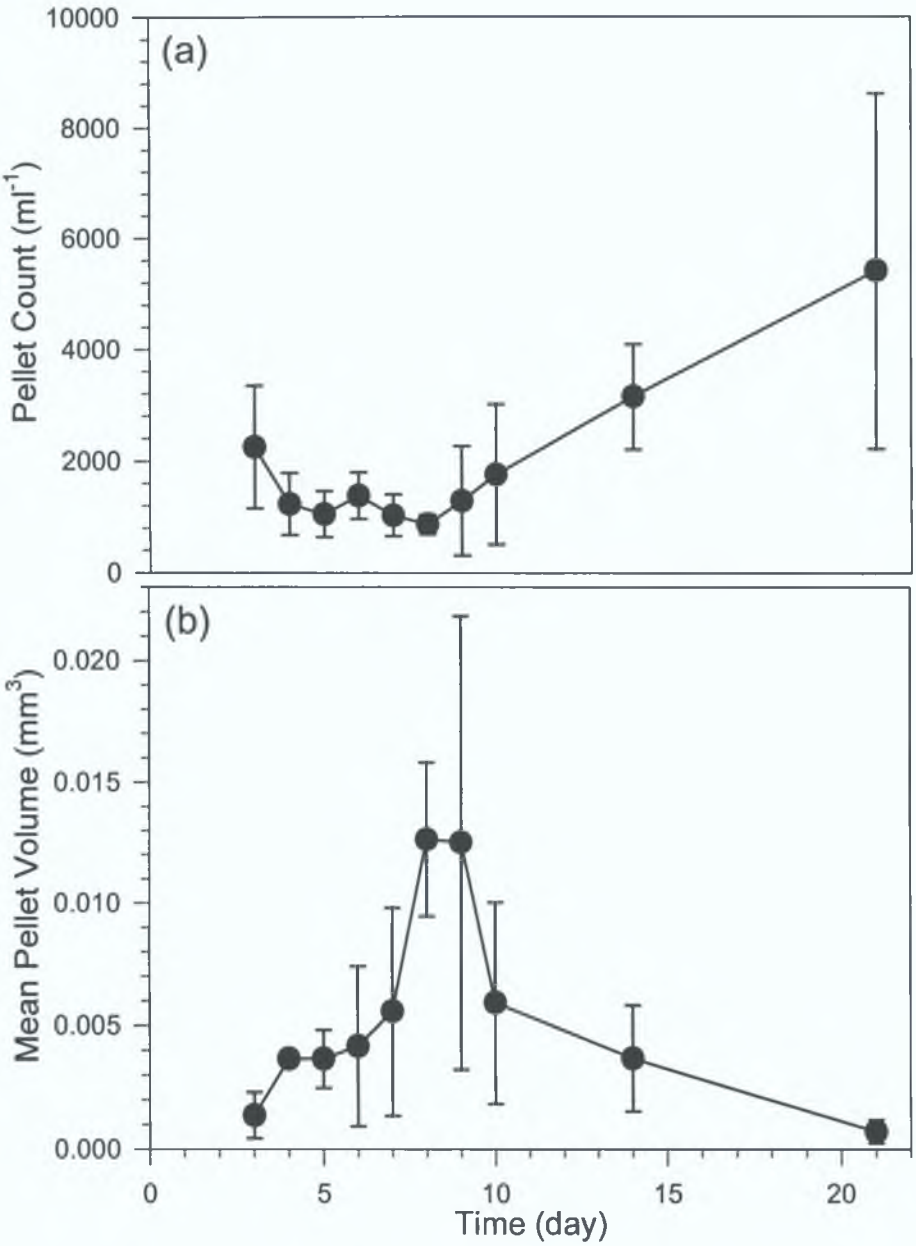


Figure 4.14. The development of *S. hygroscopicus* morphological parameters (a) pellet count and (b) mean pellet volume in shake flask culture with respect to time. Error bars represent the +/- standard deviation of the mean obtained from a set of five flasks.

Figure 4.14 (a) and (b) shows that between days 8 and 14 the pellet count increases from 867 to 3152 ml^{-1}, the mean pellet volume decreases from 0.0126 to 0.0036 mm^3 and Figure 4.16 shows that biomass continues to increase from 1.05 to 1.95 gl^{-1} indicating that the influence of particle aggregation on the morphological development of the organism is lessening.

The size distribution profiles, shown in Figure 4.15, indicate that a shift occurs from a single population of medium to large particles (0.75 - 1.5 mm diameter) to a mixed one containing a higher proportion of small particles (0.25 - 0.75 mm diameter). The shear forces experienced by the large particles, although relatively low in shake flask systems, have undoubtedly played a part in the disruption of these pellets. As illustrated in Figure 4.15, by day 10, 70% of the volume fraction is in the 1.0 – 2.0 mm range and as particles enlarge both the probability of collision and the energy that the will have should they collide increases.

Between days 14 and 21 biomass death begins to occur as a result of nutrient deprivation, as shown in Figure 4.16 (a). Cultures cannot survive without the adequate nutrients and as is illustrated in Figure 4.15 the volume fraction declines rapidly as a result of pellet core death bringing about fragmentation. This process of disruption and fragmentation results in a pellet count increase and mean pellet volume decrease, as shown in Figures 4.14 (a) and (b) respectively. The potential for core death increases as pellet size increases due to difficulties with inward transport of nutrients and outward transport of toxic compounds. A loss of structural stability as a result of the formation of a hollow core increases the susceptibility of the pellet to disruption by mechanical forces (Nielsen *et al.*, 1995; Paul *et al.*, 1994). So a combination of a greater probability of collision, increased collision energy and loss of structural integrity is thought to result in pellet disruption.

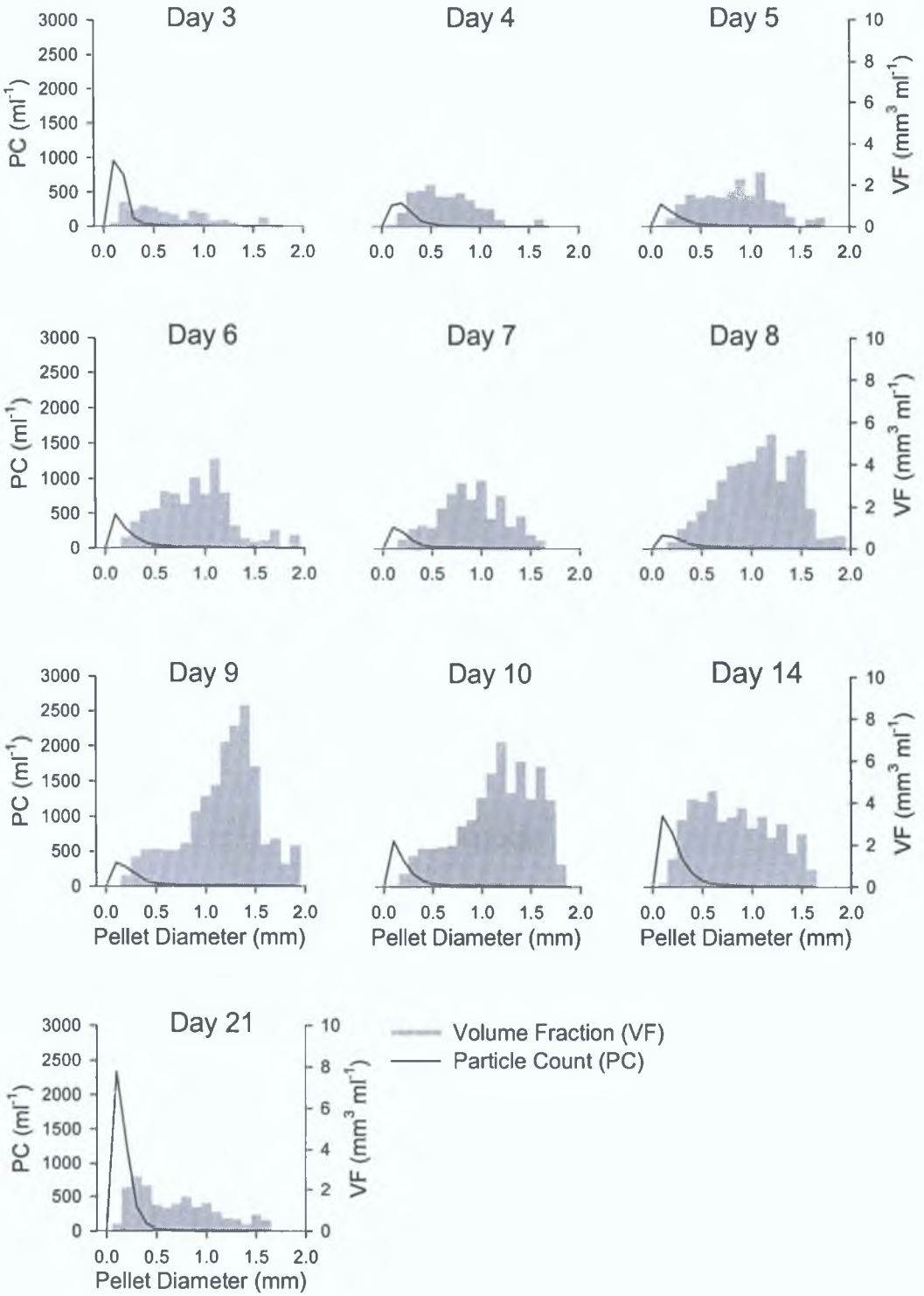


Figure 4.15. Volume fraction and particle count distributions for *S. hygroscopicus* in shake flask over 21 days.

The growth of *S. hygroscopicus* in Bennett's broth under the defined conditions is believed to be diauxic. The first phase of biomass growth (0.0 - 0.84 gl^{-1}), day 0 - 6, as illustrated in Figure 4.16 (a), appears to be achieved

using minimal amounts of glucose (0.1 gl^{-1}). This is believed to be as a result of the preferential utilisation of complex substrates present in broth constituents such as the yeast and beef extracts and the n-z amine a. In this initial phase the organism appears to be actively concentrating on reproduction. Between days 4 and 6 it appears that the biomass begins to run out of the preferentially utilised substrates and switches metabolic pathways in order to use the abundant glucose present in the system. From day 7 onwards the biomass actively utilises glucose, probably as its sole carbon source. As illustrated in Figure 4.16 (a) biomass growth of 1.05 gl^{-1} and glucose consumption of 7.96 gl^{-1} from day 7 – 14 are directly proportional. This glucose utilisation and concomitant increase in biomass coincides with a significant increase in the cumulative pellet volume from 24.2 mm^3 ml^{-1} at day 7 to 59.6 mm^3 ml^{-1} at day 10 and an increase in the modal point of the size distribution from approximately 0.9 mm at day 7 to 1.3 mm at day 10, as illustrated in Figure 4.16 (b) and 4.15 respectively. From day 10 – 14 the larger particles begin to disintegrate, as shown by the decline in the cumulative pellet volume from 59.6 mm^3 ml^{-1} to 44.2 mm^3 ml^{-1} and the shift of the modal point of the volume fraction distribution from 1.3 mm to 0.7 mm, as illustrated in Figures 4.16 (b) and 4.15 respectively. By day 14 the organism has utilised most of the glucose in solution, with only 0.047 gl^{-1} remaining, and consequently the biomass reaches a maximum of 1.95 gl^{-1} and declines thereafter as a result of nutrient deprivation. A decrease in biomass to 1.58 gl^{-1} and cumulative pellet volume to 18.6 mm^3 ml^{-1} at day 21 coupled with an increase in the pellet count to 5420 ml^{-1} and a decrease in the mean pellet volume to 0.0007 mm^3 suggests that the pellets are fragmenting and disrupting due to nutrient limitation.

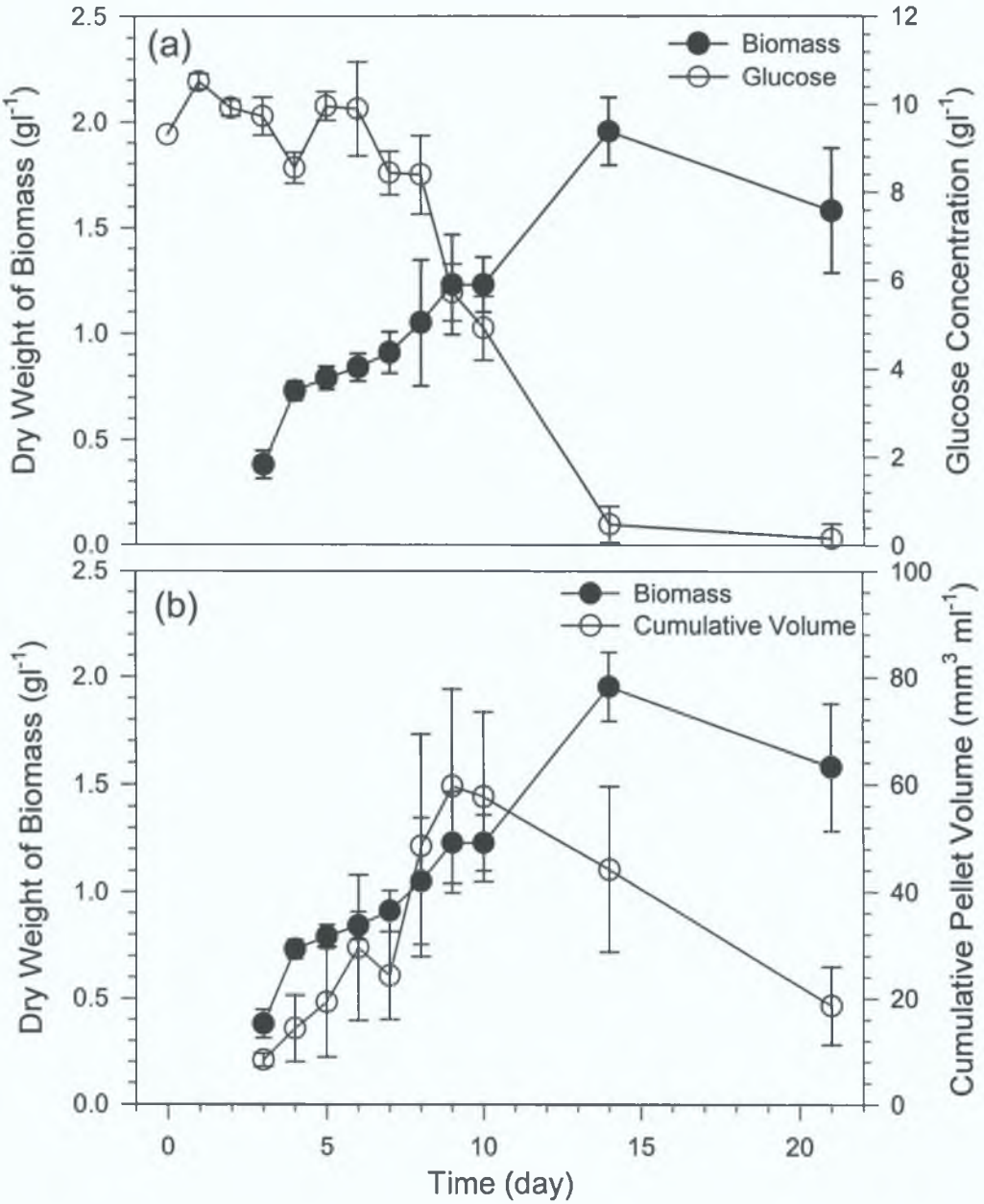


Figure 4.16. The impact of *S. hygroscopicus* development on (a) biomass production and glucose consumption profiles and (b) comparative analysis of the dry weight of biomass and cumulative pellet volume in Bennett's nutrient medium with respect to time. Error bars represent the +/- standard deviation of the mean obtained from a set of five flasks.

4.6. CONCLUSIONS

The lifecycle of a filamentous microorganism is governed by the environmental and nutritional conditions it experiences. In the case of

submerged fermentations in shake flasks, bioprocessing parameters such as media composition, inoculum concentration and shake flask agitation speed influence the morphological and physiological development of these organisms. Current knowledge with respect to the influence of these bioprocessing parameters on filamentous microorganisms suggests the following: The nutritional composition of the fermentation broth has been demonstrated to influence cell growth and hence culture morphology, with different media eliciting different responses from the organism (Choi *et al.*, 1998; Schrader & Blevins, 2001; Sinha *et al.*, 2001); Inoculum concentration has been shown to influence culture morphology whereby increasing the spore loading density increases the pellet count and decreases the pellet size (Kim & Hancock, 2000; Papagianni & Moo-Young, 2002; Vechtlifshitz *et al.*, 1990); Increasing the agitation speed of the shake flasks has been shown to increase pellet count and decrease pellet size (Amanullah *et al.*, 2001; Casas Lopez *et al.*, 2005; Cui *et al.*, 1997), indicating the potential for an analogous mode of action for both inoculum concentration and shake flask agitation.

As outlined in Table 4.1, an investigation into the influence of different nutrient media on the growth and morphological development of *S. hygroscopicus* cultures suffered experimental difficulties because, of the six media tested, growth did not occur in one, determination of dry weight of biomass was not possible in another two and image analysis was not feasible in another one. Therefore, leaving only two nutrient media, Bennett's and YEPD, suitable for further investigation. Bennett's liquid media is nutrient poor and consequently was not capable of sustaining good growth, it did however yield a highly variable mixed morphological population distribution, where typically the majority of the biomass was contained in a small number of large particles, as illustrated in Figures 4.3, 4.4 and 4.5. YEPD on the other hand is nutrient rich and thus yielded high biomass levels and a culture consisting predominantly of a large number of small to mid-sized particles, resulting in a normally distributed population within a narrow size range, as illustrated in Figures 4.3, 4.4 and 4.5.

Vechtlifshitz *et al.* (1989; 1990) proposed that the primary forces inducing cellular aggregation in cultures of *S. tendae* are the hydrophobic interactions of the vegetative cell walls. The authors reported that increased cellular hydrophobicity, which was measured by determining the contact angle of biomass, resulted in increased cellular aggregation. Similarly it is proposed that *S. hygroscopicus* biomass is hydrophobic and as a result, individual spores or hyphal elements tend to form hydrophobic interactions. For example, the organism may produce cell surface molecules responsible for aggregation, such as the hydrophobic proteins DewA and RodA produced by *A. nidulans* (Dynesen & Nielsen, 2003). Although the underlying principle of *S. hygroscopicus* pellet formation in Bennett's and YEPD is the same, the resulting morphological distributions in both cultures are dissimilar. This is most likely as a result of an altered cell physiology, due to differences in the type and concentrations of nutrients present. The Bennett's cultures exhibit greater population diversity and provide a greater platform for the further study of the kinetics of particle aggregation and subsequent pellet formation.

The inoculum concentration has been shown to influence pellet formation. An increase in the spore loading density increased the CFU to pellet ratio, as illustrated in Figure 4.7 (c), indicating an increased incidence of particle collision and hence a greater degree of aggregation. This increased incidence of aggregation, in turn, leads to faster pellet formation, as illustrated in Figure 4.8. Increased spore concentration appears to confer a competitive advantage for particle development in the early stage of fermentation, as demonstrated in Figure 4.8 by quicker particle breakthrough and the generation of a wider size distribution. The first 72 hours was shown to be the most important in the morphological development of these cultures. The establishment of a particular morphological profile at this point influences all subsequent morphological development. Increasing the inoculum spore concentration results in a larger population of smaller particles.

After 72 hours there are differences in the size distribution profiles of the resulting cultures, as illustrated in Figure 4.8, yet by day 7 there are comparable levels of biomass in all the cultures. As shown in Figure 4.9,

cultures inoculated with 2000 spores ml^{-1} produced similar levels of biomass to those inoculated with 20000000 spores ml^{-1}, which indicates that the organism is capable of producing comparable levels of biomass regardless of the initial spore loading density. Although the biomass levels are comparable at this point, the morphological distribution profiles are significantly different, especially with respect to the cumulative pellet volumes, the comparison of which in Figure 4.9 highlights an interesting result. Pellets produced in the lower spore inoculum cultures have a higher density. Inoculation of Bennett's nutrient broth with varying concentration spore inocula enabled the generation of cultures containing pellet populations with varying size distribution profiles and internal density. Similarly, Vechtlifshitz *et al.* (1990) noted that in *S. tendae* culture an inverse relationship existed between inoculum concentration and pellet size whereby increasing the order of magnitude of spores in the system produced smaller pellets. The morphological profile of the culture varied from free filamentous growth at 10^8 spores ml^{-1}, through a mixed pellet population at 10^5 spores ml^{-1} to a few large pellets at 10^2 spores ml^{-1}.

The alteration of both the spore loading density and the shake flask agitation speed are acting on the same mechanism of pellet formation. Particle collision must occur for interaction, aggregation and pellet formation to take place. The difference between the two systems is that where the spore loading density increased the number of particles and in so doing increased the probability and/or incidence of collision, the alteration of the agitation speed also increases the energy that each particle possesses. The experimental procedure demonstrated in Figure 4.11 that increased agitation resulted in a reduced CFU to pellet ratio indicating that aggregation decreases as a result of increased collision frequency and energy. The alteration of shake flask agitation speed, albeit within a narrow range, influences culture morphology with increasing agitation resulting in cultures containing a greater proportion of smaller particles by day 3, as shown in Figure 4.12.

Agitation plays a significant role in the subsequent development of these cultures from day 3 – 7 with both pellet formation and disruption closely linked to particle size and agitation speed. Pellet development through aggregation is dependent on the size of particles, the incidence and the energy of collision, whereas development through mycelial growth is dependent on the incidence and energy of collision and the potential for hyphal breakage. Pellet disruption normally occurs through hyphal fragmentation and/or pellet disintegration. The increased agitation speed increases the frequency of particles colliding and the energy that they possess when they do (Lu *et al.*, 1998), thus increasing the potential for disaggregation of temporary aggregates and fragmentation. Furthermore, an increase in the agitation speed of the shaker table will increase the energy dissipation rate and consequently decrease the scale of the smallest turbulent eddies and therefore decrease the size of aggregates formed in the system (Kaku *et al.*, 2003; Lu *et al.*, 1998). Hence, by day 7, increased agitation speed results in cultures containing a greater proportion of smaller particles. Both Nielsen & Krabben (1995) and Paul *et al.* (1994) demonstrated that the incidence of hyphal fragmentation in submerged fermentations of *P. chrysogenum* was a function of the agitation speed in the vessel, with Paul *et al.* (1994) also noting that physiological effects such as increased vacuolization, in conjunction with agitation further enhanced fragmentation.

The influence of medium composition, inoculum concentration and shake flask agitation speed on pellet formation kinetics in cultures of *S. hygroscopicus* has been demonstrated. Each of the parameters is capable of regulating the developmental cycle of the microorganism to yield vastly different size distribution profiles. The influence of these bioprocessing parameters on culture development demonstrates the potential application of this approach to the morphological and physiological engineering of *S. hygroscopicus* cultures.

Although each of the three bioprocessing parameters was varied individually to examine the impact on culture development a set of standard operating

conditions was designated for further study. These conditions were chosen primarily as they were the most interesting and would potentially provide the greatest insight into the mechanics of pellet formation within these cultures. This involved culturing the microorganism in Bennett's medium inoculated with 2 x 10^6 spores ml^{-1} and agitated at 150 rpm for an extended experimental time period in order to observe the morphological and physiological development. This investigation provides an insight into the lifecycle of the organism and is, in effect, the master control relative to which all subsequent experimental work will be assessed.

The investigation into the morphological and physiological development of *S. hygroscopicus* cultures over 21 days of culturing highlighted several issues. The first 72 hours are the most important in the morphological lifecycle of the organism; during this time the initial morphological profile is established and all subsequent development is based upon this, as illustrated in Figures 4.14 and 4.15. During the following time period (days 3 – 9) the biomass was in constant flux with pellet formation through hyphal growth aggregation and disruption through fragmentation and disintegration occurring simultaneously. Although in flux, the pellet populations within the cultures were essentially enlarging as a single phase through hyphal growth and aggregation whereby the larger particles aggregate with smaller ones. From day 10 – 14 the aggregative process declines and although the culture is still actively growing it is no longer aggregating to the same extent. By day 14, the nutrient compliment of Bennett's medium is spent and thus the organism cannot maintain the same levels of growth and so begins to die. Pellet integrity is dependent on the stability of the entire structure and with cell death comes an increased sensitivity to fragmentation and disintegration.

This investigation into environmental and nutritional factors influencing pellet formation has demonstrated the relative impacts of the nutrient medium, inoculum concentration and shake flask agitation speed on culture morphology and physiology. A set of standard operating conditions has been chosen for all subsequent experimentation. This model system provided the basis for a timecourse experiment to characterise the morphological and

physiological development of *S. hygroscopicus* cultures over a 21 day period. The impact of both inoculum concentration and agitation speed on pellet formation and development in shake flask cultures has highlighted the importance of particle collision frequency, collision energy and cell surface properties in particle aggregation and disruption. Altering fluid characteristics such as broth viscosity (Corman, 1959; Hobbs *et al.*, 1989; Hodgson, 1982) and surface tension (Jeong *et al.*, 2001; Lucatero *et al.*, 2004; Vechtlifshitz *et al.*, 1989) have been identified as potential means of regulating particle collision, interaction and subsequent aggregation, thereby influencing pellet development and will form the basis for the forthcoming work.

CHAPTER 5 – MORPHOLOGICAL ENGINEERING OF *S. HYGROSCOPICUS* THROUGH REGULATION OF FLUID ATTRIBUTES

5.1. INTRODUCTION

Filamentous organisms, both bacterial and fungal, possess the ability to grow in a range of morphological forms in submerged fermentations depending on process conditions such as shear rate (Hotop *et al.*, 1993; Tamura *et al.*, 1997), inoculum size (Vechtlifshitz *et al.*, 1990), broth rheology (Sinha *et al.*, 2001) and surface tension (Vechtlifshitz *et al.*, 1989), oxygen and nutrient availability and culture vessel design (Whitaker, 1992). Product formation in submerged fermentations of filamentous microbes is dependent not only on the level of biomass present but also the morphological profile of the culture (Braun & Vechtlifshitz, 1991; Treskatis *et al.*, 1997; Vechtlifshitz *et al.*, 1992).

The kinetics of particle aggregation often depends on the existence of an energy barrier between colliding particles; if the collision energy is not sufficient then the particles will not aggregate. Once a collision has occurred the aggregation mechanism of fine particles in turbulent systems is either; coagulation by means of compression of the electrical double layer, polymer aggregation by means of ion bridging or hydrophobic aggregation by means of interaction between particles (Lu *et al.*, 1998). Collision energy is primarily a function of fluid turbulence, which is in turn impacted on by broth rheology, the control of which enables regulation of particle interaction.

Bacterial cell walls are hydrophobic by nature and when biomass is placed in an aqueous fermentation system it tends to aggregate. When hydrophobic particles are introduced into an aqueous system, aggregation is as a result of two forces. Firstly, weak van der Waals attractive forces are responsible for particle-to-particle interaction and secondly, given the hydrophobic nature of the biomass particles, the aqueous phase withdraws to a region around the particles and forms a rigid hydrogen bonded network with itself. The

formation of groups of hydrophobic particles clustering in an aqueous solution is primarily as a result of the orientation of the aqueous solvent (Zubay, 1998).

Surface-active agents, better known as surfactants, adsorb at the surface or interface of two immiscible phases, an example being an oil/water system. Surfactants posses both polar (hydrophilic) and non-polar (hydrophobic) regions on the same molecule. The hydrophilic region of the molecule interacts with the aqueous phase and the hydrophobic region with the organic, thus forming a link between the two previously immiscible phases (Attwood & Florence, 1983). The introduction of a surfactant into a system containing hydrophobic particles in an aqueous phase results in the formation of a spherical cluster of surfactant molecules, known as a micelle, around the hydrophobic particle; thereby solubilising the once insoluble and altering the attractive forces between neighbouring hydrophobic particles (McMurray, 1996). Regulation of particle interaction using surfactants and its effect on particle size and morphology has been observed in such diverse systems as the synthesis of nanoscale alpha alumina by sol-gel processing (Sharma *et al.*, 2002) and culturing of a *Trichoderma* species (Lucatero *et al.*, 2004).

The impact of three non-ionic surfactants, Pluronic F68, Brij 58 and Triton X-100 on pellet formation in cultures of *Streptomyces tendae* was investigated by Vechtlifshitz *et al.* (1989). Both Pluronic F68 and Brij 58 in the range of 0.0001 – 0.01 % w/w brought about increased pellet formation and resulted in larger pellets with increasing surfactant concentration. A further increase in the concentration beyond 0.01 % w/w resulted in a corresponding decrease in pellet formation until it ceased completely and dispersed mycelia were obtained. It would appear from the experimental data that comparable levels of biomass were obtained throughout, yet culture morphology was altered. The inclusion of low concentrations of surfactant was shown to increase biomass hydrophobicity and hence aggregation, whereas higher concentrations decreased hydrophobicity and aggregation. Of the three surfactants used, Triton X-100, although enhancing pellet formation was observed to decrease both the growth rate and the cellular yield with

increasing concentration in the range 0.0015 - 0.03 % w/w, potentially due to toxicity effects.

Biosurfactants are surface active agents of microbial origin, which typically act in the same manner as chemical surfactants. Just like chemical surfactants, biosurfactants possess both hydrophobic and hydrophilic moieties within their molecular structure, enabling them to interact with both types of compounds and reduce the surface or interfacial tension between two immiscible phases (Karanth *et al.*, 1999). Biosurfactant molecules are typically glycolipids, lipopeptides and lipoproteins, fatty acids and phospholipids, polymeric biosurfactants or particulate biosurfactants (Desai & Banat, 1997). This grouping encompasses a wide variety of structurally different molecules. Glycolipids usually consist of a combination of a carbohydrate and a long-chain aliphatic or hydroxylaliphatic acid, such as rhamnolipid; lipopeptides and liposproteins consist of a combination of peptides or proteins and lipid groups of varying length, such as surfactin; phospholipids are made up of long chain fatty acids and a phospho or amine group, such as Phosphatidylethanolamine; polymeric biosurfactants are polymers containing a polysaccharide backbone with attached fatty acids and proteins, such as emulsan; particulate biosurfactants are typically extracellular membrane vesicles.

Rosenburg & Zen (1999) state that the natural role of biosurfactants may be two-fold. Firstly, these compounds may be involved in increasing the surface area or bioavailability of hydrophobic water-insoluble substrates, for example, the increased biodegradation of *n*-alkanes in the presence of a biosurfactant BS-UC by *Candida antartica* (Hua *et al.*, 2003). Secondly, the regulation of the attachment-detachment of microorganisms to and from surfaces, for example, the presence of rhamnolipid greatly increased the hydrophobicity of *Pseudomonas aeruginosa* cells and regulated their interaction with surfaces (Zhang & Miller, 1994). Furthermore, biosurfactants have been shown to regulate the formation of aerial hyphae in many filamentous bacteria and fungi, for example, the hydrophobic protein SapB in the case of *S. coelicolor*

(Tillotson *et al.*, 1998) and the hydrophobic peptide streptofactin in the case of *S. tendae* (Richter et al., 1998).

The majority of surfactants in use today are chemically derived from petroleum, a non-renewable raw material (Banat *et al.*, 2000). The authors note that surfactants are an important class of industrial chemicals, the use of which is widespread in almost every sector of modern industry today. Greek (1991) notes that the total worldwide production of surfactants at the beginning of the 1990's was approximately three million tonnes per year, with an estimated value of US $4 billion. Furthermore, of the total volume of surfactant produced approximately 54% is used for household or laundry detergents and 32% is used for industrial applications. Consequently, biosurfactant production is attracting significant of attention due to the diverse range of the molecules produced, the environmentally friendly nature and biodegradability of the compounds and the potential for production in industrial fermentation processes using renewable raw materials (Banat *et al.*, 2000; Mulligan, 2005).

In Chapter 4, selected bioprocessing parameters (nutrient medium, inoculum concentration and shake flask agitation speed) were shown to influence pellet formation and development in *S. hygroscopicus* cultures. It is hypothesized that the fluid properties of viscosity and surface tension also influence this process. The aim of the experimental work performed was to assess the impact of artificially regulating broth viscosity and surface tension on the morphological development of *S. hygroscopicus* cultures.

5.2. REGULATION OF PELLET DEVELOPMENT THROUGH MANIPULATION OF BROTH VISCOSITY

Fermentation broth rheology can be influenced by the composition of the nutrient broth, synthesis of extracellular polymers or the morphological profile of the biomass. A change in the rheological characteristics of a fermentation fluid can cause significant mixing and aeration problems due to altered fluid flow patterns. This may be responsible for regulating particle aggregation and subsequent pellet development within the culture. The composition of the nutrient medium is the only parameter capable of regulating broth rheology from the beginning of the fermentation, the most important phase in the morphological development of the organism.

The alteration of fluid viscosity affects the motion of particles within a fermentation broth. Increasing apparent viscosity in a fermentation broth through the addition of xanthan gum, for example, will decrease the Reynolds number, a measure of fluid turbulence. This in turn should regulate particle collision and aggregation, leading to reduced pellet formation in submerged fermentations. The experimental procedure undertaken was designed to assess the impact of altering broth apparent viscosity on the morphological development of *S. hygroscopicus* cultures.

The experimental procedure was as described section 2.6 except for the addition of varying concentrations of Xanthan gum (Sigma) to artificially regulate the apparent viscosity of the fermentation broth. Xanthan gum was added to the media prior to autoclaving at the concentrations shown in Table 5.1 to regulate the rheological characteristics of the resulting fermentation broths. Bennett's nutrient medium acted as the control for experimental procedure. The duration of the experimental procedure was 7 days, with sampling for image analysis purposes occurring on the third, fifth and seventh day and sampling for dry weight of biomass determination occurring on the seventh day. A single set of five flasks was prepared for each xanthan gum concentration and was used throughout the experimental time period for the aforementioned sampling. All data shown is based on that obtained from

the set of five replicate flasks. Rheological measurements were performed on the autoclaved xanthan gum supplemented Bennett's fermentation media prior to inoculation and the cell-free broths at day 7, as described in section 2.3.4. Likewise, the gas-liquid mass transfer coefficients for the xanthan gum supplemented Bennett's fermentation media was determined using the autoclaved media prior to inoculation, as described in section 2.3.5.

Table 5.1. Concentration range of xanthan gum supplemented Bennett's fermentation broths and the resulting rheological characteristics.

Xanthan Gum (gl^{-1})	Flow Behaviour Index (n) (-)	Fluid Consistency Index (K) (Ns^nm^{-2})	Apparent Viscosity (μ_a) (10^{-3} Nsm^{-2}) @ 28 s^{-1} shear rate
Control (Bennett's)	0.99	0.91	0.90
0.1	0.90	1.70	1.38
0.5	0.89	2.84	1.95
1.0	0.82	7.19	3.79
2.0	0.86	8.53	5.12
3.0	0.84	19.57	11.34
4.0	0.75	49.21	21.52
5.0	0.62	119.62	36.21

The apparent viscosity (μ_a) of any pseudoplastic solution is dependent on the rheological characteristics, flow behaviour index *n*, fluid consistency index *K*, and the shear rate γ experienced in the system, as shown in equations 2.1, 2.2 and 2.3. The apparent viscosity data shown in Table 5.1 were calculated at a shear rate of 28 s^{-1}; the shear rate calculated by Fujita *et al.* (1994) for a similar shake flask system. Given the concentration dependence of *n* and *K* as shown in Table 5.1 and the shear rate experienced in the system it is possible to estimate the apparent viscosity of a variety of xanthan gum suspensions over a range of concentrations and shear rates. The

dependence of apparent viscosity on both concentration and shear rate enabled the formulation of fermentation media with desirable rheological characteristics. Table 5.1 contains the range of apparent viscosity values for each of the concentrations of xanthan gum used.

The introduction of polysaccharides such as xanthan gum into the nutrient broth potentially presents the organism with another utilisable nutrient. Should the organism consume or degrade this compound then the apparent viscosity of the solution should, in theory, alter over the course of the fermentation. However, comparison of cell-free broth rheological characteristics pre- and post-fermentation, as illustrated in Figure 5.1, showed no significant change, indicating that xanthan gum had not been consumed by the organism.

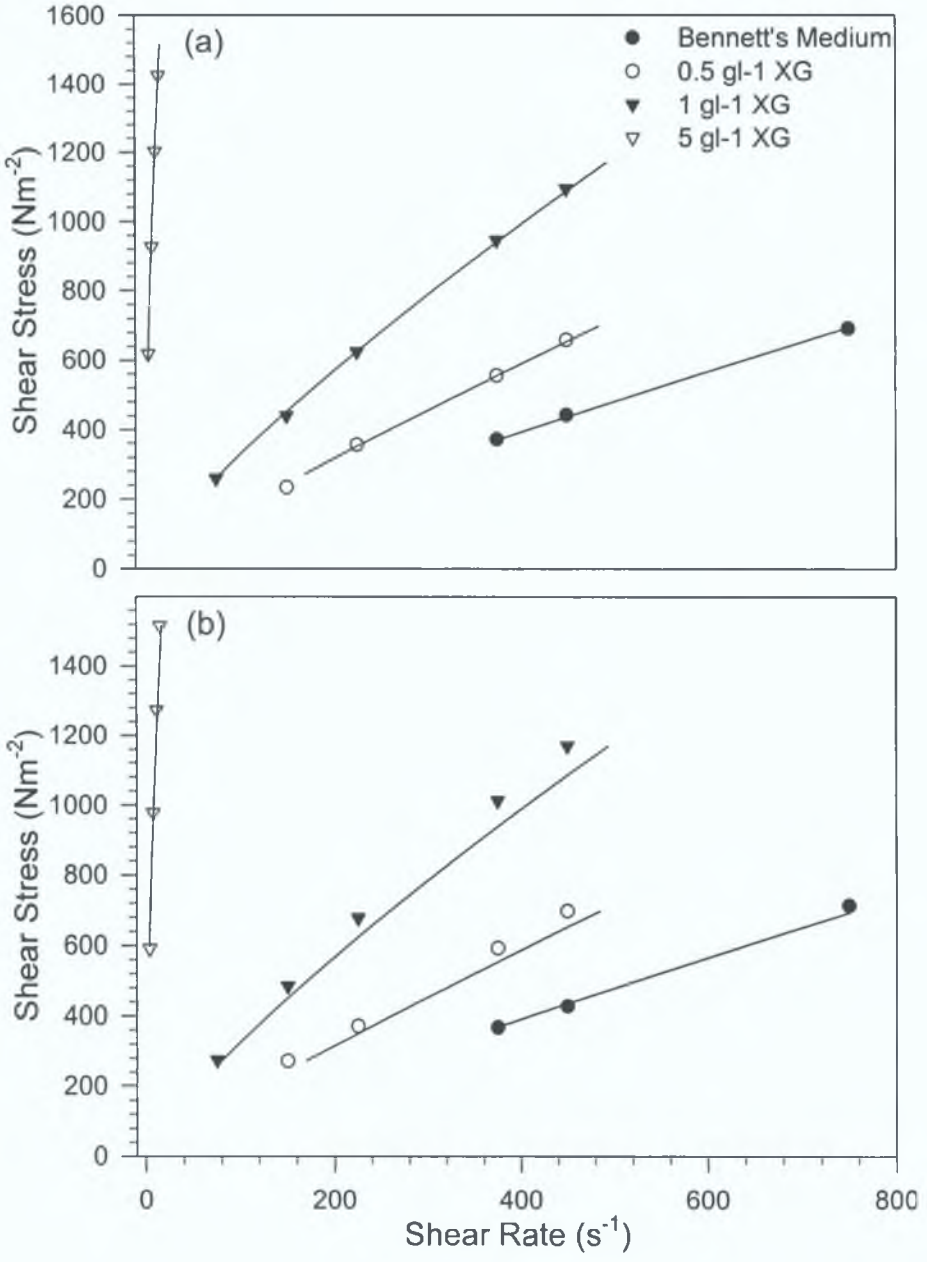


Figure 5.1. Relationship between shear rate and shear stress with respect to varying xanthan gum (XG) concentration in cell-free broths (a) prior to inoculation (b) at day 7 of a *S. hygroscopicus* fermentation.

5.2.1. Regulation of *S. hygroscopicus* morphology using xanthan gum

The day 7 pellet count per ml and mean pellet volume are given in Figure 5.2. The pellet count increases to a maximum, at a concentration of 3 gl^{-1} xanthan gum and then decreases at higher xanthan concentrations. The mean pellet volume decreases with respect to increasing xanthan gum

concentration. The addition of 3 gl^{-1} xanthan gum to the fermentation media increases the pellet count by a factor of approximately four while correspondingly decreasing the mean pellet volume by a factor of three and a half. As is the case with the pellet count per ml of solution, the maximum concentration of biomass is produced in the 3 gl^{-1} xanthan gum cultures. The biomass concentration increases to an optimal two and a half fold at 3 gl^{-1} xanthan gum and decreases for higher concentrations.

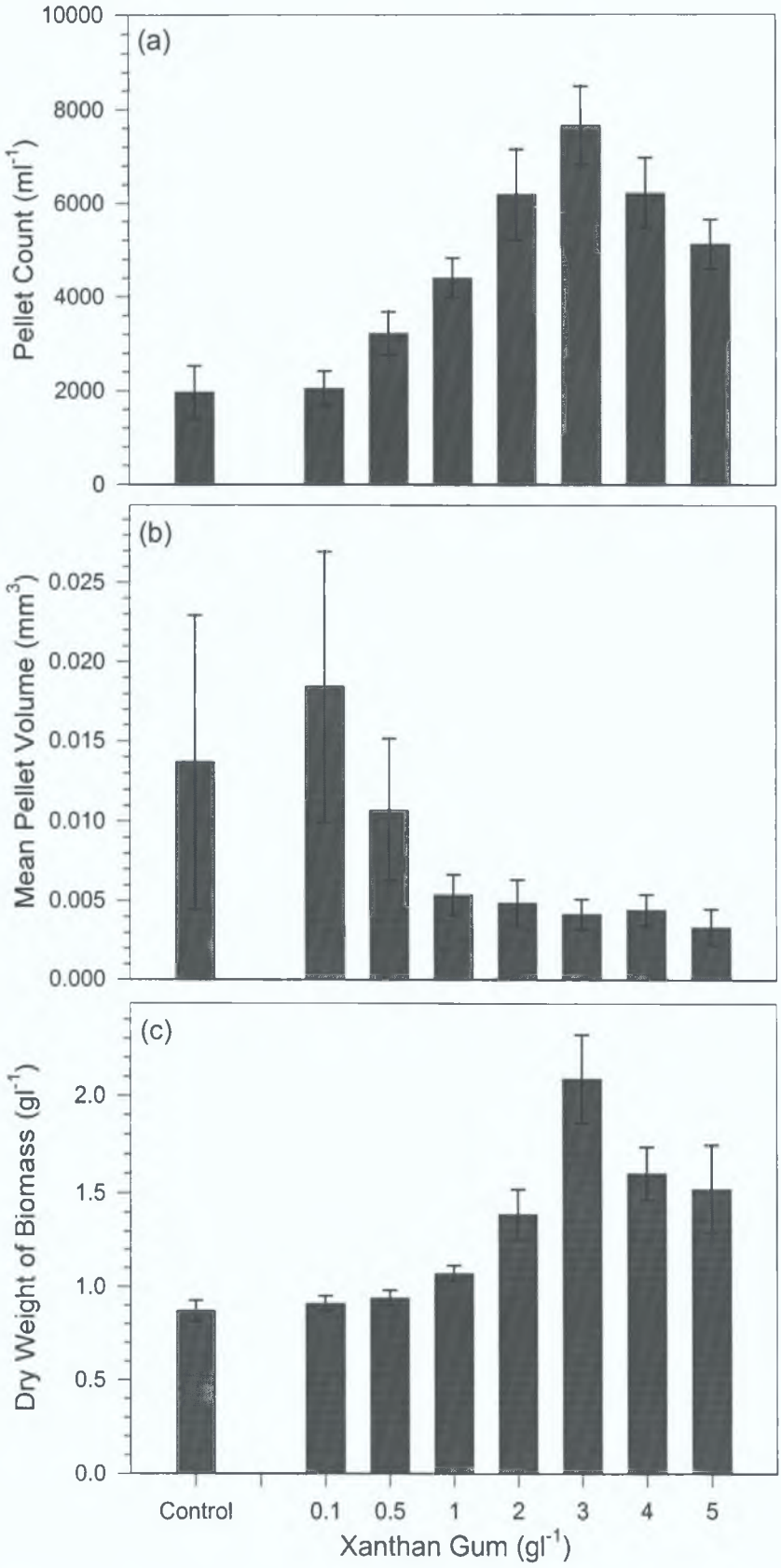


Figure 5.2. Impact of varying concentration of xanthan gum on *S. hygroscopicus* fermentation broths on day 7 (a) pellet count, (b) mean pellet volume and (c) dry weight of biomass.

The correlation between increased pellet count, reduced mean pellet volume and the concentration of xanthan gum may also be seen in the morphological profiles shown in Figure 5.3. This figure contains both images of the stained microbial biomass and the corresponding size distribution profiles for a range of xanthan gum concentrations. Increasing xanthan gum concentration and hence apparent viscosity results in a narrower size distribution with a single mode, i.e. more pellets with a reduced mean volume. Decreasing the apparent viscosity returns the system to control-like behaviour with a broader distribution and the potential for multi-modality, as can be seen in the culture supplemented with 0.5 gl^{-1} xanthan gum, as shown in Figure 5.3. Increasing apparent viscosity also increases the homogeneity of the cultures as can be seen by the increased overlap on the normalised particle and volume frequencies in the cultures supplemented with 3 and 5 gl^{-1} xanthan gum.

Increasing xanthan gum concentration in *S. hygroscopicus* shake flask fermentations, up to an optimal value of 3 gl^{-1}, results in a four-fold increase in pellet count and a two and a half fold increase in biomass with respect to the Bennett's control, as indicated in Figures 5.2 (a) and (c). This is assumed to be due to reduced particle-to-particle interaction and subsequent aggregation. Furthermore, as a result of reduced particle-to-particle interaction, the mean pellet volume decreases. This results in more individual spores or smaller hyphal aggregates developing into pellets without subsequent aggregation. Figure 5.3 indicates that the formation of pellets and the resulting population size distribution is regulated by the apparent viscosity of the culture. As well as ensuring a narrower, more normal distribution, increasing the apparent viscosity ensures a more homogeneous population, as evidenced by the overlapping of the normalised particle and volume frequencies in the 3 and 5 gl^{-1} distributions in comparison to the Bennett's control.

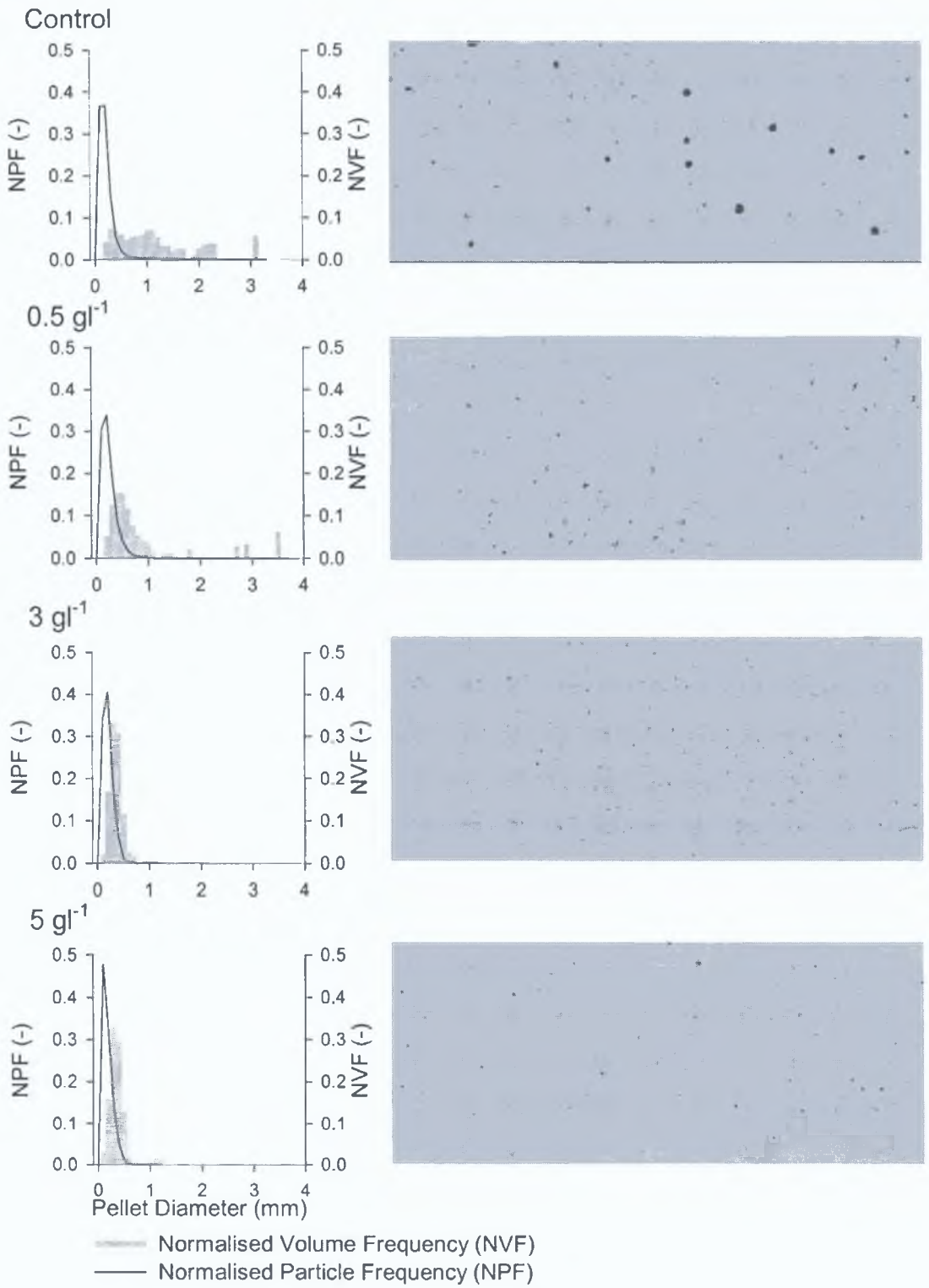


Figure 5.3. Stained microbial biomass and morphological distributions for Control (1 in 5 Dilution), 0.5 gl^{-1} (1 in 10 Dilution), 3 gl^{-1} (1 in 10 Dilution) and 5 gl^{-1} xanthan gum (1 in 10 Dilution) for day 7 fermentation samples.

The influence of altering broth rheology, through the introduction of viscous agents, on the morphological development of filamentous microorganisms has been examined by a number of authors. For example, Corman (1959) used nutrient thickening agents to increase the apparent viscosity of *Blakeslea* and *Choanephora* cultures to prevent clumping and to regulate the extent of pellet formation. The compounds used were a mix of carbohydrates, such as carboxymethyl cellulose and acacia gum, and proteinaceous material, such as soybean flour and corn steep liquor. The author noted that varying the type and concentration of compounds added enabled the generation of nutrient broths with (apparent) viscosity levels in the range of 1 to 30 Nsm^{-2}. Increasing apparent viscosity resulted in the development of cultures with an increased proportion of dispersed mycelia and consequently less pelleted growth. The addition of polymeric compounds, such as polyethylene-glycol, to nutrient media has been reported to influence the morphological development of *Streptomyces coelicolor* A3(2) (Hodgson, 1982) and *Streptomyces lividans* (Hobbs *et al.*, 1989), whereby the introduction of this agent into the solution favours the generation of a population of dispersed mycelia as opposed to pellets. Although neither Hodgson (1982) nor Hobbs *et al.* (1989) measured the apparent viscosity of the resulting solutions both authors do conclude that the increased apparent viscosity was responsible for regulating pellet formation.

5.2.2. Influence of xanthan gum on apparent viscosity and gas-liquid mass transfer

The morphological regulation of *S. hygroscopicus* cultures through the addition of varying concentrations of xanthan gum artificially controls broth apparent viscosity as shown in Figure 5.4. Increasing xanthan concentration results in an exponential increase in broth apparent viscosity, which does not alter over the course of the experiment, as shown in Figure 5.1. The addition of xanthan gum to the fermentation broth and its impact on apparent viscosity also affects the gas-liquid mass transfer coefficient, K_La, of the system. Counter-intuitively, it is observed in Figure 5.4 (b) that increasing apparent viscosity steadily increases K_La by up to 37% at 2 gl^{-1} xanthan gum. At levels

in excess of 2 gl^{-1}, a steady decrease in K_La is observed with respect to xanthan gum concentration.

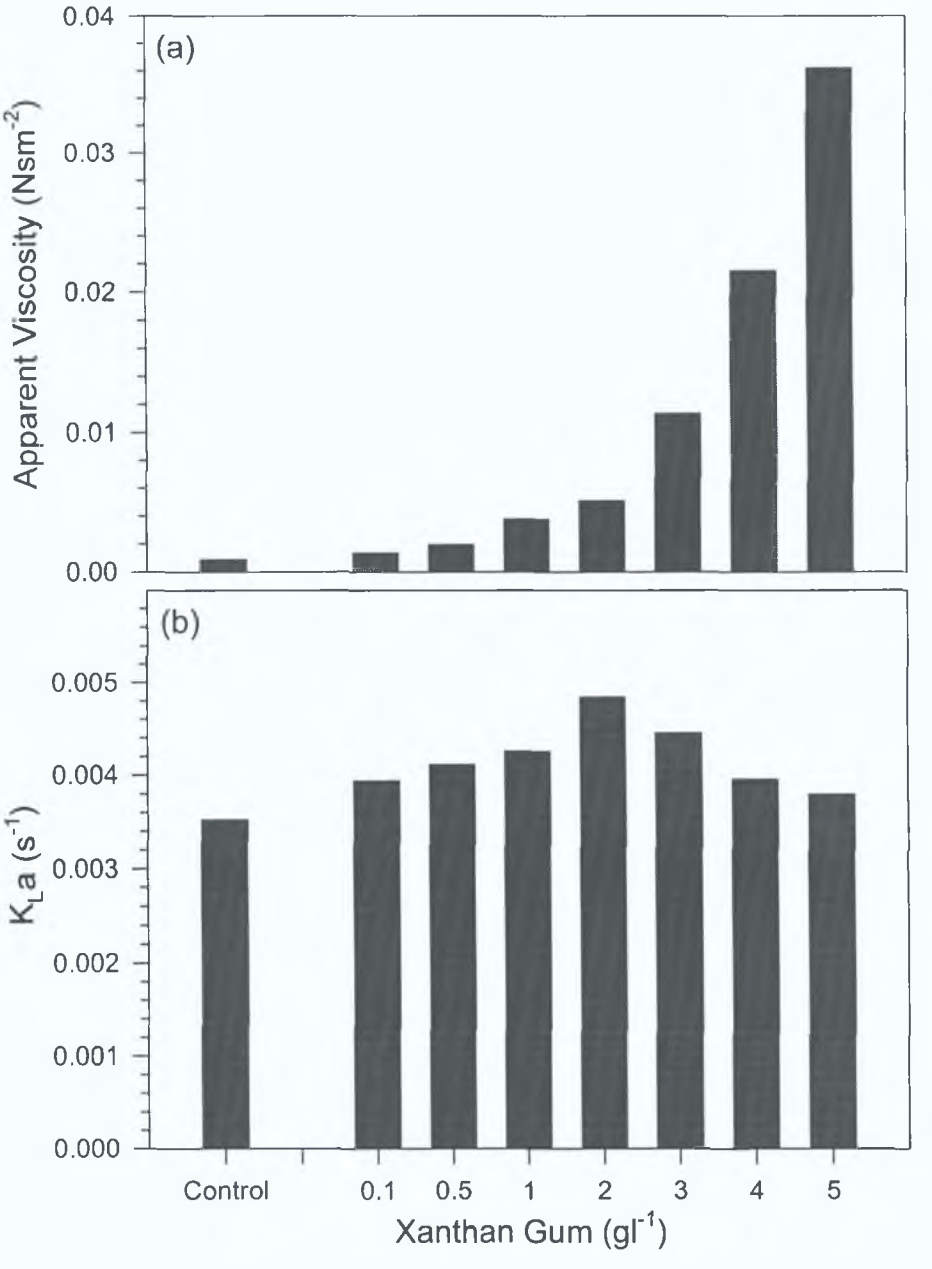


Figure 5.4. Impact of adding varying concentrations of xanthan gum to *S. hygroscopicus* fermentation broths on (a) apparent viscosity at a shear rate of 28 s^{-1} and (b) gas-liquid mass transfer coefficient in 100 ml of broth contained in a 250 ml shake flask agitating at 150 rpm.

Increasing viscosity results in a reduction in the Reynolds number, and hence fluid mixing, this in turn should also mean a decrease in the rate of gas-liquid

mass transfer. Previous research has shown that increasing broth apparent viscosity using xanthan gum has resulted in a decreasing rate of oxygen transfer in stirred tank reactors (Garcia-Ochoa & Gomez, 1998; Garcia-Ochoa *et al.*, 2000). However this does not appear to be the case with shake flask systems where the addition of xanthan gum increases the gas-liquid mass transfer coefficient in the range investigated, as shown in figure 5.4. It has been previously shown that xanthan gum concentrations in the range 2.5 to 20 gl^{-1} can increase the rate of oxygen diffusion in non-agitated solutions (Ho *et al.*, 1988). The authors note that the "ice-like" structures of the dipolar water molecules are believed to be weakened or broken by the presence of xanthan gum molecules. The disruption of the structure of water molecules in this manner is thought to lower the resistance to solute diffusion and therefore increase the diffusion of oxygen from the gaseous to the liquid phase. Furthermore, Ho *et al.* (1988) observed that increasing xanthan gum concentration resulted in a concomitantly increasing oxygen diffusion coefficient.

In an aerated, stirred tank reactor, agitation is achieved by means of an impeller and oxygen transfer is achieved by means of bubble dispersion, whereas in shake flasks, agitation is by means of shaking the flask and oxygen transfer is at the air-liquid interface. It is proposed that in agitated shake flask systems at the shear rate investigated, the driving force behind oxygen transfer is diffusion based in much the same way as the system examined by Ho *et al.* (1988). However, the difference between the system examined by Ho *et al.* (1988) and the shake flask system is that the fluid is in motion in shake flasks and consequently there is mixing within both the bulk liquid and gaseous phases, which will influence the oxygen concentration at the air-liquid interface and, in turn, influence the gas-liquid mass transfer coefficient. An increase in xanthan gum concentration increases the gas-liquid mass transfer coefficient up to an optimum at 2 gl^{-1} Xanthan gum. At concentrations in excess of this point the viscosity of the broth increases sharply as illustrated in figure 5.4 (b) and results in the reduction of bulk fluid mixing and consequently a decrease in the gas-liquid mass transfer coefficient.

5.3. REGULATION OF PELLET DEVELOPMENT THROUGH MANIPULATION OF BROTH SURFACE TENSION

The addition of polymeric substances such as surfactants or antifoams to nutrient media has traditionally been used as a counteractive measure to prevent both foaming and/or wall growth in submerged fermentations. However, the addition of surfactant molecules such as Span 20, Tween 40 and Tween 80 has been demonstrated by a number of researchers to influence the morphological development of filamentous microorganisms in submerged fermentations (Domingues *et al.*, 2000; Jeong *et al.*, 2001; Lucatero *et al.*, 2004; Znidarsic *et al.*, 2000). The mode of action and the morphological influence of individual compounds have been shown to be dependent on the compound itself, the concentration at which it is added and the organism it is targeting. Surfactants affect the interaction of particles within a fermentation broth through their impact on surface tension and hence hydrophobic interactions between biomass particles. Alteration of cell surface hydrophobicity within the system through the control of broth surface tension enables the regulation of particle interaction and subsequent aggregation. The manipulation of broth surface tension prior to inoculation through the introduction of varying concentrations of Tween 80, Triton X100 and Silicone Antifoam provided an insight into the impact of hydrophobic interactions on the morphological development of *S. hygroscopicus* cultures.

The shake flask culturing for this experimental procedure was as described in section 2.6 except for the addition of varying concentrations of chemical surfactants Tween 80, Triton X100 and Silcorel AFP 20 Silicone Antifoam (BDH Laboratory Supplies, UK) to artificially regulate the surface tension of the fermentation broth. The concentrations at which the surfactants were added ranged from 0.01 - 0.000001 % v/v Tween 80 and 0.1 - 0.00001 % v/v Triton X100 in orders of magnitude and 0.1, 0.05, 0.01, 0.005, 0.001 % v/v Silicone Antifoam. Each of the compounds was added to the medium post-autoclaving at the concentrations shown in Figure 5.5 and the effects on broth surface tension were as illustrated. Bennett's nutrient medium acted as the control for experimental procedure. The duration of the experimental

procedure was 7 days, with sampling for image analysis purposes occurring on the third, fifth and seventh day and sampling for dry weight of biomass determination occurring on the seventh day. A single set of five flasks was prepared for each surfactant concentration and was used throughout the experimental time period. All data shown is based on that obtained from the set of five replicate flasks. Surface tension measurements were performed on the cell-free surfactant supplemented Bennett's fermentation medium post-autoclaving.

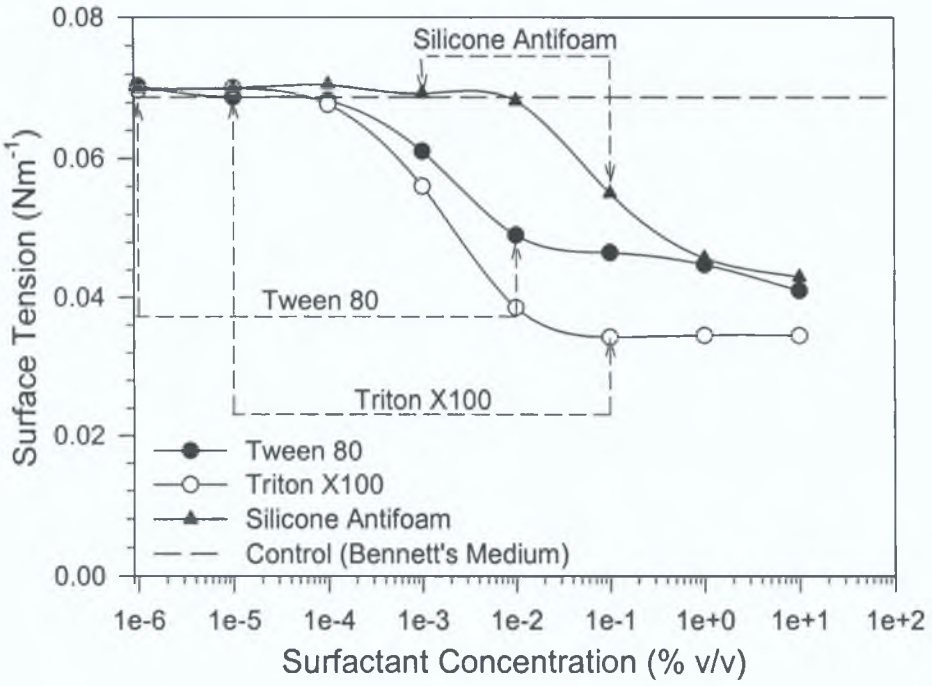


Figure 5.5. The impact of increasing levels of Tween 80, Triton X100 and Silicone Antifoam on the surface tension of cell-free Bennett's medium. Measurements were taken at 20^0c using a Tensiometer. Selected regions represent the concentrations of each of the compounds used in the experimental procedure.

5.3.1. Compound Specificity

Figure 5.6 illustrates the morphological impact of 0.01% (v/v) Tween 80, 0.01% (v/v) Triton X100 and 0.1% (v/v) Silicone Antifoam on cultures of *S. hygroscopicus*. Each of the three compounds is responsible for inducing a

different morphological profile at the concentrations examined. Tween 80 acts as a binding/flocculating agent capable of producing a single population of large particles early on in the fermentation, which increase in size not only through growth but also through agglomeration. Triton X100 and Silicone Antifoam are responsible for producing more uniform distributions of smaller particles with a comparable or decreased pellet count, which increase in size through growth and, to a lesser extent, through agglomeration. Both Tween 80 and Silicone Antifoam had equivalent or slightly higher levels of biomass than the control whereas Triton X100 had a 16% reduction as shown in Table 5.2. Triton X100 was also shown to have a toxic affect on *S. tendae* biomass production, with the growth rate decreasing with increasing concentration in the region of 0.0015 – 0.03 % (w/w) (Vechtlifshitz *et al.*, 1989).

Although all three compounds are surfactants, the mode of action of Tween 80 differs from that of Triton X100 and Silicone Antifoam, as it is responsible for producing cultures consisting of a small number of large pellets whereas the other two produce cultures consisting of a large number of small to medium sized pellets. The addition of each of the three surfactants resulted in the generation of varying population distributions indicating the potential use of surfactants in the morphological engineering of *S. hygroscopicus* cultures.

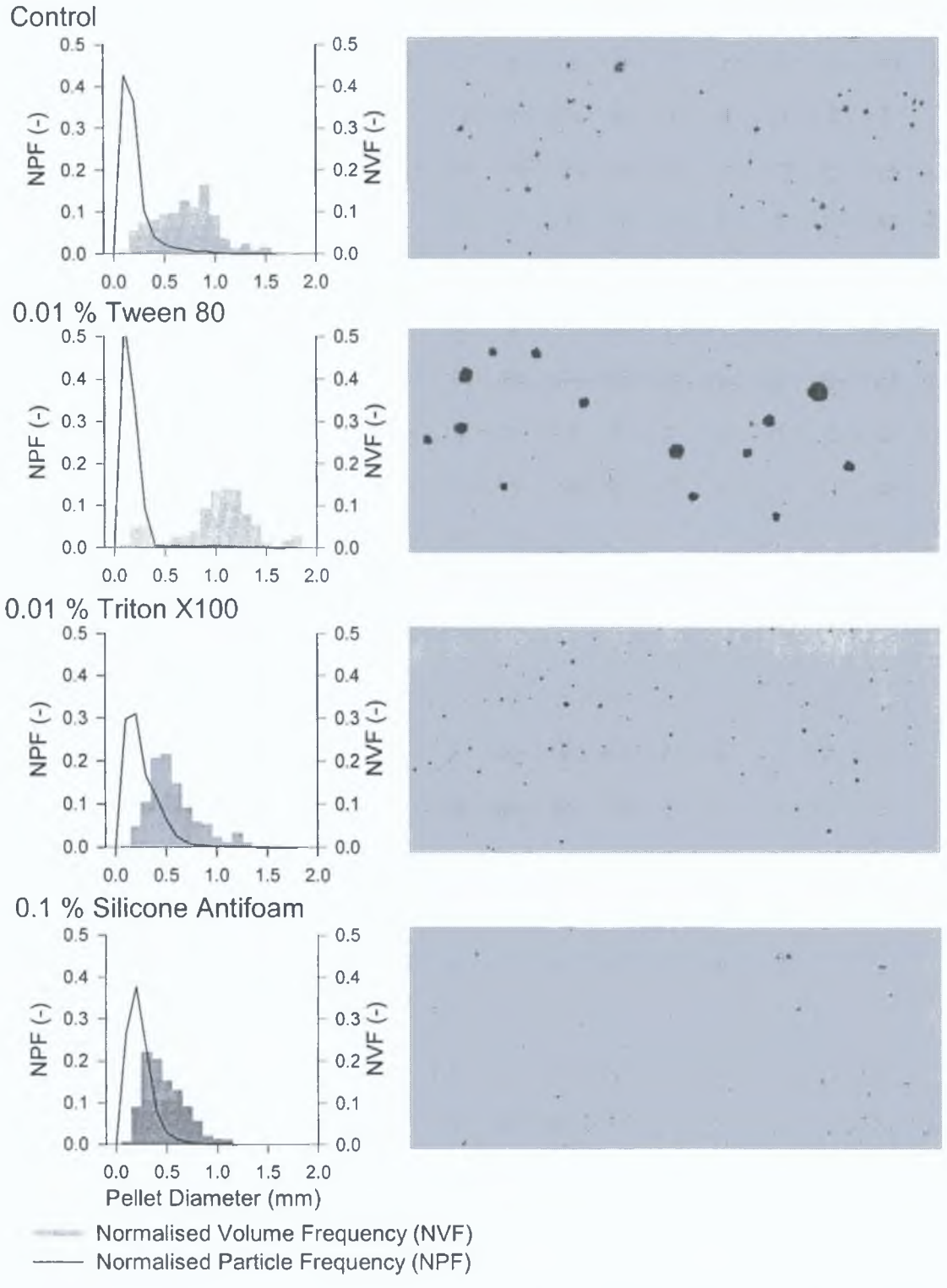


Figure 5.6. Day 7 stained microbial biomass and morphological distributions for Control (1 in 10 Dilution), 0.01% (v/v) Tween 80 (1 in 5 Dilution), 0.01% (v/v) Triton X100 (1 in 10 Dilution) and 0.1% (v/v) Silicone Antifoam (1 in 10 Dilution).

Table 5.2. Compound specific influence of surfactants on the morphological and physiological characteristics of *S. hygroscopicus* fermentations at day 7

Sample	Pellet Count (ml^{-1})	Mean Pellet Volume (mm^3)	Dry Weight (gl^{-1})
Control	1793	0.0118	0.72
0.01% Tween 80	1449	0.0122	0.71
0.01% Triton X100	1013	0.0149	0.60
0.1% Silicone Antifoam	1730	0.0080	0.80

5.3.2. Concentration Specificity

The influence of surfactants on the morphological profile of *S. hygroscopicus* cultures depends on the amount of the compound present. The surfactant concentration has been discovered to be critical to the development of the population distribution, as shown in Figure 5.7 and Table 5.3. For each of the compounds in question, an effective concentration range was determined within which culture morphology was influenced. At concentrations exceeding the effective range, two surfactants (Tween 80 and Triton X100 are both detergents) became toxic to the organism and at concentrations below the effective range all three systems returned to control-like behaviour. The effective range for Tween 80, in which the coagulative effect may be seen, extends from 1% – 0.001% (v/v). In the case of Triton X100 the effective range extends from 0.1% - 0.001% (v/v), within which the compound was capable of limiting biomass growth by up to 31%. The effective range for Silicone Antifoam extends from 1% - 0.01%.

Figure 5.7 illustrates the morphological impact of varying Tween 80, Triton X100 and Silicone Antifoam concentrations on the morphological development of the population distribution at day 3. For each of the compounds in question a different morphological impact is being induced as demonstrated in section 5.2. High concentrations bring about the formation of

a single population of pellets with a narrow distribution within a compound specific size range. Decreasing concentration results in a return to control-like behaviour within the cultures.

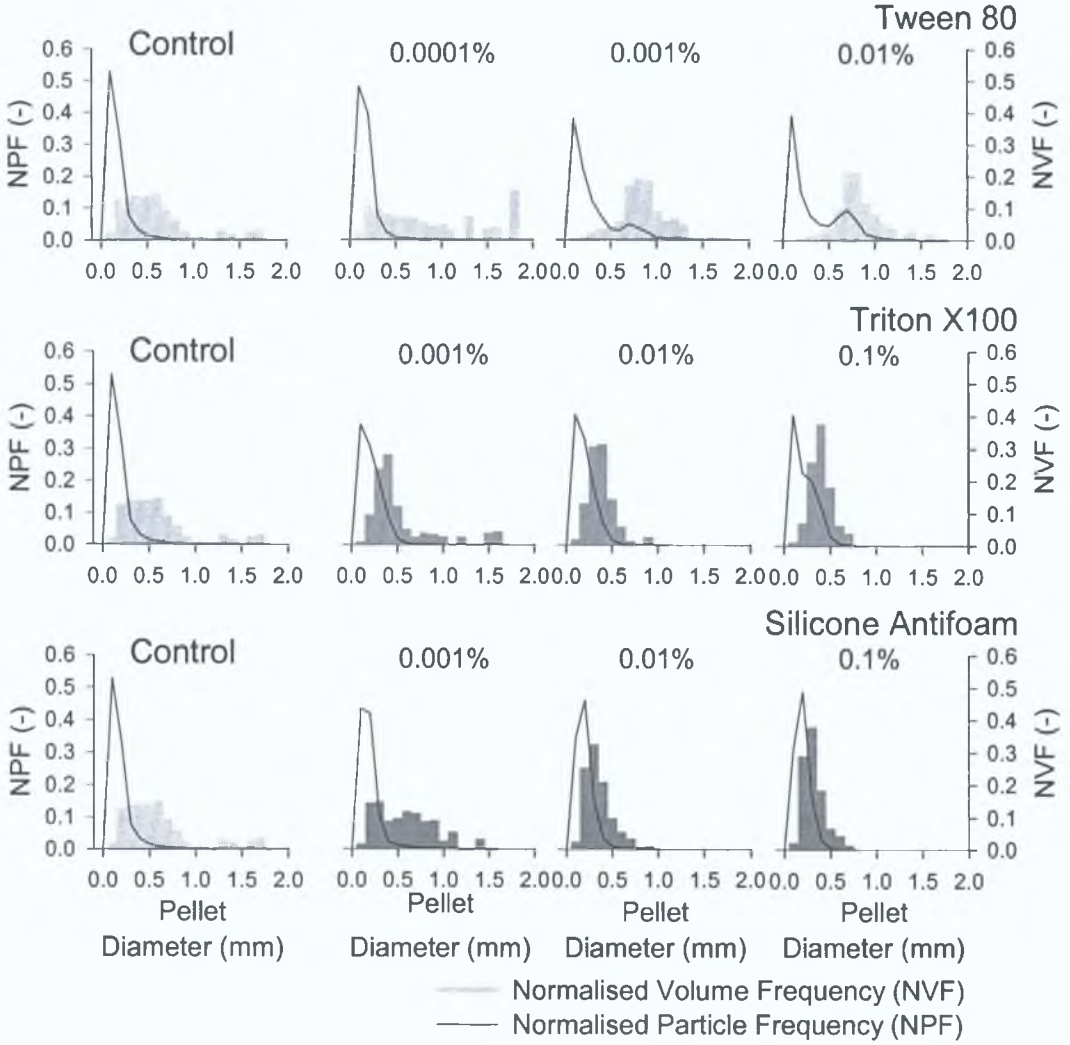


Figure 5.7. Day 3 normalised volume and particle frequency distributions for varying concentrations of Tween 80, Triton X100 and Silicone Antifoam with respect to the Control.

Table 5.3. Concentration specific influence of surfactants on the morphological and physiological characteristics of *S. hygroscopicus* fermentations at day 3.

Sample	Pellet Count (ml^{-1})	Mean Pellet Volume (mm^3)
Control	1535	0.0010
0.0001% Tween 80	1346	0.0011
0.001% Tween 80	85	0.0085
0.01% Tween 80	141	0.0178
0.001% Triton X100	1223	0.0022
0.01% Triton X100	1951	0.0018
0.1% Triton X100	1092	0.0027
0.001% Silicone Antifoam	1696	0.0013
0.01% Silicone Antifoam	2169	0.0015
0.1% Silicone Antifoam	2662	0.0016

Surfactants act by reducing the surface tension of a liquid. Figure 5.4 illustrates the impact of the addition of varying concentrations of surfactants on the surface tension of nutrient media. The regulation of broth surface tension is dependent on the type and concentration of the surfactant present, which, in turn, regulates hydrophobic interactions between biomass particles and thus controls the potential for particle aggregation and pellet formation.

5.3.3. Time Dependency

The morphological and physiological development of *S. hygroscopicus* cultures is influenced by the type and concentration of surfactant present in the system throughout the 7 day experimental period, as demonstrated in sections 5.3.1 and 5.3.2. Furthermore, as has been previously mentioned,

the morphological impact of the type and concentration of the individual surfactant is greatest in the first 72 hours. From day 3 to day 7 all cultures will increasingly tend towards control-like behaviour, as illustrated in Figure 5.8 and Table 5.4, with respect to the pellet count, mean pellet volume and morphological distributions. The presence of surfactants in the nutrient broth is thought to regulate the hydrophobic interactions of particles and thus the formation of pellets throughout the course of the fermentation.

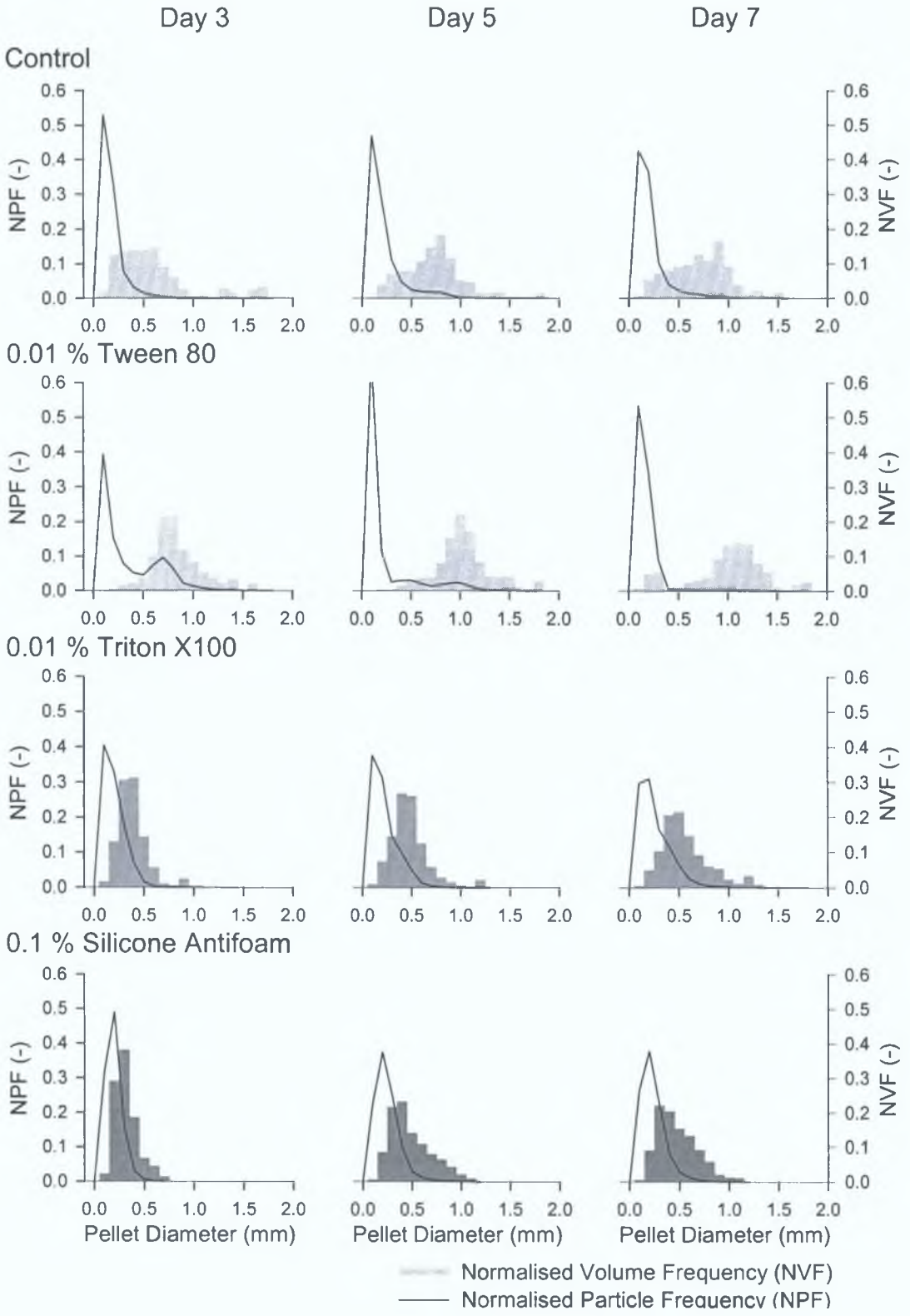


Figure 5.8. Day 3, 5 and 7 normalised volume and particle frequency distributions for Control, 0.01% Tween 80, 0.01% Triton X100 and 0.1% Silicone Antifoam.

Table 5.4. Time dependent influence of surfactants on the morphological and physiological characteristics of *S. hygroscopicus* fermentations.

Sample	Pellet Count (ml^{-1})	Mean Pellet Volume (mm^3)	Biomass Conc. (gl^{-1})
Control - Day 3	1535	0.0046	
Control - Day 5	990	0.0152	
Control - Day 7	1793	0.0118	0.72
0.01% Tween 80 - Day 3	141	0.0538	
0.01% Tween 80 - Day 5	215	0.0565	
0.01% Tween 80 - Day 7	1449	0.0123	0.71
0.01% Triton X100 - Day 3	1951	0.0046	
0.01% Triton X100 - Day 5	1403	0.0084	
0.01% Triton X100 - Day 7	1013	0.0149	0.60
0.1% Silicone Antifoam - Day 3	2662	0.0030	
0.1% Silicone Antifoam - Day 5	1911	0.0091	
0.1% Silicone Antifoam - Day 7	1730	0.0080	0.80

5.3.4. Sample Variability

Submerged fermentations of filamentous organisms are prone to flask-to-flask variability within a supposedly identical population as demonstrated in section 3.5. Both Figure 5.9 and Table 5.5 demonstrate the pellet count, mean pellet volume and size distribution variability experienced in three of the five identical control flasks. One of the major problems encountered when culturing filamentous organisms is the flask-to-flask variability inherent in these processes. This sample variability is of concern given the lack of reproducibility in systems of this type and the dependence of product formation on culture morphology. The random nature of particle collision in

submerged fermentations and thus the interactions that form between individuals in a single population bring about flask-to-flask variability. The introduction of the surfactants Tween 80, Triton X100 and Silicone Antifoam into the system reduces the broth surface tension. By regulating the surface tension it is possible to regulate the morphology and physiology of the organism and reduce flask-to-flask variability.

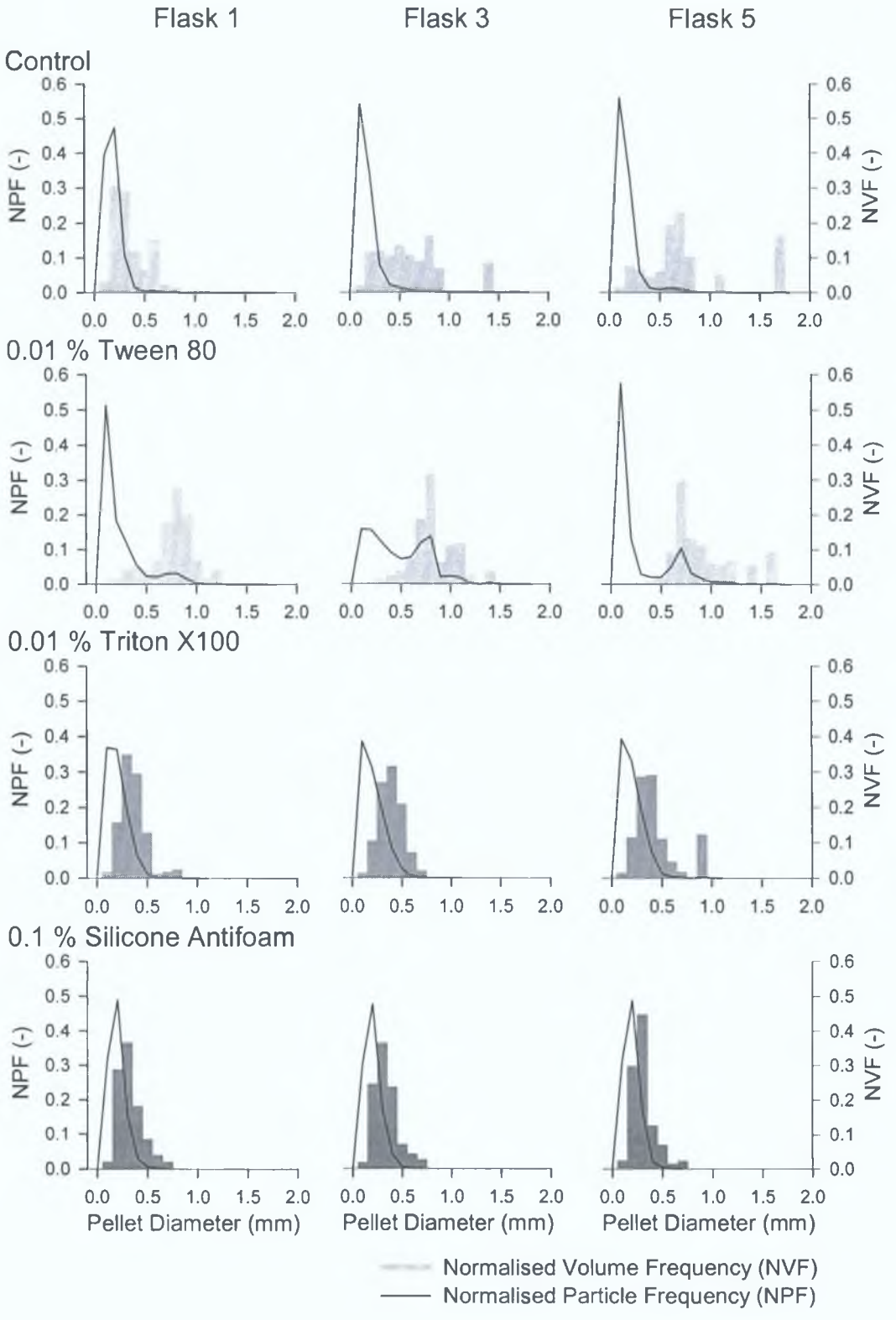


Figure 5.9. Day 3 normalised volume and particle frequency distributions for Control, 0.01% Tween 80, 0.01% Triton X100 and 0.1% Silicone Antifoam flasks 1, 3 and 5.

Table 5.5. Comparison of morphological variability in *S. hygroscopicus* fermentations at day 3.

Sample	Pellet Count (ml^{-1})	Mean Pellet Volume (mm^3)
Control 1	3189	0.0026
Control 3	1800	0.0047
Control 5	1279	0.0065
0.01% Tween 80 1	169	0.0247
0.01% Tween 80 3	55	0.0952
0.01% Tween 80 5	288	0.0585
0.01% Triton X100 1	2648	0.0043
0.01% Triton X100 3	1539	0.0055
0.01% Triton X100 5	2101	0.0051
0.1% Silicone Antifoam 1	2610	0.0032
0.1% Silicone Antifoam 3	2341	0.0035
0.1% Silicone Antifoam 5	2622	0.0029

5.3.5. Biosurfactant Production

As a consequence of measuring surface tension in control cultures it became apparent that the organism produces a compound capable of altering the surface tension of the fermentation broth. Figure 5.10 illustrates the reduction in the surface tension of the control fermentation broth over 7 days. The native Bennett's nutrient broth has a surface tension of approximately 0.068 – 0.069 Nm^{-1}, which remains constant over the first two days of fermentation as illustrated in Figure 5.10. From day 2 onwards the surface tension of the cultures drops significantly until it reaches approximately 0.043 Nm^{-1} by day 7, with the greatest incidence of variability occurring at day 3. No artificial surfactants are present in this system and the surface tension measurement

was made on a centrifuged cell-free supernatant. The production of this indigenous biosurfactant controls broth surface tension, the potential of which to regulate culture morphology is recognised. Subsequent experimental work will endeavour to elucidate the relationship if any between biosurfactant production and pellet formation.

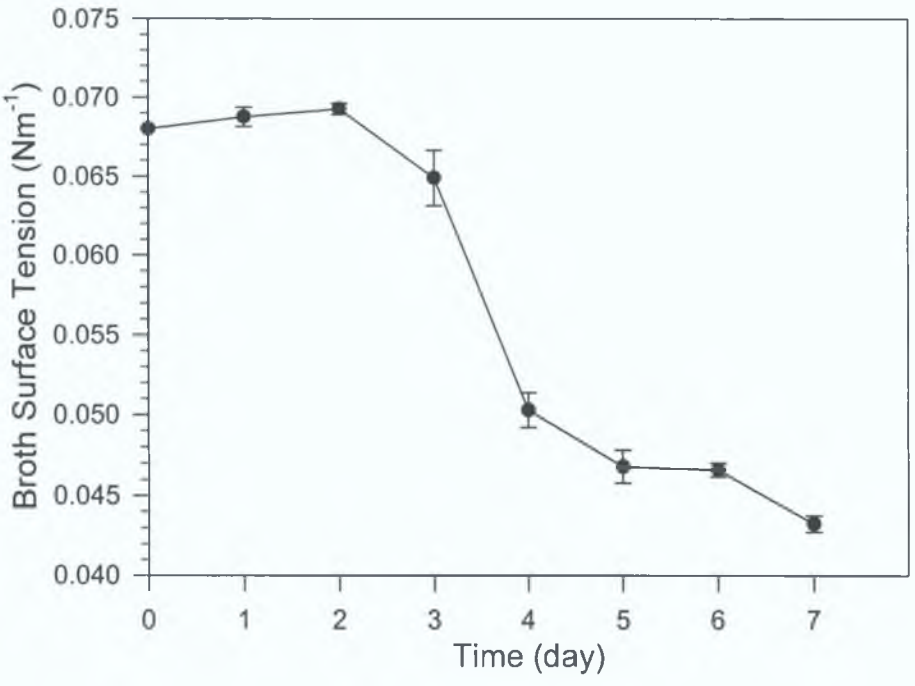


Figure 5.10. Surface tension profile of cell-free *S. hygroscopicus* cultures grown in Bennett's nutrient media over a 7 day experimental period. Error bars represent the +/- standard deviation of the mean obtained from a set of five flasks. Measurements were taken at 20^0c using a Tensiometer.

5.4. MORPHOLOGICAL DEPENDENCE OF BIOSURFACTANT PRODUCTION

In section 5.3.5 it was shown that submerged fermentations of *S. hygroscopicus* exhibit reduced broth surface tension due to the production of a surface active agent(s) by the organism. This compound(s), a biosurfactant(s), is thought to be a primary metabolite given that the organism produces it during the first phase of growth, day 0 - 3 as shown in Figure 4.16 (a). Furthermore, Figure 5.10 indicates that biosurfactant

production exhibits a certain degree of flask-to-flask variability within *S. hygroscopicus* shake flask cultures. Further investigation requires examination of biosurfactant production with respect to culture morphology and physiology on a flask-by-flask basis to test this hypothesis.

In section 3.5 the morphological and physiological variability within *S. hygroscopicus* cultures was thought to be as a result of random particle aggregation and subsequent pellet formation. The experimental procedure undertaken involved performing dry weight of biomass determination, image analysis and broth surface tension measurement for each of fifty identical flasks at a single time point (72 hours after inoculation). The broth surface tension was measured for each flask and the results are presented in Figure 5.11.

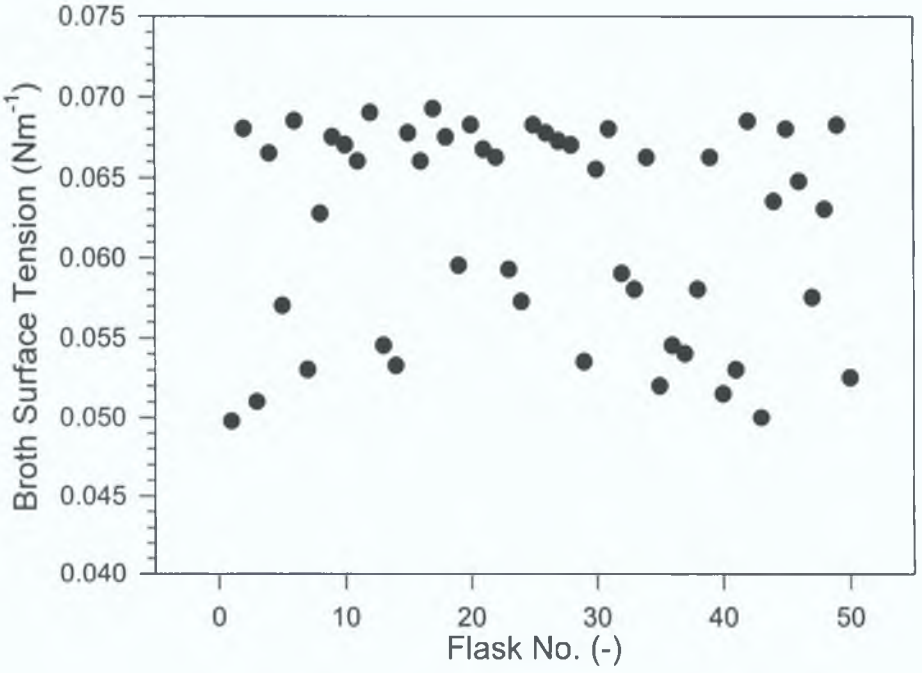


Figure 5.11. Broth surface tension flask-to-flask variability in *S. hygroscopicus* cultures at 72 hours.

The flask-to-flask variability experienced in the pellet count, mean pellet volume, dry weight of biomass and size distribution profiles in Figures 3.6 and 3.7 was also observed in the broth surface tensions as illustrated in

Figure 5.11. These values vary from a minimum of 49.75 Nm^{-1} to a maximum of 69.25 Nm^{-1} in an apparently random fashion. Given that biosurfactant production is variable in *S. hygroscopicus* shake flask cultures that have themselves been shown to be morphologically and physiologically variable this leads to the hypothesis that biosurfactant production is potentially related to culture morphology and/or physiology.

As illustrated in Figure 5.12 (a), biosurfactant production is not directly correlated to biomass concentration, with cultures of comparable levels of biomass having variable surface tension. In general increased biomass does result in a greater incidence of biosurfactant production. Figure 5.12 (b) illustrates the relationship between pellet count and biosurfactant production. Similarly, the pellet count is not directly correlated to compound production, nevertheless cultures with a smaller pellet count also have a higher incidence of compound formation. However, as illustrated by Figure 5.12 (c), a correlation does appear to exist between mean pellet volume and broth surface tension reduction, whereby cultures with increasing mean pellet volume exhibit lower surface tension. Biosurfactant production is not directly proportional to biomass concentration or the pellet count but is related mean pellet volume; it would appear to be linked to culture morphology.

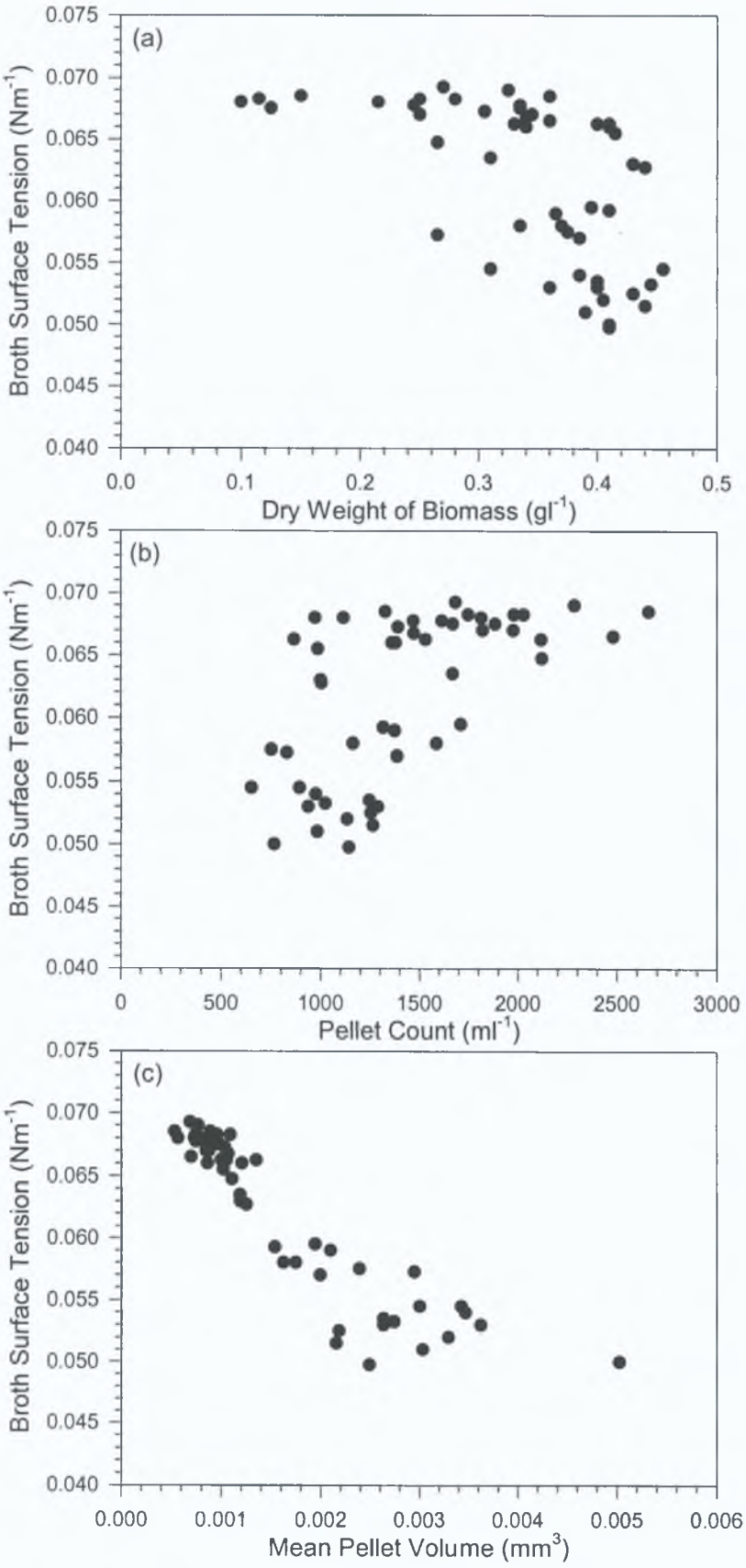


Figure 5.12. Broth surface tension flask-to-flask variability with respect to (a) dry weight of biomass, (b) pellet count and (c) mean pellet volume at 72 hours.

Given that biosurfactant production appears to be dependent to a certain extent on the mean pellet volume, a morphological parameter, and is dependent on the biomass concentration it was decided to investigate the possible existence of a relationship between all three. Figure 5.13 represents a three-dimensional scatter plot of broth surface tension, mean pellet volume and dry weight of biomass for the 72 hour shake flask population. It clearly illustrates that cultures with a small mean pellet volume, regardless of the biomass concentration, contain less biosurfactant. However, increasing mean pellet volume in cultures with high biomass levels brings about increased compound formation. It would appear that the incidence of biosurfactant production increases in cultures that have high biomass levels and a large mean pellet volume; culture morphology appears to be the driving factor.

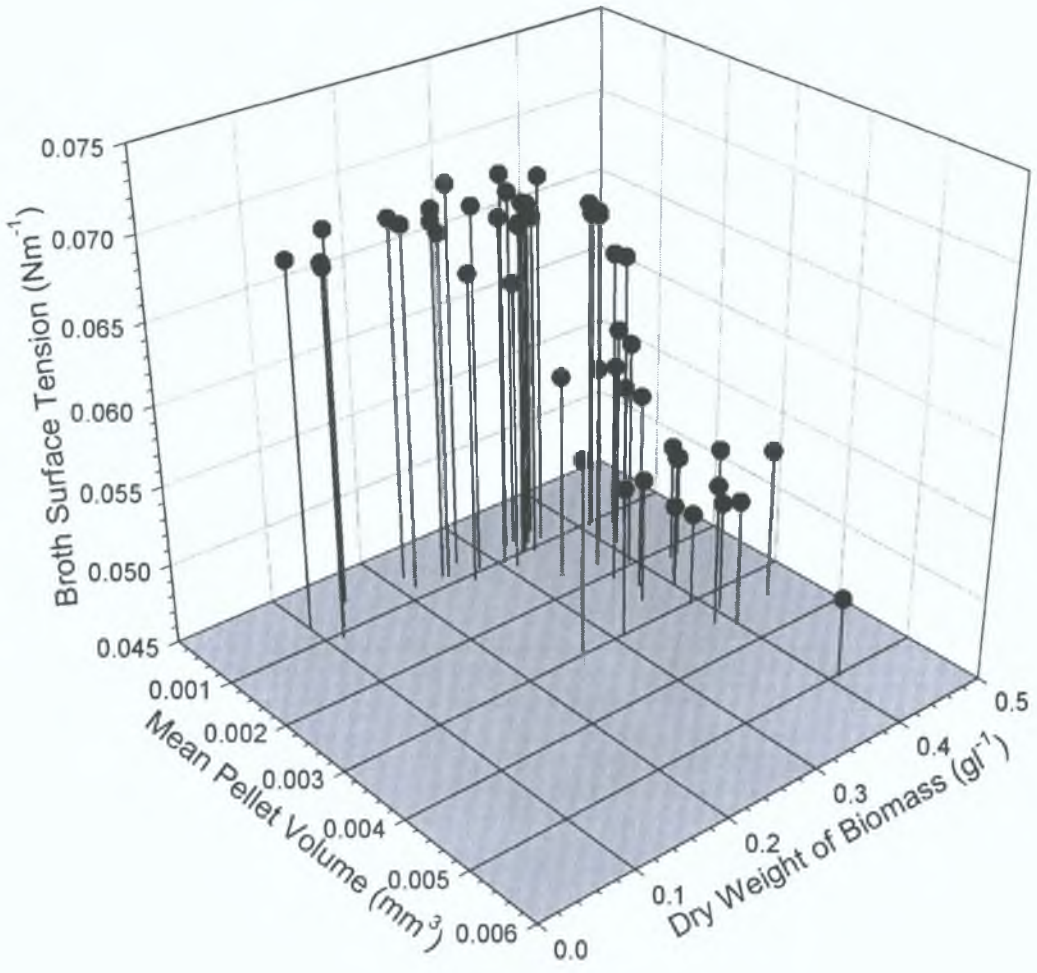


Figure 5.13. Dependence of broth surface tension flask-to-flask variability on mean pellet volume and biomass concentration at 72 hours.

The mean pellet volume measurement is derived from the morphological distribution, calculated by dividing the cumulative pellet volume by the pellet count for a sample. This measurement is not a true representation of culture morphology given that it is based on the numerical pellet count. The only true representations of culture morphology are the particle count and volume fraction distributions shown in Figure 5.14 where the morphological profiles for individual flasks have been arranged with respect to increasing surface tension. The incidence of biosurfactant production is greatest in cultures with uniform pellet size distributions in a narrow range with a mode of approximately 0.5 mm. Cultures exhibiting a different morphology do not have the same incidence of compound formation. It would appear that a strong correlation exists between the incidence of biosurfactant production

and the presence of a sufficient volume of pellets with a diameter of approximately 0.5 mm.

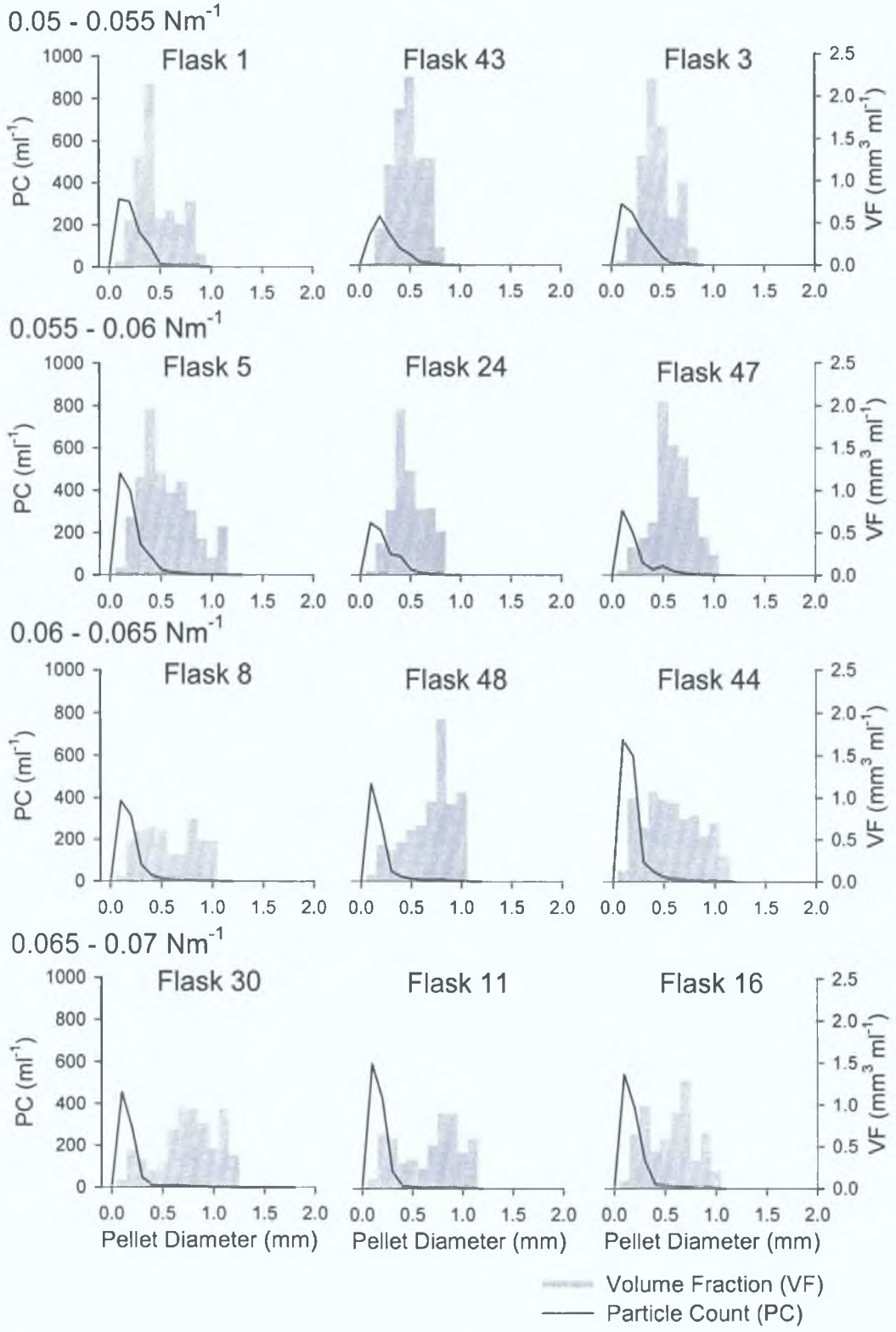


Figure 5.14. Broth surface tension flask-to-flask variability with respect to volume fraction and particle count distributions at 72 hours.

Figure 5.14 illustrates the potential existence of a relationship between culture morphology and biosurfactant production. On closer inspection of Figure 5.14 it would appear that cultures with uniform distributions in a narrow size range with a mode of approximately 0.5 mm exhibit the lowest broth surface tension. Furthermore, the presence of biosurfactant appears to be related to the volume of biomass present in or around 0.5 mm in diameter. Figure 5.15 (a) contains the total pellet volume for each of the flasks with respect to its corresponding broth surface tension; no correlation is seen to exist. However, if the correlation between the total volume of pellets of approximately 0.5 mm in diameter and the production of biosurfactant were to exist then this subset of the population needed to be examined further. Therefore, on selecting a subset of the entire population based on the modal value of 0.5 mm a potential correlation becomes apparent whereby cultures with high cumulative volume in the size range, 0.3 – 0.7 mm, exhibit lower broth surface tension. Increasing biosurfactant production appears to correlate with an increasing cumulative pellet volume fraction in the 0.3 – 0.7 mm range.

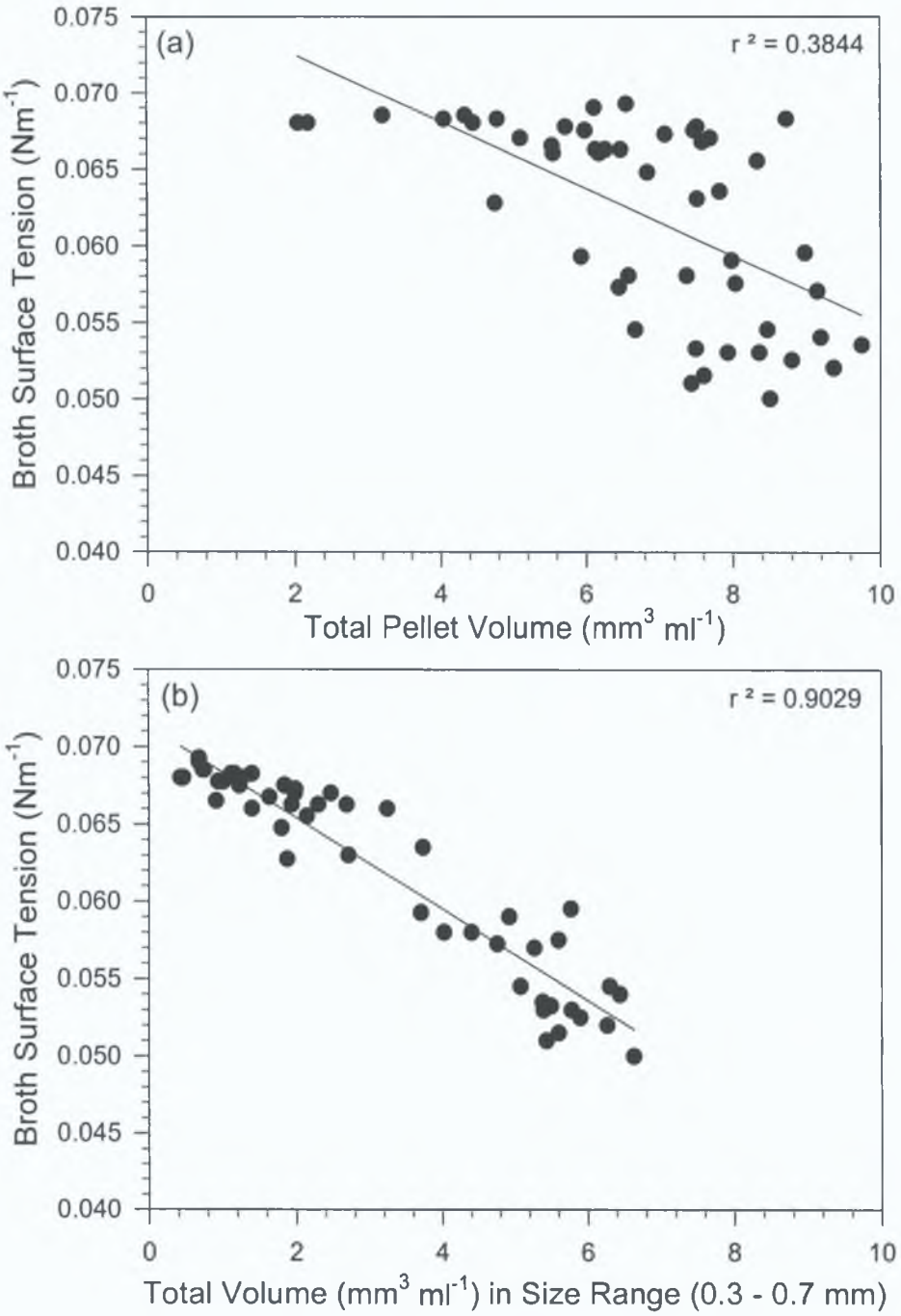
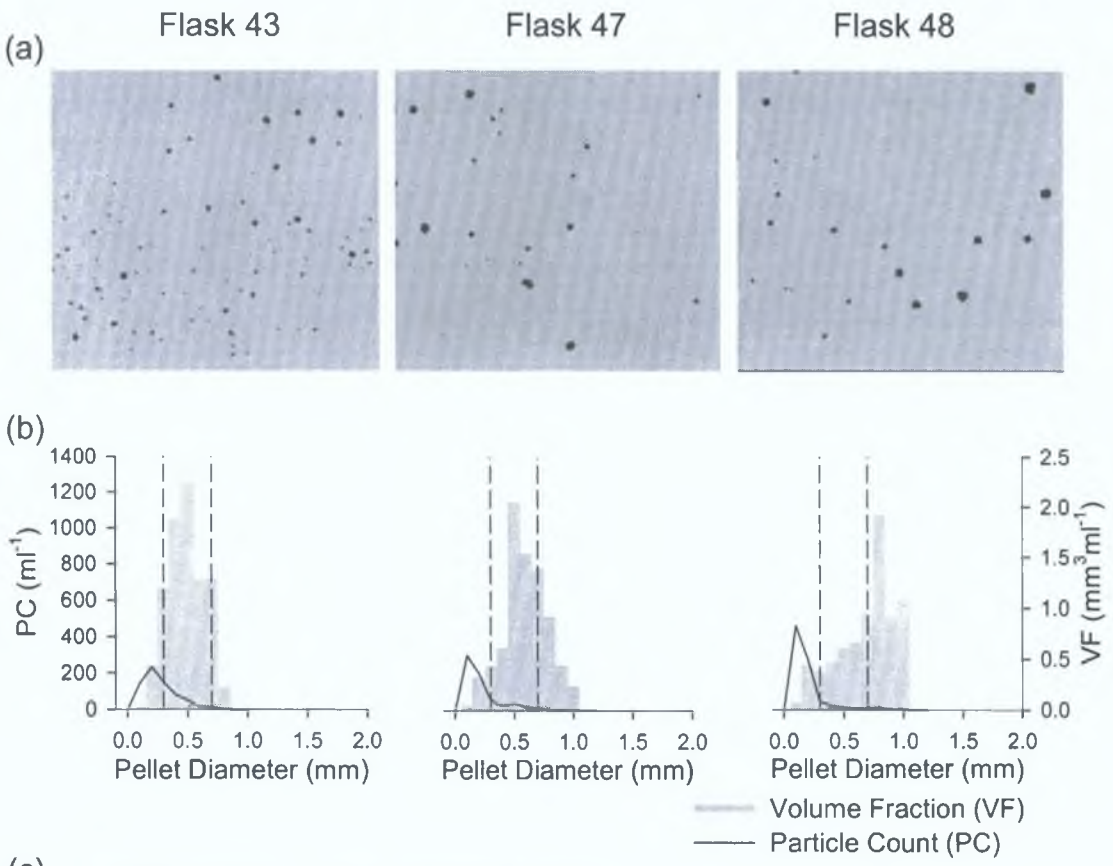


Figure 5.15. Broth surface tension flask-to-flask variability with respect to (a) total pellet volume and (b) total pellet volume in the size range (0.3 – 0.7 mm).

Perhaps the best illustration of the potential relationship between morphology and biosurfactant production is given in Figure 5.16 for flasks 43, 47 and 48, where it is clearly shown that cultures of comparable biomass and total volume per ml have different broth surface tensions. Biosurfactant production

is thought to be correlated with the amount of pelleted biomass in the 0.3 – 0.7 mm size range, as shown in Figure 5.15. For example, flasks 43, 47 and 48 have broths surface tensions of 0.050, 0.0575 and 0.063 Nm^{-1} respectively and the percentage of biomass present in the 0.3 to 0.7 mm size range for each of the flasks is 77, 57 and 28% respectively.



(c)

Total Biomass (gl^{-1})		
0.410	0.375	0.430
Surface Tension (Nm^{-1})		
0.0500	0.0575	0.0630

Figure 5.16. A comparison of culture morphology and biosurfactant production for flasks 43, 47 and 48, showing (a) stained microbial biomass, (b) volume fraction and particle count distributions and (c) dry weight of biomass and broth surface tension levels. The dashed line (- -) in (b) indicates 0.3 to 0.7 mm pellet diameter.

Pellet formation in *S. hygroscopicus* systems and the resulting morphological and physiological state of the culture is dependent on particle aggregation. The underlying mechanisms of pellet formation and development appear to be random and accordingly so is the generation of any given morphological distribution. After 72 hours there is a high degree of flask-to-flask variability within the population that coincides with a high degree of broth surface tension variability as a result of different levels of biosurfactant production. The formation of this compound(s) is thought to be a morphologically driven process with cultures containing a larger proportion of pellets in the 0.3 – 0.7 mm diameter size range coinciding with higher biosurfactant production.

5.5. BIOSURFACTANT SEPARATION

Data presented in section 5.4 suggested the production of an unknown indigenous biosurfactant by *S. hygroscopicus* in shake flask cultures. The potential for the morphological regulation of *S. hygroscopicus* cultures through the inclusion of varying concentrations of Tween 80, Triton X100 and Silicone Antifoam in the fermentation broth was demonstrated in section 5.3. Given that the inclusion of chemical surfactants in the broth prior to inoculation morphologically alters the culture and the fact that the organism produces its own biosurfactant during the course of the fermentation, it is highly likely that this indigenous molecule is also capable of regulating morphology. Furthermore, it is hypothesized that the organism may be producing this biosurfactant as part of a morphological auto-regulatory mechanism. In order to demonstrate the efficacy of the indigenous molecule, it must first be purified and then its morphological impact, if any, can be assessed in fresh nutrient broth.

As with all separation processes biosurfactant recovery is based on knowledge of the compound, identification of a distinctive molecular structure or biochemical trait and the exploitation of these to isolate the compound of interest from contaminant material. Biosurfactant molecules are typically glycolipids, lipopeptides and lipoproteins, fatty acids and phospholipids, polymeric biosurfactants or particulate biosurfactants (Desai & Banat, 1997;

Karanth *et al.*, 1999). Some of the techniques commonly used isolate and purify biosurfactant molecules are ammonium sulphate, acetone or acid precipitation, solvent extraction, crystallisation, adsorption or chromatography (Desai & Banat, 1997). The authors note that: precipitation steps are predominantly applicable to proteinaceous material such as lipoproteins, polymeric or particulate biosurfactants; solvent extraction and adsorption both require the use of organic solvents and are applicable to glycolipids or phospholipids; crystallisation is applicable to glycolipids. Chromatographic techniques are applicable to virtually all biosurfactant material. The design of an isolation and purification procedure for the biosurfactant(s) produced by *S. hygroscopicus* will take into account the suitability of each of the aforementioned techniques to the compound(s) in question. Precipitation techniques are not applicable as the compound(s) is heat stable and consequently not thought to be proteinaceous. Solvent extraction techniques are not applicable as the purified material is subsequently required for microbial fermentations, as described in section 5.6, with the solvents potentially posing a toxicity issue. Crystallisation is not applicable as high concentration pure solutions are required and the fermentation broths used contain low concentrations of the impure compound. Therefore, chromatographic techniques were deemed to be the best option for isolating the compound of interest.

Compound isolation and purification by chromatography is typically based on one of the following methods: gel filtration, affinity, hydrophobic interaction or ion-exchange chromatography. The selection of one or more of these techniques will depend on the specific compound of interest and its molecular traits, which will be exploited for separation purposes. All that was initially known about the biosurfactant of interest is that it is probably an organic compound with both hydrophobic and hydrophilic groups. Knowing this, Hydrophobic Interaction and Gel Filtration Chromatography are the most logical choices, with affinity and ion-exchange systems discounted due to insufficient knowledge of the compound(s) to be separated. The separation and purification of this molecule(s) facilitated assessment of the impact, if

any, of this indigenously produced compound on the morphological development of *Streptomyces hygroscopicus* var. *geldanus* cultures.

As illustrated in Figure 5.5, the relationship between Tween 80, Triton X100 and Silicone Antifoam concentration and broth surface tension is non-linear. The same type of non-linear relationship is exhibited by the indigenously produced biosurfactant as shown in Figure 5.17. The stock broth used for the experiment has a surface tension of 0.041 Nm^{-1}, whereas a 50% dilution of the stock has a surface tension of 0.0435 Nm^{-1} indicating a reduction in concentration of 50% resulted in only a 0.0025 Nm^{-1} change. Broth surface tension reduction is not a direct measure of biosurfactant concentration.

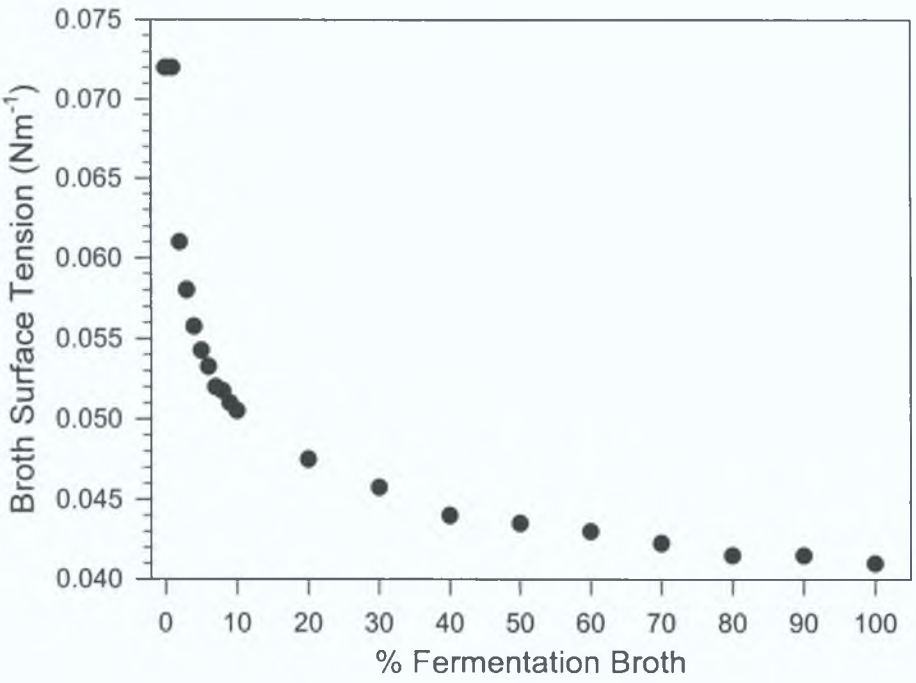


Figure 5.17. The influence of cell-free broth dilution with distilled water on the liquid surface tension for a day 12 fermentation sample. For example, the 10% point refers to the surface tension of a solution containing 10% fermentation broth and 90% distilled water.

Autoclaving the biosurfactant-containing broth had little or no affect on the surface tension. This high temperature and pressure stability testing indicated that the biosurfactant was likely to be a non-proteinaceous organic

compound. Identification of physical and/or chemical attributes of the compound can be exploited for separation purposes. Surfactants contain both hydrophobic and hydrophilic groups making them ideal candidates for hydrophobic interaction chromatography, whereby hydrophobic molecules in a sample will interact with hydrophobic ligands on a chromatographic matrix, depending on the ionic strength of the solution. A hydrophobic interaction chromatography system operates on the basis that an impure sample is applied to a column containing hydrophobic packing material (Phenyl-Sepharose) in a high concentration salt solution. Under such high salt conditions the hydrophobic compounds will, ideally, bind to the packing material while non-hydrophobic compounds will pass through unimpeded. A washing solution, usually containing the same high salt concentration as the sample, is then applied to wash out any remaining contaminants. Elution of the bound compounds is usually achieved by decreasing the salt concentration of the liquid phase until the compound of interest no longer binds and is eluted off the column. At this point the eluent is collected at the bottom of the column for further examination.

An investigation into the various binding and elution conditions and their impact on compound separation resulted in system optimisation, as illustrated in Figure 5.18. The sample used for this procedure was a cell-free, day 12, *S. hygroscopicus* broth cultured in Bennett's nutrient medium. The approach taken in optimising the hydrophobic interaction chromatography system involved varying the NaCl concentration in both the binding and washing phases and varying the liquid flowrate in the binding, washing and elution phases. By inspection of the resulting binding and elution profiles for the compound(s) of interest with respect to the contaminant material the optimal operating conditions, as shown in Figure 5.18, were determined.

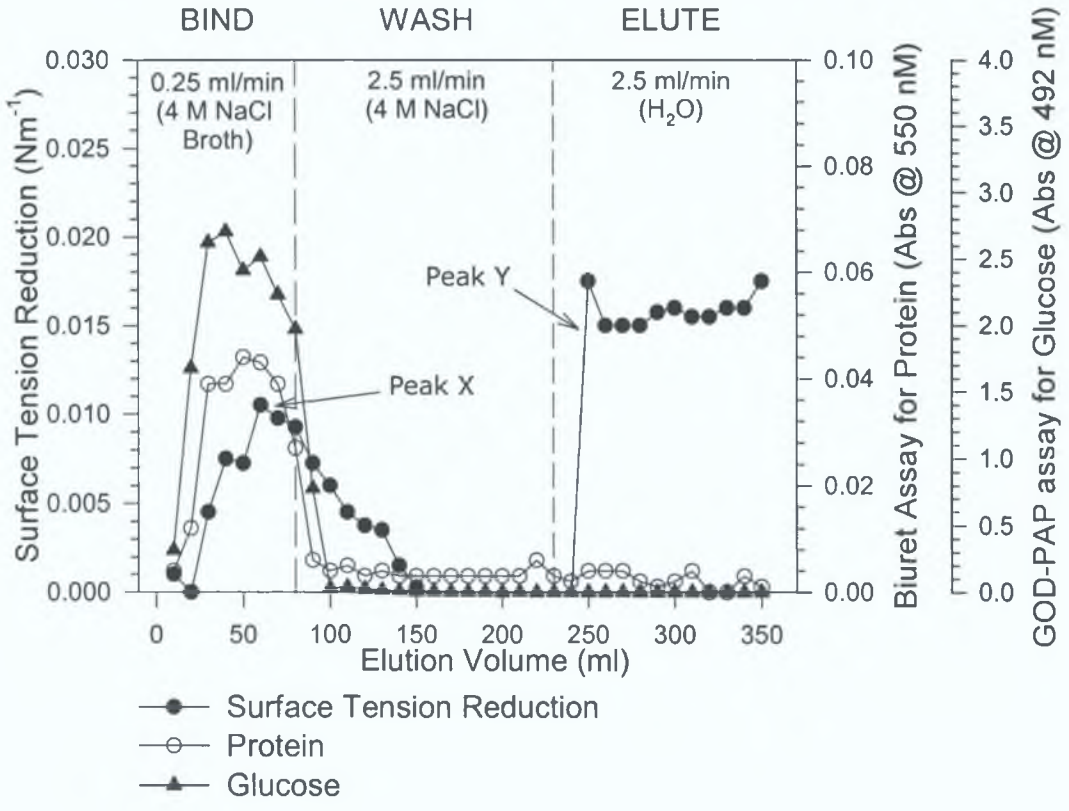


Figure 5.18. Hydrophobic interaction chromatography of 50 ml of a cell-free, day 12, *S. hygroscopicus* broth on a Phenyl-Sepharose column.

A number of system specific observations may be made. Firstly, a high concentration of salt is required to effectively bind biosurfactant to the column. When using an aqueous mobile phase the biosurfactant material will not bind to the hydrophobic ligands and will be eluted off the bottom of the column. In the presence of a 4 M NaCl mobile phase, biosurfactant will reversibly bind to the column and can be subsequently eluted by reducing the ionic strength of the solution, as demonstrated in Figure 5.18. As illustrated in Figure 5.18 the broth is made up of at least two biosurfactant molecules, one of which, peak X, will not bind to the column regardless of ionic strength, whereas the other molecule, peak Y, will bind to Phenyl-Sepharose reversibly. The inclusion of a wash step between the bind step and the elute step in the form of 2.5 ml min^{-1} 4 M NaCl provides for the removal of contaminating material.

The hydrophobic interaction chromatography system separated a biosurfactant-containing fraction from glucose but not from all the proteinaceous material. In order to remove this proteinaceous material it was proposed to use a gel filtration chromatography column. This technique operates on the basis of molecular weight fractionation, a process whereby molecules of or below a certain size pass into porous beads while larger molecules are excluded. Those that pass through the beads are retained on the column for a longer period of time than those that do not and thus are fractionated. A two-step process incorporating a combination of both hydrophobic interaction and gel filtration chromatography was attempted to further purify the compounds of interest.

Once the optimum binding, washing and eluting conditions for the hydrophobic interaction chromatography column had been determined, as illustrated in Figure 5.18, the separation process was scaled up from 50 ml to 2 L of broth. Process scale up was desirable for the purification of sufficient material for a subsequent investigation into the influence of the indigenous biosurfactant on culture morphology in section 5.6. As illustrated in Figure 5.19, 2 L of 4 M NaCl broth was used in the bind phase, followed by 1 L of 4 M NaCl wash, followed by distilled H_2O for elution. As in the previous section, a certain amount of the biosurfactant did not bind to the column. Furthermore, the consistent increase in surface tension during the binding phase indicates that the binding efficiency of the column for the second biosurfactant decreases. The column does not bind glucose and selectively binds protein as illustrated by the continuous elution of these compounds during this time period. In the wash phase compounds not bound to the resin but still resident in the column are removed by passing 1 L of 4 M NaCl through. Changing the ionic strength of the mobile phase by introducing distilled H_2O results in the removal of the remaining NaCl and consequently the elution of bound compounds.

The highest proportion of biosurfactant and contaminants emerge in the first ~ 1 L of eluent, from 3 – 4 L. The eluent in this region has a high concentration of biosurfactant but also a high level of contaminants and is

thus relatively impure, making it ideal for further purification steps. This material was further purified by gel filtration chromatography in order to further assess the biosurfactants contained therein. The sample obtained at 3055 ml, as illustrated in Figure 5.19, was chosen for this purpose.

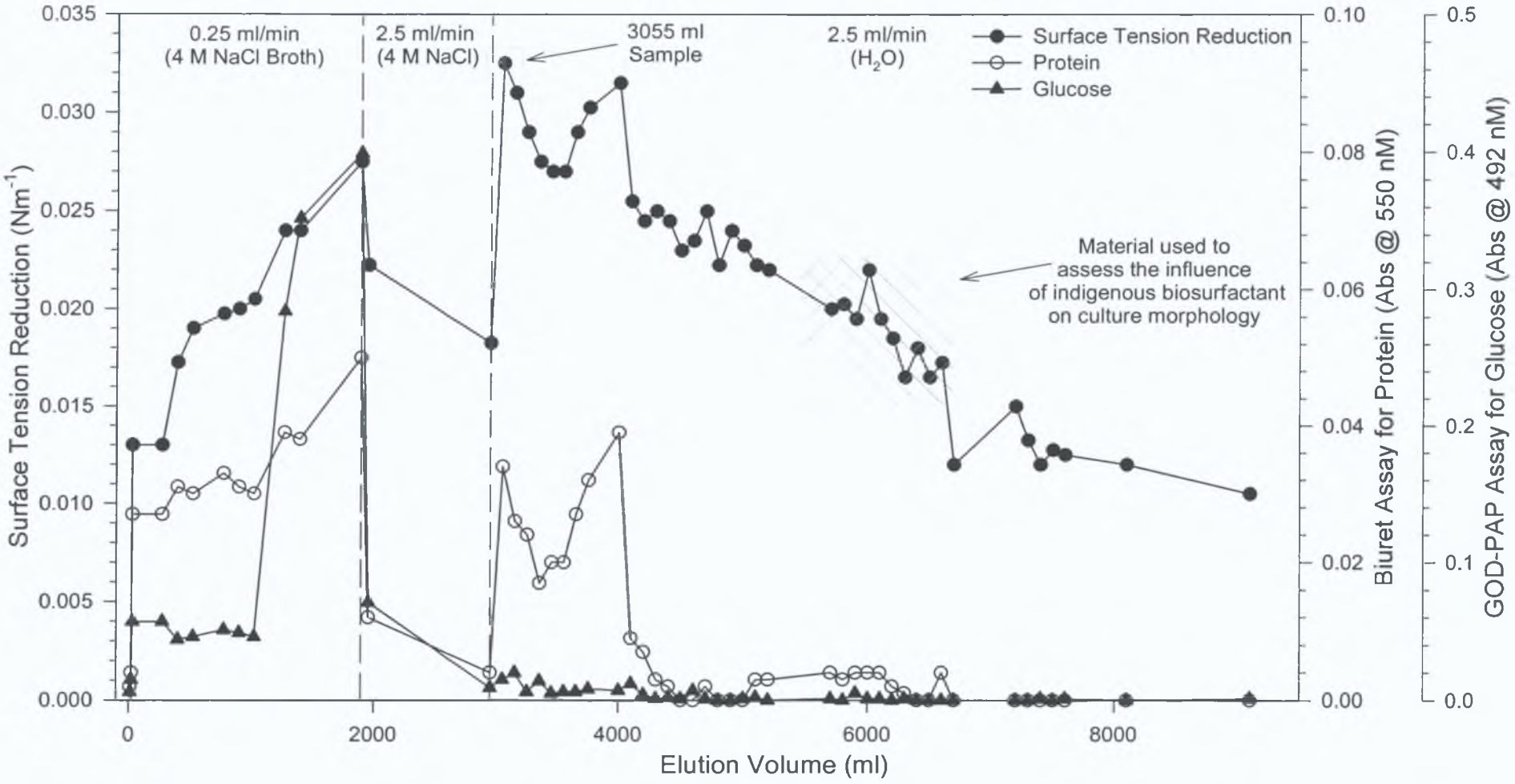


Figure 5.19. Hydrophobic interaction chromatography using 1895 ml of a cell-free day 12 *S. hygroscopicus* broth sample on a Phenyl-Sepharose column, using 4 M NaCl solution to bind / wash and H_2O to elute.

Optimisation of the Sephadex G-25 system involved altering the residence time of the sample on the column, which was achieved by controlling the column length and the mobile phase flowrate. In order to achieve better separation the cell free broth samples were run on a 75 cm column with varying flowrates. Increasing column length from 20 cm to 75 cm and decreasing the sample flowrate increased residence time and facilitated a greater separation of small molecular weight material. Gel filtration chromatography on the systems described provides good compound separation from the large molecular weight material such as polysaccharides and proteins as demonstrated in Figure 5.20.

1 ml of the selected sample, from the hydrophobic interaction chromatography column, was applied to two gel filtration systems, Sephadex G-25 and Sephadex G-10, for further purification as illustrated in Figure 5.20. Both systems succeeded in removing a high proportion of the contaminant material and purifying the biosurfactant further. The G-10 column, with a molecular weight fractionation range of <700, provided better separation than the G-25 column, highlighting the presence of a single biosurfactant peak at 120 ml and a cluster of peaks from 670 to 810 ml. The reduction in surface tension of *S. hygroscopicus* fermentation cultures appears to be due to a complex mixture of many different low molecular weight biosurfactants.

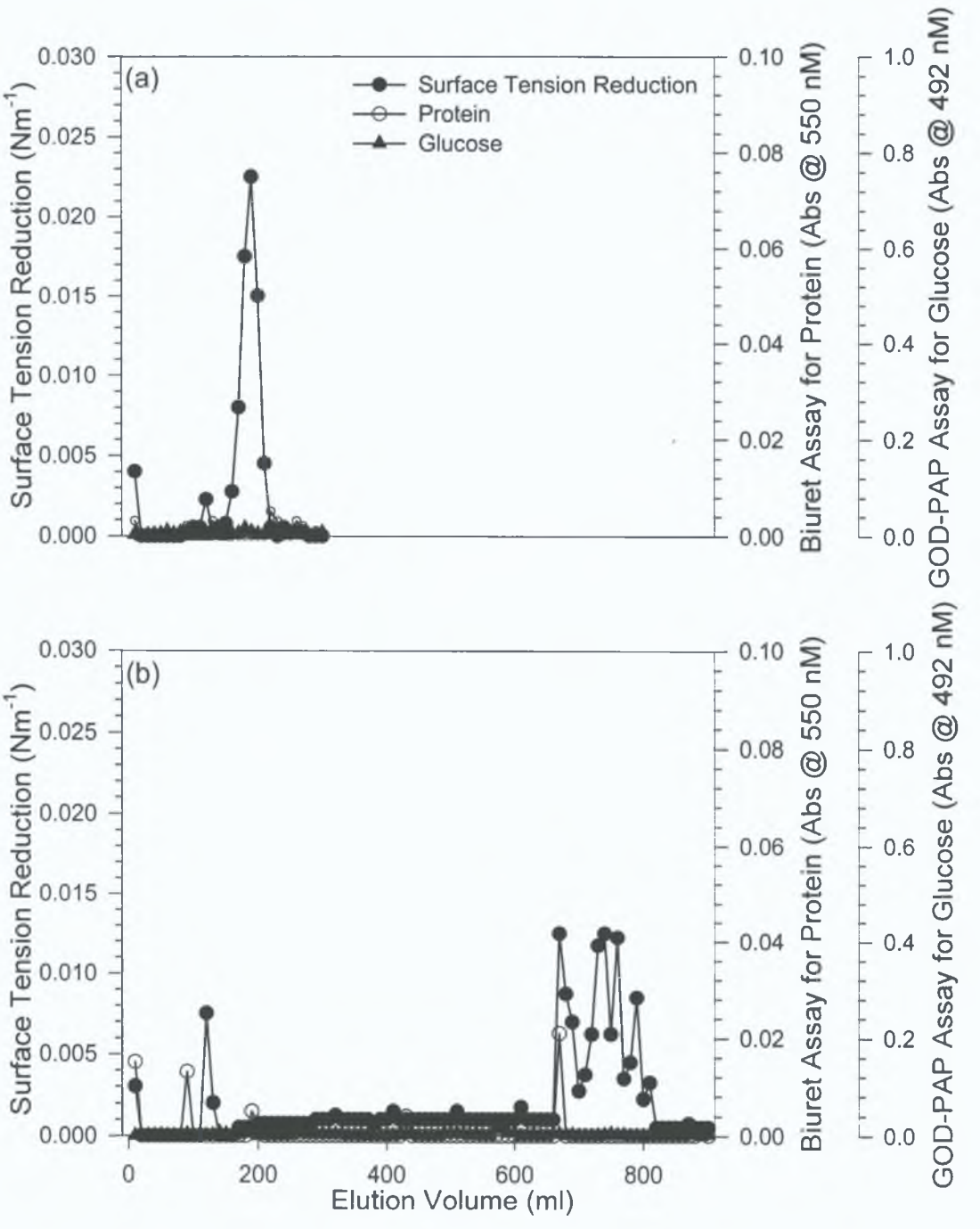


Figure 5.20. Gel filtration chromatography of the 3055 ml hydrophobic interaction column sample on (a) Sephadex G-25 (molecular weight fractionation range 100-5000) and (b) Sephadex G-10 (molecular weight fractionation range <700) in a 75 cm length column at a flowrate of 0.25 ml min^{-1}.

The identification and purification of the novel biosurfactant streptofactin, produced by the filamentous bacteria *S. tendae*, has been achieved by

Richter *et al.* (1998). Streptofactin is a mixture of structurally related extracellular hydrophobic peptides, which is believed to be responsible for regulating the production of aerial mycelium in *S. tendae* colonies grown on solid medium. The purification of streptofactin by Richter *et al.* (1998) involved extraction from agar into acetone, rotary evaporation to concentrate the solution, and lyophilization to remove residual liquid. The resulting crude extract was redissolved in distilled water and ammonium sulphate was added to precipitate the compound, which was subsequently separated from the solution by centrifugation. Another biosurfactant molecule involved in the regulation of aerial mycelium formation is the hydrophobic protein SapB, which has been shown by Tillotson *et al.* (1998) to be involved in the erection of aerial structures by *S. coelicolor*. The purification of SapB by Tillotson *et al.* (1998) involved centrifugation, removal of the hydrophobic material and allowing it to air dry before solubilising it in 100% tri-flouroacetic acid. The resulting solution was transferred to a glass tube and N_2 gas was bubbled through it to evaporate the tri-flouroacetic acid, with the resulting material subsequently solubilised in either water or PBS buffer. The mode of action of biosurfactants in regulating the formation of aerial mycelia is believed to be through the reduction of surface tension at the colony-air interface, which is thought to facilitate the upward growth of mycelia from the colony surface (Willey *et al.*, 1991). As with all separation processes biosurfactant recovery is based on knowledge of the compound, identification of a distinctive molecular structure or biochemical trait and the exploitation of these to isolate the compound of interest from contaminant material. In the examples given above the authors used this approach to devise system specific purification processes suited to compounds of interest.

In Figure 5.19 the material from approximately 5.5 L onwards has a low concentration of biosurfactant but it also has a much lower concentration of contaminants. This material was used in the subsequent investigation into the impact of the indigenous biosurfactant on culture morphology in section 5.6.

The isolation and purification of the indigenous biosurfactant compound is necessary to obtain sufficient quantities to facilitate further investigation. A combined two-step process incorporating both hydrophobic interaction and gel filtration chromatography provides a powerful purification tool. The major drawback of the two-stage process is that although it provides good purification it is of limited applicability due the bottleneck of sample throughput on the gel filtration columns and as such is purely for analytical purposes. This combined approach did highlight the presence of multiple potential biosurfactant compounds in solution. In order to purify sufficient material for further investigation into the morphological auto-regulation of *S. hygroscopicus* by an indigenous biosurfactant(s), a single hydrophobic interaction chromatography step must be used, which removed in excess of 92% of proteinaceous material and 99% of glucose. The resulting material will facilitate the preparation of fresh nutrient broth as seen in section 5.6. The incorporation of this material will regulate broth surface tension.

5.6. AN ASSESSMENT OF THE INFLUENCE OF INDIGENOUS BIOSURFACTANT ON CULTURE MORPHOLOGY

In section 5.4 the production of a biosurfactants by *S. hygroscopicus* has potentially been shown to be morphologically related, with pellets of a specific size thought to be associated with the production of compounds of interest. In section 5.3 the addition of chemical surfactants to the fermentation broth was shown to control culture morphology through the regulation of particle interaction and aggregation. This regulatory capacity of surfactants, coupled with the fact that the organism produces its own indigenous compound early in the fermentation has lead to the hypothesis that biosurfactant synthesis may potentially be a morphological auto-regulation mechanism. In order to ascertain the morphological impact of one or more of the biosurfactant compounds their efficacy with respect to a control system must be assessed. Given that the compounds in question are indigenous to the organism, the only time they are not present in the system is before they have been produced, i.e. 0 – 72 hours after inoculation, as

shown in Figure 5.10. Based on previous work this time period appears to be the most sensitive for morphological regulation. Further investigation into the potential morphological auto-regulation of *S. hygroscopicus* by indigenously produced biosurfactant molecules requires the isolation and purification of one or more of these compounds for inclusion in fresh nutrient broth prior to inoculation. The biosurfactant material used was taken from the latter stages of a hydrophobic interaction chromatography process (section 5.5) and contained sufficient quantities of biosurfactant for our purposes with only residual contaminant material.

The biosurfactant material from the hydrophobic interaction chromatography step had a surface tension of 0.0525 Nm^{-1}. This solution formed the basis for the fermentation broth. Bennett's nutrient medium acted as the control for this experimental procedure and the proportion of biosurfactant material added to each of the nutrient broths is as given in Table 5.6, for example Broth 1 contained 1 part biosurfactant material to 5 parts distilled water. The same amount of the requisite nutrient material was added to each of the solutions prior to autoclaving, as described in section 2.2.1. The surface tension of the resulting broths is illustrated in Figure 5.21. The experimental procedure performed involved culturing 100 ml of the relevant broth in a 250 ml Erlenmeyer flask with 1% (v/v) of 2 x 10^6 spores ml^{-1} *S. hygroscopicus* spore suspension and incubating on an orbital shaker at 150 rpm and 28°C. The duration of the experimental procedure was 7 days, with sampling for image analysis purposes occurring on the third, fifth and seventh day and sampling for dry weight of biomass determination occurring on the seventh day. A single set of three flasks was prepared for each agitation speed and was used throughout the experimental time period for the aforementioned sampling. All data shown is based on that obtained from the set of three replicate flasks.

Table 5.6. Ratio of biosurfactant containing material to distilled water in the nutrient media prepared for the investigation of the influence of indigenously produced biosurfactant on culture morphology.

Fermentation Media	Ratio of Biosurfactant Material : Distilled Water
Control (Bennett's)	0 : 6
Broth 1	1 : 5
Broth 2	2 : 4
Broth 3	3 : 3
Broth 4	4 : 2
Broth 5	5 : 1
Broth 6	6 : 0

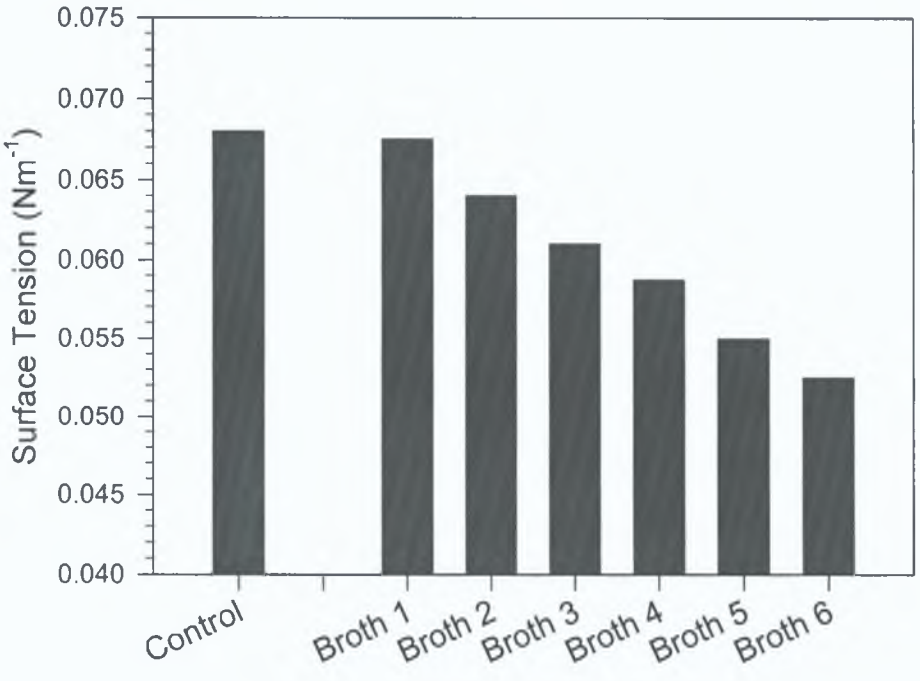


Figure 5.21. Surface tension of fermentation broths prepared with biosurfactant material prepared using hydrophobic interaction chromatography.

The introduction of the indigenous biosurfactant material has a marked influence on the morphology of the resulting cultures. As illustrated in Figure 5.22 decreasing broth surface tension resulted in a decreasing pellet count and an increasing mean pellet volume, for broths 1, 2 and 3 with respect to the control. This suggests that decreasing surface tension in this range, 0.060 – 0.068 Nm^{-1} is responsible for regulating particle interaction and subsequent aggregation to produce a population containing a small number of uniformly sized pellets within the first 72 hours. The subsequent development of these cultures, as illustrated by the relative stability within the pellet counts and a large increase in the mean pellet volume, indicates that the population as a whole is enlarging. Broths 4, 5 and 6 do not behave in a similar manner, which was subsequently shown to be a toxicity issue.

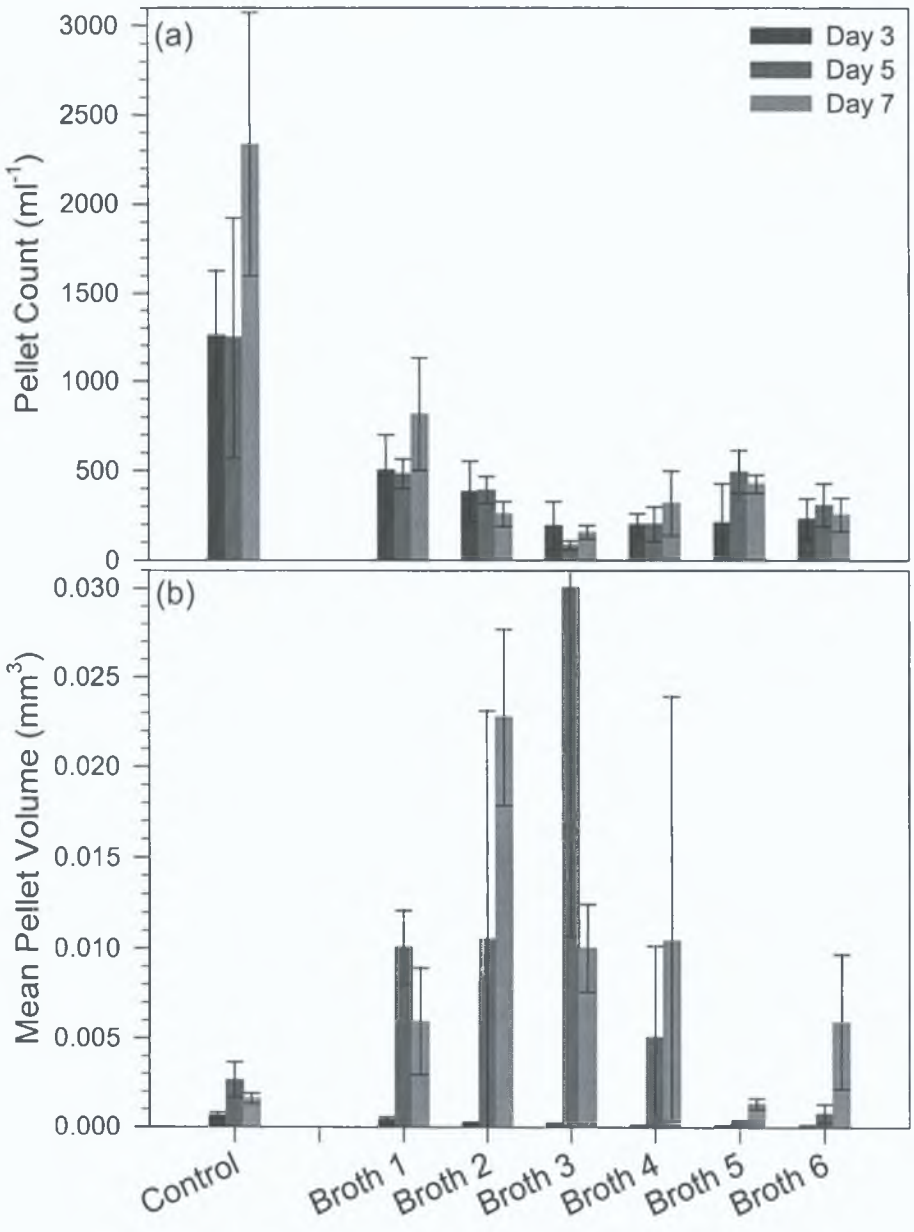


Figure 5.22. The development of the morphological parameters (a) pellet count and (b) mean pellet volume in artificially regulated surface tension cultures over 7 days.

The size distribution profiles, as illustrated in Figure 5.23, demonstrate the morphological impact of altering the broth surface tension by using the indigenously produced biosurfactant compounds. As previously stated for Figure 5.22 the introduction of a biosurfactant alters the morphological behaviour of the organism compared to that observed in the control flasks. Decreasing broth surface tension results in a narrower population distribution

consisting of a smaller number of uniformly-sized pellets in each of the broths at day 3. The morphological and physiological development of these cultures from day 3 to 7 involves the continual enlargement of a single population of pellets in comparison to the bi-modal distribution observed in the control. The presence of indigenous biosurfactant in fresh nutrient broth increases the rate of particle interaction and subsequent pellet formation thus influencing culture morphology.

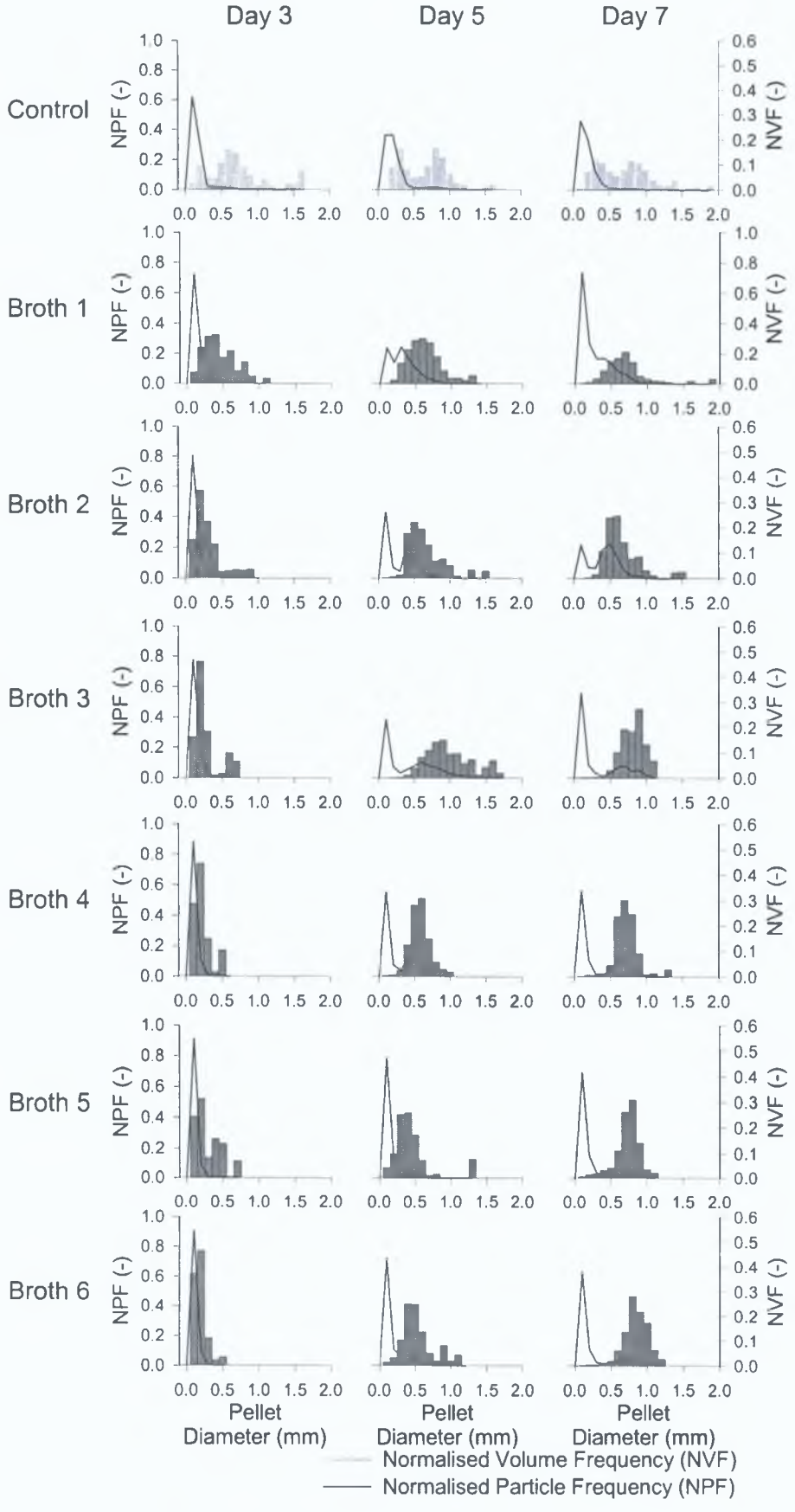


Figure 5.23. Normalised volume and particle frequency distributions for *S. hygroscopicus* in artificially regulated surface tension cultures over 7 days.

The addition of the indigenous biosurfactant into the fermentation broth also influences the production of biomass within the cultures as shown in Figure 5.24. In general, an increasing proportion of the biosurfactant material results in reduced biomass production, indicating potential retardation of *S. hygroscopicus* growth within these cultures, which highlights the presence of a toxic component. A number of the biosurfactant compounds are low molecular weight organic molecules. Many antibiotic compounds also fall into this category; indeed *S. hygroscopicus* is noted for producing a range of these compounds (Deboer & Dietz, 1976). The authors note that the organism produces the antibiotic compounds Geldanamycin, Nigericin, Nocardamine and another unidentified compound similar to Libanamycin. Gel filtration chromatography performed in section 5.5 indicated the presence of multiple biosurfactant compounds in the eluent from the hydrophobic interaction chromatography step. It is thought that one or more of these compounds has been separated from the original broth, carried through and may subsequently be inhibiting biomass growth.

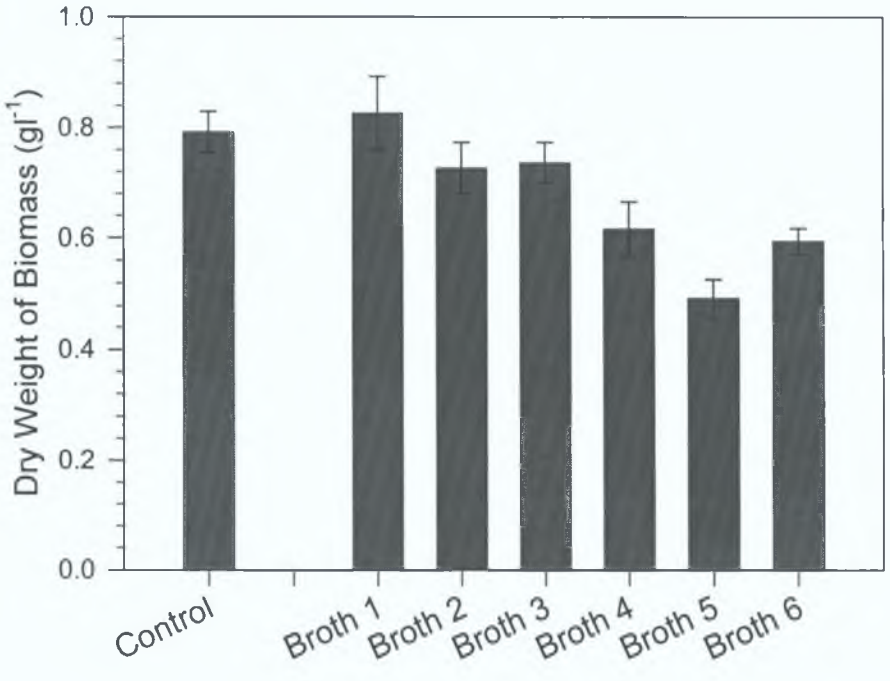


Figure 5.24. The impact of artificially regulating broth surface tension on *S. hygroscopicus* dry weight of biomass concentration after 7 days.

The presence of indigenous biosurfactant material in the nutrient medium is responsible for controlling broth surface tension, which in turn regulates the morphological and physiological development of *S. hygroscopicus* cultures. The microorganism produces compound(s) that are capable of regulating pellet morphology within submerged fermentations. It is thought to be likely that the production of such compounds by the organism may be a morphological auto-regulation mechanism.

5.7. CONCLUSIONS

The cultivation of filamentous bacteria is of particular interest given the ability of these microorganisms to produce a wide variety of commercially valuable secondary metabolites including compounds such as Geldanamycin, a product of the actinomycete *Streptomyces hygroscopicus* var. *geldanus* (Deboer et al., 1970; Deboer & Dietz, 1976). The morphological profile of filamentous microorganisms frequently influences productivity with compound formation often taking place within pellets of a given size or density (Braun & Vechtlifshitz, 1991; Vechtlifshitz *et al.*, 1992). The formation and development of pellets within cultures of many filamentous microorganisms is dependent not only on hyphal extension through apical and branched growth but also through hyphal aggregation. The aggregative processes that occur are thought to be as a result of cell-to-cell interactions that are typically due to the presence of exopolymeric substances, hydrophobicity and/or charge of the cell surface (Atkinson & Daoud, 1976). Alteration of certain fluid attributes has been proposed as a potential control mechanism to regulate particle aggregation, in particular manipulation of broth viscosity and surface tension. The introduction of a viscous agent into solution will modify bulk fluid flow and thus alter the motion of particles contained within it, thereby affecting particle collision. The introduction of a surfactant into solution will modify the broth surface tension and thereby change the interactions experienced by hydrophobic biomass within the aqueous system. The alteration of fluid attributes and its knock-on impact on particle interaction should theoretically

influence particle aggregation and hence pellet formation thereby regulating culture morphology.

Fermentation broths are typically Newtonian fluids whereby the viscosity remains constant regardless of shear rate or time. Non-newtonian behaviour in fermentation broths is normally the result of the inclusion of a viscous nutrient compound (Cho *et al.*, 2002; Choi *et al.*, 1998; Sinha *et al.*, 2001), the production of an extracellular viscous agent (Garcia-Ochoa *et al.*, 2000) or the biomass itself (Choi *et al.*, 2000; Riley *et al.*, 2000), all of which have been shown to alter the morphological development of the filamentous cultures. Altering broth rheology, through the introduction of viscous agents, has been shown by a number of authors to regulate the morphological development of filamentous microorganisms (Corman, 1959; Hobbs *et al.*, 1989; Hodgson, 1982). In the work reported the addition of varying concentrations of the microbial polysaccharide Xanthan gum in fermentation media was shown to be responsible for altering broth rheology and inducing pseudoplastic behaviour in the fluid. The inclusion of this compound and its corresponding impact on broth apparent viscosity influenced the morphological development of *S. hygroscopicus* cultures. At the optimum concentration of 3 gl^{-1} the pellet size distribution profiles indicated that increased apparent viscosity resulted in the formation of a population, containing a larger number of smaller pellets, with increasing biomass concentration. Along with culture homogeneity lower flask-to-flask morphological variability was also observed. Introduction of a viscous agent into the solution increases the resistance to motion within a fluid, thereby altering the flow characteristics and decreasing turbulence, which in turn is thought to decreases random particle collision and subsequent aggregation.

Introduction of the viscous agent xanthan gum into fermentation broth alters fluid flow and mixing. The presence of this compound in a nutrient broth within a stirred tank reactor has previously been shown to reduce the gas-liquid mass transfer coefficient (Garcia-Ochoa & Gomez, 1998; Garcia-Ochoa *et al.*, 2000). However on closer examination of this phenomenon within shake flask cultures a different result was obtained. Counter intuitively

it was observed that increasing apparent viscosity increased the gas-liquid mass transfer coefficient up to a maximum at 2 gl^{-1} Xanthan gum. Given that the mechanisms of gas-liquid mass transfer are different in both stirred tank reactors (Garcia-Ochoa & Gomez, 1998; Garcia-Ochoa *et al.*, 2000) and static solutions (Ho *et al.*, 1988) it is hypothesized that the process of gas-liquid mass transfer in agitated shake flasks is diffusion based. An increase in broth viscosity is not only responsible for regulating particle aggregation, but also gas-liquid mass transfer within shake flasks, both of which are believed to be responsible for the elevated levels of biomass in cultures supplemented with xanthan gum. The regulation of broth apparent viscosity through the addition of a non-Newtonian agent, such as xanthan gum, may be used to morphologically and physiologically engineer submerged cultures of filamentous organisms.

Another parameter identified as a potential target for investigation was broth surface tension. The inclusion of three different chemical surfactant molecules, Tween 80, Triton X100 and Silicone antifoam, at a range of concentrations enabled the regulation of broth surface tension. The incubation of the organism in the presence of these three surfactants resulted in variations in the morphological profile of the organism. Each of the three compounds altered the culture morphology with respect to both the control and each other. Tween 80 acts has a flocculative effect and produces cultures predominantly made-up of small numbers of large pellets while Triton X100 and Silicone Antifoam produce cultures containing populations of small to medium sized pellets. These compound specific profiles are thought to be as a result of the different modes of action of each of the surfactants and the way in which they influence particle interaction and thus pellet formation. As well as compound specificity, concentration specificity is also observed whereby the morphological impact of these compounds is seen to be dependent on the amount present. A decrease in concentration results in a decrease in morphological control and a return to control-like behaviour. The identification of an effective range and optimal concentration for each of the surfactants will aid in maximising the desired morphological and physiological affect. Reducing surfactant concentration will increase the broth

surface tension, which in turn increases particle hydrophobicity and the incidence of interaction thereby influencing pellet formation.

Although demonstrated to be at its most potent in the first 72 hours of the culture the influence of surfactant addition on the morphological profile of *S. hygroscopicus* cultures is observed throughout the experimental time period. The inclusion of these compounds in the fermentation media influences not only the aggregation of hyphal elements in the initial stages of pellet formation but also the continual development of pellets in the later stages of the fermentation. The system will tend to return to control-like behaviour as the cultures mature, regardless of which surfactant is used or at what concentration it is added. The reduced impact of these compounds in the latter stages of fermentation is thought to probably be as a result of increased biomass concentration, increased particle size due to hyphal growth and the increasing influence of bioprocessing parameters such as agitation. The addition of surfactants is responsible for reducing broth surface tension, decreasing biomass hydrophobicity and regulating particle aggregation and pellet formation in filamentous cultures. The regulation of particle aggregation in this manner is not only responsible for the type of pellets that are formed but also the manner in which it happens. Pellet formation in shake flask cultures of *S. hygroscopicus* has been shown in section 3.5 to be a disordered affair resulting in a high degree of flask-to-flask variability. The inclusion of surfactants in the fermentation media prior to inoculation regulates the inherent variability in pellet formation and results in increased homology within the flask population.

The addition of chemical surfactants to the fermentation media as a means of regulating broth surface tension and hence hydrophobic interactions between biomass particles was decided upon after researching the fields of fluid mechanics and cell surface biology. The inclusion of these molecules in the fermentation broth has been shown to influence culture development. However it appears that the organism may have already been exploiting this mechanism for the same purpose. *S. hygroscopicus* has been shown to produce a biosurfactant(s) during the course of the experimental procedure.

Furthermore, it has been noted that sample-to-sample variability tended to reduce in the latter stages of the culture, possibly as a result of the presence of this compound(s). It is hypothesized that the organism produces this compound(s) as a morphological auto-regulatory mechanism.

The morphological variability in early stage culture development, as shown in section 3.5, was accompanied by variability in the broth surface tension indicating differential levels of compound formation. This variability of both the culture and the compound it produced lead to the speculation of the potential existence of a relationship between culture morphology/physiology and compound formation. No correlation was observed between biosurfactant production and either biomass concentration or pellet count, but one did appear to exist with the mean pellet volume, a crude measurement of culture morphology. On closer inspection, it was observed that a higher incidence of biosurfactant production occurred in cultures with increased mean pellet volumes and biomass concentrations. Further examination indicated that cultures containing a higher proportion of pellets in the size range of 0.3 – 0.7 mm diameter also had a higher incidence of biosurfactant, which lead to the hypothesis that production may possibly be morphologically dependent. The morphological dependence of compound production is not uncommon for filamentous microorganisms (Vechtlifshitz *et al.*, 1992) and is often brought about by mass transfer limitations and the resulting cellular differentiation that it incurs (Nielsen & Carlsen, 1996). Given that the production of this compound(s) is thought to be morphologically dependent this highlights the possibility that compound formation may potentially be a means of morphological auto-regulation.

In order to assess the morphological impact of the indigenously produced biosurfactant(s) molecules it must first be separated before being used to make fresh media. Fermentation broths contain a complex mixture of compounds from which the specific compound(s) of interest was isolated. Utilisation of both hydrophobic interaction and gel filtration chromatography techniques proved useful in the separation of these biosurfactant molecules. Extensive optimisation was achieved and in excess of 92% of proteinaceous

material and 99% of glucose was removed resulting in relatively pure solutions containing the compound(s) of interest. The presence of multiple different biosurfactant molecules was highlighted. Utilisation of the hydrophobic interaction and gel filtration techniques in series provided a much-improved degree of separation. The separation and purification of a component molecule from a crude fermentation broth is not a simple process, but in this case it has been made harder due to the presence of multiple compounds of unknown molecular structure, all of which are responsible for reducing surface tension. Further investigation and process optimisation remains to be done in order to isolate and purify each of the compounds of interest. The hydrophobic interaction chromatography column provided material of sufficient purity and surface tension reducing potential for subsequent incorporation into fresh nutrient media in order to assess the morphological impact of the indigenously produced biosurfactants.

The utilisation of the purified material from the hydrophobic interaction chromatography step enabled the artificial regulation of broth surface tension using the indigenously produced biosurfactant. The presence of this material was responsible for regulating culture morphology and physiology thereby indicating that compounds contained within this mixture are responsible for regulating the organism that produces them. Thus given the proposed relationship between culture morphology and biosurfactant production and ability of these indigenous compounds to regulate culture morphology it is thought that this system may be a means of morphological auto-regulation.

The process of pellet formation in submerged fermentations of filamentous microorganisms is partly dependent on cellular aggregation, which is in turn a function of particle collision and interaction and cell surface properties. Given the dependence of the aggregative process on particle collision and the subsequent cell surface interactions the identification of the fluid mechanics properties of viscosity and surface tension as potential control strategies was made. The artificial control of broth viscosity using Xanthan gum and surface tension using Tween 80, Triton X100 and Silicone Antifoam enable the regulation of particle collision and interaction respectively. Both approaches

allowed for the morphological engineering of *S. hygroscopicus* cultures. The identification and subsequent separation of an indigenously produced biosurfactant, the formation of which is thought to be morphologically dependent, enabled the assessment of its impact on the morphological development of its parent organism. It is proposed that *S. hygroscopicus* is capable of morphological auto-regulation.

CHAPTER 6 – SUMMARY AND RECOMMENDATIONS FOR FUTURE WORK

6.1. SUMMARY

The genus *Actinomyces* is an important group of microbes due to ability of many of its members to produce commercially valuable secondary metabolites. Product formation in submerged fermentations of filamentous microorganisms is often not only dependent on the level of biomass present but also the morphological profile of the culture (Braun & Vechtlifshitz, 1991; Vechtlifshitz *et al.*, 1992), necessitating a better understanding of the mechanisms of pellet formation and development. Conventional microscopic image analysis methods have traditionally provided a valuable, though limited, toolset for the morphological assessment of filamentous fermentation broths (Paul & Thomas, 1998). Nielsen (1996) concluded that there was a need for the development of image analysers that allow for the rapid quantification of large populations of pellets in order to assess the growth kinetics of filamentous microorganisms.

The development of an image analysis technique has enabled the high throughput morphological characterisation of *S. hygroscopicus* pelleted fermentation samples, at a rate hitherto difficult using conventional microscopic imaging methods. The availability of high performance desktop scanners has allowed for a cost effective image acquisition system for images of moderate resolution and large field of view. This combined with the advent of high performance desktop computing has facilitated the handling, processing and analysis of such images. The subsequent validation of this technique using 109 µm and 644 µm NIST traceable size calibration particles demonstrated the reproducibility of the method to within 5%. Once validated this technique was utilised for the morphological quantification of *S. hygroscopicus* fermentation samples and was demonstrated to be applicable in the quantification of culture morphology. The technique described, although developed for the study of pelleted fermentation broths, is

applicable for any procedure that necessitates the macroscopic image analysis of non-translucent spherical particles of diameter greater than 100 µm.

The image analysis technique was applied to the morphological quantification of *S. hygroscopicus* fermentation samples. The degree of pellet count, mean pellet volume, biomass concentration and pellet size distribution variability within a population of identical flasks was highlighted. The flasks were prepared using the identical Bennett's nutrient medium, inoculated with identical spore stock and incubated on identical shaker tables in the same warm room. The cause of this flask-to-flask variability is not fully understood, however, it is thought that this variation is as a result of a random process of particle collision, interaction and aggregation, leading to variable pellet formation. The inherent flask-to-flask variability in this system highlights the requirement for a sufficient experimental sample size to obtain as accurate statistical representation of culture morphology and physiology as possible. Enlargement of the sample size to fifty flasks enabled the accurate demonstration of the true extent of morphological and physiological variability. However, continuously performing experiments of this scale is prohibitively unfeasible given the constraints of equipment and manpower in multi-parameter timecourse experiments. In this case a finite selection of specimens from the population must be used to determine the most statistically accurate representation of the whole population possible. A sample size of five flasks provided an experimental outcome to within ± 12% of the population mean, an acceptable approximation.

The growth of filamentous microorganisms is greatly influenced by the environmental and nutritional conditions experienced, especially the nutrient medium composition (Glazebrook *et al.*, 1992; Kojima *et al.*, 1995), the type and concentration of inoculum used (El-Enshasy *et al.*, 2000; Vechtlifshitz *et al.*, 1990) and the shake flask agitation speed (Amanullah *et al.*, 2001; Casas Lopez *et al.*, 2005). An investigation into the impact of bioprocessing parameters on pellet formation illustrates the potential for regulation of the morphological and physiological development of *S. hygroscopicus* cultures. A

direct comparison of Bennett's and YEPD fermentation media demonstrated a nutrient dependence on pellet formation and biomass growth with Bennett's being judged the more interesting of the two, given the increased morphological heterogeneity present. The different morphological profiles between Bennett's and YEPD are thought to be due to increased growth rate and/or alterations in cell surface properties responsible for aggregation. Inoculum loading density was also illustrated to influence pellet formation, most likely through regulation of the frequency of collision, upon which subsequent aggregation is based. An inverse relationship was demonstrated to exist between spore concentration and culture morphology, whereby, increasing the spore concentration resulted in a population containing a larger number of smaller pellets, a trend previously reported for S. tendae by Vechtlifshitz *et al.* (1990). Furthermore, the organism was shown to be capable of overcoming variations in loading density to produce cultures of comparable biomass levels after 7 days. The morphological profile of cultures by day 3 governed the subsequent development in the remainder of the fermentation.

The frequency of collision is thought to depend not only on the number of particles present in the system but also the rate at which they are moving, which is a function of agitation speed. An investigation into the impact of agitation on morphology revealed it influences both particle aggregation and fragmentation. It is thought to do this through the regulation of the frequency of particle collision and the collision energy, upon which subsequent aggregation is based. In this case also, an inverse relationship was shown to exist between increasing agitation and decreasing pellet size distributions. Culture similarity at day 3 had given way to different morphological profiles by day 7 indicating that agitation has a greater influence on the development in the latter stages. Each of the three bioprocessing parameters was shown to influence the development of *S. hygroscopicus* in a different manner. A set of standard operating conditions incorporating Bennett's media, inoculated with 10^6 CFU ml^{-1} and agitated at 150 rpm provides a model system for further study. Under these conditions culture development was shown to be constantly changing with pellets thought to be continuously forming,

enlarging and disrupting through aggregation, hyphal growth and fragmentation. The size distribution profiles provided a good representation of the overall morphology of these cultures throughout the 21 day fermentation and illustrated these processes. The growth of *S. hygroscopicus* in Bennett's nutrient broth was shown to be diauxic with the organism preferentially consuming complex substrates for biomass production followed by a switch to glucose metabolism. An investigation into the influence of certain bioprocessing parameters, such as medium composition, inoculum concentration and shake flask agitation speed, on culture development has enabled the assessment of traditional biochemical engineering approaches to morphological regulation. The study of the organisms' lifecycle has provided invaluable information regarding behaviour under the defined standard operating conditions. This system formed the basis for all subsequent morphological engineering investigations.

Morphological engineering through the regulation of pellet formation and development enables the generation of a defined particle size distribution within cultures of filamentous microorganisms. Pellet formation and development is dependent not only on the mechanisms of hyphal extension but also hyphal aggregation and/or entanglement (Vechtlifshitz *et al.*, 1990). The aggregative processes that occur are thought to be as a result of cell-to-cell interactions due to the presence of exopolymeric substances, hydrophobicity and/or charge of the cell surface (Atkinson & Daoud, 1976). Pellet formation has been proposed to be dependent on particle collision, interaction and aggregation/entanglement. The manipulation of fluid mechanics have been identified as a potential control mechanism to regulate particle aggregation, in particular broth viscosity (Corman, 1959; Hobbs *et al.*, 1989) and surface tension (Dynesen & Nielsen, 2003; Vechtlifshitz *et al.*, 1989).

Pellet formation and development through aggregation relies primarily on the collision of particles within the fermentation broth. The viscosity of a solution governs the resistance to motion within the fluid and in the case of the biological system in question the motion of particles in solution. The addition

of the viscous agent xanthan gum to the fermentation broth increased the resistance to motion within the fluid, thereby altering the flow characteristics and decreasing turbulence, which in turn is thought to decrease the degree particle collision. The inclusion of varying concentrations of this compound and its corresponding impact on broth apparent viscosity, influenced the morphological development of *S. hygroscopicus* cultures through regulation of pellet formation. Similarly, Corman (1959) used nutrient thickening agents to increase the apparent viscosity of *Blakeslea* and *Choanephora* cultures, which in turn prevented clumping and regulated the extent of pellet formation. An increase in broth viscosity was shown to be not only responsible for regulating particle aggregation, but also the gas-liquid mass transfer coefficient within shake flasks. Artificially regulating broth apparent viscosity was shown to be capable of morphologically and physiologically engineering submerged cultures of S. hygroscopicus.

Once particle collision has occurred, the process of aggregation then becomes dependent on cell surface attractive forces such as hydrophobic interactions (Dynesen & Nielsen, 2003), which are governed by the broth surface tension (Vechtlifshitz *et al.*, 1989). The inclusion of three different surfactant molecules, Tween 80, Triton X100 and Silicone Antifoam, at a range of concentrations, enabled the regulation of broth surface tension. The incubation of the organism in the presence of these three surfactants resulted in variations in the size distribution profiles of the organism with each being responsible for altering culture morphology with respect to both the control and each other. The impact varied not only from one compound to another but also for different concentrations of the same compound. Surfactant molecules are capable of regulating broth surface tension and hence the interaction of hydrophobic particles, which, in turn, influences the aggregation of hydrophobic biomass particles throughout the fermentation resulting in decreased flask-to-flask variability and greater culture homogeneity. Artificially regulating broth surface tension was shown to be capable of morphologically and physiologically engineering submerged cultures of S. hygroscopicus.

The addition of chemical surfactants to the fermentation broth has been demonstrated to be capable of morphologically regulating *S. hygroscopicus* cultures. The organism also produces its own indigenous biosurfactant during the course of the fermentation process. The production of this compound thought to be morphologically dependent. The crude purification of the fermentation broth using gel filtration and hydrophobic interaction chromatography columns revealed the presence of many different compounds capable of reducing broth surface tension, most of whom are thought to be low molecular weight organic molecules. The re-introduction of purified material into fresh nutrient broth prior to inoculation facilitated the investigation into the impact of this indigenous compound on the morphology of its parent organism. The biosurfactant material was responsible for altering culture morphology thus leading to the hypothesis that these compounds are produced as a means of morphological auto-regulation.

6.2. OVERALL CONCLUSIONS

To propose a hypothesis for the formation and development of *S. hygroscopicus* pellets all the evidence gathered in this work has to be examined carefully. Firstly, filamentous organisms will always tend to form pellets through the three dimensional nature of apical and branched growth. The evidence from the inoculation concentration experiment suggests that at low seeding densities aggregation does not occur and initial pellet formation arises from a single spore. However, increased loading densities demonstrate that if sufficient particles are present they will aggregate, with increased concentration comes increased aggregation. Increased inoculum concentration and the concomitant increase in aggregation results in the formation of pellets over a broader morphological range in a shorter time period. In Bennett's cultures under the standard operating conditions approximately 90% of the spores aggregate. This high degree of aggregation is thought to be responsible for the degree of morphological variability observed in Bennett's cultures. Furthermore, given the high degree of particle aggregation in this system a 10% reduction in the numbers of particles aggregating from 10 spores per pellet to 9 spores per pellet would result in a

100% increase in the numbers of pellets formed from approximately 2000 to 4000 pellets ml^{-1}, which may be responsible for the degree of flask-to-flask variability observed.

The process of particle aggregation is thought to depend on particle collision and subsequent interaction. Many attempts have been made to control this process concentrating primarily on regulating the frequency of particle collision and once collision has occurred regulating cell surface interaction. The regulation of particle collision frequency has been achieved in two ways. Firstly, by varying inoculum concentration and hence the number of particles in the system and secondly, by altering broth viscosity and hence decreasing turbulent fluid flow it has been possible to regulate the incidence of particle collision. Both of these approaches significantly influenced pellet formation and development. Once collision has taken place particles either entangle or interact due to cell surface properties such as hydrophobicity, cell surface charge and the presence of exo-polymers. Each of these result in the formation of a temporary aggregate that may subsequently develop and become permanent as a result of hyphal intertwining. The regulation of cell surface interactions, namely hydrophobicity has been attempted using surfactants in order to control broth surface tension. Each of the compounds used induced a different morphological impact with the most notable being Tween 80, which acted as a flocculating agent and increased particle aggregation. Neither Triton X100 nor Silicone Antifoam succeed in reducing the extent of particle aggregation, but they did succeed in regulating the chaotic nature of interaction resulting in the formation of cultures with a greater degree of homogeneity in the pellet population.

The regulation of particle collision frequency afforded greater morphological control than regulation of cell-to-cell hydrophobic interactions indicating that the former is more fundamental to the process of aggregation. Given the dependence of aggregation on particle collision frequency it is suggested that this process is initially mechanically driven. The investigation into the influence of shake flask agitation demonstrated that increased shear was responsible for disrupting temporary aggregates and resulting in a decreased

CFU to pellet ratio. Increasing agitation not only disrupts the formation of temporary aggregates, but is also responsible for an increased incidence of particle fragmentation.

The experimental work described in this thesis has aided in the quantification, regulation and morphological engineering of pellet formation and development in *Streptomyces hygroscopicus* var. *geldanus* fermentations. It is possible to use these various approaches to generate morphologically defined cultures of the organism in question. Although particle aggregation was not fully prevented by any of the methods investigated, it was, to a certain degree controlled.

6.3. RECOMMENDATIONS FOR FUTURE WORK

6.3.1. Influence of culture morphology on product formation

The development, validation and application of an image analysis technique in chapter 3 demonstrated the usefulness of this method in the morphological quantification of pelleted fermentation broths. Product formation in submerged fermentations of filamentous microorganisms is often dependent on the morphology of the biomass population. The application of this technique in conjunction with compound production profiling will enable direct comparisons of culture morphology and productivity. *S. hygroscopicus* is responsible for manufacturing many compounds of interest such as Geldanamycin, Nigericin and Nocardamine, the production of any of which is potentially morphologically dependent.

6.3.2. Morphological variability in *S. hygroscopicus* cultures

The degree of variability observed from flask-to-flask in chapter 3 highlighted the apparently random nature of pellet formation and development in *S. hygroscopicus* cultures. Information regarding this degree of variability in filamentous microbial systems is scant. Given that aggregation is thought to

be quite sensitive to the starting conditions it may be useful to examine and characterise the extent of these parameters on the morphological development of the cultures.

6.3.3. Morphological variability in other filamentous organisms

As mentioned in the previous section, information regarding this degree of variability in filamentous microbial systems is scant. Given that the image analysis technique developed enables high throughput screening of pelleted microbial fermentation broths it may be useful to apply this technique to the assessment of morphological variability within cultures of other filamentous microorganisms. An investigation of this type would help determine whether or not morphological development is such an apparently random event in other systems and to what extent if any it occurs.

6.3.4. Influence of cell surface properties on culture morphology

The influence of cell surface properties, specifically hydrophobicity, on particle aggregation and pellet formation was demonstrated in Chapter 5. However, hydrophobicity is not the only cell surface property responsible for aggregation, other researchers have pointed to the cell surface charge and the production of exo-polymeric substances as potential influences. Regulation of the ionic strength of the fermentation media will influence the cell surface charge and is thought to be capable of altering the kinetics of pellet formation. The production of exo-polymeric substances by streptomycetes is not a widely reported phenomenon and the references to it in the literature are scant. However, preliminary experimental work performed point to the potential production of such compounds, specifically Alginate by *S. hygroscopicus*. The introduction of alginate lyase into the fermentation media was responsible for altering the morphological development of the culture. This work is still only at a preliminary stage but it is proposed to use image analysis techniques to assess the morphological influence of cell surface charge and exo-polymers on culture development.

6.3.5. Compound specific surfactant mode of action

The compound specific action of the different surfactant molecules was shown in chapter 5. The three different surfactants not only altered the morphological profile with respect to the control but also each other. Further study is required into the specific modes of action of each of these compounds, the way in which they influence particle aggregation and the potential differences from one to another. For example what is driving the aggregative process observed in the Tween 80 cultures.

6.3.6. Gas-liquid mass transfer in *S. hygroscopicus* fermentation broths

A more quantitative approach to the impact of altering broth viscosity and surface tension, both through the introduction of chemical surfactants and the indigenously produced biosurfactant, on the gas-liquid mass transfer coefficient and the overall oxygen transfer rate needs to be performed. Once the extent to which the gas-liquid mass transfer coefficient is influenced is known an assessment of the potential impact on biomass growth and the morphological development of the culture may be possible.

6.3.7. Biosurfactant characterisation

The morphologically dependent production of multiple biosurfactant molecules was demonstrated in Chapter 5. The crude purification of biosurfactant material followed by re-introduction into *S. hygroscopicus* cultures demonstrated the potential existence of a morphological auto-regulatory mechanism. The purification of each of the biosurfactant molecules from the crude fermentation broths and subsequent inclusion in fresh nutrient broths will provide more information on the potential morphological influence of each. The purification and identification of multiple biosurfactant compounds, each of which appears to have different molecular characteristics, from crude *S. hygroscopicus* fermentation cultures will prove a daunting task.

6.3.8. Modelling the morphology of filamentous microorganisms

A more quantitative assessment of the various environmental and nutritional conditions on the morphological distribution of *S. hygroscopicus* cultures is required. The analysis based on population distributions may potentially benefit from a curve-fitting approach where correlations might be better represented. The experimental data from the existing set of procedures performed may be suitable for modelling purposes and may facilitate the study of the growth kinetics of filamentous microorganisms in more detail.

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APPENDIX A - IMAGE ANALYSIS ALGORITHM

The following is the code for the image analysis algorithm developed in Chapter 3 and used in conjunction with Optimas 6.5 ® for the morphological quantification of pellets in *S. hygroscopicus* broths using a flatbed scanner.

```
OpenConfiguration ("C:\\Program Files\\Optimas 6.5\\Config\\cormac.cfg");
Calibrate (Calibration5);
ScalarToList (255.0);
ScalarToList (0.0);

Calibrate (One_Pixel_per_Centimeter);
myroi=ROI;
ROIToBuffer();
NewImage ("SoftwareFixed", "ScaledFilter", (myroi[1,0]/10) : (myroi[0,1]/10),
1 : 1 : 8 : 1 : 3 : 3, , ModelImage, );
ScaledFilter.ActivateImage ();
Calibrate (One_Pixel_per_Centimeter);
ArithmeticOp ("Copy", "BUFFER", , , "Scale X and Y", FALSE, FALSE);
for(i=0;i<20;i++)
Filters ( Average5x5 );
ROIToBuffer ();
CloseImage();
ArithmeticOp ("Divide", "BUFFER", , 200.0, "Scale X and Y", FALSE,
FALSE);

Calibrate (One_Pixel_per_Centimeter);
myroi=ROI;
ROIToBuffer();
NewImage ("SoftwareFixed", "ScaledFilter", (myroi[1,0]/10) : (myroi[0,1]/10),
1 : 1 : 8 : 1 : 3 : 3, , ModelImage, );
ScaledFilter.ActivateImage ();
Calibrate (One_Pixel_per_Centimeter);
ArithmeticOp ("Copy", "BUFFER", , , "Scale X and Y", FALSE, FALSE);
for(i=0;i<20;i++)
Filters ( Average5x5 );
ROIToBuffer ();
CloseImage();
ArithmeticOp ("Divide", "BUFFER", , 200.0, "Scale X and Y", FALSE,
FALSE);

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;

volume=pow(mArAreaEquivDiameter,3)*3.1417/6.0;

delete(volume);

if (ChanID = DDEInitiate ("Excel", "Sheet1"))
{

        statval=Moments(mmararea);

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


        for(i=0;i<vectorlength(mmararea);i++)
        {

            output="R":totext(i+2):"C1";
            DDEPoke (ChanID,output, marareaequivdiameter[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);
}
```