CHARACTERISATION AND GROWTH OPTIMISATION OF *STREPTOMYCES NATALENSIS*

A thesis presented for the
degree of M.Sc.

by

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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 MSc. in Biological Sciences, 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

The morphological development and growth characteristics of the actinomycete *Streptomyces natalensis* were studied in submerged culture. The morphology of the organism was monitored using semi-automated image analysis. A protocol was developed for taking geometric measurements of the cell population in terms of pellet area, perimeter and diameter.

The organism was found to be pH sensitive and, under certain circumstances, was shown to suffer from oxygen limitation. The optimum inoculum for a 100 mL fermentation (in a 250 mL shake flask) was found to be a 5 mL aliquot of a 96 hour old culture. However, a 100 mL culture was employed, despite superior performance at the 50 mL scale, due to reproducibility problems encountered with the smaller volume. When *S. natalensis* was cultivated in a 150 mL culture (in a 250 mL flask) the organism was found to be subject to oxygen limitation.

Medium composition also affected the growth of the organism, with a YEPD-based medium and an actinomycete medium developed by Sarra *et al.* (1996) resulting in the greatest levels of biomass production. With the exception of $CaCO_3$, concentrations of the individual components in a medium developed by Struyk and Waisvisz (1975) were found to be optimal, although yielding lower levels of biomass than the YEPD or Actinomycete media. If the calcium carbonate concentration was reduced from the recommmended 8 g/L to 4 g/L, biomass production was enhanced.

The organism grows predominantly in a pelleted form with some filamentous clumps present under certain conditions. The pellets increase in size until the culture is approximately 73 hours old, at which time the pellets undergo some form of disintegration which may be caused by lysis or breakage due to pellet-pellet collisions. After this time, the average pellet area decreases sharply to a stable value. This pattern is relatively insensitive to changes in media components or concentrations. The only exceptions are during cultivation in either YEPD or actinomycete media (Sarra *et al.*, 1996), where the percentage of filamentous clumps present in the sample increases consistently.

NOMENCLATURE

Symbol	Definition	Units
C_1	Flexibility constant	-
C_m	Biomass concentration	g/L
Cn	Concentration of pimaricin in standard	μg/mL
Cs	Concentration of pimaricin in sample	μg/mL
C_W	Ratio of mycelial wet to dry weight	-
D	Impeller diameter	m
d_m	Hyphal diameter	μm
H	Liquid height in fermenter	M
K	Consistency index	Ns^n/m^2
k_c	Casson constant	$(Pa\ s)^{0.5}$
k_La	Volumetric oxygen mass transfer coefficient	s^{-1}
l_m	Length of a mycelial hypha	μm
M_F	Apparent morphology factor	-
n	Flow behaviour index	-
Pn	Peak height of pimaricin standard	Mm
Po_2	Partial pressure of oxygen	N/m^2
Ps	Peak height of sample at 317nm	Mm
R_{vb}	Specific biomass production rate	g/L.h
T	Vessel diameter	m
$Y_{x/s}$	Aerobic yield of cells per gram substrate	g/g substrate
α	Constant	-
β	Constant	-
δ	Morphology factor	-
Γ	Constant	-
ρ_m	Density of mycelium	Kg/m^3
τ	Shear stress	Pa

ABBREVIATIONS

AA	Arachidonic acid
atm	Atmosphere
ATCC	American Type Culture Collection
CBS	Central Bureau Voor Schimellcultures
CMC	Carboxymethlycellulose
CMS	Counterflow mixing system
CSP	Corn Steep Powder
DNS	3,5-dinitrosalycylic acid
GA	Gibberellic acid
HON	5-hydroxy-4-oxonorvaline
HPLC	High Performance Liquid Chromatography
IPYA	Indole-3-pyruvic acid
KHCF	Potassium hexacyanoferrate II – trihydrate
NCIMB	National Collections of Industrial and Marine Bacteria
NRRL	Northern Regional Research Laboratory
psi	Pounds per square inch
RP	Rheological parameters
rpm	Revolutions per minute
STR	Stirred Tank Reactor
TMS	Turbine mixing system
UV	Ultra violet
vvm	Volumes/volume/minute
YEPD	Yeast Extract, Peptone, Dextrose

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CHAPTER 1

INTRODUCTION

Streptomyces natalensis is an actinomycete which was first isolated in 1955 in Natal, South Africa. It produces pimaricin (natamycin) which is an anti fungal agent. Pimaricin has been used in preserving food and beverages which are particularly prone to fungal contamination, *e.g.* to protect the surface of salami (Holley, 1981). However, very little research has been reported on the characterisation of this organism in submerged culture or on optimisation of cultivation conditions.

The morphology of filamentous organisms is of interest both from a biological and biochemical engineering viewpoint. Biologically, morphology can be used in the identification of an organism and can reflect its pathogenic potential and adaptation to its environment. In relation to biochemical engineering, the morphological characteristics exhibited by the organism in submerged culture can influence the viscosity of the broth, and thus impact on oxygen transfer, growth, productivity and downstream processing for recovery of the desired product. The morphology of submerged cultures varies greatly, depending both on the organism and on the growth conditions, with suspensions of unicells, long hyphal filaments and pellets among the possible forms observed (Edelstein and Hadar, 1983).

Currently, there is a strong trend towards quantifying microbial morphology in suspension cultures using image analysis (Thomas and Paul, 1996). Allowing rapid, accurate and detailed analysis of morphology, image analysis has clear advantages over earlier approaches to morphological measurements *e.g.* digitising tables and Coulter counters.

In this project, both shake flasks and stirred tank reactors (STRs) were used to cultivate the organism and the concentrations of the principal media components were varied. The effects of these changes on the growth and morphological characteristics of *S. natalensis* were monitored with a view to optimising the cultivation conditions.

CHAPTER 2

CHARACTERISATION AND GROWTH OPTIMISATION OF FILAMENTOUS MICRO-ORGANISMS

2.1 INTRODUCTION

A number of economically important industrial fermentations employ filamentous micro-organisms. Examples of these processes include the production of antibiotics (*e.g.* penicillin by *Penicillium chrysogenum*), the production of enzymes (*e.g.* rifamycin oxidase by *Curvularia lunata*), and the production of exopolysaccharides (*e.g.* pullulan by *Aureobasidium pullulans*). The physical properties of these systems differ significantly from those of non-filamentous bacteria and yeast cultures. This is due primarily to the fact that filamentous systems can exhibit two extreme types of morphology in submerged fermentation, namely, the pelleted and the free filamentous forms (Metz and Kossen, 1977; Olsvik *et al.,* 1993). Between these two extremes can lie a wide range of mycelial morphology, the existence of which is frequently ignored, particularly in rheological studies.

The rheological properties of a filamentous fermentation fluid are normally considered to be determined by both the biomass concentration and the morphological form of the mycelia (Roels *et al.*, 1974; Kim *et al.,* 1983). Therefore, the control of mycelial morphology during fermentation is often a prerequisite for industrial applications as it is well established that the broths involved in such processes are highly viscous with non-Newtonian rheological properties.

Hence, when optimising a filamentous fermentation, great care must be taken to prevent, if possible, both the mass transfer and downstream processing complications associated with the formation of highly viscous filamentous broths. By controlling both the fermentation media components and operating parameters, a delicate balance can be achieved between optimising biomass and product yields, and maintaining broth viscosity at an acceptable level. This review outlines some of the research into the control of biomass and morphology development, including media optimisation studies and the effects of fermentation parameters such as agitation and

aeration. Correlations relating biomass concentration and morphological characteristics to the rheological profiles of filamentous fermentations are also discussed.

2.2 THE ACTINOMYCETES

Until the late 1950's the actinomycetes were regarded, on the basis of their macroscopic and microscopic morphology, as an unusual group of fungi. However, application of electron microscopy showed that the actinomycetes were prokaryotic, gram-positive bacteria (Williams, 1990). Growth of actinomycetes is filamentous on solid substrate. In submerged cultures, the preferred choice for industrial fermentations, growth can occur in different modes, *i.e.* pellets, mycelial fragments and well-dispersed mycelia. Oxygen requirements of actinomycetes can vary (Piret and Demain, 1988). Some genera are fermentative; others are oxidative.

Although actinomycetes occur in a wide range of environments, the vast majority are saphrophytes, found in terrestrial and aquatic habitats. The genus *Streptomyces* predominates in almost all soil types. They are important colonisers of soil organic material, the bulk of which consists of insoluble polymers. Some genera are more common in freshwater environments. Others are thermophilic and play an important role in the decomposition of composts and fodders, which are self-heating processes (Williams, 1990).

Actinomycetes play both detrimental and beneficial roles in nature. Human diseases such as 'farmer's lung' (caused by a number of organisms including *Thermoactinomyces vulgaris*) and hypersensitivity pneumonitis (*Streptomyces albus*) are due to production of spores (Lacey, 1988). A number of domestic animal diseases also arise from these organisms (Lechevalier, 1988), *e.g.* mastitis (*Actinomyces pyogenes*), foot rot (*Dermatophilus congolenis*), and tuberculosis (*Mycobacterium bovis*). They are also responsible for 'bulking' in sewage treatment plant and cause the formation of scums and foams.

Actinomycetes are extremely active in biodegradation and have been known to cause spoilage of hay, straw, cereal grains, seeds, bagasse, plant fibres, wood, pulp, paper, wool, hydrocarbons, rubber and plastics (Demain, 1988). These biodegradative properties, however, can be channelled into waste removal activities and are extremely important in the recycling of materials in nature. Most agricultural and urban wastes primarily consist of cellulose complexed with varying amounts of hemicellulosic polysaccharides and lignin, and the cellulases and xylanases of a number of actinomycete species are well characterised (Crawford, 1988).

A large number of actinomycete enzymes are currently of interest in areas such as enzyme processes, clinical chemistry, and medical therapy. Glycosidases *e.g.* amylase, are exploited for the degradation of plant biomass; proteases serve as additives to detergents and are used in the tanning industry. Glucose isomerases have been successfully used to obtain fructose heavy syrups. Enzymes produced by actinomycetes have been used for beer and wine clarification and also serve as non-toxic food preservatives. Cholesterol oxidase (*Streptomyces violascens* and *Norcardia* spps.) has been used for the rapid determination of total cholesterol levels in blood (Peczyńska-Czoch and Mordarski, 1988).

However, the greatest contribution that actinomycetes have made to science is through their production of antibiotics and anti-tumour agents. About two-thirds of the known antibiotics are produced by actinomycetes and approximately 80% of these have been produced by the *Streptomyces* species (Williams, 1990; Haque *et al.*, 1995). Streptomycin is produced by *Streptomyces griseus* and has antibiotic activity against *Mycobacterium tuberculosis*. This was the first commercially successful antibiotic produced by an actinomycete (Claridge, 1979). Tetracyclines, which have had a significant impact on both chemotherapy and non-medical fields, are also produced by a number of *Streptomycete* species. (Hostálek *et al.*, 1979). The actinomycete *Amycolatopsis orientalis* produces the glycopeptide antibiotics vancomycin and *N*-demethylvancomycin, which continue to be the antibiotics of choice against β-lactam resistant enterobacteria (McIntyre *et al.*, 1996).

2.2.1 *Streptomyces natalensis* AND THE PRODUCTION OF PIMARICIN

The polyene macrolide, pimaricin, was first discovered in 1955 when a group of Dutch investigators isolated a new, highly active antibiotic from the culture medium of a *Streptomyces* strain (Struyk and Waisvisz, 1975). The *Streptomyces* strain originated from a soil sample taken in Natal, South Africa and was named *Streptomyces natalensis*. The World Health Organisation did not accept the name pimaricin and the antibiotic's name was subsequently changed to 'natamycin'. Both names appear in the literature. The antibiotic is also commercially produced by *Streptomyces gilvosporeus* (McCabe, 1990). Morphologically, *S. natalensis* grows filamentously on agar. The hyphae are branched, irregularly twisted, generally of uniformly thickness (Struyk and Waisvisz, 1975). There is little information in the literature on the morphology of the organism in submerged culture.

Pimaricin is a white to creamy white, almost tasteless, and almost odourless, crystalline powder (De Ruig *et al.*, 1987). The chemical formula is $C_{33}H_{47}NO_{13}$ and the molecular weight is 665.74. Temperatures of up to 120°C do not impair the antibiotic activity, as long as the exposure time is less than 1 hour (Raab, 1972). Pimaricin may be inactivated under the influence of UV light with wavelengths of 300 – 350 nm (McCabe, 1990). The compound is of particular potential interest in the food industry because of its effectiveness in inhibiting fungal growth, without interfering with bacterial fermentation or ripening processes. The low solubility of natamycin makes it suitable for surface treatment of foods, because it does not readily migrate into the interior and does not adversely affect flavour or appearance (Tuinstra and Traag, 1982; Fletouris *et al.*, 1995). More recently, natamycin has been used in the treatment and prevention of a number of poultry diseases (Raghoenath and Webbers, 1997).

Despite its recognised potential, there has been limited research on commercialisation of natamycin, because of the extremely high cost of its manufacture, with recovery and purification requiring multiple steps, including pH changes and concentration steps (Millis *et al.*, 1991). The high cost is predominantly due to the use of organic solvents *e.g.* butanol (Struyk and Waisvisz, 1975), in the

extraction of pimaricin from fermentation broth. Borden *et al.* (1995) patented the extraction of pimaricin from fermentation broth, using a substantially water-miscible solvent at alkaline pH. More recently, however, Raghoenath and Webbers (1997) developed a recovery process which does not require the use of any organic solvents.

Bridger (1968) obtained antibiotic yields of 750 mg/L during fermentation. Struyk and Waisvisz (1975) observed concentrations of between 535 and 640 mg/L of pimaricin in a number of fermentation broths. Millis *et al.* (1991) noted pimaricin concentrations of up to 2 g/L.

A number of techniques are employed at present to detect pimaricin at residue levels in fermentation broths and on some food products. These include bioassays, UV spectroscopy and, the most common, HPLC. The latter technique has been used to detect pimaricin penetration in cheese (Tuinstra and Traag, 1982). Mahon (1990) developed a HPLC method for the routine estimation of pimaricin levels in fermentation broths. De Ruig (1987) developed a UV spectrophotometric method to measure pimaricin content in cheese and cheese rind and the technique was advanced by Fletouris *et al.* (1995). This UV method has many advantages over the HPLC approach as it does not require concentration or purification of the sample prior to analysis. The analysis is simple and is completed in 6 minutes, compared to 13.5 minutes with the HPLC technique.

In DCU to date a two previous studies have been completed in the optimisation of pimaricin production by *S. natalensis*. Mahon (1990) studied pimaricin production during cultivation in a 10L fermenter. Yields of up to 1g/L of pimaricin were observed. A HPLC assay method was also developed in this study. McCabe (1990) studied the effect of some media components on pimaricin production in both shake flasks and a 10L fermenter. Two methods were used to assay for the presence of pimaricin; a bioassay and UV spectroscopy. Yields of up to 700 mg/L were observed during this study.

2.3 MORPHOLOGICAL CHARACTERISATION

2.3.1 MORPHOLOGY - THE CLASSICAL DEFINITIONS

Morphology is the description of the external form and structure of an organism, but can also describe the nature of the mycelial network and the homogenous phase. The morphology of a micro-organism influences mycelial growth and, consequently, in submerged cultures, affects production kinetics, by determining the rheology of the broth and by causing diffusion limitation of nutrients within mycelial flocs. Usually, microbial morphology is characterised by visual observation using a microscope.

Hyphal flocs exhibit several morphological forms. In submerged culture, mycelial growth can be in the form of disperse filamentous mycelia (*e.g. Fusarium graminearium*), spherical colonies or 'pellets' (*e.g. Aspergillus niger*) or any combination of these extremes depending on the fermentation conditions (Cox and Thomas, 1992). Some micro-organisms are dimorphic, *e.g. Kluyveromyces marxianus*. This phenomenon may be described as the process by which the filamentous form is transferred into a yeast-like form or vice versa as a result of environmental conditions (Walker and O'Neill, 1990).

In pelleted form, the mycelium develops as stable spherical aggregates, comprising a branched and partially intertwined network of hyphae. These pellets can have diameters of several millimetres. The most important advantage of growth in pelleted form is a considerable reduction in broth viscosity, in comparison to growth in filamentous form (Hersbach *et al.*, 1988).

Several mechanisms have been proposed for pellet formation: agglomeration of spores and hyphae, agglomeration of hyphae and agglomeration of solid particles and hyphae. Pellet-pellet and pellet-wall (*i.e.* fermenter wall, baffles *etc.*) interactions are also considered to be of importance in pellet formation (Metz and Kossen, 1977). Pellet morphologies have been classified into three loosely banded groups encompassing the variations observed under different fermentation regimes (Hersbach *et al.*, 1988).

- Fluffy loose pellets: pellets that have a compact central core with a loosely packed filamentous outer zone
- Compact smooth pellets: pellets that have a compact core with limited lateral growth
- Hollow pellets: pellets that have developed from the previous two forms, and have a hollow core

2.3.2 THE DEVELOPMENT OF MORPHOLOGICAL STUDIES

Characterisation of mycelial morphology is important for the design and operation of fermentations involving filamentous micro-organisms. Initial investigations of mycelial morphology relied upon inaccurate and time-consuming manual measurements from photographs. One of the earliest semi-automated methods for morphological characterisation employed an electronic digitiser (Metz *et al.*, 1981). Photographs of the micro-organism were projected onto a digitising table, where the images could be manually traced with a cursor. Co-ordinates along an individual hypha were recorded and used to calculate morphological measurements such as main hyphal length, total hyphal length and the number of hyphal tips. This method, however, was slow and labour intensive. Quantification of morphology throughout the fermentation ideally requires an automated method of analysing a significant number of mycelia from each sample. Methods based on digital image analysis have now been developed (Adams and Thomas, 1988), and will be discussed in further detail in Section 2.3.3.

Fractal models have been used for the characterisation of morphology (Patankar *et al.*, 1993). A fractal is an object in which similar structural patterns are repeated at different length scales, with the result that small sections of the object, upon magnification, appear very similar to the original object. In mycelia, the repeated branching of the mycelial hyphae in a tree-like form during growth gives rise to self-similarity and the fractal structure. The fractal model yields a single quantitative index of morphology, which has been found to have general applicability and can be used in empirical or theoretical correlations between morphology and other properties. Patankar *et al.* (1993) successfully applied a fractal model to the

characterisation of *P. chrysogenum*, *Streptomyces tendae*, and *Streptomyces griseus* cultures in a bioreactor. Experimental observations and simulations of mycelial growth validated the fractal model. Jones *et al.* (1993) also investigated the use of fractal geometry in the quantitative analysis of a mycelial inoculum of *Pycnoporus cinnabarinus*.

Morphologically based models for the development of hyphal structures and pellets have been proposed by a number of workers including Yang *et al.* (1992) and Nielsen (1993). Filtration has been used to monitor the morphological development of microbial broths (Oolman and Liu, 1991).

2.3.3 IMAGE ANALYSIS

Image analysis is a technique which can be used for the rapid and automatic measurement of the morphological characteristics of micro-organisms. It has been used to characterise freely dispersed hyphae in terms of main hyphal length, total hyphal length and number of tips (*e.g.* Packer and Thomas, 1990; Tucker *et al.*, 1992; Vanhoutte *et al.,* 1995). Researchers have also developed protocols to measure the morphological parameters of pelleted micro-organisms in terms of the area, diameter and roughness of individual pellets (*e.g.* Cox and Thomas, 1992; Reichl *et al.*, 1992a). In addition to the morphological characterisation of filamentous organisms, image analysis techniques have also been used to monitor the morphology of budding yeast cells (Pons *et al.*, 1993), dimorphic yeast (O'Shea and Walsh, 1996) and plant cells (Kieran *et al.*, 1993).

The principle steps in image analysis are:

- Image capture: the conversion of an image into an electronic signal suitable for digital processing and storage. The image is captured using a television camera, which may be mounted on a microscope, and converted into a grid of pixels.
- Segmentation: the act of separating the regions of interest within a captured image from the background. The most common method used is called thresholding, which works well in situations where illumination can be carefully controlled. If illumination is a problem then other techniques such as 'edge finding' and 'region

growing' are also available.

- Object detection: a data reduction step that produces a description of each 'object' (defined in image analysis terms as the individual regions of interest within the whole scene). These sets of descriptions, which should fully characterise each object, are stored and used in subsequent measurement operations.
- Measurement: the gathering of quantitative data for the objects of interest.
- Analysis of the data collected.

Tucker *et al.* (1992) proposed the use of an image processing system for physiological studies on filamentous micro-organisms, with particular reference to the effect of fermentation conditions on mycelial extension and branching. Tucker and Thomas (1994) used image analysis in studies of the effect of spore concentration on the subsequent morphology of *P. chrysogenum*. In addition to determining simple morphological parameters, a direct correlation between total hyphal length and dry weight was obtained. Morphological characteristics could, therefore, be used to determine the growth kinetics of filamentous fungi (Lejeune and Baron, 1995).

2.4 THE FACTORS WHICH AFFECT MORPHOLOGY

The morphology of a filamentous micro-organism developing in any fermentation system may be represented as a final result of competing influences: equilibrium between forces of cohesion and disintegration. As can be seen from Figure 2.1, a wide range of factors influence the formation of flocs, clumps and pellets. A more comprehensive list of the factors affecting morphology is presented in Table 2.1 (Atkinson and Daoud, 1976).

There is substantial evidence in the literature, to suggest that engineering variables have considerable influence upon the morphology of moulds. Factors such as oxygen tension, growth rate and shearing forces can influence the growth, morphogenesis and productivity of a filamentous micro-organism (Van Suijdam and Metz, 1981).

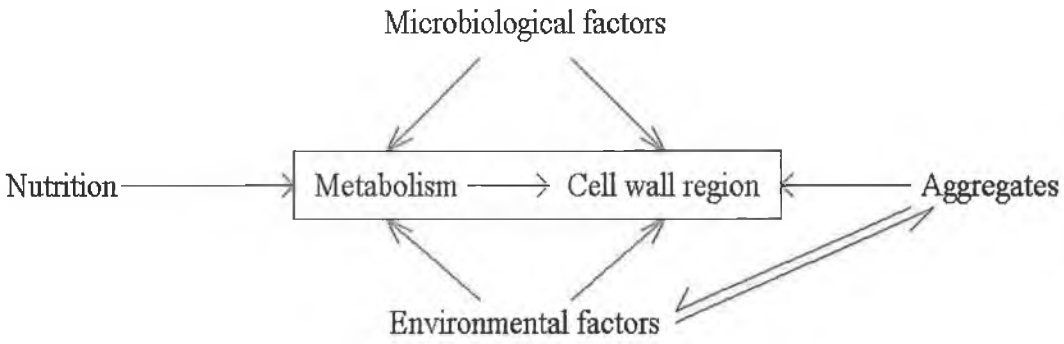


Figure 2.1: Factors affecting aggregation *(Adapted from: Atkinson and Daoud, 1976)*

Table 2.1: Factors affecting the formation of flocs (*From: Atkinson and Daoud, 1976*)

Microbiological	**Environmental**		
	Physical	**Chemical**	**Biological**
Cell wall	Hydrodynamic Properties	Presence of	Inoculum size
Genetics	aeration	chelating agents	Presence of
Growth rate	dilution rate	C/N ratio	other
Nutrition	fermenter size	enzymes	organisms or
Physiological age	time and intensity of	ferrocyanide	strains
	agitation	nitrogenous	
	viscosity	substances	
	Interfacial Phenomena	oils	
	gas bubbles	sugars	
	surface active agents	trace metals	
	suspended solids		
	Ionic Properties		
	organic solvents		
	pH		
	presence of calcium ions		
	Temperature		

2.4.1 ENVIRONMENTAL FACTORS - PHYSICAL

2.4.1.1 AERATION CONDITIONS

In fermentations conducted in sparged vessels, the role of aeration is two-fold: it satisfies the oxygen needs of the micro-organisms and contributes to fluid mixing. Relatively small changes in the dissolved oxygen concentration in the medium of aerobic cultures can result in changes in the physiological state of micro-organisms. In pellets, oxygen limitation can cause autolysis, eventually leading to the formation of hollow pellets (Hersbach *et al.*, 1988). It has been reported (Schügerl *et al.*, 1983) that the dissolved oxygen concentration in pellets which have diameters greater than 400μm is reduced below the critical value in the centre of the pellet, even when the bulk medium is saturated with oxygen. In studies with *P. chrysogenum*, hyphae cultivated under oxygen aeration were found to be thicker, shorter and more highly branched than in an air-sparged system (Van Suijdam and Metz, 1981). Carter and Bull (1971), when investigating the morphology of *Aspergillus nidulans*, found that abnormal morphologies, such as large, thick-walled cells, were observed at oxygen tensions below 18 mmHg.

2.4.1.2 DILUTION RATE

Weibe and Trinci (1991) investigated the effect of dilution rate on the morphology of *F. graminearium* and found that, for the two strains used in the study, hyphal diameter increased with increasing dilution rate. For strain A3/5, hyphal diameter increased from 1.8μm to approximately 2.8μm, when the dilution rate increased from $0.07h^{-1}$ to $0.19h^{-1}$. For strain C106, hyphal diameter increased from 1.7μm to approximately 4.0μm for the same increase in dilution rate.

2.4.1.3 AGITATION EFFECTS

During cultivation in a conventional stirred tank reactor, aggregates are subject to agitation-related effects at all times, from formation through the presence of stable flocs, to subsequent break up. Morphology can be significantly influenced by agitation. However, the published results are contradictory, which indicates that the effect of stirrer speed on fungal morphology is influenced by various parameters such as preculture conditions, medium composition and viscosity (Schügerl *et al.*, 1998). For example, Makagiansar *et al.* (1993), working with *P. chrysogenum*, found that

mean hyphal length decreased with increasing agitation rate from 350μm at 265 rpm to 120μm at 1150 rpm. However, in chemostat studies of *P. chrysogenum*, Van Suijdam and Metz (1981) found that the influence of shear stress upon the hyphal length of the mycelium branches was very limited. A substantial reduction in length could be achieved only through an impractically large increase in energy input.

Seichert *et al.* (1982) found that, at low impeller frequencies, *A. niger* formed long sparsely branched hyphae with thin walls while at higher frequencies, heavily branched thick hyphae with thick walls were formed. When cultivated on a rotary shaker, *A. niger* grew as pellets, approximately 3mm - 4mm in diameter; in baffled flasks it grew in filaments or in spheres of less than 1mm in diameter (Musílková *et al.*, 1982). Mitard and Riba (1988), also working with *A. niger*, noted that no aggregates were observed when a rotational speed of 1500 rpm was used.

In a study of *Streptomyces clavuligerus*, it was found that increasing stirrer speed accelerated the fragmentation rate (Belmar-Beiny and Thomas, 1991). Ohta *et al.* (1995) noticed an agitation effect on the morphology of *Streptomyces fradiae*, with hyphal length decreasing with increasing agitation rate. In studies of *Saccharopolyspora erythraea*, Martin and Bushell (1996) found that particle size distribution was significantly affected by stirrer speed, with mean particle diameters of 70μm and 124μm recorded at 1500 rpm and 750 rpm, respectively. McNeil and Kristiansen (1987) found that the percentage of yeast-like cells, as opposed to filamentous cells, present in an *A. pullulans* fermentation increased from approximately 5% to 100% when the impeller speed was increased from 100 rpm to 750 rpm. In studies of a number of *Absidia* species, Davoust and Hansson (1992) found that a low impeller speed generally resulted in pulpy growth and that elevated stirrer speeds caused the formation of a more compact morphology.

2.4.1.4 pH

In filamentous organisms such as *A. niger* and *P. chrysogenum*, pH has been found to play an important role in the overall morphology, particularly in the formation of pellets (Metz and Kossen, 1977). When studying the effects of pH variations on the

morphology of *A. niger,* Papagianni *et al.* (1994) reported that when the pH was increased from 2.1 to 4.5, the average filament length and diameter increased from 242.21μm and 1.65μm to 265.10μm and 2.54μm, respectively. Both McNeil *et al.* (1989) and Reeslev *et al.* (1991) found that with *A. pullulans* the proportion of yeast-like cells rose with increasing pH, in the range 3.5 - 6.3. When investigating the influence of pH on the morphology of *Streptomyces akiyoshiensis*, Glazebrook *et al.* (1992) observed that an initial pH of 5.5 minimised aggregation of the mycelium while supporting adequate biomass production. At pH 6.5 and above, the suspensions consisted of well dispersed single spores. The morphology of *S. tendae* was found to be significantly effected by pH (Vecht-Lifshitz *et al.*, 1990). At a pH optimal for growth (pH 7 - 8), the average pellet diameter was 0.1mm - 0.2mm, whereas when the pH was reduced to pH 4 the pellet diameter was significantly larger (1.2mm - 1.5mm).

2.4.1.5 CARBON DIOXIDE

In a study on *P. chrysogenum* (Smith and Ho, 1985), cultivation at influent carbon dioxide (CO_2) concentrations of 15% and 20% resulted in morphological abnormalities. Swollen and stunted hyphae predominated, with a significant quantity of spherical or yeast-like cells. Ho *et al.* (1987) noticed a similar effect, when cultivating *P. chrysogenum*; at CO_2 concentrations of between 0% and 8%, the organism grew in filamentous form. In research on *P. chrysogenum*, Ju *et al.* (1991) found that at an influent CO_2 concentration of 20%, the organism grew in pelleted form. McIntyre and McNeil (1997) investigated the effects of CO_2 on the morphology of *A. niger*. It was observed that, in processes with high dissolved CO_2 levels, (12% and 15%), a deviation from the pelleted growth desirable for citrate production occurred after 16 hours of cultivation. Longer hyphae were visible around the edges of the pellets. The short, stubby hyphae characteristic of citrate production became less prevalent as the CO_2 levels were increased.

2.4.1.6 TEMPERATURE

While investigating the morphology of *S. tendae,* Vecht-Lifshitz *et al.* (1990) found that incubation temperatures of approximately 31°C resulted in pulpy growth, while pelleted growth was observed at incubation temperatures of between 26°C and 30°C.

However, Reichl *et al.* (1992b) found that temperature variations between 27°C and 37°C had no effect on the mean hyphal growth unit of *S. tendae*. Lindberg and Molin (1993) noted a considerable temperature dependency by the morphology of *Mortierella alpina*. At temperatures of 12°C and 18°C, stable pellets were formed, with diameters of 0.2mm and 0.4mm, respectively. At 25°C, large pellets (10mm diameter) were formed which exhibited mycelial growth after 24 hours of cultivation. In their studies on *A. awamori*, Schügerl *et al.* (1998) observed that at a cultivation temperature of 25°C, only pellets were observed. When the organism was cultivated in 35°C, clumped growth predominated.

2.4.2 ENVIRONMENTAL FACTORS – CHEMICAL

The influence of the ammonium-glucose ratio on the morphology of *A. pullulans* in continuous cultivation was investigated by Reeslev *et al.* (1996). The yeast-like fraction of the biomass was about 40% when the fermentation was nitrogen-limited but was reduced to 5% - 15% when glucose-limited. A more comprehensive review of the effects of media components on the morphology of filamentous micro-organisms can be found in Section 2.4.4.1.

2.4.3 ENVIRONMENTAL FACTORS – BIOLOGICAL

Tucker and Thomas (1994) investigated the effects of inoculum spore concentration on the subsequent morphology of *P. chrysogenum* cultivated in both shake flasks and a 6L agitated bioreactor. The study revealed that, even at the same inoculum concentration, differences were found between the two systems in terms of the final morphology of both the freely dispersed mycelia and the mycelial aggregates. Inocula concentrations of greater than 10^5 spores mL^{-1} were required for the development of filamentous as opposed to aggregated forms. Nielsen *et al.* (1995) reported that for *P. chrysogenum*, at high spore concentrations, hyphal elements agglomerate and develop into large diameter pellets, but for very high spore concentrations, above 10^8 spores mL^{-1}, the final hyphal element is small and agglomerates are not, therefore, formed. Schügerl *et al.* (1998) observed that at high inoculum spore concentrations ($>10^5$ spores mL^{-1}), *A. awamori* forms filamentous mycelia and small pellets whereas with low spore concentrations (10^2 spores mL^{-1})

large pellets are formed. It has been noted that in *S. tendae*, the average pellet size decreased with increasing inoculum concentration (Vecht-Lifshitz *et al.,* 1990), reaching a plateau at large inoculum sizes; for example with an inoculum of $10^1 - 10^4$ spores mL^{-1}, the average pellet size remained close to 1mm.

The use of a 0.1% vegetative inoculum in the cultivation of *S. akiyoshiensis* yielded cultures with relatively large mycelial pellets (Glazebrook *et al.*, 1992). Although the average pellet size decreased as the inoculum size was increased, it remained much larger than those produced from a spore inoculum.

2.4.4. MICROBIOLOGICAL FACTORS

2.4.4.1 MEDIA COMPOSITION

The simplest method of altering the growth of any organism is to alter its food source. The organism must adapt to changes in the nutrients available and in their concentrations. As well as resulting in changes in growth rates, biomass concentrations and substrate utilisation profiles, a change in nutrients can also have a significant effect on both the macroscopic and microscopic morphology of the organism.

Reichl *et al.* (1992b) found that a reduction in glucose concentration from 40g/L to 1g/L in the cultivation medium for *S. tendae*, resulted in an increase in branching rate and specific growth rate, while the apical extension rate of individual hyphae was unchanged. In studies with *S. akiyoshiensis*, Glazebrook *et al.* (1992) found that glucose and lactose yielded the smallest mycelial aggregates (0.18mm and 0.2mm, respectively).

When carboxymethlycellulose (CMC) was used as the sole carbon source in the cultivation of *C. lunata*, the organism formed mycelia as opposed to the pellets formed in the presence of glucose (Banerjee, 1993). When *Rhizopus arrhizus* is grown in glucose-peptone and glucose-CSL-salts media, the organism forms pellets (Morrin and Ward, 1990). Supplementation of these media with CMC or Carbopol results in the production of dispersed or clumped mycelial growth.

Joung and Blaskovitz (1985) observed a significant influence of ammonium nitrate on the morphology of *A. niger* in submerged culture. At an ammonium nitrate concentration of 0.33g/L, pellets comprising mycelia hyphal stems, vesicles, stigmata and conidial spores were observed. All media containing 1g/L ammonium nitrate yielded only long, smooth filaments. Furthermore, media containing 3g/L ammonium nitrate produced fungal mycelia which were short and branched.

Byrne and Ward (1989) studied the effect of peptone concentration on the morphology of *R. arrhizus* and found that as the concentration was increased from 1g/L to 20g/L, the morphology changed from pelleted form to clumped mycelia. Glazebrook *et al.* (1992) also examined the effects of varying nitrogen sources on the morphology of *S. akiyoshiensis* and found that the largest mycelial aggregates, (0.27mm), were formed when the organism was cultivated in glutamate, the smallest, (0.07mm), with ammonium sulphate.

The formation of yeast-like cells and yeast-like growth in *A. pullulans* fermentations has been controlled by the initial concentration of yeast extract in the medium (Reeslev *et al.*, 1991). In the presence of sufficient yeast extract the growth form was filamentous until glucose became exhausted at the onset of stationary phase, after which the presence of yeast-like cells predominated.

The effects of the concentration of peanut flour on the cell morphology of *Cephalosporium acremonium* were investigated by Schügerl *et al.* (1988). The mould, which forms long hyphae, adhered to the surface of the solid particles. However, as the fermentation progressed, the peanut flour dissolved. Simultaneously, the long hyphae became swollen and gradually decomposed into spherical arthrospores.

2.4.4.2 GROWTH RATE

For the dimorphic micro-organism, *K. marxianus*, Walker and O'Neill (1990) found that in aerobic liquid culture both morphology and metabolism changed in a dramatic fashion over the course of a growth cycle. The results indicated that, in addition to

oxygen availability, growth rate also influences the physiology of the organism. Metz and Kossen (1977) reported that the number of pellets in chemostat cultures of *P. chrysogenum* increased with increasing growth rate. It was postulated that this effect might be due to the presence of shorter, more branched hyphae, leading to a reduction in the interaction between hyphae and an increased likelihood of pellet formation.

The degree of clumping exhibited by filamentous bacteria and fungi has been reported to increase with the age of the culture during the exponential phase, but subsequently decrease as the culture enters the stationary phase (Atkinson and Daoud, 1976).

2.5 GROWTH OPTIMISATION - CASE STUDIES

In any fermentation involving filamentous micro-organisms, a number of elements have to be taken into consideration in order to optimise the overall process. Where appropriate a delicate balance must exist between optimal biomass yield and secondary metabolite production. Moreover, the effects of growth optimisation on the fermentation parameters and downstream processing must not be ignored, in particular, the effects of increased biomass levels and, where relevant, viscous extracellular products, on broth rheology.

2.5.1 *Aspergillus niger*

Musílkova *et al.* (1982) investigated the effects of aeration on both biomass and citric acid yields in *A. niger* and found that when the organism was cultivated in baffled shake flasks, the biomass yield (13g/L) was similar to that produced in an unbaffled shake flask (12.5g/L). Although dissolved oxygen concentrations were not measured, the implication was that biomass production in *A. niger* was relatively insensitive to aeration level as the oxygen transfer rate in baffled flasks was estimated to be about four times that in unbaffled flasks. A similar result was reported by Friedrich *et al.* (1989). However, Musílkova *et al.* (1982) noted that the use of baffled flasks caused an appreciable increase in citric acid biosynthesis.

Friedrich *et al.* (1989) noted that if, in an *A. niger* fermentation at maximal growth rate, the aeration rate was increased from 0.5 to 1.2 vvm and agitation increased from 300 to 500 rpm, the depectinising activity of the proteolytic enzymes produced was double that obtained when the original conditions are maintained throughout the fermentation.

Papagianni *et al.* (1994) investigated the influence of agitation on the production of citric acid by *A. niger*. In these studies it was noted that as the agitation rate in the bioreactor was increased from 100 rpm to 300 rpm, citric acid production increased to a maximum value and was not further affected by increasing agitation rate.

McIntyre and McNeil (1997) studied the effect of carbon dioxide concentration on biomass and citrate yields by *A. niger*. It was observed, in this study, that as the inlet CO_2 concentration was increased from 0% to 3%, the yield factor for biomass decreased from 0.141 to 0.120g biomass/g substrate. As the concentration was further increased to 12% the $Y_{x/s}$ increased to a maximum value of 0.234. Further increases in CO_2 concentration led to a reduction in the biomass yield. The maximum product yield, 0.643g product/g substrate, was observed at 5% CO_2 while the minimum, 0.290, occurred at 12% CO_2.

In studies on the production of citric acid by *A. niger,* Papagianni *et al.* (1994) found that increasing pH reduced citric acid production rates: at pH 2.1 the citric acid yield was 60.52 g/L, whereas at pH 4.5, the yield was 11.73 g/L.

Friedrich *et al.* (1990) studied the effects of inoculum size on both biomass concentration and proteolytic enzyme production by *A. niger*, using the Apple Juice Depectinising Assay. It was observed that biomass levels were unaffected by inoculum size. However, as the inoculum size was increased from 100 spores/L to 10^8 spores/L, the final proteolytic enzyme activity decreased from 60 U/mL to 30 U/mL.

McIntyre and McNeil (1997) observed that citrate production in *A. niger* began only after all nitrogen was utilised in the fermentation medium. Friedrich *et al.* (1990) reported that while biomass levels in *A. niger* fermentations were unaffected by the presence of potassium hexacyanoferrate II – trihydrate (KHCF), supplementation with KHCF doubled the proteolytic enzyme concentration compared to the concentration in unsupplemented medium.

2.5.2 *Aureobasidium pullulans*

A. pullulans is capable of growing and synthesising pullulan on a wide range of carbon sources, although sucrose and glucose support the highest yields (Seviour *et al.*, 1992).

When studying the effect of C: N ratio on the morphology and productivity of *A. pullulans*, Reeslev *et al.* (1996) noted that as the feed concentration of ammonium sulphate was increased from 0.3g/L to 1.45g/L the steady state concentration of biomass increased from 1.9g/L to 7.0g/L. When the ammonium concentration was further increased, subsequent increases of biomass were achieved at the expense of pullulan productivity. It was concluded that nitrogen is a limiting nutrient. It was also observed that if the fermentation was nitrogen-limited, the pullulan productivity was 36 mg/g/h; no exopolysaccharide production was observed when the system was glucose-limited.

The formation of exopolysaccharides by *A. pullulans* is known to be affected by the initial concentration of yeast extract in the medium (Reeslev *et al.,* 1991). The product yield decreases as yeast extract concentration increases. In continuous culture, the highest rates of polysaccharide production by *A. pullulans* occurred under conditions of nitrogen-limitation and constant dilution rate, at a pH of 4.5 (Seviour *et al.,* 1992).

Reeslev *et al.* (1991) investigated the effect of pH on the production of exopolysaccharides by *A. pullulans* and found that product yield at pH 3.5 is nearly double that at pH 6.5. However, there appears to be no link between culture pH and

the rate of ethanol production by *A. pullulans* (Madi *et al.*, 1996).

The final biomass level in batch cultures of *A. pullulans* increased from 4.5g/L to 6.5g/L as the operating pressure was increased from 0 MPa to 0.5 MPa (Dufresne *et al.,* 1990). At a pressure of 0.8 MPa, the final biomass levels decreased sharply to 0.5g/L and remained at this value for all higher pressures investigated. The pressure exerted on the system also influenced pullulan production. The quantity of pullulan produced increased as the pressure was increased from 0.1 MPa to 0.65 MPa, to a maximum value of 13.75g/L. This effect was attributed to increased oxygen transfer rates at higher pressures. Beyond this critical pressure the pullulan concentration decreased sharply to 1.0g/L. The authors postulated that this was due to the competing effects of oxygen availability and pressure inhibition.

2.5.3 *Streptomyces akiyoshiensis*

In a wide-ranging study of *S. akiyoshiensis*, Glazebrook *et al.* (1993) investigated the effects of medium composition, pH and inoculum size on system performance. The highest biomass yield was obtained using asparagine as the nitrogen source; the lowest yields were obtained with ammonium sulphate. The production of 5-hydroxy-4-oxonorvaline (HON) by *S. akiyosheinsis* is also greatly influenced by the choice of nitrogen source. Potassium nitrate or aspartic acid resulted in optimal HON production (4.5mM and 4.0mM, respectively); with asparagine or ammonium sulphate, a HON concentration of 0.1mM was obtained.

The choice of carbon source is influenced by the nitrogen source employed and starch appears to be the optimal carbon source for the production of HON, (5.6mM), by *S. akiyoshiensis*, when cultivated with KNO_3 (Glazebrook *et al.*, 1993). However, using aspartic acid as the nitrogen source, glucose was found to be optimal (4.2mM HON produced).

S. akiyoshiensis optimally produces HON (17 mM) after 7 days at pH 7.2, whereas at pH 5.7 and pH 6.0 the HON concentration is less than 5 mM.

Glazebrook *et al.* (1993) also observed that the production of HON becomes more rapid with increasing vegetative inoculum levels. However lower production rates allowed a higher product yield (*e.g.* 13.9 mM compared to 12.5 mM produced during rapid growth).

2.5.4 *Streptomyces armentosus*

He *et al.* (1995) extensively investigated the effects of medium composition on biomass yields and secondary metabolite production by *S. armentosus.* Starch yielded the highest biomass concentrations (6.1g/L) while the lowest level (2.4g/L) was observed with galactose. When starch was used as the carbon source, the armentomycin yield was double that obtained with other carbon sources.

Biomass production by *S. armentosus* also appears to be affected by the choice of nitrogen source used in the cultivation medium and the highest level (6.7g/L) was observed when cultivated with lysine. Armentomycin yields were highest when either lysine, alanine or glycine were used as the nitrogen sources.

An initial phosphate concentration of 3mM in *S. armentosus* fermentations gave the highest armentomycin titre; increasing the phosphate concentration beyond this value lowered the titre, but the reduction was not significant.

2.5.5 *Mortierella alpina*

Lindberg and Molin (1993) studied the influence of a number of parameters on production formation by *M. alpina.* It was found that biomass was unaffected by glucose levels. Optimisation of the production of arachidonic acid (AA) was found to be dependent on glucose-limitation. When the organism was cultivated at 18°C the production of AA was significantly improved if glucose was limited (57% of total fatty acids present in glucose-limited cultures as opposed to 33% with excess glucose). A similar trend was observed when the organism was cultivated at 25°C, with AA constituting 44% of total fatty acids in glucose-limited cultures compared to 30% with excess glucose.

Lindberg and Molin (1993) also investigated the effect of pH and incubation temperature on the biomass yields of *M. alpina.* It appears that the optimum pH for the cultivation of the organism is 6.5, yielding a dry weight of 23g/L. If the pH is further increased, the biomass yield decreases and growth ceases when the pH reaches 8.5. It was observed that the time taken to reach a biomass concentration of 21g/L increased from 45 hours at 25°C to 138 hours at 12°C. The percentage of arachidonic acid (AA) produced by *M. alpina* in fermentation was also found to be affected by pH, increasing from 26% of total fatty acid content at pH 5.5 to 31% at pH 6.5.

The proportion of AA produced was also shown to be temperature dependent, with an optimum temperature of 18°C (32% AA), dropping to 30% at 25°C and to 24% at 12°C. These cultures were cultivated in non-limited nutrient conditions.

2.5.6 *Amycolatopsis orientalis*

A. orientalis appears to be affected by the phosphate concentration in the medium (McIntyre *et al.,* 1996). As the phosphate concentration increases from 2mM to 4mM, the final biomass concentration increases from approximately 8.5g/L to 9.9g/L. Further increases to 8mM yield a biomass level of 8.75g/L. The maximum specific production rate of vancomycin (0.45 mg/g.h) was obtained at the lowest phosphate feed concentration investigated (2 mM).

McIntyre *et al.* (1996) also studied the effect of dilution rate on biomass concentrations of *A. orientalis*. Biomass yields remained unchanged as the dilution rate was increased from $0.02h^{-1}$ to $0.09h^{-1}$ in phosphate-limited chemostat cultures. However the biomass decreased dramatically as the dilution rate was further increased to $0.14h^{-1}$. In the case of glucose-limited chemostat cultures, a similar effect was observed except that as the dilution rate was increased from $0.02h^{-1}$ to $0.09h^{-1}$, biomass levels increased slightly. It was observed that in phosphate-limited chemostat cultures, the maximum vancomycin production rate (0.37 mg/g/h) occurred at a dilution rate of $0.087h^{-1}$. A similar trend was also apparent when the fermentations were operated under glucose-limited conditions, although the

corresponding maximum vancomycin production rate (0.12 mg/g/h) was three times lower than under phosphate-limited conditions.

Clark *et al.* (1995) reported that vancomycin was produced by *A. orientalis* only if oxygen limitation was prevented.

2.5.7 *Streptomyces griseoflavus*

El-Abyad *et al.* (1994) performed a number of studies on the effects of medium composition and medium supplementation on both biomass and product formation by *S. griseoflavus*. Asparagine was found to be the optimal nitrogen source for biomass production. However, ammonium nitrate proved to be the most favourable nitrogen source for the production of indole-3-pyruvic acid (IPYA), yielding 9.5mg/L. Supplementation of nutrient media with a number of indole compounds affected biomass production; maximum biomass levels were obtained using indoleacetic acid (0.8g/L) and minimum levels with tryptophan (0.12g/L). It was also observed that when nutrient medium was supplemented with NaCl (7g/L) the final IPYA concentration increased 6-fold to a value of 13.37mg/L. Supplementation of the medium with indole compounds reduced IPYA production in all cases, with the most dramatic reduction, from 2mg/L to 0.25mg/L observed on addition of indole acetamide.

2.5.8 *Streptomyces antibioticus*

Haque *et al.* (1995) found glycerol to be the best carbon source, in studies on the optimisation of the cultivation of *S. antibioticus*. In this case, arginine was the best nitrogen source for the optimisation of both biomass and antibiotic production. Glycerol was also found to promote synthesis of a broad-spectrum antibiotic resulting in an inhibition zone of 24mm in plate cultures of *E. coli*. The same workers reported an optimal pH of 6.8 for antibiotic production, with minimal production at pH 6.0.

2.5.9 *Streptomyces clavuligerus*

S. clavuligerus was found to grow best when glycerol was used as the carbon source, while the most effective nitrogen sources were glutamate, glutamine and histidine (Vining *et al.*, 1987). Cephamycin production by *S. clavuligerus* was also monitored in this study and it was noted that succinate resulted in a cephamycin titre (65mg/L) over twice that observed with maltose (32mg/L) and three times that observed with glycerol (20mg/L). Trypticase soy broth as a nitrogen source resulted in 142mg/L cephamycin; the lowest titre, 22mg/L, was observed with urea.

Brana *et al.* (1986) also noted, in studies with *S. clavuligerus,* that the production of cephalosporins in a chemically defined medium was strongly influenced by the nature of the nitrogen source used. Some amino acids such as proline (0.8 units antibiotic/mg biomass) and arginine (0.9 units/mg biomass) inhibited antibiotic production. The optimal nitrogen sources for the production of cephalosporins are asparginine (25 units/mg) and aspartate (22.9 units/mg). Biomass production (3.4 g/L) in these fermentations was optimal when histidine was used; those nitrogen sources which result in low antibiotic production also result in poor biomass concentrations.

Production of cephamycin and clavulanic acid by *S. clavuligerus* is controlled by phosphate concentrations in the medium (Lebrihi *et al.*, 1987). In the presence of 2mM phosphate, the levels of cephamycin (60mg/L) and clavulanic acid (80mg/L) were higher than in the presence of 75mM phosphate, with concentrations of approximately 10mg/L of both products under the latter conditions. The phosphate inhibition of cephalosporin production was also noted by Lübbe *et al.* (1985). Production of cephalosporin was reduced from 0.062 g/L to 0.028 g/L, when the phosphate concentration was increased from 20 mM to 100 mM; biomass levels were observed to be unaffected.

When 130 μg/mL of ferrous iron was added to the fermentation medium for *S. clavuligerus*, the production of cephamycin C increased from 98 μg/mL to 200 μg/mL (Rollins *et al.*, 1987).

When cultivated in a 7L STR, biomass concentrations by *S. clavuligerus* were lower at 490 rpm than at either 990 rpm or 1300 rpm (Belmar-Beiny and Thomas, 1991). This may have been due to the levels of dissolved oxygen in the broth. It was noted that with the higher agitation rates, the dissolved oxygen did not fall below 90%. However, when an agitation rate of 490rpm was used, the dissolved oxygen tension fell to 60% saturation.

2.5.10 *Rhizopus arrhizus*

Byrne and Ward (1989) found that, during the cultivation of *R. arrhizus*, as the peptone concentration was increased from 1.0g/L to 20.0g/L the biomass level increased from 0.45g/L to 4.95g/L. At a constant initial peptone level of 5g/L, when the glucose concentration was increased from 5g/L to 45g/L, the dry weight increased from 1.75g/L to 1.95g/L. The nitrogen source had a greater effect on biomass yields than other components in the medium.

Morrin and Ward (1990), also studying the cultivation of *R. arrhizus*, noted that supplementation of the medium with CMC promoted production of biomass at the expense of fumaric acid. Supplementation of a glucose-peptone medium with CMC increased fumaric acid production from 1.78g/L to 5.36g/L. However, fumaric acid levels were considerably reduced (from 1.78g/L to 0.92g/L), on the supplementation of the medium with Carbopol.

2.5.11 PRODUCT OPTIMISATION STUDIES

The productivity of *C. acremonium* has been shown to increase with increasing peanut flour concentration (Schügerl *et al.*, 1988). Cephalosporin C concentrations of 5.5 - 6.0 kg/m^3 were obtained at a flour concentration of 30 kg/m^3. At a flour concentration of 100 kg/m^3, the Cephalosporin C concentration increased to 10 - 11 kg/m^3. If the oxygen partial pressure did not drop below 20% of the saturation value, then no oxygen limitation occurred.

Kaiser *et al.* (1994) found that biomass production in submerged cultivation of *Streptomyces parvulus* was adversely affected by high dissolved oxygen

concentrations. Increasing dissolved oxygen concentration to an equivalent of 1260 mbar did not effect biomass concentration. However, when the dissolved oxygen concentration was increased further to an equivalent of 2205 mbar, the observed biomass concentration decreased by 40%. Also the production of manumycin also decreased with increasing dissolved oxygen concentration. As the dissolved oxygen level increased from 305 mbar to 1670 mbar (partial pressure), the production of manumycin decreased from 27 mg/L to 2.5 mg/L.

It was observed that the production of erythromycin by *S. erythraea* was unaffected by oxygen limitation; production continued even at an oxygen concentration of 0% (Clark *et al.*, 1995). Investigations into the effect of agitation on the production of erythromycin by *S. erythraea* indicated that at an agitation rate of 1500 rpm, production was lower than at 750 rpm, with yields of 0.867 mg/mg biomass and 0.913 mg/mg biomass, respectively (Martin and Bushell, 1996). Lynch and Bushell (1995) found that the production of erythromycin in *S. erythraea* was significantly enhanced in cyclic fed batch culture (maximum production - 75 mg/L) compared to batch culture (maximum production - 40 mg/L). Chemostat culture resulted in reduced production with maximum levels (10.36 mg/L) at a dilution rate of $0.04h^{-1}$. As the dilution rate increased, the productivity fell.

Priede *et al.* (1995) investigated the growth of *Fusarium moniliforme* (a.k.a. *Gibberella fujikuroi)* in two dissimilar stirred bioreactors, namely STRs equipped with a turbine mixing system (TMS) and a counterflow mixing system (CMS). The agitator speed in the CMS was adjusted to yield the same specific power input as in the TMS. Thus, the agitator speed in the TMS was varied between 200 rpm and 300 rpm, and in the CMS between 410 rpm and 565 rpm. It was found that the levels of Gibberellic acid (GA) produced were significantly higher in the TMS (380 mg/L), than in the CMS (270 mg/L). While traces of GA were produced before exhaustion of nitrogen from the medium, the onset of significant production corresponds to full nitrogen depletion (Vass and Jefferys, 1979). With regard to the influences of temperature, it was reported that the maximum specific growth rate occurs at 31°C, decreasing rapidly at higher temperatures and less rapidly at lower temperatures. The

specific growth rate is independent of pH in the range 4 – 6. However, moving outside this range causes a reduction in the specific growth rate (Vass and Jefferys, 1979).

2.6 THE SUBSEQUENT EFFECTS OF GROWTH OPTIMISATION

The behaviour of fermentation broths is of considerable importance, from the viewpoint of cellular kinetics and in the engineering design of fermenters. The economic cost of a fermentation process can be greatly increased by allowing the viscosity of the fermentation broth to increase unchecked. The power input required to effectively agitate the broth and to ensure adequate gas transfer, and the cost of downstream processing in the recovery of the final product, are both increased beyond economic viability by highly viscous pseudoplastic fermentation broths (Charles, 1978). The rheological characteristics of fermentation broths must be monitored carefully throughout the course of the fermentation and if possible, manipulated to yield the most suitable compromise between biomass yields and economic considerations. Another consideration in the fermentation process is possible feedback inhibition occurring due to the accumulation of the product of interest in the fermentation broth. Both of these factors are further discussed in this section.

2.6.1 RHEOLOGY

2.6.1.1 EFFECTS OF MORPHOLOGY AND BIOMASS ON RHEOLOGY

The concentration and the morphological state of the mycelium normally determine the rheological properties of mycelial fermentation broths. High biomass concentration will, in general, lead to high viscosity (Olsvik and Kristiansen, 1994). However, describing viscosity as a function of biomass concentration alone is of limited value, as process variables such as dissolved oxygen and specific growth rate have been shown to influence the relationship between the biomass concentration and the consistency index (Olsvik and Kristiansen, 1992a; 1992b). Moreover, factors such as hyphal-hyphal interactions, which are difficult to quantify and which may

vary with fermentation conditions, impacting upon rheology, (Van Suijdam and Metz, 1981).

Roels *et al.* (1974) proposed a correlation between morphology and rheology. This correlation introduced a morphology factor, δ, defined as:

$$\delta = \left(\frac{16 \cdot (C_l)^3 \cdot C_W}{3 \cdot \rho_m} \cdot \left(\frac{l_m}{d_m} \right)^{0.5} \right)^2 \qquad \textbf{(2.1)}$$

where C_l is a flexibility constant, C_W is the ratio of mycelial wet to dry weight, l_m is the length of a mycelial thread, ρ_m is the mycelial density, d_m is the hyphal diameter. The value of δ was found to depend on the ratio of mycelial length to diameter, or more generally, its morphology. Using a Casson model to describe the rheological behaviour of such broths, it was assumed that both k_c and $\tau_{0.5}$ (Casson parameters) were functions of the volume fraction of the mycelial particles, and thus of biomass concentration and the morphology factor. In a review of the work of Roels *et al.* (1974), Metz *et al.* (1981) concluded that, although the introduction of the morphology factor was of great value, the correlation presented in equation (2.1) could not be used for the following reasons:

- Network interaction between the branched hyphae is neglected.
- The hyphae are treated as flexible chains and are supposed to form a spherical coil. This is in conflict with microscopic observations.
- The assumption inherent in the Casson model, that the coils present in the form of 'rouleux' orientated at 45° to the direction of flow, is questionable.
- The randomising effect of the Brownian motion, essential for the validity of polymer rheology theories, is essentially absent in mould suspensions.

In studies of *A. niger* broths, Fatile (1985) observed power-law (pseudoplastic) behaviour and showed that the power-law constants could be related to biomass concentration and the shape of the mycelial aggregates. Mitard and Riba (1988), also working with *A. niger,* showed that the size and peripheral structure of the pellets clearly had an impact on the rheology of the fermentation suspension.

From studies of *C. acremonium*, Kim and Yoo (1992) proposed a correlation for the consistency index, which could be estimated from the cell concentrations corresponding to different morphological types. Theoretical values of consistency index derived from the correlation were in close agreement with experimental values.

With the use of image analysis, Packer and Thomas (1990) showed that in submerged culture, *P. chrysogenum* existed as clumps, which could not be easily disrupted by dilution. They suggested that in such cases the hyphal dimensions of free mycelia might influence the broth viscosity to a lesser extent and that the rheological properties of the broth could be correlated with morphological parameters describing the size and shape of these clumps. Similar conclusions were reported for *P. chrysogenum* by Tucker *et al.* (1993) and for *A. niger* by Olsvik *et al.* (1993). In both cases, it was found that the power law consistency index could be correlated with biomass concentration and the roughness factor of the clumps. During studies on the fermentation characteristics of *Trichoderma harzianum*, Serrano-Carreón *et al.* (1997) observed that the morphological development of the organism appeared to influence the rheological properties of the broth. As the fermentation progressed, the viscosity increased, despite a reduction in biomass concentration. The authors postulated that the increased viscosity was related to changes in hyphal flexibility and hyphal-hyphal interactions, although no corroborating quantitative data were available.

Tucker and Thomas (1993) found that the rheological parameters of *P. chrysogenum* broths correlated well with two morphological variables: mean clump compactness and roughness. The following generalised correlation was proposed:

$$RP = \text{constant} * C_m^{\alpha} * (\text{roughness})^{\beta} * (\text{compactness})^{\Gamma} \qquad \textbf{(2.2)}$$

where RP represents rheological parameters (*e.g.* K and n), C_m is the biomass concentration and α, β, and Γ are constants. Values for the relevant parameters were presented for *P. chrysogenum* broths but the model has yet to be applied to other systems.

In order to relate rheological behaviour to cell morphology for broths of *Aspergillus awamori*, Ruohang and Webb (1995) derived two empirical correlations, where the only variable was cell concentration. This approach is obviously very attractive in that it allows for simple broth rheology prediction during the fermentation process; conversely, biomass concentration could be inferred from rheological measurements. However, such correlations seldom have widespread applicability. Queiroz *et al.* (1997) attempted to correlate biomass concentrations in *A. awamori* fermentations with the rheological characteristics of the broth. It was observed that the flow behaviour index, n, was less affected by initial substrate concentration than the consistency index, K. As the biomass concentration increased throughout the fermentation, the broth became pseudoplastic, exhibiting a considerably increased apparent viscosity. This trend reflected the predominance of the filamentous, as opposed to pelleted, growth form. It was noted that the most significant increases in K occurred when the biomass concentration was in the range 4 - 12 g/L, the upper limit corresponding to carbon source exhaustion.

In a study of *P. chrysogenum,* Liu and Yu (1993) found that two of the morphological parameters inferred from rheology measurements (δ, defined by the Casson model, and δ^*, defined by intrinsic viscosity) varied systematically with broth age and with the morphology which was microscopically observed. Because different fermentations require different growth morphologies for optimum product yield, and the morphology of mycelial broths varies with broth age, it was suggested that these morphological parameters might be used to quantitatively represent the morphology of mycelial broths.

Despite the proliferation of combined rheological and morphological studies on specific fermentation systems, the rheological characteristics of a fermentation broth cannot always be related to the cell morphology. For example, Warren *et al.* (1995) found no relationship between broth rheology and cell morphology in studies of three actinomycetes, *Streptomyces rimosus, S. erythraea* and *Actinomadura roseorufa*.

2.6.1.2 EFFECTS OF RHEOLOGY ON DOWNSTREAM PROCESSING

As observed in Section 2.6, the rheological characteristics of a fermentation broth can impact significantly on downstream processing operations. One example of this is the production of viscous polysaccharides *e.g.* pullulan, dextran, and xanthan gum (McNeil and Harvey, 1993).

Separation of micro-organisms from high viscosity fermentation broths is difficult and time-consuming using conventional methods. While studying *A. pullulans* as a source of pullulan, Yamasaki *et al.* (1993a, 1993b) attempted to use cross-flow membrane filtration to isolate the organism from the fermentation broth. *A. pullulans* assumes two different morphologies and their findings showed that the yeast-like form (which produces pullulan) was suited to cross-flow filtration in terms of both permeation flux and transition of pullulan. With this knowledge, the fermentation conditions could be optimised both to increase pullulan production and to improve the separation of biomass from the pullulan. If this were attempted for other viscous fermentation broths, more production processes might become feasible and viscosity-related problems could be minimised during the recovery stages (Yamasaki *et al.*, 1993a, 1993b).

2.6.2 BIOCHEMICAL EFFECTS

Biosynthesis of many antibiotics is known to be sensitive to accumulation of the antibiotic. For example, during studies of josamycin production by *S. narbonensis*, Eiki *et al.* (1988) observed that mycelial growth and josamycin production were interrupted concomitantly in the course of the fermentation even when sufficient glucose was provided. The organism was examined for antibiotic sensitivity and it was noted that mycelial growth was inhibited on the addition of 0.25 – 0.4 mg josamycin/mL broth. This growth inhibition could be partially reversed in a slightly acidic environment and with the addition of yeast extract and magnesium phosphate.

Xylanase production by a selection of actinomycetes was also found to be subject to end-product inhibition (Ball and McCarthy, 1989). When *Streptomyces viridificans*

is cultured in media containing glucose, production of chitinase is subject to both end-product inhibition and catabolite repression (Gupta *et al.*, 1995).

2.7 SUMMARY

On the basis of the work reviewed here, it can be seen that it is of crucial importance to be able to understand and predict the complex relationships that exist between the morphological development of a filamentous organism and accumulation of both biomass and metabolites of interest. Of equal importance is the need to predict the effect that both morphology and biomass can have on broth rheological characteristics and downstream processing requirements. Despite this importance, very few authors have taken both morphology and rheology simultaneously into consideration simultaneously when studying filamentous microorganisms.

In relation to the optimisation of secondary metabolite production, both rheological and biochemical effects must ideally be taken into consideration as well as the influence of changes in media components or the fermentation parameters on the biomass, morphology and growth characteristics of the organism. A delicate compromise must be achieved between these varying factors in order that any fermentation involving microorganisms is truly optimised.

CHAPTER 3

MATERIALS AND EXPERIMENTAL METHODS

3.1 THE ORGANISMS

The organism used for all studies was *Streptomyces natalensis*, NCIMB number 10038 (NCIMB Ltd., UK). This organism is also listed as ATCC 27448 (American Type Culture Collection, USA), CBS 700.57 (Central Bureau Voor Schimellcultures, Holland) and NRRL 2651 (Northern Research Laboratory, USA). The organism was obtained as a freeze-dried culture and was resuspended in liquid medium. Both plate and liquid cultures were established using the resuspended organism.

3.2 AUTOCLAVING PROCEDURE

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

3.3 MEDIA

The components for all media were dissolved in deionised water.

3.3.1 MAINTENANCE MEDIA

The organism was maintained on both Oatmeal and YEPD agar plates. The components and relevant concentrations for both media are shown in Table 3.1.

3.3.2 INOCULUM PREPARATION MEDIUM

As recommended by Struyk and Waisvisz (1975), the organism was cultivated in liquid culture medium prior to inoculation of the fermentation media, to ensure adequate biomass for inoculation. The concentrations of the relevant components of this medium are shown in Table 3.2. Prior to autoclaving, the medium was filtered through Whatman No. 1 filter papers using a Buchner funnel apparatus to remove any particulate matter.

Table 3.1 Solid media formulation

Medium	Component	Manufacturer	Concentration (g/L)
Oatmeal agar	Oatmeal (food grade)	Flahavans and Sons (Waterford, Ireland)	30
	Technical agar No. 3	Oxoid (Basingstoke, UK)	20
YEPD agar	Yeast extract	Oxoid	10
	Bacteriological peptone	Oxoid	20
	Glucose monohydrate	BDH (Poole, UK)	20
	Technical agar No. 3	Oxoid	10

Table 3.2 Inoculum preparation medium

Components	Manufacturer	Concentration (g/L)
Glucose monohydrate	BDH	10
Sodium Chloride	Merck (Germany)	10
Bacteriological Peptone	Oxoid	5
Corn Steep Powder	Merck	3

3.3.3 FERMENTATION MEDIA

3.3.3.1 YEPD LIQUID MEDIUM

The components and concentrations used in the formulation of this medium are described in Table 3.1. Technical agar No. 3 was omitted from the liquid medium.

3.3.3.2 PRODUCTION MEDIUM

The components of the production medium are shown in Table 3.3. The majority of media used were based on this production medium formulation which was used by both Mahon (1990) and McCabe (1990). The variations employed during media studies are described in Chapter 4.

3.3.3.3 UNBUFFERED MEDIUM

This medium was formulated as described in Table 3.3 with the omission of calcium carbonate.

Table 3.3 Production medium formulation

Component	Manufacturer	Concentration (g/L)
Glucose monohydrate	BDH	30
Corn steep powder	Merck	1
Ammonium sulphate	BDH	5
Potassium chloride	Riedel-de Haën (Germany)	4
Potassium dihydrogen phosphate	May and Baker (Dagenham, UK)	0.2
Calcium carbonate	Riedel-de Haën	8

3.3.3.4 BUFFERED MEDIA

A number of buffered media were employed, based on the formulation of the production medium, described in Table 3.3. In all cases, the calcium carbonate was replaced with a phosphate buffer (Table 3.4). After the relevent components were dissolved in deionised water, each solution was filtered as described in Section 3.3.2, to remove residual particulate matter. The concentrations of the components of these media were varied in media studies.

3.3.3.5 PEPTONE MEDIUM

The formulation of this medium is as described in Table 3.3 with ammonium sulphate substituted with an equal concentration of peptone, 5g/L.

Table 3.4 Phosphate buffer formulation (made up to 1L)

Component	Manufacturer	Molarity	Concentration (mL/L)
Sodium hydroxide	Merck	0.2 M	500
Sodium dihydrogen phosphate	Riedel-de Haën	0.2 M	300
Deionised water			200

3.3.3.6 CARBONATED MEDIUM TYPE 1

This medium is as described in Table 3.3, with the calcium carbonate replaced with 8g/L sodium carbonate (Riedel-de Haën).

3.3.3.7 CARBONATED MEDIUM TYPE 2

This medium is as described in Table 3.3, with the calcium carbonate replaced with 8g/L of sodium hydrogen carbonate (Riedel-de Haën).

3.3.3.8 ACTINOMYCETE MEDIUM

This medium is as described by Sarra *et al.* (1996). The components are detailed in Table 3.5.

Table 3.5 Formulation of Actinomycete medium (Sarra *et al.*, 1996)

Component	Manufacturer	Concentration (g/L)
Glucose monohydrate	BDH	40
Yeast extract	Oxoid	6
Bacteriological peptone	Oxoid	4
Glycine	Riedel-de Haën	2
$MgSO_4.H_2O$	May and Baker	0.50
KH_2PO_4	May and Baker	0.68

3.4 CULTIVATION OF THE ORGANISM

3.4.1 CULTIVATION ON SOLID MEDIA

The organism was maintained on both YEPD and Oatmeal agar plates. Subculturing was performed on a fortnightly basis to ensure viable vegetative cultures. The organism was maintained on Oatmeal plates and was cultured on YEPD was to indicate that the fermentation was uncontaminated. Each inoculum sample was also examined microscopically for contamination prior to use.

3.4.1.1 YEPD AGAR

A loop full of cells was taken from a stock plate and aseptically streaked onto a fresh YEPD plate. These plates were then incubated at 30°C for 4 days and stored at 4°C. After inoculating liquid medium with these plate cultures, it was found that the viability of the organism was adversely affected after 2 weeks.

3.4.1.2 OATMEAL AGAR

A 3 mL aliquot of sterile Ringers solution was used to suspend a lawn of the organism taken from a maintenance plate and 1 mL of this suspension was then used to inoculate a fresh plate. These plates were incubated at 30°C for 4 days for vegetative growth and for 14 days for spore formation. Vegetative growth results in a creamy yellow lawn of growth. A characteristic grey colour indicates the presence of spores. These plates were then stored at 4°C.

3.4.2 SHAKE FLASK CULTURES

All shake flask cultures were grown in 250 mL Erlenmeyer flasks, containing between 50 and 150 mL of medium as specified. The flasks were incubated at 30°C and at a rotational speed of 180rpm on a two-tier LH engineering orbital shaker, with a throw of 50 mm. Cotton wool bungs were used to close the flasks and prevent contamination, while still allowing an oxygen supply to the organism.

3.4.2.1 INOCULUM SHAKE FLASK CULTURES

For inoculum cultures, 50 mL aliquots of inoculum preparation medium were placed in 250 mL Erlenmeyer flasks and inoculated with 1 mL of suspended culture from an Oatmeal maintenance plate. The flasks were incubated for 96 hours under the conditions described in Section 3.4.2. Similar conditions were employed by Mahon (1990).

3.4.2.2 SHAKE FLASK FERMENTATIONS

For all fermentations, with the exceptions of the volume study cultures, 100 mL aliquots of a fermentation medium were placed in 250 mL Erlenmeyer flasks and each inoculated with 5 mL from an inoculum culture. The flasks were then incubated for up to 8 days at 30°C on an orbital shaker (180rpm). In the case of volume study fermentations, 50 mL, 100 mL and 150 mL aliquots were variously employed. The inoculum volume was scaled accordingly. These flasks were incubated in the same manner as all others.

3.4.3 1L STR FERMENTATIONS

In these fermentations, the organism was cultivated in a baffled 'Micogen' fermentation pot. Air was supplied to the fermentation broth by an aquarium air

pump (Interpet Ltd., Surrey, U.K.). Prior to being introduced to the broth the air was filtered through a sterile air filter. The aeration rate in all cases was 0.75vvm (based on initial broth volume) and air was introduced by a sparger, centrally positioned, 1 cm below the impeller. Temperature control was implemented using a circulating water bath with the water circulated through the vessel baffles. A working volume of 800 mL was used. The medium was autoclaved in the glass fermentation pot (New Brunswick Scientific Ltd.), by placing the vessel (including associated air filters) into the Tomy SS-325 autoclave, at 121°C (15psi) for 20 minutes. Agitation was implemented using a Biolafitte agitator motor, coupled to a shaft fitted with a turbine impeller. The organism was cultivated for up to 10 days and the broth was sampled on alternate days. Further details on the design of the fermenter are provided in Table 3.6.

Table 3.6 Configuration of the 1L fermenter

Vessel diameter (T) (cm)	10.50
Liquid height (H) (cm)	9.25
Impeller diameter (D) (cm)	4.75
Clearance of impeller above base (cm)	2.00
D/T	0.45
H/T	0.91

3.4.4 10 LITRE FERMENTATIONS

A 10L "Micogen" fermenter was also used for batch fermentations and had one major advantage over both shake flask and 1L STR work. Due to the large culture volume, samples could be taken without significantly reducing the culture volume. This removed the risk of flask-flask variations encountered with shake flask work, and increased the accuracy of the data collected.

3.4.4.1 FERMENTER CONFIGURATION

The fermenter used was a 16L stainless steel vessel equipped with a self-sterilising sample port at the base. Agitation was supplied by a belt driven stainless steel shaft, on which three Rushton turbine impellers were mounted. Aeration was supplied via

a nozzle sparger centrally positioned below the bottom impeller. Further details are provided in Table 3.6.

Table 3.7 Configuration of "Micogen" fermenter

Vessel diameter (T) (cm)	22.0
Height of the liquid (H) (cm)	26.5
Impeller diameter (D) (cm)	7.50
Clearance of lowest impeller above base (cm)	7.00
Impeller spacing on shaft (cm)	10.00
D/T	0.34
H/T	1.20

3.4.4.2 FERMENTATION SETUP AND STERILISATION

Prior to use, the vessel was cleaned thoroughly and rinsed with deionised water. 10L of prepared medium was then added through one of the access ports on the top-plate. 10 mL of silicone antifoam was added prior to sterilisation in the fermenter. The dissolved oxygen probe (Mettler Toledo) polarised according to manufacturers' instructions was inserted into one of the top ports. The vessel was sealed and the agitation rate set to 600 rpm. Sterilisation was performed *in situ* by passing steam at 15 - 20psi through the vessel baffles. Once the pressure in the vessel had reached 5psi (corresponding to a temperature of approximately 105°C) steam was introduced to the medium through the main sparger. This flow was halted when the temperature reached approximately 118°C as, if the steam injection was prolonged, the vessel temperature would overshoot the desired 121°C. When the temperature in the vessel reached 121°C, it was held constant for 25 - 30 minutes, to ensure effective sterilisation of the medium. Once the sterilisation cycle was complete, the steam supply was terminated and the vessel cooled by passing water through the baffles. As the vessel temperature fell towards 100°C, the vessel was sparged with sterile air to prevent the pressure dropping below 5psi. This pressure was then maintained throughout the fermentation.

3.4.4.3 FERMENTER OPERATION

Once the vessel had stabilised at cultivation temperature and the dissolved oxygen probe calibrated, the vessel pressure was dropped briefly to allow inoculation via one of the top ports. A blowtorch was used to sterilise the neck of the Erlenmeyer flask and the port, immediately prior and subsequent to the transfer.

3.4.4.4 FERMENTER VALIDATION

A number of problems were encountered in maintaining sterility during the cultivation of *S. natalensis* in the 16L fermenter. Frequently, in the fermentations attempted, the fermentation would become contaminated after approximately 24 hours with a *Bacillus* strain. Several tests were performed to isolate the contamination source.

Initially, it was thought that the excessive foaming encountered during normal operation compromised the integrity of the seals and/or filters and thereby caused the contamination problem. However by varying the antifoam addition profile, it was observed that the foaming occurred as a result of contamination and not as a precursor, as was originally suspected.

The next step of the validation process focused on the sterilisation operation. 10L of production medium was placed in the fermenter and sterilised as outlined in Section 3.4.4.2. After the medium was cooled to 27°C, a sample was taken aseptically and microscopically examined. When the sample appeared free from any microbial presence, a loopful was steaked on a YEPD agar plate and incubated for three days at 30°C. This plate subsequently indicated that the sample was sterile when removed from the vessel.

The integrity of the seals was then examined. Again, 10L of production medium was autoclaved *in situ* as described in Section 3.4.4.2. After the medium was cooled, the fermenter was allowed stand for four days at a pressure of 5psi, agitated at 600rpm. The vessel was not aerated. At the end of the four days, a sample was aseptically removed and analysed microscopically. A loopful was also streaked onto a YEPD agar plate. Again the sample was proven to be sterile.

The fermenter was filled with production medium which was sterilised and allowed to cool. The vessel was then aerated with compressed air at 1 vvm and agitated at 600rpm. No inoculum was added. However, after 24 hours the fermentation medium was found to be contaminated. The air filter was replaced and a number of successful runs at 400rpm were accomplished. There was, however, a recurrence of this sterility problem after a few weeks. It appeared to be associated with the inlet air line and/or the connections to the vessel. It was not possible to remedy the problem and no further work in the 16 L vessel could be undertaken.

3.5 MEASUREMENT OF FERMENTATION PARAMETERS

3.5.1 DETERMINATION OF BIOMASS

Whatman No. 1 filter papers (70mm diameter) were pre-dried overnight at 105°C. This temperature was previously used to dry actinomycete, yeast and bacteria samples. Deionised water (100 mL) was passed through the filter paper in a Buchner funnel apparatus and the filter was then weighed on an analytical balance. A predetermined volume of culture fluid was passed through the wet filter paper in the Buchner funnel. The resultant supernatant was again filtered through the filter cake to ensure removal of all cellular material. The filter cake was then washed with 100 mL of deionised water to remove any residual extracellular material from the cake mass. The paper was reweighed and the increase in filter weight used to calculate sample wet weight (g/L).

To determine the sample dry weight (g/L), the pre-dried filters were quickly weighed on an analytical balance prior to use. The protocol followed is as described for the wet weight calculations with the exception that prior to weighing the filter cake, the paper was then returned to the oven and dried overnight. Again the increase in filter weight was used to calculate sample dry weight (g/L).

3.5.1.1 ATTEMPTS TO REMOVE CARBONATE FOR BIOMASS DETERMINATION

Due to the fact that the production medium described in Section 3.3.3.2 contained calcium carbonate, it was not possible to accurately determine the biomass yields

using this medium. Therefore, a number of strategies were investigated with a view to removing the suspended solids in order to aid in the analysis of fermentation broth samples.

While it is known (Perry and Green, 1997) that calcium carbonate is only sparingly soluble in water (0.0014%), the first test involved establishing the solubility of calcium carbonate in the medium. A known quantity of pre-dried calcium carbonate was added to an aliquot of medium, mixed thoroughly and the resulting suspension filtered. The supernatant was passed through the filter cake again, to eliminate the possibility of some of the carbonate escaping through the filter paper. The dry weight of the filter cake was determined. It can be seen, from Figure 3.1, that calcium carbonate is slightly soluble in the medium. In the fermentation medium, the calcium carbonate acts as a buffering agent and some dissolution occurs during the course of the fermentation, as the broth pH changes. Therefore, knowledge of the initial calcium carbonate concentration in the production medium is not sufficient to permit the accurate determination of the contribution of solid $CaCO_3$ to the biomass concentration, as estimated by dry weight measurements.

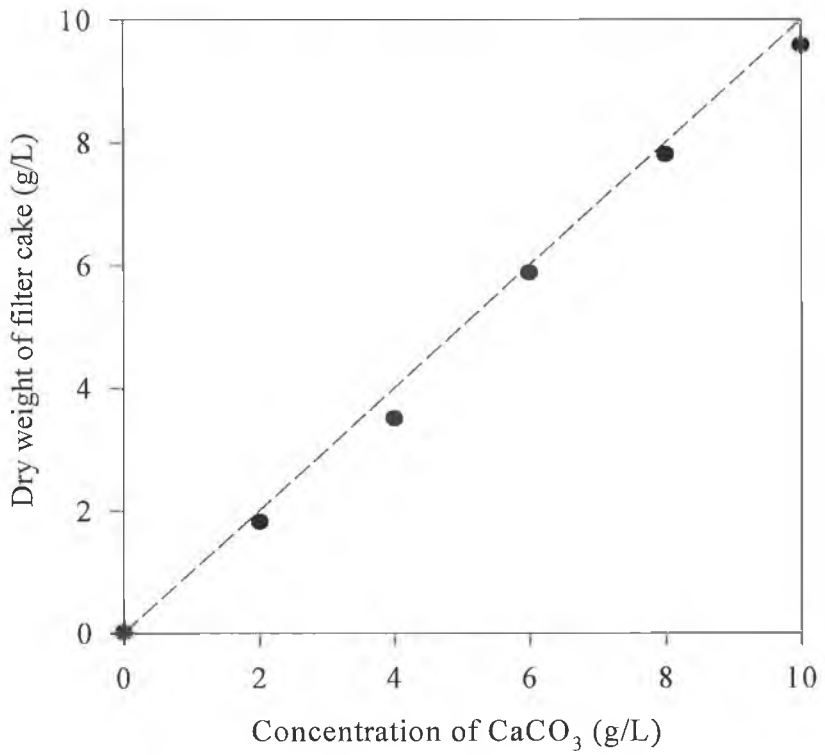


Figure 3.1 Data obtained during calcium carbonate solubility tests

A second test was performed in an attempt to separate the suspended solids from the biomass by centrifugation. However, the density of the carbonate was similar to that of the biomass and the two could not be separated in this way.

Calcium carbonate becomes soluble as the pH of the medium is lowered. Therefore, the third experiment involved reducing the pH of a broth sample to pH 3.0 by the addition of concentrated phosphoric acid. This resulted in the full dissolution of calcium carbonate in the medium. In order to apply this technique to broth samples, it was necessary to determine whether changing the pH of a broth sample would result in cell lysis, leading to lower dry weight readings and/or variations in morphological data. Table 3.8 shows the dry weight and morphological data for both control flasks and samples in which the pH was reduced to pH 3.0. This test was performed a minimum of three times. It can be seen that the imposed pH variation does not significantly affect the characteristics of the sample. This method was, therefore, adopted for the removal of suspended solids in production medium, and was used in the collection of all data presented in this thesis.

Table 3.8 Effect of pH adjustment on fermentation broth sample characteristics

Characteristics measured	Control	pH adjusted flask
Overall pH	6.26	3.01
Dry weight (g/L)	1.04	1.06
Pellet area (mm^2)	$7.87 * 10^{-3}$	$7.52 * 10^{-3}$
Pellet diameter (mm)	0.08	0.10
% Clumps	37.73	36.84

3.5.2 DETERMINATION OF SUGAR CONCENTRATION

All reducing sugar concentrations were determined using a variation of the dinitrosalycyclic acid method (Miller, 1959). A 0.5 mL sample of the supernatant recovered on filtering the broth through a Whatman No. 1 filter paper was added to 1.5 mL of DNS reagent. In some cases, it was necessary to dilute the supernatant sample with deionised water. The samples were then boiled for 10 minutes in a waterbath. A 5 mL aliquot of deionised water was added to each vial and the samples were then allowed to cool to room temperature. The optical density of the

samples was read at 540nm on a uv/vis spectrometer (Pye Unicam 8625, ATI Unicam, Cambridge, UK). A standard curve for the concentration range 0.0g/L - 1.0g/L was found to be linear and was repeated for each set of samples analysed. A representative standard curve is presented in Figure 3.2. The sugar concentration in each sample was determined from the measured absorbance, using the standard curve.

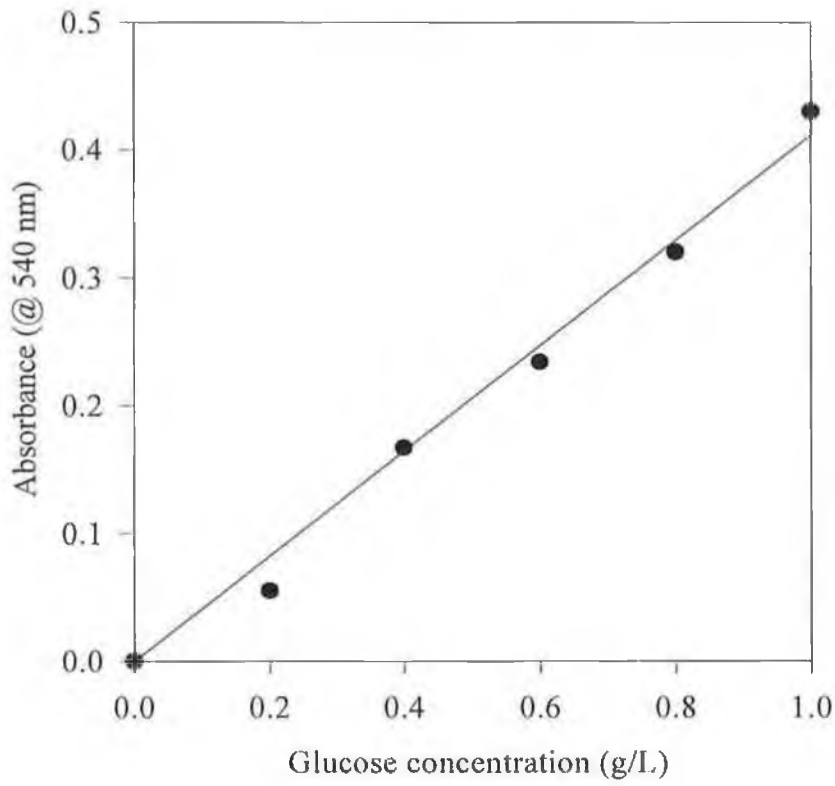


Figure 3.2 Sample of glucose standard curve for DNS assay

3.5.3 DETERMINATION OF pH

Broth pH was determined using a WTW Microprocessor Precision-pH/mV-Meter meter. Samples of the supernatant generated from the dry weight analysis were used in preference to cell samples.

3.5.4 DETERMINATION OF DISSOLVED OXYGEN CONCENTRATION

The dissolved oxygen concentration was monitored in the 16L 'Micogen' system using a Mettler Toledo dissolved oxygen probe connected to a Mettler Toledo dissolved oxygen transmitter (Model 4300). After connection to the transmitter, the probe was allowed to polarise according to manufacturer's instructions for 1 - 2 hours. It was then calibrated prior to inoculation.

3.6 DETECTION OF PIMARICIN

Three separate methods were used in an effort to detect pimaricin in the fermentation cultures of *S. natalensis*. These methods varied in both sensitivity and accuracy.

3.6.1 BIOASSAY

This assay, developed by Raab (1972), is the least sensitive and least accurate of the methods employed in the detection of pimaricin. An overnight culture of *Saccharomyces cerevisiae*, listed as ATCC number 9763, was prepared and 0.1 mL of this culture was used to seed 300 mL of molten YEPD agar. This agar was then dispensed into sterile petri dishes (Sterilin) - exactly 20 mL per plate - and these were allowed to set. Using a flame sterilised 7mm cork borer, a well was made in the centre of each plate. A 150µL aliquot of either supernatant sample or pimaricin standard was placed in the well. The plates were then covered and allowed to stand at room temperature for 2 hours, to permit sample diffusion to take place. They were then incubated overnight at 30°C. After incubation, clear zones on the plates, highlighted using a light box, indicated the presence of pimaricin and could be compared to the range of standards. The pimaricin standards used for this assay were in the range 5µg/mL to 400µg/mL. The pimaricin was diluted in a 65:35 mixture of methanol and distilled water. Supernatant samples were also diluted in methanol-water (65:35).

The accuracy of this method is limited as it relies on the human eye to differentiate between the varying diameters of the clear zones. The absence of a visible clear zone at a pimaricin concentration of 5 µg/mL suggests that the method may be insensitive

to low levels of pimaricin. However it is an easy and inexpensive method for qualitatively establishing the presence of pimaricin at concentrations in excess of 5 μg/mL. For the conditions investigated, a linear relationship was found to exist between pimaricin concentration in the range 50 -350 μg/mL and the diameter of the resulting clear zone. Figure 3.3 shows the standard curve for this bioassay, while Figure 3.4 demonstrates the distinction between positive and negative results.

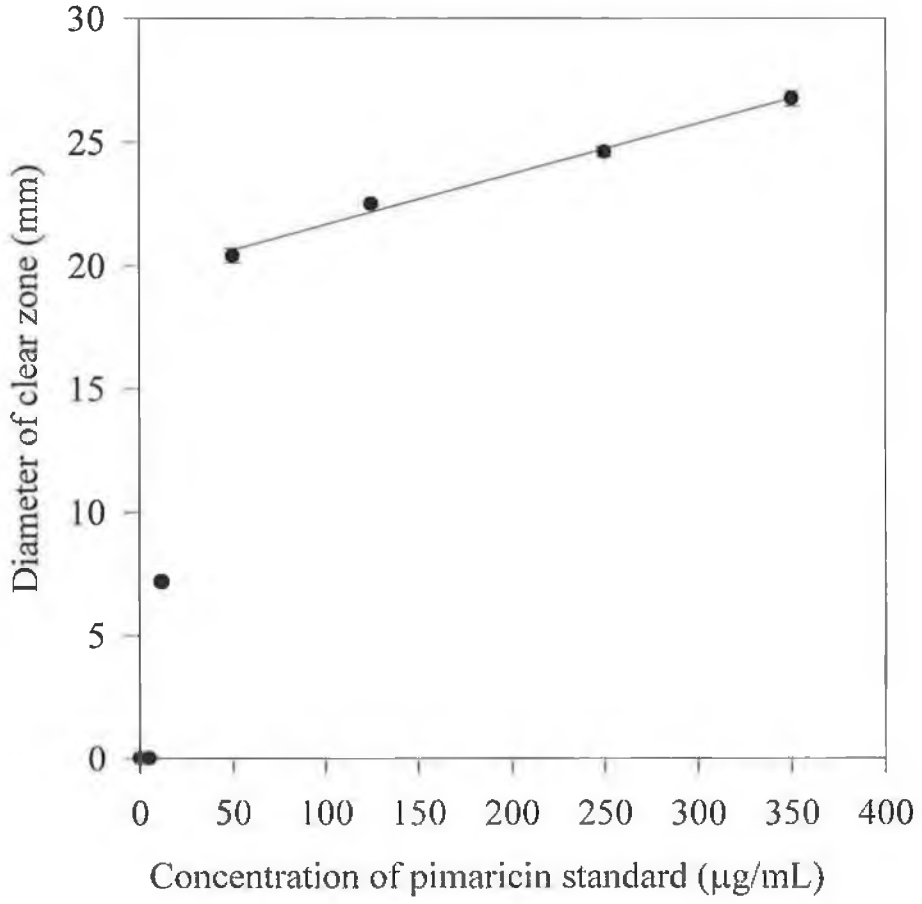


Figure 3.3 Sample of standard curve for pimaricin bioassay

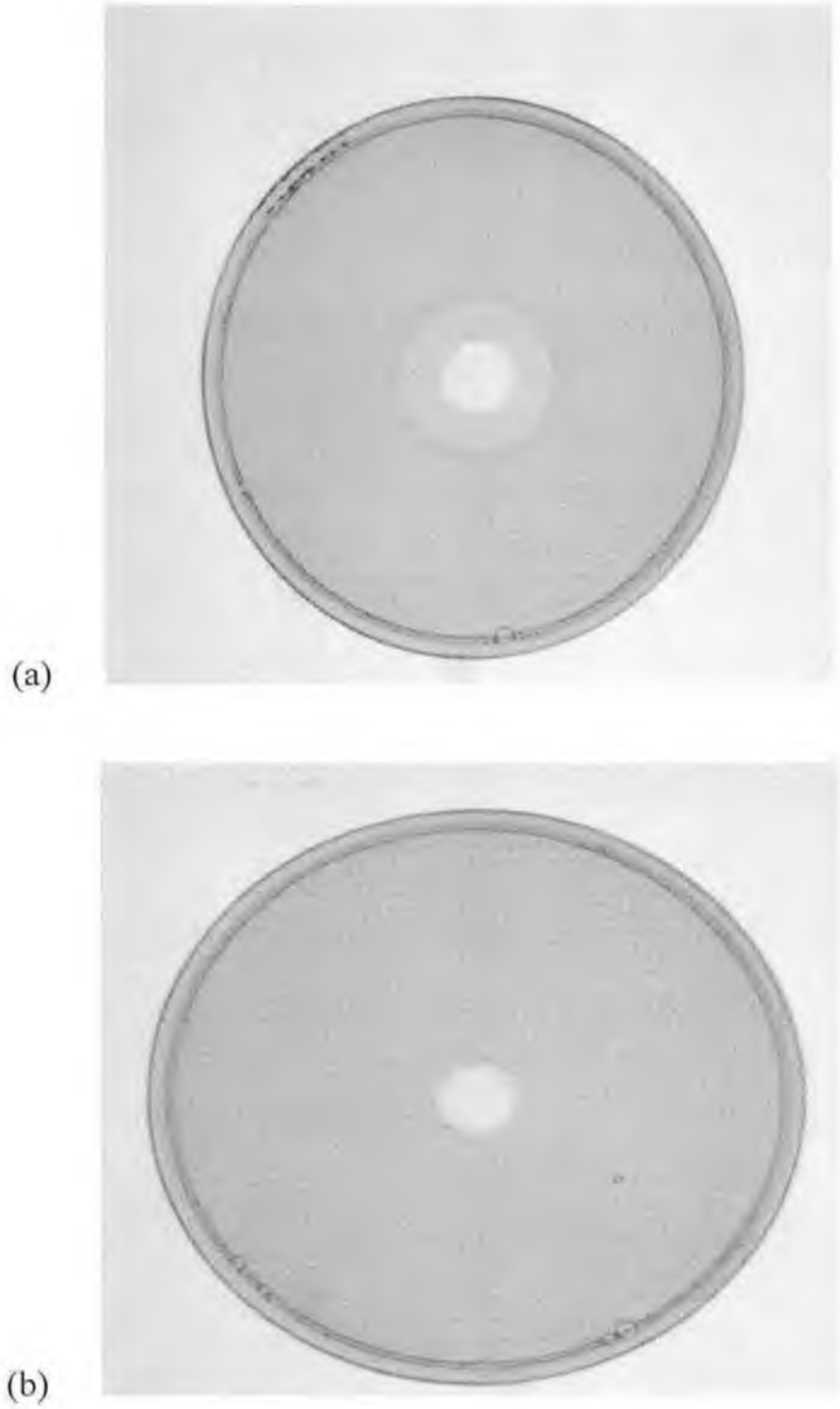


Figure 3.4 Sample of (a) positive (50 μg/mL) and (b) negative results for pimaricin bioassay

3.6.2 UV METHOD

This method was adapted from the work of De Ruig *et al.* (1987). It is significantly more sensitive than the bioassay as it can detect concentrations of pimaricin as low as 1μg/mL. Samples of supernatant were diluted in a methanol water mix (65:35). Standard pimaricin solutions of 5μg/mL and 15μg/mL were run with each batch of samples. The UV absorbance of the samples in the range 340nm to 300nm were scanned using an UV-160A Shimadzu spectrophotometer. Aqueous methanol was used as a blank. The height of the characteristic pimaricin peak at 317nm has been shown to be directly proportional to the concentration between 1 and 17μg/mL (McCabe, 1990), and these peak heights can be used to calculate the concentration of pimaricin in broth supernatant samples. Figure 3.5 illustrates the characteristic pimaricin peak on the absorption spectrum.

Using equation (3.1) the pimaricin concentration in a sample can be calculated.

$$Cs = \frac{Ps \times Cn}{Pn} \times dilution\ factor \qquad (3.1)$$

where

Cs = Concentration of pimaricin in sample (μg/mL)

Ps = Peak height of sample at 317nm (mm)

Pn = Peak height of pimaricin standard (mm)

Cn = Concentration of pimaricin in standard (μg/mL)

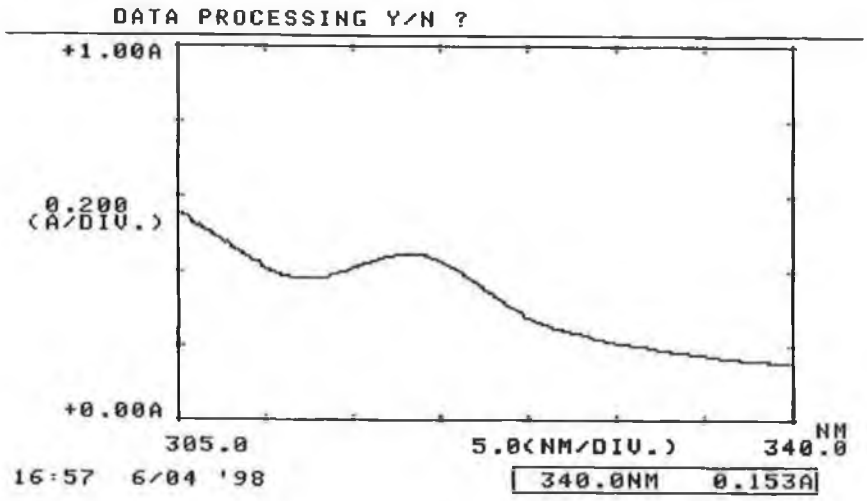
3.6.3 HPLC METHOD FOR THE DETECTION OF PIMARICIN

The following method was developed by Mahon (1990), for the pre-treatment of fermentation samples prior to pimaricin detection using a HPLC based method.

A 2 mL aliquot of fermentation sample was placed in a glass universal containing 2 mL of 2M NaOH. These samples were vortexed and left to stand overnight to facilitate the leaching of pimaricin from the mycelium. The pH of the samples was then adjusted to approximately pH 3.0 with 0.5 mL concentrated phosphoric acid (BDH) and the samples were immediately extracted with 5 mL of butan-1-ol (BDH).

Figure 3.5 (a) Positive (10 μg/mL) and (b) negative UV profiles for pimaricin detection.

(a)



(b)

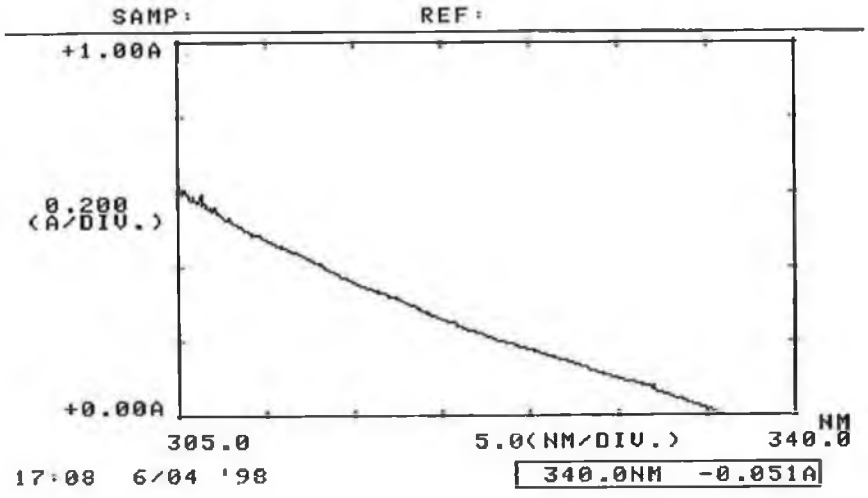


Figure 3.5 (a) Positive (10 µg/mL) and (b) negative UV profiles for pimaricin detection.

A Shimadzu Liquid Chromatograph equipped with an LC-9A pump and an SPD-6AV uv/vis spectrophotometric detector was used for analysis, at a wavelength of 303nm. A flowrate of 0.5 mL/min was employed and the pressure cut-off point was set at 1300Pa. A guard column packed with C8 packing was used to protect the analysis column, a LiChrosorb RP8-10 column, of length 25 cm and diameter 4.6 mm. Both the guard column and the analysis column were obtained from Hichrom (Reading, U.K.). The chromatograph was attached to a computer for data acquisition purposes.

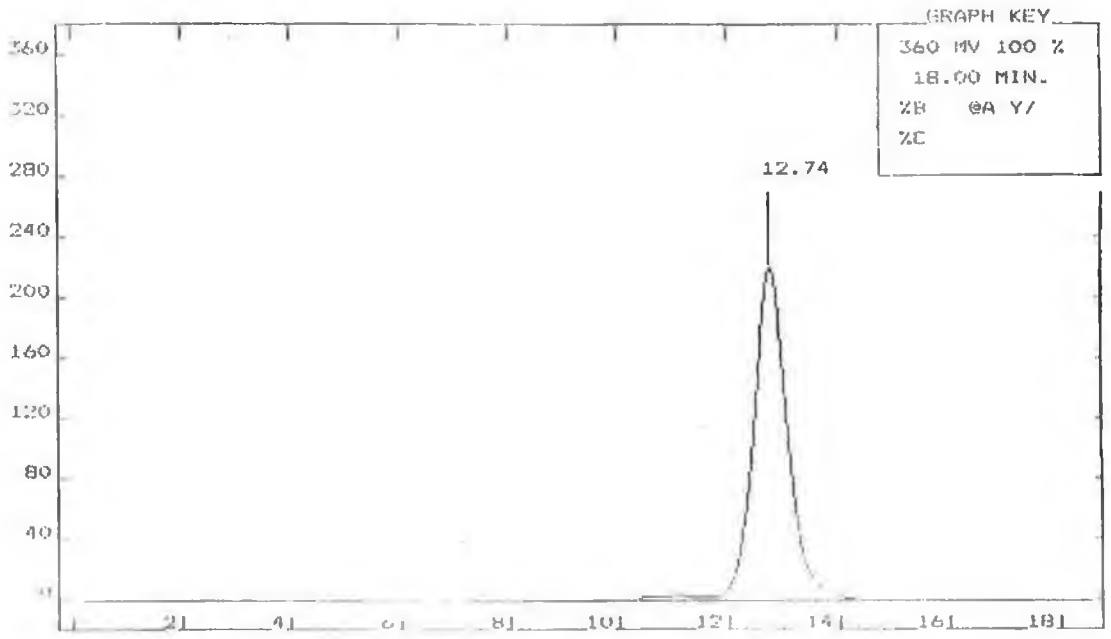
The mobile phase used in this study was a 65:35 mixture of HPLC grade methanol and aqueous potassium dihydrogen phosphate solution (3.03g/L). This mobile phase was filtered through 0.45μm Millipore filters and degassed prior to use under vacuum. It was then passed through the column for approximately 1 hour to equilibrate the column to the mobile phase.

A 10 μL aliquot of sample was injected into the HPLC system using a 100 μL microliter syringe (Hamilton Bonaduz Ltd., Switzerland). The trace plotted on the computer was used to calculate the concentration of pimaricin levels. The areas under the peaks obtained by injecting pimaricin standards (0.005g/L - 0.05g/L) were used to quantify the concentration in the injected samples. Figure 3.6 outlines (a) a pimaricin positive trace and (b) a negative trace.

3.6.3.1 VALIDATION OF TECHNIQUE AND PROBLEMS ENCOUNTERED

Despite using all three methods for all fermentation samples collected during the studies of *S. natalensis*, all results were negative for pimaricin. Attempts were made to validate the HPLC extraction method as this is the most sensitive of the three detection methods described. All tests performed were completed a minimum of four times. The first step was to identify the peaks associated with the solvents used in the extraction process and those corresponding to the fermentation medium. A simple visual comparison confirmed that the peaks observed with the extracted fermentation samples did not correspond to pimaricin.

(a)



(b)

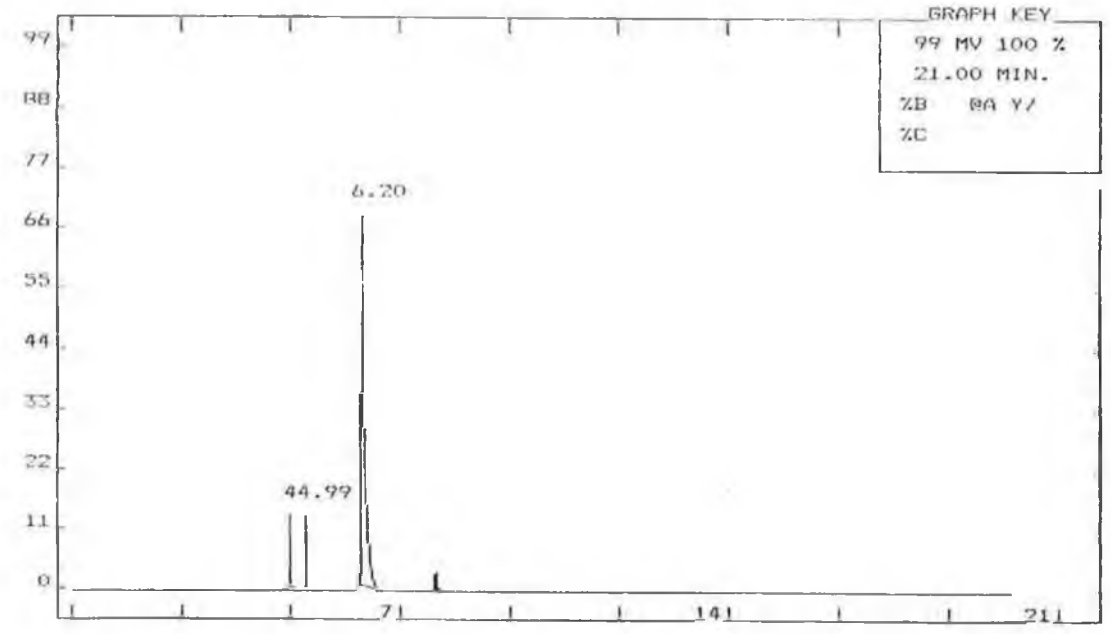


Figure 3.6 Pimaricin (a) positive (0.05 g/L) and (b) negative HPLC profiles

For the UV method, only extracellular pimaricin (*i.e.* that present in the supernatant) is detected. Accordingly, no pre-treatment of the biomass should, in theory, be necessary for the HPLC method. However, a pre-treatment stage was introduced by Mahon (1990) with a view to maximising pimaricin concentration for detection. As all broth samples for analysis were frozen for storage, a sample pimaricin solution was frozen overnight and, when thawed, analysed using HPLC. The results indicated that the pimaricin was unaffected by freezing.

1 mL of supernatant was spiked with 1 mL of pimaricin standard (0.05 g/L) and extracted as described in Section 3.6.3. This also yielded a negative trace. It was then attempted to ascertain whether this result was associated with one of the chemical species previously employed, *i.e.* phosphoric acid, 2M NaOH, or the butanol extraction step. Pimaricin was found to be unaffected by the presence of the phosphoric acid. However when a standard was placed in an equal volume of 2M NaOH overnight, it was found that the pimaricin was partially degraded, resulting in a detection of 0.0055g/L instead of 0.0125g/L pimaricin. This pH sensitivity of pimaricin was previously mentioned by Borden *et al.* (1995). Using a one-step extraction into butanol yielded in a negative trace. While Mahon (1990) succeeded in detecting pimaricin in broth samples, it is now felt that the results were affected by the pre-treatment process. It is likely that the detected levels of pimaricin presented by Mahon (1990) underestimate the actual concentrations present in the broth samples analysed. It was, therefore, necessary to alter both these steps in the extraction technique.

Two alterations in the butanol extraction procedure were attempted using pimaricin standard solutions. Initially the duration of extraction was increased to both 1 hour and 2 hours. These one step extractions both proved ineffective. A three step extraction was then attempted. 1 mL of the standard was added to 1.5 mL of butanol and handshaken. This was allowed to stand for 15 minutes and was shaken again. The butanol layer was at this time removed and placed in a separate glass universal. Fresh butanol (1.5 mL) was added to the original sample and the extraction step repeated. Again the butanol layer was removed and added to the butanol from the

first step. Fresh butanol (2 mL) was added to the sample and the steps were again repeated. This resulted in a very small peak at the time of interest but was still ineffective.

Another, more effective, solvent was then sought to replace butanol. Acetone and methanol were both examined. Acetone, when used to extract the supernatant spiked with pimaricin, resulted in a 90% extraction. Methanol however resulted in a 98% extraction and was employed in all subsequent studies. With biomass samples, the sample for HPLC was filtered through a 0.45μm filter prior to injection.

It was postulated that, by reducing the exposure time of the pimaricin to the 2M NaOH, the degradation of the pimaricin would be reduced. However, even by reducing the exposure time to 1 hour the sample still yielded a negative trace. It was then decided to reduce the concentration of the NaOH to 0.5M while the exposure times were varied between 15 minutes and 12 hours. Although the extent of degradation was reduced, only 24% extraction efficiency was achieved after an exposure time of 15 minutes, using 0.5M NaOH. Using 0.2M NaOH for exposure times of up to 1 hour, a maximum of 45% of the original pimaricin was detected after an exposure time of 30 minutes.

A substitute method for cell lysis was then investigated. 2M Na_2CO_3 was examined for its effect on pimaricin. This was a much more effective technique, resulting in an 83% recovery after 10 minutes exposure. Reducing the molarity of Na_2CO_3 to 0.2M did not affect the efficiency of the assay. In an effort to facilitate cell lysis and the leaching of pimaricin from the mycelia, a 0.05g/L solution of pimaricin and 2M Na_2CO_3 were boiled for 1 hour. This resulted in a negative HPLC trace, suggesting that pimaricin might be heat sensitive. To investigate this possibilty further, the standard solution was heated to 50°C for 2 hours. Subsequent HPLC analysis indicated 90% pimaricin recovery. However when 2M Na_2CO_3 was added to the pimaricin standard prior to heating, the pimaricin detected during HPLC analysis was 2% of the concentration used. When fermentation samples were tested using this method, negative traces were also observed.

Physical extraction was also attempted with the biomass samples being processed in a bead mill prior to filtration and extraction with methanol. This also yielded a negative trace. It was therefore decided to abandon attempts to monitor pimaricin in fermentations as the organism did not appear to produce the antibiotic under any of the cultivation conditions attempted.

In summary, it is postulated that the use of organic solvent, combined with an alkaline treatment, as praticed by Mahon (1990), does not accurately detect the level of pimaricin present in fermentation broth. A gentler technique should be undertaken, using for example a physical method of cell disruption (*e.g.* bead mill) followed by addition to methanol or acetone. While Mahon was successful in detecting levels of pimaricin, it can be assumed that a proportion of pimaricin present in the supernatent would have been adversely affected by the technique employed. However, further work must be undertaken in both extraction and purification from fermentation broth to ensure that the highest recovery possible is achieved.

3.7 STATISTICAL ANALYSIS OF DATA

Statistical analysis of the data collected during this study was performed using the statistical package included in Sigma Plot Scientific Graph System (Jandel Corporation), Version 1.02. Plotted values represent the mean of the data, with error bars representing the standard error.

CHAPTER 4

RESULTS AND DISCUSSION: MORPHOLOGICAL CHARACTERISATION

4.1 INTRODUCTION

When biologists first began investigating micro-organisms, only qualitative analysis could be performed using a microscope. The use of digitising tables in conjuction with microscopes represented a considerable improvement in terms of quantitative measurements. However, the main disadvantages of these early methods for measuring the morphological characteristics of an organism lay in the fact that they were time consuming and subject to human error. The advent of image analysis provided a faster, more accurate, potentially automated, or semi-automated, method of extracting pertinent morphological measurements from stored images and slides; if correctly performed, there is a complete lack of bias in the measurements.

4.2 IMAGE ANALYSIS HARDWARE

The three main hardware components used in the image analysis system employed in this work were a personal computer, a CCD type TV camera and a microscope.

The computer supporting the processing and analysis software contained a 66MHz Intel 486 DX2 processor. An AcerView 56L Low radiation 15" colour monitor (Acer Peripherals Inc., Taiwan) was used. The system also contained an ELViS frame-grabber board, to facilitate the capture of live and frozen video-based images.

The camera employed was a JVC TK-1280E colour video camera (Victor Company of Japan, Ltd., Japan), which was attached to the microscope via a C-mount adapter.

All studies were performed at a magnification of 40×, using an Olympus BX40 brightfield microscope (Olympus Optical Company, Japan). At this magnification, each pixel corresponded to 2.08μm. Both lighting and condenser settings were maintained at constant levels to ensure that the conditions, under which the images were analysed, were reproducible.

4.3 IMAGE ANALYSIS SOFTWARE

The Leica Q500MC software employed was a Windows-based package, which has a number of image processing/ analysis facilities, the most important being:

1. the ability to acquire either a live or stored grey image and display that image on screen. Each grey image is composed of 720x512 pixels, in which each pixel has a grey level ranging from 0 (representing black) to 255 (representing white). Initially a live image is displayed on the screen. Most applications work with monochrome images where grey level differences are exploited to distinguish critical features. Colour systems are also available, but their handling requires more expensive equipment and specialised software (Paul and Thomas, 1998). At this stage the lighting and focus may be adjusted until the image is of a suitable quality, at which point the image is frozen and is then available for processing. The package allows for the short-term storage of up to seven images, in regions known as planes. These planes are also extremely useful for the modification and processing of an image without overwriting or losing the original.
2. the ability to modify and enhance the acquired image until suitable for further analysis. Such modifications include edge enhancers and detectors, image smoothing, sharpening and inversion. These processes are only used if the quality of the frozen image hinders the separation of the regions of interest from the rest of the grey image.
3. the ability to segment regions of interest from the grey image resulting in a binary image. This feature is also known as detection. In order to isolate the regions of interest, the software package uses detectable differences in grey levels between the regions of interest, defined as '1', and the background, defined as '0'.
4. the ability to subsequently facilitate both manual and automatic modification of the binary image. Due to poor detection or the presence of dust or debris, it is sometimes necessary to manipulate the detected image. Six binary planes are provided to facilitate the modification of binary images.
5. the ability to measure a number of selected features contained in the binary image. The software allows the measurement of multiple parameters on any given feature in a binary image, such as feature area, or dimensions such as

diameter and perimeter. Also, measurements can be used to remove features from an image *e.g.* by specifying a minimum acceptable area for regions of interest, debris can be simply removed from a binary image.

6. the ability to present the measured data in a form suitable for further processing or statistical analysis.
7. the ability to program routines to automatically complete complex series of the above events (QUIPS); these routines can be designed to run without manual intervention. Due to the provision of programming operations facilitating data input and output and the ability to conditionally execute certain operations, QUIPS proved very flexible and user friendly.

4.4 THE MORPHOLOGY OF THE ORGANISM

In submerged culture, *Streptomyces natalensis* has been observed to grow in two distinct forms - pelleted growth and filamentous clumped growth. In any fermentation sample, the population contains varying percentages of these morphological forms. Depending on the fermentation medium used and the fermentation conditions to which the organism is exposed, as can be seen from Figure 4.1, pellets may be further reclassified as 'smooth' (a) or 'hairy' (b). Filamentous clumps are shown in Figure 4.1 (c). These images were collected at 100× magnification to highlight the differences between the three forms. The effects of varying the medium components and fermentation parameters on the organism morphology are discussed in more detail in Chapters 5 and 6.

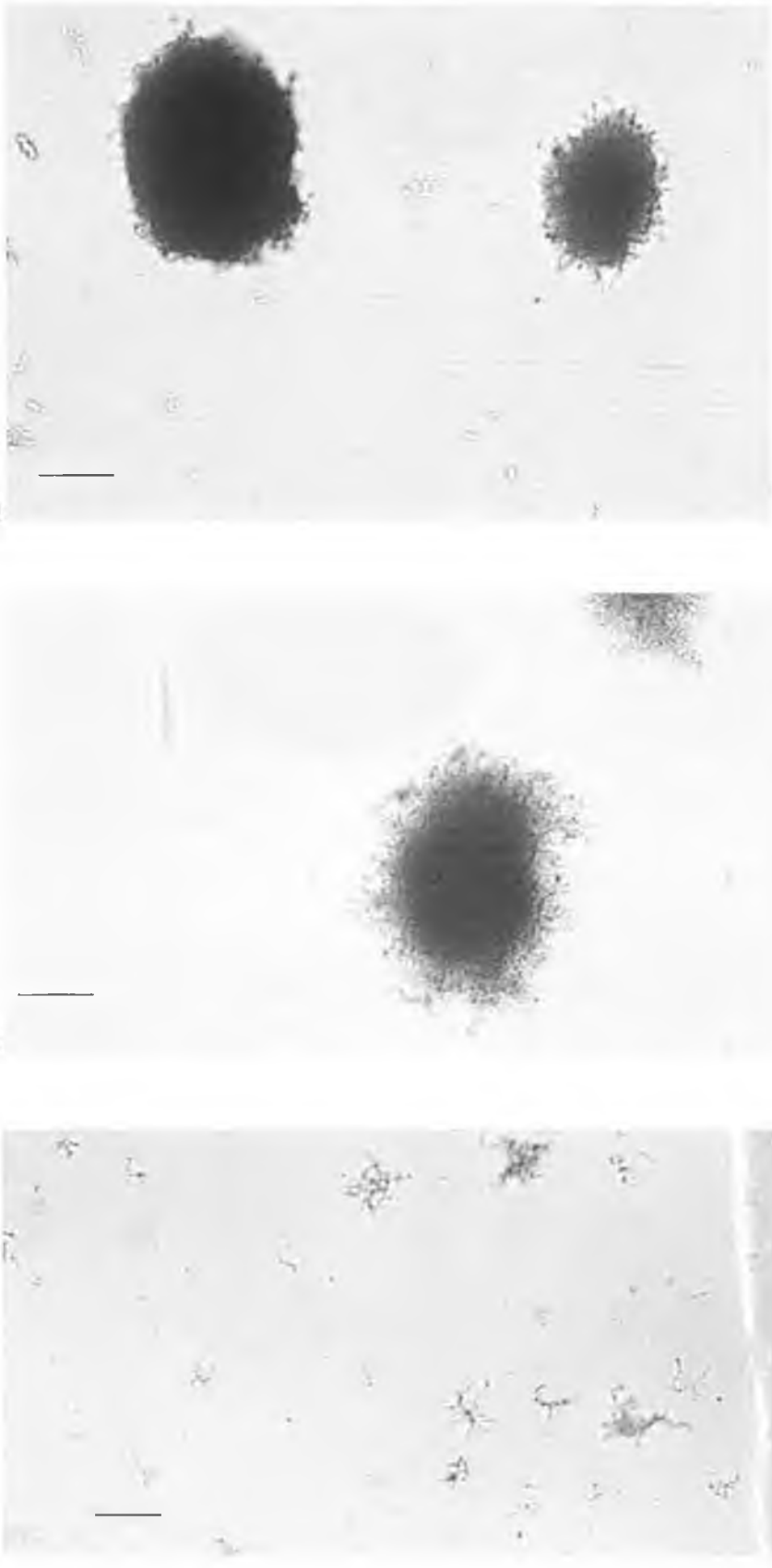


Figure 4.1 Example of *Streptomyces natalensis* morphology (a) typical pellets, 100×; (b) 'hairy' pellets, 100× and (c) filamentous clumps, 100× (Bar = 50μm)

4.5 SLIDE PREPARATION PRIOR TO IMAGE ANALYSIS

Due to the morphology of the organism, the use of glass microscope slides was found to yield inaccurate results. This was due to the fact that when the coverslip was placed on the slide, the particles were flattened and therefore appeared much larger during analysis than in suspension. A Sedgewick Rafter chamber (Graticules Ltd., U.K.) was therefore used as it provided the depth necessary to allow accurate morphological measurements. A 1 mL aliquot of broth was placed in the chamber using a 1 mL Gilson pipette, with a tip diameter of 1 mm, which is approximately 10 times greater than the diameter of the largest biological entity encountered in a typical broth. This sample was allowed to settle for approximately one minute. The samples were diluted, when necessary, with distilled water.

4.6 IMAGE ANALYSIS PROTOCOL FOR THE CHARACTERISATION OF S. NATALENSIS

An example of a typical morphological field acquired during characterisation can be seen in Figure 4.2. It is apparent that the sample contains some debris as well as various combinations of morphological groups. Also, the divisions on the Sedgewick Rafter cell are detectable and interfere with the measurements. Therefore, a great deal of image pre-treatment must be performed prior to the measurement of the morphological parameters.

The first step is to isolate the large pellets from smaller pellets, clumps, media debris and the Sedgewick Rafter divisions, using a size filter and a roundness filter. This step is necessitated by the fact that, when detected, the larger pellets frequently appear attached to the divisions or to smaller pellets, and must be separated, to ensure accurate measurement. This is achieved by a series of erosions and dilations of the binary image. The isolated large pellets are then returned to the binary image storing the smaller pellets and clumps. This binary image must undergo further editing to remove the debris and divisions. The debris is removed by including a minimum area feature accept (all particles less than $2.15 * 10^{-4}$ mm^2 are removed) in the program, while the Sedgewick Rafter divisions are removed by including a roundness feature accept.

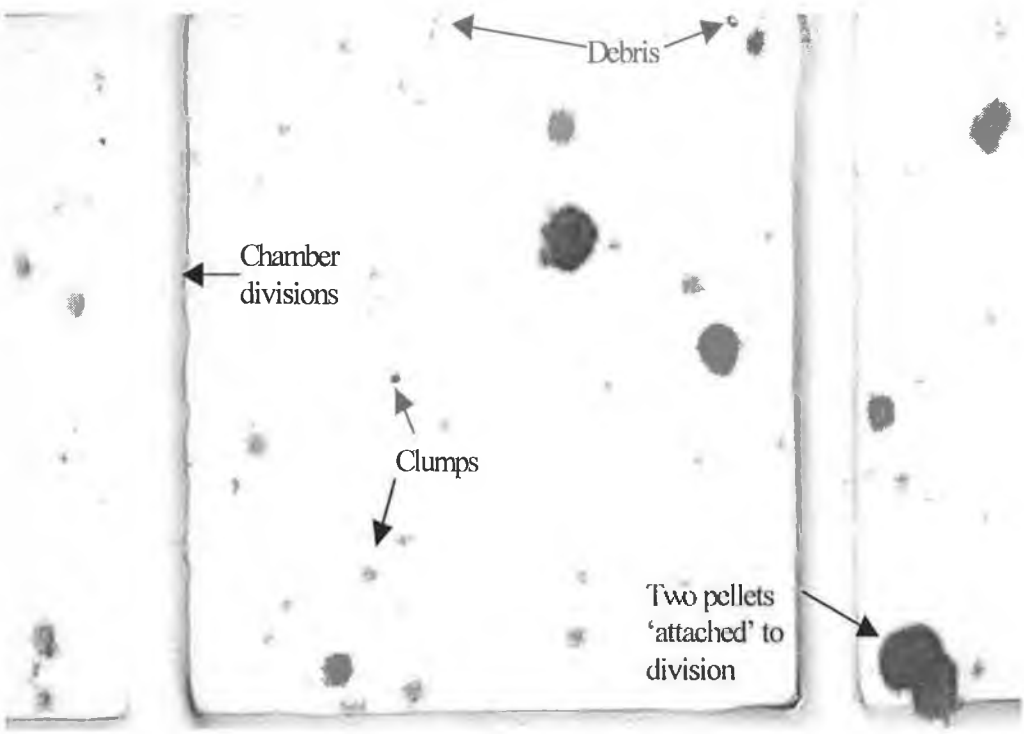


Figure 4.2 Example of typical image analysis field, 40×

A manual edit stage is included to remove any objects which evaded the automatic edit functions *e.g.* air bubbles, and which would otherwise be included in the final binary image. For each detected object, the following parameters are then measured: area, diameter, perimeter, and convex area. The area and convex area of the pellet cores are also measured. The convex area of the pellet is the area of a convex hull surrounding the pellet. The value used for the diameter is the mean of the measurements taken in six different directions (Figure 4.3). The circular outline in Figure 4.3 indicates the convex area of the pellet.

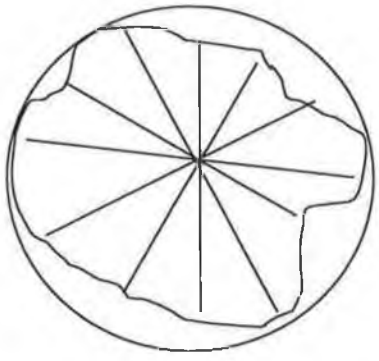


Figure 4.3 Schematic diagram outlining the calculation of the mean pellet diameter used during *S. natalensis* studies.

Using these data, the pellet roughness and the percentage of filamentous clumps in the sample can be calculated. Pellets were defined as any entity with a distinct core; clumps were defined as those without a clear core. The core is defined as any particle with an area less than 2.29 * 10^{-3} mm^2. The percentage of clumps present in a broth sample was calculated on a volume basis, using equation 4.1. Pellet roughness or "hairiness" is calculated using equation 4.2.

$$\%\ clumps = \frac{\sum clump\ volume}{\sum total\ particle\ volume} \times 100 \qquad \textbf{(4.1)}$$

$$roughness = \left(\frac{Core\ area - Core\ convex\ area}{Pellet\ area - Pellet\ convex\ area} \right) \times 100 \qquad \textbf{(4.2)}$$

A minimum of 100 entities were measured in each sample. This limit was tested for statistical accuracy and reproducibility. For a single broth sample the image analysis protocol was run four times. The first run measured 50 objects; the second measured 100; the third, 150; and the fourth 200. It was found that the values recorded for the morphological parameters did not vary for sample sizes greater than than 100. This test was repeated and the same findings were observed.

Figure 4.4 shows an outline of the image analysis protocol used. Appendix A contains a copy of the protocol code.

4.7 MORPHOLOGICAL CHARACTERISATION

4.7.1 MORPHOLOGICAL DEVELOPMENT DURING CULTIVATION

As outlined in Section 4.6, the morphological parameters measured during the fermentation of *S. natalensis* were particle area, particle convex area, particle diameter, particle perimeter, pellet core area, pellet core convex area and percentage clumps. Figure 4.5 illustrates the development of (a) area, (b) convex area and (c) diameter over the course of a batch growth cycle in 250 mL shake flasks containing buffered fermentation medium. Figure 4.6 shows (a) pellet perimeter, (b) particle

roughness and (c) percentage clumps. The development of the pellet core is shown in Figure 4.7. The data presented in Figures 4.5 – 4.7 are the mean values for data acquired from ten independent runs; error bars represent the standard error.

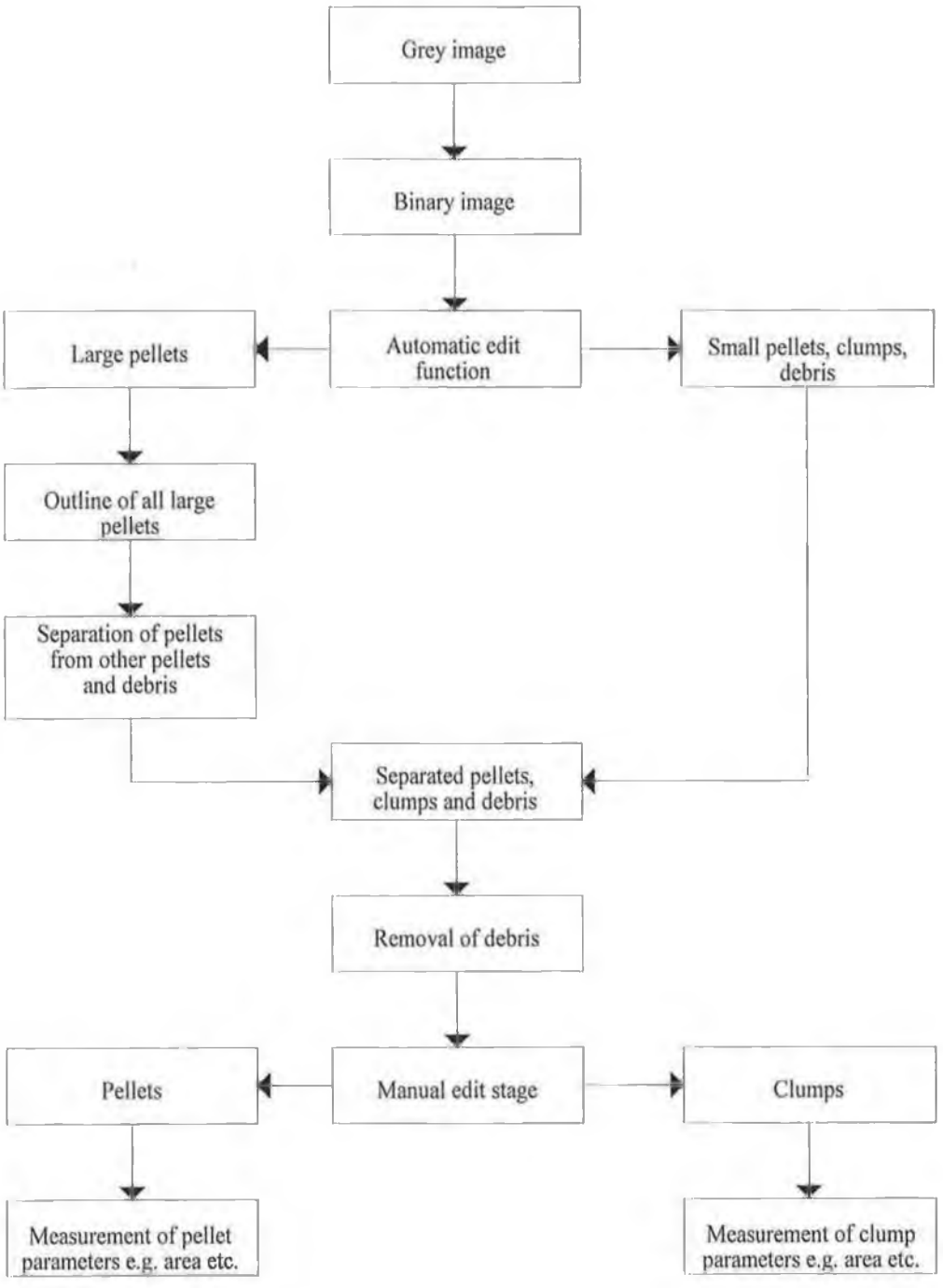


Figure 4.4 Image analysis protocol

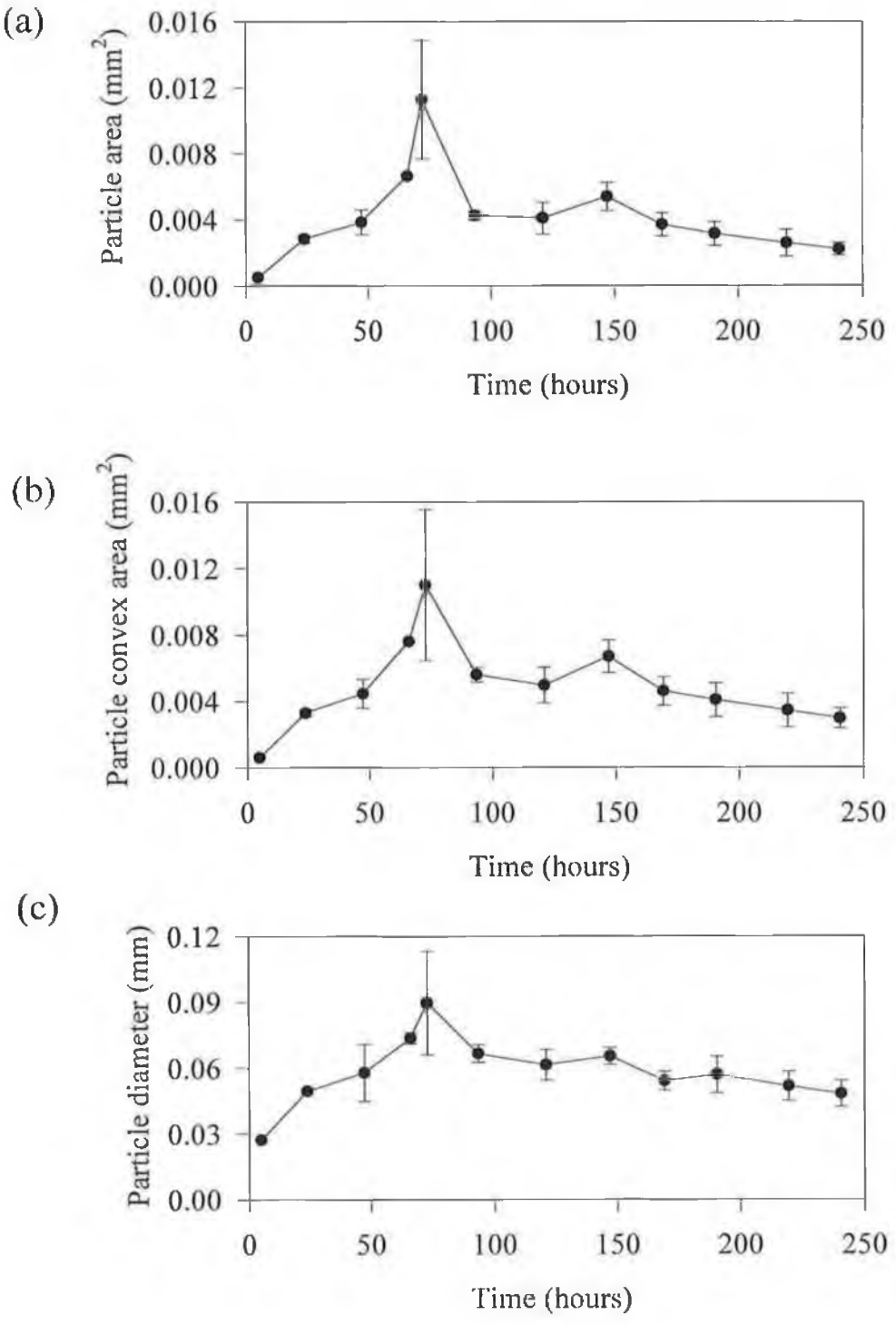


Figure 4.5 Morphological development of *S. natalensis* during batch cultivation in buffered medium (a) particle area, (b) particle convex area and (c) particle diameter

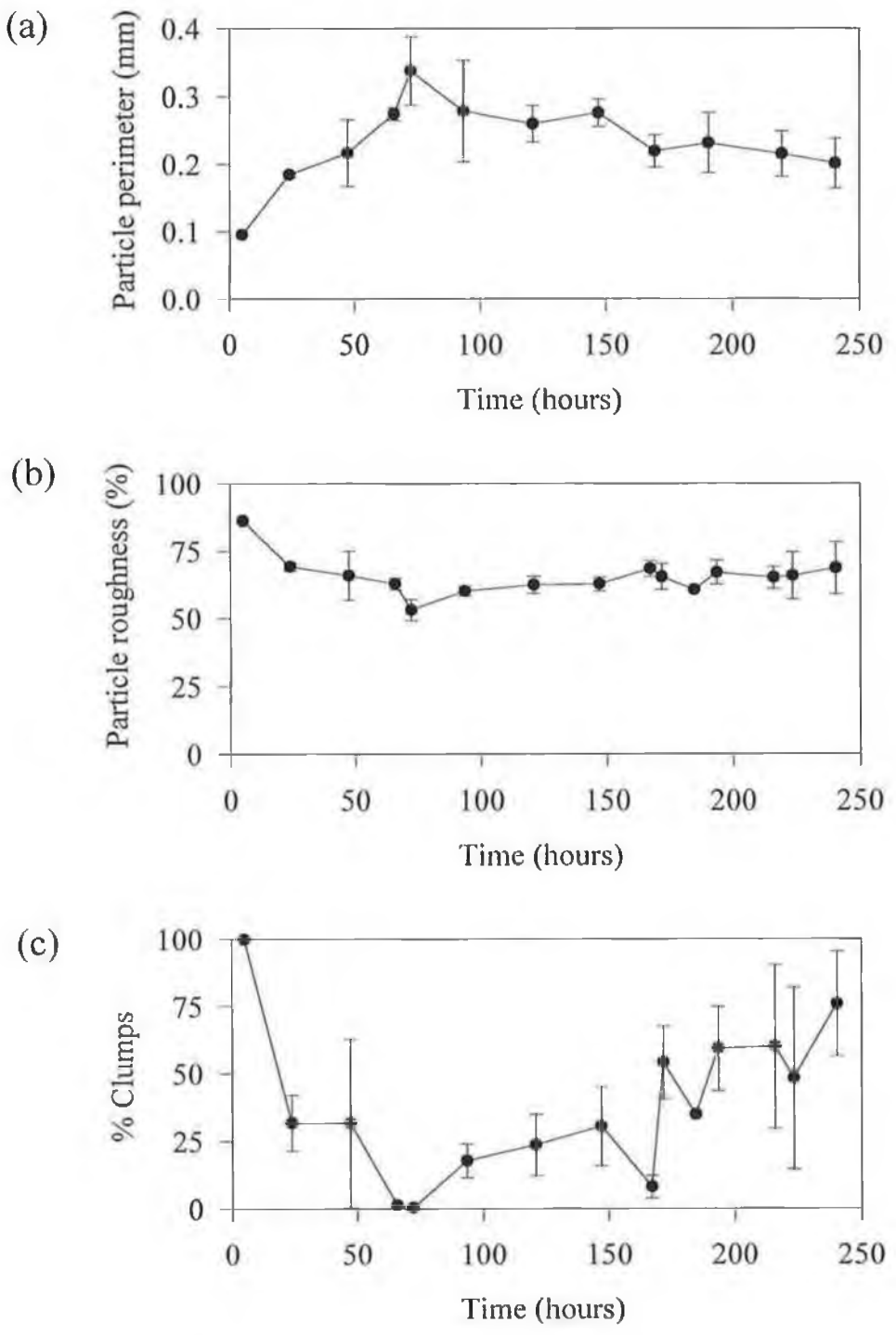


Figure 4.6 Development of (a) particle perimeter, (b) particle roughness and (c) percentage clumps (volume based) in shake flask cultures

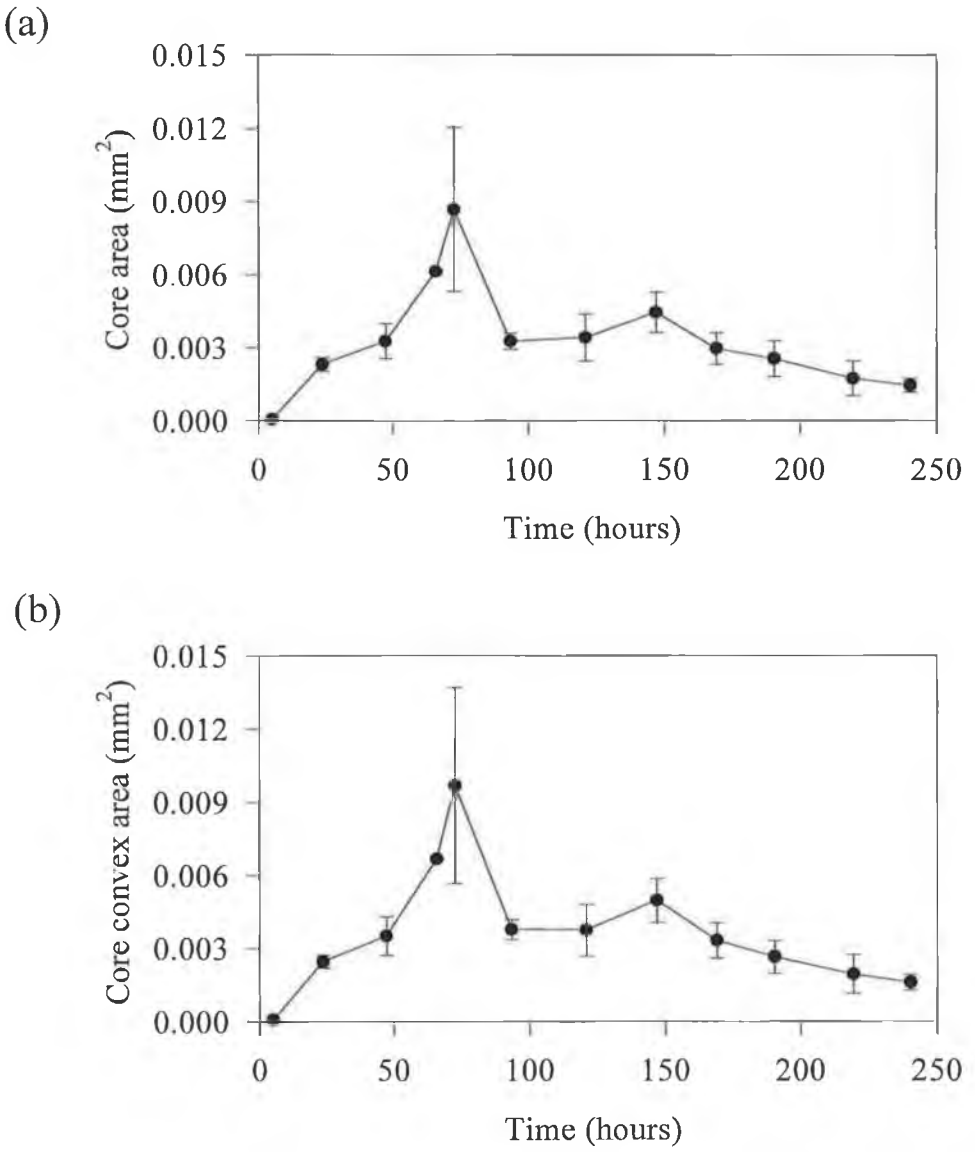


Figure 4.7 Morphological development of pellet core in shake flasks - (a) core area and (b) core convex area

It can be seen from Figure 4.5 (a) that during the first 80 hours after inoculation, the particles present in the broth grow significantly in size, with the average area increasing approximately 20 fold, from 5.2×10^{-4} mm^2 to 0.01 mm^2. At about 80 hours, the particles present in the sample appear to undergo some form of disintegration with the average area plummeting to 4.1×10^{-3} mm^2 at 120 hours and only falling slightly for the remainder of the cultivation period. Coincident with particle area disintegration, the volume based percentage of clumps present in the broth increases from 0.35% to 30.4%, accounted for by fragments of the disintegrated particles. Initially the percentage of clumps present in the sample

(Figure 4.6 (c)) decreases during the early stages of the fermentation. This appears to be due to the fact that the clumps are growing and expanding to form pellets. Also, as outlined in Figure 4.6 (b) the roughness of the particles present decreases until approximately 73 hours. This may be due to a particle consolidation period at the beginning of the fermentation. As can be seen from Figure 4.7, the development of the pellet core area behaves in a similar fashion to the development of the particle area.

There are two possible explanations for the observed pellet disruptions. The first is that the biomass concentration has increased to a level at which the pellets are colliding with each other, causing breakage. This results in a decrease in the mean pellet area observed in these fermentation samples. As the biomass level does not fall for the remainder of the cultivation period, individual pellets in the shake flask never get the opportunity to regrow to the previously observed extent.

The second explanation is that the particles initially increase to this maximum value, through rapid radial expansion. However, as will be discussed in Chapter 5, *S. natalensis* appears to be sensitive to the dissolved oxygen levels in the fermentation broth. The maximum particle area observed was 0.015 mm^2, corresponding to a mean diameter of 90 μm, when the organism was cultivated in buffered fermentation medium. Although this diameter is significantly lower than the value of 400 μm suggested by Schügerl *et al.* (1983) for limitation in *P. chrysogenum* pellets, it is possible that in cultivations of *S. natalensis*, these pellets may be subjected to oxygen limitation.

Given that the biomass concentration in the flasks did not exceed 1.4g/L (d.w.) during the first 100 hours of cultivation, it is unlikely that the first scenario is wholly applicable, and it is postulated that a combination of these two scenarios results in the observed morphological profile in shake flask fermentations.

4.7.2 MORPHOLOGICAL DISTRIBUTIONS

In a number of studies and mathematical models, a common misconception is that the culture consists of uniformly sized pellets. Since the collection of pellet sizes defines the amount of mycelial surface exposed to the nutrient laden medium, it implicitly determines the proportion of mass that actually contributes to growth. Fragmentation can increase this proportion by shifting pellet size distributions to smaller radii, for which there is more surface area exposed per unit volume (Edelstein and Hadar, 1983). A number of factors influence these variations in distributions. The first is a reseeding phenomenon whereby miniature pellets arise from the fragments broken from the larger pellets due to collisions. Other features include pellet lysis and cell death.

Figure 4.8 shows (a) number and (b) volume-based size distribution profiles for *S. natalensis* during batch cultivation in buffered fermentation medium in shake flasks. Because smaller clumps predominate, on a number basis throughout the growth cycle, the contribution of larger pellets to the total biomass present is less obvious than when the data are presented as a volume-based distribution.

As is evident from Figure 4.8, the size distribution profiles of vary significantly during cultivation. Initially the population is relatively homogeneous, all of the biomass appearing in the form of small particles with a volume of less than 0.016 mm^3. As the fermentation progresses, the population becomes more heterogeneous, with the percentage volume occupied by larger particles increasing. Analysis of the number-based distributions (Figure 4.8(a)) confirms the reduction in average pellet size between 78 hours and 96 hours observed in Figure 4.5(a) with comparatively little variation in the size distributions thereafter. However, the corresponding volume-based profiles are less clear. It is obvious that the larger pellets, >0.3 mm^3, constitute a significant fraction of the biomass during the latter stages of the cycle (> 144 hours). It is also possible that during these times, a larger number of entities should be analysed than the minimum of 100, established on the basis of validation trials performed during the earlier stages of the growth cycle (120 hours).

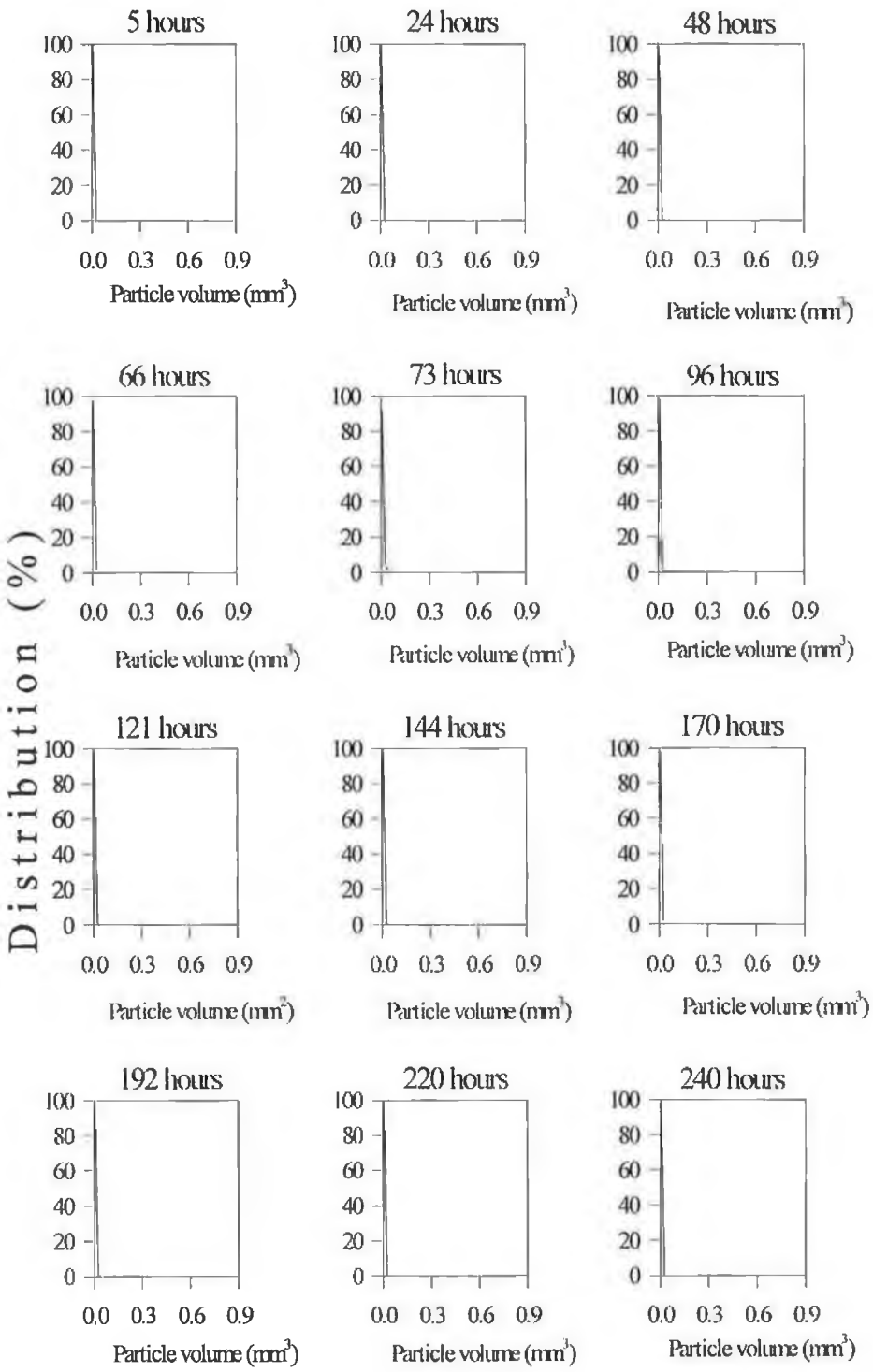


Figure 4.8(a) Development of number based particle size distributions during cultivation in buffered fermentation medium in shake flask cultures.

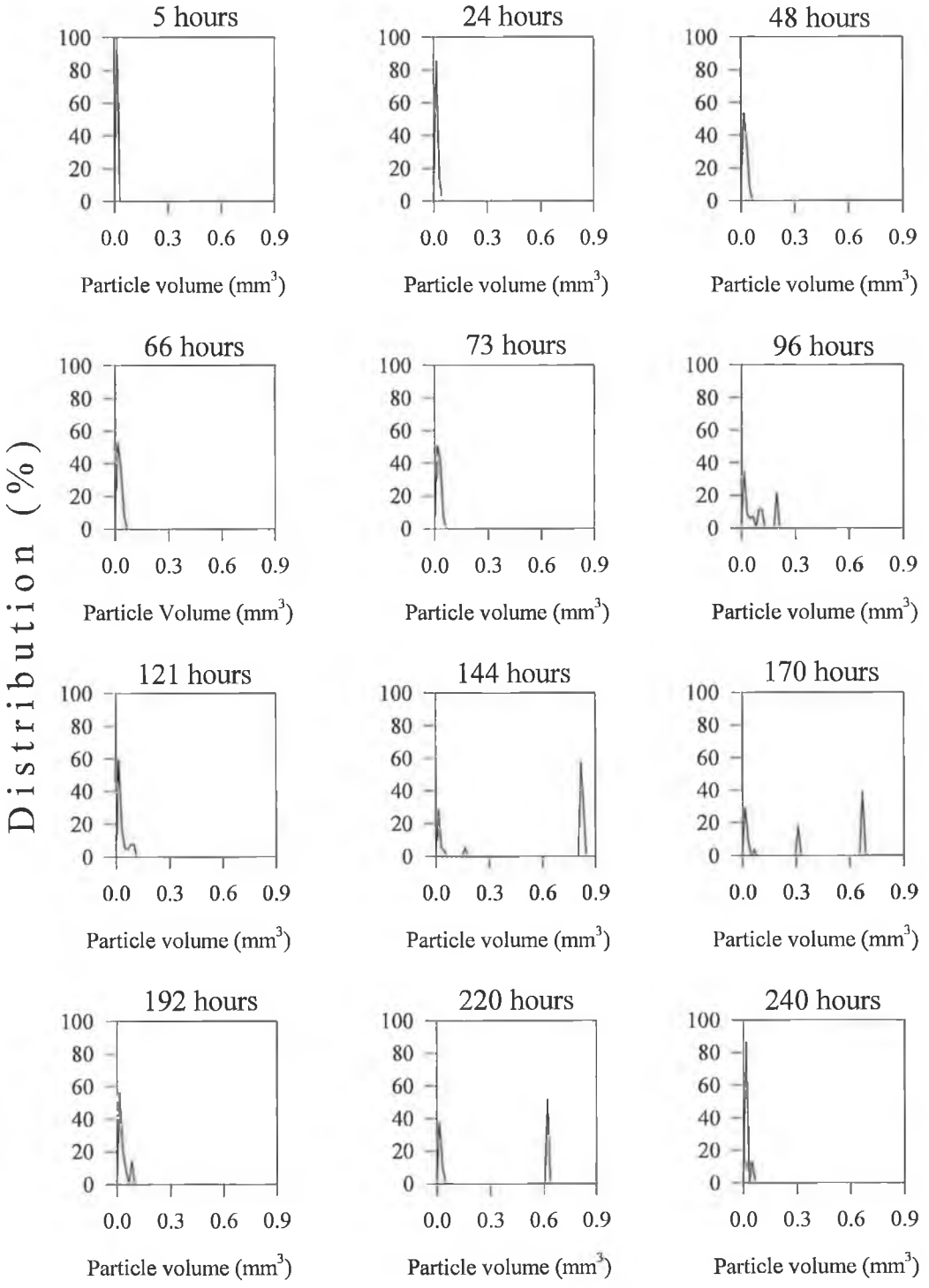


Figure 4.8(b) Development of volume based particle size distributions during cultivation in buffered fermentation medium in shake flask cultures.

It is postulated that as the fermentation progresses the particle area increases as the particles expand and grow. When they reach a critical area, breakage occurs, due to collisions and/or oxygen limitation phenomena and the subsequent reseeding described by Edelstein and Hadar (1983) ensues.

CHAPTER 5

RESULTS AND DISCUSSION: SHAKE FLASK AND STIRRED TANK REACTOR STUDIES

5.1 BATCH GROWTH CHARACTERISTICS OF Streptomyces natalensis

The organism was initially cultivated in unbuffered fermentation medium as described in Section 3.3.3.3, in order to facilitate measurement of biomass yields, in the absence of suspended carbonate particles. However, in this medium the organism was found to be pH-limited; growth was inhibited when the pH fell below pH 4.0. The organism was then cultivated in buffered fermentation medium, as detailed in Section 3.3.3.4. The observed differences in biomass yield for buffered and unbuffered media are presented in Figure 5.1. The data presented in this and all subsequent figures represent the mean values for samples taken from a number of replicate runs. Each sample represents one shake flask. In Figure 5.1, the error bars on the unbuffered fermentation medium data represent the standard error for four runs, while those on the buffered fermentation data represent the standard error for ten runs. All biomass data refer to dry weight values.

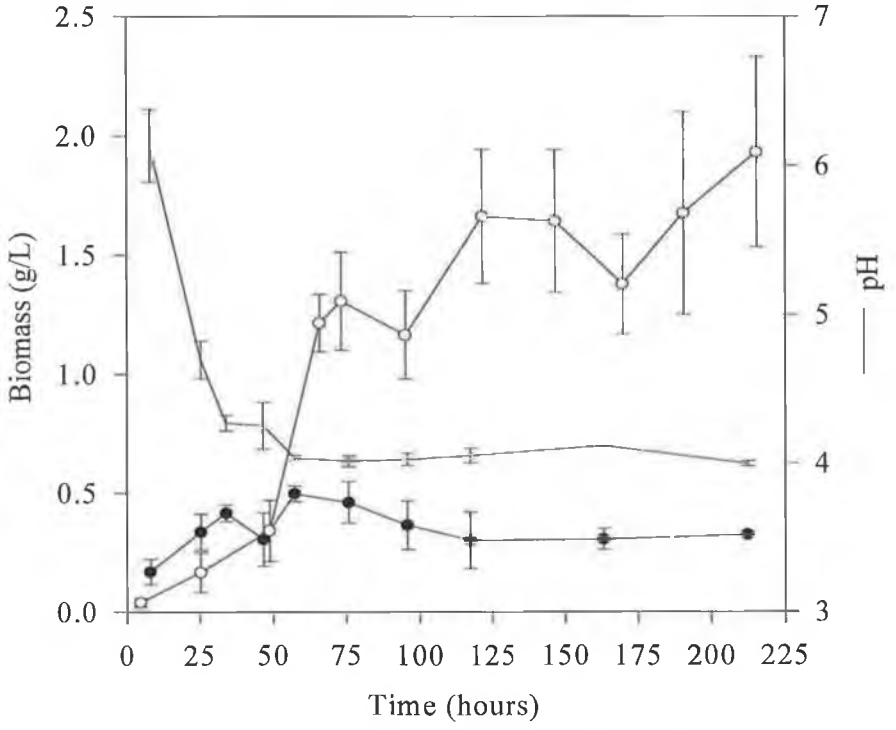


Figure 5.1. Biomass profiles for *Streptomyces natalensis* grown in both unbuffered and buffered media (● unbuffered medium; ○ buffered medium; — pH of unbuffered broth).

After approximately 66 hours of cultivation in unbuffered medium, by which stage the broth pH has fallen to a value of 4.0, growth ceases. Biomass levels do not exceed 0.5 g/L. In the corresponding buffered system, a biomass concentration of 2.61 g/L was achieved after 225 hours of cultivation.

As these effects may be associated with the presence of the phosphate buffer, the influence of medium components and concentrations is discussed further in Chapter 6.

Typical biomass and pH profiles for *S. natalensis* grown in buffered fermentation medium are presented in Figure 5.2(a). Glucose concentration in the medium is outlined in Figure 5.2 (b), with the corresponding morphological characteristics of the organism shown in Figure 5.2(c).

It is not possible to compare these profiles with the results presented by either McCabe (1990) or Mahon (1990) which are the only sources of comparative data on *S. natalensis*. Neither had the use of an image analyser and so their morphological observations were purely qualitative. McCabe did not monitor biomass production, as no method for removing calcium carbonate from the fermentation sample was employed. Mahon presented biomass concentration as wet weight, containing calcium carbonate. Moreover, as the strain used by both authors produced pimaricin while that strain used in this study did not, it is difficult to estimate the effect that this would have on either morphology or biomass production.

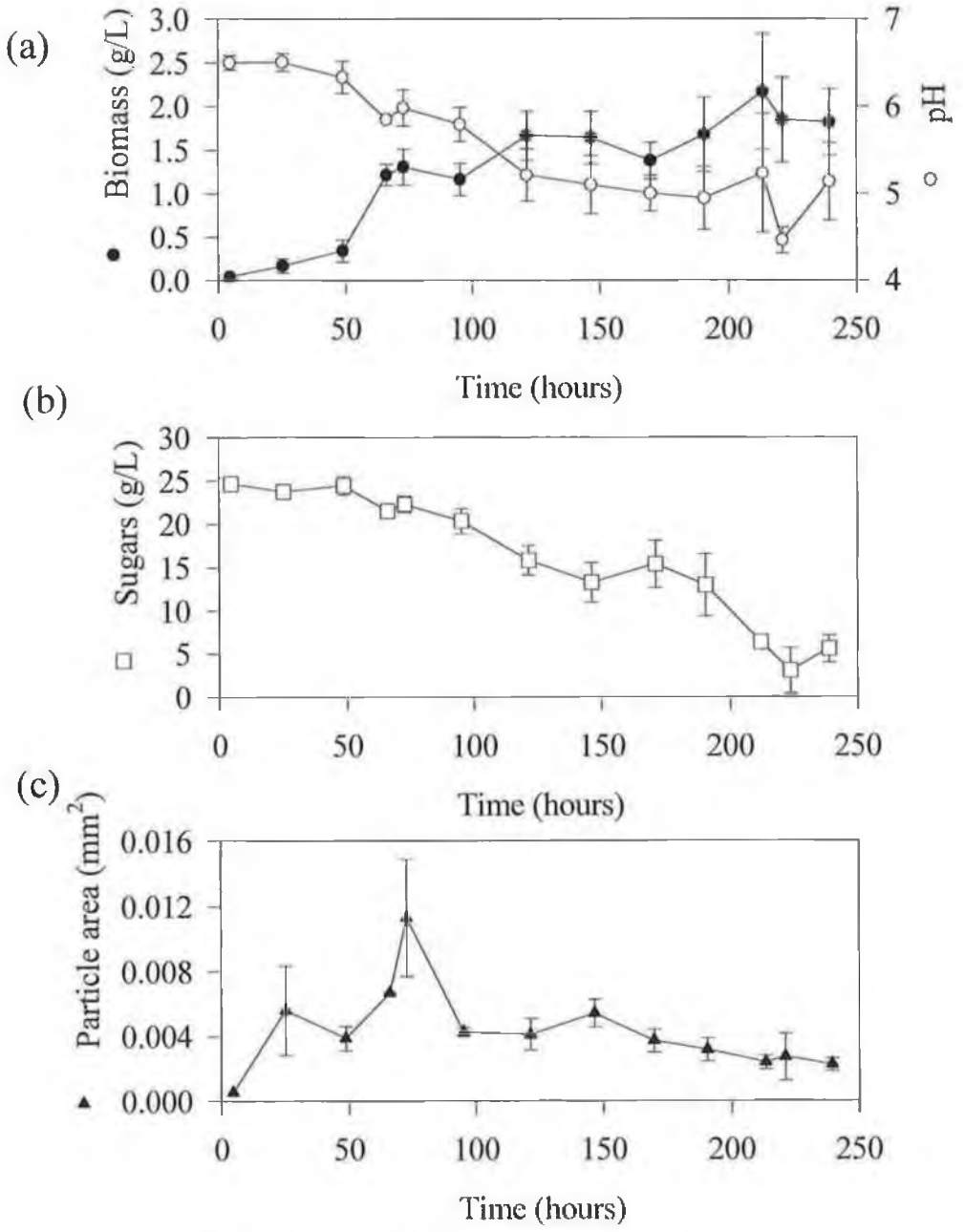


Figure 5.2. Growth curves for *S. natalensis* grown in buffered fermentation medium: (a) biomass and pH profiles, (b) extracellular total reducing sugars concentration and (c) morphological variations throughout the batch growth cycle.

5.2 INOCULUM STUDIES

The following studies were performed in order to identify the optimum inoculum conditions in terms of size and age. Eight flasks, each containing 50 mL of inoculum medium, were inoculated with 1 mL of plate suspension and incubated at 30°C. At 24 hour intervals, a single flask was removed and the contents of this flask used to inoculate three other flasks, each containing 100 mL of buffered fermentation medium. The first flask was inoculated with 1 mL, the second with 5 mL and the third with 10 mL. The flasks were incubated under normal growth conditions and after 96 hours the broth was analysed, as described in Section 3.6. When the inoculum was older than 144 hours, no growth was observed in the inoculated flasks. Accordingly, only the data obtained from the first six inoculum cultures harvested are discussed. These studies were performed in duplicate.

Figure 5.3 shows (a) biomass and (b) morphological profiles for these inoculum systems. It is evident from the data presented that both the inoculum age and volume employed have a significant effect on overall biomass yield. Dealing initially with the volume of inoculum used, it can be seen that, with regard to biomass yield, the use of a 1 mL inoculum is very inefficient regardless of culture age. The maximum biomass obtained using a 1 mL inoculum (0.98g/L) was observed with a 120 hour inoculum but this was smaller than the biomass levels observed for either of the other inoculum volumes of any age, except for the 10 mL inoculum at 144 hours (0.44g/L). It is also clear that of the inoculum volumes studied, a 1 mL aliquot of inoculum culture (120 hours) resulted in the largest particle area observed, 0.063 mm^2.

For larger inocula it can be seen that when the inoculum cultures are young (72 hours or less), a 10 mL inoculum yields higher biomass concentrations. For the older cultures, a 5 mL inoculum is more effective, with the highest biomass concentration (1.65g/L) obtained at 96 hours. No underlying relationship between particle area and either inoculum volume or age could be discerned. On the basis of these studies, an optimum inoculum size of 5 mL taken from a 96 hour inoculum culture was identified for 100 mL shake flask cultures and was employed in all subsequent work.

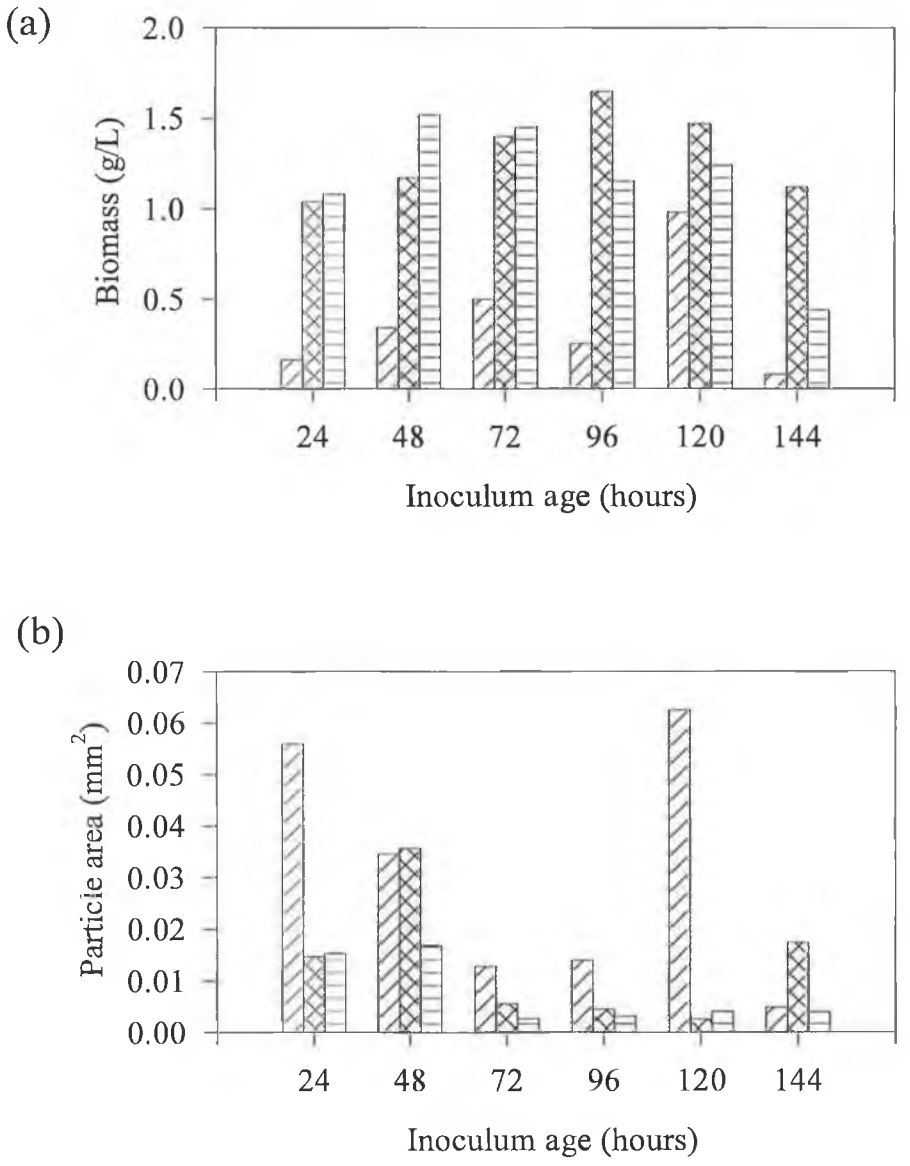


Figure 5.3. The effect of inoculum age and volume on (a) biomass accumulation and (b) morphology after 96 hours of cultivation (☑ 1 mL inoculum; ☒ 5 mL inoculum; ⊟ 10 mL inoculum).

5.3 VOLUME STUDIES

In order to ascertain the effect of broth volume on the cultivation of *S. natalensis*, a number of shake flask studies were performed. Cultures were established in 250 mL Erlenmeyer flasks, using buffered fermentation medium, with broth volumes of 50 mL, 100 mL and 150 mL. The optimal inoculum age and volume determined in Section 5.2 were employed with the inoculum volume scaled appropriately. The data

presented in this study represent the mean values obtained from six replicate runs. Error bars have been excluded for clarity but the maximum error during any fermentation was 29%, observed after 48 hours using 150 mL of fermentation medium.

It can be seen from Table 5.1 that for the systems investigated, the maximum values of biomass concentration (3.92g/L), specific biomass production rate, R_{vb} (0.027g/L.h) and biomass yield, $Y_{x/s}$ (0.175g/g glucose) were obtained using a 50 mL working volume and all values decrease as the broth volume increases.

Table 5.1 Growth characteristics of shake flask cultures cultured in buffered fermentation medium - volume studies

Volume (mL)	Max. biomass (g/L)	Max. R_{vb} (g/L.h)	Max. $Y_{x/s}$ (g/g glucose)
50	3.920	0.027	0.175
100	2.116	0.018	0.134
150	1.125	0.016	0.096

Figure 5.4 shows the biomass profiles obtained during these volume studies. Clearly the smaller the liquid volume employed, the higher the accumulated biomass concentrations. The overall biomass concentration in the 50 mL culture is almost four times that obtained in the flask containing 150 mL. Specific surface area available for O_2 mass transfer increases with decreasing liquid volume. This appears to suggest that when *S. natalensis* is cultivated in larger volumes of medium, the organism may be subject to oxygen deprivation, resulting in diminished growth. This oxygen sensitivity exhibited by *S. natalensis* was also noticed by other researchers (Ives, P., personal communication, 1996). Biomass concentrations in fermentations of *S. clavuligerus* are known to be adversely effected by low oxygen levels (Belmar-Beiny and Thomas, 1991). It is possible that baffled shake flasks could be used to overcome or at least reduce this oxygen limitation problem.

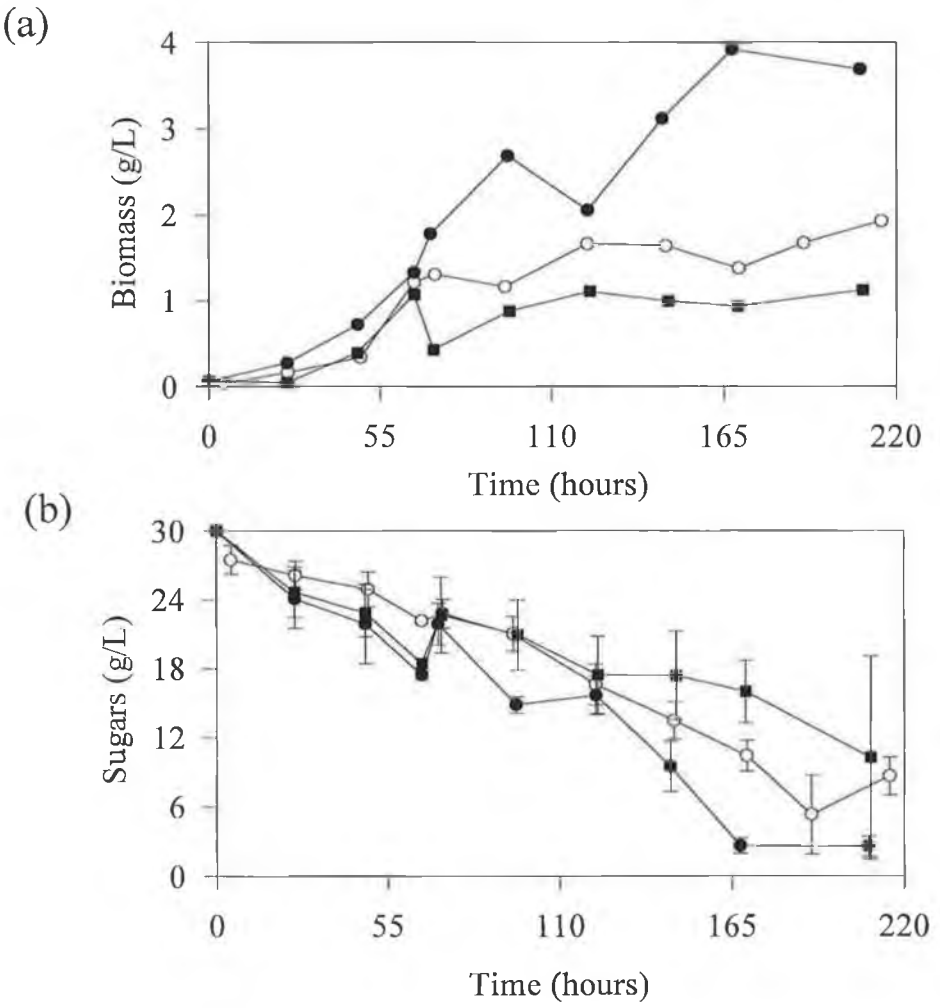


Figure 5.4. (a) Biomass and (b) sugar profiles obtained during volume studies (● 50 mL volume, ○ 100 mL volume, ■ 150 mL volume).

The biomass yields per gram of glucose utilised, $Y_{x/s}$, for each system are shown in Figure 5.5. It can be seen that when a 100 mL volume is used, $Y_{x/s}$ increases to the maximum value of 0.134g/g glucose, at approximately 96 hours. The biomass yield then decreases to a value which is maintained for the remainder of the growth cycle. In the cases of the 50 mL and 150 mL systems, the maximum biomass yield occurs much later in the fermentation, at approximately 170 hours and 144 hours, respectively. However, at all stages, the biomass yield in the 150 mL system is, in general, lower than those observed in either of the other two systems. Assuming that O_2 may be limiting, this data suggests that glucose is being diverted elsewhere, as less biomass is produced per unit substrate. Unlike the other volumes, the $Y_{x/s}$ values

for the 50 mL system increase throughout the first 120 hours, before appearing to stabilise at approximately the maximum $Y_{x/s}$ value of 0.175g/g glucose.

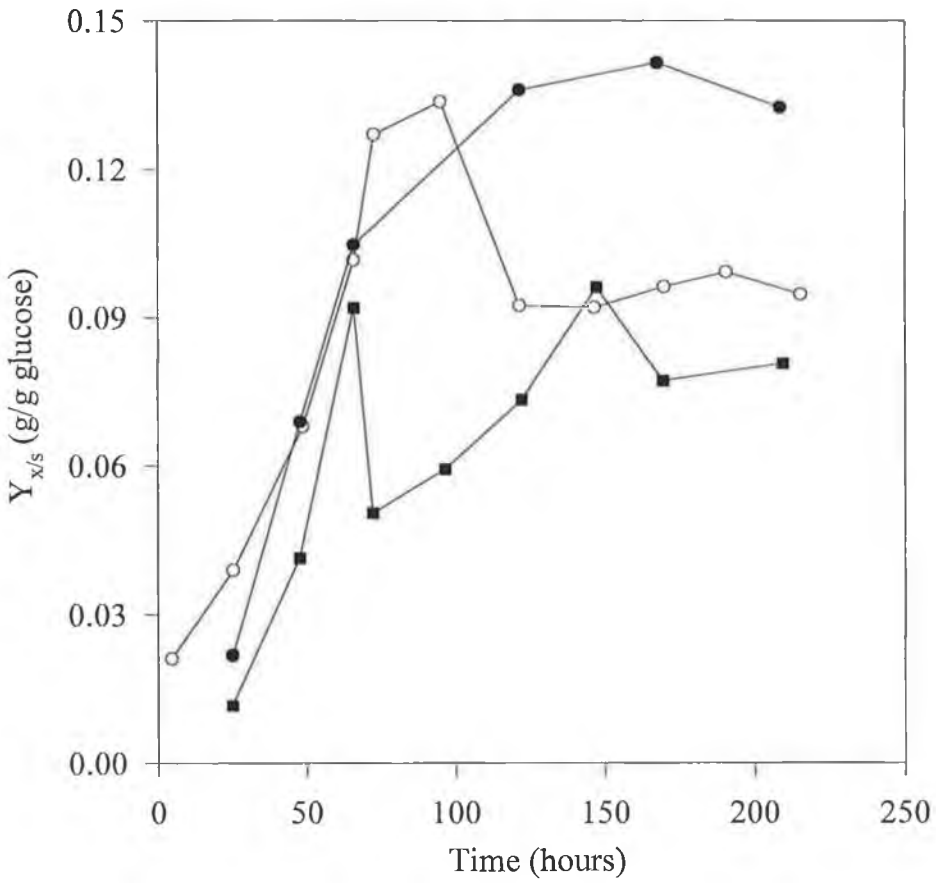


Figure 5.5. Biomass yield per gram of glucose utilised during volume studies. (● 50 mL volume, ○ 100 mL volume, ■ 150 mL volume).

With regard to the R_{vb}, the maximum values observed during the fermentation are found at 96 hours in the 50 mL system and 66 hours in both the 100 mL and 150 mL systems. Table 5.2 shows the overall $Y_{x/s}$ and R_{vb} values obtained for these systems and all are seen to decrease with increasing volume.

Table 5.2 Overall values for $Y_{x/S}$ and R_{vb} during volume studies

Volume (mL)	Overall R_{vb} (g/L.h)	Overall $Y_{x/s}$ (g/g glucose)
50	0.018	0.132
100	0.010	0.101
150	0.005	0.081

Morphologically, it can be seen from Table 5.3 that, for the range of conditions investigated, the greatest overall particle area and perimeter were observed at a working volume of 150 mL. Moreover, it was noted that pellet perimeter and diameter both increase with increasing culture volume. However, there was no apparent relationship between culture volume and average particle area. This indicates that the pellets are less compact when a working volume of 100 mL is used, compared to those produced with either of the other two volumes. The morphological parameters listed in Table 5.3 have been defined in Section 4.5 and the values reported are those observed at the end of the fermentation, normally between 190 hours and 200 hours.

Table 5.3 Final average morphological characteristics for shake flask cultures in buffered fermentation medium - volume studies

Volume (mL)	Particle area (mm^2)	Perimeter (mm)	Diameter (mm)
50	$4.329 * 10^{-3}$	0.185	0.047
100	$2.637 * 10^{-3}$	0.216	0.052
150	$4.288 * 10^{-3}$	0.239	0.061

Figure 5.6 outlines the development of particle area with time in the three systems. All three cultures behaved in a broadly similar fashion. In the 50 mL culture, the particle area increased during the first 24 hours and then decreased to a minimum at 66 hours. The particle area increased for a second time to the maximum area observed at 95 hours and then decreased to a stable value which did not vary for the remainder of the fermentation. With both the 100 mL and 150 mL cultures, the particle area increased to the maximum observed value at 73 hours and then decreased, although the effect is more pronounced at the 100 mL scale than for 150 mL. In the case of the 100 mL system, the area subsequently decreased to a stable value which was maintained for the remainder of the fermentation. However in the case of the 150 mL working volume, the particle area decreased slowly throughout the rest of the growth curve and did not stabilise as was observed with the 50 mL and 100 mL cultures.

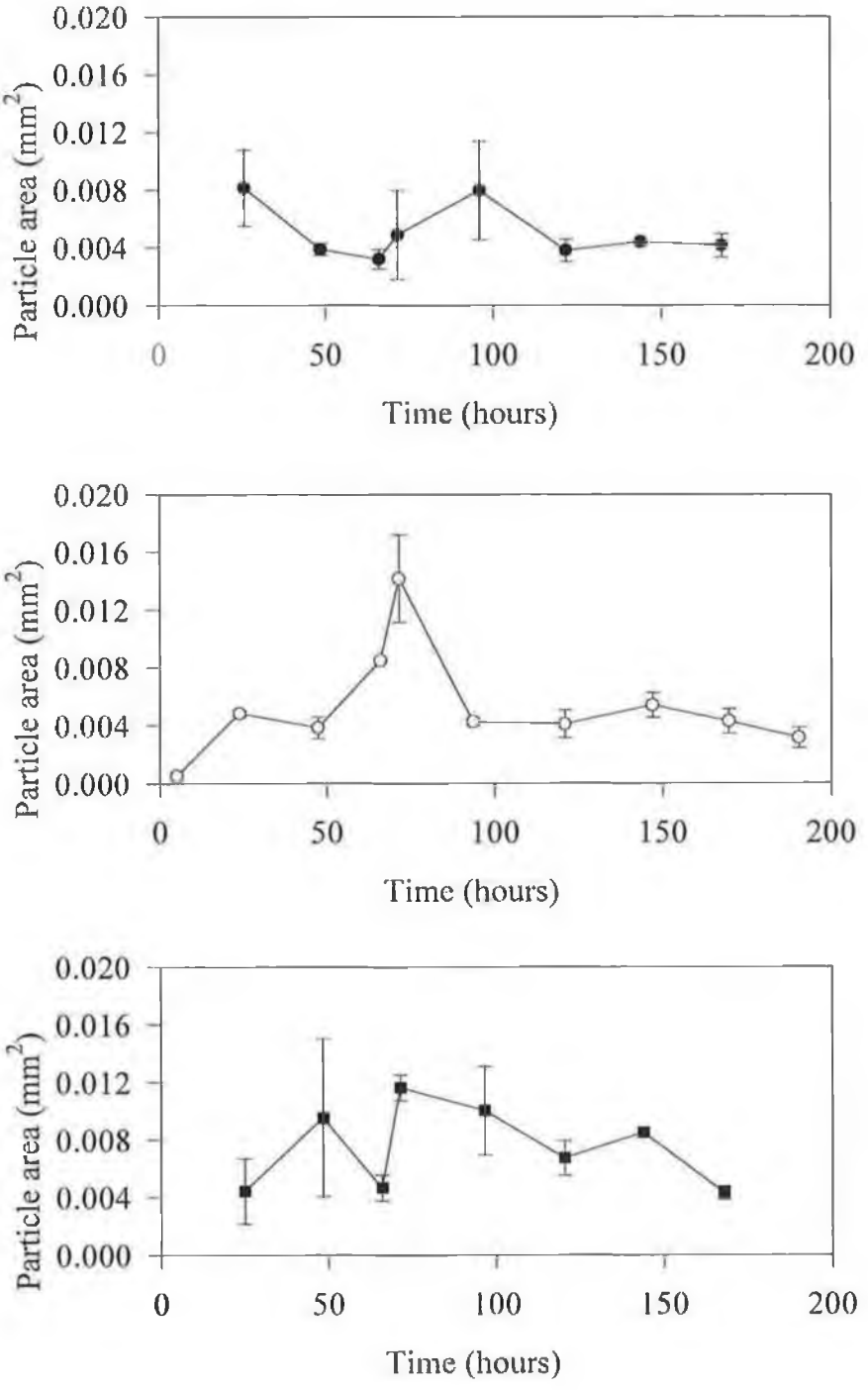


Figure 5.6 Morphological characteristics during volume study fermentations (● 50 mL volume, ○ 100 mL volume, ■ 150 mL volume).

It is postulated that the differences observed between these area profiles may be, in part, attributable to the intensity of the agitation conditions prevailing in the flasks.

As all flasks were maintained on the same shaker table, the power input per unit volume increases with decreasing liquid volume. Accordingly, the environment in the 50 mL system is most severe, which may account for the reduction in average particle area during the early stages of cultivation and the failure of particles to expand to the same extent as in the other systems. However, it is evident from the corresponding biomass profiles that growth is not adversely effected under these conditions. In the 150 mL system, conversely, the largest pellets were formed. However, the biomass profiles suggest that the organism was subject to oxygen limitation under these conditions.

Despite the fact that the highest biomass was observed with a fermentation culture volume of 50 mL, a 100 mL working volume was used in all shake flask studies. This was due to the practical difficulties associated with comprehensive analysis of the smaller broth volume.

5.4 STIRRED TANK REACTOR STUDIES

As can be seen from Figures 5.4 - 5.6, the growth and morphological profiles for *S. natalensis* show evidence of fluctuations which are obviously associated with the cultivation conditions in the shake flask at various liquid volumes *e.g.* due to oxygen limitation or agitation effects, or which may be due to the fundamental limitations of shake flask cultivation procedures. Because the broth volumes employed are relatively small, (50 - 100 mL), it is necessary to harvest the full flask contents each time. This leads to the possibility of flask/flask variations, although these effects should be minimised by the use of a minimum of four replicates in all cases. However, scale-up to 1L and 10L STRs was undertaken with a view to overcoming the inherent limitations of shake flask systems.

5.4.1 1 LITRE SYSTEM

The organism was cultivated at two agitation rates, 250 rpm and 500 rpm at a constant aeration rate of 600 mL/min (corresponding to a value of 0.75vvm on the basis of the original broth volume. A working volume of 800 mL (buffered

fermentation medium) was used. At each agitation rate, two fermentations were undertaken.

Figure 5.7 shows the biomass profiles for these two sets of conditions. At both speeds the biomass production in the STR system is greater than that observed with a 100 mL shake flask system (Figure 5.4). At an agitation rate of 250rpm the biomass concentration observed increased consistently throughout the first 240 hours of growth. However, at 500rpm, the biomass level fluctuated, with an initial peak (3.74 g/L at 92 hours). This was followed by a significant reduction to 2.0 g/L at 144 hours. As there is no replicate data for this time point, it is unclear if this biomass profile is accurate. The maximum biomass concentration achieved in the system at 500rpm, (3.74g/L), was 24% greater than that 250rpm, (2.84g/L). It is postulated that this effect is attributed to the increased oxygen transfer rates occuring at the higher agitation rate. Table 5.4 details the maximum values of $Y_{x/s}$ and R_{vb} values obtained for these 1L fermentations.

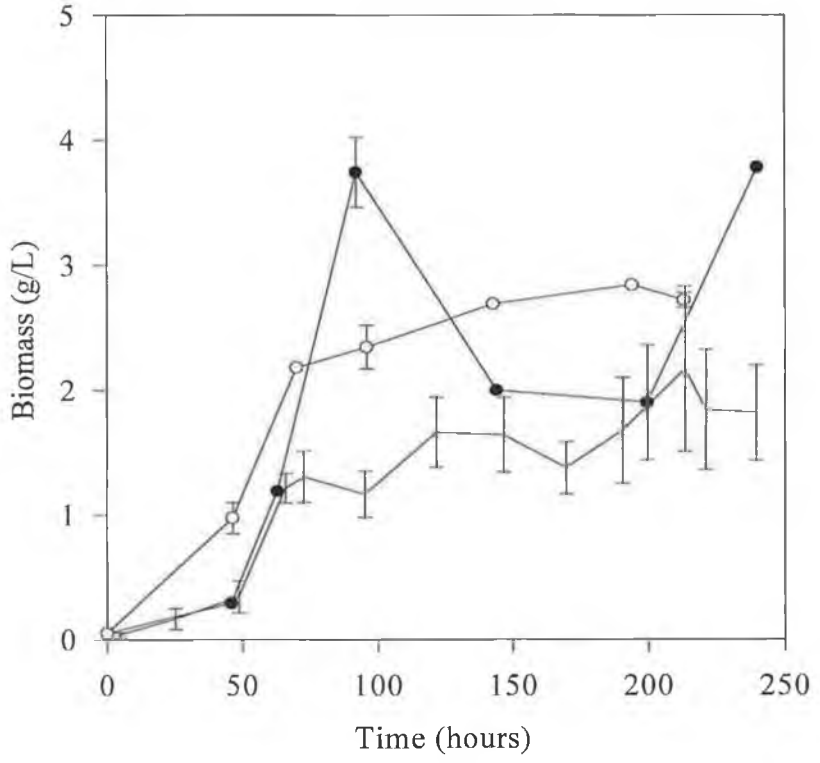


Figure 5.7 Biomass profiles for *S. natalensis* cultivated in a 1L fermenter. (● 500rpm, ○ 250rpm,— shake flask system (100 mL volume))

Table 5.4 Maximum growth parameters observed - 1L fermentations

Agitation rate (rpm)	Max. $Y_{x/s}$ (g/g glucose)	Max. R_{vb} (g/L.h)
250	0.197	0.031
500	0.221	0.041

Due to the limited data available, particularly during the 500 rpm runs, it is difficult to draw any conclusions about substrate consumption profiles or biomass production rates. However on the basis of the available data it is obvious that as the system is scaled up from shake flask to STR, higher biomass levels are achieved and that higher biomass concentrations are supported by the higher agitation rates investigated.

The morphological development of *S. natalensis* in the 1L STR is outlined in Figure 5.8. As can be seen, at an agitation rate of 250rpm, the particle area decreases steadily to the minimum observed value of $1.555 * 10^{-3}$ mm^2, at the end of the fermentation. Again, as with the biomass profiles, this morphological profile is much smoother than that observed at 500rpm. At the higher speed, the particle area increases to the maximum area observed, $4.580 * 10^{-3}$ mm^2, at the end of the fermentation with one sharp reduction at 144 hours corresponding to a similar reduction in the biomass profiles (Figure 5.7). However as previously mentioned only one sample was taken at this time point and the data are not, therefore, reliable.

It should be noted that during each of these 1L runs, five samples, each of 35 mL, were harvested. This volume constituted approximately 22% of the total working volume. As was evident from the volume studies (Section 5.3), morphological profiles obtained during batch cultivation are strongly dependent on the prevailing hydrodynamic and/or oxygen transfer conditions. As the broth volume was reduced by sampling, both oxygen transfer rates and specific power input to the system might reasonably be expected to increase, with consequences for both growth and morphological characteristics. As sample volumes of at least 35 mL were required for comprehensive broth analysis, this is an inherent limitation of batch cultivation studies at the 1L scale.

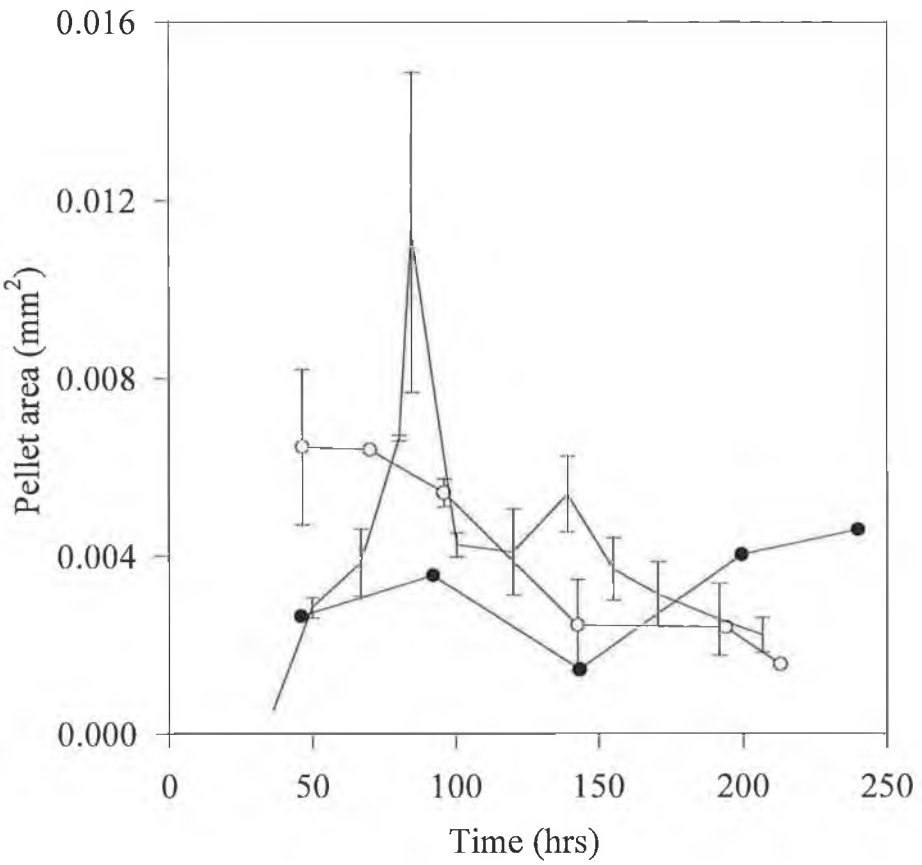


Figure 5.8 Morphological profiles of *S. natalensis* in 1L fermenter (● 500 rpm, ○ 250 rpm, — shake flask system (100 mL volume))

5.4.2 10 LITRE FERMENTATIONS

Cultivation of the organism was scaled up to the 'Micogen' 16L fermenter. A working volume of 10L of production medium was used. The purpose of this scale up was to facilitate repeated sampling of the fermentation broth. Because of the larger volumes employed in the 'Micogen' system, multiple sampling does not affect the volume to any great extent, thereby minimising the volume-related variations in oxygen mass transfer and agitation intensity encountered in the 1L vessel. As detailed in Section 3.4.3.4, an agitation rate of 400rpm and an aeration rate of 1.0vvm were used in all cases. Three fermentations were performed under these conditions and the data presented in this section represent the mean values for these runs. Biomass, dissolved oxygen and sugar profiles are presented in Figure 5.9. Biomass

concentration data only exists for one of the three runs (Figure 5.10 (a)) as there were initial problems with suspended solids in the medium. Error bars are omitted from Figure 5.10 (b) for clarity.

As can be seen, the dissolved oxygen concentration decreases gradually to approximately 40% while the biomass concentration increases to a maximum value of 2.61g/L. From Figure 5.9(c) it can be seen that after approximately 76 hours, the sugars present in the medium are exhausted and therefore, the fermentations were terminated at this time. The biomass concentration, observed at this agitation rate, is low compared to both the 1L fermenter and the shake flask results. Visually, it appeared that an agitation rate of 400 rpm was sufficient to prevent cell settling, and therefore the oxygen concentrations presented in Figure 5.9(b) are representative of the concentrations to which the organism was exposed. Thus, if intra-pellet diffusion limitations can be discounted, it appears as if oxygen limitation should not be an issue for this broth. Ideally, however, further runs should be undertaken at higher aeration rates, or at higher operating pressure with a view to investigating the influence of dissolved oxygen levels on system performance. However, for the reasons outlined in Section 3.4.4.4, no further runs could be undertaken with the 'Micogen' vessel.

As can be seen from a comparison of Figure 5.10 and Figure 5.5, $Y_{X/S}$ (and R_{vb}) profiles deviate significantly from those observed in shake flasks. In both cases the values for the 10L fermentations increase constantly to the maximum values observed. It is postulated that the smoother trends observed for these runs are attributable to the maintenance of uniform conditions for the duration of the cultivation period coupled with the elimination of flask/flask variations.

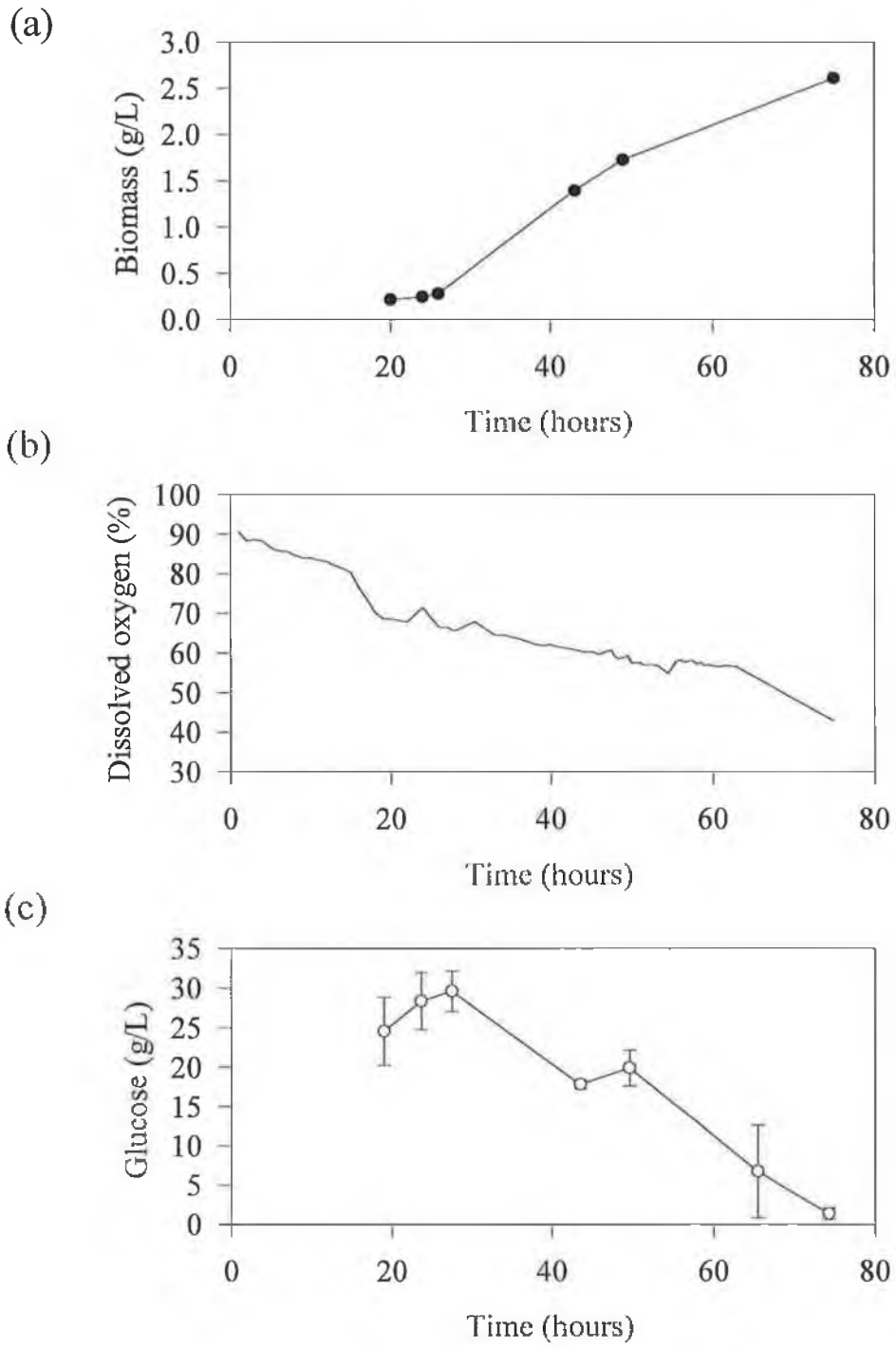


Figure 5.9 Profiles for (a) biomass, (b) dissolved oxygen and (c) total extracellular reducing sugars during a 10L fermentation. (N = 400 rpm, aeration rate = 1 vvm)

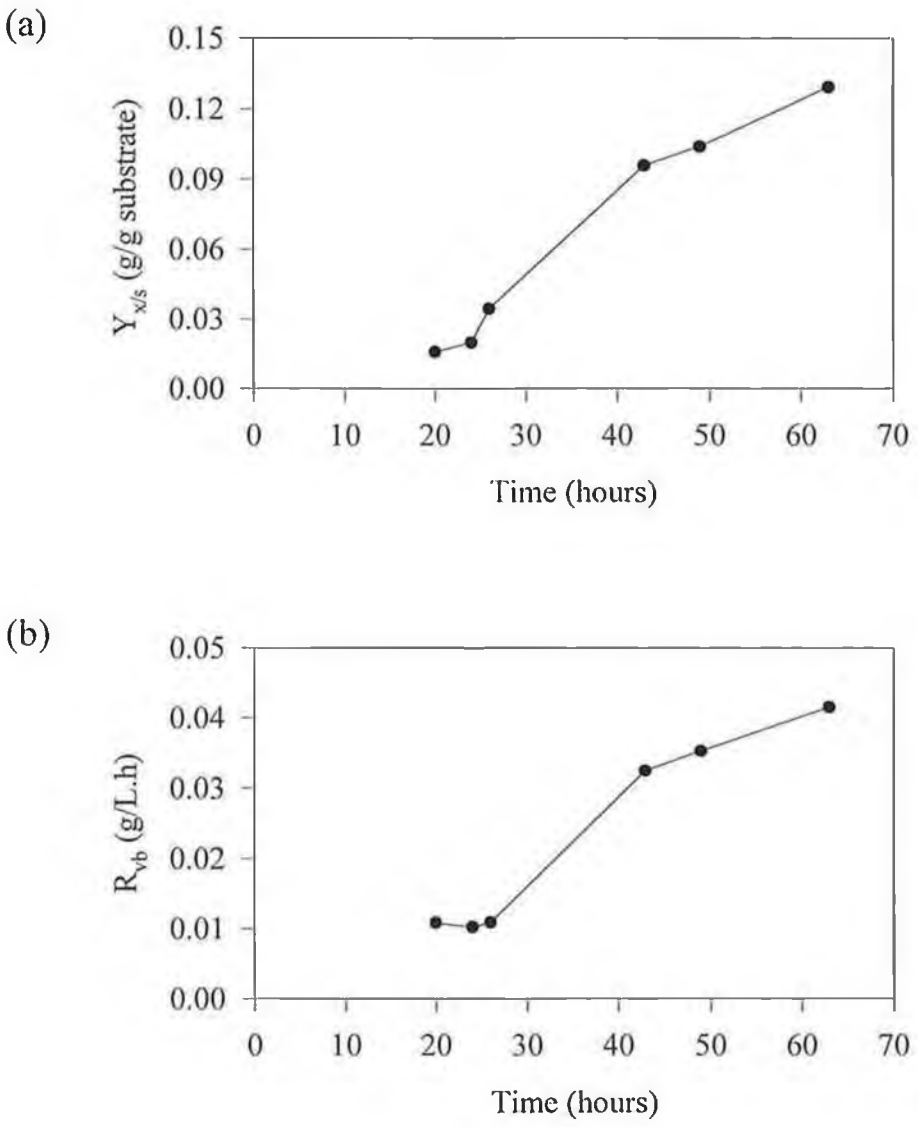


Figure 5.10 Observed values for (a) $Y_{X/S}$ and (b) R_{vb} for 10L fermentations

Figure 5.11 shows the morphological profile for *S. natalensis* when cultivated in the 16L fermenter. Clearly, the particle area increases as the fermentation progresses, with the maximum particle area observed at approximately 66 hours. After this maximum has been reached the particle area begins to decrease slightly. This trend is similar to those observed in shake flask fermentations over the same time period.

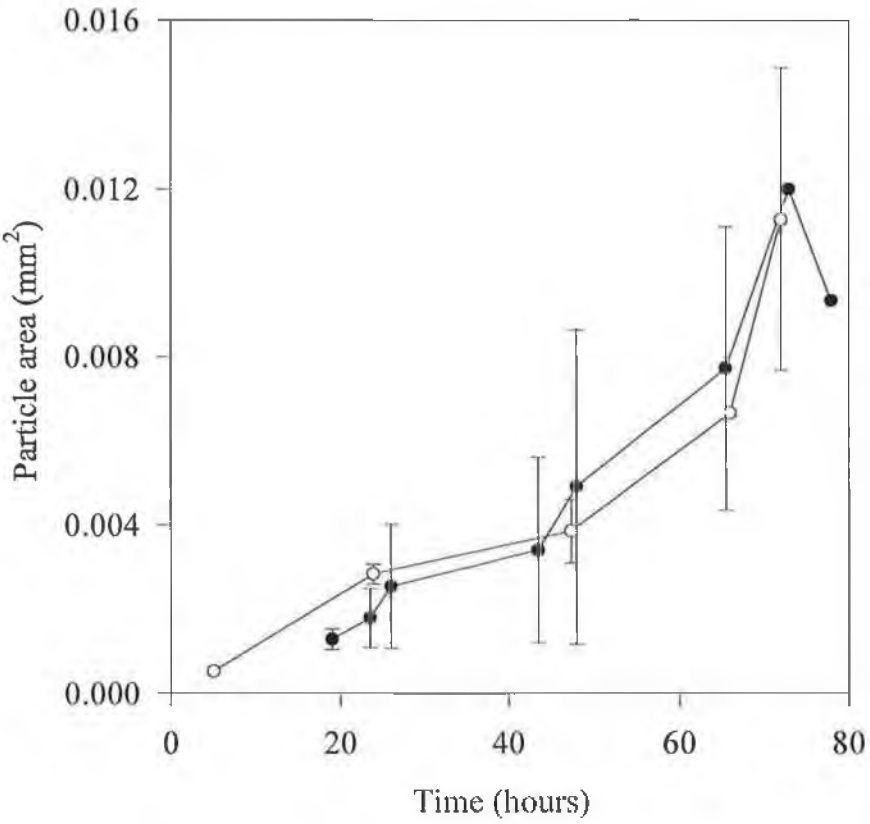


Figure 5.11 Morphological development of *S. natalensis* during 10L fermentations (●) and shake flask cultures (○)

CHAPTER 6

RESULTS AND DISCUSSION: MEDIA STUDIES

6.1 THE EFFECT OF MEDIA COMPONENTS ON THE GROWTH AND MORPHOLOGICAL CHARACTERISTICS OF S. NATALENSIS

These studies were undertaken to determine the effects of medium composition on the growth and morphology of the organism. In all cases, 250 mL Erlenmeyer shake flasks were employed, with a working volume of 100 mL. Both Mahon (1990) and McCabe (1990) used inoculum medium and production medium in their studies of *S. natalensis*. In this study, as mentioned in Section 5.1, to avoid the presence of suspended solids in the medium, both unbuffered and buffered fermentation media were used. The effect of nitrogen source on the cultivation of filamentous micro-organisms is well documented (*e.g.* Brana *et al.*, 1986; Vining *et al.*, 1987; Glazebrook *et al.*, 1993; He *et al.*, 1995). Therefore it was decided, in this study, to study the effects of YEPD medium and peptone medium on the growth and morphological characteristics of *S. natalensis*. Sarra *et al.* (1996) reported high biomass yields in fermentations of *Saccharopolyspora erythraea*, and it was also decided to cultivate *S. natalensis* in the medium employed. The various media are described in Section 3.3.

6.1.1 BIOMASS PRODUCTION

Figure 6.1 outlines the biomass profiles observed with each of the seven media used in the cultivation of *S. natalensis*. A minimum of two replicate runs were performed using the actinomycete medium (Sarra *et al.*, 1996) and the inoculum medium, with duplicate flasks sampled at each point; four replicate runs for YEPD, peptone and unbuffered fermentation media; five runs using production medium and ten using buffered fermentation medium. All runs were terminated after 200 hours, with the exceptions of those employing buffered fermentation medium and production medium which were allowed to run for 240 hours. The data presented in this section are the mean values for the relevant runs.

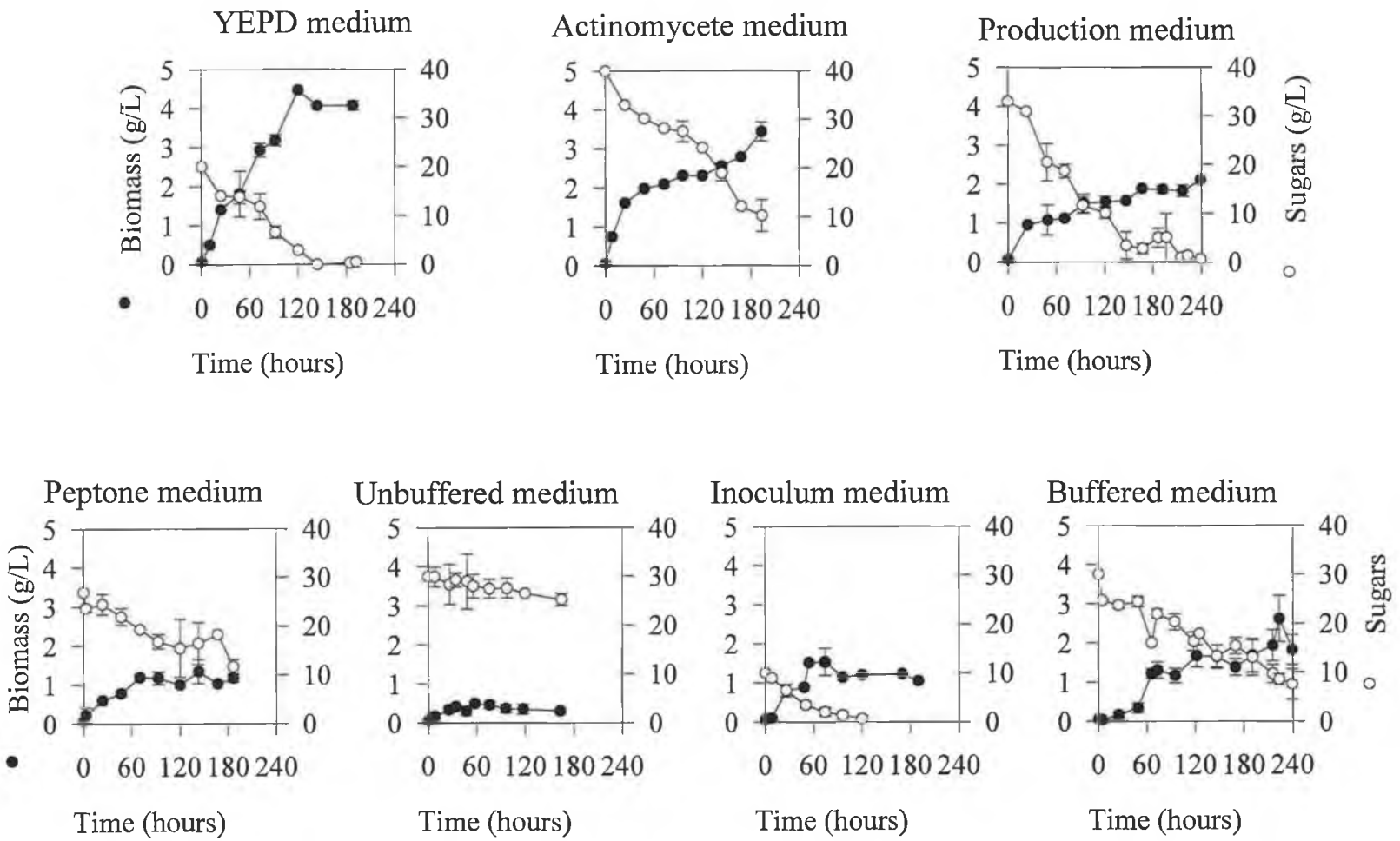


Figure 6.1 Effect of medium composition on batch growth curve (● biomas, ○ extracellular total reducing sugars)

Table 6.1 outlines the maximum biomass concentrations obtained with each medium and the time taken to reach these biomass levels. The highest biomass levels were obtained with YEPD medium (4.47g/L) and the actinomycete medium (3.43g/L) described by Sarra *et al.* (1996) suggesting that the yeast extract contained in both media contributes to elevated biomass production. The lowest biomass level, 0.496g/L, was observed using the unbuffered fermentation medium; as stated in Section 5.1, poor biomass accumulation in this case was attributed to pH limitation. It appears as if the choice of buffer employed has little effect on maximum biomass concentration as both buffered fermentation medium (phosphate buffer) and production medium (buffered by calcium carbonate) yield approximately the same biomass concentration.

Table 6.1 Maximum biomass concentrations for media studies

Cultivation medium	Maximum biomass (g/L)	Time taken to reach maximum biomass (h)
YEPD	4.47	120.3
Actinomycete	3.43	193.7
Buffered fermentation	2.61	223.5
Production	2.11	239.0
Inoculum	1.53	74.33
Peptone	1.34	143.0
Unbuffered fermentation	0.50	57.50

Although it can also be seen from Table 6.1 that unbuffered fermentation medium resulted in the shortest time to reach the maximum biomass for the fermentation, virtually no growth occurred and thus this medium cannot be compared to the other six employed.

Inoculum medium and YEPD medium, which do not contain added buffers, resulted in maximum biomass levels at 74.3 hours and 120.25 hours, respectively. Although the actinomycete medium developed by Sarra *et al.*, (1996) is not buffered, the organism takes longer to reach its maximum biomass than the peptone medium. This may be associated with an inhibitory effect of the high initial

glucose concentration (40g/L) in the actinomycete medium. This glucose effect is examined in more detail in Section 6.5. Production medium, designed to support the production of pimaricin (Struyk and Waisvisz, 1975), resulted in the longest time to reach maximum biomass, 239 hours. However, this is only slightly longer than for the buffered fermentation medium (223.5 hours).

6.1.2 EFFECT OF MEDIA COMPONENTS ON BIOMASS YIELD COEFFICIENTS

Figure 6.2 outlines the biomass yield coefficients observed with each of the media employed. The maximum biomass yield (0.665g/g glucose) was also obtained with YEPD medium; the lowest (0.122g/g glucose) with the production medium.

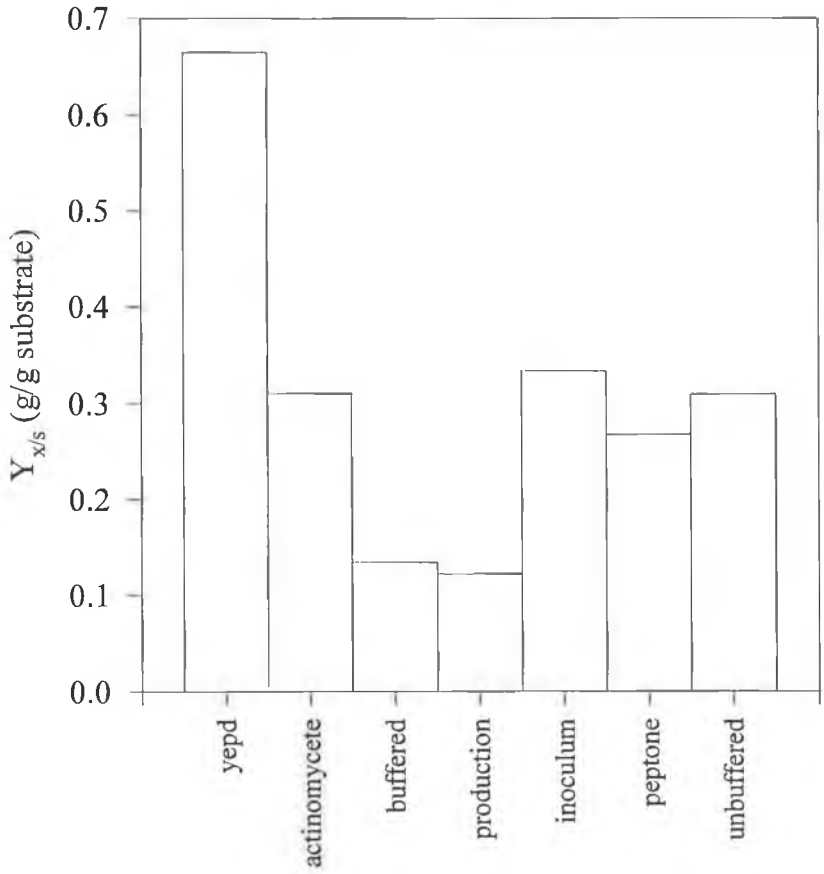


Figure 6.2 Maximum biomass yield per gram of glucose utilised during media studies

With regard to the specific biomass production rate, R_{vb}, it can be seen from Figure 6.3 that the maximum value observed during the media studies was with

actinomycete medium (0.067g/L.h). The lowest value, 0.018g/L.h, was noted with buffered fermentation medium.

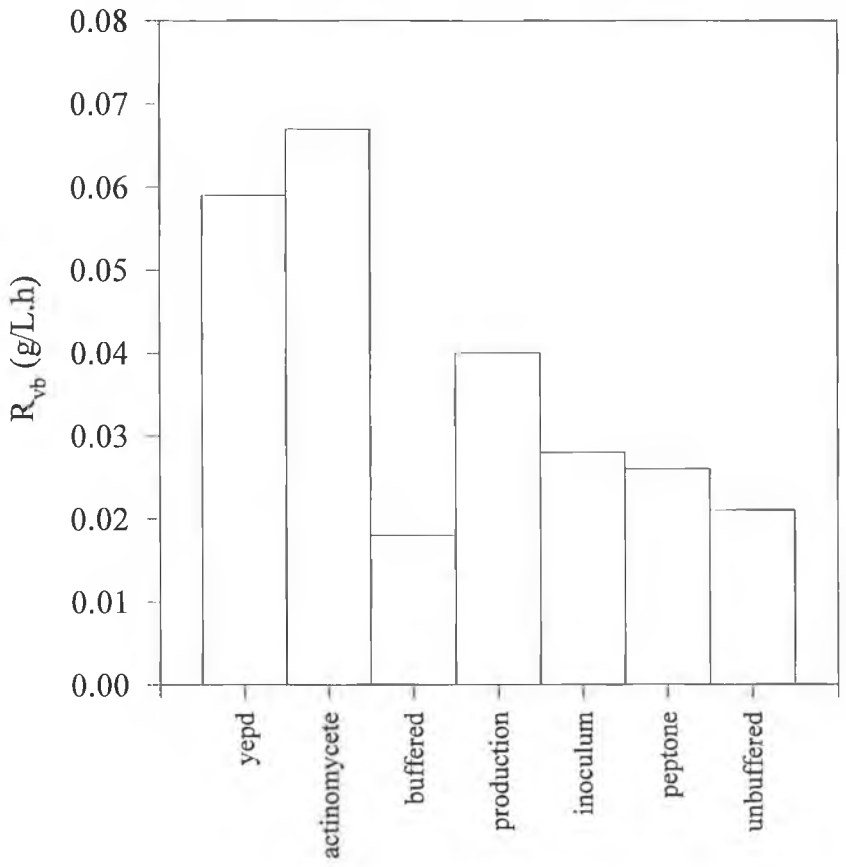


Figure 6.3 Maximum specific biomass production rates observed during media studies

As stated in Section 5.3, the $Y_{x/s}$ values for the volume study fermentations decrease from the maximum value to stabilise at a value that is maintained for the remainder of the fermentation. A similar trend occurs for most of the fermentations in this study with two exceptions. When unbuffered medium is used in the cultivation of *S. natalensis*, the $Y_{X/S}$ values decrease and do not stabilise. This is due to the suspension of biomass production after the pH drops below pH 4.0. All substrate use also ceases at this point. The $Y_{x/s}$ values for the fermentation using the actinomycete medium developed by Sarra *et al.* (1996) also decrease and do not stabilise after the maximum values. From Figure 6.1 it can be seen that the biomass concentrations continued to increase throughout the duration of cultivation; the system did not reach stationary phase. This resulted in the observed lack of stabilisation in $Y_{x/s}$ values.

Also, as stated in Section 5.3, the R_{vb} values for each of the fermentations outlined decreases after the maximum recorded value to a stable value that is maintained for the duration of the cultivation periods. Table 6.2 lists the overall values for $Y_{x/s}$ and R_{vb} recorded for each medium. Clearly when using phosphate buffer, the overall R_{vb} value is very low.

Table 6.2 Overall $Y_{X/S}$ and R_{Vb} values for shake flask cultures - media studies

Medium	Overall $Y_{x/s}$ (g/g glucose)	Overall R_{Vb} (g/L.h)
YEPD	0.219	0.022
Actinomycete	0.188	0.018
Buffered	0.099	0.008
Production	0.081	0.009
Inoculum	0.164	0.010
Peptone	0.048	0.005
Unbuffered	0.019	0.003

6.1.3 MORPHOLOGICAL CONSEQUENCES OF MEDIA MANIPULATION

As very little morphological characterisation of *S. natalensis* has been performed, semi-automated image analysis was used in this study to monitor the morphological development of the organism. Medium components have been observed to affect the morphology of actinomycetes, and other filamentous organisms (Reeslev *et al.*, 1991; Glazebrook *et al.*, 1992; Reichl *et al.*, 1992b; Banerjee, 1993). During the media studies reported here it was observed that no significant changes in the overall morphological form of *S. natalensis*, were associated with the choice of cultivation medium.

Table 6.3 shows the values for particle diameter and perimeter observed in each medium after approximately 200 hours of culitvation. Figure 6.4 outlines the development of particle area during cultivation in the seven media used. As with the biomass values (Section 6.1.1), the values presented in this section are the average values obtained for all relevent runs performed.

Table 6.3 Average morphological characteristics for shake flask cultures in various media after 200 hours of cultivation

Medium	Perimeter (mm)	Diameter (mm)
YEPD	0.099	0.026
Actinomycete	0.113	0.030
Buffered	0.216	0.052
Peptone	0.190	0.047
Inoculum	0.113	0.030
Production	0.220	0.051
Unbuffered	0.435	0.067

On the basis of average particle perimeter, diameter and area, after 200 hours of cultivation, the smallest particles were observed in the YEPD and actinomycete media, which supported the highest levels of growth. When buffer is added, the mean particle area increases, with all unbuffered media (YEPD, actinomycete and inoculum media) yielding smaller particle dimensions than either buffered media or production medium. While the final particle measurements observed when the organism is cultivated in unbuffered fermentation medium are greater than those for any other medium, this may be due, in part, to the severe pH conditions the organism experienced in this fermentation and is discounted due to the lack of growth. The organism formed the largest pellets when grown in production medium ($3.377 * 10^{-3} mm^2$)(Figure 6.4). The presence of yeast extract and peptone in media result in the formation of small 'hairy' pellets. It was also observed that, as the nitrogen source was changed from inorganic to organic, the particle area decreased.

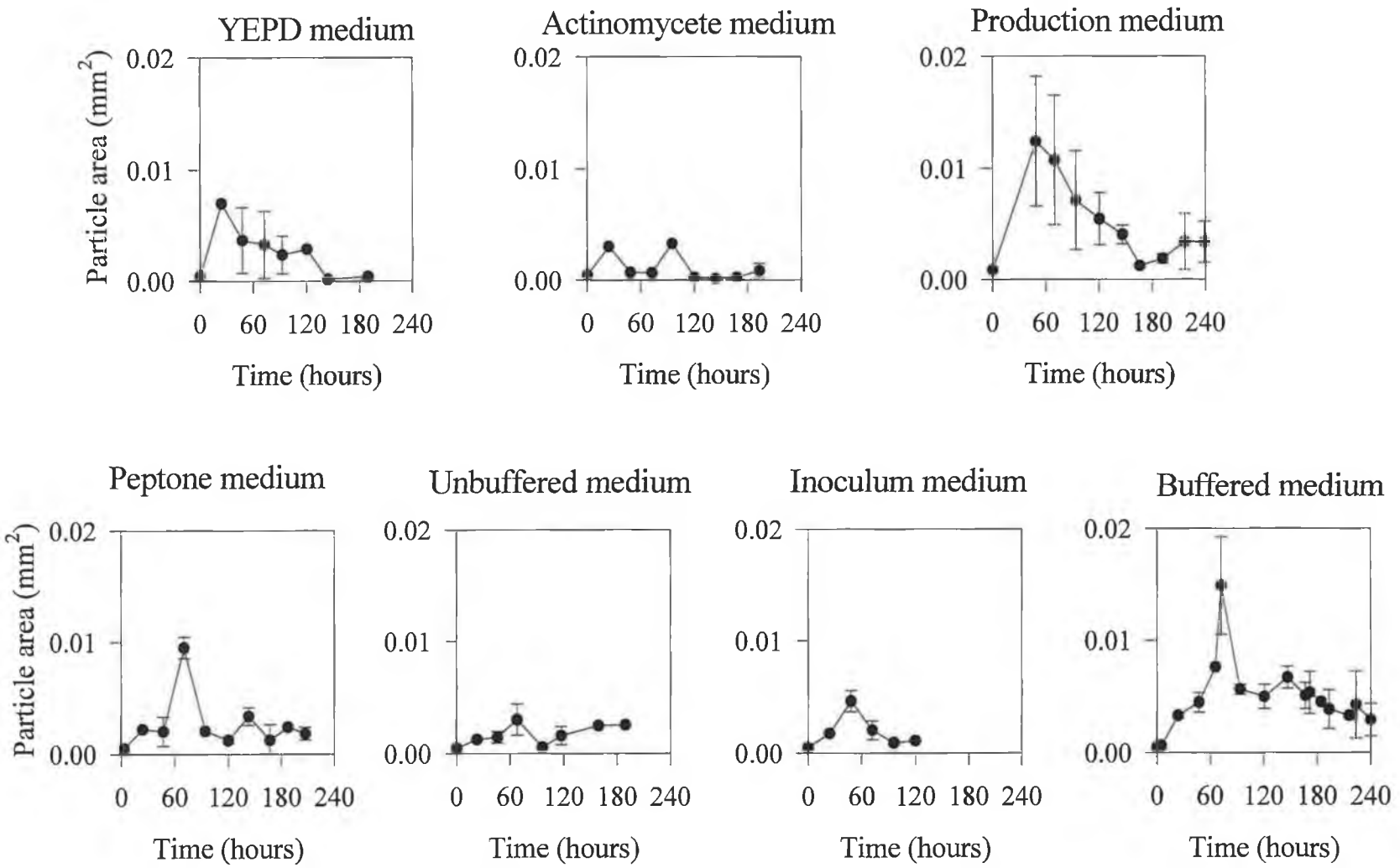


Figure 6.4 Particle area development during batch cultivation

Analysis of the system morphology after a specified cultivation period is a convenient and useful way of comparing the influence of various media. However, these results would obviously be enhanced by more comprehensive media profiles (*e.g.* nitrgen, phosphate) which might reveal evidence of nutrient limitation at earlier stages in the cultivation period which would impact on subsequent morphological profiles.

6.1.4 RELATIONSHIP BETWEEN MORPHOLOGY AND GROWTH PARAMETERS

As can be seen in Figure 6.4 the particle areas observed during the medium studies fluctuate, and in all cases, the maximum particle area is observed within the first 100 hours of cultivation. It was decided to compare the morphological development with the biomass production rate profiles observed for the peptone run. This medium was only taken as an example to illustrate the relationship between biomass and pellet morphology in shake flask cultivations.

Figure 6.5 compares particle diameter development with both $Y_{x/s}$ and R_{vb} profiles during the cultivation of the organism in peptone medium. Clearly, the initial development of the particle diameter cannot be correlated with $Y_{x/s}$ and R_{vb} values. However, when the organism begins to stabilise morphologically (at approximately 120 hours), both the $Y_{x/s}$ and R_{vb} values also reach their stable levels. Therefore a possible relationship between the final morphology of the organism and the overall values for $Y_{x/s}$ and R_{vb} was further investigated.

Figure 6.6 compares the final particle diameter of *S. natalensis* in all shake flask cultures with the corresponding overall $Y_{x/s}$ values. As is clear from this graph, the scatter of the data is too great to indicate a relationship between these two parameters.

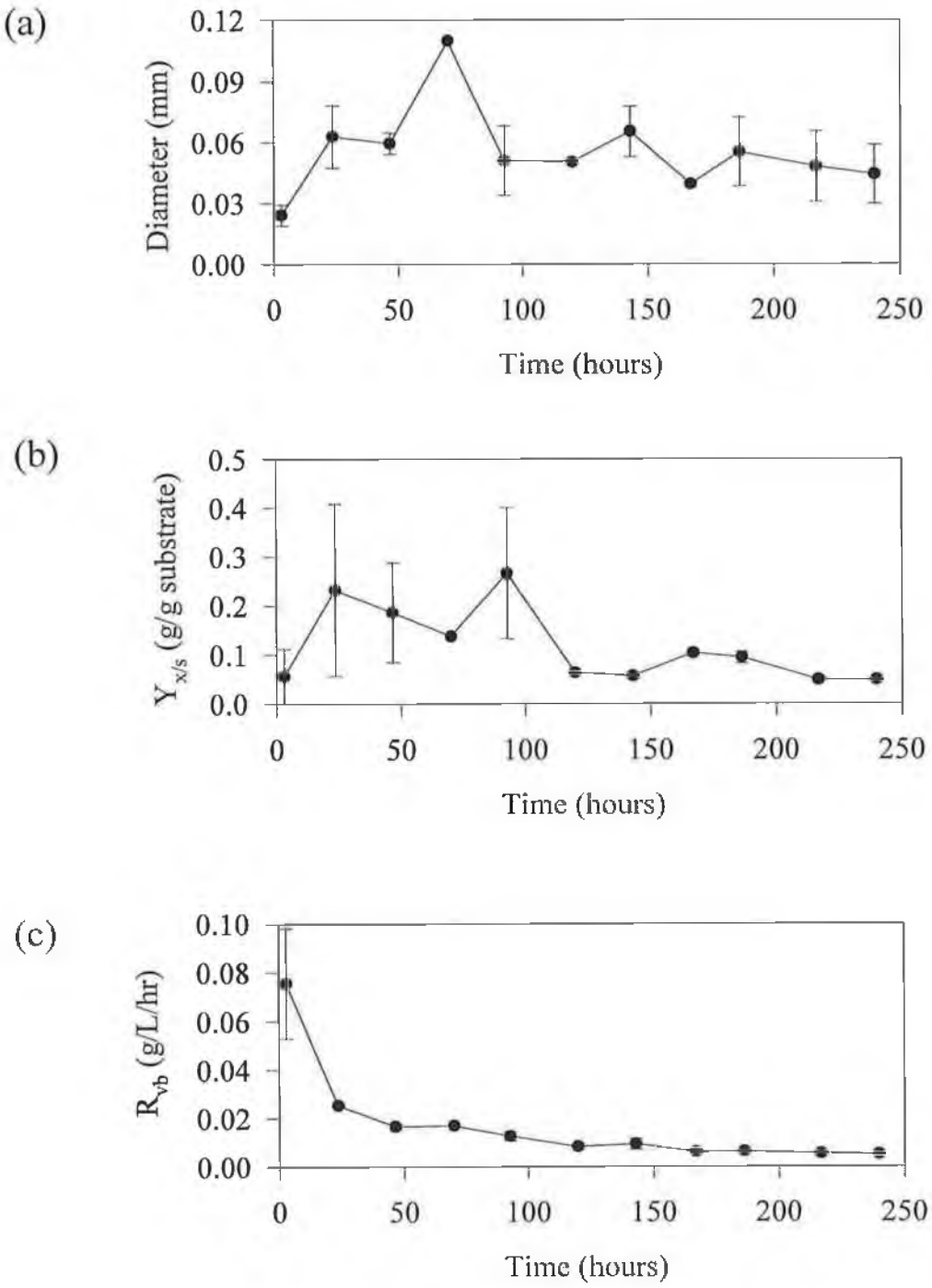


Figure 6.5 Variation in (a) particle diameter, (b) $Y_{X/S}$ and (c) R_{vb} for *S. natalensis* in peptone medium.

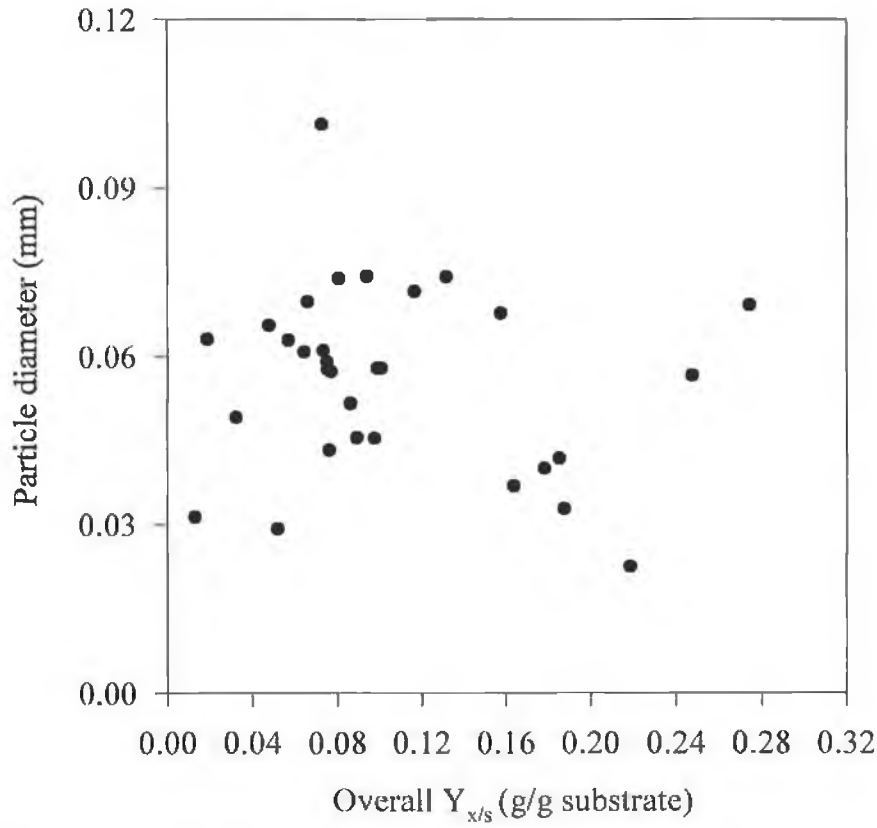


Figure 6.6 Relationship between overall $Y_{X/S}$ values and final particle area for all investigated media.

However, as outlined in Figure 6.7, it can be postulated that a relationship exists between the overall R_{vb} values observed for shake flask cultivation of *S. natalensis* and the corresponding final morphological parameters. This trend is independent of medium composition or their concentration. The particle diameter increases sharply as the R_{vb} increases initially to a value of approximately 0.07 g/L.h. This seems to indicate that at either very low or very high specific biomass production rates, the average area of individual particles is also small.

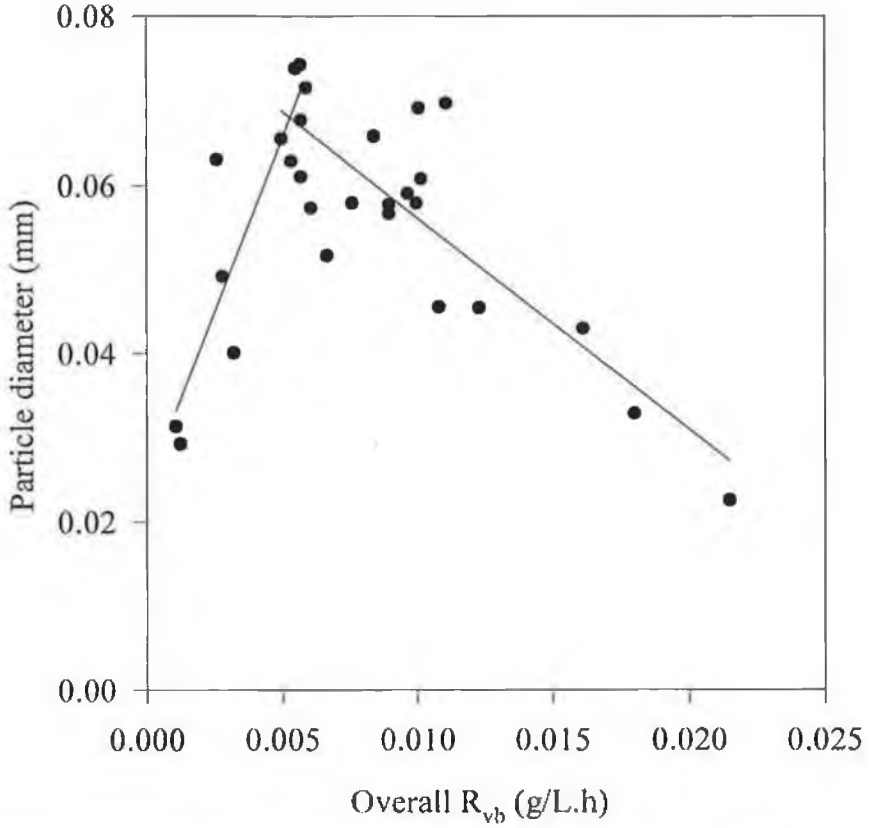


Figure 6.7 Relationship between overall specific biomass production rate and final particle area for all investigated media

The specific growth rates for the seven media studied, were also calculated. A possible correlation between these data and the corresponding morphological measurements was investigated but, as can be seen from Figure 6.8, there was no apparent relationship. This difficulty in relating the particle morphology as characterised by average particle diameter to the growth rate is thought to be due to the increasing heterogeneity of morphological forms present in the fermentation broth throughout the cultivation period (Section 4.7.2).

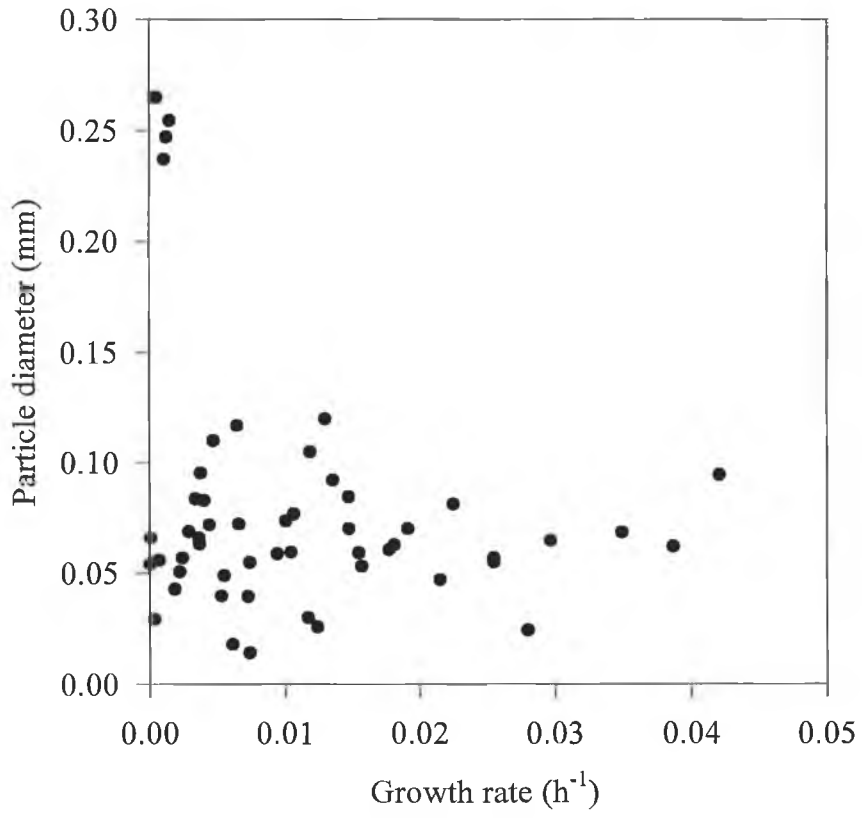


Figure 6.8 Investigation of relationship between growth rate and particle morphology

6.2 EFFECT OF MEDIA MANIPULATION ON S. natalensis

In an attempt to optimise the buffered fermentation medium used in the majority of shake flask studies on *S. natalensis*, and originally recommended by Struyk and Waisvisz (1975), the concentrations of a number of key components were varied. The subsequent effects on both the morphological and growth characteristics of the organism were monitored. The concentration ranges studied in this and all subsequent studies are not complete but provide an indication of the influence of various components on system performance. Table 6.4 summarizes the concentrations of the media components used in these studies. All fermentations were duplicated while the control fermentation, using buffered fermentation medium, was run ten times. In the case of the carbonate studies, production medium, also recommended by Struyk and Waisvisz (1975), was used as the control medium.

Table 6.4 Summary of media studies

Component	Medium	Control Concentration (g/L)	Range Investigated (g/L)
Nitrogen $(NH_4)_2SO_4$	Buffered fermentation	5	2.5 – 10
Nitrogen Peptone	Peptone	5	2.5 – 10
Carbonate $CaCO_3$	Production	8	4 – 16
Carbonate Na_2CO_3	Production	$CaCO_3$ 8g/L	8
Carbonate $NaHCO_3$	Production	$CaCO_3$ 8g/L	8
Phosphate	Buffered fermentation	0.2	0.2 – 0.8
Corn Steep Powder	Buffered fermentation	1	1 – 4
Glucose	Buffered Fermentation	30	10 – 40

6.2.1 ORGANIC VERSUS INORGANIC NITROGEN SOURCES

From a cursory examination of the data presented in Table 6.1 and Figures 6.2 and 6.3, clear differences exist between the growth characteristics of *S. natalensis* when cultivated in buffered fermentation medium, containing inorganic nitrogen, and peptone medium, where the main nitrogen source is organic. These effects may be due, not just to the choice of nitrogen source, but also the nitrogen concentrations in the media. In the buffered fermentation medium, nitrogen constituted 1.06 g/L of the 5 g/L ammonium sulphate added. In the peptone medium, only 0.7 g/L of the 5 g/L peptone was accounted for by nitrogen. Ideally, trials should have been performed at equal initial nitrogen concentrations. All other components in these media are identical. Peptone medium, although yielding a lower maximum biomass concentration than the buffered fermentation medium (Table 6.1), resulted in a greater $Y_{x/s}$ value (Figure 6.2) and the greatest overall R_{vb} value (Figure 6.3).

Figure 6.9 outlines the differences in the observed biomass profiles for *S. natalensis* when cultivated in buffered fermentation medium containing inorganic nitrogen - $(NH_4)_2SO_4$, peptone medium (organic nitrogen) or a medium containing a

combination of both nitrogen sources. Each of these trials was repeated a number of times: ten times for the buffered fermentation medium containing ammonium sulphate; four times for the peptone medium; twice for the medium containing both nitrogen sources, with duplicate samples taken for each time point. Error bars were omitted from Figure 6.9 for clarity, but did not exceed 20%.

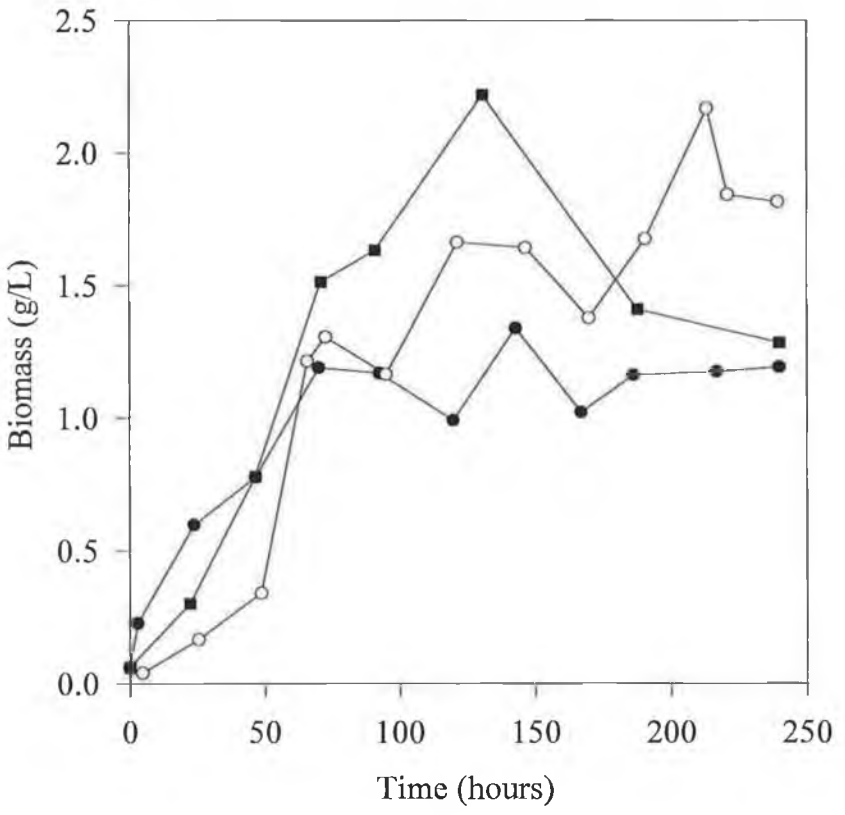


Figure 6.9 Biomass profiles during shake flask cultivations using different nitrogen sources (O $(NH_4)_2SO_4$; ● peptone; ■ $(NH_4)_2SO_4$ and peptone)

Clearly the highest final biomass concentration, (1.817g/L) is achieved with an inorganic nitrogen source. The lowest overall biomass concentration (1.193g/L) was observed when peptone medium was used to cultivate the organism. However, given that the ratio of these levels (1.52) is almost identical to the ratio of the initial nitrogen concentration in the media, (1.51), it is difficult to infer any effect independently associated with the choice of nitrogen source. The highest biomass concentration at any stage, nearly double that obtained using organic nitrogen, was observed when the organism was grown in buffered fermentation medium

supplemented peptone. This biomass level was reached faster than the maximum biomass observed with either of the other media.

Table 6.5 outlines the $Y_{x/s}$ and R_{vb} values obtained during these nitrogen studies. As was evident from Figure 6.2, the maximum biomass yield increases as the nitrogen source changes from inorganic to organic. Glazebrook *et al.* (1993) also noted, in studies on *S. akiyoshiensis*, that inorganic nitrogen resulted in lower biomass yields.

Table 6.5 Biomass yields for nitrogen studies

Nitrogen source	Max. $Y_{x/s}$ (g/g glucose)	Max. R_{vb} (g/L.h)	Overall $Y_{x/s}$ (g/g glucose)	Overall R_{vb} (g/L.h)
$(NH_4)_2SO_4$	0.134	0.018	0.099	0.0076
$(NH_4)_2SO_4$ and peptone	0.239	0.021	0.077	0.0053
Peptone	0.267	0.026	0.048	0.0050

The maximum overall $Y_{x/s}$ and R_{vb} values were observed when the peptone was the sole nitrogen source available to the organism. However, as can be seen from Table 6.5, the overall $Y_{x/s}$ and R_{vb} values behave differently to the maximum values. As the nitrogen source is changed from inorganic to organic the overall values for $Y_{x/s}$ and R_{vb} decrease. The maximum values observed at the end of the fermentations were when ammonium sulphate was used as the sole nitrogen source. This medium yielded an overall $Y_{x/s}$ value over twice that observed with peptone medium.

It can be seen, from Figure 6.10, that the morphological profiles of *S. natalensis* exhibit very similar trends regardless of the nitrogen source employed. In all three cases, a local maximum in particle area occurred at approximately 75 hours. At this stage, the smallest particles ($5.162 * 10^{-3}mm^2$) were formed in buffered fermentation medium supplemented with peptone. The largest particles ($0.011mm^2$) occurred in buffered fermentation medium (inorganic nitrogen source). These particles were over twice the size of those observed with the peptone supplemented medium.

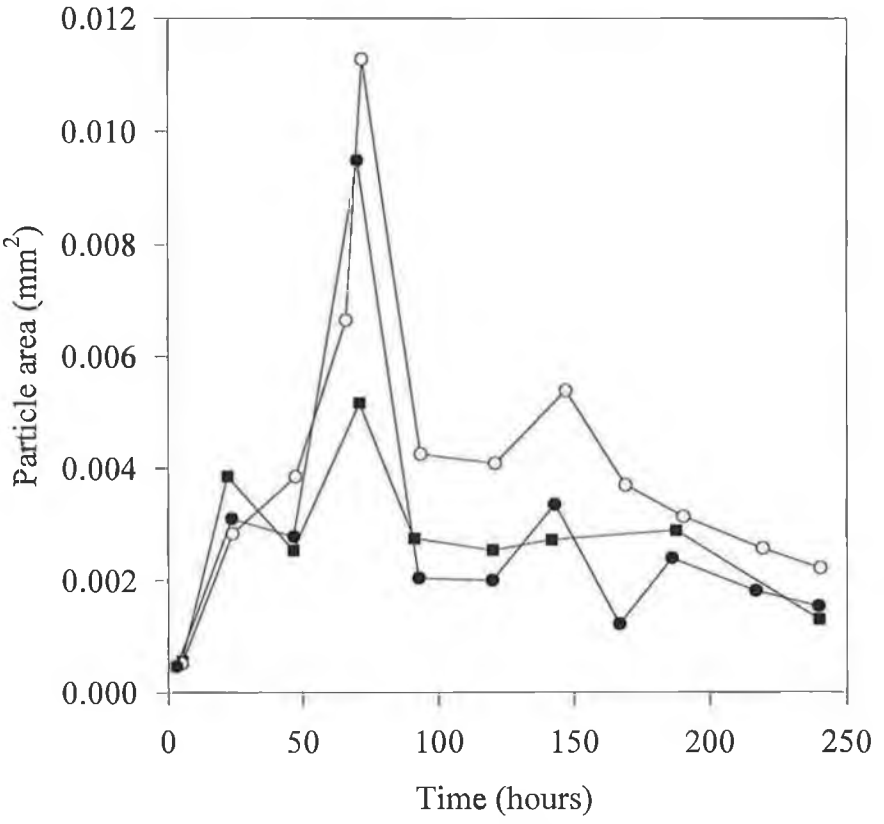


Figure 6.10 Morphological profiles for *S. natalensis* with varying nitrogen sources (O $(NH_4)_2SO_4$; ● peptone; ■ $(NH_4)_2SO_4$ and peptone)

The same trends apply to the final particle areas. The largest final mean particle area (2.214 * $10^{-3}mm^2$), occurred in the buffered fermentation medium, with the smallest (1.307 * $10^{-3}mm^2$) in the supplemented buffered fermentation medium.

6.2.2 EFFECT OF AMMONIUM SULPHATE CONCENTRATION

The concentration of ammonium sulphate used in buffered fermentation medium is 5g/L, and this was varied to ascertain the effects of inorganic nitrogen concentration on growth. It can be seen from both Table 6.6 and Figure 6.11 that of the range of conditions investigated, this concentration is optimal, yielding the maximum growth characteristics - 2.116g/L biomass, 0.134g/g glucose and 0.018g/L.h.

Table 6.6 Effect of ammonium sulphate concentration in buffered fermentation medium on shake flask culture characteristics (control conditions highlighted)

Ammonium sulphate concentration (g/L)	Max. $Y_{x/s}$ (g/g glucose)	Max. R_{vb} (g/L.h)	Overall $Y_{x/s}$ (g/g glucose)	Overall Rvb (g/L.h)
2.5	0.117	0.013	0.117	$5.888 * 10^{-3}$
5.0	**0.134**	**0.018**	**0.099**	**$7.600 * 10^{-3}$**
7.5	0.085	0.013	0.073	$5.697 * 10^{-3}$
10.0	0.060	0.012	0.057	$5.336 * 10^{-3}$

Once the concentration of ammonium sulphate is changed, the maximum biomass concentration and R_{vb} decrease, by approximately 50% in the case of biomass concentration, regardless of whether the concentration of ammonium sulphate is increased or decreased. The maximum $Y_{x/s}$ also decreases if the concentration of ammonium sulphate is varied. When the concentration was decreased to 2.5g/L the $Y_{x/s}$ value decreases slightly. When the concentration was increased beyond 5g/L, the reduction in biomass yield was even more severe, falling from 0.134g/g glucose to 0.060g/g glucose when the ammonium sulphate concentration was doubled to 10g/L.

In relation to the overall values noted for R_{vb}, the same trends are clear. 5g/L yields the maximum overall value. The overall values for $Y_{x/s}$, however, behave differently, with 2.5g/L ammonium sulphate resulting in the highest value. As the concentration increases, the $Y_{x/s}$ values decrease.

With respect to morphology, the data presented in Table 6.7 represent the final average particle areas, observed at approximately 200 hours. The smallest particles were formed when the organism was cultivated under the control conditions (5g/L of ammonium sulphate). As the ammonium sulphate concentration is increased, the particle area increases, from $2.637 \times 10^{-3} mm^2$ to $3.109 \times 10^{-3} mm^2$, observed when 10g/L is used. The greatest increase is seen when the concentration of ammonium sulphate is reduced to 2.5g/L. It can be seen from the data presented that, even though the smallest particle area was observed at an ammonium sulphate concentration of 5g/L, both the perimeter and diameter were greater than those

observed for both 2.5g/L and 10g/L. This indicates that these particles are 'hairier' than those formed in media containing other concentrations of ammonium sulphate.

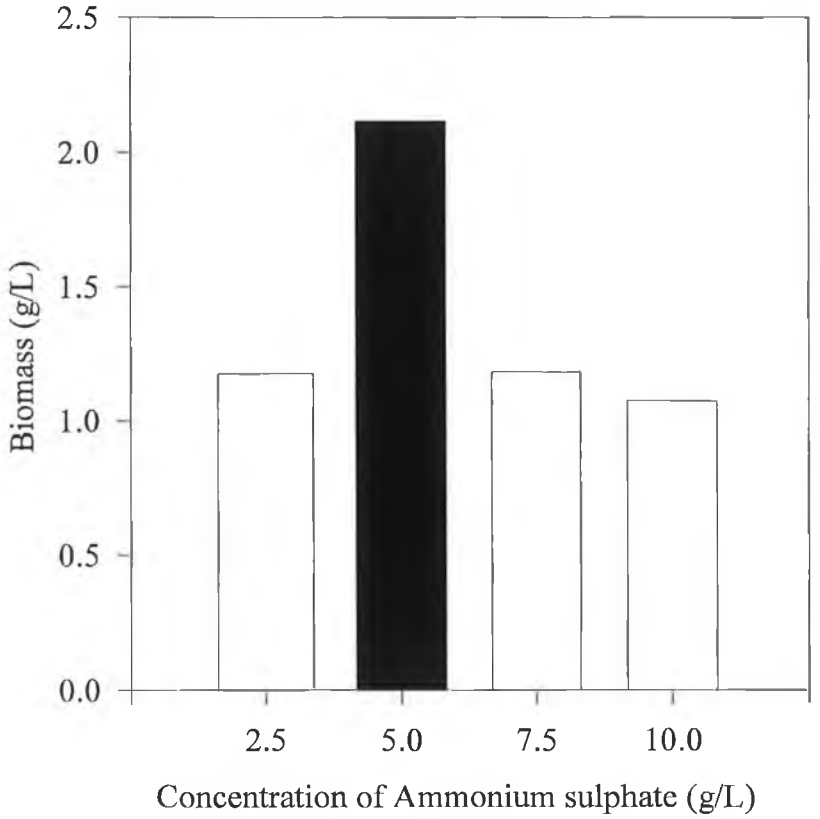


Figure 6.11 Maximum biomass concentrations observed during ammonium sulphate studies (control concentration highlighted)

Table 6.7 Morphological characteristics observed during optimisation of ammonium sulphate concentration in buffered fermentation medium (control conditions highlighted)

Ammonium sulphate concentration (g/L)	Average particle area (mm^2)	Perimeter (mm)	Diameter (mm)
2.5	$4.028 * 10^{-3}$	0.161	0.045
5.0	**$2.637*10^{-3}$**	**0.216**	**0.052**
7.5	$2.932 * 10^{-3}$	0.180	0.048
10.0	$3.109 * 10^{-3}$	0.175	0.046

6.2.3 EFFECT OF PEPTONE CONCENTRATION

To study the effect of peptone concentration in peptone medium, (Section 3.3.3.5), on the growth and morphology of *S. natalensis* the concentration was varied in the range 2.5g/L to 10g/L. Each fermentation in this study was duplicated. It can be seen, from Table 6.8 and Figure 6.12, that of the conditions investigated, a peptone concentration of 10g/L results in a maximum biomass level of 1.851g/L. As the concentration of peptone is reduced below 10g/L, the maximum biomass concentration observed during these fermentations decrease. These data, combined with the biomass profiles presented in Figure 6.9, suggest that the system may have been nitrogen limited at a pepetone concentration of 5 g/L. However, the maximum biomass concentrations for fermentations containing 7.5g/L and 5g/L peptone are very similar with only a 0.05g/L difference between the two. The biomass concentration observed when 2.5g/L peptone was used was approximately one third of that observed at a peptone concentration of 10g/L.

Table 6.8 Effect of peptone concentration in buffered fermentation medium on shake flask culture characteristics (control conditions highlighted)

Peptone concentration (g/L)	Max. $Y_{x/s}$ (g/g glucose)	Max. R_{vb} (g/L.h)	Overall $Y_{x/s}$ (g/g glucose)	Overall R_{vb} (g/L.h)
2.5	0.044	0.006	0.032	0.0028
5.0	**0.267**	**0.076**	**0.048**	**0.0050**
7.5	0.168	0.013	0.073	0.0072
10.0	0.081	0.018	0.075	0.0097

Although 10g/L peptone yields the maximum biomass concentration, a maximum biomass yield of 0.267g/g glucose and a maximum R_{vb} of 0.076g/L.h were observed at a concentration of 5g/L of peptone. The greatest overall values for $Y_{x/s}$ and R_{vb} were observed when 10g/L peptone was used, and as with the biomass concentrations, these values decrease with decreasing peptone concentrations.

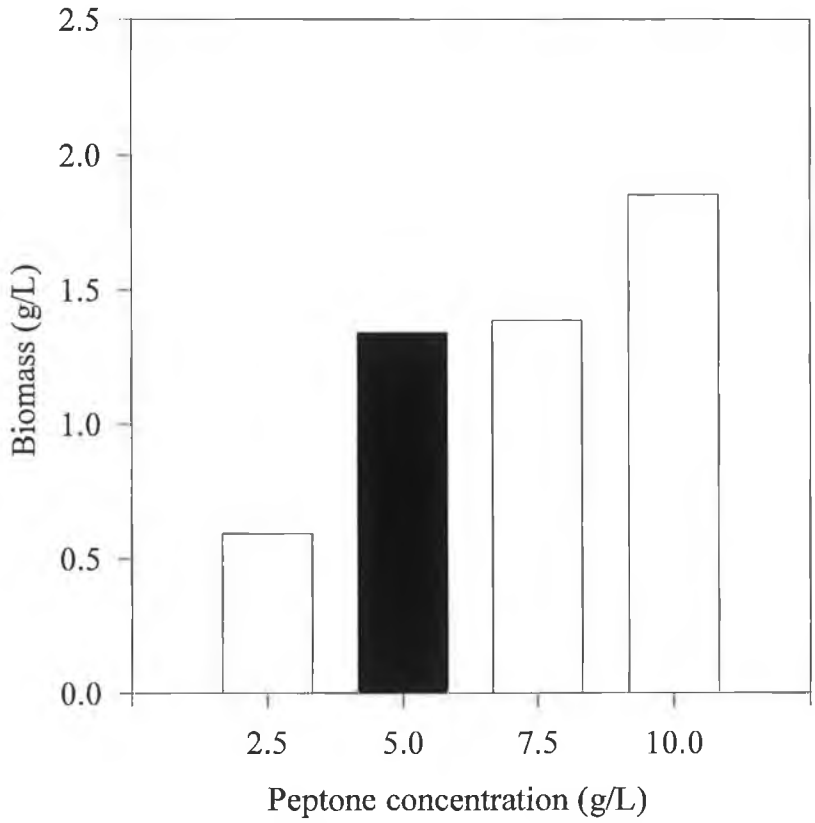


Figure 6.12 Maximum biomass concentrations observed during the optimisation of the peptone medium (control conditions highlighted)

As can be seen from Table 6.9, the largest particles with an average area of 8.082 x $10^{-3}mm^2$ were observed when the organism was cultivated in 7.5g/L of peptone. Cultivation in 5g/L of peptone resulted in the smallest particles, 1.537 x $10^{-3}mm^2$. These pellets were 'hairier' than the others observed in this study with the smoothest seen with 7.5g/L peptone. These morphological parameters were observed at approximately 200 hours.

Table 6.9 Morphological characteristics observed after 200 hours during optimisation of peptone medium (control conditions highlighted)

Peptone concentration (g/L)	Average particle area (mm^2)	Perimeter (mm)	Diameter (mm)
2.5	$1.901 * 10^{-3}$	0.150	0.041
5.0	**$1.537 * 10^{-3}$**	**0.190**	**0.047**
7.5	$8.082 * 10^{-3}$	0.351	0.092
10.0	$2.744 * 10^{-3}$	0.225	0.056

6.2.4 CARBONATE STUDIES

6.2.4.1 EFFECT OF CALCIUM CARBONATE CONCENTRATION

To observe the effect of the concentration of calcium carbonate in production medium on the growth of *S. natalensis*, concentrations of between 4g/L and 16g/L were investigated. The control value used in these studies was 8g/L (production medium). As can be seen from Figure 6.13, the maximum biomass concentration was observed at a calcium carbonate concentration of 4g/L. However the difference in the biomass concentrations obtained is not great, with approximately 0.6g/L between the maximum and minimum values observed.

The maximum biomass yield, 0.122g/g glucose, was achieved at a concentration of 8g/L (Table 6.10). As the carbonate concentration is increased to 16g/L, it can be seen that the biomass yield per gram of glucose falls from 0.122g/g glucose to 0.065g/g glucose. When the concentration of calcium carbonate is halved to 4g/L the $Y_{x/s}$ value also decreases, (0.085g/g glucose). A similar trend is observed for the R_{vb} values and the overall values for $Y_{x/s}$.

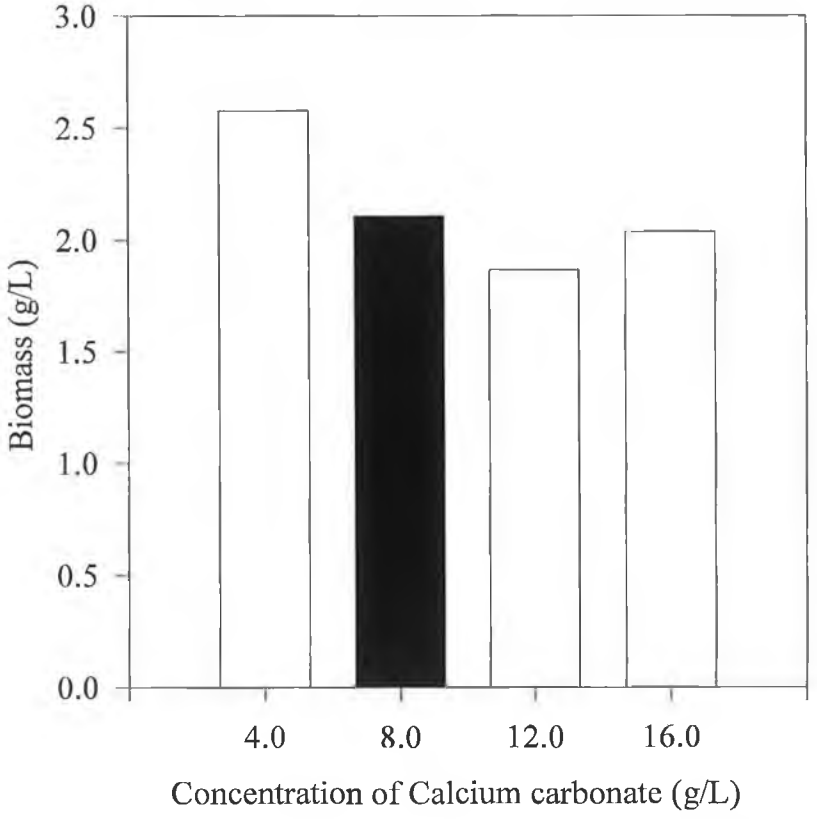


Figure 6.13 Maximum biomass concentrations observed during optimisation studies of production medium (control conditions highlighted)

Table 6.10 Effect of calcium carbonate on the growth characteristics of *S. natalensis* in production medium (control conditions highlighted)

$CaCO_3$ concentration (g/L)	Max. $Y_{x/s}$ (g/g glucose)	Max. R_{vb} (g/L.h)	Overall $Y_{x/s}$ (g/g glucose)	Overall R_{vb} (g/L.h)
4.0	0.085	0.032	0.078	0.013
8.0	**0.122**	**0.040**	**0.081**	**0.009**
12.0	0.066	0.011	0.066	0.011
16.0	0.065	0.010	0.065	0.010

Table 6.11 shows the effect of the carbonate concentration on the morphological characteristics of the organism during shake flask cultivation. It was observed that the maximum particle area occurred at a concentration of 16g/L. As the concentration of calcium carbonate was decreased below this value, the particle area, perimeter and diameter decrease.

Table 6.11 Morphological characteristics observed after 200 hours during optimisation of calcium carbonate concentration (control conditions highlighted)

$CaCO_3$ concentration (g/L)	Particle area (mm^2)	Perimeter (mm)	Diameter (mm)
4.0	$4.588 * 10^{-4}$	0.094	0.026
8.0	$\mathbf{3.377 * 10^{-3}}$	**0.220**	**0.051**
12.0	$3.508 * 10^{-3}$	0.236	0.054
16.0	$3.826 * 10^{-3}$	0.243	0.059

6.2.4.3 INFLUENCE OF CARBONATE SOURCE

In an attempt to reduce the level of suspended solids in the medium, other carbonate sources were investigated and their effects on the growth of the organism monitored. It appears, from Table 6.12, that these carbonate sources were unsuitable for the cultivation of *S. natalensis*. Final biomass levels were approximately one tenth (with either of the replacement carbonate sources) of those observed with calcium carbonate. These replacement sources also resulted in very low $Y_{x/s}$ and R_{vb} values compared to those observed when the organism was cultivated in production medium. For this data it can be concluded that no growth occurred and no further efforts to identify an alternative source were undertaken.

With the alternative carbonate sources the broth pH never fell below pH 7.7. It is postulated that *S. natalensis* may have a very narrow pH range over which growth can occur.

Table 6.12 Effect of carbonate source on the growth of *S. natalensis* in production medium (control conditions highlighted)

Carbonate source (8 g/L)	max. dry weight (g/L)	Max. $Y_{x/s}$ (g/g glucose)	Max. R_{vb} (g/L.h)
Calcium carbonate	**2.105**	**0.122**	**0.040**
Sodium carbonate	0.198	0.013	$1.081 * 10^{-3}$
Sodium hydrogen carbonate	0.229	0.052	$1.247 * 10^{-3}$

6.2.5 EFFECT OF PHOSPHATE CONCENTRATION

In an effort to optimise the growth of *S. natalensis* in buffered fermentation medium, the concentration of potassium dihydrogen phosphate was varied between 0.2g/L and 0.8g/L. The control value in these studies was 0.2g/L (buffered fermentation medium) and duplicates were performed in all cases. Table 6.13 and Figure 6.14 outline the effects that these variations had on the growth characteristics of the organism.

Table 6.13 Effect of KH_2PO_4 concentration on growth in buffered fermentation medium (control conditions highlighted).

KH_2PO_4 concentration (g/L)	Max. $Y_{x/s}$ (g/g glucose)	Max. R_{vb} (g/L.h)	Overall $Y_{x/s}$ (g/g glucose)	Overall R_{vb} (g/L.h)
0.2	**0.134**	**0.018**	**0.099**	**0.0076**
0.4	0.158	0.012	0.158	0.0057
0.6	0.085	0.014	0.077	0.0061
0.8	0.094	$5.678 * 10^{-3}$	0.094	0.0057

With the alternative carbonate sources the broth pH never fell below pH 7.7. It is postulated that *S. natalensis* may have a very narrow pH range over which growth can occur.

Table 6.12 Effect of carbonate source on the growth of *S. natalensis* in production medium (control conditions highlighted)

Carbonate source (8 g/L)	max. dry weight (g/L)	Max. $Y_{x/s}$ (g/g glucose)	Max. R_{vb} (g/L.h)
Calcium carbonate	**2.105**	**0.122**	**0.040**
Sodium carbonate	0.198	0.013	$1.081 * 10^{-3}$
Sodium hydrogen carbonate	0.229	0.052	$1.247 * 10^{-3}$

6.2.5 EFFECT OF PHOSPHATE CONCENTRATION

In an effort to optimise the growth of *S. natalensis* in buffered fermentation medium, the concentration of potassium dihydrogen phosphate was varied between 0.2g/L and 0.8g/L. The control value in these studies was 0.2g/L (buffered fermentation medium) and duplicates were performed in all cases. Table 6.13 and Figure 6.14 outline the effects that these variations had on the growth characteristics of the organism.

Table 6.13 Effect of KH_2PO_4 concentration on growth in buffered fermentation medium (control conditions highlighted).

KH_2PO_4 concentration (g/L)	Max. $Y_{x/s}$ (g/g glucose)	Max. R_{vb} (g/L.h)	Overall $Y_{x/s}$ (g/g glucose)	Overall R_{vb} (g/L.h)
0.2	**0.134**	**0.018**	**0.099**	**0.0076**
0.4	0.158	0.012	0.158	0.0057
0.6	0.085	0.014	0.077	0.0061
0.8	0.094	$5.678 * 10^{-3}$	0.094	0.0057

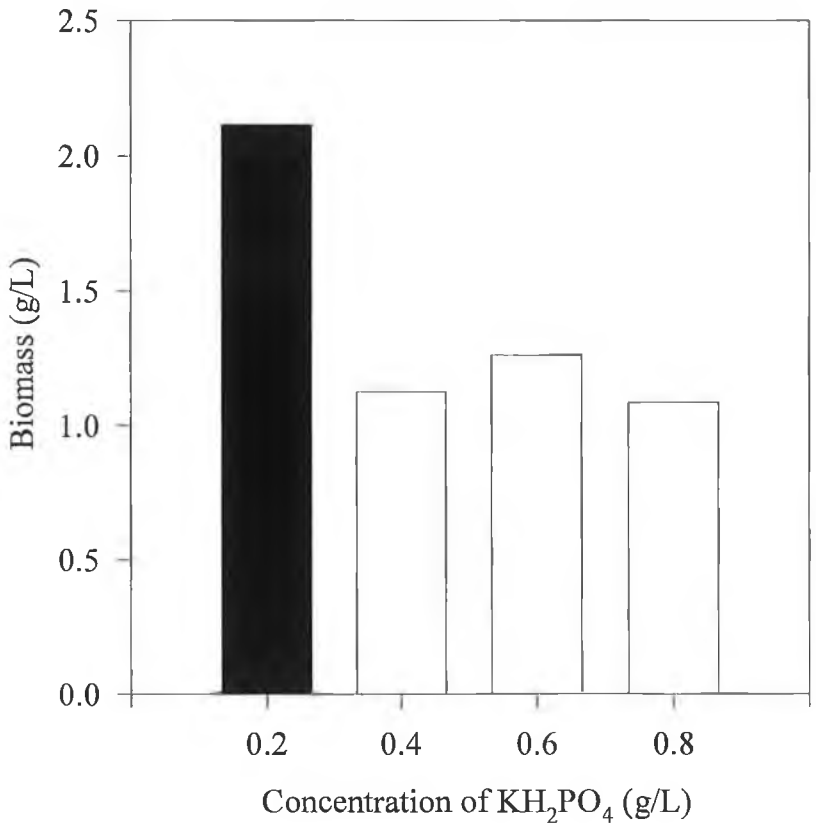


Figure 6.14 Maximum biomass concentrations observed during phosphate studies (control conditions highlighted).

For the concentration range studied, the maximum biomass concentration (2.116g/L), and highest specific biomass production rate were obtained at the control conditions. Increasing the concentration of potassium dihydrogen phosphate beyond this value results in a reduction of approximately 1g/L, regardless of the increase. The inhibitory effect of phosphate was also reported for the actinomycete, *S. clavuligerus*, (Lübbe *et al.*, 1985; Lebrihi *et al.*, 1987). As outlined in Table 6.13, the maximum biomass yield per gram of glucose utilised was observed at a potassium dihydrogen phosphate concentration of 0.4g/L. When the concentration was increased to 0.6g/L, the yield dropped from 0.158g/g to 0.085g/g. Similar trends were observed for the overall values of $Y_{x/s}$ and R_{vb}. Using 0.2g/L of KH_2PO_4, the maximum value for R_{vb} was achieved.

The concentration of potassium dihydrogen phosphate in the medium also affects the morphology observed during the fermentation as is outlined in Table 6.14. The

area, perimeter and diameter do not behave in a similar fashion, suggesting variations in the 'hairiness' of the particles. The maximum particle area was observed at 0.8g/L of potassium dihydrogen phosphate. If the concentration of KH_2PO_4 is decreased to 0.6g/L, the mean particle area decreases to the minimum value observed in this study.

Table 6.14 Effect of variations in KH_2PO_4 concentration in buffered medium on the morphology of *S. natalensis* (control conditions highlighted)

KH_2PO_4 concentration (g/L)	**Average particle area (mm^2)**	**Perimeter (mm)**	**Diameter (mm)**
0.2	**$2.637*10^{-3}$**	**0.216**	**0.052**
0.4	$3.609 * 10^{-3}$	0.195	0.052
0.6	$2.582 * 10^{-3}$	0.202	0.054
0.8	$4.340 * 10^{-3}$	0.271	0.070

6.2.6 EFFECT OF GLUCOSE CONCENTRATION

When varying the concentration of glucose used in the buffered fermentation medium it was observed that the control concentration, 30g/L, yielded the maximum biomass concentration (Figure 6.15). Reductions in the glucose concentration resulted in a reduction in overall biomass of approximately 1g/L (20g/L glucose) and 0.5g/L (10g/L glucose). The greatest reduction occurred, however, when the concentration of glucose was increased to 40g/L. McCabe (1990) also reported a similar decline in biomass production at glucose concentrations in excess of 30g/L. When cultivating *M. alpina*, Lindberg and Molin (1993) observed that when glucose was in excess the production of arachadonic acid was inhibited.

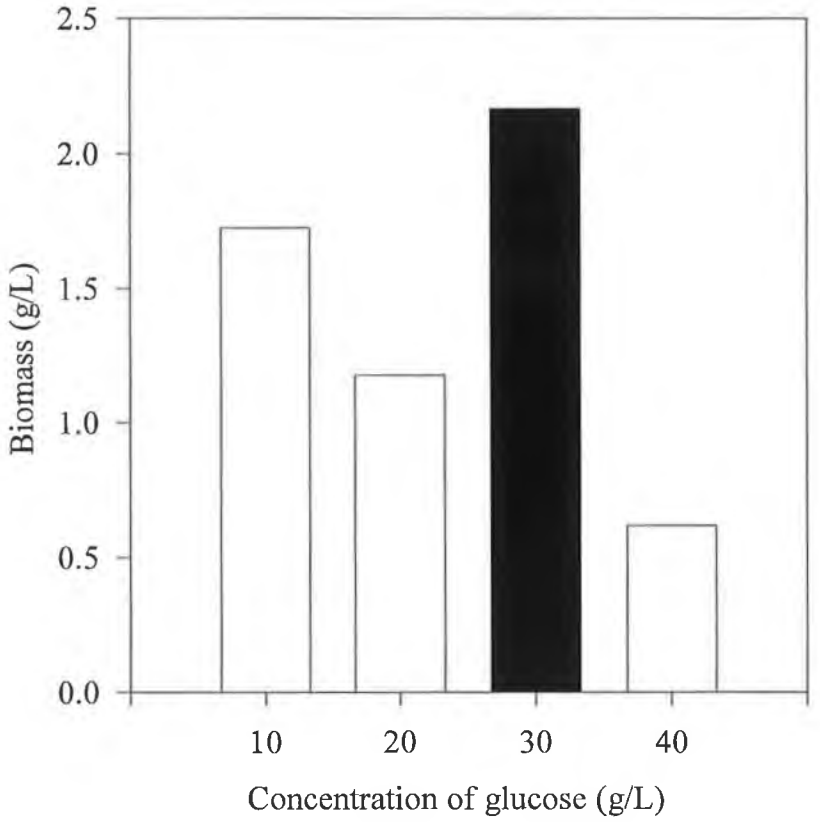


Figure 6.15 Biomass concentrations observed during glucose optimisation studies (control conditions highlighted)

However, from Table 6.15 it can be seen that 30g/L glucose yielded the lowest maximum value of $Y_{x/s}$, with 10g/L glucose yielding almost double this value. In relation to overall $Y_{x/s}$ values, the maximum was observed at 10g/L glucose. This value decreases as the concentration of glucose is increased. The maximum value for specific biomass production was observed when 30g/L of glucose was used. The minimum R_{vb} value was observed at 40g/L glucose.

Table 6.15 Optimisation of glucose concentration in buffered fermentation medium (control conditions highlighted)

Glucose concentration (g/L)	Max. $Y_{x/s}$ (g/g glucose)	Max. R_{vb} (g/L.h)	Overall $Y_{x/s}$ (g/g glucose)	Overall R_{vb} (g/L.h)
10.0	0.253	0.010	0.248	0.009
20.0	0.186	0.006	0.186	0.003
30.0	**0.134**	**0.018**	**0.099**	**0.008**
40.0	0.179	0.003	0.028	0.001

The inhibitory effects observed at a glucose concentration of 40g/L may be, in part, responsible for the length of time the organism took to reach its maximum biomass level when cultivated in the actinomycete medium developed by Sarra *et al.* (1996), which was discussed in Section 6.1.1.

The smallest mean particle areas observed during this study occurred at 40g/L glucose (Table 6.16). This concentration did not result in the greatest percentage of clumped growth in the fermentation, which occurred at 20g/L glucose. 30g/L glucose resulted in the greatest particle area.

Table 6.16 Effect of glucose concentration in buffered medium on the morphology of *S. natalensis* (control conditiond highlighted)

Glucose concentration (g/L)	Average particle area (mm^2)	Perimeter (mm)	Diameter (mm)
10.0	$2.523 * 10^{-3}$	0.188	0.051
20.0	$1.377 * 10^{-3}$	0.144	0.039
30.0	$\mathbf{2.637*10^{-3}}$	**0.216**	**0.052**
40.0	$1.261 * 10^{-3}$	0.141	0.038

6.2.7 EFFECT OF CORN STEEP POWDER (CSP) CONCENTRATION

The final medium component studied in relation to medium optimisation was CSP. As can be seen from Figure 6.16, the maximum biomass concentration was observed when 4g/L of CSP was used in buffered fermentation medium. However there was very little variation in the biomass concentrations with just 0.65g/L between the maximum, (2.366g/L) and the minimum, (1.724g/L), observed at a CSP concentration of 2g/L. As can be seen from Table 6.17, however, the reverse holds for the values of $Y_{x/s}$ observed during this study. The maximum value was observed when 2g/L CSP was used; the minimum with 4g/L. Overall R_{vb} values increase with increasing CSP concentrations.

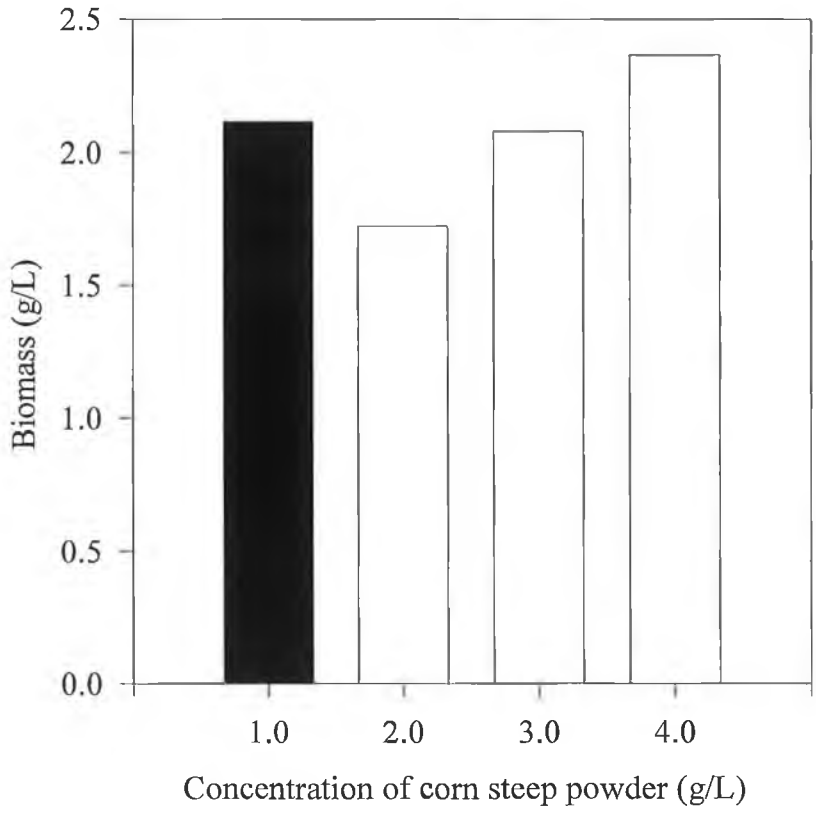


Figure 6.16 Overall biomass concentrations observed during CSP studies (control conditions highlighted)

Table 6.17 Optimisation of CSP concentration in buffered fermentation medium (control conditions highlighted)

Corn steep powder concentration (g/L)	Max. $Y_{x/s}$ (g/g glucose)	Max. R_{vb} (g/L.h)	Overall $Y_{x/s}$ (g/g glucose)	Overall R_{vb} (g/L.h)
1.0	**0.134**	**0.018**	**0.099**	**0.008**
2.0	0.155	0.015	0.076	0.009
3.0	0.131	0.020	0.090	0.011
4.0	0.108	0.020	0.098	0.012

Morphologically, the mean particle area decreases with increasing concentration of CSP. The maximum particle area, observed when 1g/L CSP was used, was over twice that observed with 4g/L.

Table 6.18 Effect of CSP concentration in buffered medium on the morphology of *S. natalensis* (control conditions highlighted).

CSP concentration (g/L)	Average particle area (mm^2)	Perimeter (mm)	Diameter (mm)
1.0	**$2.637*10^{-3}$**	**0.216**	**0.052**
2.0	$1.566 * 10^{-3}$	0.161	0.040
3.0	$1.045 * 10^{-3}$	0.136	0.035
4.0	$8.924 * 10^{-4}$	0.123	0.033

6.3 SUMMARY

From the preceding studies it is obvious that the choice of medium can have a significant effect on biomass concentration and morphology of *S. natalensis*. The use of YEPD medium results in the largest biomass concentration observed during this study. Actinomycete medium also resulted in a high biomass concentration.

There was very little difference in the morphology observed during any of the media studies. The organism still grew primarily in pelleted form, although the observed particles were slightly smaller when cultivated in YEPD or actinomycete medium.

Varying the concentrations of some of the components of buffered fermentation medium showed that, for the ranges tested, the concentrations suggested by Struyk and Waisvisz (1975) are optimal in relation to biomass levels. However, reducing the $CaCO_3$ concentration in the production medium used by Mahon (1990) and McCabe (1990) from 8g/L to 4g/L yielded a small improvement in final biomass levels. Studies involving peptone indicated that a concentration of 10g/L resulted in a higher biomass concentration than the control value of 5g/L employed. However, further trials would be required to conclusively identify optimum peptone levels.

CHAPTER 7

CONCLUSIONS AND RECOMMENDATIONS

7.1 CONCLUSIONS

The purpose of this study was to characterise the morphological development of the organism, *S. natalensis*, in submerged culture and to investigate the effects of cultivation conditions and medium components on both growth and morphology.

It was found that *S. natalensis* is a pH sensitive organism which may also be sensitive to oxygen transfer rates. In studies of *S. tendae*, Glazebrook *et al.* (1992) noted that as the volume of inoculum was increased, the size of the particles decreased. This trend was only observed with *S. natalensis* using inoculum cultures of 72 hours and 96 hours.

Morphological studies of *S. natalensis* indicate that the organism grows largely in pelleted form with a varying percentage (calculated on a volume basis) accounted for by clumps of less than $2.29 * 10^{-3}$ mm^2. For the control conditions employed (100 mL buffered fermentation medium in a 250 mL flask), the maximum average particle area was observed at approximately 73 hours, after which the particles reduce in size to a stable value which is maintained for the duration of the fermentation. Particle volume distributions indicate that there is a persistent small pellet population which is maintained as a result of the recurring disruption of larger pellets.

The organism is also affected by the hydrodynamic environment in the cultivation vessel, as characterised by liquid volume in shake flasks rotated at a constant speed, with a higher percentage of clumped growth in smaller volumes, corresponding to increased levels of biomass production. Limited studies in 1L and 10L STR configurations indicated that the organism may be sensitive to variations in agitation conditions.

Use of phosphate buffer instead of $CaCO_3$ increases the time taken to reach maximum biomass concentration. The presence of yeast extract results in the highest

biomass, $Y_{x/s}$ and R_{vb} values. Morphologically, unbuffered media result in smaller particle areas and a higher percentage of clumps present in the fermentation broth.

As with particle development, both the $Y_{x/s}$ and R_{vb} values increase to a maximum (observed at approximately 73 hours) and subsequently decrease to stable values, maintained for the remainder of the cultivation period. This trend was observed during the majority of shake flask fermentations. There appears to be a relationship between R_{vb} and particle area. However, no relationship was observed between either $Y_{x/s}$ or growth rate and the morphology of the organism as defined by average particle area. This may be due to the increased heterogeneity in the fermentations as time progresses.

Use of an organic nitrogen source results in significantly higher R_{vb} and $Y_{x/s}$ values than the inorganic nitrogen source. From the data presented, there is evidence to suggest that peptone concentration may have been limiting. Inorganic nitrogen resulted in the largest particle areas, while the smallest particles were observed using a combination of both inorganic and organic nitrogen. The organism requires calcium to grow as using alternative carbonate sources to calcium carbonate resulted in stunted growth and morphological development.

It appears that the concentration of components in buffered fermentation medium result in optimal biomass production for this medium. Increasing the concentration of CSP to 4g/L resulted in a slight increase in biomass concentration. Similarly, with respect to the production medium, decreasing the concentration of calcium carbonate to 4g/L resulted in a slight increase in the observed maximum biomass concentration. Increasing the concentration of peptone in the peptone medium to 10g/L resulted in a significant increase of biomass concentration.

7.2 RECOMMENDATIONS

- Further work should be undertaken on the pimaricin extraction and detection methods listed in Section 3.6, particularly in the HPLC technique developed by Mahon (1990). The use of other solvents and cellular disruption techniques should be examined to increase the accuracy and sensitivity of the method.
- Reinvestigation of validation protocol by which the minimum number of entites to ensure statistically representative samples was determined; analysis on a volume, rather than number basis might be more appropriate.
- Development of the image analysis program to highten the detection sensitivity in order to minimise the manual editing stage.
- Also, in relation to image analysis a second program must be written to analyse filamentous fragments formed during cultivation at high agitation rates and also when cultivated in YEPD and, to a lesser extent, Actinomycete medium.
- Baffled flasks should be introduced to volumes studies, with a view to reducing the inhibitory effect of oxygen limitation at larger volumes.
- In relation to the medium studies, further work must be done to find a substitute carbonate source in order to avoid the presence of suspended solids in the medium if possible while meeting nutritional and buffering requirements.
- Further work in the nitrogen studies is required as, on the basis of studies in buffered fermentation and peptone media, it is possible that in the latter the organism may have been subject to nitrogen limitation. Monitoring of nitrogen utilisation would be benificial.
- Lower concentrations of both calcium carbonate and phosphate should also be investigated as the maximum biomass concentrations were observed at the lowest concentrations (4 g/L and 0.2 g/L, respectively) investigated. Higher concentrations of corn steep powder should be investigated in relation to biomass production.
- Further studies into the possible alkaline sensitivity of the organism should also be undertaken as this has a major influence into the choice of possible substitute buffers.

- Further studies of the apparent inhibitory effect of high glucose concentrations are recommended. Possible suggestions for overcoming this problem would be to operate on a fed-batch basis.
- STR studies under a range of operation and agitation conditions to identify optimum environment for cultivation.

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APPENDIX 1 - IMAGE ANALYSIS PROGRAM

INITIAL SETUP

```
For  ( LOOP = 0 to 6, step 1 )
  BINEDOUT = LOOP
  Binary Edit  ( Clear BINEDOUT )
Next  ( LOOP )
Select Lens  ( Transmitted,  4 x, mag changer 1.0 x, 2.08731 microns per pixel )
Measure frame  ( x 31,  y 61,  Width 658,  Height 450 )
Setup Results Window ( Move Feature Results to x 40, y 496, w 520, h 104 )
Dim  ( X(200) )
Dim  ( FTRRES(10000,20) )
```

MEASUREMENT OF PELLETS AND CLUMPS

```
COUNTER = 1
PauseText  ( "Enter no. of fields" )
Input  ( FIELDS )
For  ( LOOPER = 1 to FIELDS, step 1 )
  Live image  ( into Image1 )
  PauseText  ( "Focus on cells of interest" )
  Pause  ( No dialog )
  ACQOUTPUT = LOOPER
  Acquire  ( into ACQOUTPUT )
  LUTINPUT = ACQOUTPUT
  LUTOUTPUT = LUTINPUT
  Grey LUT  ( Square from LUTINPUT to LUTOUTPUT )
Next  ( LOOPER )

For  ( LOOPER = 1 to FIELDS, step 1 )
```

REMOVAL OF DEBRIS

```
  DETIMAGE = LOOPER
  FERETS = 32
  Binary Logical  ( copy Binary0 to Binary1  )
  MINAREA = 1
  Detect  [PAUSE]  ( blacker than DETLOWER, from DETIMAGE into DETBINARY  )
  Binary Amend  ( Erode from Binary0 to Binary1, cycles 13, operator Octagon )
  Binary Amend  ( Dilate from Binary1 to Binary2, cycles 17, operator Octagon )
  Binary Logical  ( C = A AND B :  C Binary3, A Binary0, B Binary2 )
  Binary Logical  ( C = A XOR B :  C Binary4, A Binary0, B Binary3 )
  Binary Amend  ( Dilate from Binary3 to Binary5, cycles 1, operator Octagon )
  Binary Identify  ( Outline from Binary5 to Binary5 )
  Binary Logical  ( copy Binary5 to Binary1  )
  Feature Accept  :
    Area from 0. to 3912716.
  Feature Accept  :
    Roundness from 1. to 5.
  Measure feature  ( plane Binary4, 32 ferets, minimum area: 50, grey image: Image0 )
    Selected parameters: Area, Roundness
  Copy Accepted Features  ( from Binary4 into Binary5 )
  Binary Logical  ( copy Binary5 to Binary4  )
  Binary Logical  ( C = A OR B :  C Binary5, A Binary3, B Binary4 )
  Binary Logical  ( C = A AND B :  C Binary4, A Binary1, B Binary5 )
  Binary Logical  ( C = A XOR B :  C Binary5, A Binary5, B Binary4 )
  Binary Logical  ( copy Binary5 to Binary0  )
  Clear Accepts
  MINAREA = 50
```

```
MFEATINPUT = 0
FACCOUTPUT = 2

Measure feature(plane MFEATINPUT, FERETS ferets, minimum area: MINAREA, grey image: FTRGREY.IMAGE
feature counts into FTRCOUNT(2), results into FTRRESULTS(count,1) )
Selected parameters: Area
Copy Accepted Features  ( from MFEATINPUT into FACCOUTPUT )
Binary Logical  ( copy Binary2 to Binary1  )
Binary Edit  [PAUSE]  ( Delete from Binary1 to Binary1, nib Fill, width 1 )
Binary Logical  ( copy Binary1 to Binary0  )
```

SEPARATION OF PELLETS AND CLUMPS

```
Binary Amend  ( Erode from Binary1 to Binary2, cycles 7, operator Octagon )
Binary Amend  ( Dilate from Binary2 to Binary1, cycles 7, operator Octagon )
Binary Logical  ( copy Binary1 to Binary2  )
Binary Segment  ( Build from Binary0 to Binary1, operator Octagon )
Binary Logical  ( C = A XOR B :  C Binary3, A Binary0, B Binary1 )
```

MEASUREMENT OF PELLETS

```
MFEATINPUT = 1
Measure feature(plane MFEATINPUT, FERETS ferets, minimum area: MINAREA, grey image: FTRGREY.IMAGE
feature counts into FTRCOUNT(2), results into FTRRESULTS(count,9) )
Selected parameters: Area, Feret0, Feret90, Perimeter, Feret45, Feret67.5, Feret135, Feret157.5, ConvexArea
For  ( N = 1 to FTRCOUNT(2), step 1 )
  W = FTRRESULTS(N,2) + FTRRESULTS(N,3) + FTRRESULTS(N,5) + FTRRESULTS(N,6)
  Y = FTRRESULTS(N,7) + FTRRESULTS(N,8)
  X(N) = W+Y
  X(N) = X(N)/6
Next  ( N )
COUNT1 = COUNTER
For  ( N = 1 to FTRCOUNT(2), step 1 )
  FTRRES(COUNTER,1) = FTRRESULTS(N,1)
  FTRRES(COUNTER,2) = FTRRESULTS(N,4)
  FTRRES(COUNTER,3) = FTRRESULTS(N,9)
  FTRRES(COUNTER,4) = X(N)
  COUNTER = COUNTER + 1
Next  ( N )
MFEATINPUT = 2
MINAREA = 0
Measure feature(plane MFEATINPUT, FERETS ferets, minimum area: MINAREA, grey image: FTRGREY.IMAGE
feature counts into FTRCOUNT(2), results into FTRRESULTS(count,2) )
Selected parameters: Area, ConvexArea
For  ( N = 1 to FTRCOUNT(2), step 1 )
  FTRRES(COUNT1,5) = FTRRESULTS(N,1)
  FTRRES(COUNT1,6) = FTRRESULTS(N,2)
  COUNT1 = COUNT1 + 1
Next  ( N )
```

MEASUREMENT OF CLUMPS

```
MFEATINPUT = 3
Measure feature(plane MFEATINPUT, FERETS ferets, minimum area: MINAREA, grey image: FTRGREY.IMAGE
feature counts into FTRCOUNT(2), results into FTRRESULTS(count,9) )
Selected parameters: Area, Feret0, Feret90, Perimeter, Feret45, Feret67.5, Feret135, Feret157.5, ConvexArea
For  ( N = 1 to FTRCOUNT(2), step 1 )
  W = FTRRESULTS(N,2) + FTRRESULTS(N,3) + FTRRESULTS(N,5) + FTRRESULTS(N,6)
  Y = FTRRESULTS(N,7) + FTRRESULTS(N,8)
  X(N) = W+Y
  X(N) = X(N)/6
```

```
  Next  ( N )
  For  ( N = 1 to FTRCOUNT(2), step 1 )
   FTRRES(COUNTER,1) = FTRRESULTS(N,1)
   FTRRES(COUNTER,2) = FTRRESULTS(N,4)
   FTRRES(COUNTER,3) = FTRRESULTS(N,9)
   FTRRES(COUNTER,4) = X(N)
   FTRRES(COUNTER,5) = FTRRESULTS(0.00)
   FTRRES(COUNTER,6) = FTRRESULTS(0.00)
   COUNTER = COUNTER + 1
  Next  ( N )
 Next  ( LOOPER )

DATA OUTPUT
 For  ( N = 1 to COUNTER-1, step 1 )
  For  ( M = 1 to 6, step 1 )
   Display  ( FTRRES(N,M), field width: 8, right justified, pad with spaces, 2 digits after '.', tab follows )
  Next  ( M )
  Display Line
 Next  ( N )
```