UNIVERSITY OF
FORWARD
THINKING
WESTMINSTER⌗

Investigating Physical and Chemical Interaction of Aspergillus terreus Spores for Changes in Morphology and Physiology

Merchant, M.

Investigating Physical and Chemical Interaction of *Aspergillus terreus* Spores for Changes in Morphology and Physiology

Mahek A Merchant

A thesis submitted in partial fulfilment of the requirements of the University of Westminster for the degree of Doctor of Philosophy

June 2020

Abstract

It has been widely reported that in filamentous fungi, spore inoculum size affects culture morphology and secondary metabolite production. The reasons for this, however, have not been investigated thus far.

There are two possible explanations as to why spore inoculum size affects fungal morphology, growth and productivity. Firstly, a quorum sensing phenomenon (QS). In this case, after population densities reach a certain threshold, signalling chemicals secreted into the environment effect expression of specific genes leading to a range of physiological responses. Secondly, due to physical spore-to-spore contact, communication may develop that could trigger a physical response, altering morphology and productivity.

To explore these hypotheses, research was split into two parts: the first "supplementation", the addition of known quorum sensing molecules butyrolactone I, tyrosol and farnesol, alongside other chemicals expressed in literature as having potential effects on the physiology and morphology of a microbial culture. Furthermore, supplements from *Aspergillus terreus* MUCL 38669 obtained from I) spore supernatant, II) concentrated spore supernatant via freeze drying (FDSS) and III) high spore culture supernatants from 72-hour liquid cultures, were added to various concentrations of *A. terreus* spore inocula. The second part investigated effects of physical contact between spores by increasing spore interactions through sonication of spore suspensions and also, altering the physical space in which spores are confined to during growth, thereby forcing spore to spore contact.

Investigations showed behaviours similar to cultures affected by QS process. These included morphological changes and increased productivities. Additions of 0.01M farnesol to (low) spore inoculum concentrations of 1×10^3 spores/mL altered culture morphology to that seen of cultures of 1×10^7 spores/mL (high), lovastatin production also increased by 1208%. Similarly, the morphology of low spore cultures supplemented with FDSS were that of high spore cultures and lovastatin production increased by 67%. Sonication had no effect on cultures, however, confinement evoked changes in morphological characteristics and germination times of the culture.

Acknowledgements

Thank you to my family, friends and colleagues who have taken this journey with me. Few have been there from the beginning, but many have joined along the way. Whether you were here from day 1, or day 1460, I love you and thank you for everything.

To my supervisor, Professor Tajalli Keshavarz, without whom this project would not have been possible, thank you for your patience, dedication and wisdom throughout these past years. You have not only made me a better researcher but a better person too. To Dr Godfrey Kyazze, thank you for helping me to obtain my first degree and supporting me throughout this PhD. I also thank the lectures who have helped me, in particular Dr Claire Robertson, and the technical staff, for their assistance in the lab.

To the most important people in my life, thank you for absolutely everything and always being there for me. To my Mum, for all the positivity and strength you have given me and to my Dad, for all our "homework" sessions. Afshan, my sister, thank you for all the motivational messages and a shoulder to cry on. Ayaz, my brother, thank you for always keeping me company, even when you weren't here, you were always with me and always managed to make me laugh. I couldn't have done it without you all.

To my friend and colleague, Zain, you started as my student, and became my friend. When I was sad and alone, I was given an angel to keep me company, thank you.

Finally, to Irfaan, you joined the party late but made it amazing, I thank you and your family for all you have done, the support, the prayers, the patience, the kindness and most importantly, the love.

"We find knowledge best by admitting first what it is we do not know, and by opening our minds to what others can teach us."

The Aga Khan IV

Commencement ceremony of the American University, Cairo,

25th June 2006

Authors Declaration

I declare that all the material contained in this thesis is my own work and has been carried out in accordance with the Guidelines and Regulations of the University of Westminster. The work is original except where indicated by special reference in the text.

The submission as a whole or part is not substantially the same as any that I previously or am currently making, whether in published or unpublished form, for a degree, diploma or similar qualification at any university or similar institution.

Until the outcome of the current application to the University of Westminster is known, the work will not be submitted for any such qualification at another university or similar institution.

Any view expressed in this work are those of the author and in no way represent those of the University of Westminster.

Signed: Date:

Table of Contents

Table of Figures

Table of Tables

Table of Abbreviations

AA	Ascorbic acid
AHL	N-acyl homoserine lactone
CDW	Total cell dry weight per litre (g/L)
DMSO	Dimethyl sulfoxide
DOT	Dissolved oxygen tension
EPS	Extracellular polymeric substances
FDSS	Freeze dried spore supernatants
GPY-L medium	Lovastatin production medium
GC-MS	Gas chromatography-mass spectrometry
HPLC	High performance liquid chromatography
HSCS	High spore culture supernatants
L	Litre
M	Molar
MIC	Mean inhibitory concentration
mL	Millilitre
nM	Nano molar
NMR	Nuclear magnetic resonance
NS	No supplementation
pCO_2	Partial pressure of CO_2
PDA	Potato dextrose agar
PDB	Potato dextrose broth
pL	Picolitre
ppm	Parts per million

q_{pen}	Specific penicillin production rate
QQ	Quorum quencher
QS	Quorum sensing
QSM	Quorum sensing molecule
SS	Spore Supernatants
v/v	Volume per volume
VVM	Volume per volume per minute
YME	Yeast malt extract agar
µL	Microlitre
µM	Micrometre

1. INTRODUCTION

Many industrial processes rely on bacteria and fungi for the production of a variety of compounds that have become a necessity in our daily lives. One of the important classes of microbial products is antibiotics. Even though they have serious drawbacks, due to the widespread emergence of antibiotic resistance, their manufacture is still necessary. Some other examples of industrial products that rely on microorganisms for their production include enzymes, organic acids, statins, and steroids.

The biotechnology industry is under continuous evolution. Where production is concerned, it always looks at possible ways for enhanced production. New and novel products are highly sought after, and small-scale research is aimed for subsequent scale-up. However, one problem that arises, more often than not, is the maintenance of the yields and productivity at large-scale.

Notwithstanding the importance to achieve high productivity at large-scale, more research is required, specifically where fungal metabolites are to be scaled-up, based on successful small-scale fermentation.

1.1 Filamentous Fungi

Filamentous fungi play an import role in the industrial biotechnology sector. The versatility and their ability to produce and secrete copious amounts of enzymes, polysaccharides, organic acids, plant growth regulators, pigments, mycotoxins, alkaloids, and most commonly known, antibiotics. This

abundance, and resourcefulness that is associated with filamentous fungi cultures, makes them an important and key part of the biotechnology industry. For many years fungi have been manipulated and cultured in a variety of ways in order to improve yields and productivities.

Ways in which fermentations can be improved to better production on different scales has been immensely investigated. One such investigation has been into the effects of culture morphologies on production. It has been reported that the morphology of a culture greatly impacts the growth, the metabolism, and the life of the culture (El- Enshasy, 2007).

1.1.1 Effects of Morphology on Production

Morphological differences in the cultivation of filamentous fungi arises when the cultures are grown in submerged liquid. Culture morphologies can vary from free mycelia to dense compact pellets, it is the morphology of the culture that is linked to the growth kinetics and physiology of the species (Papagianni and Moo-Young, 2002).

The fungal cell wall acts as a barrier, it is a stable structure that determines the shape of the cell, and it protects the cells from osmotic stress. The cell wall is composed of homo and hetero – polysaccharides, proteins, lipids, melanin, protein – polysaccharide complexes and polysaccharide chains of chitin (Burnett and Trinci, 1979).

In filamentous fungi, secretion of proteins and products that are extracellular takes place at the hyphal tip, where no organelles are present. Vesicles transport materials to the surface of the plasma membrane at the hyphal tip (Wosten *et al.,* 1991).

The macro-morphological characteristics of submerged cultures of filamentous fungi depend upon the way in which the cultures are grown, this includes media composition, environmental conditions and inoculum type. The morphology of the growing culture has a large effect on the growth kinetics and the physiology of the culture (Van Suijdam and Metz, 1981 and Braun and Vecht-Lifshitz, 1991).

When growing as dispersed free mycelia in bioreactors, there is a consistent distribution of substrate and products throughout the biomass. It is the filamentous growth that causes entanglement of the hyphae which in turn increases the culture viscosity. This then results in regions with high shear, specifically at the impellers, and areas of low shear, near the vessel walls. There is no longer uniformity in the reactor, which can be counteracted by increasing agitation. This inevitably results in raised power consumption; and the increased shear, damages the cells (Van Suijdam *et al.,* 1980).

Another morphological form that filamentous fungi take on is pelleted morphology. This morphological characteristic decreases the viscosity of the broth, and therefore the culture rheology is Newtonian in nature. In terms of a bioreactor this means that there is adequate mass and heat transfer. Pelleted

morphology is also more advantageous with regards to downstream processing, as biomass removal is easier, efficient, and economically more viable. Pelleted morphology, however, as good as it is for the properties of the culture, is not the most ideal with regards to the survival/ life of the culture itself (Van Suijdam *et al.,* 1980).

Pelleted morphology is flawed due to zonation. Sufficient oxygen enables the pellet to grow in both density and diameter size, but as these increases over time, it becomes harder for oxygen to penetrate through the surface of the pellet and the centre of the pellet is oxygen deprived and starved of nutrients. On a metabolic level this can lead to alterations in metabolism and enzyme secretion kinetics (Hermersdorfer *et al.,* 1987) this therefore leads to autolysis and ultimately cell death at the core of the pellet (Pazouki and Panda., 2000).

Free dispersed mycelium   Clump   Pellet
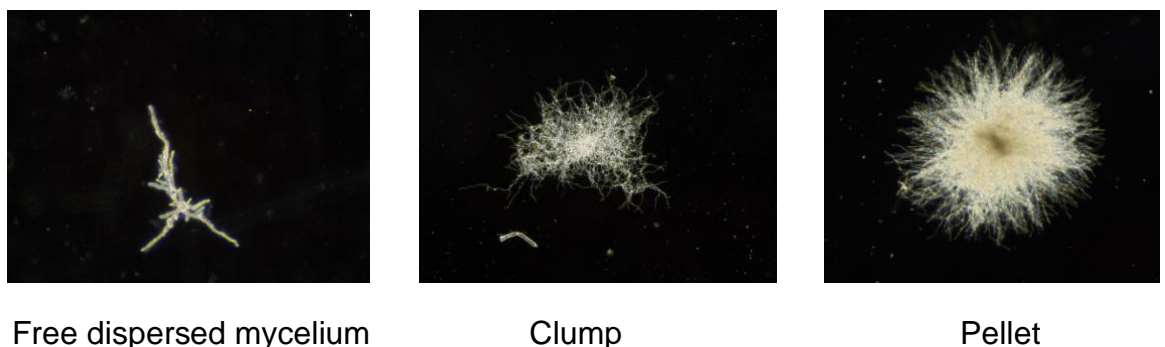
Figure 1.1 *Free dispersed mycelium, clump and pellet morphology of* A. terreus *culture*

There are differences in the physical characteristics of pellets that are formed. These differences arise due to the method by which pellet formation occurs at the time of spore germination. Pellets are formed via three different spore

interaction mechanisms. The first is coagulation, which is the coagulation of spores, during which time, the germination gives rise to a net of intertwined hyphae. The second is non-coagulation, where one spore gives rise to one pellet. The number of pellets is directly proportional to the number of spores used in the inoculum. The final mechanism is hyphal–element agglomeration. Where hyphal elements agglomerate to form a clump of hyphal elements that eventually evolve into pellets (Paul and Thomas., 1996 and Agger *et al.,* 1998).

There are also various conditions which affect the morphology of a culture in pellet formation. These are categorised into three major classifications, I) strain dependent factors, such as type of inoculum, inoculum concentration, type of strain and so on, II) nutrition dependant factors, including carbon and nitrogen sources, polymer additions, surfactants, alcohols, and finally, III) cultivation conditions such as temperature, pH, carbon dioxide, bioreactor type (El- Enshasy, 2007).

1.1.2 The Effects of Culture Parameters on Morphology

1.1.2.1 Carbon Source

In cultures of *Aspergillus niger,* Ryoo (1999) found that when sucrose concentrations in the media were increased, the diameter of pellets formed decreased. However, work by Sinah *et al., (*2001), found that for cultures of *Paecilomyces japonicas,* an increase in sucrose concentrations from 20 g/L to

60 g/L also increased pellet diameter, but concentrations of 80 g/L did not produce pelleted morphology. Cultures of *Paecilomyces sinclairii* grown with sucrose as a carbon source did not form pelleted morphology but caused long and highly branched hyphae (Cho *et al.,* 2002). In cultures of *A. niger,* glucose and xylose produced small pellets with lots of hyphae, pellets were on average 400 µm in diameter (El-Enshay *et al.,* 2001).

A later study by El Enshasy (2000) investigated the effects of using a non-glucose carbon source on morphology and glucose oxidase activity in *A. niger.* Exocellular glucose oxidase activities were detected during growth on glucose, fructose, xylose, and mannose. Glucose as carbon substrate, morphology was filamentous with small pellets, fructose as carbon source, growth was pelleted and with xylose, cultures were smaller pellets than that seen in fructose, but with more hyphae. Which was the ideal morphology as large pellets results in lower exocellular glucose oxidase activities.

1.1.2.2 Nitrogen

Overall, the type and concentration of nitrogen present in the culture has great effect on the morphological outcome. The use of corn steep liquor by Du *et al.,* (2003) in cultures of *Rhizopus chinensis* produced fluffy pellets with a compact centre, and loose outer zone. It was this morphology that they found there to be the highest antibiotic production, and highest number of pellets formed. The high nitrogen content accelerated spore germination and cell growth.

Supplementation with peptone caused dispersed mycelia morphology and yeast extract produced entangled filamentous form, whereas the use of ammonium sulphate also produced pellets that were compact but with a smooth surface.

1.1.2.3 Carbon/Nitrogen Ratio

The ratio of carbon to nitrogen affects the overall culture morphology. Byrne and Ward (1989) found that for cultures of *Rhizopus arrhizus,* grown in a peptone glucose medium, the high carbon: nitrogen ratio caused pelleted morphology, while growth in low C: N ratio media, caused mycelial growth.
Casas Lopez *et al.,* (2003) investigated the effects of C: N mass ratio on *A. terreus* biomass and lovastatin production. The composition of culture media, particularly carbon and nitrogen, affects the yield and production rate of lovastatin. Both of these components are directly linked to biomass and metabolite formation, as lovastatin biosynthesis is dependent upon the source and the ratio of carbon to nitrogen present. In this study lactose was the sole carbon source, and corn steep liquor (CSL) the nitrogen source. Three different C: N ratios were used, 14.1, 23.4 and 41.3, lactose was added at intervals during the fermentation and nitrogen was made the limiting nutrient, as this is known to increase the rate of lovastatin production. The final biomass obtained at the end of the fermentation was the same in all cases, 7 +/- 0.2 g/L. The highest lovastatin productivity was C: N ratio of 41.3: 1 and specific generation rate of 0.242 mg/ (g biomass)/ hour. The carbon

requirement for lovastatin production is high as the compound is composed solely of carbon, oxygen and hydrogen atoms. After biomass formation, surplus carbon is solely for secondary metabolite production, and is needed in excess with nitrogen in limitation, for lovastatin production to take place.

The ratio of carbon to nitrogen affects both morphology and metabolic pathways, Park *et al.,* (2001) found that for cultures of *Mortierella alpina,* different ratios of carbon to nitrogen affected morphology and arachidonic acid production. The fungus has three stages with regard to arachidonic acid production. The first phase is growth, this takes place at C: N ratio below 7, as carbon is the limiting nutrient. Once carbon concentrations increase and C: N ratio is between 7 and 15, cell growth is ceased, and biosynthesis begins. In the third phase mycelial growth is completed, and as C: N ratio increases, mycelial concentrations decrease, arachidonic acid synthesis is at its highest. Therefore, depending upon the stage at which the culture is in with regards to life cycles, the C: N ratio important.

1.1.2.4 Phosphates and Complex Organic Materials

In cultures of *Aspergillus awamori,* Gerlach *et al.,* (1998) found that the presence of low phosphate concentrations (0.3g/L) reduced the overall number of pellets formed initially; these pellets soon converted to clumped morphology. However, when the concentration of phosphate was increased to 1.05 g/L and 2.1 g/L, pelleted morphology was formed. The presence of

phosphate in the culture medium also increased the rate of substrate uptake, and the rigidity of the pellets that were formed. Ryoo (1999) reported that phosphates were an important structural constituent of the cell walls in *Aspergillus niger,* and found that phosphate in cultures of *A. niger* increased the pellet diameter, hydrophobicity and fractionation of mycelia.

Domingues *et al., (*2000) found that cultures of *Trichoderma reesei* grown in a medium composed of yeast and peptone, were unable to form pelleted cultures as seen in control media of lactose and trace elements, but instead growth was as free mycelium. They concluded that the complex medium caused higher growth rates of the fungus, which caused a rapid decrease in oxygen in the culture and therefore prevented pelleted growth.

El-Enshasy *et al.,* (1999) found that cultures of *A. niger* grown in minimal medium supplemented with yeast extract increased biomass concentrations with increased yeast extract concentration. Yeast extract concentrations between 1 to 2 g/L were the optimal for increased exocellular glucose oxidase activity. The addition of yeast extract changed morphology from filamentous to pelleted growth and the increase in nitrogen concentration caused fungal pellets to increase in size but to decrease in density and number per unit volume.

1.1.2.5 Polymers

To spore preparations of *Phanerochaete chrysosporium,* Wainwright *et al.,* (1993) added 0.2% (w/v) polyacrylic acid and 0.2% (w/v) sodium polyacrylate, to stop the effects that spore aggregation has on cultures. The addition of these polymers resulted in smaller pellets with dispersed mycelia. Similarly, Jones *et al.,* (1988) also prevented spore aggregation by addition of the polymers polyacrylic acid and sodium polyacrylate to cultures of the basidiomycetes, *Phanerochaete chrysosporium* and *Coprinus cinereus.*

Addition of other polymers have been investigated to stop spore aggregation and maintain filamentous growth. Elmayergi and Scharer (1973) added carboypolymethylene polymer to cultures of *A. niger* and found that cell growth and activity were enhanced due to changes in morphology. The cultures were dispersed in growth with higher metabolic rates, due to increase in surface area for nutrient transfer. Byrne and Ward (1987) found similar results with additions to cultures of *Rhizopus arrhizus*. Rugsaseel *et al.,* (1995) studied the effects of the viscous liquids on the morphology and citric acid production in cultures of *A. niger.* Addition of low concentrations of gelatin, carrageenan, PEG 6000, carboxymethylcellulose and agar were studied. These additions increased viscosity of the cultures and enhanced citric acid production. The morphology became spherical aggregates of thick mycelia with dense, highly branched and an entangled network of hyphae.

1.1.2.6 pH

The pH in which the culture is grown may affect its overall morphology. For cultures of *Aspergillus nidulans* a pH between 3 and 5 resulted in filamentous growth with fluffy mycelia, above pH5, growth was pelleted (Braun and Vecht-Lifshitz. 1991). Different strains of the same species may vary in morphology based on pH of the cultures. Jin *et al.,* (1999) found that *Aspergillus oryzae* DAR 1679 (strain A) formed dispersed mycelia at pH 3-4 however, strain DAR 3699 (strain B) formed compact pellets, and *A. oryzae* DAR 3863 (Strain C) at pH 3-4 formed free mycelia. An increase in pH between 4.5 and 6 produced mycelial clumps in strain A, and B, but compact pellets in strain C. However, Van Suijdam and Van Metz (1981) found that for cultures of *Penicillium chrysogenum,* pH had no effect on the morphology of the cultures for both batch and continuous fermentation.

1.1.2.7 Temperature

Anderson and Smith (1972) found that temperature affected both germination and morphology in *A. niger* cultures. The lowest rate of spore swelling was at 30 °C, and the maximum at 38 °C. Between these temperatures 97 - 99% of spores formed germ tubes. At higher temperatures between 38 and 43 °C, however, germination decreased and was inhibited at 44 °C. Hermersdorfer *et al., (*1987), found that cultures of *A. niger* at 25 °C produced small, short, branched pellets, while at 30 °C formed large and hairy pellets, and at 35 °C

growth transformed from pelleted to filamentous. Overall, high temperatures increased spore swelling and germination. Carlsen *et al.,* (1995) established that the specific growth rate of *A. oryzae* pellets could be increased with an increase in temperature from 25 to 35 °C. Miles and Trinci (1983) found that increasing temperatures from 15 to 30 °C, increased the hyphae length and wall thickness in cultures of *P. chrysogenum.* On the other hand, some studies have used decreases in temperature to alter morphologies. For example, Braun and Vecht-Lifshitz (1991) and Schugerl *et al.,* (1998) suggested that in order to evoke formation of mycelial pellets in *A. awamori* fermentations, temperatures should be reduced, for xylanase production.

1.1.2.8 Aeration – Effects of Oxygen and Carbon Dioxide

For aerobic fungi, oxygen plays an important role in both cell growth, and metabolic functions. Cho *et al., (*2002) found that in cultures of *P. sinclairii* aeration was increased from 0.5 vvm to 1.5 vvm, hyphae branching increased too. However, in cultures of recombinant *A. niger,* Wongwicharn *et al.,* (1999) found that limitation of dissolved oxygen tension (DOT) between 0 % and 10% caused long, sparsely branched hyphae, but oxygen rich media of 30% DOT formed shorter hyphal elements with more branching, and 50% formed pelleted morphology. In cultures of *A. awamori,* %DOT levels close to pure oxygen saturation, formed dense pellets, with no free mycelia. However low %DOT resulted in formation of weak and fluffy pellets (Cui *et al.,* 1998).

P. chrysogenum, is DOT sensitive, air saturation levels below 30% decreased specific penicillin production rates (q_{pen}), and at levels below 10% DOT penicillin was not formed, and oxygen uptake was significantly affected (Vardar and Lilly, 1982). Higashiyama *et al.,* (1999) found that for cultures of *M. alpine* arachidonic acid yields were increased 1.6 fold when dissolved oxygen concentrations were maintained above 7 ppm.

Carbon dioxide has also been described as essential in fungi growth (Papagianni, 2004). Bushell and Bull (1974) found that in cultures of *A. nidulans* CO_2 was necessary for fixation by pyruvate carboxylase and phosphoenol pyruvate carboxylase. Activity of enzymes and growth rate were found to be dependent upon carbon dioxide fixation. Edwards and Ho (1983) found that in cultures of *P. chrysogenum,* carbon dioxide was essential for chitin synthesis. However, increasing CO_2 levels can reduce the synthesis of chitin, and morphology of the culture becomes yeast like during growth.

McIntyre and McNeil (1997 and 1998) found that increasing partial pressure of CO_2 (pCO_2) above 7.5% in cultures of *A. niger* increased the hypha growth unit, average hypha length, branch length and produced large pellets. However, biomass, citrate concentrations and substrate consumptions decreased.

Casas Lopez *et al.,* (2004) studied the effects of oxygen availability on *A. terreus*. They found that oxygen greatly influenced the concentration of lovastatin produced. An increase of oxygen content from 20% to 80%

increased lovastatin production 4-fold. A later study by Casas Lopez *et al.,* (2005) investigated effects of increased agitation speeds from 300 to 800 rpm on lovastatin production, and found that at 300 rpm diameter of pellets increased to almost double of that of the control, but higher rpms of 600 and 800 rpm damaged fungal pellets and gave low lovastatin productivities due to pellet morphology.

1.1.2.9 Inoculum Concentration

The size of culture inoculum plays a significant role in the development of the culture with regards to both morphology and subsequent productivity. Znidarsic *et al.,* (1998), found that high spore inoculum concentrations of *Rhizopus nigricans* formed mycelium, however, all other concentrations resulted in coagulative pellet formation. Du *et al.,* (2003) found that for cultures of *R. chinesis*, high spore concentrations of 10_9 spores/mL produced hypha morphology during initial growth stages, which entangled and subsequently inhibited the formation of pellets. Large pellets were formed from low spore concentrations of 1 x 10_5 spores/mL, but with an overall lower biomass production. In cultures of *Phanerochaete chrysosporium* an increase in spore concentration from 1 x 10_3 to 1 x 10_6 spores/mL produced large pellets, lower concentrations produced larger pellets (Jimenez-Tobon *et al.,* 1997).

1.2 Spores

Spores can be considered as the legacy of a culture. It is the only part of a culture that ensures its survival, a mechanism that has been perfected over generations. Their production and dispersal, too, is a complex process that varies from each culture and species.

1.2.1 Bacterial Spores

Studies into bacterial spores was first carried out in 1876 by Cohn, who was interested in the science of bacteriology. Later that year further research was published by Koch, who had more practical interests in bacteria and diseases that were common at that time, such as anthrax, and wanted reasons as to why these bacteria were able to withstand such extreme conditions such as high temperatures of 100 °C. *Bacillus subtilis* spores have been the most researched and highly published organism with regards to spore formation and germination.

When bacterial cultures are in an environment that is no longer suitable for growth, and nutrients are in limitation (de Hoon *et al.,* 2010), sporulation mechanisms are triggered. Spore formation begins with cell division, creating a mother cell and a smaller forespore, each divide has its own genome, the mother cell then engulfs the smaller forespore and maturation takes place (Chesnokova *et al.,* 2009; Setlow, 2013 and McKenney *et al.,* 2013). Cell lysis

of the mother takes place and spores are released into the environment. These bacterial endospores are resistant to heat, radiation, pH, toxic chemicals and most environmental stress factors (Setlow, 2006) due to their composition. Spores are also described as being dormant, with little or no metabolic activity other than constantly sensing the environment in which they are in (Segev *et al.,* 2012 and Setlow, 2013) and can stay in this state for years until stimulated (Setlow, 2014).

The components of a spore are different to that of a growing cell (Setlow, 2006), as they are made up of various layers. The outermost layer, known as the exosporium and the layer within this, the spore coat, are the key factors in spore resistance, and comprise of proteins and enzymes essential for spore germination such as alanine racemase and purine nucleoside hydrolase (Henriques and Moran 2007). The space between these two layers is the interspace. After the spore coat is the outer membrane, (figure 1.2) which is the permeability barrier. Under the outer membrane is the cortex which is a peptidoglycan structure preceding the germ cell wall and during germination the cortex becomes hydrolysed through enzymatic processes. The inner spore membrane is located after the germ cell wall and houses the proteins responsible for spore germination. The centre of the spore is the core, in which DNA, ribosomes and spore enzymes are stored (Gerhardt and Marquis, 1989 and Cowan *et al.,* 2003).
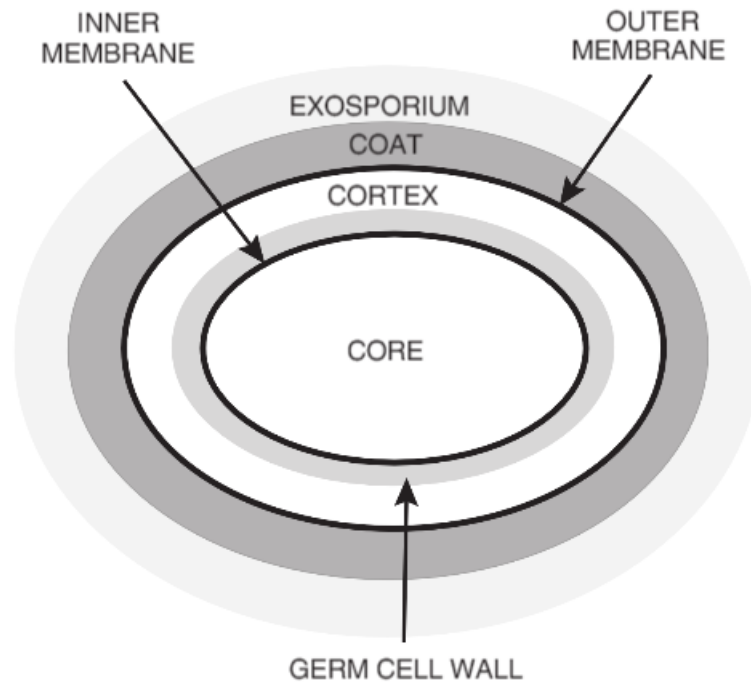
Figure 1. 2 *Structural outline of* Bacillus *spore. Layers are not drawn to scale, and the exosporium is not found in all bacterial species spores (McKenney* et al., *2013)*

Once in a hospitable environment, specific nutrients bind to germinant receptors in the inner membrane, this is the first stage of germination and is known as commitment (Yi and Setlow, 2010). During this stage the permeability of the inner membrane increases and there is a release of monovalent cations such as Na_+, H_+, K_+ and CaDPA (calcium ion (Ca_+) chelated to dipicolinic acid (DPA) (Setlow *et al.,* 2008., and Setlow, 2013). The second phase of germination is degradation of the polyglycan cortex by cortex-lytic enzymes, this process enables the germ cell wall and the core to expand and water uptake of the spore increases to a water content of 80%. The spore

is now active, and metabolism and cell growth begins (Paidhungat *et al.*, 2002 and Segev *et al.,* 2013).

1.2.2 Fungal Spores

Fungi are facultative organisms, if the conditions in which the organism is growing remains ideal, the culture will continue to grow and thrive in that environment. However, in order to ensure survival and to preserve the legacy of a fungal culture, sporulation is initiated. Filamentous fungi produce spores as part of their life cycle, spore formation and dispersal will take place if the environment is no longer suitable for growth, and nutrients are in limited supply resulting in a low growth rate of the culture.

Spores themselves provide two major roles for fungi, either for their survival or to enable them to extend their realm into new areas and habitats. Carlile *et al.,* (1994), described two types of spores. The first being dispersal spores, allowing for the fungus to conquer a new area. These types of spores have to be able to travel or be distributed over a long distance and then germinate readily. The second are survival spores, which may not separate from the original culture, but remain in a state of low metabolic activity (or dormancy as many describe). These spores are liberated from the parent mycelium via cell lysis and germinate once environmental conditions become more desirable for the needs of the fungus, i.e. the supply of nutrients increases.

Dispersal of the spores is a survival mechanism too, whereby the spores are either carried away through air currents or transported on the hides of animals or are launched by the fungal body away from the parent cells. This enables the culture to be more widely spread and therefore inhabiting new areas. The mechanism of dispersal is unique and specialized to each fungal species.

1.2.3 Sporulation and Germination

When the environment in which the culture is growing is no longer suitable for growth, the process of sporulation or spore formation is activated. Sporulation is the production of spores and can be an asexual or sexual process. It is a mechanism of genomic survival. Universally, the structure that bears spores is known as sporophores, which in Greek means spore carriers (Carlile *et al.,* 1994). In filamentous fungi when asexual sporulation takes place, conidiophores are formed on specialised hyphae and gives rise to conidia, which are asexual spores (Deak *et al.,* 2009). Sexual spores, however, are ascomycetes (Carlile *et al.,* 1994). When the mechanisms of sporulation are activated there is elongation of the apical tip of the hypha, after which a specialised thick walled cell is formed, this anchors the hyphal branch that develops into a sack, the conidiophore (Adams *et al.,* 1998). Conidiophore formation is genetically programmed, with particular genes responsible for the progression at each stage (Park *et al.,* 2012).

Spores are specialised cells that have been described or classified as being dormant. This gives the assumption that the spores are in a state of inactivity, whereby they are not metabolically active. This term is, however, ambiguous, as spores are found to be in a state of low metabolic activity, hypometabolism, carrying out basic functions of metabolism, and respiratory activity (El-Enshasy, 2007 and Feofilova *et al.*, 2012). Once in an environment of favourable conditions the spore goes through a transition of phases, from dormancy to active cell state. The first stage is germination, where the spore gradually swells due to absorbance of water from the environment (Hassouni *et al.,* 2007). There is an increase in diameter size and spore weight, the spore is now in an active state. The next stage is the spore establishes a growth polarity followed by emergence of germ tubes, the point at which the spore is classified as having germinated. The growth process continues with elongation and branching of the hyphae (Gougouli and Koutsoumanis, 2013).

1.2.4 Dormancy

There are two types of spore dormancy. The first is described as exogenous, or superficial dormancy. In this state spores can become fully active once the environment in which they are in, becomes more suitable. There is then a shift from low metabolic activity to high metabolic activity, and it is due to the environment within which the spore is placed. This form of dormancy can also be interrupted/ diminished through heat or chemical treatment.

The second type of dormancy is endogenous or constitutive dormancy. This form of dormancy is based on the internal mechanisms of the spore and relates to the enzymatic processes taking place, and specifically relates to the cAMP pathway.

1.2.5 Spore Morphology

Fungal spores are specialized cells, that are capable of withstanding harsh environments, and so have structures that are able to accommodate this. The outer layer of the spore, also called the rodlet layer, comprises of hydrophobic proteins and melanin, which act as a barrier protecting the spore (El-Enshasy, 2007). Once spores are in an ideal environment germination begins, this process usually begins with an uptake of water from the surroundings, resulting in the swelling of the spore. Proteins and enzymes are present within the spore to enable it to build and develop germ tubes, therefore the necessary structural proteins for this will vary over the germination and growth stages, but initially these proteins would include chitin synthases I and IV. Seong *et al.,* (2008) found that fresh spores produce acetyl-CoA for energy via the TCA cycle and glyoxylate cycle through degradation of fatty acids by β-oxidation. In these spore cultures there are also spore coat proteins SP96 and mannosyltransferase enzymes present, which are specific for wall production.

There are an abundance of glucosyl-phosphatidylinositol (GPI) anchored proteins, of which are glycine rich cell wall proteins, putative cell adhesion

proteins and calcineurin phosphoesterase proteins. All of which function as architectural proteins and cell wall biogenesis proteins. Fresh spore cultures contain pyruvate carboxylase, fructose 1,6-bisphosphatase and phosphoenolpyruvate carboxykinase, these enzymes are part of gluconeogenesis (Seong *et al.,* 2009).

1.2.6 Inoculum Size Effect

It is well established that the productivity of some commercially important fungal products are dependent upon the morphology of the culture (Bizukojc and Ledakowicz, 2010). One of the main factors that determine fungal morphology is the initial number of cells within the culture (El-Enshasy, 2007). This relationship between morphology and productivity has been of interest, particularly in industry, especially with regards to maximising productions (Papagianni and Mattey, 2006). Particular morphologies are more desirable for different fermentation products, and some morphologies have disadvantages during fermentation. For example, dispersed mycelial growth increases wall growth and reduces oxygen transfer-rate while culture viscosity increases too. Pellet morphologies allow for easier downstream processing, and culture broths remain well mixed with lower viscosities (Jimenez – Tobon *et al.,* 1997), however, as mentioned earlier, oxygen transfer becomes a problem as pellets grow in size.

Inoculum size has an overall effect on morphology, which in turn will affect products formed. *A. terreus* is one such example where pelleted growth yields higher concentrations of lovastatin than filamentous growth in batch fermentation (Kumar *et al.,* 2000).

The dimorphic fungus, *Candida albican*s, is an example of where cell culture numbers, can influence the morphology of a culture. If cell densities are greater than 10_6 cells/mL the cells will maintain yeast morphology, but at cell densities below this, the culture will switch to filamentous growth, with the emergence of germ tubes, and the formation of hyphae. The mechanism behind this cell density dependent morphology change is suggested to be due to quorum sensing, a communication mechanism in fungi (Hornby *et al.,* 2001; Kruppa 2009).

A similar phenomenon is seen when different spore inoculum sizes are used. A well-known example of this is in the fermentation of *A. niger* cultures for citric acid and pectic enzyme productions. Inoculum concentrations below 10_8 spores/mL generates fungal growth in the form of pellets, which is the preferred morphology for citric acid production. Spore numbers above this result in filamentous growth, which is preferred for the production of pectic enzymes (Van Suijdam *et al.,* 1980; Steel *et al.,* 1954; Papagianni 2004). Therefore, by adjusting the spore inoculum size, in a variety of species, morphology can be controlled for desirable productivity (Metz and Kossen, 1977; Gbewonyo *et al.,* 1992; Tucker and Thomas, 1992; Tucker and Thomas,

1994; Casas Lopez *et al.,* 2005; Rodriguez Porcel *et al.,* 2006; Papagianni and Matty, 2006; Bizukojc and Ledakowicz, 2010).

Ghojavand *et al.,* (2011) found that different inoculum concentrations of *Saccharopolyspora erythraea* in submerged culture produced different culture morphologies. Inoculum concentrations of $1 \times 10_3$ and $1 \times 10_4$ spores/mL produced pelleted growth, with culture broths being Newtonian in rheology. Clump morphology, however, was seen with inoculum concentrations between $1 \times 10_5$ and $1 \times 10_7$ spores/mL, and produced the highest concentration of erythromycin. Therefore, clumped morphology was preferred for erythromycin production.

Papagianni and Moo-Young (2002) found that in cultures of *A. niger,* both inoculum sizes of $1 \times 10_5$ and $1 \times 10_8$ spores/mL formed pellets after 10 hours of inoculation. The higher inoculum concentration produced a larger number of small pellets, and much larger pellets were seen in cultures of $1 \times 10_5$ spores/mL. Small pellets were more favourable for polygalacturonidase synthesis.

1.3 Quorum Sensing

1.3.1 History and Definition

Communication within species is an essential part of life. It has enabled organisms to adapt and survive under conditions which they live. In order to

deal with potential threats, and sustain future generations, communication within a culture is a vital and key activity (Barriuso, 2015). The mechanism through which microbes communicate and coordinate behaviours in both bacteria, and fungi, is termed quorum sensing (QS).

As an organism grows, it produces and secretes chemical compounds, known as messenger molecules. These messenger molecules are secreted from the cells and accumulate in the environment as population densities increase. Once a certain threshold concentration is reached, a response is evoked from the organism in response to the stimuli. The mechanism of release and uptake differs amongst bacteria (Gram positive and negative) and fungi.

1.3.2 Quorum Sensing in Bacteria

Studies into bacterial quorum sensing began with density dependent expression of bioluminescence in *Vibrio fischeri,* a symbiotic marine bacterium, and in the closely related *Vibrio harveyi.* Both species secrete and respond to acylated – homoserine lactones (3-oxo-hexanoyl-homoserine lactone) (Nealson *et al.,* 1970), these signalling molecules are called autoinducers, and accumulate in the external environment until a threshold concentration is reached and initiation of signal transduction occurs.

In all Gram negative bacteria, the quorum sensing mechanism is a LuxI to LuxR signal – response circuit (Bassler, 1999). LuxI is an autoinducer

synthase and produces acyl homoserine lactone quorum sensing molecules (QSM). Transcription factors encoded in the LuxR gene bind with the autoinducer molecules and this complex then binds to the luxICDABE operon and gene transcription commences (March and Bentley, 2004). Some examples of the response to acyl homoserine lactones, seen by Gram negative bacteria, is the regulation of virulence genes in *Pseudomonas aeruginosa,* conjugal transfer in *Agrobacterium tumefaciens* and swarming motility in *Erwini caratovora* (Fuqua and Greenberg, 1998).

In Gram positive bacteria, exact quorum sensing mechanisms differ between species, and the LuxI/R mechanism and homoserine lactones are not part of the process. The QS mechanism involves processed peptide signalling molecules that are secreted via a dedicated ATP-binding cassette exported protein (ABC), the peptide signal is recognised by cognate two-component sensor kinase protein that interacts with cytoplasmic response regulator proteins. The signal transduction mechanism is a phosphorelay cascade and is therefore similar to Gram negative QS mechanisms but with differences in regulatory factors.

1.3.3 Quorum Sensing in Fungi

Research into the mechanisms behind quorum sensing in fungi has not been as extensive as that seen for bacteria, however it has been proposed that the way the chemical signals are received is similar to that in bacteria. Fungi have the ability to produce more than one quorum sensing molecule at a time, and

the general mechanism is that the signal molecules are produced by signal-producing proteins and are then detected by signal receptor proteins which are subsequently able to regulate gene expression. For example, the signalling molecules involved in fungal development and virulence is the cyclic adenosine monophosphate (cAMP)/PKA system (Fernandes *et al.*, 2005). Mitogen-activated protein kinase, regulate cell wall construction, integrity and osmoregulation (Dohlman and Slessareva 2006; Yu *et al.* 2008)

The need for a communication system in fungi is based on survival, fungi have the ability to live in a variety of habitats, even inside living hosts, and they therefore need to communicate amongst themselves. The QS mechanism in fungi, has been hypothesized to have evolved from bacteria. As bacteria and fungi have lived side by side, it is suggested that fungi adopted the mechanism through the uptake of genes from bacteria, as a survival technique, to compete against bacteria, and avoid attack from other organisms (Sharma and Jangid 2015).

The first fungal culture to have been investigated for its QS capabilities is that of the dimorphic fungus *C. albicans.* There is an abundance of information with regards to the morphology of the dimorphic fungi as *C. albicans* is affected by inoculum size. Where cell numbers are greater than 10_6 cells/mL budding yeast cultures are formed, whereas when cell density is lower than 10_6 spores/mL, culture morphologies take the form of mycelial growth. This is a QS based mechanism that is controlled by the QSM farnesol. When present in the culture, farnesol is able to inhibit the switch from yeast to hyphal form

by inhibiting the cAMP signalling pathway. However, farnesol is unable to block elongation of pre-existing hyphae (Wongsuk *et al.,* 2016).

It is unclear how autoinducers are received in filamentous fungi, as no mechanism has been put forward yet, however, as *C. albicans* has been widely researched, the following mechanism has been put forward by Sharma and Jangid (2015) with regard to the transport and interaction of the cells to the QSM, and is shown in Figure 1.3.
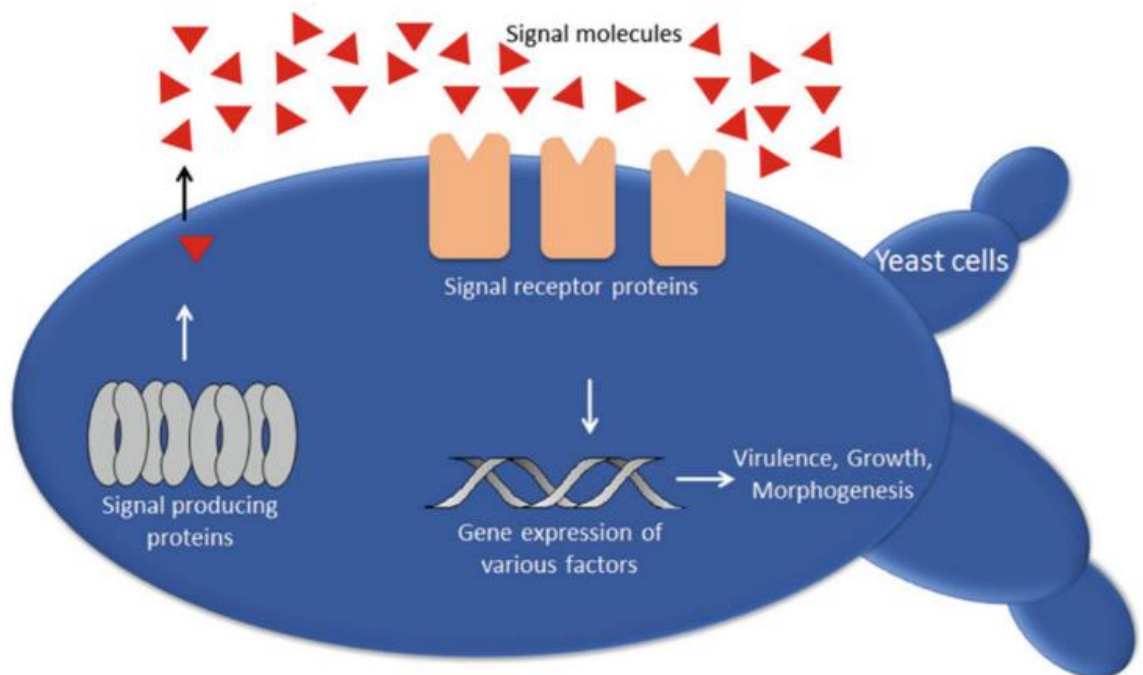
Figure 1.3: Simplified quorum sensing mechanism in fungi. Signal molecules are synthesised by signal – producing proteins, and are detected by signal receptor proteins (Sharma and Jangid 2015)

1.4 Quorum Sensing Molecules (QSM)

Different QSMs are secreted from various microbes, both bacteria and fungi. These messenger molecules evoke different responses within different culture populations. Two well-known examples of QSMs are farnesol and tyrosol. Both are produced by the species *Candida albicans,* but their effects on the microorganism differ greatly, working antagonistically against one another. Tyrosol is a promoter of hyphal development and can shorten the lag time that is created between the yeast to hyphal morphological switch (Chen *et al.,* 2004. Alem *et al.,* 2006. Cottier and Muhlschlegel, 2012). Whereas farnesol is a sesquiterpene alcohol, composed of three isoprene units (Madhani, 2011), it acts as a morphological inhibitor, preventing the morphological switch from budding yeast to hyphae by stopping the emergence of germ tubes and therefore filamentous growth. It is the filamentous form of the fungus that is responsible for virulence, and the development of biofilms (Hazen and Cutler, 1979; Hornby *et al.,* 2001; Sato *et al.,* 2004; Cottier and Muhlschlegel, 2012).

Nikerson *et al.,* (2006) found that farnesol is the more influential of the QSMs, and tyrosol cannot surpass it when the two are present, therefore with regard to QS and the responses that are produced, there are certain molecules which take precedence over others. Figure 1.4, taken from a paper by Sharma and Jangid (2015), shows the effect that these two QSMs, farnesol and tyrosol, have on the morphological characteristics of the species, and puts into context the physical changes.
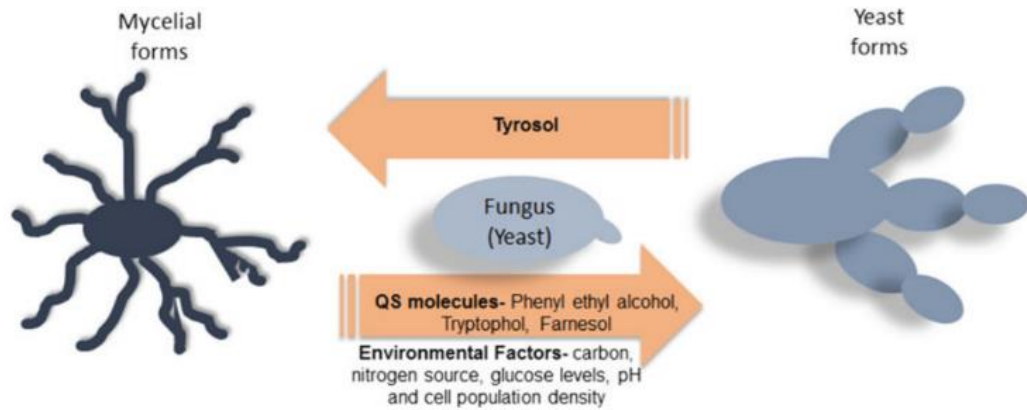
Figure 1.4: *Dimorphic switch in* C. albicans*, regulated by the quorum sensing molecules farnesol and tyrosol (Sharma and Jangid, 2015)*

Many of the quorum sensing molecules that are produced by fungi can act as an interspecies and inter-kingdom QSM; farnesol is one such molecule (Cugini *et al.,* 2007; Atkinson and Williams, 2009; Sharma and Jangid, 2015). Farnesol is widely known for its ability to inhibit biofilm formation not only in *C. albicans* but in other fungal cultures too. Biofilms are formed from the attachment of a community of microbial cells to biotic or abiotic surfaces. These surfaces are then surrounded by extracellular polymeric substances (EPS) which forms the biofilm (Sardi *et al.,* 2014; Ramage *et al.*, 2002).

In *C. albicans* cultures, biofilms are initially formed from the attachment of cells in the yeast form to surfaces, followed by filament formation and subsequent formation of mature biofilms composed of yeast cells, true hyphae, pseudohyphae and EPS. Farnesol is able to inhibit biofilm formation through

repression of the morphological switch (Cottier and Muhlschlegel, 2012; Wongsuk *et al.,* 2016).

Farnesol, not only affects *C. albicans,* but can have differing outcomes on various cultures. In high concentrations farnesol has inhibitory properties on conidia formation in *A. niger* (Lorek *et al.,* 2008; Wongsuk *et al.,* 2016), and in cultures of *Fusarium graminearum* diminished macroconidia development and germination.

Farnesol has been the most widely studied QSM from the species *C. albicans,* and has been investigated with regards to yeasts, dimorphic fungi, moulds and filamentous fungi. Results from farnesol supplementation of cultures has been very different with regards to morphologies and productivities. Table 1.1 shows some examples where farnesol has been added to different fungal cultures, and the subsequent effects of these additions the organism.

Table 1.1: *The effects of the quorum sensing molecule, farnesol, on different fungal cultures.*

Organism	Effect	Reference
A. niger	• Reduced Intracellular levels of cAMP • Conidiophore formation inhibited	Lorek *et al.,* 2008 Dichtl *et al.*, 2010
A. flavus	• Apoptosis induced	Wang *et al., 2014*
A. fumigatus	• Altered growth morphology • Altered state of cell wall	Dichtl *et al.*, 2010
C. posadasii	• Antifungal properties on culture • Reduced ergosterol production • Diminished integrity of plasma membrane	Brilhante *et al.,* 2013
H. capsulatum	• Biofilm formation inhibited • Antifungal properties on culture	Brilhante *et al.,* 2015
P. expansum	• Inhibited growth • Apoptosis induced	Liu *et al.,* 2009
P. brasiliensis	• Growth inhibition • Delayed transition to dimorphic morphology • Antifungal properties on culture	Derengowski *et al.,* 2009
P. destructans	• Inhibited growth	Raudabaugh and Miller, 2015
C. dubliniensis	• Development of hyphae inhibited • Cell morphogenesis • Formation of biofilm inhibited • Antifungal response	Henriques *et al.,* 2007 Martins *et al.,* 2007 Jabra-Rizk, 2006
C. tropicalis	• Antifungal response	Cordeiro *et al.,* 2013
D. hanseii	• Cell adhesion and motility	Gori *et al.,* 2011

Studies were carried out where farnesol from *C. albicans* was added to cultures of the bacterium *P. aeruginosa.* This quorum sensing molecule inhibited swarming mobility, which in turn enhanced biofilm formation in the culture and enhanced bacterial virulence (Gibson *et al.,* 2009).

In the cultures *A. niger* and *Candida dubliniensis*, the cell morphology is altered with regards to hyphal development and conidiophore formation. Hyphal growth has been known to be regulated by signalling pathways, and in some cases by the cAMP pathway. Farnesol acts to inhibit this pathway and therefore inhibit morphological changes. However, the inhibition of this pathway may not always prevent hyphal formation, as cultures of *A. niger,* in the presence of farnesol are still able to produce aerial hyphae but not conidiophores. When QSMs are combined with antifungal agents, the effect on cultures is much more potent than addition of antifungals alone. For example, in *C. albicans* the mean inhibitory concentration (MIC) was greatly decreased (Cordeiro *et al.,* 2013; Taff *et al.*, 2013; Chandra *et al.,* 2001). This is also associated with the decrease in biofilm formation, which is seen across multiple organisms such as *Candida krusei* and *Candida tropicalis* (Cordeiro *et al.,* 2013).

In the presence of tyrosol germ tube formation and biofilm development is promoted in *C. albicans* (Chen *et al.,* 2004; Alem *et al.,* 2006; Albuquerque and Casadevall, 2012). It also decreases the length of lag phase (Majumdar and Mondal, 2015), and inhibits neutrophil activity (Cremer *et al.,* 1999).

Studies carried out with the addition of 3-oxo-C_{12}-homoserine lactone (a QSM that is produced by the bacterium *P. aeruginosa*), to *C. albicans*, found that the effects on the culture was similar to that of farnesol. Decanoic acids, other quorum sensing molecules from cultures of *Burkholderia cenocepacia* and *Xanthomonas campestris* also had repressive effects on hyphal formation in the culture (Wang *et al.,* 2004; Davis-Hanna *et al.,* 2008*;* Boon *et al.,* 2008).

1.5 Communication between Spores

In a vast majority of experimental studies, the addition of QSMs has taken place at the time of inoculation when the spores have already germinated, and are in, or past the lag phase, whereby the culture morphology has already been formed. In some cases, subsequent additions are made to the culture at specific time increments in order to see changes to the culture morphology and productivity.

Reasons for the morphology/productivity dependence of fungi to their initial spore numbers are not reported. It has, however, been expressed that inoculum size will affect the overall morphology and productivity of the culture.

As QS plays a significant role in the physiology and morphology of fungal cultures (Porcel *et al.,* 2006; Cottier and Muhlschlegel, 2012; Sharma and Jangid, 2015), and due to the fact that QS is cell density dependent, spores, may also function in a cell density dependent manner. This hypothesis is based on the fact that it is now known that spores are active entities (Feofilova

et al., 2012). Therefore, whether it be QS or another mechanism, some form of communication is likely to be taking place between spores.

1.6 Confinement – Diffusion Sensing

It is undisputed that bacteria and fungi produce, secrete and sense small chemical compounds and that they produce signalling molecules known as auto inducers that are able to regulate gene expression and can control many functions within the organism.

Alongside QS, another mechanism is also postulated. The premise behind QS is that it is cell density dependent, and that QSMs are secreted into the environment, once a critical threshold concentration is reached, a regulatory response is triggered in the cells, which results in a coordinated response by the target genes (Wongsuk *et al.,* 2016). However, Redfield (2002) hypothesised that cells are able to sense the auto inducers secreted, but because of the dilution of the secreted molecules, the cells detect them only when they arrive at a critical concentration close to the cells. When the large volume of medium is stirred, the QSMs are dispersed, and hence the signals are not effective. Therefore, if the diffusion and mixing were to be limited, cells would be able to interact even at low concentrations of QSMs and evoke a response. Boedicker *et al.,* (2009) tested this theory by confining a single bacterial cell of *P. aeruginosa*. They found that QS was initiated, and response mechanisms were turned on in the cells. Figure 1.5 shows schematically the

process involved. These results show that by confining the cells, and allowing the auto inducers to accumulate in the environment, QS responses can be evoked, and therefore the number of cells is not as important as once described, but it is the concentration of QSMs in the environment that is key to regulation of cellular functions.

Choudhary *et al.,* (2010) stated that the QS phenomena is associated with large populations, but it is also important to be aware that the confinement of a few cells to a small volume allows for the accumulation of QS signalling molecules, which can be sufficient to activate QS.

Figure 1.5 The confinement of P. aeruginosa *(grey rod) in a small volume, secreting QS molecules (orange dots) that are unable to diffuse away from the cell, once the threshold concentration is reached the response is evoked in the cell, in which the fluorescence genes are switched on (green rod) (Boedicker* et al., *2009)*

Confinement therefore has two functions, to restrict the space in which the spores are a) able to move in and affect bumping into one another and b) to limit the distance with which diffusion of any cell signalling or QSM can move to, therefore forcing spores to come into contact with these molecules.

1.7 Quorum Sensing Applications

QS has a key role in the functionality of microorganisms in that it can regulate cell activities, for example QS can control virulence, biofilm formation, and antibiotic production. The mechanism of QS is the binding of signalling compounds to the receptor, followed by binding to the promoter sequence, enabling transcriptional regulation of the target genes. The inhibition of cell signalling is called quorum quenching (QQ). There are three methods of cell signalling inhibition, I) stopping the production of signalling molecules, II) degradation of signalling molecules by enzymes and III) blocking signalling molecules from binding to receptor molecules. QQ has many applications, for example it has been employed as a technique in agriculture to stop the spread of disease, in wastewater treatment and in medical applications, to stop biofilm formation (Sharma and Jangid, 2015).

In agriculture, bacteria have been used to evoke defence mechanisms in plants. *Pseudomonas* spp.*, Pantoea* and *Erwinia* spp. are able to manipulate plant pathogenesis, causing plant cells to express defence mechanisms (Kalia and Kumar, 2015). Similar responses are seen with the addition of *P. syringae*, which helps tobacco plants to become resistant to pathogenic attack. Potato plant-rot is protected by *Microbacterium testaceum*, which lives on the surface of the leaves, defending against the plant pathogen *Pectobacterium carotovsorum* (Quinones *et al.,* 2004).

The secondary metabolites produced by *Delisea* sp.*, Asparagopsis* sp. and *Bonnemaisonia* sp. have been used as antifouling agents against bacteria in wastewater treatments (Kinley *et al.,* 2016; Paul *et al.,* 2006). Fisheries have used both natural and synthetic QS inhibitors to protect sea life such as brine shrimp (*Artemia franciscana*) and rainbow trout from pathogenic *Vibrio* spp. by the use of brominated furanones (Defoirdt *et al.,* 2006; Rasch *et al.,* 2004). Furanone is also used medically to inhibit pathogenicity of *P. aeruginosa*. (Hentzer *et al.,* 2003). The QSM 3OC12-HSL from *P. aeruginosa,* was used by Li *et al.,* (2004) to inhibit proliferation of human breast cancer cell lines and induce apoptosis. Also shipping industries have combined kojic acid, produced by *Aspergillus* spp., with nontoxic paint to be used to control bacteria and diatoms found at the base of ships (Dobretsov *et al.,* 2011).

1.8 Model Fungus for This Research

The name *Aspergillus is* derived from the Latin word *aspergillum*, which is a tool used by the Roman Catholic Church to sprinkle Holy water. *Aspergillus* was given this name by Pier Antonio Micheli, a priest and biologist, in 1729, as the conidiophores produced by the species reminded him of an aspergillum.

Aspergillus spp. have been used for decades throughout the biotechnology industry. The species is saprophytic, found living in the soil or on decaying matter and has great ecological and metabolic variety. For example, *A. oryzae* is used in the food industry, particularly in the Far East, for food fermentation.

It has been used in the manufacture of miso, also known as soya bean paste, for sake or rice wine production, and in the making of soy sauce (Machida and Gomi, 2010). *A. niger*, on the other hand, has been utilised for the production of citric acid. Another important organism for one of its secondary metabolites is *Aspergillus terreus*, as it has the ability to produce the cholesterol lowering drug, lovastatin, which was one of the first statins to be identified, and approved by the Food and Drug administration in 1987, lovastatin is also precursor to, the now commercially available semi synthetic statin, simvastatin (Chaynika and Srividya, 2014).

1.8.1 *Aspergillus terreus* – Species Background

Aspergillus terreus is a filamentous fungus, belonging to the Ascomycetes family. Cultures can be found in abundance throughout the environment in desert soils, compost, marine environments, and grasslands (Kozakiewicz, 1989). It is a robust and well investigated organism. Its growth, productivities and gene expression has been well researched and greatly published. The organism was one of the first to be utilised for its many metabolites. *A. terreus* is an important organism in the pharmaceutical industry, due to the nature of the secondary metabolites that it produces.

A. terreus has been chosen as the model for this study because of its high spore producing ability, its differences in morphologies with different inoculum concentrations and also because of the poor capacity in maintaining lovastatin

productivity in scale-up from shake flasks to bioreactors (Pawlak *et al.,* 2012). These manifestations have made this fungus an ideal organism to investigate spore interactions, subsequent fungus morphologies and lovastatin productivities.

1.8.2 Growth and Morphology

Cultures of *A. terreus* are commonly yellow – brown in colour and form globular structures known as conidia, these contain asexual spores (figure 1.6). Aerial hyphae are also produced, along with aleurospores, which are lateral cells formed in the absence of conidiophores. This is a characteristic feature unique to *A. terreus* (Casas Lopez *et al.,* 2005). Cultures are also hydrophobic in nature as are the spores that are produced; they are difficult to wet but are highly dispersible.
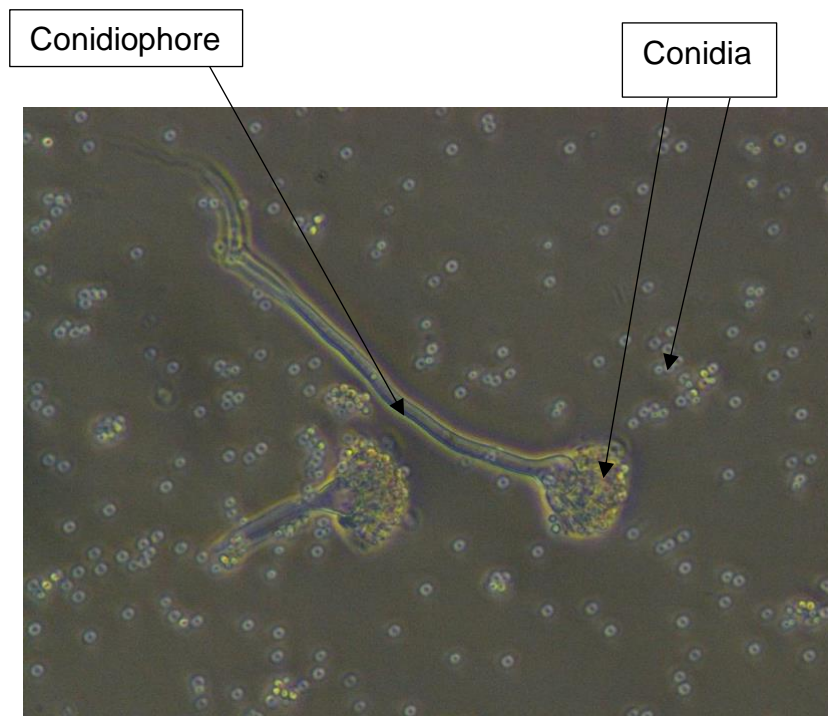
Figure 1.6: *Conidiophore and liberated spores from the species Aspergillus terreus (captured on Nikon light microscope x40 magnification)*

A single hypha is composed of three different regions or zones. The first is the apical region, located at the tip of hypha, where elongation and substrate uptake takes place. The second region is the subapical zone located behind the apical region sections. The last zone is the distal hypha, responsible for metabolic product formation (Bizukojc and Ledakowicz, 2010; Gougouli and Koutsoumanis, 2012).

Morphology and subsequent productivity of *A. terreus* cultures have also been controlled through supplementation of culture broths with various additives. For example, Lai *et al.,* (2003)*,* investigated additions of methionine, and found

that supplementation of 100 mg/L of DL-methionine to 72 hours old cultures increased production of lovastatin by 20%. Bizukojc *et al.,* (2007), investigated additions of B group vitamins with regard to a variety of carbon sources, and found lovastatin productivity to have increased.

While the differences in morphology and productivity have been repeatedly reported between cultures from low and high spore inocula, to my knowledge there has been no systematic study to address the reason(s) for these differences which can be decisive in the type and productivity of products at large (industrial) scale.

As there have been differences in the morphology of the cultures, and the subsequent productivities based on the starting number of fungal spores, it is possible that the number of spores as inocula present within a given environment may represent a QS process.

1.8.3 Secondary Metabolite Production

A. terreus produces a wide variety of secondary metabolites that have a range of pharmaceutical and biological importance. Over 165 secondary metabolites have been reported (Boruta and Bizukojc, 2016) for this organism. Some of these include lovastatin, also known as Mevinolinic acid.

Many of these secondary metabolites are by-products of lovastatin production and have not been extensively researched but have been identified as being produced by *A. terreus.* The by-product (+)-Geodin, is produced in abundance alongside lovastatin and studies have been carried out in order to limit its production (Bizukojc and Ledakowicz, 2008). Boruta and Bizukojc, (2016) found the following by-products produced in abundance, using lactose (as the primary carbon source) and rapeseed oil, asterric acid (2.96 mg/L), butyrolactone I (10.32 mg/L), terrein (9.37 mg/L) and (+)-geodin (77.53 mg/L), and high lovastatin concentrations were also obtained (106.57 mg/L).

1.8.4 Lovastatin

Lovastatin ($C_{24}H_{36}O_5$), also known as Mevinolin, Mevacore$_{TM}$ and Monacolin K, is part of a group of compounds known as statins. It is composed of two polyketide chains, connected by an ester linkage, (figure 1.7). Lovastatin acts as a competitive inhibitor for the enzyme 3-hydroxy-3methyl-glutaryl coenzyme A (HMG-CoA) reductase. The enzyme is the rate limiting step in the biosynthesis of cholesterol and it catalyses the reduction of HMG-CoA to mevalonate during cholesterol synthesis (Lingappa *et al.,* 2004, Chaynika and Sridya. 2014). Lovastatin is therefore manufactured for the treatment of atherosclerosis and ischemic heart disease. Lovastatin has also shown to have anticancer properties, in breast cancer it has the ability to inhibit cellular proliferation and induce apoptosis and necrosis.
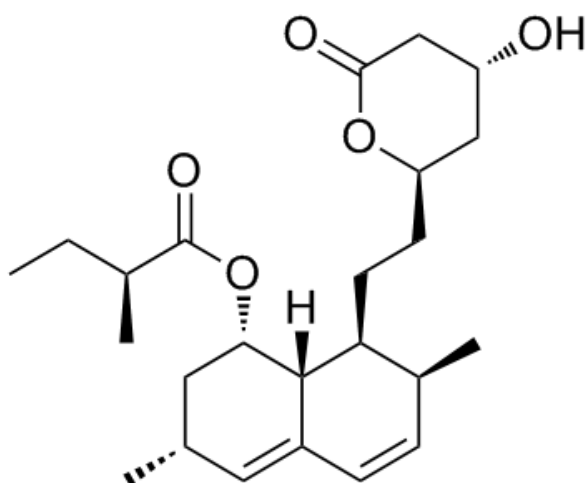
Figure 1.7: Chemical structure of the compound lovastatin part of the group of cholesterol lowering drugs, statins (Goswami *et al.,* 2012)

A. terreus is the largest producer of lovastatin, but it can be produced by *Penicillium* sp. and *Monascus ruber* too. Lovastatin production by *A. terreus* has been widely investigated, and production methods have been manipulated in many ways in order to increase productivities on an industrial scale (Casas Lopez *et al.,* 2003). The main requirement for production of lovastatin via *A. terreus* is a carbon source, most commonly glucose, and a complex nitrogen source, as this is required for the synthesis of the polyketide (Hajjaj *et al.,* 2001). Production of lovastatin by *A. terreus* is not an easy process. Some difficulties arise in fermentation design and upholding high yields necessary for large scale production. Downstream processing is also an extensive and expensive process (Chaynika and Srivdya 2014) and increases the cost of production. Different techniques such as using agricultural waste as carbon source has been employed to increase and improve productivities

on a large scale whilst keeping production costs low (Jaivel and Marimuthu, 2010).

Ways in which lovastatin yields can be increased have been widely researched, the most widely investigated area is media composition, particularly carbon sources. No one specific carbon source has been agreed upon as being the optimal for lovastatin production. Sources have included glucose, lactose, fructose and crude oil. In depth research has also been carried out with regard to the optimum ratio between carbon and nitrogen for the production media. But it has been agreed the media should contain a complex nitrogen source, as this is a requirement for lovastatin metabolism (Lopez *et al.,* 2003).

Investigations into the addition of inducers has also been undertaken, whereby compounds such as nicotinamide, calcium panthenate, thiamine, methionine, riboflavin, and butyrolactone I (Schimmel *et al.,* 1998; Lai *et al.,* 2003; Bizukojc *et al.,* 2007 and Rahim *et al.,* 2015) have been shown to increase productivities. Culture conditions have also been investigated. These include temperature, dissolved oxygen tension and pH, all in an attempt to increase productivities. Research has also been carried out into the type of inoculum used, whether it is spores or hyphal culture. Lopez *et al.,* (2003) found that neither inoculum type had an effect on lovastatin yields. However, little research goes into the explanation as to why and how additions of these compounds, or changes in inoculum size affect productivities.

Butyrolactone I, also produced by *A. terreus,* has QS effects. Molecules containing γ-butyrolactone, such as butyrolactone I are known for their QS properties as they act as diffusible self-regulating factors in a number of bacteria, and control different functions, including antibiotic production, biofilm formation, bioluminescence, virulence factor production and plasmid conjugal transfers. In the *Streptomyces* species, it is involved in both morphological differentiation and secondary metabolism. Schimmel *et al.* (1998) found that additions of butyrolactone I to *A. terreus* cultures increased hyphal branching, sporulation and secondary metabolism. More specifically, the production yields of lovastatin improved three fold. Raina *et al.* (2012) found that addition of 100 nM butyrolactone I to cultures of *A. terreus* at 96 hours post inoculation, increased lovastatin production 2.5-fold.

1.9 Aim and Objectives

Research carried out on fungal spores suggests that spore inoculum size affects the overall culture morphologies and the formation of secondary metabolites, but there is no information as to why this is the case, why the initial spore concentration of the inoculum has such an influence on the culture, and what mechanism(s) are involved that enable these differences to take place.

The overall aim of this work is to investigate the interactions between *A. terreus* spores with regards to a QS based communication mechanism. In this context, the aim is to explore the behaviour of *A. terreus*, for sustained/improved productivity, in relation to the inoculum size at very small scale to larger scales.

In order to fulfil the aim, the following objectives will be addressed:

- To investigate the effects of different spore inoculum sizes on culture morphology grown on solid agar.
- To investigate the possibility of a QS or chemical communication based mechanism between *A. terreus* spores.
- To explore the behaviour of *A. terreus* at different inoculum sizes from very small to large scale for sustained/ improved productivity.
- To investigate spore to spore contact through space confinement, in order to encourage spores to increase spore to spore contact frequencies.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

All chemicals and reagents were obtained from Sigma-Aldrich Company Limited, Dorset, United Kingdom, unless otherwise stated.

2.2 Strains

The lovastatin producer strain *Aspergillus terreus* (*A. terreus*) MUCL 38669 was obtained from CABI Biosciences UK Centre, Surrey, United Kingdom and used throughout this study.

2.3 Media and Growth Conditions

2.3.1. Maintenance Medium for *Aspergillus terreus* MUCL 38669

A. terreus cultures were maintained on potato dextrose agar (PDA) slopes and sub-cultured onto petri dishes as required. In 1 L of distilled water, 15.6 g of PDA was dissolved and autoclaved at 121 °C for 15 minutes. Slants and petri dishes were subcultured and incubated at 27 °C for 7 days and subsequently stored at 4 °C.

2.3.2 Sporulation Medium

In order to obtain high spore numbers *A. terreus* cultures were grown on yeast and malt extract (YME) agar (Table 2.1). The medium was prepared in 1 L of distilled water, stirred continuously and autoclaved at 110 °C for 10 minutes.

To the previously prepared PDA plates of *A. terreus* cultures (section 2.3.1), 10 mL of sterile 0.01% Tween 80 and 20 glass beads were added. The plates were swirled carefully to avoid contamination, and surface-scraped with sterile spreader. The washings were collected, and 1 mL was transferred to YME plates and incubated at 27 °C for 5 days. After this time, plates were stored at 4 °C until use for spore harvest.

Table 2.1: *Yeast Malt Extract (YME) Agar*

Ingredients	g/L
Malt extract	10.0
Yeast extract	4.0
Glucose	4.0
Agar	20.0

2.3.3 Submerged Liquid Fermentation Medium

Submerged liquid fermentation was carried out using complex, and defined media in shake flasks. Production of lovastatin from *A. terreus* was determined when grown in a medium designed for the production of lovastatin (2.3.3.3).

2.3.3.1 Complex Medium

Initial 250 mL shake flask experiments were carried out using potato dextrose broth (PDB) of 24 g/L. Sterilisation of medium was carried out at 121 °C for 15 minutes.

2.3.3.2 Defined Medium

Submerged liquid cultures of *A. terreus* were carried out in 250 mL shake flasks with defined medium (table 2.2). The medium was prepared as three separate components, a) glucose b) Mono-hydrate sodium glutamate (MSG) and trace elements c) Iron and potassium. Components a and b were autoclaved separately at 110 °C for 10 minutes, component c was filter sterilised through a 0.22 µm mixed cellulose ester membrane (Millipore (U.K.) Limited, Hertfordshire, United Kingdom). Before sterilisation the pH of component b was adjusted to 6.5 by addition of KOH (2N). After sterilisation all components were aseptically mixed and used as the defined medium.

Table 2.2: *Defined medium*

Components	Ingredients	g/L
A	Glucose	45.0
B	Mono-hydrate sodium glutamate	12.500
	$MnSO_4.H_2O$	0.076
	$ZnSO_4.7H_2O$	0.200
	$MgSO_4.7H_2O$	0.100
	$CaCl_2.2H_2O$	0.020
	$CuCl_2.H_2O$	0.005
	H_3BO_3	0.011
	$(NH_4)_6Mo_7O_{24}.4H_2O$	0.005
C	KH_2PO_4	5.0
	K_2HPO_4	5.0
	$FeSO_4.7H_2O$	0.2

2.3.3.3 Lovastatin Production Medium

For the production of lovastatin by *A. terreus,* cultures were grown in a complex medium, known as GPY – L medium (table 2.3) (Schimmel *et al*., 1999), composed of glucose, lactose, peptonised milk and yeast extract. The pH of the solution was adjusted to 7.4 by addition of 0.1 M NaOH prior to sterilisation at 110 °C for 10 minutes.

Table 2.3: Lovastatin Production Medium (GPY – L medium)

Ingredients	g/L
Glucose	25.0
Lactose	50.0
Peptonised milk (Oxoid)	24.0
Yeast extract	2.5

2.4 Inoculum Preparation Techniques

2.4.1. Preparation and Provision of Spores (Spore Harvest)

To a lawn of *A. terreus* (grown over 5 days on YME agar), 5 mL of sterile 0.01% Tween 80, supplemented with 20 glass beads, was added to the petri dish and swirled aseptically, the washings were collected. An additional 10 mL of 0.01%

tween 80 was added, swirled and the surface scraped with an L shaped sterile spreader to break open the conidiophores and release the spores. The washings were collected, used plates and glass beads discarded. This process was repeated using three petri dishes of *A. terreus,* to obtain a total volume of 50 mL of washings.

The washings were centrifuged at 2800 x *g* for 10 minutes. The supernatant was then removed (and kept for use during supplementation experiments, section 2.5). The spun down spores were re-suspended in 50 mL sterile distilled water.

2.4.2 Determining Spore Number

A haemocytometer was used to determine the number of spores in the stock spore suspension. The haemocytometer was loaded with 0.1 µL of the spore suspension, the top left, right and bottom left, right, and central grids were counted, and an average calculated, then multiplied out to determine the overall numbers of spores on the whole haemocytometer grid. The number calculated was for 0.1 µL, this was then multiplied out by 10000 to determine the number of spores per 1 mL. Spore suspension was diluted with sterile distilled water to obtain desired spore numbers where necessary.

2.5 Supplementation of Spores on Solid Substrate

To separate YME plates, 500 µL of the following were added. a) the QSM, Butyrolactone I (Enzo Life Sciences (UK) LTD., Exeter, United Kingdom), dissolved in ethanol to a concentration of 100 nM and filter sterilised through 0.2 µm cellulose acetate membrane filter (VWR International, Pennsylvania, United States). b) Spore supernatant (SS): During the spore harvest process (Section 2.4) the surface of *A. terreus* lawn cultures were washed with Tween 80. The washings were collected and centrifuged to separate spores from the liquid. Once centrifuged, two layers were formed, the lower layer contained the spun-down spores, and the supernatant (upper layer), contained the cell culture plate washings. After centrifugation the supernatant was removed and filter sterilised through a sterile 0.22 µm mixed cellulose ester membrane filter (VWR International, Pennsylvania, United States), and denoted as spore supernatant (SS).

Control conditions were the addition of, absolute ethanol, filter sterilised through a sterile 0.22 µm mixed cellulose ester membrane, and buffer solution of 0.01% Tween 80 made in distilled water and autoclaved at 121 °C for 15 minutes.

Plates were inoculated with various spore concentrations ranging from 1×10^1 to 5×10^7. YME plates of 5 day old cultures were harvested for spores (Section 2.4) spore suspensions were counted to determine spore number and diluted to the following concentrations (Table 2.4) with sterile distilled water.

Table 2.4: *Spore Concentrations*

	Spores/ mL
1	1×10^1
2	4×10^2
3	1×10^3
4	4×10^3
5	5×10^3
6	1×10^7
7	5×10^7

Inoculum of 1 mL was added to each supplemented plate and incubated for 5 days at 27 ˚C. Table 2.5 summarises plate additions and inoculum concentrations. Visual inspection, and where possible, microscopic inspection took place every 24 hours. Plates were observed using Nikon eclipse Ci microscope.

All tests were carried out in duplicate. Table 2.5 below summarises the conditions in which *A. terreus* spores were cultured.

Table 2.5: *Plate additions: - no supplementation (NS), ethanol, tween80,*

butyrolactone I and spore supernatant (SS)

Inoculum size (Spore number)	Additions				
	Control			Test	
1×10^1	NS	Tween 80	Ethanol	Butyrolactone I	SS
4×10^2	NS	Tween 80	Ethanol	Butyrolactone I	SS
1×10^3	NS	Tween 80	Ethanol	Butyrolactone I	SS
4×10^3	NS	Tween 80	Ethanol	Butyrolactone I	SS
5×10^3	NS	Tween 80	Ethanol	Butyrolactone I	SS
1×10^7	NS	Tween 80	Ethanol	Butyrolactone I	SS
5×10^7	NS	Tween 80	Ethanol	Butyrolactone I	SS

2.6 Shake Flask Studies (250 mL flask)

2.6.1 Supplementation of Spores in Complex Medium

Shake flasks of 250 mL (20% working volume) were used for submerged liquid cultures of 1×10^3 (low) and 1×10^7 (high) spores/mL of *A. terreus* spores. PDB medium was supplemented with 1 mL of the following: 100 nM of Butyrolactone I (QSM) filter sterilised through 0.22 µm cellulose esters membrane filter (VWR International, Pennsylvania, United States). Spore supernatant, collected during spore harvest processes and filter sterilised through the sterile 0.22 µm mixed cellulose ester membrane filter prior to use.

Ascorbic acid derived from Vitamin C tablet was also added, the vitamin C tablet was dissolved in distilled water to produce a solution of 10 mg/mL, and filter sterilised through the 0.22 μm mixed cellulose esters membrane filter. Control of filter sterilised ethanol (0.22 μm mixed cellulose ester membrane) and spores with no additions were also prepared.

As described in section 2.4, 5 day old *A. terreus* cultures, grown on YME agar plates, were harvested for spores. Spore number determined and diluted to 1×10^4 and 1×10^8 spores/mL with sterile distilled water, upon addition to the medium, the final spore concentrations in the flasks were 1×10^3 and 1×10^7 spores/mL, respectively.

Table 2.6 below summarises the additions made to the culture flasks, and the initial concentration of the inoculum added. Final spore concentrations per mL stated. The concentrations of 1×10^7 spores/mL and 1×10^3 spores/mL are referred to throughout the study.

Table 2.6: *Culture flask content*

Flask	Medium volume (mL)	Inoculum concentration (spores/mL)	Inoculum volume (mL)	Additive	Additive volume (mL)	Final spore concentration (spores/mL)
1	45	1×10^8	5	-	-	1×10^7 (High)
2	45	1×10^4	5	-	-	1×10^3 (Low)
3	44	1×10^4	5	QSM	1	1×10^3 (Low)
4	44	1×10^4	5	SS	1	1×10^3 (Low)
5	44	1×10^4	5	AA	1	1×10^3 (Low)
6	44	1×10^4	5	Ethanol	1	1×10^3 (Low)

Flasks were prepared in triplicate and incubated at 27°C and 220 rpm for 5 days. Samples from flasks were taken every 12 hours. Per each sample taken, a minimum of 30 fungal pellets were analysed for morphology.

2.6.2 Supplementation of Spores in Defined Medium

2.6.2.1 Supplementation with High Spore Culture Supernatant

Defined medium was supplemented with high spore culture supernatants (HSCS) prior to inoculation with low spore numbers. Controls of high and low spore numbers were also inoculated in the defined medium.

High spore culture supernatants were obtained from the cultivation of three 250 mL flasks containing 45 mL sterile defined medium inoculated with 5 mL of 10_8 spores/mL. Flasks were incubated at 27 °C and 220 rpm for 72 hours.

After incubation, contents of the flasks were aseptically filtered through 0.2 μm nylon filter system (Corning B.V. Life Sciences, Amsterdam, The Netherlands), to remove the biomass. The process was carried out under laminar flow hood. The final volume was noted, and filtrate was kept for later analyses.

As described in section 2.4, 5 day old *A. terreus* cultures, grown on YME agar plates, were harvested for spores. After the number of spores in the spore suspension had been calculated, spore concentrations were diluted to 1 x 10_4 and 1 x 10_8 spores/mL. After additions to the medium, concentration of spores were 1 x 10_3 and 1 x 10_7 spores/mL respectively.

Table 2.7 summarises the preparation of 250 mL shake flasks

Table 2.7: *summary of shake flask preparations*

Flask	Medium volume (mL)	Inoculum concentration (spores/mL)	Inoculum volume (mL)	Additive	Additive volume (mL)	Final spore concentration (spores/mL)
1	45	1×10^8	5	None	-	1×10^7 (high)
2	45	1×10^4	5	None	-	1×10^3 (low)
3	15	1×10^4	5	HSCS	30	1×10^3 (low)

Flasks were incubated at 27 °C and 220 rpm for 5 days and prepared in triplicate. Samples from flasks were taken every 12 hours. A minimum of 30 pellets per sample were analysed for morphology.

2.6.2.2 Supplementation with Quorum Sensing Molecules

2.6.2.2.1 Tyrosol

A 1 M solution of tyrosol was prepared in DMSO, filter-sterilised through the 0.2 μm cellulose acetate membrane filter and added to 250 mL flasks with defined medium to obtain final tyrosol concentrations of 0.04 and 0.01 M. Flasks were inoculated with 5 mL of 1×10^4 and 1×10^8 spores/mL to obtain a final spore concentration of 1×10^3 and 1×10^7 spores/mL, respectively. Control flasks were prepared containing the two different spore sizes (5 mL)

and the defined medium only. Another control was the medium and spores with 0.04 and 0.01 M DMSO. Flasks were incubated over a 5 day period at 27 °C and 220 rpm. Experiments were carried out in triplicate; samples were taken daily and a minimum of 30 pellets per sample were analysed microscopically.

2.6.2.2.2 Farnesol

Farnesol was supplied as 98% pure, filter sterilised through a 0.2 µm cellulose acetate membrane filter and added to 250 mL flasks with the defined medium to obtain concentrations of 0.1, 0.08, 0.04, and 0.01 M. Flasks were inoculated with 5 mL of 1×10^4 and 1×10^8 spores/mL to obtain a final spore concentration of 1×10^3 and 1×10^7 spores/mL, respectively. Control flasks were prepared containing the two different spore sizes (5 mL) and the defined medium. Flasks were incubated at 27 °C and 220 rpm for 5 days. Experiments were carried out in triplicate. Samples were taken daily and a minimum of 30 pellets per sample were analysed microscopically.

2.7 Large Scale Supplementation (1L Shake Flask) – Lovastatin Production

2.7.1 Supplementation with Farnesol

To 1 L shake flasks, 180 mL of lovastatin production medium was added, alongside 20 mL of spore inoculum concentration to obtain a final spore concentration of 1 x 10_3 and 1 x 10_7 spores/ mL, respectively. To each flask 504 µL of sterile farnesol was added to prepare a final concentration 1 M of farnesol.

Control conditions had no farnesol present. Flasks were maintained at 27 ˚C and 220 rpm, and samples were taken in duplicate over 14 days.

2.7.2 Supplementation with Concentrated Spore Supernatants

Cultures of *A. terreus* were grown on YME plates, as described in section 2.4 after 5 days of growth, plates were scraped, and spores harvested. The spore washings that were obtained during spore harvest was centrifuged at 2800 x *g* for 10 minutes, the resulting supernatant was removed and separated into 10 mL aliquots in falcon tubes. These were subsequently frozen for 12 hours and then freeze dried for 24 hours. This freeze dried spore supernatant was used as an addition to high and low spore numbers.

To 1L shake flasks, 200 mL of lovastatin production medium was added, in addition to the freeze dried spore supernatants. To separate flasks 1 x 10^7 spores/mL and 1 x 10^3 spores/mL were added. This formed the test subject, high and low spores containing freeze dried spore supernatant (FDSS), respectively. Control conditions did not contain the FDSS. Flasks were incubated at 27 °C and 220 rpm for 14 days. Samples were taken daily and analysed for lovastatin production, biomass production and substrate consumption. Experiments were carried out in duplicate.

2.8 Spore Contact Studies

2.8.1 Confinement

To shake flasks of 1 L and 100 mL, 20% working volume of defined medium was added, spores were harvested as described in section 2.4, and 2000 spores were added to each flask. Flasks were maintained in a reciprocating water bath at 27 ˚C and 60 rotations/minute and aerated at 0.5 vvm. At 2 hour increments, post inoculation, 50 µL of sample was analysed microscopically, to identify germination, changes in spore morphology and subsequent fungal morphology. Samples were taken over 48-hour period. Experiments were carried out in triplicate. Figure 2.1 presents a schematic diagram and photo of the process set up. Table 2.8 summarises the parameters used.
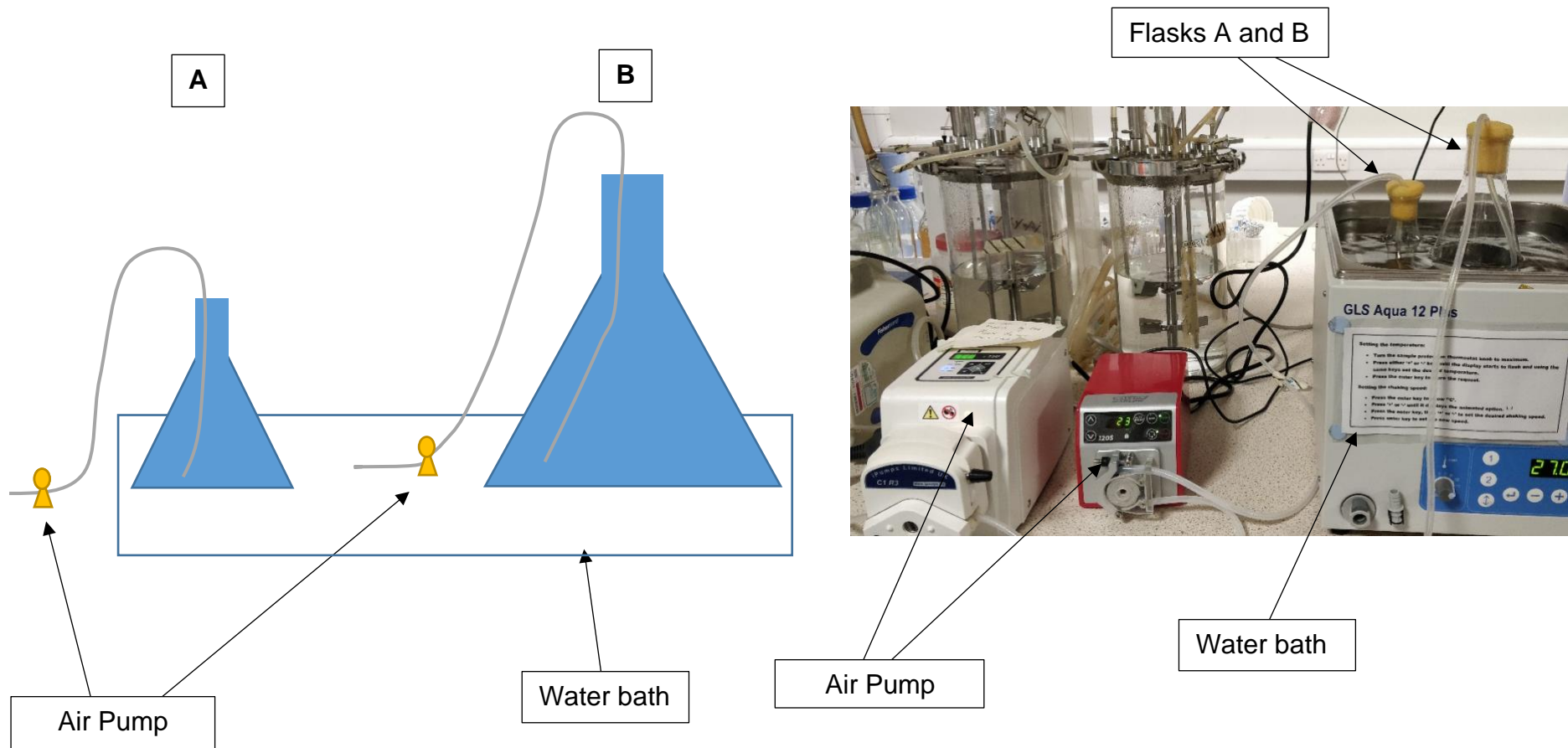
Figure 2.1 *Schematic diagram and photograph of the set-up for the confinement experiment. Flask A, is 10 mL and flask B is 1L in size. Both are kept in* reciprocating *water bath, and aerated.*

***Table 2.8** Parameters used for the set up for each flask*

Flask	A	B
Flask size (mL)	100	1000
Volume of medium (mL)	20	200
Number of spores	1000	1000
VVM	0.5	0.5
Air flow rate (mL/min)	10	100
Temperature (°C)	27	27
Rotations (lateral movement in water bath)	60	60

2.8.2 Sonication

Universal bottles containing 5 mL of 1×10^4 spores/mL (low spore numbers) and 5 mL of 1×10^8 spores/mL (high spore numbers), were placed in a sonicated water bath at 45 kHz and sonicated for 1, 2 and 5 minute intervals. The control samples were not subjected to sonication. Spore suspensions were prepared in triplicate and used as inocula with 45 mL defined medium in 250 mL shake flasks.

Table 2.9 below summarises the content of the 250 mL shake flasks.

Table 2.9: *Summary of culture conditions*

Flask	Inoculum concentration (spores/mL	Sonication duration (minutes)	Final spore concentration (spores/mL)
1	1×10^8	0	1×10^7
2	1×10^8	1	1×10^7
3	1×10^8	2	1×10^7
4	1×10^8	5	1×10^7
5	1×10^4	0	1×10^3
6	1×10^4	1	1×10^3
7	1×10^4	2	1×10^3
8	1×10^4	5	1×10^3

Flasks were prepared in triplicate and incubated at 27 °C, 220 rpm for 5 days. Samples from flasks were taken every 12 hours, and a minimum of 30 pellets per sample were analysed for morphology.

2.9 Morphological Analysis – Microscopy

2.9.1 Slide preparation

Approximately 1 mL of sample was removed from 250 mL culture flask (section 2.6) and using a plastic loop, pellets were placed onto the glass slides. Samples from each flask were analysed. Where fungal mass was large and saturated with medium, they were allowed to dry slightly before a cover slip was placed over the top, this prevented a liquid ring from being formed around the pellet.

2.9.2 Data Collection

Nikon Eclipse Ci-L light microscope, alongside different phase-contrast filters, and magnifications of x4, x10 and x40 were used for imaging and analysis of culture morphologies. Hyphae tip numbers were counted manually, and the diameter of each pellet was measured using the NIS Elements software package built into the system.

2.10 Assays

2.10.1 Biomass Assay

Biomass production was measured as total cell dry weight (CDW) per litre (g/L) of culture broth at the end of the experiments where relevant. CDW was determined by filtration of flask content on oven dried, pre-weighed, filter paper (Whatman No.1). The filter paper and biomass was kept in a desiccator for 24 hours and dried to a constant weight at 50 °C, in an oven. The filter paper was re-weighed, and cell dry weight determined by the difference between the combined weight of the filter paper and the weight of the filter paper alone.

2.10.2 Total Carbohydrate

A total carbohydrate assay was carried out using the phenol-sulphuric acid method of DuBois *et al* (1956). Shake flask samples were centrifuged at 14000 rpm for 5 minutes. The supernatant was collected and diluted prior to use to concentrations no greater than 200 mg/L with HPLC grade water.

Into glass test tubes, 200 µL of 5% (w/v) phenol was aliquoted, followed by 200 µL of sample, glucose standard or water as blank, 1 mL of concentrated sulphuric acid was rapidly added centrally to the liquid surface. Tubes were maintained at room temperature for a minimum of 10 minutes, after which

tubes were covered and vortexed. From each sample, 200 µL was transferred to a 96-well plate, absorbance was measured at 490 nm in plate reader.

Standard curve was prepared using concentrations between 0 and 200 mg/L of glucose in HPLC grade water, the blank was prepared using HPLC water (Figure 8.2). Tests were carried out in triplicate.

2.10.3 Lovastatin Detection and Quantification (HPLC)

2.10.3.1 Lovastatin Standard Preparation

Mevinolinic acid, is the open hydroxyl acid form of lovastatin. It is produced and secreted into the fermentation broth by *A. terreus*. This form of lovastatin is unstable, it was therefore prepared freshly from the lactone form, when required. The conversion of lovastatin from the lactone form to the β-hydroxy acid form was performed as described by Yang and Hwang (2006). Lovastatin (2 mg) was dissolved in 10 mL of 0.1 N NaOH, prepared in 50% aqueous acetonitrile solution. The lovastatin standard was maintained in 45 °C water bath for 1 hour, followed by neutralisation to pH 7 with the addition of 0.1 N HCl prepared in acetonitrile. For calibration purposes, standard solution of lovastatin was prepared at concentrations between 0.0125 – 0.15 mg/mL.

2.10.3.2 Sample Preparation

Lovastatin was extracted from samples collected during shake flask fermentation by addition of equal volume of HPLC-grade methanol to the sample culture. Samples were then shake for 2 hours at 250 rpm and 22 ˚C. The organic phase was removed and filtered through 0.2 µm cellulose acetate filter (Watman TM, Maidstone, UK) prior to HPLC analysis.

2.10.3.3 HPLC Method

Lovastatin standards and samples were quantitatively analysed using HPLC system (Dionex) with UV-VIS detector using reverse phase chromatography on a Phenomenex C18 column (C18, 5 µm x 4.6 mm x 150 mm) equilibrated at 25 °C. Samples were analysed via isocratic method, the mobile phase was composed of HPLC-grade acetonitrile and 0.1% aqueous phosphoric acid (H_3PO_4) at a ratio of 55:45 (v/v). The flow-rate was 1 mL/min, injection volume was 25 µL and peak detection was at 238 nm.

2.11 Statistical Analysis

Average hyphal tip numbers and diameters were observed; from this the standard deviation of the means were calculated. T-tests carried out and statistical analysis were done to determine whether differences obtained in the

mean values were significant, with 95% confidence interval. Where appropriate, a one way ANOVA was carried out to determine variance between groups. This was carried out using Microsoft Excel, and or in IBM SPSS software.

3. RESULTS

3.1 Introduction

Scalability of production is one of the key challenges in the microbial biotechnology sector. In many cases where optimum conditions are established at bench scale, production cannot be increased or even maintained at large-scale despite better controlled culture conditions. There is little investigation of this issue in literature despite clear economic benefits.

An important issue related to the production from filamentous fungi, is related to the inoculum size. It has been widely reported that the size of fungal inocula (mainly spores) affects the secondary metabolite production, largely because of the morphology that arises from the cultivation of different inoculum sizes. The reasons for this have not been investigated thus far. This project explored the effects of fungal inoculum size, and this chapter reports the results of these investigations.

There are two possible explanations as to why the differences in spore inoculum size have caused differences in fungal morphology, growth and productivity. These explanations form the basis of the investigations that are presented in this chapter.

The first explanation for these observations is based on the quorum sensing phenomenon, whereby when the microbes' population density reaches a

certain threshold, the signalling chemicals secreted into the environment are detected and then processed triggering a range of responses. The second is that there is some form of physical interaction taking place between the spores, enabling them to communicate or interact in a manner that manifests in a physical response such as specific development of morphology leading to changes in productivity.

In order to explore these hypotheses, the work was split into two main parts:

Part 1

"Supplementation" which entailed the addition of known chemicals that would evoke a response from the cells, into the culture at the time of inoculation. This involved the addition of known quorum sensing molecules, butyrolactone I, tyrosol and farnesol, alongside other chemicals that have been expressed in literature as having potential effects on the physiology, morphology and metabolic processes of a microbial culture. One such example is ascorbic acid.

The chemicals that were secreted during *Aspergillus terreus* growth were also used to supplement fresh spores at the time of inoculation. These chemicals were obtained from spore washings – a by-product of the spore collection process, when *A. terreus* agar plates were washed to liberate spores. The washings are assumed to contain the extracellular compounds secreted by the organism during the growth cycle, referred to throughout the thesis as spore

supernatants (SS). The spore supernatants were used as an addition to supplement the spores being inoculated, in both solid and liquid medium. The spore supernatants that were collected were concentrated through freeze drying and also used as an additive to supplement spore inoculum.

In addition to using spore supernatants, larger quantities of *A. terreus* growth culture supernatants were obtained by inoculating a defined medium with high spore numbers (1×10^7 spores/mL) of *Aspergillus terreus*, and culturing for 72 hours at shake flask scale. After which, the biomass was filtered, and the spent medium (referred to as high spore culture supernatants, HSCS) used to supplement the spores that were subsequently inoculated in liquid medium.

Part 2

The second section of this chapter was directed to the investigation of the effects of physical contact between spores. For this, two approaches were attempted. The first was via sonication of spore suspensions of 1×10^3 and 1×10^7 spores/mL prior to inoculation and the second, by altering the space within which the spores were confined to by inoculating 2000 spores in a small 10 mL flask and in a larger 1 L flask. All parameters remain constant, the only factor being altered in the space within which spores are able to move freely. Smaller flasks restrict the space and therefore increases spore to spore interactions. The theory behind subjecting 1×10^3 and 1×10^7 spores/mL to various time periods of sonication was to: a) force spores to separate from one another, b) increase the number of "hits" between the spores as they impinged

on each other, and c) possibly scratch/rupture the spore coats to enable faster germination times.

Throughout these experiments samples were taken and analysed for morphological differences of the cells, including the number of hyphal tips and the diameter of pellets formed. Where possible, the production of the secondary metabolite lovastatin was also assayed for, as was substrate consumption and total biomass. Statistical analysis was carried out throughout.

3.2 Supplementation

3.2.1 Supplementation of Plate Cultures

Solid media was used as the substrate for inoculation of *A. terreus* spores. Spore concentrations between 1×10^1 and 5.17×10^7 spores/mL were used as inocula. The following additions were made to the spores at the time of inoculation in order to see the effect on overall fungal morphology. The controls were, a) spores with no additions (no supplementation, NS), b) spores with ethanol, c) and spores with tween 80. The test cultures were spores with 100 nM of the quorum sensing molecule butyrolactone I, and spores with spore supernatant, collected during spore harvest. Plates were prepared in duplicate, and over a period of 4 days (95 hours) visual observations were made twice daily post inoculation.

Overall, the results showed different inoculum sizes inoculated on plates formed different morphologies. Low spore numbers, once germinated, produced larger colonies that had long, thin highly branched hyphae (figure 3.7). Whereas the high number or spores (5×10^7) produced a lawn of growth, with no defined colony boundaries (figure 3.2). Additions to plates did not produce a large difference in morphologies, therefore liquid culture studies were adopted as more appropriate medium for morphology and productivity analysis.

3.2.1.1 Morphological Studies of Spore Numbers on Solid Media

In this experiment the controls were: a) no additions to the plates, b) ethanol, and, c) tween 80. As previously stated, the quorum sensing molecule butyrolactone I as supplement was dissolved in ethanol, therefore ethanol was also used as a control. Another control was the tween 80, as the spores were initially scraped from the lawn cultures using tween 80.

Figure 3.1 shows images taken of yeast malt agar plates that have been inoculated with 5×10^3 spores/mL with and without various additions. Plates show no real difference in growth in all cases, except where the quorum sensing molecule butyrolactone I was added. Growth is less dense and individual colonies are smaller. Germination was observed 48 hours after inoculation for all conditions.

In plates that were inoculated with the highest spore concentration (figure 3.2), 5×10^7 spores, germination was observed after 18 hours of growth, except where the spore supernatant and quorum sensing molecules were added. The latter began to germinate after 25 hours of growth. Germination was seen as a cloudy layer over the plate surface with no distinct boundaries around colonies. After 48 hours from inoculation these plates had full growth over the surface, and individually germinated spores were undetectable.
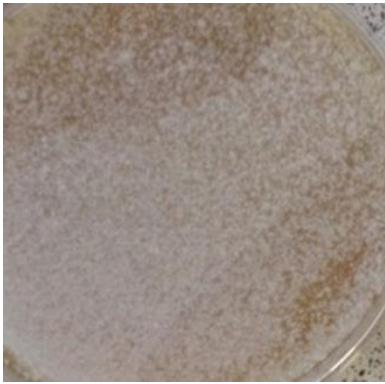
No Supplementation (NS)	Butyrolactone I	Spore Supernatant
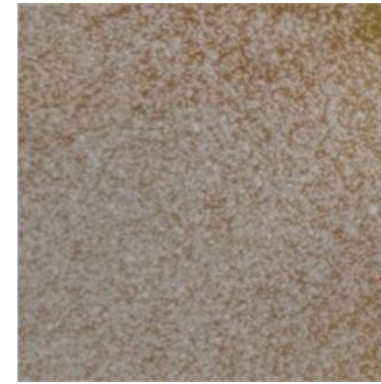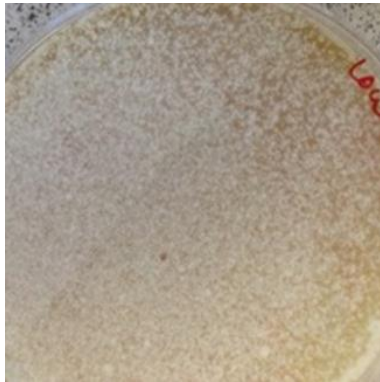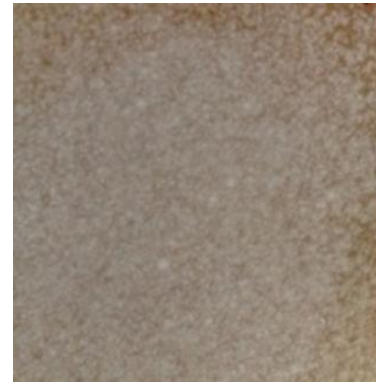
Tween 80	Ethanol

Figure 3.1: *YME plates, inoculated with 5 x 10$_3$ spores (no supplementation, NS), QSM butyrolactone I, Spore supernatants, Tween 80 and ethanol. Images were taken 48 hours after inoculation.*

No Supplementation (NS)   Butyrolactone I   Spore Supernatant
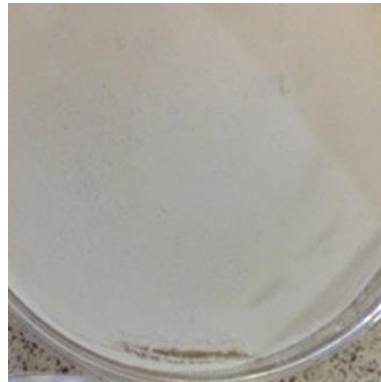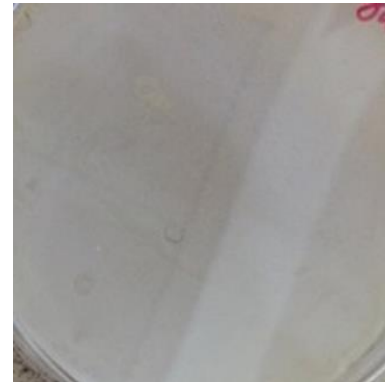
Tween 80   Ethanol

Figure 3.2: *YME plates, inoculated with 5 x 10₇ spores (no supplementation, NS), QSM butyrolactone I, Spore supernatants, Tween 80 and ethanol. Images have been taken 48 hours after inoculation.*

Figures 3.3 and 3.4, show microscopic growth for 4 x 10_3 spores, after 48 and 70 hours, respectively. At 48 hours of growth, in all cases, hyphal tips were long and in abundance. In samples of no supplementation and tween 80, conidiophores were observed at the tips of the hyphae; these conidiophores represent spore production. At 70 hours post inoculation there was an increase in conidiophores, and less free hyphae.

A similar outcome can be seen in the cultivation of 4 x 10_2 spores. Figure 3.5 shows more hyphal branching with no conidiophores at 40 hours post inoculation. Similarly, figure 3.6, shows 4 x 10_2 spores, 70 hours post-inoculation, with a high amount of conidiophores but more visible hyphal branching than that seen in figure 3.3. Figure 3.7 shows the plates 70 hours after inoculation. The growth on the control plates is larger and has more aerial hyphae compared to the test subjects for the same time period.

Conidiophore

No supplementation (NS)

Butyrolactone I

Spore supernatant

Hyphae

Tween 80

Ethanol

Figure 3.3: *Microscopic images, 48 hours post inoculation, of plates inoculated with 4 x 10$_3$ spores (no supplementation, NS), butyrolactone I, spore supernatant, Tween 80 and ethanol. (x40 magnification Nikon eclipse Ci)*

Conidiophore

No Supplementation (NS)   Butyrolactone I   Spore Supernatant

Tween 80   Ethanol

Figure 3.4: *Microscopic images, 70 hours post inoculation, of plates inoculated with 4 x 10$_3$ spores (no supplementation, NS), butyrolactone I, spore supernatant, Tween 80 and ethanol. (x40 magnification Nikon eclipse Ci)*

Hyphae

No supplementation (NS)

Butyrolactone I

Spore Supernatant

Tween 80

Ethanol

Figure 3.5: *Microscopic images, 48 hours post inoculation, of plates inoculated with 4 x 10$_2$ spores (no supplementation, NS), butyrolactone I, spore supernatant, Tween 80 and ethanol. (x40 magnification Nikon eclipse Ci)*

No supplementation (NS)

Butyrolactone I

Spore supernatant

Conidiophore

Tween 80

Ethanol

Figure 3.6: Microscopic images, 70 hours post inoculation, of plates inoculated with 4 x 10$_2$ spores (no supplementation, NS), butyrolactone I, spore supernatant, Tween 80 and ethanol. (x40 magnification Nikon eclipse Ci)

No supplementation (NS)	Butyrolactone I	Spore supernatant
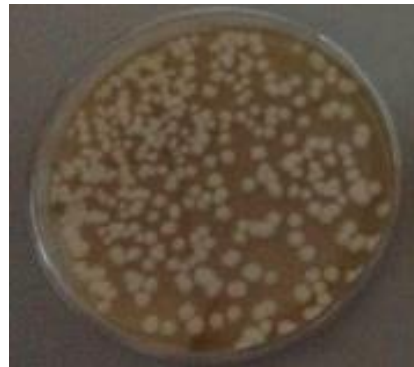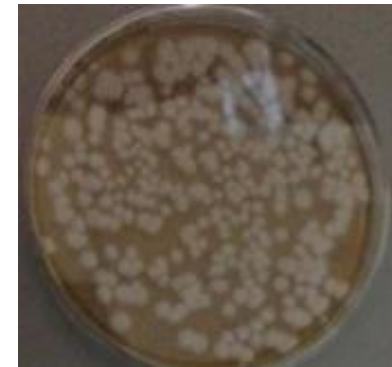
Tween 80	Ethanol

Figure 3.7: *Photographic images, 70 hours post inoculation, of plates inoculated with 4 x 10$_2$ spores (no supplementation, NS),*

butyrolactone I, spore supernatant, Tween 80 and ethanol.

Figures 3.8, 3.9 and 3.10, shows the growth of 1×10^1 (10) spores. In figure 3.8 it can be seen that at 48 hours post inoculation, fungal morphology was of longer hyphae with increased branching and a tight interwoven centre. This becomes clearer at 70 hours post inoculation, as figure 3.9 shows dense growth with an abundance of hyphal tips. In figure 3.10 it is clear that in the control with no supplementation, colonies are larger in size with more hyphae as the growth appears to be 'fluffy'.
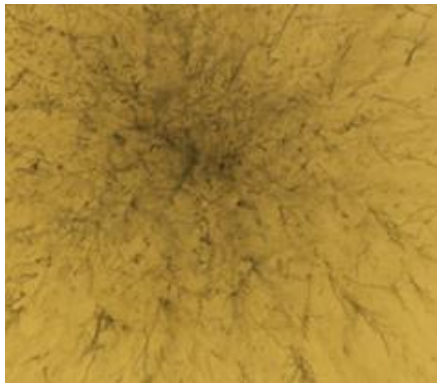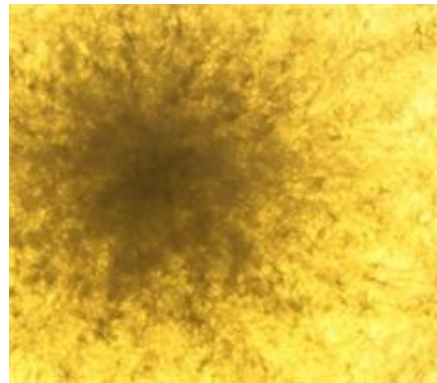
No supplementation (NS)　　　　Butyrolactone I　　　　Spore supernatants

Tween 80　　　　Ethanol

Figure 3.8: *Microscopic images, 48 hours post inoculation, of plates inoculated with 1 x 10₁ (10) spores (no supplementation, NS), butyrolactone I, spore supernatant, Tween 80 and ethanol. (x40 magnification Nikon eclipse Ci)*

No supplementation (NS)

Butyrolactone I

Spore supernatant

Tween 80

Ethanol

Figure 3.9: *Microscopic images 70 hours post inoculation, of plates inoculated with 1 x 10$_1$ (10) spores, (no supplementation, NS), butyrolactone I, spore supernatant, Tween 80 and ethanol. (x40 magnification Nikon eclipse)*

No supplementation (NS) Butyrolactone I Spore supernatant
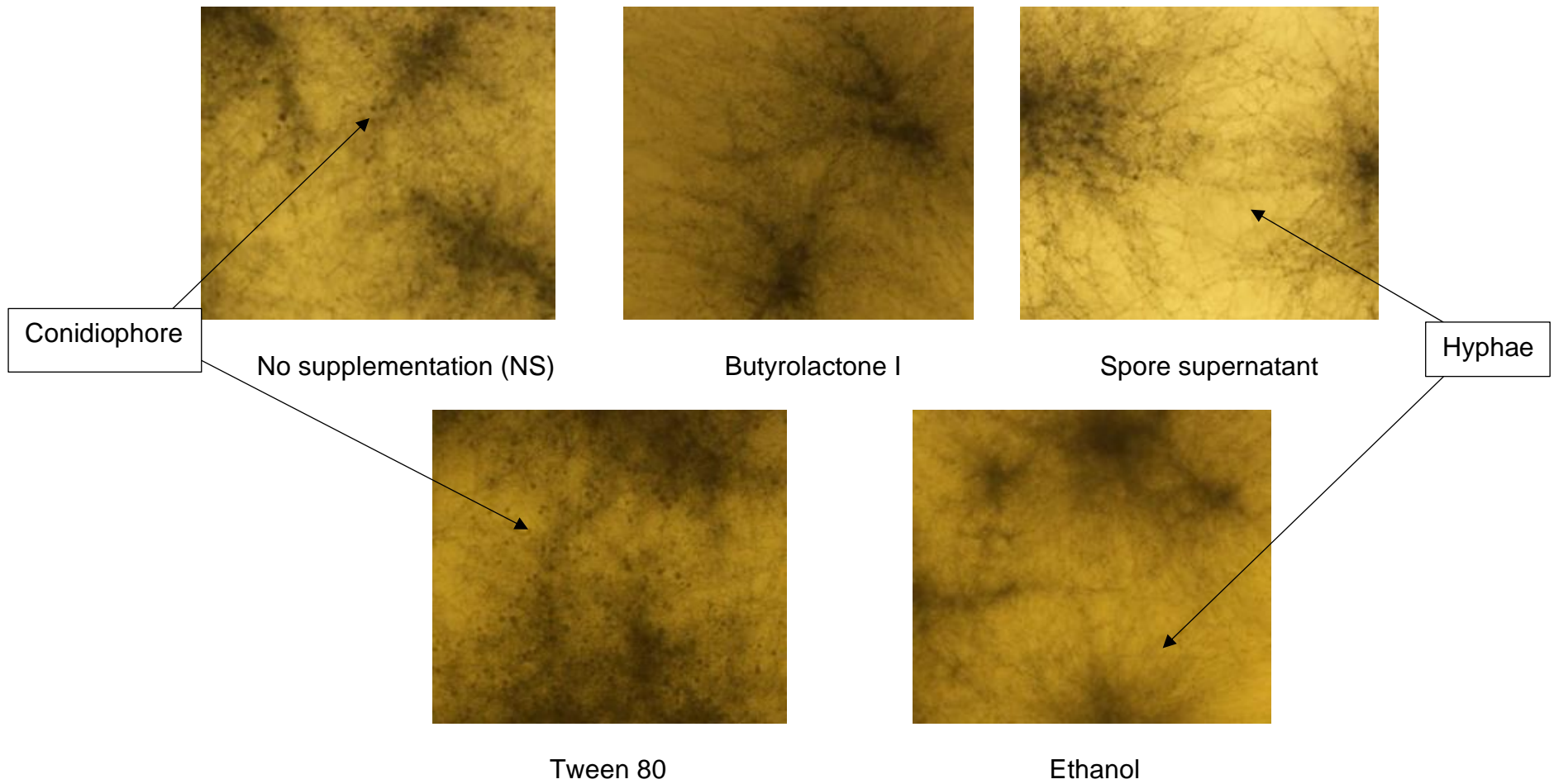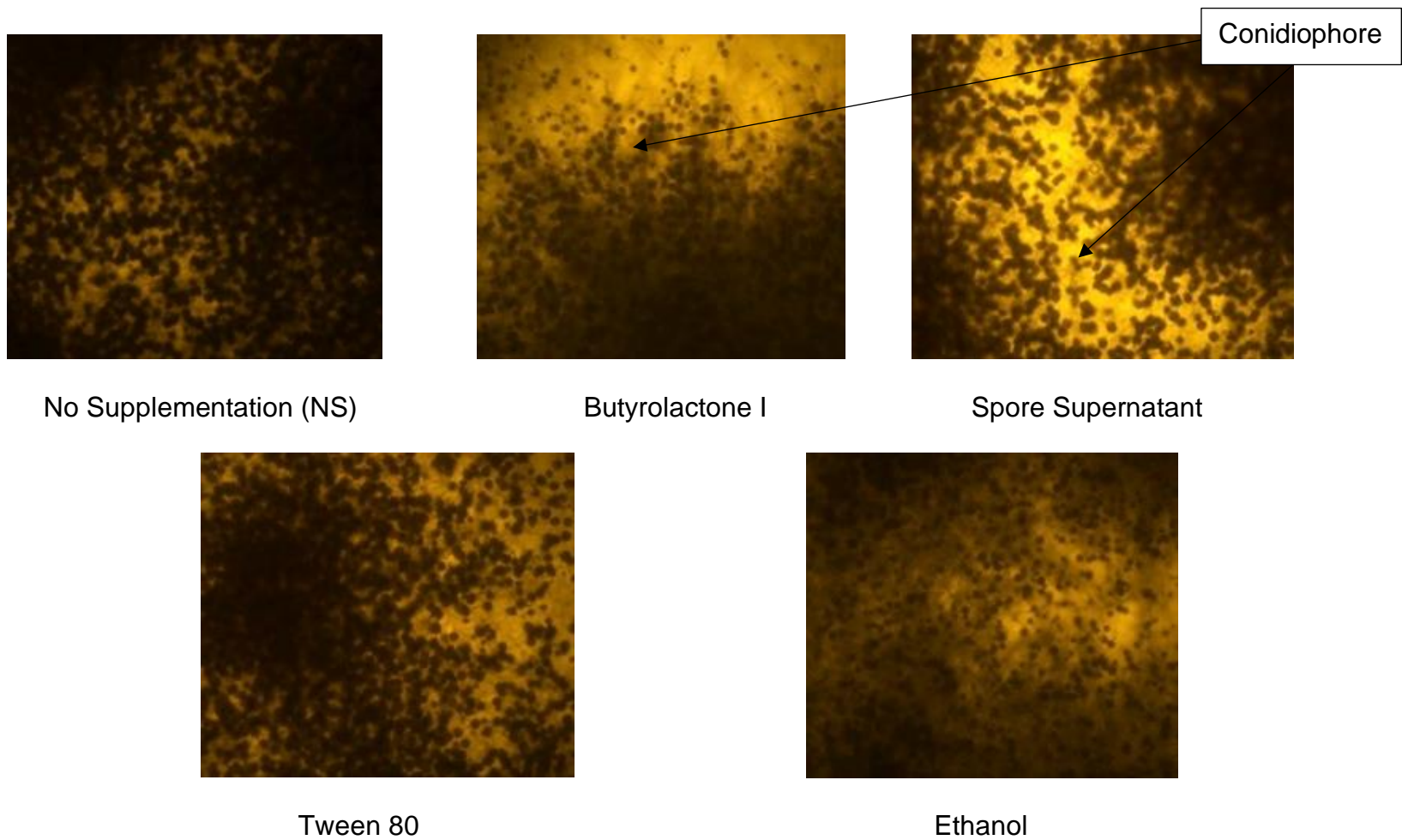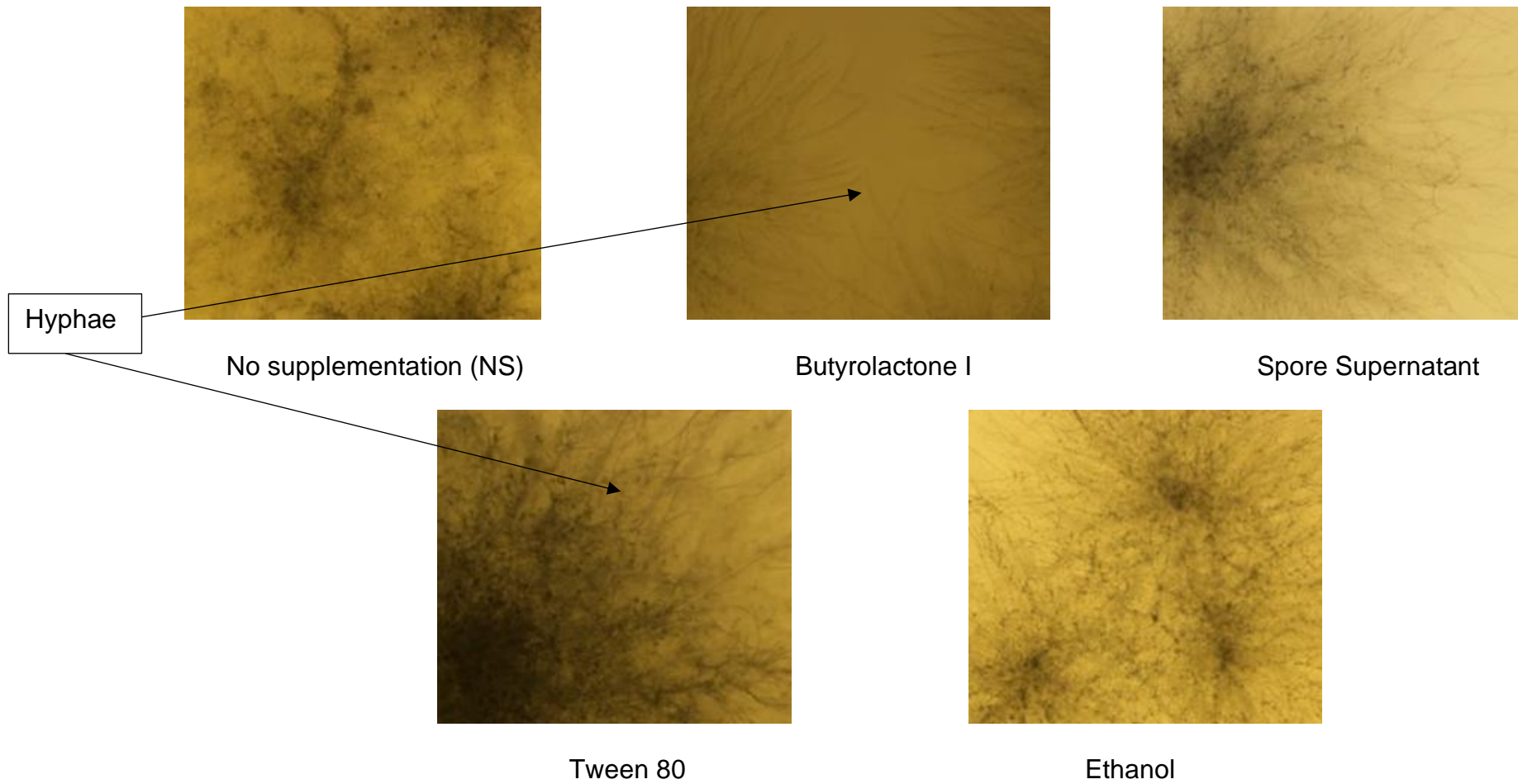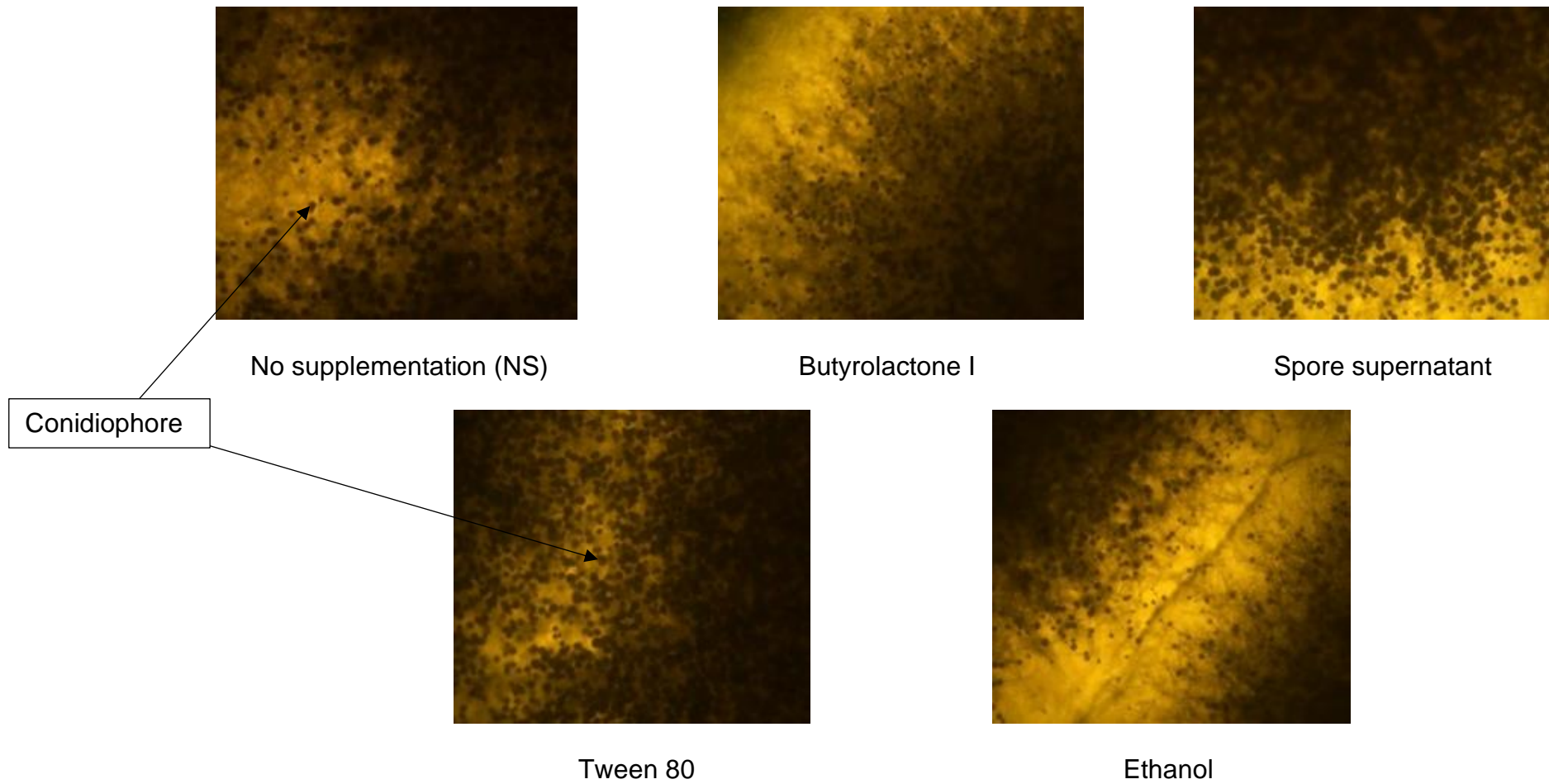
Tween 80 Ethanol

Figure 3.10: Plate images, 70 hours post inoculation, of plates inoculated with 1 x 10$_1$ (10) spores (no supplementation, NS), butyrolactone I, spore supernatant, Tween 80 and ethanol.

3.2.2 Supplementation in Submerged Liquid Fermentation

Following on from solid media studies, liquid cultures were used as culture media, as this method of growth of ideal for extraction of metabolites and is best practice in industry. It also allows for morphological comparisons to be determined. Three different media were adopted and inoculated with High (1 x 10^7 spores/mL) and low (1 x 10^3 spores/mL) with various supplementations made at the time of inoculation so as to analyse for differences in culture morphology and productivity between the spore sizes, media and additives.

Initial work was carried out using the widely available complex medium, potato dextrose broth, to which, butyrolactone I, ascorbic acid, ethanol, tween 80, and spore supernatants (obtained during spore harvest) were added. This was followed by a defined medium rich in glucose and trace elements, in which high spore culture supernatants (spent medium obtained from 72 hour high spore inoculum liquid culture), tyrosol and farnesol were added to test for the effects on fungal morphology. Finally, a medium with high concentrations of the carbohydrate's glucose and lactose, specifically designed for the production of the secondary metabolite lovastatin was used, in which the inoculum was supplemented with 0.01 M farnesol and freeze dried spore supernatants (obtained during spore harvest and concentrated via freeze drying). Samples obtained were analysed for lovastatin production, biomass production and substrate consumption. In each case the additions were made at the time of inoculation.

3.2.2.1 Supplementation of Spores in Complex Medium

Two different spore concentrations of 1 x 10_3 (low) and 1 x 10_7 (high) spores/mL were used to inoculate the complex medium potato dextrose broth. Low spore cultures were supplemented with 100 nM butyrolactone I (prepared in ethanol), spore supernatants, ascorbic acid, and ethanol. Control groups of high and low spore numbers were prepared with no medium supplementation.

Butyrolactone I has been identified as a secondary metabolite and as a QSM in *A. terreus.* Schimmel *et al.,* (1998) reported that in some bacterial cultures small γ-butyrolactone containing molecules are produced that act as self-regulating factors, and can control a variety of functions, including antibiotic production, bioluminescence, biofilm formation and virulence factor production (Beppu, 1995; Davies *et al.,* 1998). In eukaryotes butyrolactones have the ability to inhibit cyclin dependent kinases (protein kinases that have the ability to control cell cycle progression in all eukaryotes, regulated by phosphorylation and dephosphorylation of serine, threonine and tyrosine residues) (Nishio *et al.,* 1996; Kanemitsu *et al.,* 1998). This gives a basis for why butyrolactone I is added to spore cultures, as it is widely known to have effects on cultures, and in particular cultures of *A. terreus,* it is theorised that cultures will respond to its presence.

Research by Raina *et al.,* (2012) found that culture supernatants from *A. terreus* contained butyrolactone I, and concentrations would continue to increase up to 48 hours post inoculation. This therefore gives a sound basis

for the use of spore supernatants as an additive to spore inoculum concentrations.

Experimental data in figures 3.11 and 3.12 show pellet diameters and hyphal tip numbers for low spore numbers that have been supplemented with butyrolactone I, spore supernatants, ascorbic acid, and ethanol. The number of hyphal tips and pellet diameters for cultures inoculated with a high spore concentration of 1×10^7 spores/mL was also plotted on the same graphs so as to allow for comparison. Ideal morphology is that of small loose pellet, with a high number of hyphal tips, such as that seen when high concentrations of spores are used as inoculum.

In each of the cases, pellet diameters increased significantly in size compared to the low spore inoculum control. Hyphal tip numbers increased slightly when compared to the low spore number control cultures, but where the quorum sensing molecule butyrolactone I was added, the hyphal tip numbers were slightly more than that of the other test samples. At 40 hours diameters of low spore cultures supplemented with SS and AA were smaller than that of low spore control cultures. At 90 hours post inoculation ideal morphology seen with addition of butyrolactone I. Overall ethanol additions decreased pellet diameters by almost half the size of that seen in low spore control cultures.

This study compared low spore culture with additions to the high spore control group and low control group. At 65 hours post, cultures with additions of SS, butyrolactone I, AA and ethanol decreased in pellet size, smaller than that of

low spore control cultures, but pellets were larger than that of high spore control cultures. At the end of run, 90 hours post inoculation, diameters of cultures with additions of butyrolactone I, SS, and AA increased in size to greater than that of low spore control cultures and were greater than that of high spore control cultures (figure 3.11). In all conditions, 90 hours post inoculation, hyphal tip numbers were greater than of both high and low spore control cultures. However, increase was greater in cultures with the quorum sensing molecule (figure 3.12).

Figure 3.11: *Pellet diameter (µm) against time for high spore number inoculum (1 x 10$_7$ spores/mL) control cultures (red), low spore number inoculum (1 x 10$_3$ spores/mL) control cultures (blue), low spore number cultures supplemented with SS (purple), butyrolactone I (green), A.A (orange) and ethanol (yellow). Experiments were carried out in triplicate, 30 pellets per sample were analysed microscopically. T-test carried out comparing test conditions against controls for both high and low spores where p<0.05, therefore the data is significantly different. Standard deviation error bars shown.*
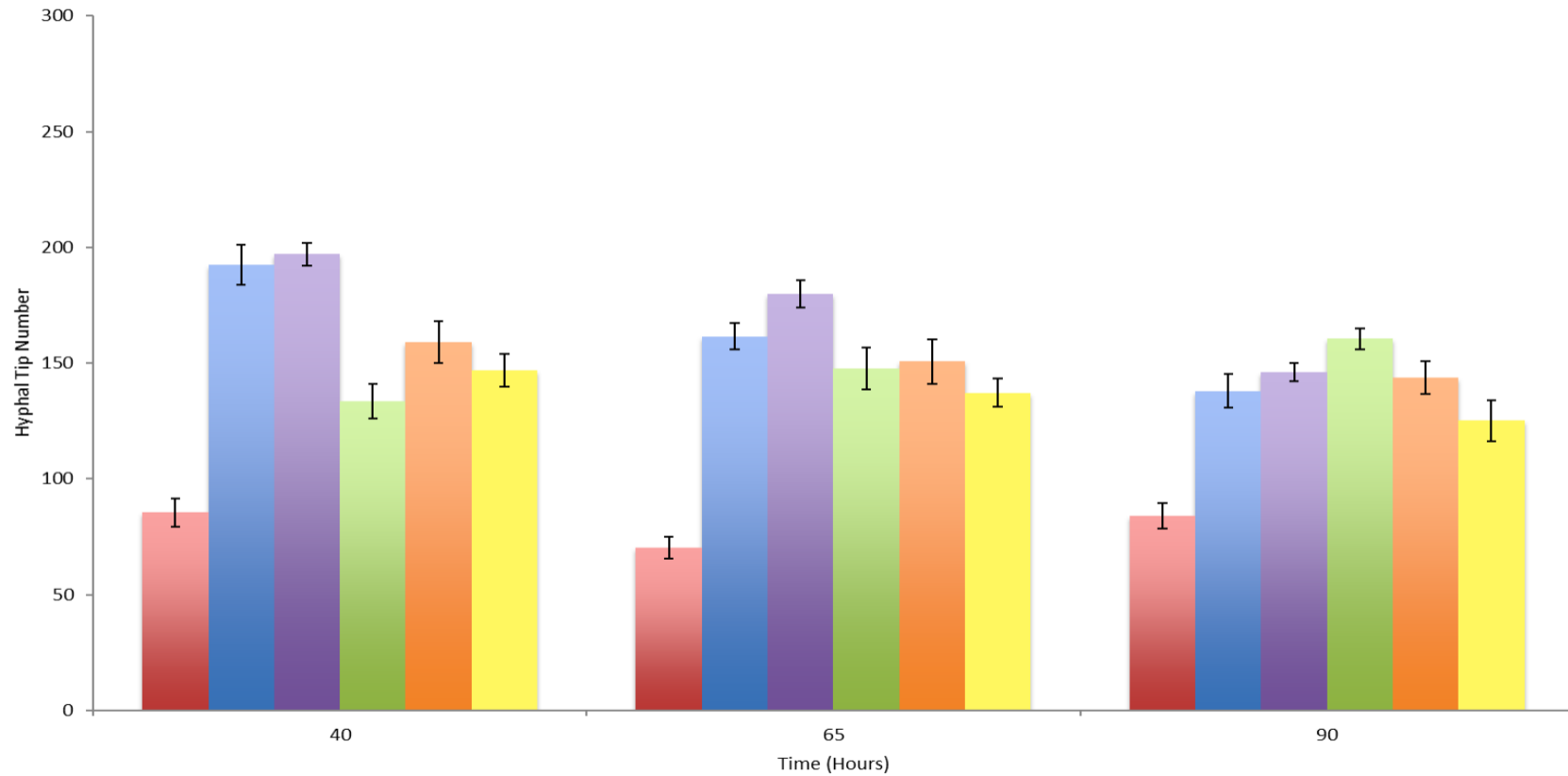
Figure 3.12: *Hyphal tip number against time for high spore number inoculum (1 x 10$_7$ spores/mL) control cultures (red), low spore number inoculum (1 x 10$_3$ spores/mL) control cultures (blue), low spore number cultures supplemented with SS (purple), butyrolactone I (green), A.A (orange) and ethanol (yellow). Experiments were carried out in triplicate, 30 pellets per sample were analysed microscopically. T-test carried out comparing test conditions against controls for both high and low spores where p<0.05, therefore the data is significantly different. Standard deviation error bars shown.*

3.2.2.2 Supplementation of Spores in a Defined Medium

The spent medium from a 72 hour, high spore (1 x 10^7 spores/mL) culture was added to the defined medium and then inoculated with 1 x 10^3 spores/mL. Morphological characteristics were assayed. Hyphal tip numbers and pellet diameters were plotted against time for 1 x 10^3 spores/mL with and without the high spore culture supernatant (HSCS), and 1 x 10^7 spores/mL (figure 3.13).

Figures 3.13 and 3.14 show pellet diameter and the number of hyphal tips against time, respectively. Inoculum concentrations of 1 x 10^7 spores/ mL (high and, 1 x 10^3 spores/mL (low) and 1 x 10^3 spores/mL (low) supplemented with high spore culture supernatants (HSCS) is represented in each of the graphs. In low spore cultures pellet formation is observed 20 hours post inoculation, whereas in high spore cultures pellet formation is observed 18 hours post inoculation. As time progresses pellet diameters of low spores with HSCS increase, from 20 to 70 hours post inoculation, diameters are smaller than that of low spore control cultures. Between 40 and 45 hours diameters are closer to that observed in high spore controls. Specifically, at 40 hours, diameters of HSCS is same as that of high spore control.

Figures 3.15, 3.16 and 3.17, show some of the specimens that were analysed. The images obtained vary greatly. In figure 3.15, germination of high spore numbers was earlier than that of both low spores numbers (figure 3.16) and low spore numbers supplemented with HSCS (figure 3.17). As germination is

observed 18 hours post inoculation in high spore cultures, and at 20 hours post inoculation for both low spore cultures and low spore cultures supplemented with HSCS.

The images and the graphs show that cultures from high spore inoculum are small in size with a high abundance of hyphal tips. However, in the test sample of low spore numbers with HSCS, diameters are smaller than the control of low spore numbers (until 70 hours), and the number of hyphal tips are higher (after 70 hours).

This experiment showed that the addition of high spore culture supernatants increased pellet diameters and hyphal tip numbers compared to the low spore control group ($p < 0.05$) at 90 hours post inoculation.
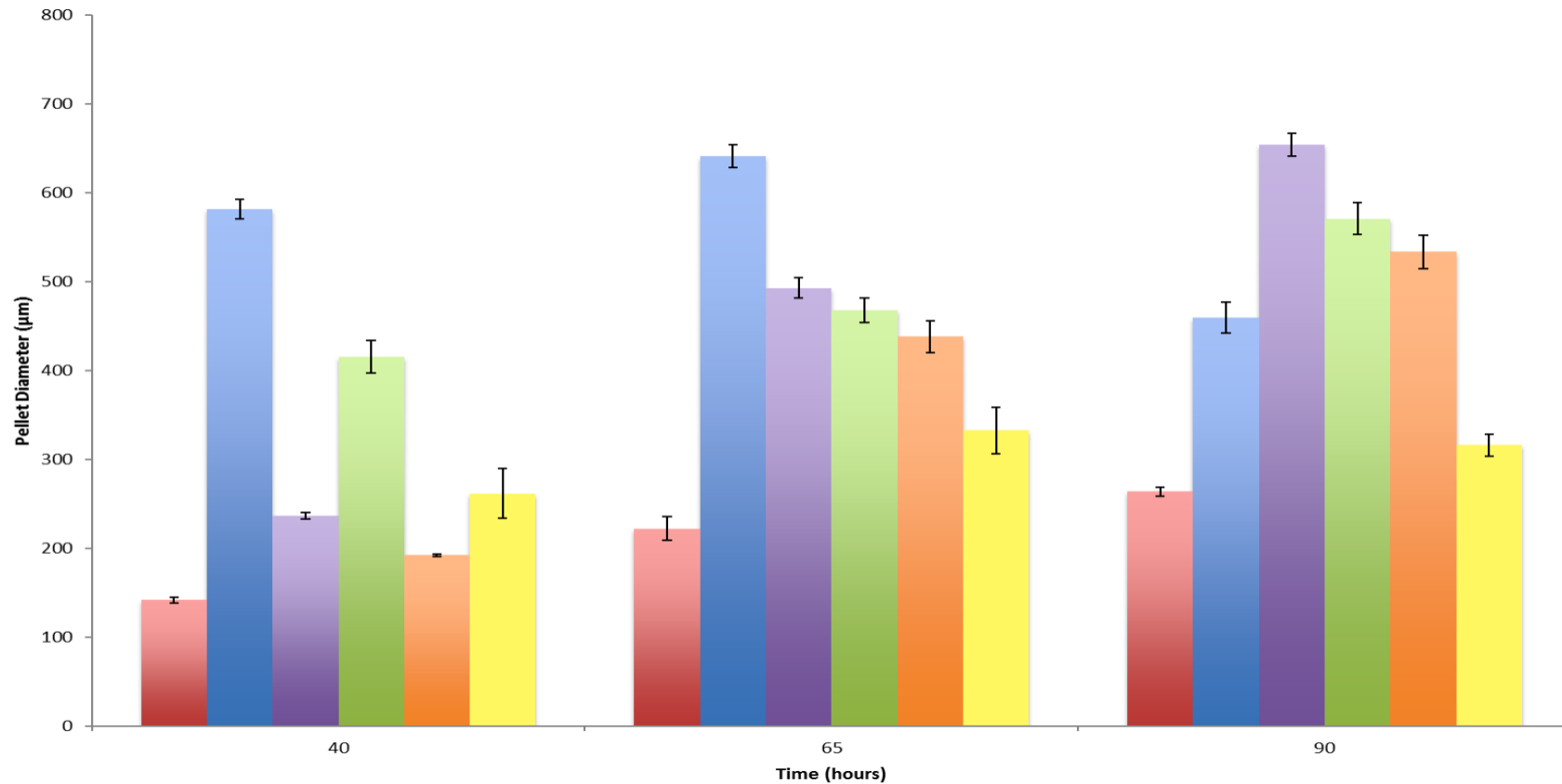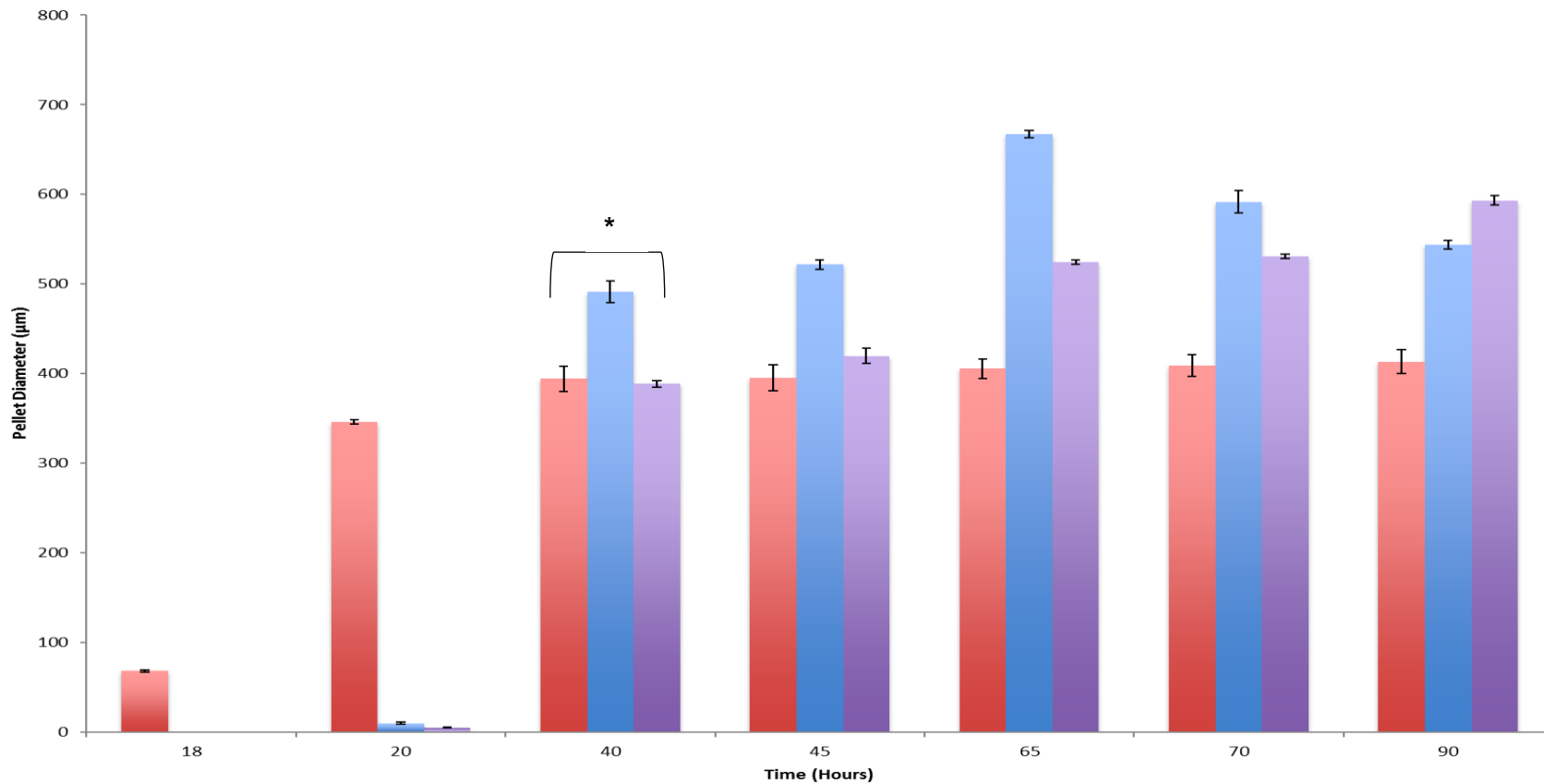
Figure 3.13: *Pellet diameter (μm) against time for high spore number inoculum (1x10^7 spores/mL) control cultures (red), low spore number inoculum (1x10^3 spores/mL) control cultures (blue) and low spore number cultures supplemented with HSCS (purple). Experiments were carried out in triplicate, 30 pellets per sample were analysed microscopically. T-test carried out comparing all data of HSCS against control group of high and low spores where p<0.05, therefore the date is significantly different, however at 40hours *T-test shows no difference between data sets. Standard deviation error bars shown.*
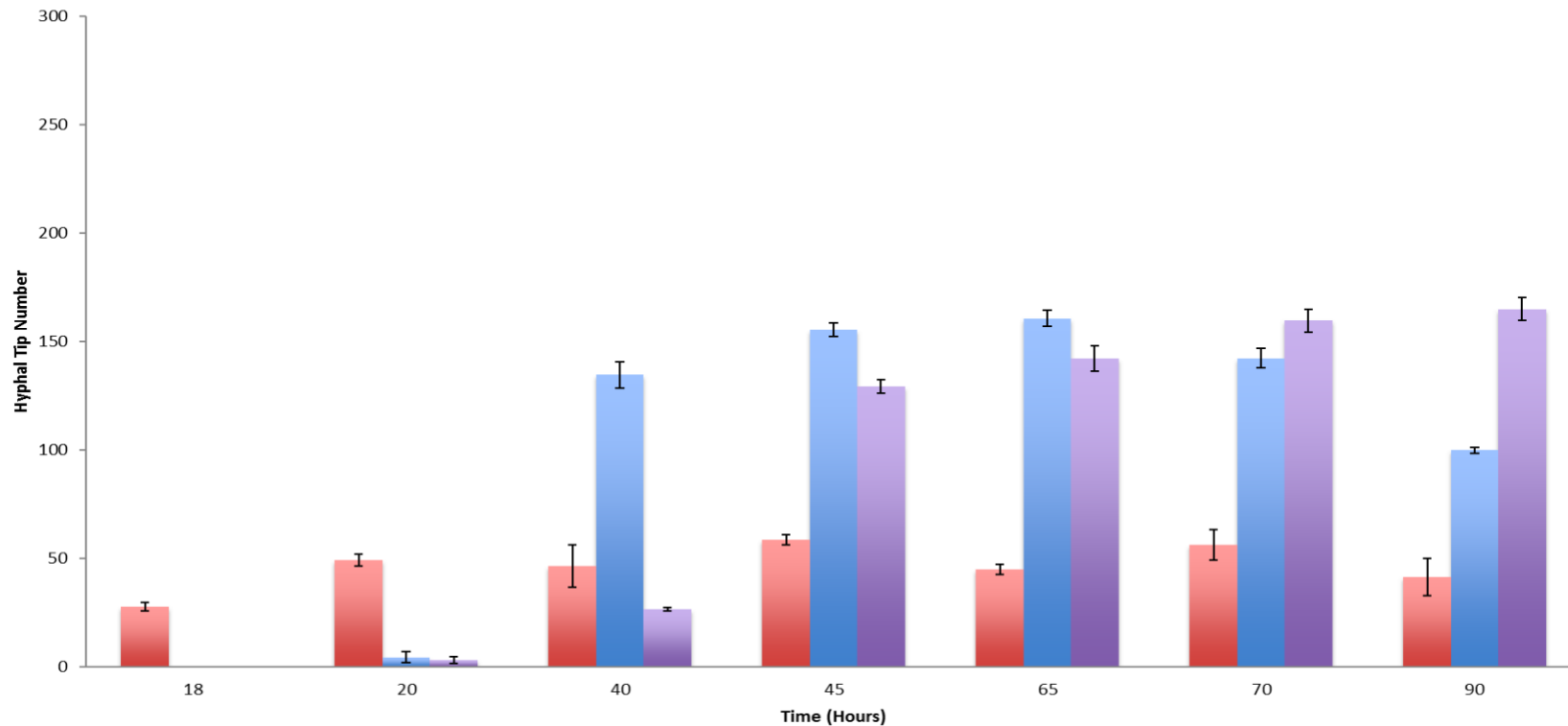
Figure 3.14: *Hyphal tip numbers against time for high spore number inoculum ($1x10^7$ spores/mL) control cultures (red), low spore number inoculum ($1x10^3$ spores/mL) control cultures (blue) and low spore number cultures supplemented with HSCS (purple). Experiments were carried out in triplicate, 30 pellets per sample were analysed microscopically. T-test carried out comparing all data of HSCS against control group of high and low spores where $p<0.05$, therefore the data is significantly different. Standard deviation error bars shown.*
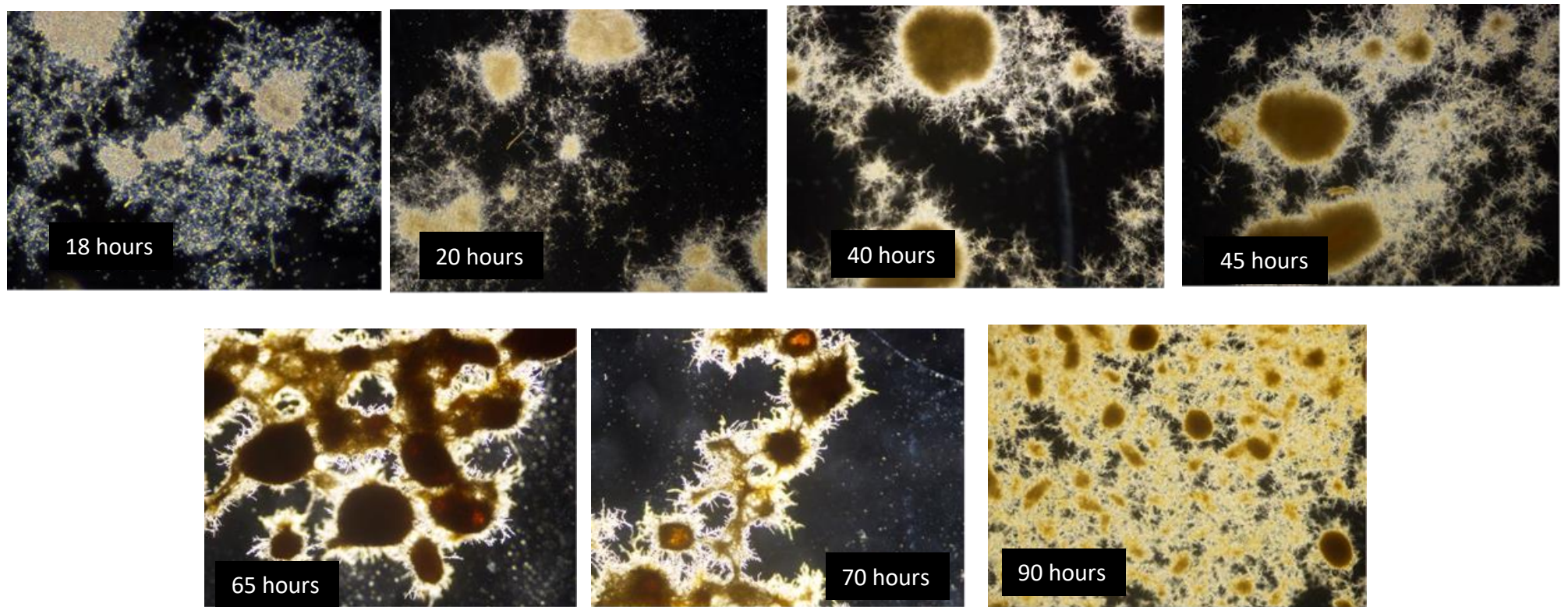
Figure 3.15: *The progression from 18 to 90 hours of the morphology of submerged cultures of $1x10_7$ (high) spores/mL were used as inoculum. Images are a representation of the overall population sampled. Images taken at x4 magnification. Phase contrast used throughout.*
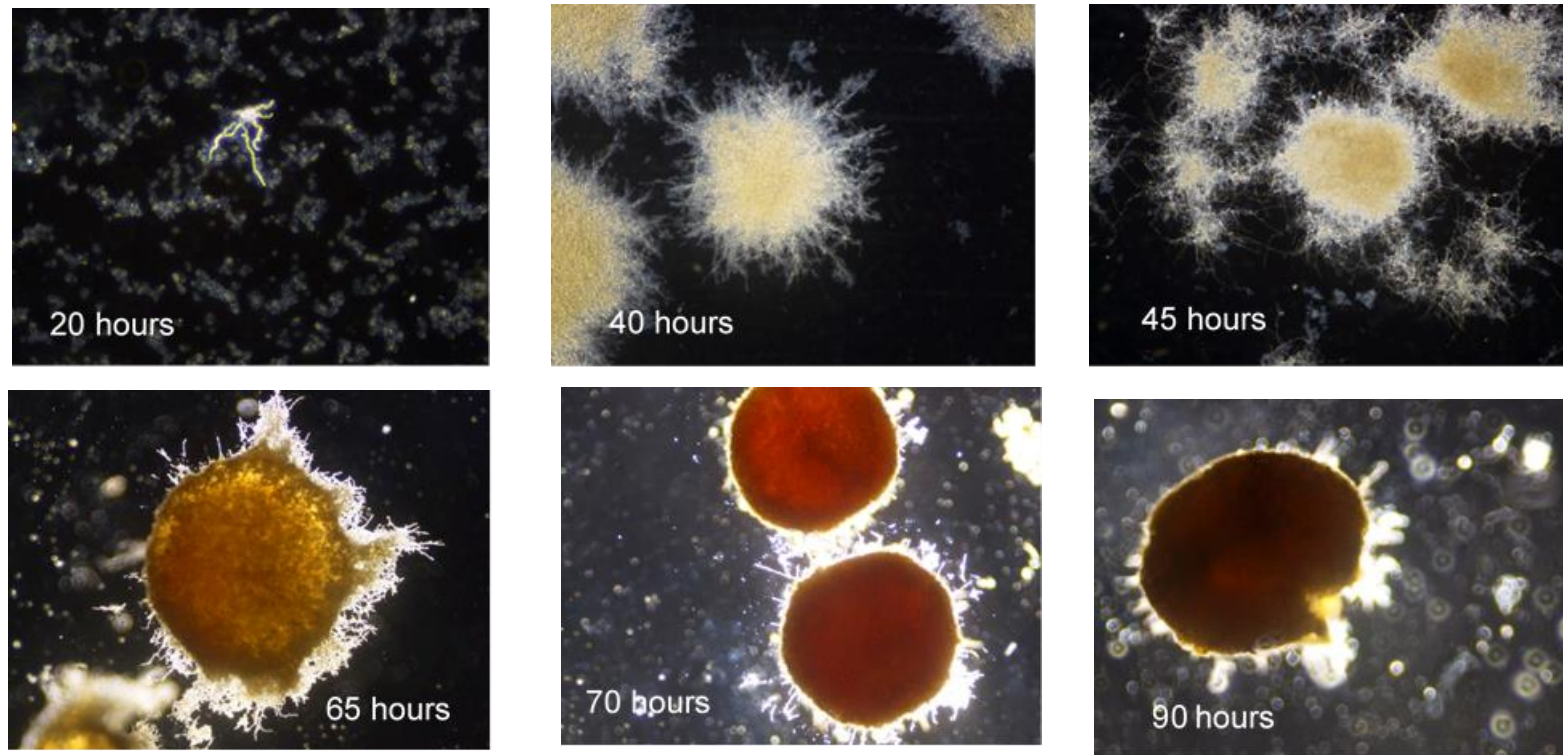
Figure 3.16: *The progression from 20 to 90 hours, of the morphology of submerged cultures of $1x10_3$ (low) spores/mL were used as inoculum. Images are a representation of the overall population sampled. Image at 20 hours taken at x10 magnification, all other images at x4 magnification. Phase contrast used throughout.*
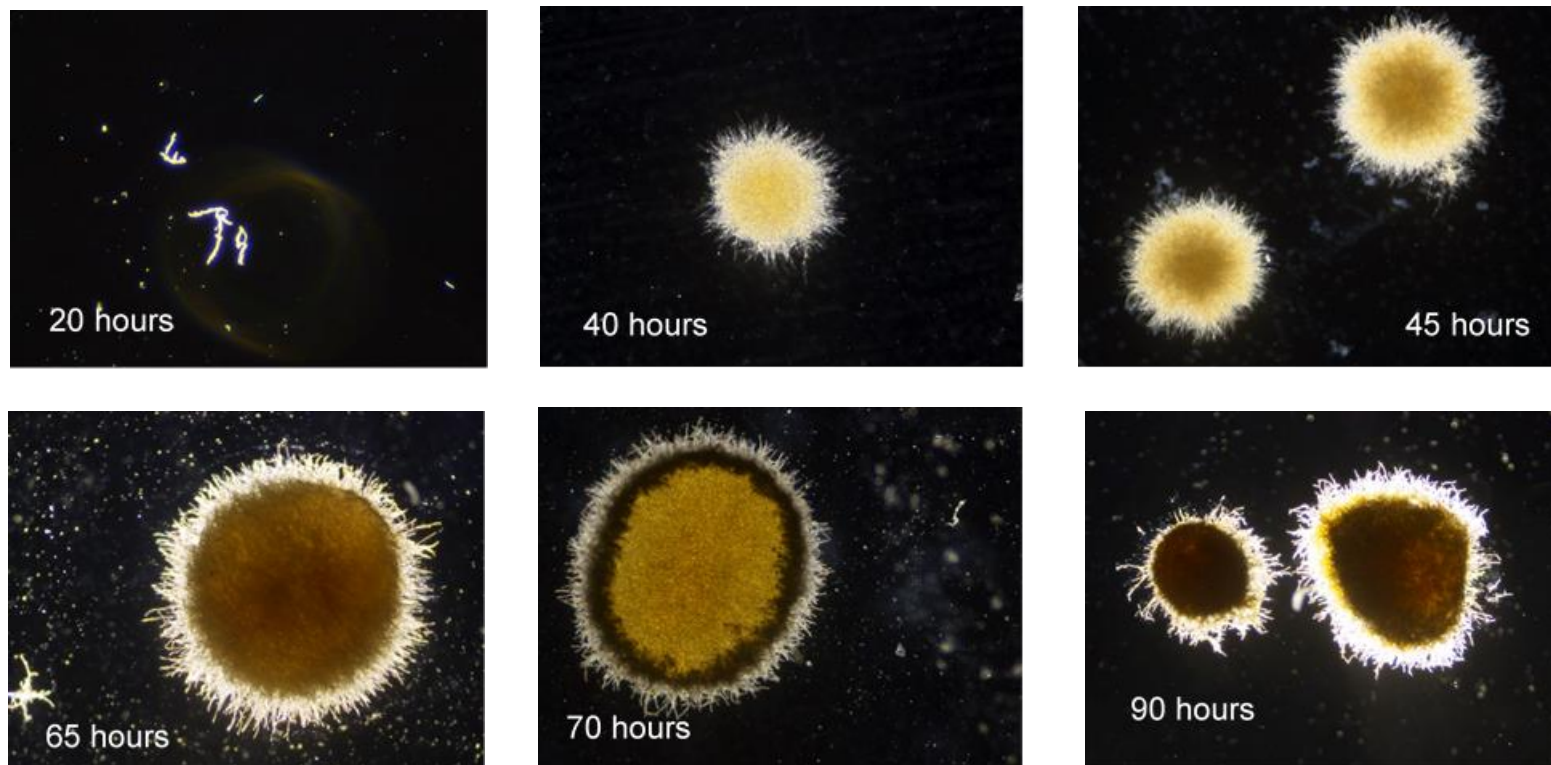
Figure 3.17: *The progression from 20 to 90 hours, of the morphology of submerged cultures of 1x10₃ (low) spores/mL supplemented with high spore culture supernatant were used as inoculum. Images are a representation of overall population sampled. Image at 20 hours, taken at x10 magnification, all other images x4 magnification. Phase contrast used throughout.*

3.2.3 Supplementation of the defined medium with Quorum Sensing Molecules

Research suggests a trans-species QS process involving QS molecules. On this basis, this research focused on the QS molecules farnesol and tyrosol as additives to high and low spore inocula, so as to see the effects that these molecules have on the morphology of the culture.

3.2.3.1 Tyrosol

Investigations carried out by Chen *et al* (2004) found that additions of tyrosol to highly diluted cultures enabled exponential growth without a substantial lag phase. Chemically synthesised tyrosol (minimum concentration of >10 µM) also shortened lag phase.

Additions of 0.04 and 0.01 M tyrosol were made to high and low spore concentrations at the time of inoculation. DMSO concentrations of 0.04 and 0.01 M were also made to determine effect on the culture as tyrosol was dissolved in this. Additions of tyrosol to both high and low spore cultures resulted in pellet diameters smaller than that of high and low spore control cultures (respectively) (figure 3.18 and 3.19). However, in high spore cultures hyphal tip numbers decreased (figure 3.20), similarly in low spore cultures tip numbers also decreased, except with additions of 0.01 M tyrosol, where hyphal tip numbers were greater than that of low spore control cultures and

that of high spore control cultures (figure 3.21). Additions of DMSO as control should not have affected the culture, however in high spore cultures pellet diameters increased (figure 3.18) and hyphal tip numbers decreased (figure 3.20). In low cultures at 60 hours, DMSO caused diameters to be smaller than that of low spore control cultures, but at 85 hours addition of 0.01 M DMSO, caused pellets to be greater in size than that of low control cultures (figure 3.19) the same pattern in seen with regard to hyphal tip numbers (figure 3.21).

Figure 3.18: Pellet diameter against time for high spore numbers (1x10₇ spores/mL) control culture (red), high spore numbers inoculated with 0.04 M tyrosol (purple), 0.01 M tyrosol (green), 0.04 M DMSO (orange) and 0.01 M DMSO (yellow). Experiments carried out in triplicate, 30 pellets per sample taken was analysed. T-Test carried out comparing test conditions to control where p<0.05, data significantly different. Standard deviation error bars plotted.
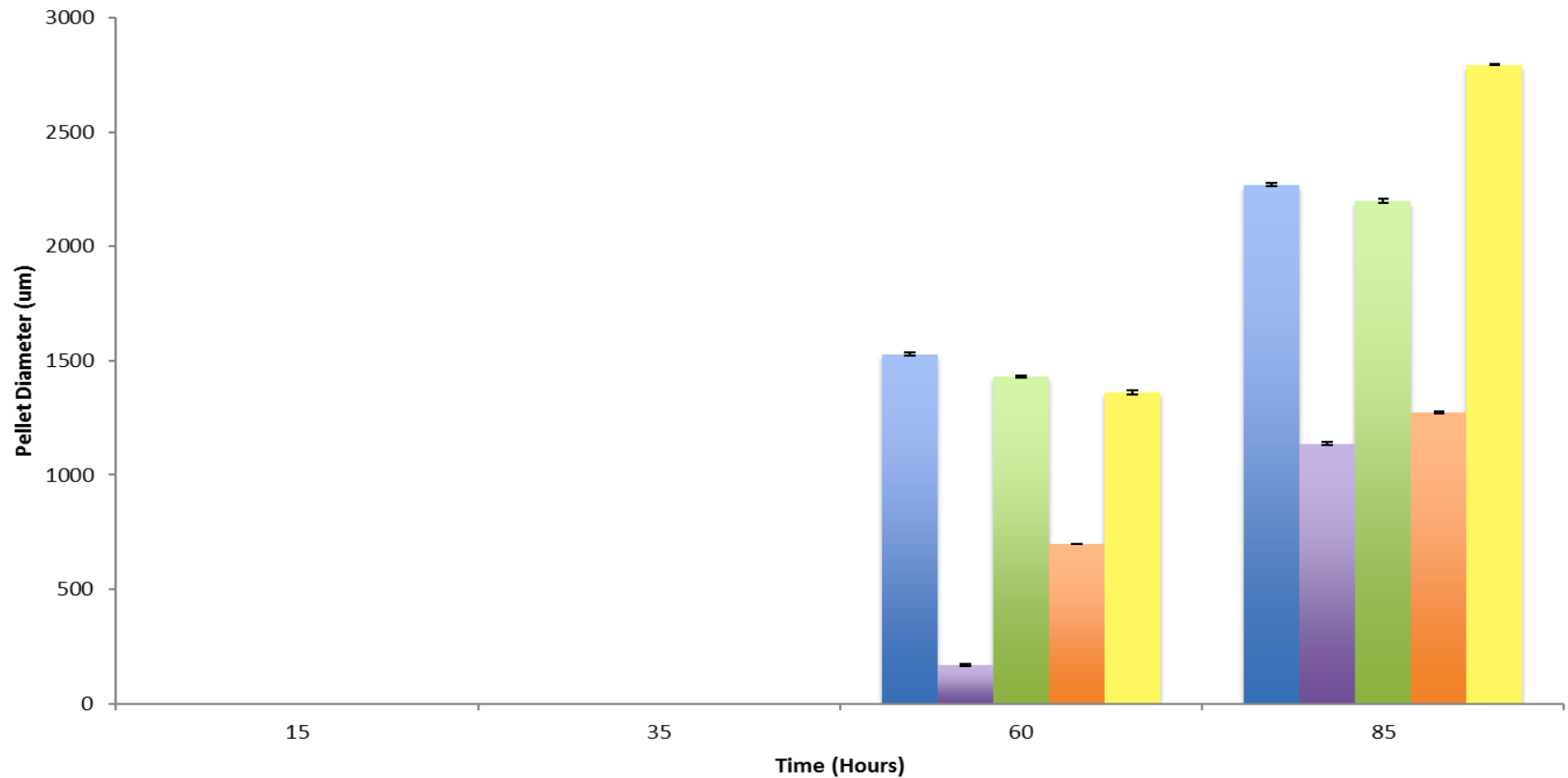
Figure 3.19: *Pellet diameter against time for low spore numbers ($1x10_3$ spores/mL) control culture (blue), low spore numbers inoculated with 0.04 M tyrosol (purple), 0.01 M tyrosol (green), 0.04 M DMSO (orange) and 0.01 M DMSO (yellow). Experiments carried out in triplicate, 30 pellets per sample taken was analysed. T-Test carried out comparing test conditions to control where $p<0.05$, data significantly different. Standard deviation error bars plotted.*
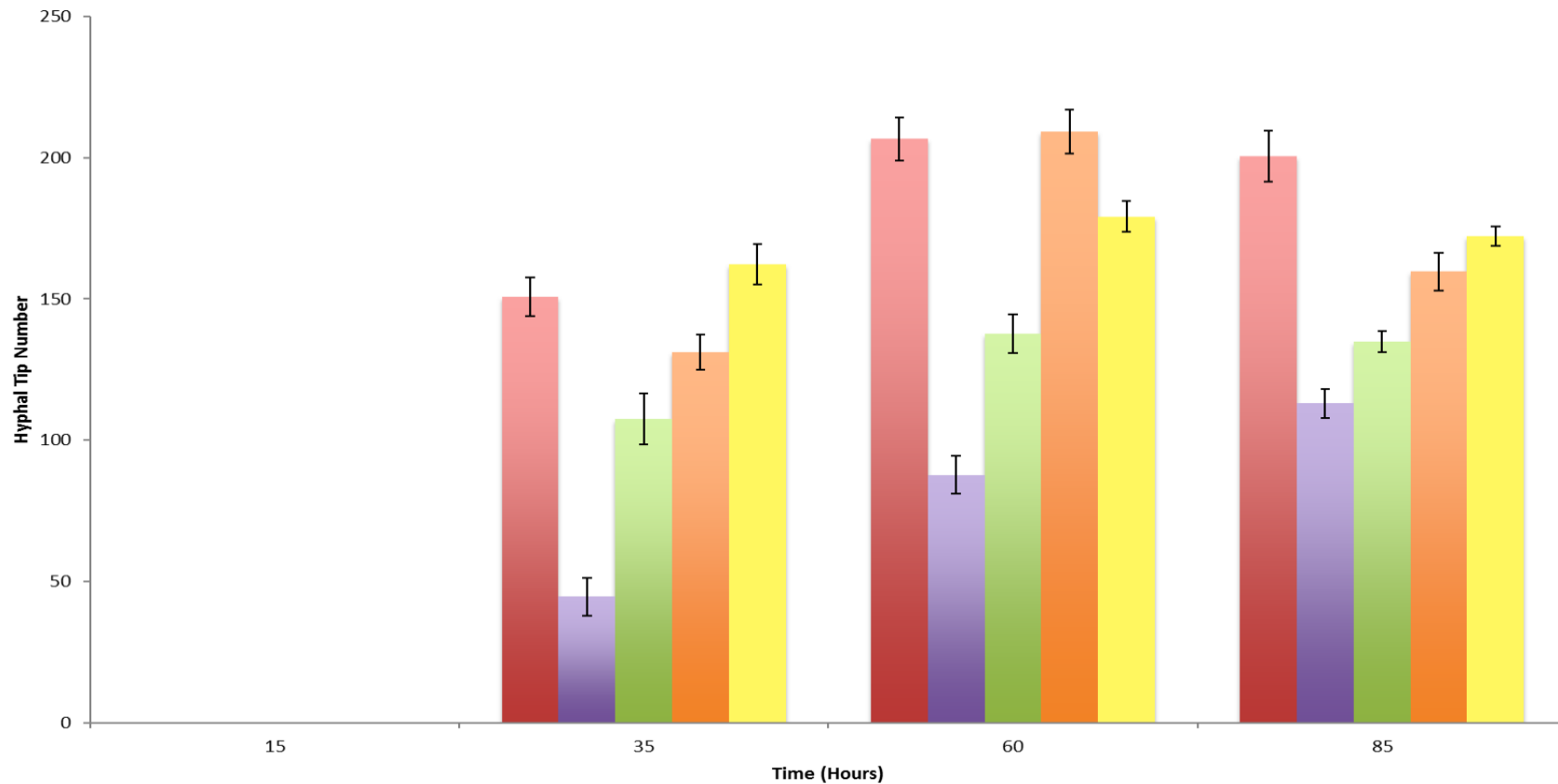
Figure 3.20: *Hyphal tip numbers against time for high spore numbers ($1x10_7$ spores/mL) control culture (red), low spore numbers inoculated with 0.04 M tyrosol (purple), 0.01 M tyrosol (green), 0.04 M DMSO (orange) and 0.01 M DMSO (yellow). Experiments carried out in triplicate, 30 pellets per sample taken was analysed. T-Test carried out comparing test conditions to control where p<0.05, data significantly different. Standard deviation error bars plotted.*
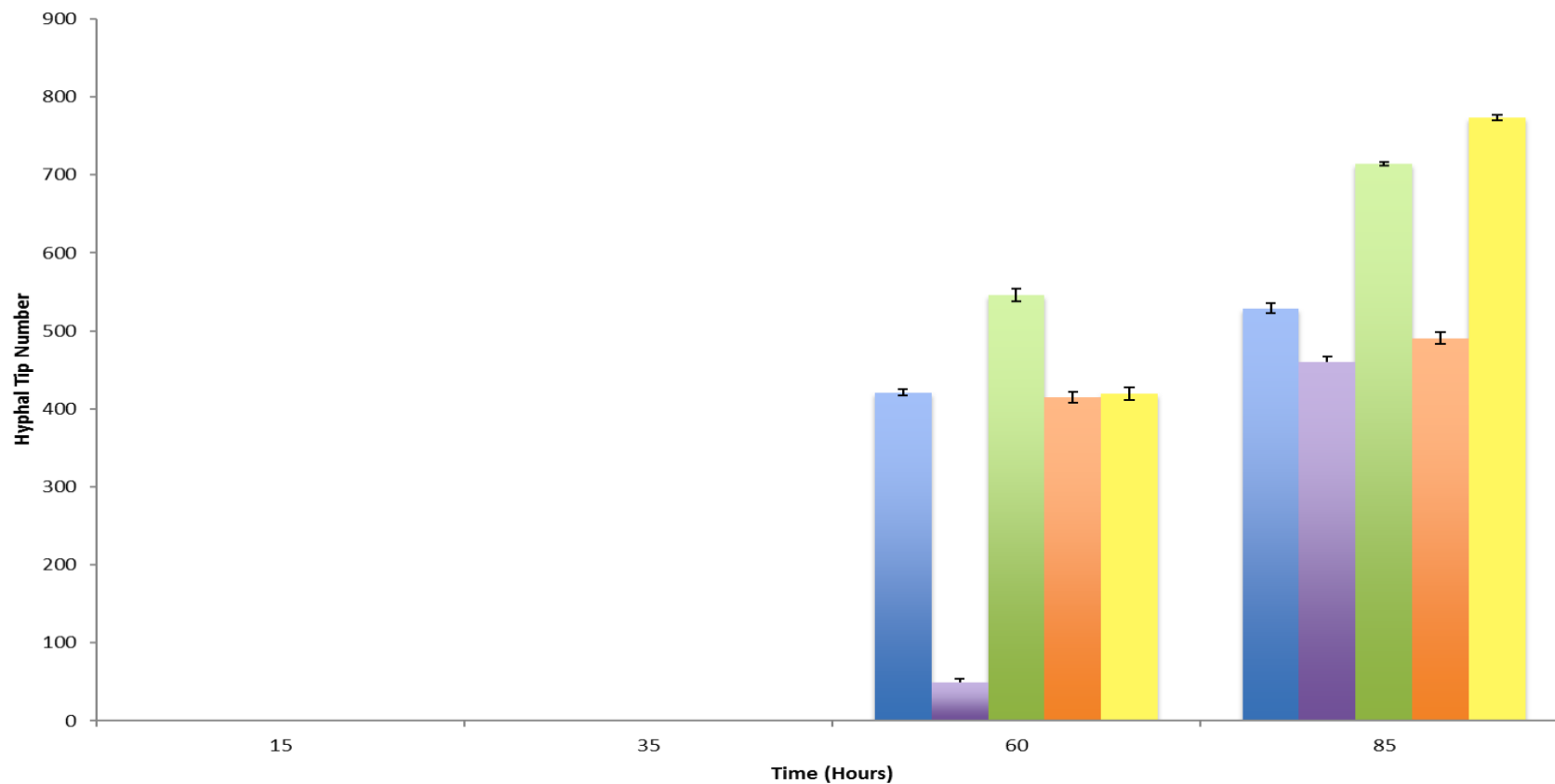
Figure 3.21: *Hyphal tip numbers against time for low spore numbers (1×10^3 spores/mL) control culture (blue), low spore numbers inoculated with 0.04 M tyrosol (purple), 0.01 M tyrosol (green), 0.04 M DMSO (orange) and 0.01 M DMSO (yellow). Experiments carried out in triplicate, 30 pellets per sample taken was analysed. T-Test carried out comparing test conditions to control where $p < 0.05$, data significantly different. Standard deviation error bars plotted.*
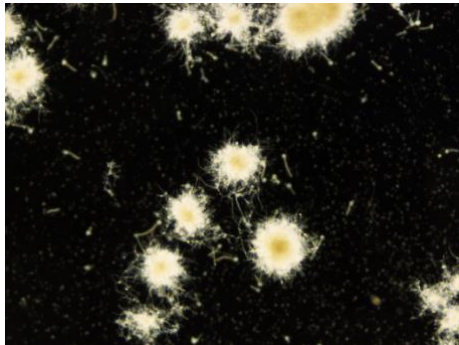
0.04 M Tyrosol
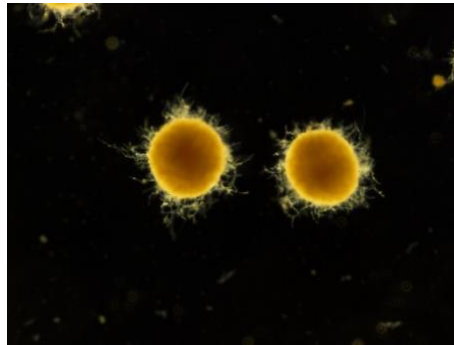
0.01 M Tyrosol
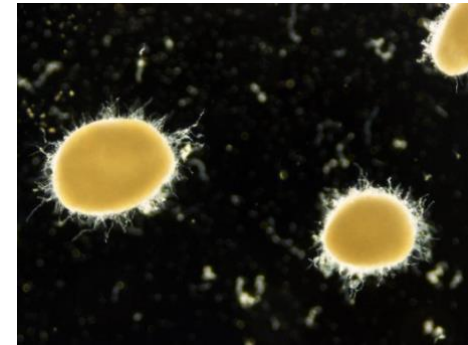
0.04 M DMSO
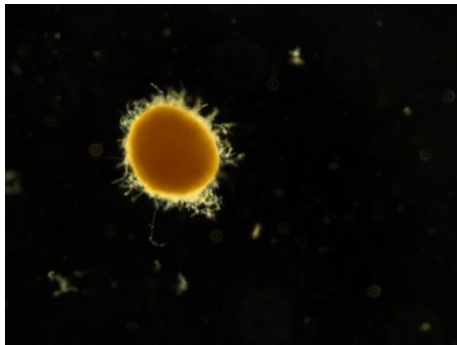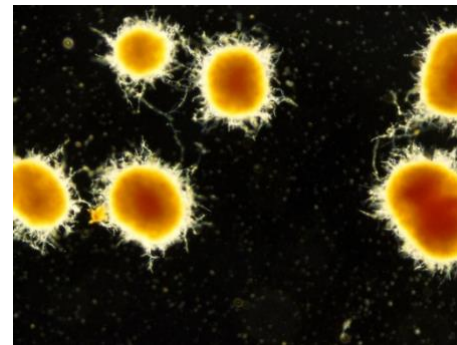
0.01 M DMSO

Control (No additions)

Figure 3.22: *Microscopic images, 85 hours post inoculation of high spore numbers (1 x 10$_7$ spores/mL) supplemented with 0.04 and 0.01 M tyrosol, 0.04 and 0.01 M DMSO and control with no supplementation. Images are a representation of samples taken. Magnification of x4 with phase contrast used.*

0.04 M Tyrosol   0.01 M Tyrosol   0.04 DMSO

0.01 M DMSO   Low spores (Control)
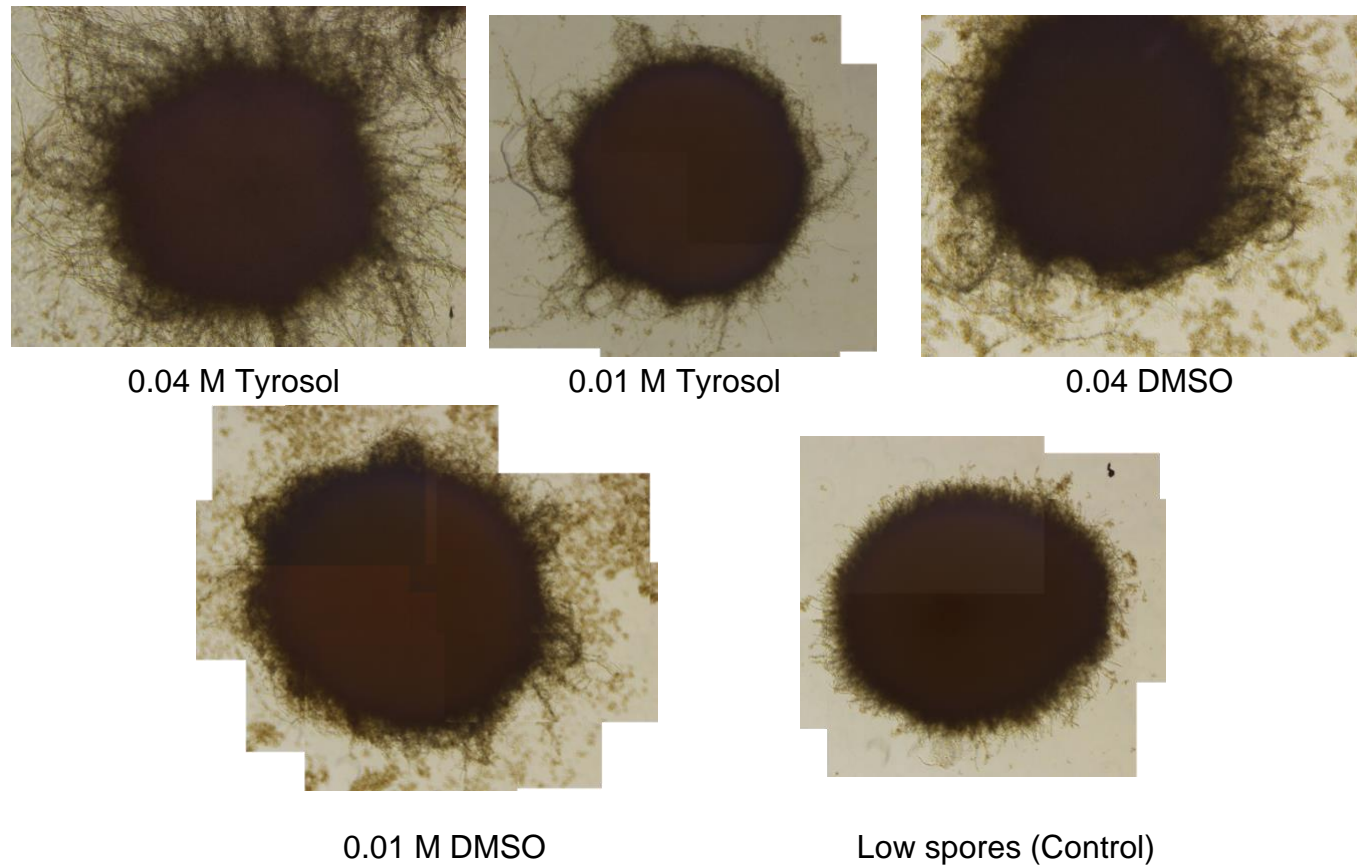
Figure 3.23: *Microscopic images, 85 hours post inoculation of low spore numbers (1×10^3 spores/mL) supplemented with 0.04 and 0.01 M tyrosol, 0.04 and 0.01 M DMSO and the control with no supplementation. Images are a representation of samples taken. Images have been brought together to form one pellet as whole. Magnification of x4 with phase contrast used.*

Table 3.1: *Percentage change in hyphal tip number and pellet diameter for test samples of high and low spore inoculum with tyrosol or DMSO compared to the control samples of high and low spore inoculum concentrations, where no additions to the inoculum was made. Diameters, tip numbers, and % changes are for cultures at the end of the experimental run, 85 hours post inoculation.*

Inoculum size (spores/mL)	Parameter	0.04 M tyrosol	0.01 M Tyrosol	0.04 M DMSO	0.01 M DMSO
1×10^7 (High)	Pellet Diameter (µm)	26% Decrease	28% Decrease	11% Increase	2% Increase
1×10^7 (High)	Hyphal Tip (number)	44% Decrease	33% Decrease	20% Decrease	14% Decrease
1×10^3 (Low)	Pellet Diameter (µm)	50% Decrease	3% Decrease	44% Decrease	23% Increase
1×10^3 (Low)	Hyphal Tip (number)	13% Decrease	35% Increase	7% Decrease	46% Increase

3.2.3.2 Farnesol

The first fungal culture to have been investigated for its QS capabilities is that of the dimorphic fungus *C. albicans.* When present *C. albicans* culture, farnesol is able to inhibit the switch from yeast to hyphal form by inhibiting the cAMP signalling pathway. However, farnesol is unable to block elongation of pre-existing hyphae (Wongsuk *et al.,* 2016).

Farnesol, not only affects *C. albicans,* but can have differing outcomes on various cultures. In high concentrations farnesol has inhibitory properties on conidia formation in *A. niger* (Lorek *et al.,* 2008; Wongsuk *et al.,* 2016), and in cultures of *Fusarium graminearum* diminished macroconidia development and germination.

Farnesol has been the most widely studied QSM from the species *C. albicans,* and has been investigated with regards to yeasts, dimorphic fungi, moulds and filamentous fungi.

In this study concentrations of 0.1, 0.08, 0.04 and 0.01 M farnesol was added to both high and low spore concentrations at the time of inoculation. In high spore cultures additions of farnesol reduced pellet diameters (figure 3.24) and increased hyphal tip numbers (figure 3.26). Additions to low spore numbers greatly reduced pellet diameters to diameters closer to that of high spore cultures (figure 3.25). Hyphal tip numbers increased in low spore cultures with all concentrations of farnesol (figure 3.27). Table 3.3 shows percentage

changes in pellet diameters and hyphal tip numbers for all farnesol concentrations compared to controls. With regard to low spore cultures the best result obtained was with the addition of 0.01 M farnesol, as results obtained were closer in morphology to that of high spore cultures.
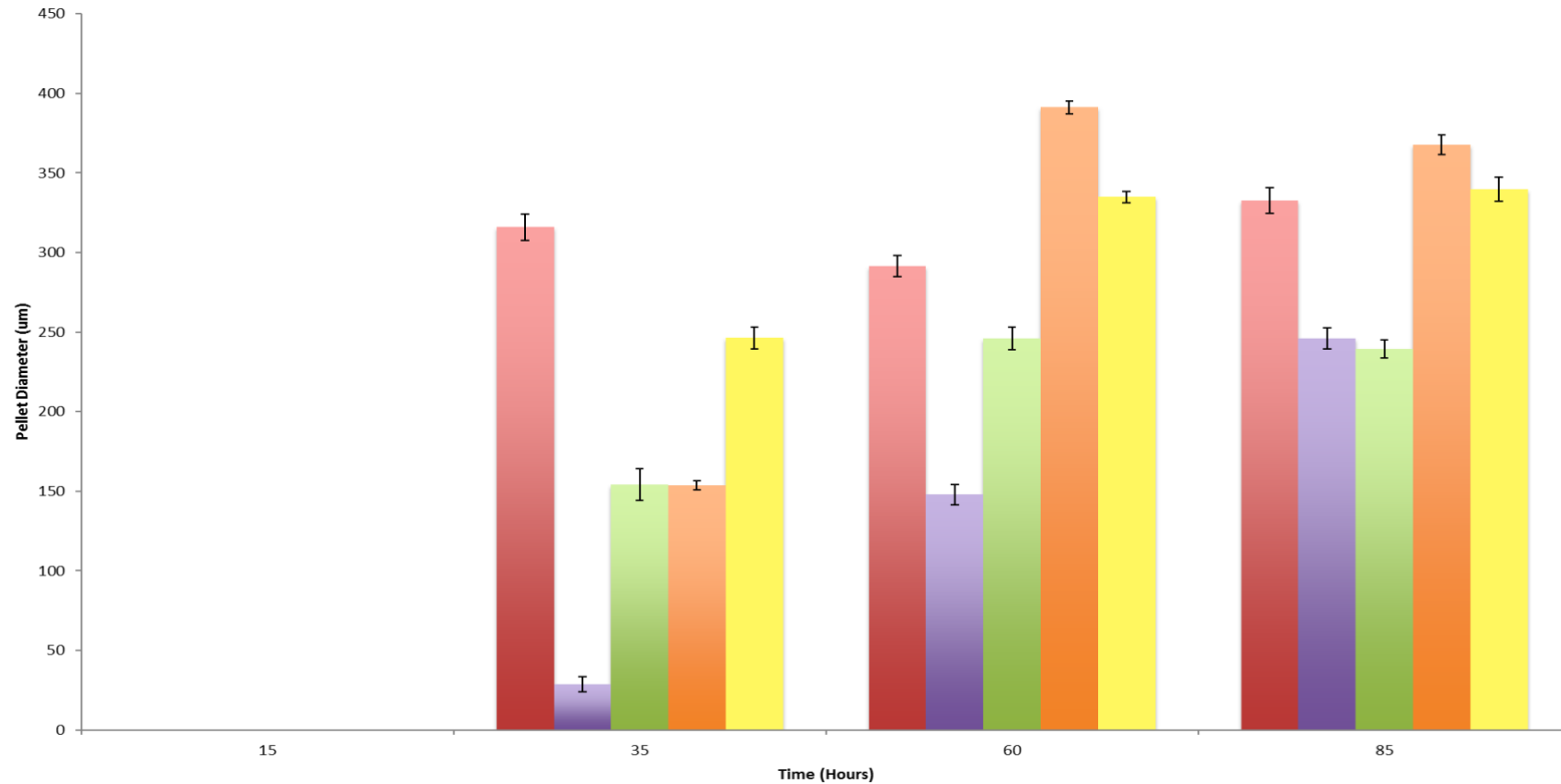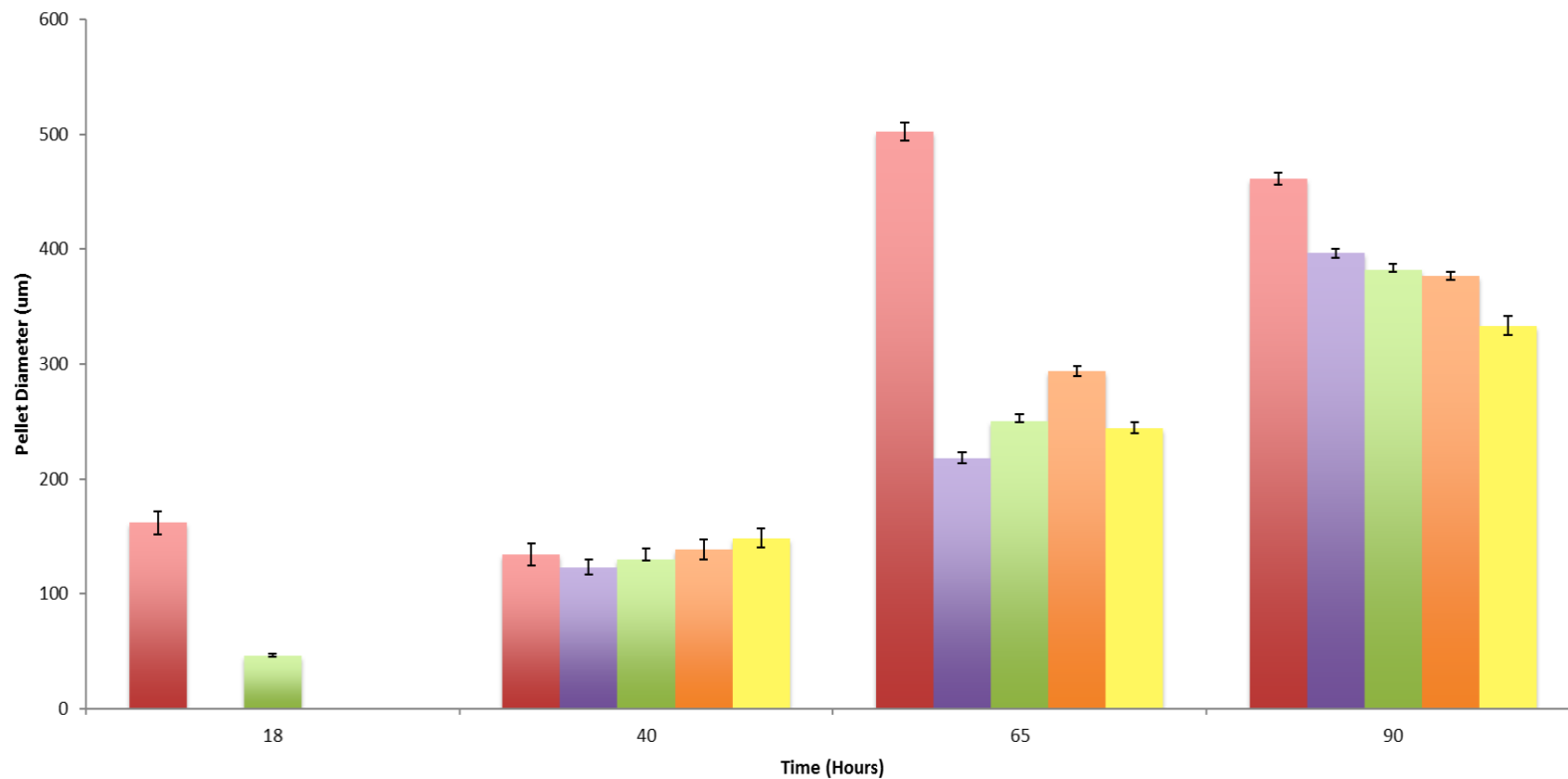
Figure 3.24: *Pellet diameter against time for high spore numbers (1x10₇ spores/mL) control culture (red), high spore numbers supplemented with 0.1 M farnesol (purple), 0.08 M (green), 0.04 M (orange) and 0.01 M (yellow). Experiments carried out in triplicate, 30 pellets per sample taken was analysed. T-Test carried out comparing test conditions to control where p<0.05, data significantly different. Standard deviation error bars plotted.*
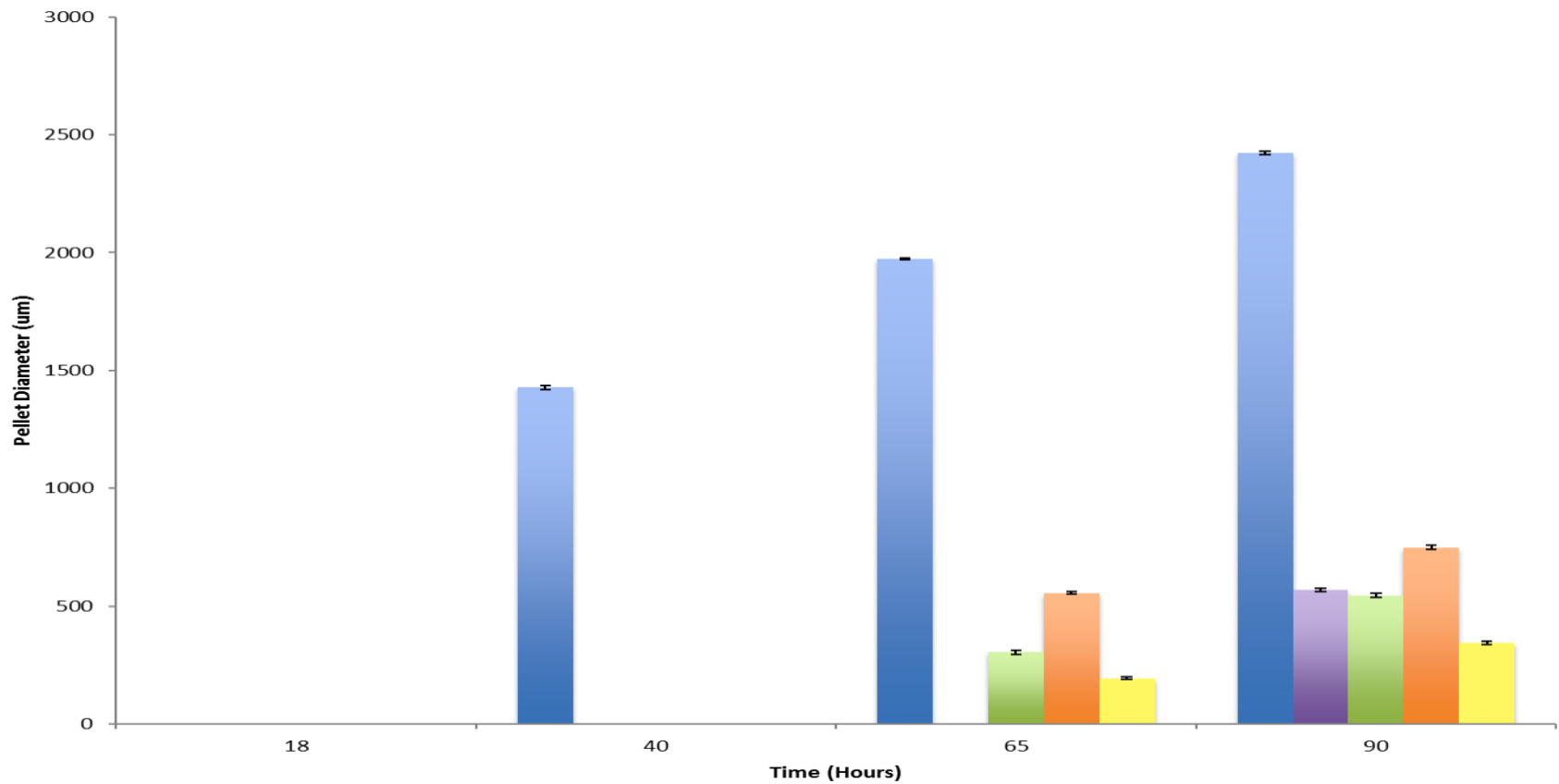
Figure 3.25: *Pellet diameter against time for low spore numbers (1x10$_3$ spores/mL) control culture (blue), low spore numbers supplemented with 0.1 M farnesol (purple), 0.08 M (green), 0.04 M (orange) and 0.01 M (yellow). Experiments carried out in triplicate, 30 pellets per sample taken was analysed. T-Test carried out comparing test conditions to control where p<0.05, data significantly different. Standard deviation error bars plotted.*
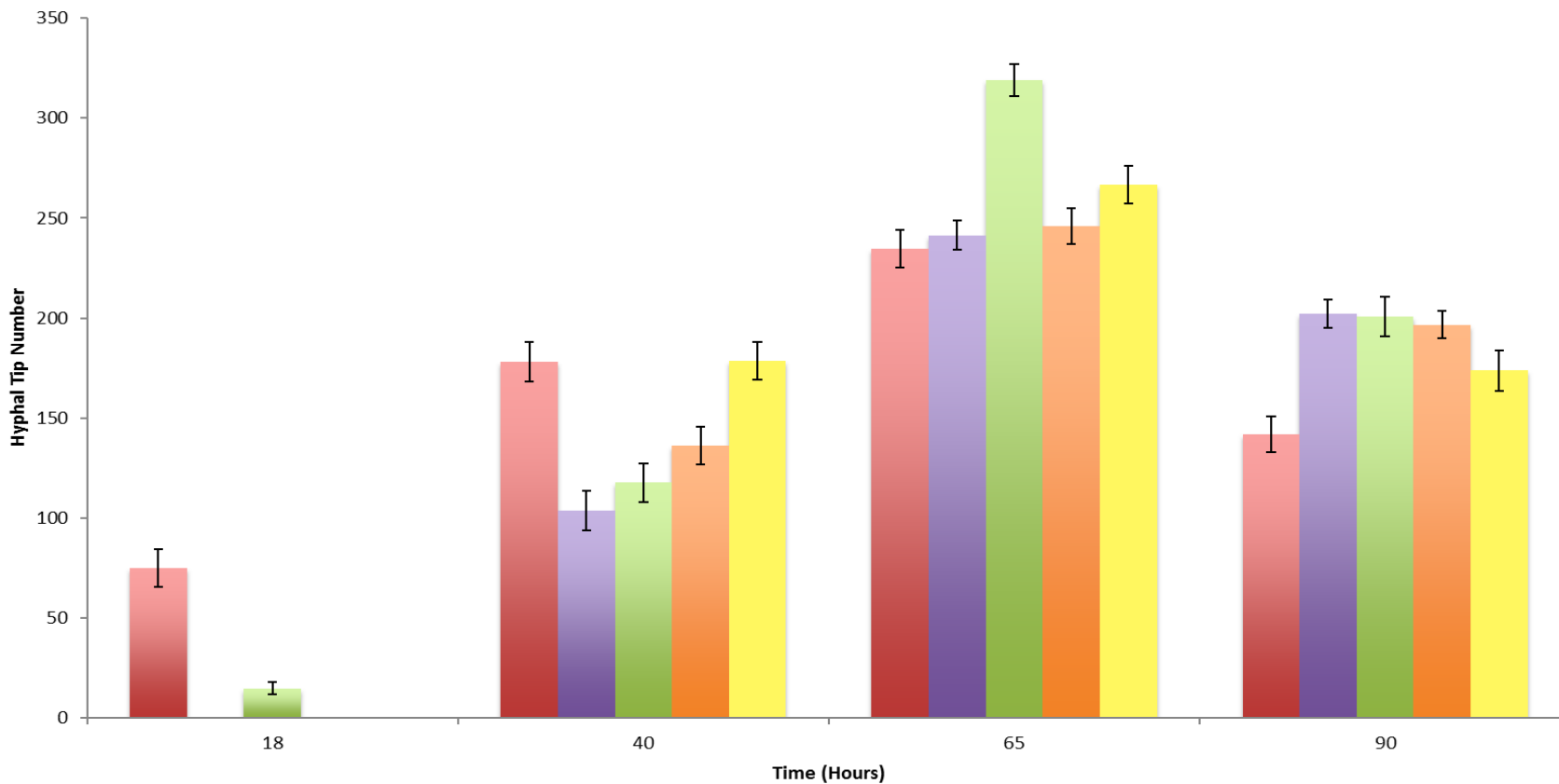
Figure 3.26: *Hyphal tip number against time for high spore numbers ($1x10^7$ spores/mL) control culture (red), high spore numbers supplemented with 0.1 M farnesol (purple), 0.08 M (green), 0.04 M (orange) and 0.01 M (yellow). Experiments carried out in triplicate, 30 pellets per sample taken was analysed. T-Test carried out comparing test conditions to control where $p<0.05$, data significantly different. Standard deviation error bars plotted.*
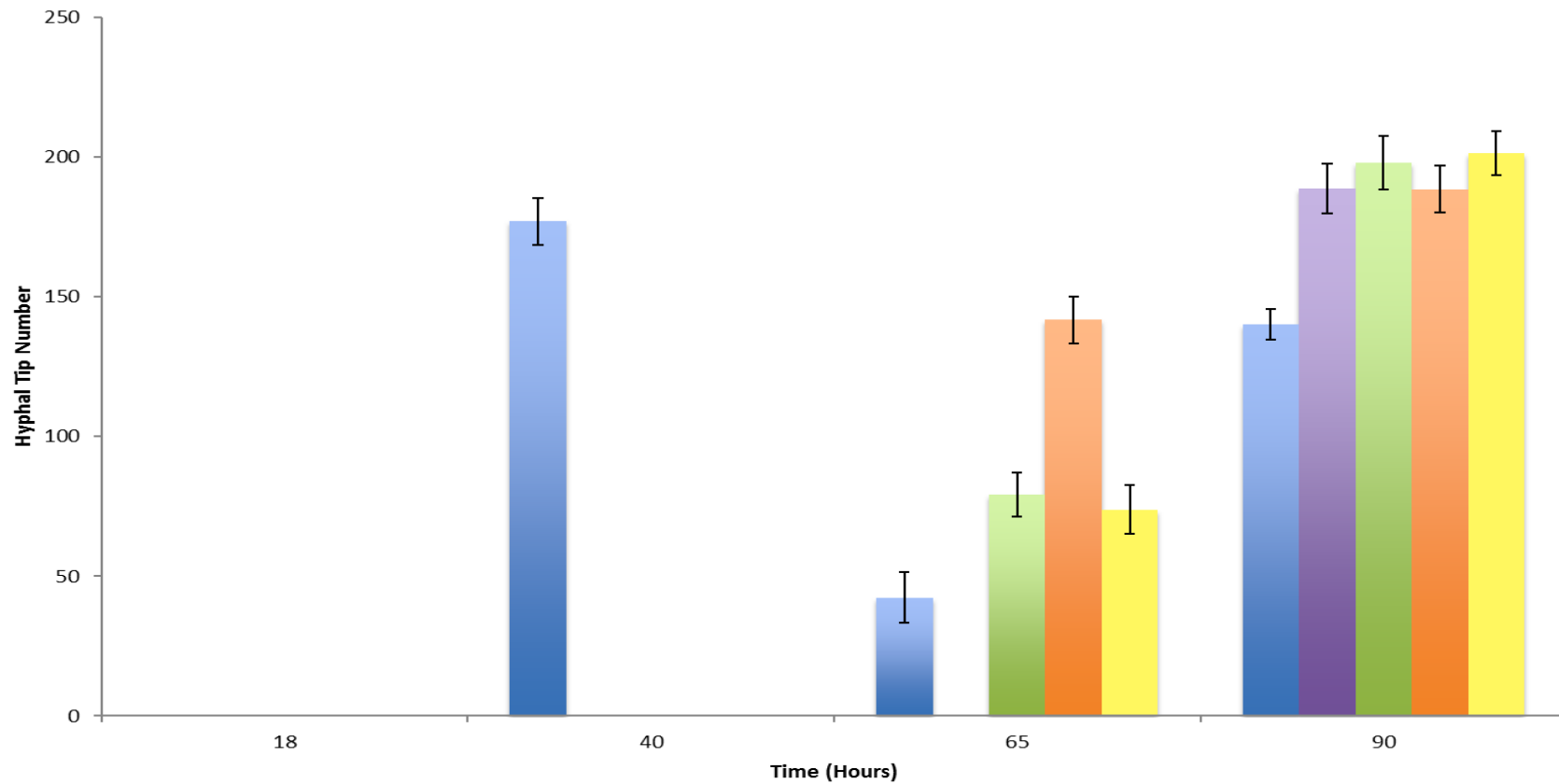
**Figure 3.27:** *Hyphal tip number against time for low spore numbers ($1x10_3$ spores/mL) control culture (blue), low spore numbers supplemented with 0.1 M farnesol (purple), 0.08 M (green), 0.04 M (orange) and 0.01 M (yellow). Experiments carried out in triplicate, 30 pellets per sample taken was analysed. T-Test carried out comparing test conditions to control where $p<0.05$, data significantly different. Standard deviation error bars plotted.*
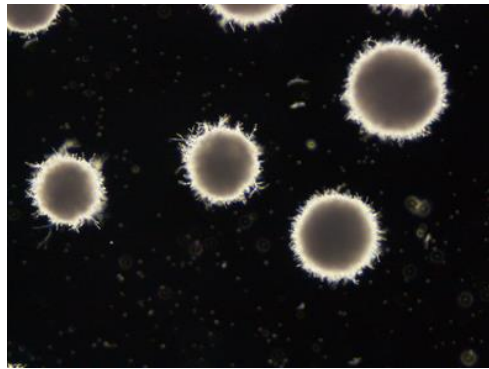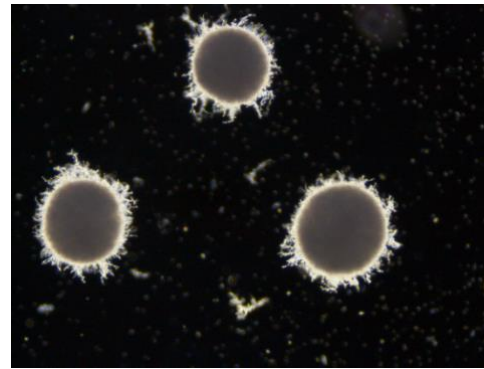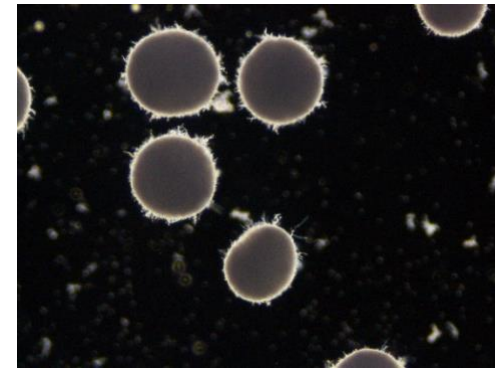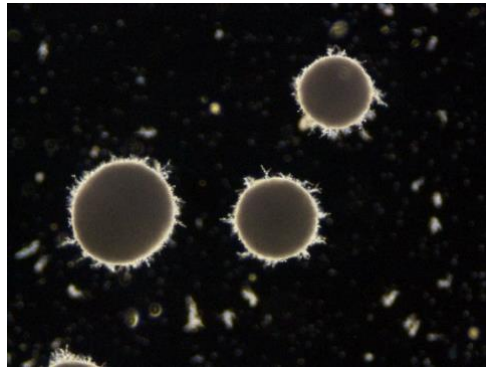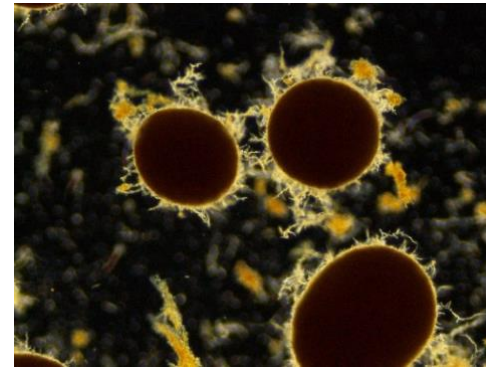
Figure 3.28: *Microscopic images of pellets formed from high spore numbers (1×10^7 spores/mL) supplemented with 0.1, 0.08, 0.04 and 0.01 M farnesol and control with no supplementation, 90 hours post inoculation. Images were taken at x4 magnification, with phase contrast. Images are a representation of the culture group.*
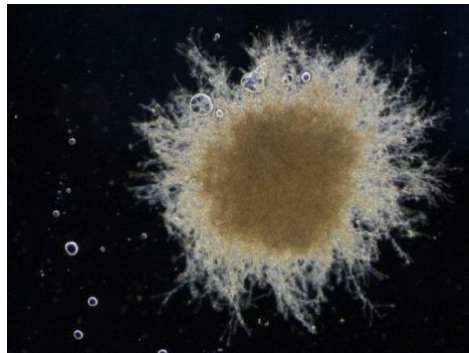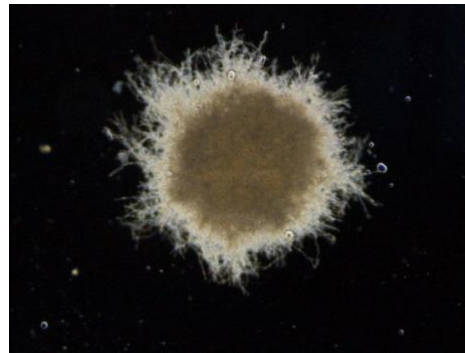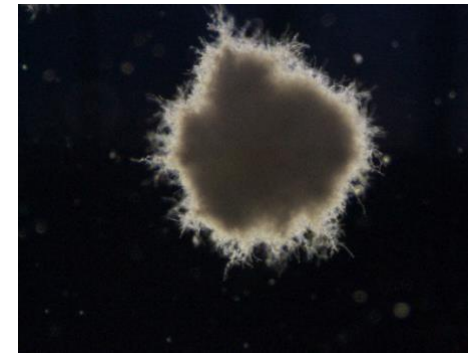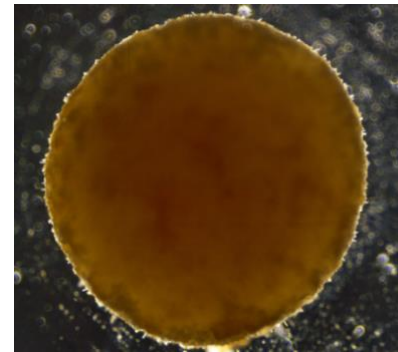
Figure 3.29: *Microscopic images of pellets formed from low spore numbers (1x10$_3$ spores/mL) supplemented with 0.1, 0.08, 0.04 and 0.01 M farnesol and control with no supplementation, 90 hours post inoculation. Images were taken at x4 magnification, with phase contrast. Images are a representation of the culture group.*

Table 3.2: *Percentage change in hyphal tip number and pellet diameter for test samples of high and low spore inoculum with farnesol concentrations between 0.1 and 0.01 M, compared to control samples of high and low spore inoculum concentrations where no additions to the inoculum was made. Diameters, tip numbers, and % changes are for cultures at the end of the experimental run, 90 hours post inoculation.*

Inoculum size (spores/mL)	Parameter	0.1 M	0.08 M	0.04 M	0.01 M
1×10^7 (High)	Pellet Diameter (µm)	14% Decrease	17% Decrease	18% Decrease	28% Decrease
1×10^7 (High)	Hyphal Tip (number)	42% Increase	42% Increase	39% Increase	23% Increase
1×10^3 (Low)	Pellet Diameter (µm)	77% Decrease	77% Decrease	69% Decrease	86% Decrease
1×10^3 (Low)	Hyphal Tip (number)	35% Increase	41% Increase	35% Increase	44% Increase

3.2.4 Large Scale Supplementation

In order to determine productivities, larger scale (1 L shake flasks) cultures were carried out. Results from 250 mL shake flask with defined medium section 3.2.3.2, showed that additions of 0.01 M farnesol to inoculum had the greatest effect on fungal morphology. In addition to large scale production with farnesol, additions of concentrated freeze dried spore supernatants, FDSS, were prepared and used as supplements with low spore inoculum.

3.2.4.1 Supplementation with Farnesol – Large Scale

In this study the addition of 0.01 M farnesol to low spore culture numbers greatly increased lovastatin production. In cultures of high spore inoculum, lovastatin production increased by 87% with the addition of farnesol, concentrations increased from 615 mg/L to115 mg/L. In low cultures lovastatin concentrations increased by 170% from 175 mg/L to 472 mg/L (figure 3.30 and table 3.4)

Figure 3.30 shows lovastatin concentrations of high and low spore numbers (controls) supplemented with Farnesol. At 6 days post inoculation lovastatin production is seen in high spore control cultures, at 7 days production is seen in low spore cultures. Production of lovastatin is seen in low spore cultures supplemented with farnesol 14 days post inoculation, at this point high spore control cultures produced 615 mg/L of lovastatin, high spore cultures with

farnesol produced 1151 mg/L, low spore control cultures produced 175 mg/L and low spore cultures supplemented with farnesol produced 472 mg/L.

Over the course of the experimental run, high spore cultures consumed the most amount of carbohydrate compared to low spore control cultures (figure 3.31) and produced less biomass than low spore control cultures (figure 3.32).

Overall additions of farnesol to high spore cultures increased lovastatin production by 87%, and increased production in low spore cultures by 170%. However, when low spore cultures with addition of farnesol is compared to high spore control cultures, there is a decrease of 23% lovastatin produced.
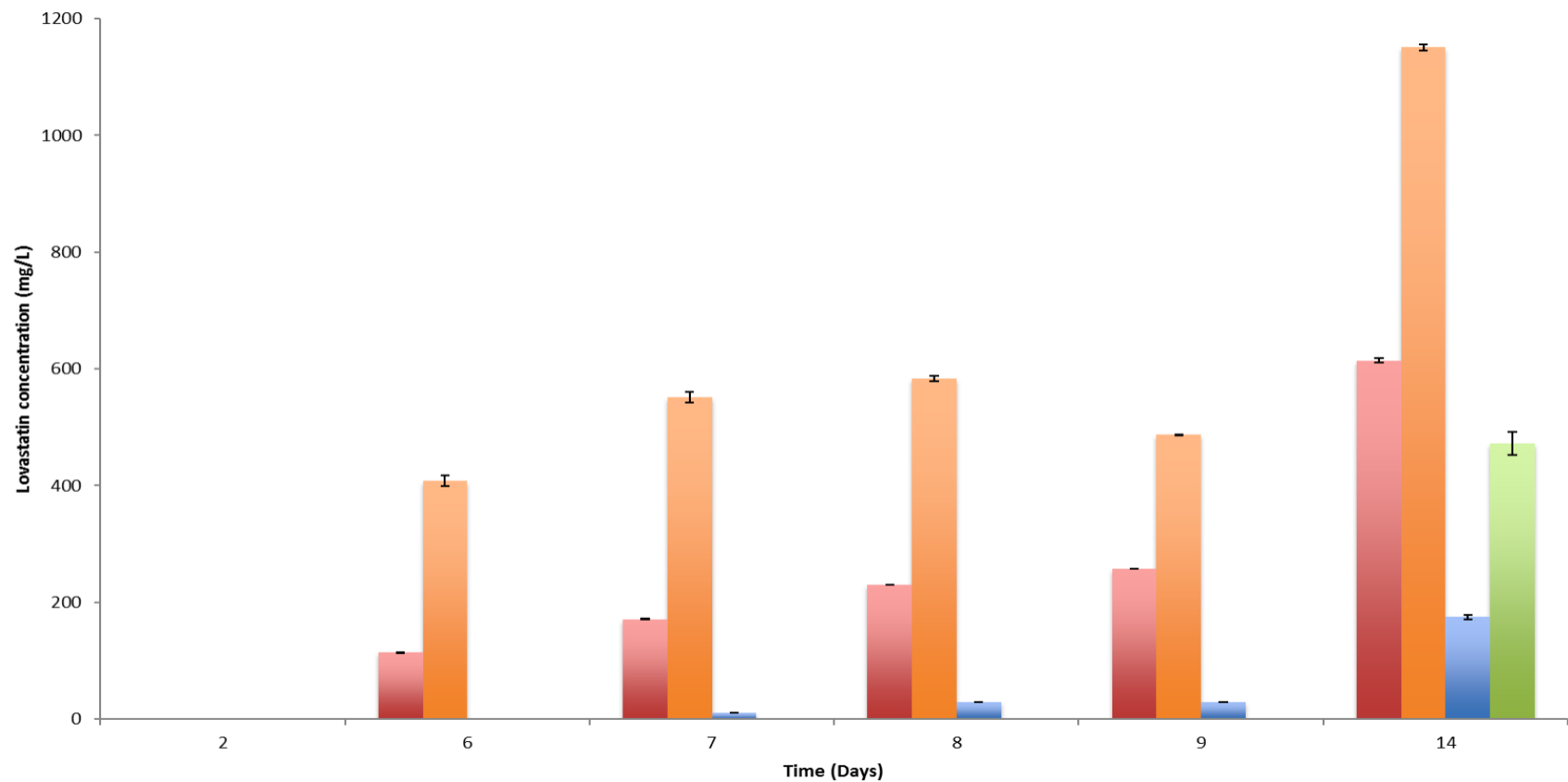
Figure 3.30: *Concentration of lovastatin (mg/L) produced in 1L shake flasks by high spore (1x10$_7$spores/mL) control cultures (red), high spore cultures supplemented with 0.01 M farnesol (orange), low spore (1x10$_3$ spores/mL) control cultures (blue) and low spore cultures supplemented with 0.01 M farnesol (green). Experiments carried out in duplicate, T-Test carried out comparing test conditions to control where p<0.05, data significantly different. Standard deviation error bars plotted.*

Figure 3.31: *Carbohydrate concentration (g/L) for high spore ($1x10_7$spores/mL) control cultures (red), high spore cultures supplemented with 0.01 M farnesol (orange), low spore ($1x10_3$ spores/mL) control cultures (blue) and low spore cultures supplemented with 0.01 M farnesol (green). Experiments carried out in duplicate. Standard deviation error bars plotted.*

Figure 3.32: *Overall biomass (Dry Cell Weight g/L) for high spore ($1x10_7$spores/mL) control cultures (red), high spore cultures supplemented with 0.01 M farnesol (orange), low spore ($1x10_3$ spores/mL) control cultures (blue) and low spore cultures supplemented with 0.01 M farnesol (green). Standard deviation error bars plotted.*

Table 3.3: *Comparison of percentage of lovastatin produced by high and low spores supplemented with 0.01 M farnesol, compared to high and low control cultures. (Data obtained from figure 3.30).*

Inoculum Size (spores/mL)	Percentage Comparison of Test Against Control Cultures
1×10^7 (High) + Farnesol	**87%** increase compared to high control
1×10^3 (Low) + Farnesol	**170%** increase compared to low control
1×10^3 (Low) + Farnesol	**23%** decrease compared to high control

Table 3.4: *Product yield (Δ P/S) for high spore (1×10^7 spores/mL) control cultures, low spore (1×10^3 spores/mL) control cultures and high and low spore cultures inoculated with 0.01 M farnesol.*

Inoculum Size (spores/mL)	Product Yield (mg/g)
1×10^7 (high)	33
1×10^7 (high) & farnesol	66
1×10^3 (low)	13
1×10^3 (low) & farnesol	236

3.2.4.2 Supplementation with Concentrated Spore Supernatant

Spore supernatants were concentrated through freeze drying processes and added as supplements to cultures of high and low spore numbers. Lovastatin productivities, carbohydrate consumption and biomass production were determined. The aim of this study was to encourage low spore numbers to behave in a similar manner to high spore numbers, in regard to both morphology and productivities.

The highest concentration of lovastatin obtained from low spore cultures with FDSS surpasses that of high spore control cultures and high spore cultures inoculated with FDSS (figure 3.33). However, low spore cultures with FDSS did not consume as much carbohydrate as that seen in high spore cultures (figure 3.34). Table 3.6 shows percentage change of lovastatin produced in cultures supplemented with FDSS compared to control groups. High spore cultures with FDSS produced 7% more lovastatin than high spore control cultures. Low spore cultures with FDSS produced 1208% more than low spore control cultures. When lovastatin production by low spore cultures with FDSS is compared to high spore control cultures, 62% more lovastatin is produced in low cultures with FDSS than high spore control cultures. Figure 3.36 – 3.38 shows morphology of low spore cultures supplemented with FDSS as being similar to that seen in high spore control cultures.
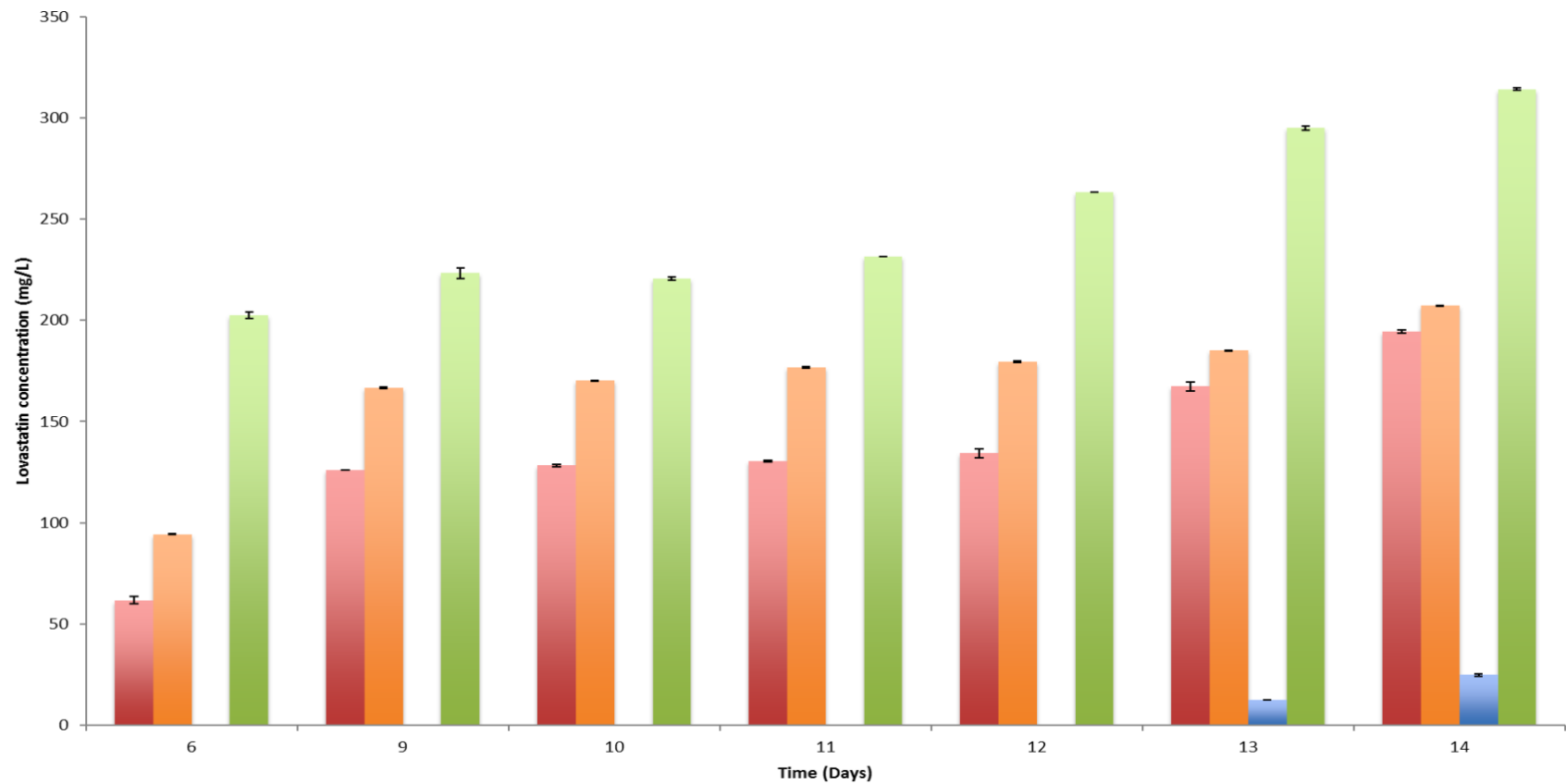
Figure 3.33: *Concentration of lovastatin (mg/L) produced in 1L shake flasks by high spore (1x10₇spores/mL) control cultures (red), high spore cultures supplemented with FDSS (orange), low spore (1x10₃ spores/mL) control cultures (blue) and low spore cultures supplemented with FDSS (green). Experiments carried out in duplicate, T-Test carried out comparing test conditions to control where p<0.05, data significantly different. Standard deviation error bars plotted.*
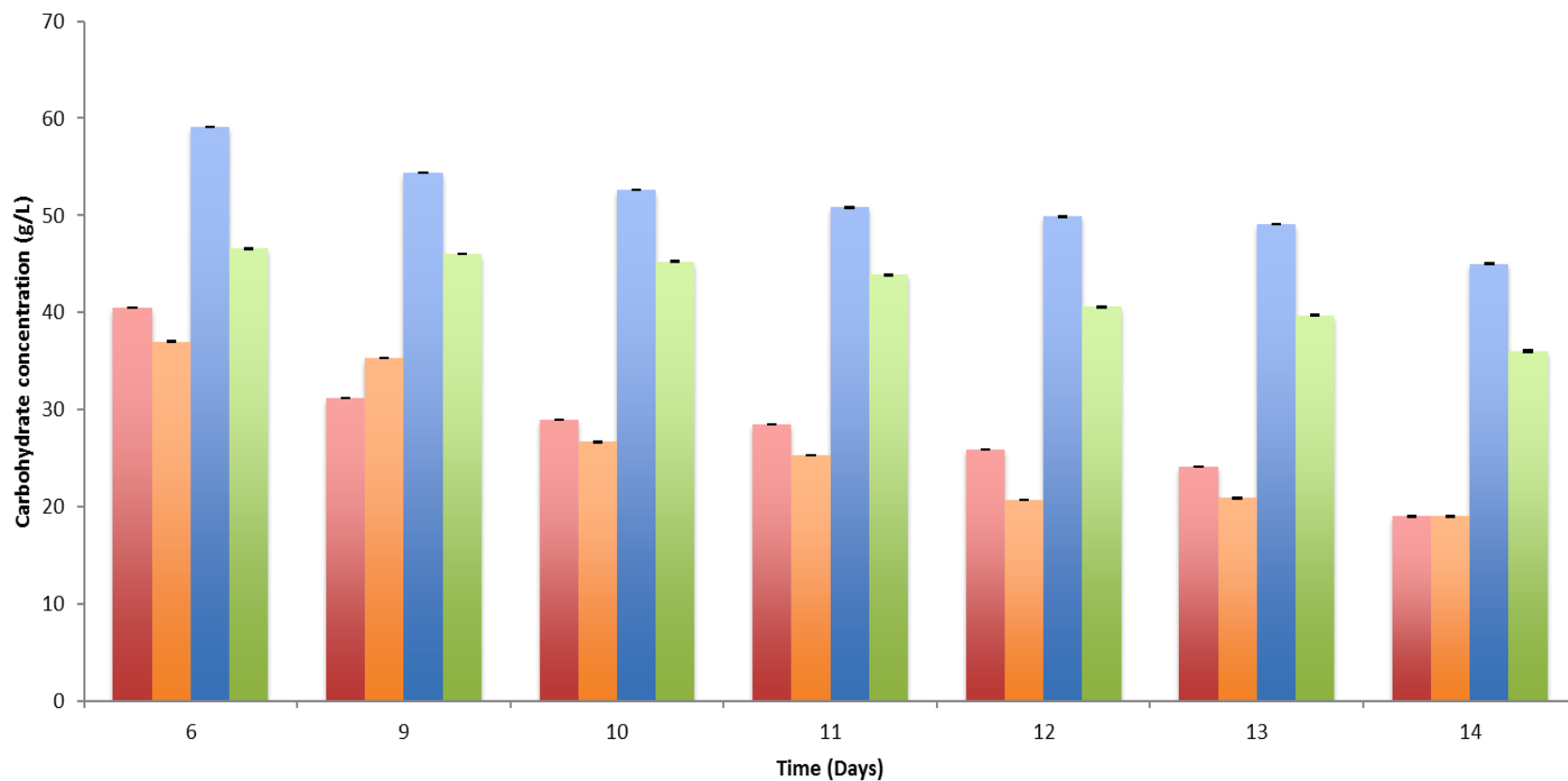
Figure 3.34: *Carbohydrate concentration (g/L) for high spore (1x10$_7$spores/mL) control cultures (red), high spore cultures supplemented with FDSS (orange), low spore (1x10$_3$ spores/mL) control cultures (blue) and low spore cultures supplemented with FDSS (green). Experiments carried out in duplicate. Standard deviation error bars plotted.*
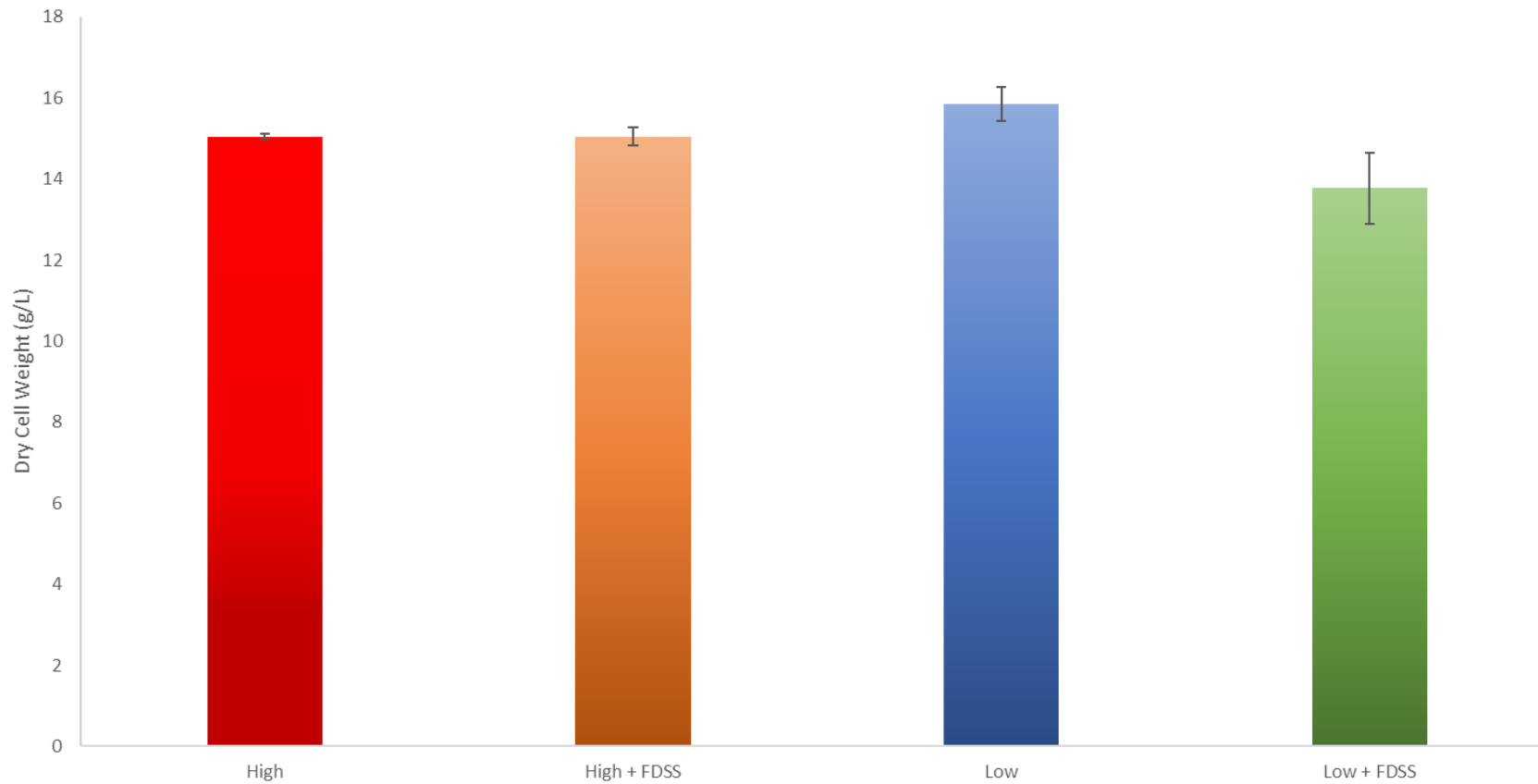
Figure 3.35: *Overall biomass (Dry Cell Weight g/L) for high spore (1x10^{7}spores/mL) control cultures (red), high spore cultures supplemented with FDSS (orange), low spore (1x10$_3$ spores/mL) control cultures (blue) and low spore cultures supplemented with FDSS (green). Standard deviation error bars plotted.*
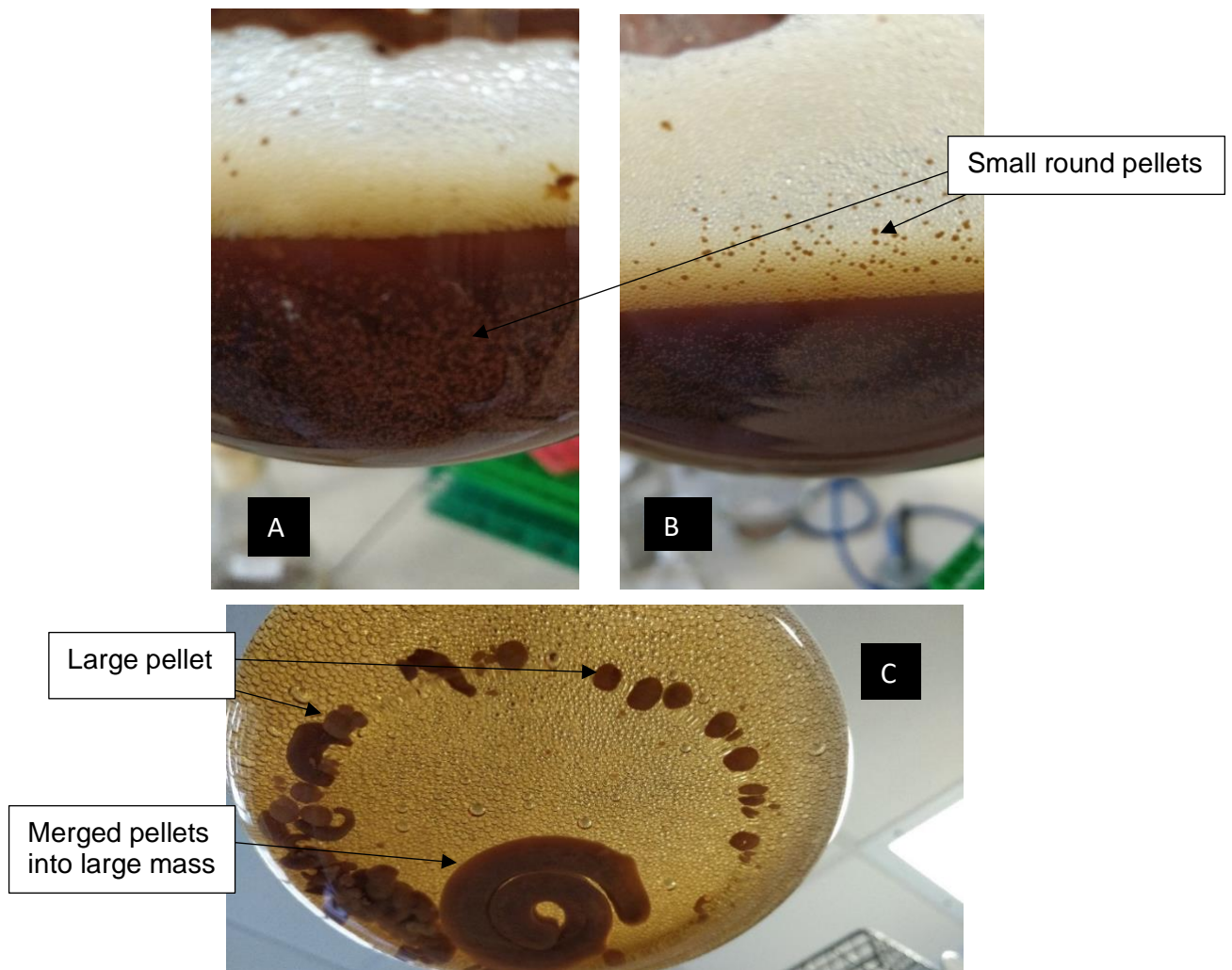
Figure 3.36: *Photographic images comparing morphological differences between high spore (1 x 10^7 spores/mL) control cultures (A), low spore (1 x 10^3 spores/mL) cultures supplemented with FDSS (B), and low spore control cultures (C), 6 days post inoculation. Images at different angles to show the morphology clearly, A and B are side profile of the flasks, and C is from the bottom of the flask. Morphology of flasks A and B are similar in physical form with small round pellets. However, flask C, the pellets are large in size and have merged together to form a large mass.*
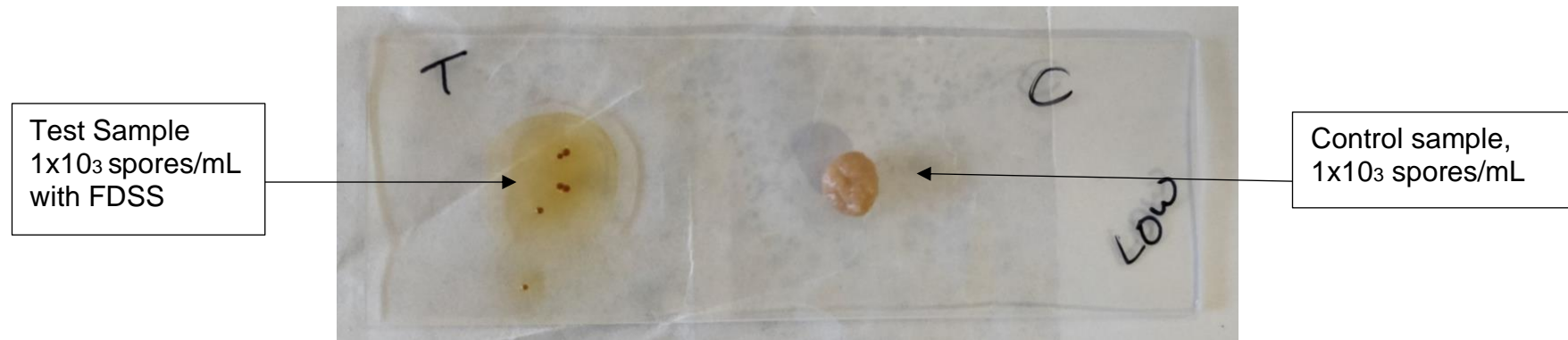
Figure 3.37: Morphological comparison of pellets formed 6 days post inoculation in low spore (1 x 10$_3$ spores/mL) cultures supplemented with FDSS (left) compared to low spore control cultures (right). Pellets formed with FDSS addition are small in size compared to large mass in control.
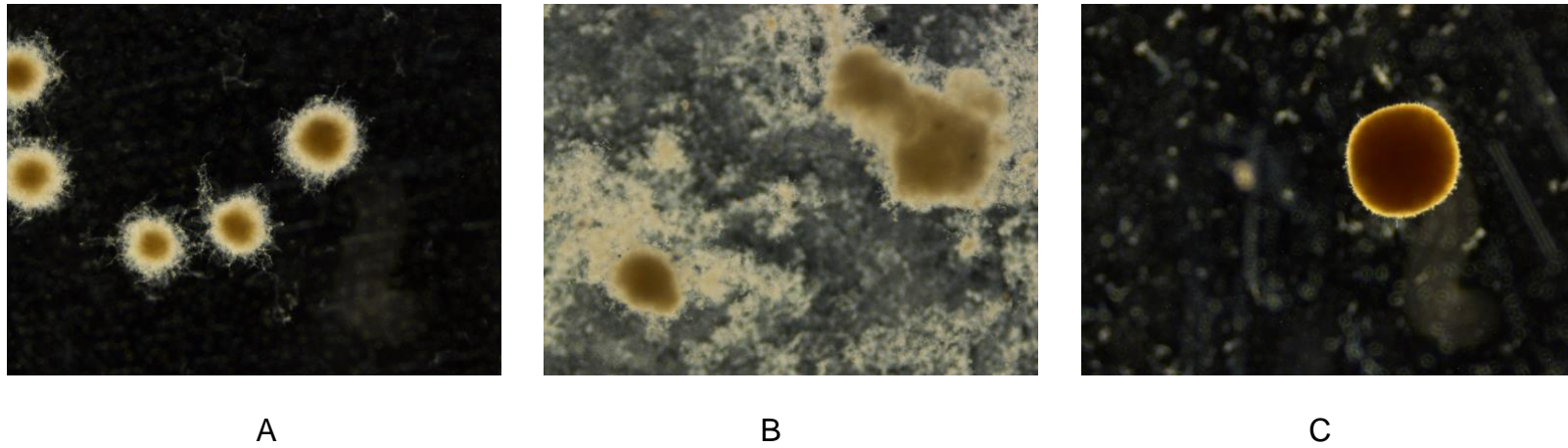
Figure 3.38: *Microscopic images of high spore (1 x 10$_7$ spores/mL) control cultures (A), high spore cultures inoculated with FDSS (B) and Low spore culture (1 x 10$_3$ spores/mL) inoculated with FDSS (C). Images taken at x4 magnification, 3 days post inoculation.*

Table 3.5: *Comparison of percentage of lovastatin produced by high and low spores supplemented with FDSS, compared to high and low control cultures. (Data obtained from figure 3.33)*

Inoculum Size (spores/mL)	Percentage Comparison of Test Against Control Cultures
1 x 10^7 (High) + FDSS	**7%** increase compared to high control
1 x 10^3 (Low) + FDSS	**1208%** increase compared to low control
1 x 10^3 (Low) + FDSS	**62%** increase compared to high control

Table 3.6: *Product yield (Δ P/S) for high spore (1 x 10^7 spores/mL) control cultures, low spore (1 x 10^3 spores/mL) control cultures and high and low spore cultures inoculated with FDSS.*

Inoculum Size (spores/mL)	Product Yield (mg/g)
1 x 10^7 (high)	5.4
1 x 10^7 (high) + FDSS	11
1 x 10^3 (low)	3
1 x 10^3 (low) + FDSS	5

3.3 Spore Contact Studies

3.3.1 Sonication

In order to increase the interactions between spores, the spore suspensions were subjected to sonication prior to inoculation. Sonication separates spores that are attached together, whilst increasing the number of 'hits' (impingements) that the suspended spores are subjected to. The spore suspensions were subjected to either 1, 2 or 5 minutes of sonication, to investigate the effects on germination times and morphologies. Microscopic observations of sonicated spores showed spores to be separate from one another, viability studies deemed spore unaffected by sonication.

This study showed that sonication had little effect on the germination times and culture morphologies for both spore inoculum sizes of 1×10^7 and 1×10^3 spores/mL. Figure 3.39 and 3.41 show no difference in pellet diameters and hyphal tip numbers of sonicated high spore cultures, similarly hyphal tip numbers of low spore cultures show no difference when spores are subjected to sonication (figure 3.42). However, pellet diameters of low spore cultures did show slight differences in diameter compared to control cultures when subjected to sonication (figure 3.40).
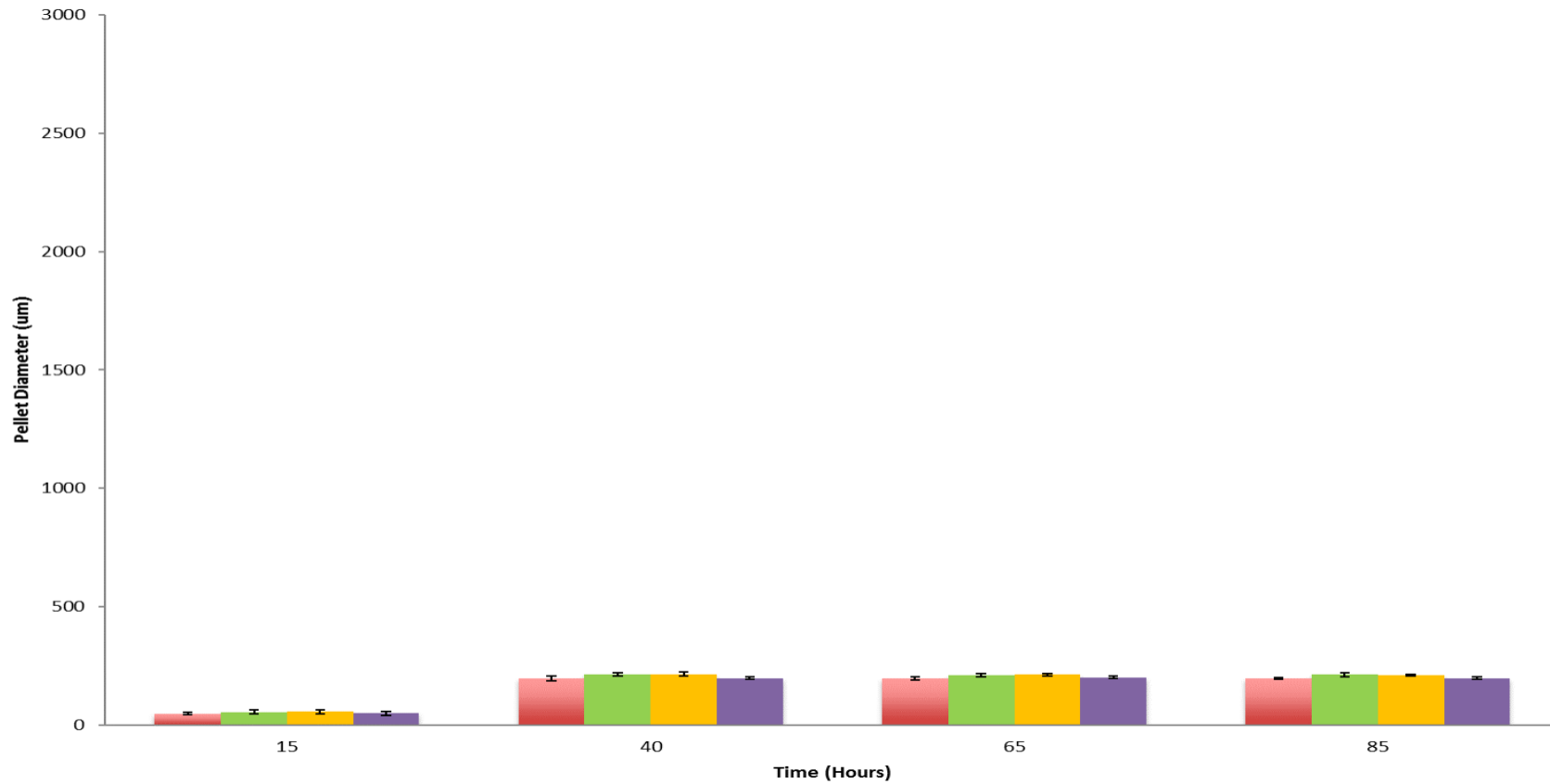
Figure 3.39: *Pellet diameter against time for high spore (1 x 10₇ spores/mL) control culture (red) and high spores subjected to 1 minute sonication (green), 2 minutes sonication (yellow) and 5 minutes sonication (purple). Experiments carried out in triplicate and 30 pellets per sample analysed. T-Tests show no significant difference in mean values. Standard deviation error bars plotted.*
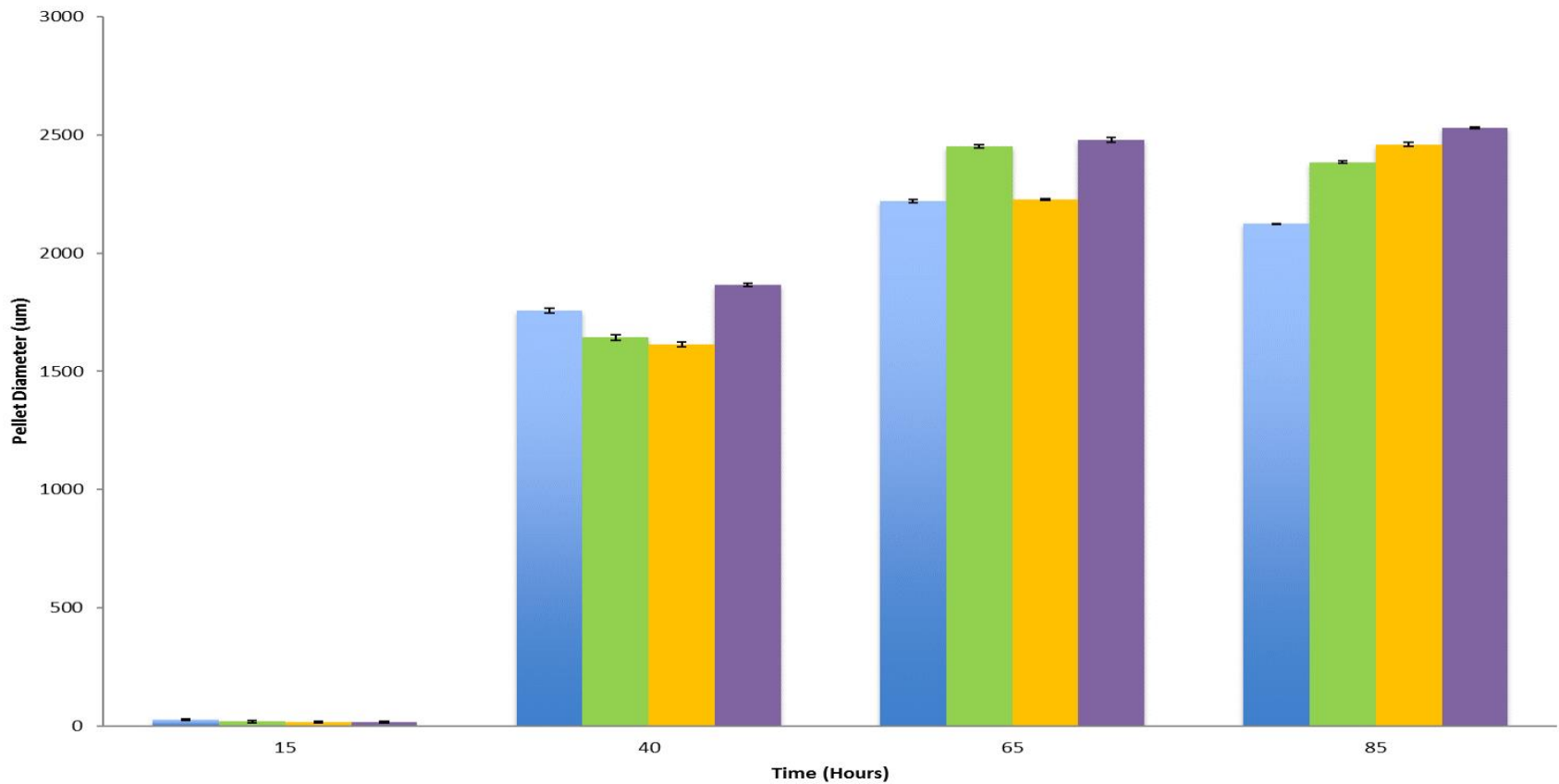
Figure 3.40: *Pellet diameter against time for low spore ($1 \times 10_3$ spores/mL) control culture (blue) and low spores subjected to 1 minute sonication (green), 2 minutes sonication (yellow) and 5 minutes sonication (purple). Experiments carried out in triplicate and 30 pellets per sample analysed. T-Tests show differences in mean values plotted $p<0.05$. Standard deviation error bars plotted.*
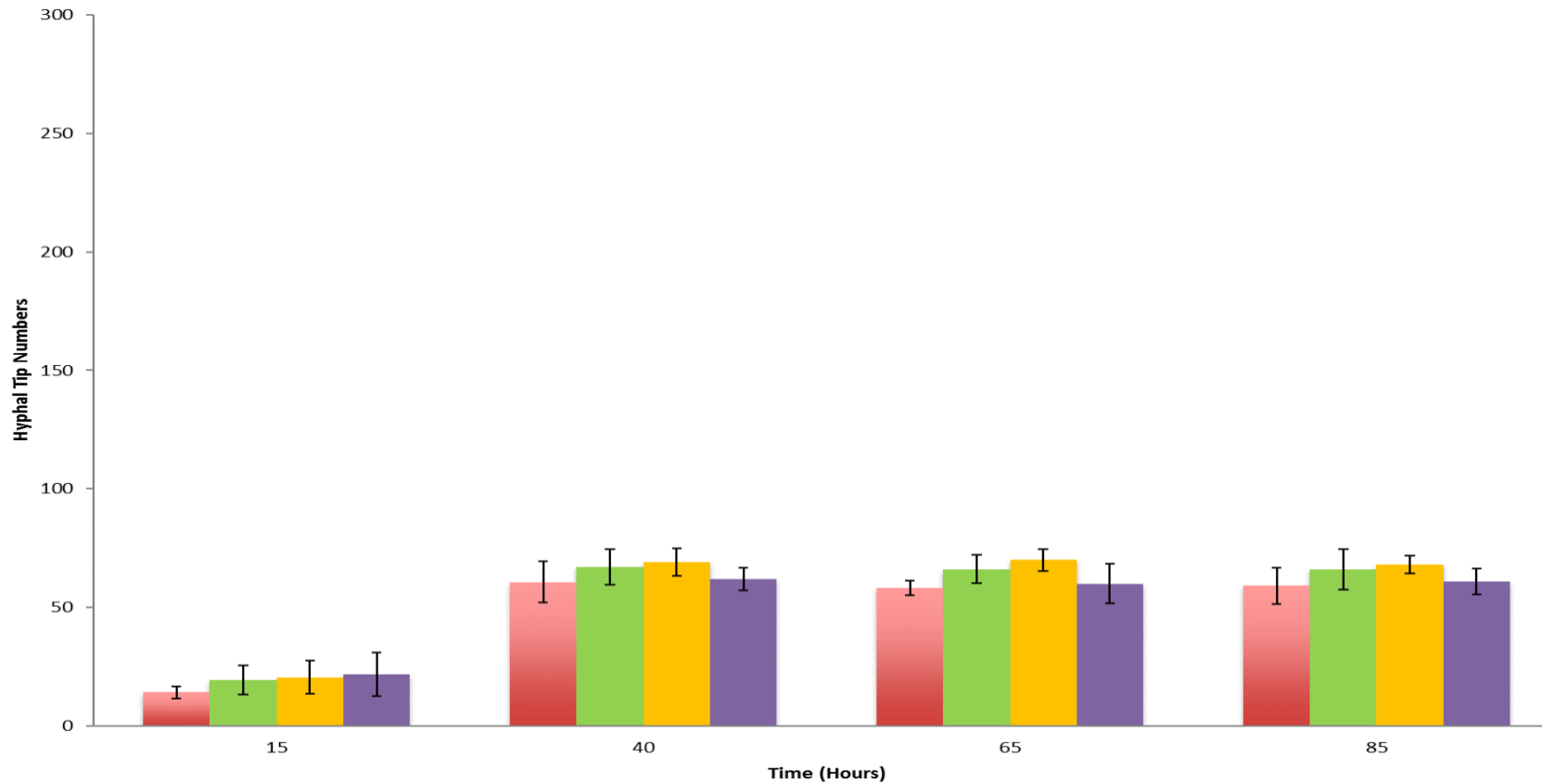
Figure 3.41: *Hyphal tip number against time for high spore (1 x 10_7 spores/mL) control culture (red) and high spores subjected to 1 minute sonication (green), 2 minutes sonication (yellow) and 5 minutes sonication (purple). Experiments carried out in triplicate and 30 pellets per sample analysed. T-Tests show differences in mean values, $p<0.05$. Standard deviation error bars plotted.*
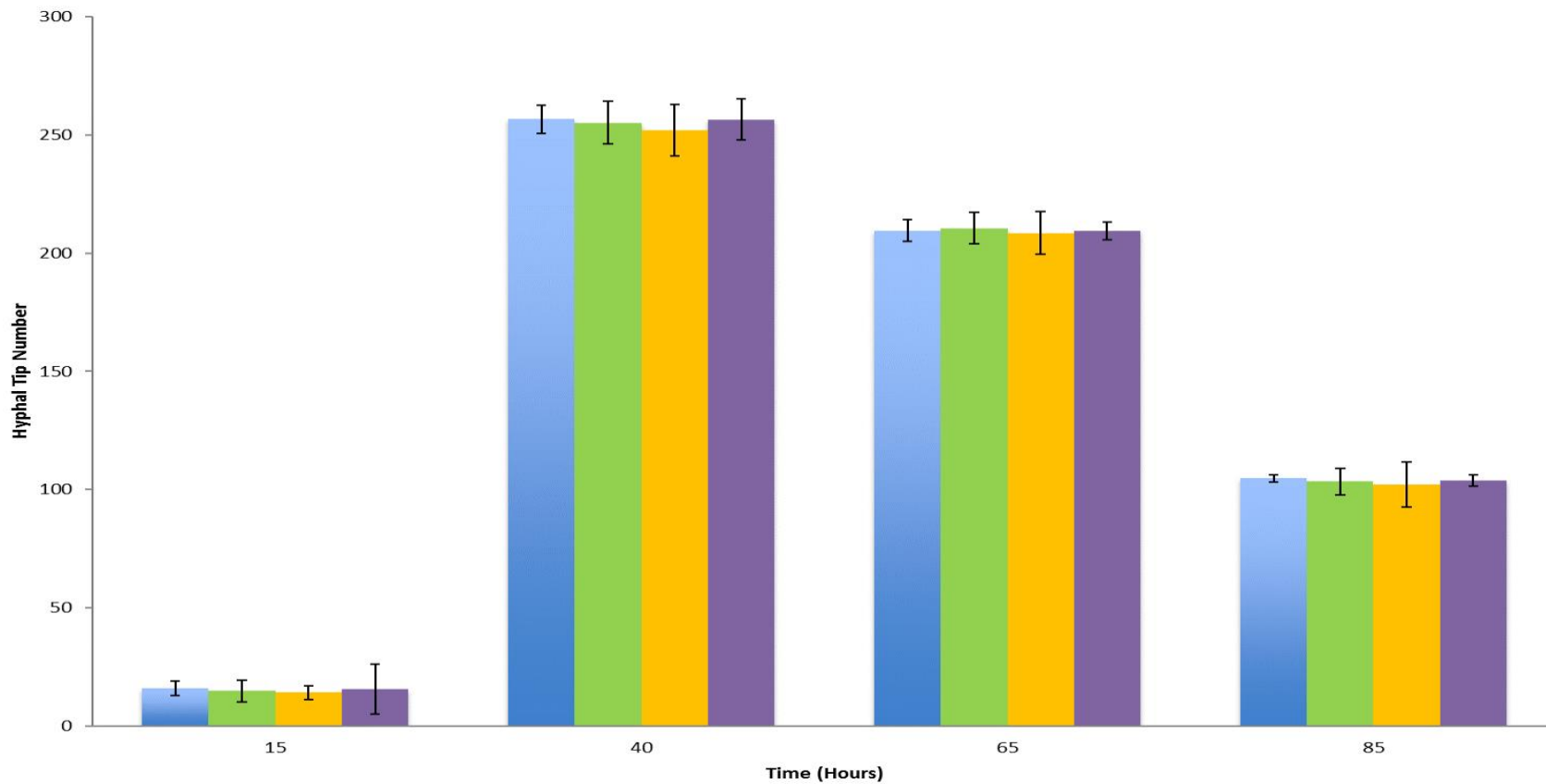
Figure 3.42: *Hyphal tip number against time for low spore (1 x 10$_3$ spores/mL) control culture (blue) and low spores subjected to 1 minute sonication (green), 2 minutes sonication (yellow) and 5 minutes sonication (purple). Experiments carried out in triplicate and 30 pellets per sample analysed. T-Tests show no differences in mean values. Standard deviation error bars plotted.*

144
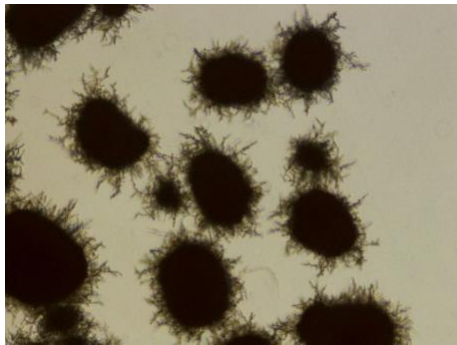
Control (0 min)
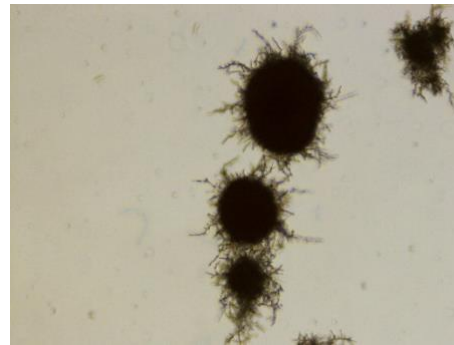
1 minute
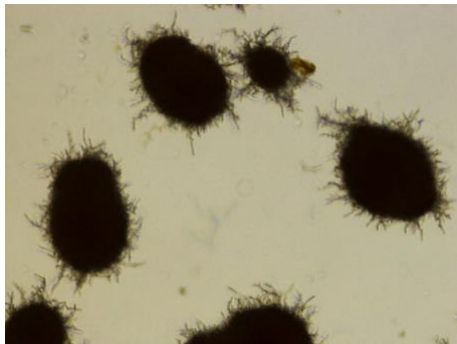
2 minutes
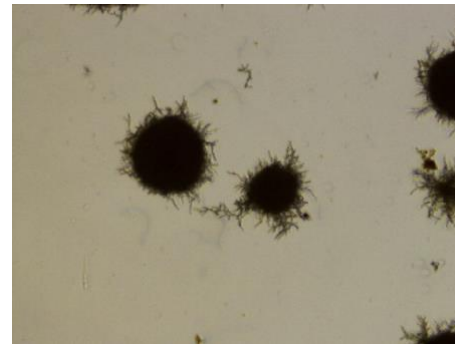
5 minutes

Figure 3.43: Microscopic images of pellets of high spore (1 x 10$_7$ spores/mL) control cultures and high spore cultures subjected to 1 minute, 2 minutes and 5 minutes sonication, 65 hours post inoculation at x4 magnification. Images shown, are a representation of images collected.
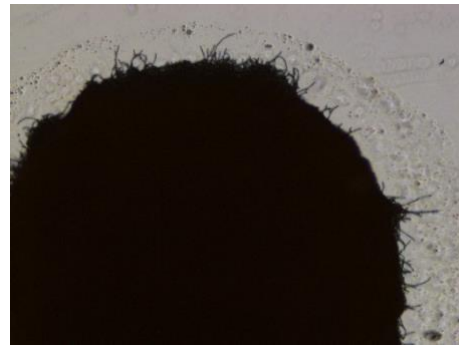
Control (0 minutes)
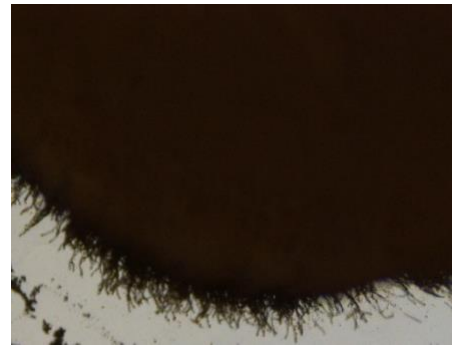
1 minute
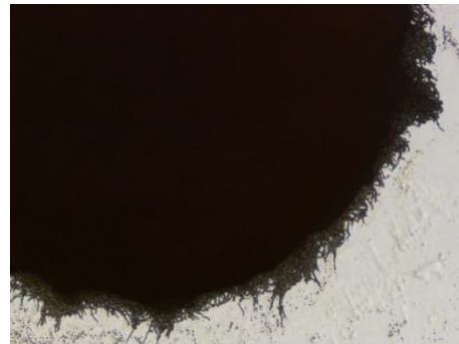
2 minutes
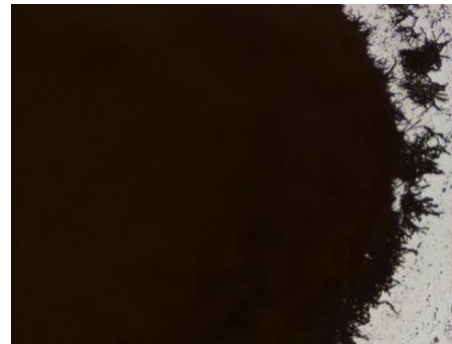
5 minutes

Figure 3.44: *Microscopic images of pellets of low spore (1 x 10$_3$ spores/mL) control cultures and low spore cultures subjected to 1 minute, 2 minutes and 5 minutes sonication, and 65 hours post inoculation at x4 magnification. Images shown, are a representation of images collected.*

3.3.2 Confinement Studies

In order to increase spore interactions, the space available in which spores could interacted with one another was manipulated, allowing spores to be further apart (1 L flask) and restricted in space (10 mL flask). Therefore, the same inoculum size of 2000 spores was used as inoculum for 10 mL and 1 L shake flask fermentations. Both fermentations were carried out under the same conditions. The flasks were kept at a constant temperature and shaken continuously. Air was sparged into the flasks at the same flow rate. The germination time and the fungal morphology were investigated over a period of 48 hours.

The results showed that the time taken for the spores to germinate was shorter in the smaller 10 mL shake flask compared to the larger 1L flask. Culture morphologies were also different. In the smaller flask cultures were dense mycelial clumps, but in the larger flask, cultures were loose and small mycelial clumps
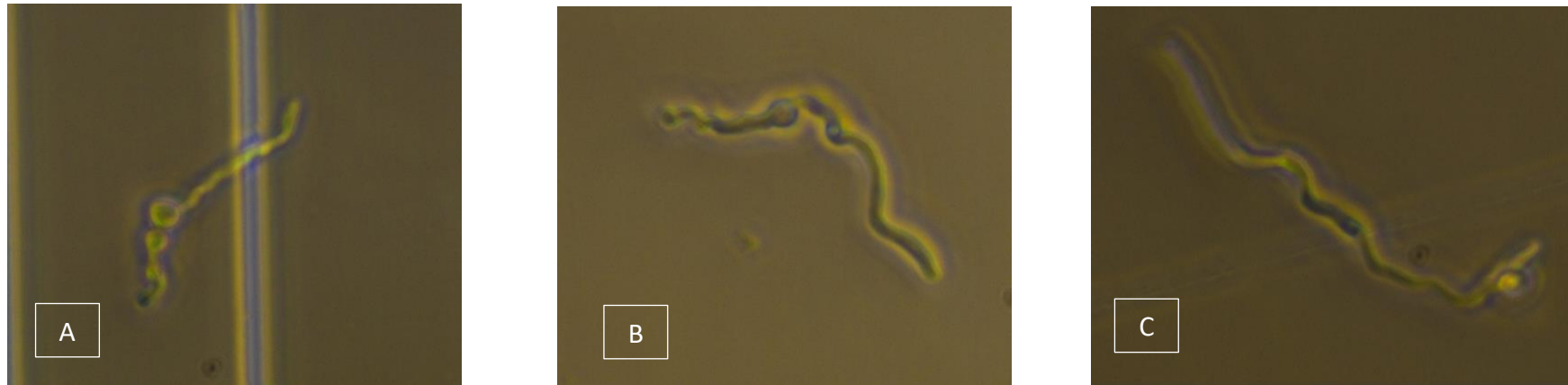
Figure 3.45: *Images A & B both show germination of two spores in the small (10 mL) flask. Germination began 18 hours post inoculation. Image C shows the germination of spores in the large (1 L) flask, 23 hours post inoculation. A haemocytometer was initially used to make spore observation. x40 magnification with phase contrast. Experiment was carried out in duplicate, and samples were taken every two hours. A minimum of 10 spores (pre and post germination) were observed per sample over a 48 hour period.*
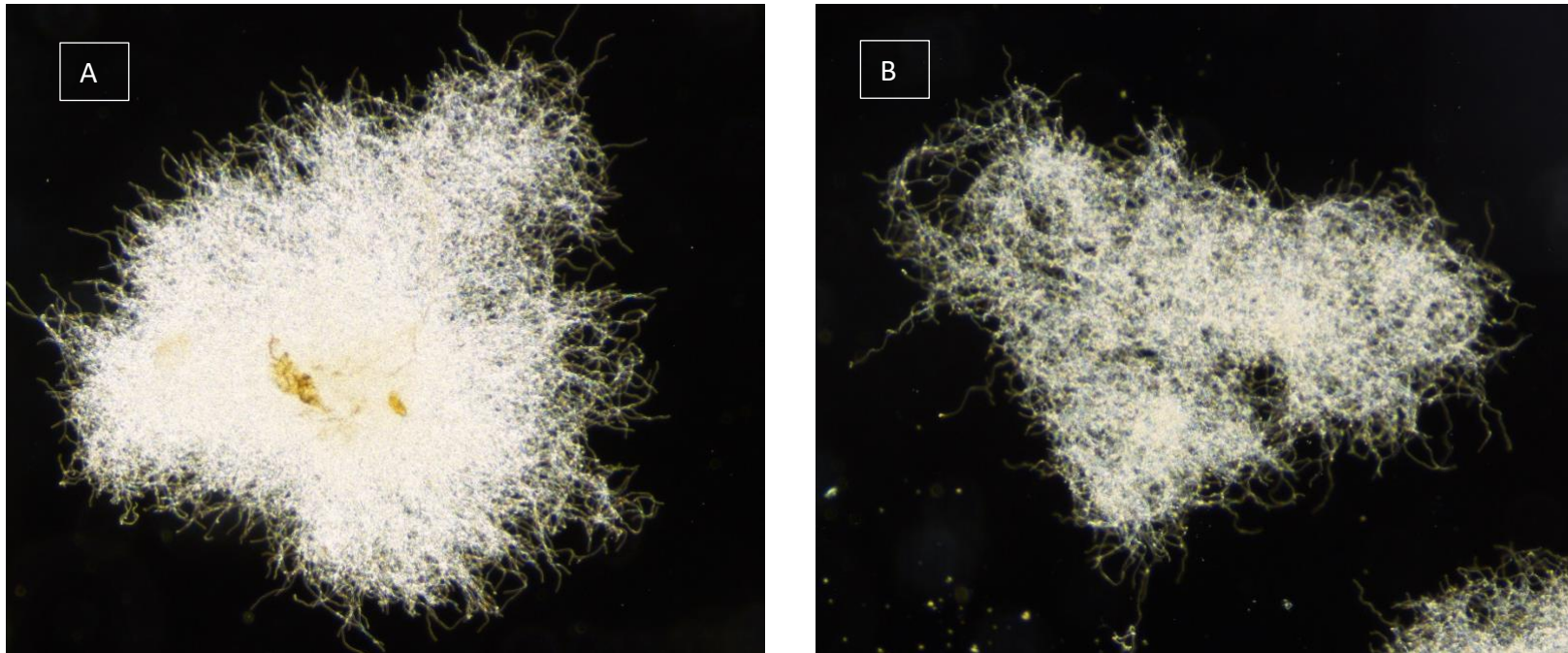
Figure 3.46: Microscopic images of the fungal morphology 38 hours post inoculation. Image A represents the morphology of cultures grown in the small *(10mL) flasks, and image B represents that of the large (1L) flasks. Both images were taken microscopically at x4 magnification, phase contrast.*

4. DISCUSSION

4.1 Introduction

The morphology of filamentous fungi in submerged liquid fermentation varies depending upon different environmental factors, as described in chapter 1. Particular morphologies are desirable for the production of certain metabolites, and some morphologies are also better suited for fermentation in bioreactors. Mycelial growth, forms dense biomass and wall growth, affecting the homogeneity of the culture and efficient mass and heat transfer. Pelleted growth provides homogeneous culture, but mass transfer within pellets is an issue as they grow in size.

For *Aspergillus terreus* cultures, the production of the secondary metabolite, lovastatin is dependent upon pellet size, and is favoured when pellet diameters are small. Inoculum of low (1 x 10_3 or below) spore numbers produces large pellets with little or no hyphae and very small concentrations of lovastatin. However, with inoculum concentrations of 1 x 10_7 spores/mL or higher, morphologies are small pellets with lots of hyphae that produce high quantities of lovastatin (Bizukojc and Ledakowicz 2007).

Another specific morphological requirement is to produce pellets with a high number of hyphal tips. The reason for this is that it is in the tips that secondary metabolism takes place and where secondary metabolites are secreted (Nielsen and Krabben, 1995; Paul and Thomas, 1996).

In this chapter, the results obtained will be discussed in relation to the number of spores, their interactions and subsequent morphology of *A. terreus* cultures and lovastatin production.

4.2 Supplementation

4.2.1 Supplementation of Plate Cultures

Inoculum concentrations between 1×10^1 and 5×10^7 spores were cultured on YME agar plates to determine changes in culture morphology over time, when different spore numbers were used as inoculum. Cultures were also supplemented with butyrolactone I, spore supernatant (SS), ethanol (control group as the QSM butyrolactone I was dissolved in ethanol) and tween 80 (control group to determine if tween 80 affected spores and it was used during spore harvest). Differences in growth morphology was observed and analysed.

Growth of cultures of 5×10^7 spores/mL were too thick and dense for variations in morphology to be identified. In cultures of 5×10^3 spores/mL, at 48 hours post inoculation (figure 3.1), all samples showed thick growth, however in cultures supplemented with butyrolactone I, growth was less dense.

Comparison of growth in all cases showed similarities, and growth was very dense with intertwined hyphae, inoculum concentrations of 1×10^1 showed

distinct individual colonies. Inoculation of 10 spores allowed for individual growth to be observed, with differences in diameter and hyphae growth to be determined. Cultures of high spore numbers (> 1 x 10$_7$ spores/mL) germinated earlier than that of low (< 5 x 10$_3$ spores/mL) spore cultures. There was no differentiation between growths of individual spores, as there was a limitation of space (figure 3.2) available for growth.

It is not possible, however, to draw conclusions solely from plate work, but the information allowed for a basis of understanding of morphological differences that arise from different spore inocula. Supplementation at this scale was inconclusive and therefore the next logical steps taken were submerged liquid cultures, as this is best practice for the study of morphological differences and production.

4.2.2 Supplementation of submerged liquid fermentation

Similar to plate culture studies, spores of *A. terreus* were cultured in both complex and defined liquid medium. Supplemented with spore supernatants, ascorbic acid, quorum sensing molecule butyrolactone I, and ethanol. A comparison of the resulting morphologies from inoculum sizes of 1 x 10$_7$ spores/mL (high) and 1 x 10$_3$ spores/mL (low) was carried out. The aim of this study was to investigate what effects the different compounds have on the morphology of the culture, and specifically whether addition of QS

compounds can cause low spore numbers to behave in a similar manner to that of high spore numbers.

4.2.3 Supplementation of Spores in Complex Medium

In this study the complex medium, potato dextrose broth, was used as carbon source. Low spore numbers were supplemented with the quorum sensing molecule butyrolactone I, ascorbic acid, spore supernatants (SS) and ethanol.

In *A. terreus,* butyrolactone I was found by Schimmel *et al.,* (1998) as being able to induce morphological changes, increase spore formation and increase secondary metabolite production. They found that additions between 200 – 500 μM of butyrolactone I (dissolved in ethanol), to 18 hour old submerged cultures, increased hyphal branching but decreased the average length of hyphae associated with each branch, whilst cell mass remained constant, secondary metabolite production of lovastatin also increased. The highest lovastatin yield was obtained when additions of butyrolactone I were made 120 hours post inoculation. Similarly, Raina *et al.,* (2012) added 100 nM butyrolactone I at 24, 96, and 120 hours post inoculation to submerged cultures of *A. terreus* in 5 L bioreactors. The additions at 96 hours produced the highest lovastatin yields with a 2.5 fold increase compared to control samples of the same time point.

This was the basis for additions of butyrolactone I to spore inoculum cultures, in the thought that it would alter the morphology of the culture and thereby improve lovastatin production. However, 100 nM additions of butyrolactone I to low spore cultures, did not drastically alter the morphology of the culture to match that of high spore cultures, there were changes to the morphology of the culture. Pellet diameters of low spore cultures with the addition of butyrolactone I, 40 hours post inoculation, were smaller in diameter compared to the low spore control, but still larger than high spore numbers, similarly at 65 hours. Overall, however, there was a decrease in diameter, but at 90 hours, diameters increased to greater than that of low spore control diameters (figure 3.11). Throughout the study, tip numbers gradually increased and at 65 hours post inoculation, were lower than that of low spore number control cultures, but higher than that of high spore number control cultures. At 90 hours, tip numbers were greater than that of both high and low control groups (figure 3.12).

Ideal morphology is for low spore numbers to be small pellets with large number of hyphal tips, similar to that of high spore numbers. Ethanol decreased pellet diameters and tip numbers; this could potentially be due to inhibition. As butyrolactone I was dissolved in ethanol, it would be ideal to see that ethanol have no effect on the culture, but as it decreased both pellet diameter and tip numbers, it cannot be determined whether the effects seen with the addition of butyrolactone I could be due to ethanol inhibition, and therefore the butyrolactone I would be working antagonistically with the

ethanol. But this cannot be said for certain, as further investigations would be required.

The results obtained were not as expected, the reasons for this may have been that the number of germinated cells had surpassed the threshold concentration for QS response to butyrolactone I, to address this, higher concentrations of butyrolactone I could be added at the time of inoculation to determine if this changes the effect on morphology. Raina *et al.,* (2012) found that over a period of 48 hours of growth, the concentration of butyrolactone I in the culture supernatant would steadily increase. After 48 hours however, there was a decline in butyrolactone I concentrations. This may suggest that after 48 hours the culture is no longer responding to butyrolactone I concentrations present in the media, or that the culture is no longer producing the QSM molecule, as it has rendered it useless.

Overall the ideal morphology was obtained with the addition of butyrolactone I, with reduced diameters and increased tips. This experiment was limited by the concentration of butyrolactone I added. It would have also been useful to identify if lovastatin production was increased with the addition of butyrolactone I, irrespective of morphology.

Spore supernatants (the spore free liquid) were obtained during spore harvest (section 2.5). In both bacterial and fungal research on quorum sensing, culture supernatants and spent medium have been used to supplement fresh media as they contain potential QSMs that are secreted during growth by the

organism, therefore research has been carried out using different organisms in identifying the quorum sensing molecules present in the cultures supernatants and spent media.

For a molecule to be deemed as a QSM, there are certain criteria that must first be fulfilled, these include, I) the ability to regulate its own production. II) The production of the molecule should be continuous throughout the growth of the organism, accumulation of which is proportional to cell density. III) The point at which the QS response is initiated during the growth stage is determined by cell density, which is species specific, and therefore the QSM qualifies as being cell density dependant (Swift *et al.,* 2001; Miller and Bassler., 2001; Bassler, 2002; Winzer *et al.,* 2002). IV) The molecule must evoke a coordinated response in the organism that does not include metabolising or detoxifying the molecule itself, some examples include sporulation (Weinrauch *et al.,* 1990), morphological differentiation (Ochi, 1987), secondary metabolite production (Bainton *et al.,* 1992) and virulence determination (Zhu *et al.,* 2002). V) Exogenous addition of the QSM to mutant strains that are deficient in the ability to produce the QSM, should restore the QS response (Albuquerque and Casadevall, 2012).

Results from addition of SS showed an increase in pellet diameters, that were larger than the control runs with low spore numbers, hyphal tip numbers were also abundant, which is an ideal morphological characteristic, but not in conjunction with large pellet diameters. At 40 hours diameters were much smaller than low spore number control cultures, closer to diameter sizes of

high spore control cultures. However, as time progressed diameters increased, still lower than that of low spore control cultures, but greater than high spore control cultures. At the end of the run the diameter is greater than that of both high and low. From the time of inoculation until 65 hours post, diameters were as expected and as desired, smaller than low spore numbers and closer to that of high spore control (figure 3.12).

The desired outcome was achieved, with an increase in tip numbers surpassing both high and low spore numbers. Although a decrease in tip numbers was observed at the end of run, this was expected due to shear in the flasks. Further investigations are required so as to determine the cause of changes in spore morphology. There could potentially be QSM secreted into the environment that is then used to supplement the spores.

Ascorbic acid was used as an additive because of the work by Van Hauwenhuyse *et al.,* (2014) he found that ascorbic acid interfered with the yeast – to hypha transformation in *C. albicans.* Ojha *et al.,* (2009) also found that concentrations of 50 – 100 mg/mL ascorbic acid on *C. albicans* delayed growth as ascorbic acid concentrations increased. Addition of 125 mg/mL caused cells to remain in lag phase for 12 hours, double the time for control cultures of the same cell concentration. However, the log phase was shortened to 2- 4 hours, as opposed to control of 8-10 hours. Treatments with ascorbic acid at concentrations above 15 mg/mL, resulted in the cultures remaining as yeast form and hyphae like extension was not observed. Similar findings of hyphae differentiation inhibition were obtained by Nasution *et al.,* (2008).

At 45 hours post inoculation additions of AA reduced pellet size and was closest in size to that of high spore numbers. However, after this, diameters increased, at 65 hours pellets were smaller than low spore control cultures, but were greater than that of high cultures, and smaller than diameters seen where butyrolactone I and SS were present. By the end of the run at 90 hours pellet diameter was greater than that of low spore controls but still smaller than that seen with QSM and SS additions. Hyphal tip numbers were higher than that of high spore numbers but lower than low spore numbers, and remained consistent throughout growth. Therefore, addition of ascorbic acid to spore inoculum concentration did not have any notable effect on pellet diameter, nor increase hyphal tip numbers in comparison to the controls.

Overall addition of butyrolactone I had the highest number of hyphae tips at the end of the 90 hour run, but the desired morphology was that obtained with SS additions and these morphologies remained during the course of growth.

4.2.4 Supplementation of Spores with High Spore Culture Supernatant

In this study supernatants from 72 hour *A. terreus* cultures, grown using high spore inoculum (1×10^7 spores/mL), were added as supplements (high spore culture supernatants, HSCS).

Chen *et al.,* (2004) found that the addition of cell – free supernatants from high density *C. albicans* cultures of inoculum concentrations of > 10_8 cells/mL to low cell densities of 5 x 10_3 cells per mL, abolished their lag phase but increased exponential growth. However, supernatants from low density cultures had no effect on lag phase. Alternatively, addition of supernatants from stationary phase of *Histoplasma capsulatum* to low density cells induced α-(1,3)-glucan, a compound which is usually in low amounts in the cell wall (Kugler *et al.,* 2000). Morphological changes were observed in cultures of *Ceratocystis ulmi* as spent medium added to fresh cultures encouraged morphological switch from hyphae to yeast growth (Hornby *et al.,* 2004).

In this study the addition of HSCS to low spore inoculum concentrations reduced pellet diameters to a size similar than high spore cultures. In low spore cultures, pellet diameters at 40 and 45 hours were the same as that seen in high cultures (figure 3.13). There is a trend that is observed, post 45 hours diameters increased, which is also seen in previous experiments, figure 3.11, with the addition of butyrolactone I and SS, where at 60 hours diameters increased. Pellet diameters remained constant as time progressed after 40 hours in high spore cultures, suggesting that in high spore numbers the compound responsible for the morphological differences (potential QSM) is in abundance and may be secreted constantly throughout growth.

Figure 3.14 shows hyphal tip numbers, at 40 hours, the number of tips is lower than that of high spore numbers. At 45 hours, the number of tips surpasses

that of both high and low spore numbers. The ideal morphology is achieved at 45 hours, small pellets with an abundance of hyphal tips.

With addition of HSCS the concentration of compounds present may be surpassed by the culture, and so there is more culture than compounds present. Therefore, a more concentrated spore supernatant is required for investigation, such as FDSS.

4.2.5 Supplementation of Spores with Freeze Dried Spore Supernatants

The aim of this study was to investigate the effects that FDSS have on the morphology of low spore cultures, so as to obtain small pellets similar to that of high spore cultures with abundance of hyphae, with a comparable lovastatin production.

With the data and results obtained, there are compounds within the spore supernatants that when at high concentrations evoke a response in the low spore cultures, causing changes in growth to that similar of high spore cultures, from which the washings have been obtained. Further investigations into the specific composition of the FDSS needs to be carried out, and cross referenced to determine if QSMs are present, and if not, what the cause of the change is.

Section 3.2.4.2 looked at the effects of high spore culture supernatants on the morphology of the culture and found there to be changes to the culture at 45 hours post inoculation, but at the end of the run the changes were not as great or of a big impact. Therefore, the next step was to concentrate spore washings further and use this to supplement the culture. Therefore, increasing the amount of potential QSMs with which the spores are able to interact. Spore washing obtained during harvest were freeze dried to become more concentrated and these were added to low spore numbers, in order to see effects on lovastatin production. Figure 3.34 showed that low spore controls consumed the least amount of substrate, but also produced least amount of lovastatin. Low cultures supplemented with FDSS consumed less substrate than both high and low spore control groups, even though it produced the highest concentration of lovastatin, and had the lowest biomass (figure 3.35).

As stated by Schimmel *et al.*, (1998), additions of butyrolactone I, a QS compound found in *A. terreus*, altered morphology of the culture by increasing hyphae branching, whilst maintaining cell mass (section 4.2.3). However, additions of FDSS to low spore cultures (assuming QSMs present) changed morphological characteristics to that of high spore cultures, with decreased pellet diameters and increased hyphal tip numbers (figure 3.38) but biomass decreased slightly (figure 3.35).

It is possible that SS, HSCS and FDSS may contain different compounds as they are extracted from different parts of the culture's growth phase. SS and FDSS would contain similar, except FDSS is more concentrated than SS.

HSCS are from high spores grown for 72 hours, so composition may be different. Could potentially concentrate HSCS and also analyse each of the additives.

The highest concentration of lovastatin produced (table 3.5) was in low spore cultures with FDSS, 1208% increase compared to low spore control cultures. When compared to high spore control cultures, low spore cultures with FDSS produced 62% more lovastatin, which is the desired outcome, as the intention was for low spore cultures to behave in manner similar to high spore numbers, with regards to both physical morphology and lovastatin production, which has been achieved. In particular low cultures with FDSS look more similar to high spore control cultures, as observed in figures 3.36 and 3.37.

When compared to lovastatin concentrations obtained by Raina *et al.,* (2012), where butyrolactone I was added to cultures over a period of 120 hours, the highest concentration obtained was 3.2 g/L, a 3.5 fold increase compared to the control samples. The overall concentration obtained in this study was lower than that obtained by Raina *et al.,* (2012)*,* however the amount produced in comparison to the control in this study is significantly higher. Culture morphologies were also greatly altered.

 With this amount of lovastatin production increase, yields obtained and low carbohydrate consumption, this method would be ideal in industry, obtaining high quantities of product, more than if high spore cultures were used, can allow for fermentations to be carried out irrespective of spore inoculum size,

but with high productivities. However, a limitation that arises is the carbohydrate wastage, as pure glucose and lactose were used as substrate, and with the amount remaining at the end of the run it is not very efficient.

4.2.6 Supplementation with Quorum Sensing Molecules

One of the most highly researched fungal QSM is farnesol, produced and secreted by the dimorphic fungus, *Candida albicans*. Farnesol regulates the dimorphic switch from yeast to filamentous growth form based on cell culture densities. Alongside farnesol, another QSM, tyrosol, is produced. It works antagonistically to farnesol.

When the culture is in an environment that favours filamentous growth, tyrosol acts as a cell signalling molecule produced by the culture, causing a reduced lag phase in the culture and stimulation of germ tube formation which results in filamentous formation (Chen *et al.,* 2004).

4.2.6.1 Tyrosol

As described in the introductory chapter (section 1.4), addition of tyrosol to cultures of low densities reduces lag phase and promotes filamentous growth of *C. albicans* cultures.

An objective of this study was to investigate the effects to spore germination time and culture morphology when different spore inoculum sizes were supplemented with QSMs. In this study different concentrations of tyrosol were added to spore inocula.

Results from figure 3.18 show that additions of 0.04 and 0.01 M tyrosol reduced pellet size in both high and low spore cultures. Figure 3.20 shows hyphal tip numbers for high spore cultures and that there is a decrease in tip numbers which is not ideal or desirable. In low spore cultures (figure 3.19) with addition of tyrosol, pellets were not observed until 60 hours post inoculation, and pellet diameters were smaller than that of low spore controls. Figure 3.21 show tip numbers increased at 60 hours with 0.01 M tyrosol, and additions of 0.04 M DMSO caused a decrease in both tip numbers and diameter and 0.04 M tyrosol decreased tip numbers, however, the addition of 0.01 M DMSO increased tip numbers and diameters.

DMSO was added to cultures as control, but it is not possible to definitively determine its effect on high spore cultures from the data obtained. DMSO has been found to effect fungal cultures, Randhawa and Aljabre (2007) found that DMSO of 1% (v/v) inhibited growth of *Microsporum canis,* however

concentrations below 1% (v/v) had no effect on cultures and were deemed safe for use as solvent for antifungal drugs. In *C. albicans* Rodriguez-Tudela *et al.,* (2001) found that concentrations as low as 2% (v/v) DMSO slowed down the growth.

The effects of tyrosol on filamentous fungi has not been widely investigated. With regard to *C. albicans* tyrosol promotes growth and reduces lag phase, in this study this is not the case, and results are conflicting as tyrosol was dissolved in DMSO, and therefore it cannot be determined whether the results observed are due to an element of QS via tyrosol, or due to and an inhibitory effect from DMSO which has been widely reported as a fungal growth inhibitor. Results may also be due to the receptors in *A. terreus*, as tyrosol is not a known QSM for the culture and therefore its response may be a survival method, as cultures would only come into contact with tyrosol when grown in a co-culture. However, this is speculation as studies on this area have not been carried out.

Overall tyrosol did change morphology of the culture, however it was not the desired morphology. Table 3.1 shows percentage change of pellet diameters and hyphal tip numbers in both high and low spore cultures. It can be seen that DMSO does have an effect on the culture, which is not adequate of a control. Therefore, this work is limited by the carrier of tyrosol, and if investigations were to continue a different method for tyrosol dilution would need to be implemented.

4.2.6.2 Farnesol

Nikerson *et al.,* (2006) found that in *C. albicans* the most efficient farnesol concentration to have an effect on germ tube formation was between 1-2 µM. This concentration reduced germ tube formation by 50%. Studies carried out have found that farnesol is produced by different organisms, for example, in *Saccharomyces fermentati,* farnesol is produced during wine making processes (Fagan *et al.,* 1981). Granshaw *et al.,* (2003) found that during the growth cycle of *Neurospora crassa* farnesol is produced as part of conidiation. Similarly, in *A. niger* cultures treated with > 10 mM farnesol conidia formation was abolished (Lorek *et al.,* 2008).

Other investigations have been carried out into the effects of farnesol addition to organisms that do not produce it. Semighini *et al.,* (2006) found that in *A. nidulans,* 100 µM triggered apoptosis, and when grown in a co-culture with *C. albicans*, CFU/mL of *A. nidulans* were reduced by 180 fold within 24 hours. This is a survival technique in response to farnesol found naturally in the surrounding environment, produced by other organisms.

The aim of this investigation was to determine the effects of farnesol on spore germination and morphology. Overall, farnesol additions decreased pellet sizes, and increased the number of hyphal tips, and delayed germination.

From figure 3.24 the addition of 0.08 M of farnesol to high spore cultures caused pellet formation at 18 hours post inoculation, similar to high spore control cultures. At 40 hours post inoculation, pellets diameters of high cultures

were similar in size for all concentrations of farnesol added. However, from 60 hours to 90 hours pellet diameters remained smaller than that of high spore control cultures. The addition of farnesol to low spore inoculum reduced pellet size, in particular, additions of 0.01 M had the greatest reduction on pellet diameter formation (figure 3.25).

At the end of the run, 90 hours post inoculation, in high spore cultures, hyphal tip numbers were greater than high spore control cultures for each farnesol concentration (figure 3.26). However, in low spore cultures (figure 3.27) tip numbers were greatly increased, specifically with the addition of 0.01 M farnesol. Table 3.2 shows that that 0.01 M farnesol increased tip numbers and decreased pellet diameters, which is the desired morphology.

Low spore cultures with addition of 0.01 M farnesol produced pellets that were small with lots of hyphae (figure 3.25/3.26), morphology described as ideal for lovastatin production. In order to determine if this was the case, 1 L shake flasks with lovastatin production medium and high and low spore inoculum were supplemented with 0.01 M farnesol, and production of lovastatin determined.

At 14 days post inoculation (figure 3.30), lovastatin concentrations were much higher in low spore cultures than in low spore control cultures. Low spore control cultures produced 175 mg/L of lovastatin, 424 mg/L was produced in low cultures with farnesol, an increase of 170%. Control group of high spore numbers produced 615 mg/L of lovastatin, and high spore cultures

supplemented with farnesol produced 1151 mg/L, an increase of 87%. The experimental run was stopped after 14 days, but as carbohydrate concentrations were still relatively high (figure 3.31) the run could have continued until depletion to determine true final concentrations of lovastatin produced based on substrate availability. All conditions were given the same carbohydrate concentrations initially. From figure 3.31, high spore cultures consume the most amount, which coincides with biomass quantity (figure 3.32). But for low cultures with farnesol, increased carbohydrate consumption takes place. This limitation would need to be addressed if work were to continue. Culture morphologies is comparable between the two culture sizes, but concentration of lovastatin is different. Based on results obtained, additions of farnesol to low spore cultures delays lovastatin production, therefore continuing the fermentation beyond 14 days would be of interest regarding production levels.

Research states that farnesol inhibits growth in *C. albicans*, however in cultures of *A. terreus* it seems to have a positive effect on growth and improves lovastatin productivity.

4.3 Lovastatin Comparison of FDSS to Farnesol

The addition of farnesol and the addition of FDSS both had a positive impact on culture morphology and lovastatin productivity in low and high spore cultures.

With the addition of FDSS to 1×10^7 spores/mL (high spore inoculum) there was an 87% increase in the amount of lovastatin produced compared to control cultures of the same inoculum size. The addition of farnesol, to the same spore inoculum size increased lovastatin production by 7%. Moreover, the addition of FDSS to 1×10^3 spores/mL (low spore inoculum) increased lovastatin concentrations by 170% compared to control cultures of the same inoculum size. The addition of farnesol to the same spore inoculum size increased production by 1208%. Production levels by low spore inoculum size should aim to produce the same or more lovastatin than that produced by high spore control cultures. Therefore, when compared, low spore inoculum with FDSS added produced 23% less lovastatin than high spore control cultures, however, low spore cultures with farnesol added produced 62% more lovastatin than high spore control group (as summarised in table 4.1).

Table 4.1 *Percentage differences of lovastatin production test cultures of high (1 x 10^7 spores/mL) and low (1 x 10^3 spores/mL) spore numbers inoculated with either FDSS or farnesol compared to control cultures of high and low spore numbers*

Test Vs Control Spore number	FDSS	Farnesol
High Vs High	87% Increase	87% Increase
Low Vs Low	170% Increase	1208% Increase
Low Vs High	23% Decrease	62% Increase

Therefore, farnesol had the greatest impact with regard to lovastatin production, where other research had found it to inhibit growth. These results support the notion that the FDSS possibly contain a QSM and comparisons to results of a known QSM support that overall finding. It can therefore be deduced that there is a QS process (or similar) between spores. However further investigations need to be carried out in order for these findings to be considered definite (section 6.1)

Similarly, in cultures where FDSS were present, lovastatin production increased. But for low cultures production does not reach levels to that seen in high spore numbers which is the ultimate goal. However as effects on the culture are similar to that seen with farnesol, further investigations need to be carried out as there is a possibility that within the FDSS, there is a compound

of similar structure to farnesol or a collection of compounds that can evoke a similar response as seen with farnesol additions.

4.4 Spore contact studies

4.4.1 Sonication

Sonication was carried out in order to increase impingement. That is the numbers of individual hits. But also to stop spores from 'sticking' together, as spores that are agglomerated or 'stuck together' germinate and form large clumps and pellets, the vibrations that are created between spores force separation, dislodging them from one another, and therefore eliminating the chances of pellet formation via agglomeration, which is one mechanism for large pellet formation (Nielsen *et al.,* 1995; Dynesen and Nielsen, 2003)

Ultrasound has been used in specifically designed sonobioreactors as a method to increase the productivity of a biological process. At high acoustic power inputs (> 20 kHz) ultrasound ruptures cells, methods employed in the laboratory technique use ultrasonication for cell disruption. However, intermittent power ultrasound for a short duration of time have shown productivity enhancing effects (Chisti, 2003).

Francko *et al.,* (1990 and 1994) found that in cultures of *Anabaena flos-aquae* growth rate and final biomass yields increased up to 46% when subjected daily to low sonication levels of 20 kHz, 50 W pulse for 5-minute periods. However,

in microalgae, *Selenastru capricornutum,* sonication reduced growth rates but in both cases the production of secondary metabolites increased (Francko *et al.,* 1990).

Lin *et al.,* (2001) and Wu and Lin (2002) found that suspensions of *Panax ginseng*, when subjected to low doses, sonication of 100 kW m$_{-3}$ for a period of between 0.5 to 6 minutes found ion fluxes across cell membranes and secondary metabolism were stimulated without reducing biomass yield. Intermittent sonication doubled ethanol yield in *Saccharomyces cerevisiae,* however, continuous sonication did not have this effect and lower frequencies reduced fermentation times in beer, sake and wine fermentation by the same yeast (Matsuura *et al.,* 1994. Schlafer *et al.,* 2000).

The reasons and mechanism behind increases in metabolic activity when subjected to ultrasound are not known (Sainz Herran *et al.,* 2008), but Liu *et al.,* (2005) hypothesised that the cause is due to the increase in particle movements within the liquid, accelerating mass transfer rates in the reactor and therefore production is increased.

In this study sonication was performed on 5 mL spore suspension prior to inoculation, spore suspensions of 1×10^7 (high) and 1×10^3 (low) spores/mL were subjected to 0 (control), 1, 2 and 5 minutes of sonication (test conditions). Viability of spores was determined microscopically, as sonication may have rendered the spores unable to grow. Spores were sonicated for the maximum time used in the study of 5 minutes, and plated, 10 spores were used, and

after 5 days of incubation, 10 individual clusters of growth was observed, therefore all spores were viable. Microscopic observations of spores after sonication showed that spores were separate from one another.

No difference was observed between pellet diameters of high spore cultures at various sonication times (figure 3.39). However, pellet diameters of low spore cultures increased initially at 40 and 65 hours for spores subjected to 5 minutes of sonication. Spores subjected to 1 minute of sonication, pellet diameters of cultures increased at 65 hours post inoculation, but at the end of the run at 85 hours, in all cases, spore diameters increased surpassing that of low spore control cultures (figure 3.40). No real difference in hyphal tip numbers was observed in high spore cultures and low spore cultures subjected to various sonication time periods compared to control groups (figures 3.41 and 3.42).

In the current study sonication did not have any tangible overall effect on germination or culture morphology. However, Sainz Herran *et al.,* (2010) found that for *A. terreus,* sonication did not affect biomass growth rate and total biomass concentration at high or low sonication intensities, and that ultrasound was useful for influencing metabolite production, fungus morphology and broth rheology, whilst not damaging the microorganism.

Sonication separated spores, but the separation did not change the morphology in any way. Therefore, increasing spore to spore contact did not

alter growth morphologies, but it cannot be determined whether sonication affected secondary metabolite production.

4.4.2 Confinement Studies

One of the objectives of this project was to investigate the effects of confinement on spore germination and morphology. Spores were confined to two different flask sizes, 100 mL and 1 L flasks, working volume of 20%. Each flask was aerated. Spores were observed over a 48 hour period, differences in morphology were significant. Spores confined to the smaller 100 mL flask germinated first after 18 hours post inoculation, then after 5 hours, germination was observed in the larger flask. Morphology in both flasks differed as the smaller flask produced thick, dense mycelia, whereas in the larger flask spores took longer to germinate, growth started as loose mycelia and then became denser, and rounder forming a tight sphere. In the smaller flask there was short germination period, common to that observed in large inoculum sizes, and morphology was small loose pellets.

In a small space, a small number of spores behave similar to that of large spore numbers. The reasons for this may be due to spore to spore interactions in which spores sense one another and act in a manner that is seen when in abundance. Another explanation put forward by Boedicker *et al.,* (2009) suggest that the outcome is due to QS, and the confining of the spores to a small space also restricts the QSM to a small space and the spore and QSM are in constant contact, unlike in a large volume where there is a greater

diffusion gradient, and the concentration of QSM is diluted by the medium that it is in, and the molecules become dispersed reducing the interaction of the spore to the QSM.

Similarly, research has been carried out with bacterial cells of *P. aeruginosa* in which a single cell was confined to a small volume of 1 pL and QSM that were secreted were unable to disperse away from the bacterial cells, and as the molecules accumulated in the vicinity of the bacterial cell, response mechanisms were evoked (the QSM secreted by the bacterial cells were trapped in the small volume alongside the bacterial cells, and so the cells then responded to its own QSM that it secreted into its environment)

In this study, the restricted environment means that the QS molecules that are secreted are unable to disperse, and instead remain in the vicinity of the spores, therefore the spores interact. The number of molecules secreted into the small space would be the same number secreted into the large space the difference is the concentration. The smaller space has less liquid therefore the more concentrated the molecules will be. In the smaller flask the threshold concentration is reached faster. But a specific quantity is the threshold. The smaller volume caused a larger concentration, therefore the spores 'thought' there was a high number of them present in the media.

A. terreus is known to produce QSM, and itself is affected by QS. It is not known whether there is a QS process involved with fungal spores, but this confinement study does lead one in that direction suggesting there is a form

of communication, whether through physical contact or chemical signals that takes place between the spores. Therefore, considering all the factors it can be suggested that it is highly likely that a QS molecule is involved, and QS processes takes place between *A. terreus* spores where it causes changes in the morphology of the culture.

5. CONCLUSION

Previous research carried out on fungal spores has suggested that spore numbers affect overall culture morphologies and the production of secondary metabolites. However, it is not clear why initial spore concentration of the inoculum has such an impact on the subsequent culture, and what mechanism(s) are involved that enables these differences. This work aimed to investigate the reasons behind these differences in culture morphology and physiology based on potential communication between spores.

There were two main hypotheses set out in the beginning of this work with regards to explanations associated with why the differences in spore inoculum size can lead to differences in fungal morphology, growth and productivity. The first hypothesis for these differences was due to a quorum sensing phenomenon, whereby a chemical (QS) process. High quantity of spores are constantly producing and secreting chemical compounds into their environment, causing cells to behave in a particular manner, of which morphological characteristics and secondary metabolite production. The second was based a form of physical interaction taking place between the spores, that a high quantity of spores are able to physically interact as there are more of them to come into contact with, enables them to communicate or interact in a manner that manifests as a physical response, altering morphology and productivity.

Chemical interactions were investigated to see what effect these have on the overall morphology and productivity of the culture, through the supplementation of a small quantity of *A. terreus* spores with QSMs, spent

medium from high spore cultures, spore supernatants and concentrated spore supernatants (FDSS).

The addition of known QSMs tyrosol and farnesol, impacted the way in which the culture evolved. Farnesol in particular positively impacted the culture morphology. Low quantities of spores with the addition of farnesol formed small pellets with an abundance of hyphae, the ideal and desired morphology for *A. terreus* cultures, seen in high spore cultures. Farnesol also improved lovastatin production in the culture of both high and low spore numbers. Additions to low spore cultures increased lovastatin production 1208%, 62% more than that produced by high spore cultures. There is, therefore, promising compounds within the FDSS that need more investigation, so as to determine whether these compounds are truly QSMs.

Freeze dried spore supernatant (FDSS) were also investigated for effects on both culture morphology and productivity. FDSS additions to low spore numbers affected the overall morphology of the culture, to be as that of high spore cultures. Lovastatin production in low spore cultures was also greatly increased by 170%. Comparisons to effects observed with farnesol additions showed similarities in the way in which the QSM farnesol, and FDSS affected both the morphology and lovastatin productivity.

In addition to this, investigations into the effects of physical interactions between spores was carried out by forcing interactions between spores through sonication to encourage spore to spore contact. Through this

investigation, it was found that sonication had no effects on the spore integrity, nor the growth of the culture.

Studies into the impact that the area within which spores are able to disperse, and chemicals to diffuse was also investigated. A small quantity of spores were restricted to and cultured in a small physical space; comparisons were made to the same spore number cultured in a larger space. By restricting the space spores germinated faster and morphology was more like that seen when large quantities of spores were cultured. Reasons for this need to be further investigated, whether spores are able to interact better with each other or with the chemicals that are secreted.

From this work it can be said that spores are able to interact both chemically and physically with one another, exact processes, however, are undetermined. However, this work has brought together some important findings with regard to communication and spore-to-spore interactions. Through the use of spore supplementation, and confinement studies, the differences observed in morphology and productivity may be attributed to QS process.

These observations can be exploited and used in the first instance for improving large-scale production for example lovastatin using *A. terreus* cultures. These concepts may be extended to and explored in other fungal cultures. These findings not only have an impact on industry with regards to enhancement of production, but also in terms of broadening research knowledge, and understanding the nature and physiology of spores.

6. FUTURE WORK

During the progression of this work, different aspects of investigation and various lines of inquiry have arisen. Therefore, the following lines of research have been posed and, if pursued, could lead to further developments and understanding of the mechanisms of interaction between *Aspergillus terreus* spores, giving reasons as to why the inoculum size of the culture is so important with regard to culture morphology and physiology.

6.1 Identification of Compounds in Spore Supernatants

This research has shown that freeze dried supernatants from *A. terreus* spore preparations have a significant effect on spore germination, morphology and productivity of the culture. Therefore, it is important to identify molecules within the supernatant responsible for these changes. Subsequently, the mechanism of action of the molecule(s) can be investigated.

To further understand the metabolic activities that take place pre-germination, the spore supernatant can be purified for various compounds, not only potential QSM, but also, for example, other proteins. In order for a molecule to be deemed a QSM it should have the ability to regulate its own production. Auto-induction is the most important property of a QSM. Studies have shown that QSM auto-induce their own synthesis. Genes regulating the synthesis of enzymes are activated by components within the media.

In order to purify and identify the unknown compounds ethyl acetate extraction would be undertaken. GC-MS of the ethyl acetate extract of a high cell density *A. terreus* culture supernatant, should reveal numerous amounts of compounds. Followed by purification and identification of the molecules by fractionation via HPLC.

Once the active compound(s) are purified and isolated, its structural elucidation would then be carried out by nuclear magnetic resonance spectroscopy (NMR). Once compounds have been identified and analysed, the culture is subjected to the compound so that changes in culture morphology can all be analysed for. Molecules can then be cross-examined with other known QSM for similarities in structure and function. Compounds of interest, chemically synthesised and added to spore cultures to compare outcomes.

6.2 Application of Methods to Other Cultures

The effects of freeze dried spore supernatants on germination, morphology and secondary metabolite production should be further investigated in other *Aspergillus* species, for example *A. niger* and *A. flavus.* These effects can then be cross examined with the addition of QSM/compounds identified to be present within spore supernatants. The effects of FDSS can also be investigated in other industrially important fungi, such as *Monascus purpureus.*

6.3 Butyrolactone I Studies

A. terreus is widely known for its production of butyrolactone I, and its QS effect on the culture, initial studies carried out were not in depth and inconclusive to state whether spores also produced and responded to butyrolactone I.

Butyrolactone I is a well-known QSM in *A. terreus* cultures and is used to improve productivity. Therefore, further investigations into the effects of this QSM on *A. terreus* spores needs to be carried out. Work carried out by Raina (2008) found that additions of 100 nM butyrolactone I made over the growth period to *A. terreus* cultures increased lovastatin production.

Investigations into the effect of butyrolactone on spores would give an insight into the relationship between spores and cultures, whether they share similarities in QS mechanisms. Further investigations also carried out into the life of the QSM, whether these are consumed, broken down or accumulate in the environment. This again would give invaluable insight into the ways in which spores interact with these communication molecules.

6.4 Scale – Up

Scale-up study of processes carried out at bioreactor scale to determine whether productivities can be maintained or improved at large scale with implementation of FDSS, and also with the use of compounds identified through processes carried out during identification (section 6.1).

Following this process optimisation of bioreactor studies would be carried out. Large scale production of FDSS for QSM extraction would need to be produced in a cost effective manner and therefore less wastage would need to occur. The current process requires high quantities of expensive pure carbohydrates such as glucose ad lactose. The use of a cheap carbon source, where all carbohydrates are consumed, with a high product yield, would be both cost effective and sustainable. However, an optimum process would need to be developed.

The mechanisms carried out in this study can also be used with cultures that are not studied extensively; for example, basidiomycetes, in order to establish process method transferability.

7. REFERENCES

Adams, T.H., Wieser, J.K., Yu, J. (1998) asexual sporulation in *Aspergillus nidulans. Microbiology and Molecular Biology Reviews.* 35 – 54

Agger, T., Spohr, A. B., Carlsen, M., and Nielsen, J. (1998) Growth and product formation of *Aspergillus oryzae* during submerged cultivations: verification of a morphologically structured model using fluorescent probes*. Biotechnology and Bioengineering.* **57** (3) 321 – 329

Albuquerque, P., Casadevall, A. (2012). Quorum sensing in fungi – a review. *Medical Mycology* **50** 337 – 345

Alem. M. A., Oteef. M. D., Flowers. T. H., Douglas. L.J. (2006) Production of tyrosol by *Candida albicans* biofilms and its role in quorum sensing biofilm development. *Eukaryotic Cell.* **5** (10) 1770 – 1779

Anderson, J. G., Smith, J. E. (1972) The effects of elevated temperatures on spore swelling and germination in *Aspergillus niger. Canadian Journal of Microbiology.* **18** (3) 289 -297

Atkinson. S., Williams. P. (2009) Quorum sensing and social networking in the microbial world. *Journal of The Royal Society Interface.* **6** 969 – 978

Bainton, N. J., Bycroft, B. W., Chhabra, S. R., Stead, P., Gledhill, L., Hill, P.J., Rees, C. E., Winson, M. K., Salmond, G. P., Stewart, G. S. (1992). A general

role for the lux autoinducer in bacterial cell signalling: control of antibiotic biosynthesis in *Erwinia. Gene* **116** (1) 87 – 91

Barriuso, J. (2015) Quorum sensing mechanism in fungi. *AIMS Microbiology.* **1** (1) 37 – 47

Bassler, B. L. (1999) How bacteria talk to each other: regulation of gene expression by quorum sensing. *Current Opinion in Microbiology.* **2**, 582 – 587

Bassler, B. L. (2002). Small talk. Cell-to-cell communication in bacteria. *Cell.* **109** (4) 421 – 424

Beppu T. (1995) Signal transduction and secondary metabolism: prospect for controlling productivity. *Trends Biotechnology.* **13** 264–269

Bizukojc, M., Ledakowicz, S. (2007) A macrokinetic modelling of the biosynthesis of lovastatin by *Aspergillus terreus. Journal of Biotechnology.* **130** (2007) 422 – 435

Bizukojc, M., Ledakowicz, S. (2008) Biosynthesis of lovastatin and (+)-geodin by *Aspergillus terreus* in batch and fed-batch culture. *Biochemical Engineering Journal.* **42** (3) 198 – 207

Bizukojc, M., Pawlowska, B., Ledakowicz, S. (2007) Supplementation of the cultivation media with B-group vitamins enhances lovastatin biosynthesis by *Aspergillus terreus. Journal of Biotechnology.* **127** 258 – 268

Bizukojc. M. and Ledakowicz. S. (2009) Physiological, morphological and kinetic aspects of lovastatin biosynthesis by *Aspergillus terreus. Biotechnology Journal.* **4**, 647 – 664

Bizukojc. M., Ledakowicz. S. (2010) The morphological and physiological evolution of *Aspergillus terreus* mycelium in the submerged culture and its relation to the formation of secondary metabolites. *World Journal of Microbiology and Biotechnology.* **26** 41 – 54

Boedicker. J. Q, Vincent, M. E., Ismagilov, R. F. (2009) Microfluidic confinement of single cells of bacteria in small volumes initiates high-density behaviour of quorum sensing and growth and reveals its variability. *Angewandte Chemie International Edition.* **48** 5908 – 5911

Boon, C., Deng, Y., Wang, L., He, Y., Xu, J., Fan, Y., Pan, S. Q., Zhang, L. (2008) A novel DSF-like signal from *Burkholderia cenocepacia* interferes with *Candida albicans* morphological transition. *The ISME Journal.* **2** 27 – 36

Boruta, T., Bizukojc, M. (2016) Induction of secondary metabolites of *Aspergillus terreus* ATCC 20542 in the batch bioreactor cultures. *Biotechnological Products and Process Engineering.* **100** 3009-3022

Braun, S. and Vecht-Lifshitz, S. E. (1991) Mycelial morphology and metabolite production. *Trends in Biotechnology.* **9** 63- 68

Brilhante, R. S. N., de Lima, R. A. C., Caetano, E. P., Leite, J. J. G., Castelo-Branco, D. C. M., Ribeiro, J. F., Bandeira, T. P. G., Cordeiro, R., Monteiro, A. J., Sidrim, J. J. C., Rocha, M. F. G. (2013) Effect of farnesol on growth, ergosterol biosynthesis, and cell permeability in *Coccidioides posadasii. American Society for Microbiology.* **57**(5) 2167 – 2170

Brilhante, R. S., de Lima, R. A., Marques, F. J., Silva, S. F., Caetano, E. P., Castelo-Branco, D., Bandeira, T., Moreira, J. L., Cordeiro, R., Monteiro, A. J. (2015) *Histoplasma capsulatum* in planktonic and biofilm forms: in vitro susceptibility to amphotericin B, itraconazole and farnesol. *Journal of Medical Microbiology.* **64** 394 – 399

Bryne, G. S and Ward, O. P. (1987) Effect of polymers on pelleting of *Rhizopus arrhizus. Transactions of the British Mycological Society.* **89** 367 – 371

Bushell, M. E., Bull, A. T. (1974) Anaplerotic carbon dioxide fixation in steady and non-state fungal cultures. *Archives of Microbiology.* **1** (23)

Byrne, G.S. and Ward, O.P. (1989) Effect of nutrition on pellet formation by *Rhizopus arrhizus. Biotechnology and Bioengineering.* **33** (7) 912 – 914

Carlile, M. J., Watkinson, S. C., Gooday, G. W. (1994) The fungi, 2nd ed. United Kingdom: Elsevier.

Carlsen, M., Spohr, A. B., Nielsen, J., Villadsen, J. (1995) Morphology and physiology of an α-amylase producing strain of *Aspergillus oryzae* during batch cultivations. *Biotechnology and Bioengineering.* **49** 266 – 276

Casas Lopez, J. L., Sanchez Perez, J. A., Fernandez Sevilla, F. G., Acien Fernandez, F. G., Molina Grima, E., Chisti, Y. (2003) Production of lovastatin by *Aspergillus terreus*: effects of the C:N ratio and the principle nutrients on growth and metabolite production. *Enzyme and Microbial Technology.* **33** 270 -277

Casas Lopez, J. L., Sanchez Perez, J. A., Fernandez Sevilla, J.M., Rodriguez Porcel, E. M., Chisti, Y. (2005) Pellet morphology, culture rheology and lovastatin production in cultures of *Aspergillus terreus. Journal of Biotechnology.* **116** 61 – 77

Casas Lopez, J. L., Sanchez Perez, J. A., Fernandez Sevilla, J.M., Acien Fernandez, F. G., Molina Grima, E., Chisti, Y (2004) Fermentation optimization for the production of lovastatin by *Aspergillus terreus*: use of the response surface methodology. *Journal of Chemistry Technology and Biotechnology.* **79** (10) 1119 – 1126

Chandra, J., Mukherjee, P. K., Leidich, S. D., Faddoul, F. F., Hoyer, L. L., Douglas, L. J., Ghannoum, M. A. (2001) Antifungal resistance of *Candida* biofilms formed on denture acrylic in vitro. *Journal of Dental Research.* **80** (3) 903 – 908

Chaynika. P. and Srivdya. S. (2014) Bioprospecting of lovastatin producing fungi isolated from soil samples. *International Research Journal of Biological sciences.* **3** (9), 42 - 46

Chen, H., Fujita, M., Feng, Q., Clardy, J., Fink, G. R. (2004) Tyrosol is a quorum-sensing molecule in *Candida albicans. PNAS* **101** (14) 5048 – 5052

Chesnokova, O.N., McPherson, A.H., Seichen, C.T. and Turnbough, C.L. Jr. (2009) The spore-specific alanine racemace of *Bacillus anthracis* and its role in supressing germination during spore development. *Journal of Bacteriology.* **191,** 1303 – 1310.

Chisti, Y. (2003) Sonobioreactors: using ultrasound for enhanced microbial productivity. *Trends in Biotechnology.* **21** (2) 89 – 93

Cho, Y. J.,Hwang, H. J., Kim, S. W., Song, C. H., and Yun, J. W.(2002) Effect of carbon source and aeration rate on broth rheology and fungal morphology during red pigment production by *Paecilomyces sinclairii* in a batch bioreactor*. Journal of Biotechnology.* **1** 13 – 23

Choudhary, S., Dannert, C. S. (2010) Applications of quorum sensing in biotechnology. *Applied Microbiology Biotechnology.* **86** 1267 – 1279

Cordeiro, R. A., Teixeira, C. E., Brilhante, R. S., Castelo-Branco, D. S., Paiva, M. A., Giffoni-Leite, J. J., Monteiro, A. J., Sidrim, J. J Rocha, M. F. (2013) Minimum inhibitory concentrations of amphotericin B, azoles and caspofungin against *Candida* species are reduced by farnesol. *Medical Mycology.* **51** 53 – 59

Cottier. F., Muhlschlegel. F.A., (2012) Review article: communication in fungi. *International Journal of Microbiology.* **2012** 1 – 9

Cowan, A.E., Coppel D. E. Setlow, B and Setlow P (2003) A soluble protein is immobile in dormant spores of *bacillus subtilis* but is mobile in germinated spores: implication for spore dormancy. *Proceedings of the National Academy of Sciences **100,*** 4209 - 4214

Cremer, J., Vatou. V., Braveny, I. (1999) 2,4-[hydroxyphenyl]-ethanol, an antioxidative agent produced by *Candida* spp., impairs neutrophilic yeast killing *in vitro. FEMS Microbiology letters.* **170** 319-325

Cugini. C., Calfee. M. W., Farrow. J. M., Morales. D. K., Pesci. E. C., Hogan. D. A. (2007) Farnesol, a common sesquiterpene, inhibits PQS production in *Pseudomonas aeruginosa. Molecular Microbiology.* **65** 896 – 906

Cui, Y. Q., Ouwehand, J. N. W., van der Lans, R. G. J. M., Giuseppin, M. L. F., and Luyben, K. C. A. M. (1998) Aspects of the use of *Aspergillus awamori. Enzyme and Microbial Technology.* **23** 168 – 177

Davies D G, Parsek M R, Pearson J P, Iglewski B H, Costerton J W, Greenberg E P. (1998) The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science.* **280** 295–298

Davis-Hanna, A., Piispanen, A. E., Stateva, L. I., Hogan, D. A. (2008) Farnesol and dodecanol effects on the *Candida albicans* Ras 1-cAMP signalling pathway and the regulation of morphogenesis. *Molecular Microbiology.* **67** (1) 47 – 62

de Hoon, M.J., Eichenberger, P. and Vitkup, D. (2010) Hierarchical evolution of the bacterial sporulation network, *Current Biology.* **20,** R735 – R745.

Deak E, Wilson SD, White E, Carr JH, Balajee SA. (2009) Aspergillus terreus accessory conidia are unique in surface architecture, cell wall composition and germination kinetics. *Public Library of Science.* **4**

Defoirdt, T., Boon, N., Sorgeloos, P., Vestraete, W., Bossier, P. (2006) Quorum sensing and quorum quenching in *Vibrio harveyi:* lessons learned from *in vivo* work. *Nature.* **2** 19 – 26

Derengowski, L.S., De-Souza-Silva, C., Braz, S. V., Mello-De-Sousa, T. M., Bao, S. N., Kyaw, C. M., Silva-Pereira, I. (2009) Antimicrobial effect of farnesol, a *Candida albicans* quorum sensing molecule, on *Paracoccidioides brasiliensis* growth and morphogenesis. *Annals of Clinical Microbiology and Antimicrobials.* **8** (13)

Dichtl, K., Ebel, F., Dirr, F., Routier, F. H., Heesemann, J., Wagener, J. (2010) Farnesol misplaces tip-localized Rho proteins and inhibits cell wall integrity signalling *Aspergillus fumigatus. Molecular Microbiology.* **76** 1191 – 1204

Dobretsov, S., Teplitski, M., Bayer, M., Gunasekera, S., Proksch, P., Paul, V. J. (2011) Inhibition of marine biofouling by bacterial quorum sensing inhibitors. *National Institutes of Health.* **27** (8) 893 – 905

Dohlman, H. G., Slessareva, J. E (2006) Pheromone signalling pathways in yeast. *Science Signalling.* **2006** (364)

Domingues, F. C., Queiroz, J. A., Cabral, J. M. S., Fonseca, L. P. (2000) The influence of culture conditions on mycelial structure and cellulase production by *Trichoderma reesei* Rut C – 30. *Enzyme and Microbial Technology.* **26** (5) 394 – 401

Du, L. X., Jia, S. J. and Lu F. P. (2003) Morphological changes of *Rhizopus chinesis* 12 in submerged culture and its relationship with antibiotic production. *Process Biochemistry.* **38** (12) 1643 – 1646

DuBois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., Smith, F. (1956) Colorimetric method for determination of sugars and related substances. *Analytical Chemistry.* **28** (3) 350 – 356

Dynesen, J., Nielsen, J. (2003) Surface hydrophobicity of *Aspergillus nidulans* conidiophores and its role in pellet formation. *Biotechnology Progress* **19** (3) 1049 – 1052

Edwards, A. G., Ho, C. S. (1983) Effects of carbon dioxide on *Penicillium chrysogenum*: an autoradiographic study. *Biotechnology and Bioengineering.* **32** 1 – 7

El – Enshasy, H. A. (2007) Chapter 9 – Filamentous fungal cultures – process characteristics, products and applications. *Bioprocessing for Value-Added Products from Renewable Resources.* **2007** 225 – 261

El-Enshasy, H., Hellmuth, K., Rinas, U (1999) Fungal morphology in submerged cultures and its relation to glucose oxidase excretion by recombinant *Aspergillus niger. Applied Biochemistry and Biotechnology.* **81**

El-Enshasy, H., Hellmuth, K., Rinas, U (2000) GpdA- promoter-controlled production of glucose oxidase by recombinant *Aspergillus niger* using nonglucose carbon sources. *Applied Biochemistry and Biotechnology.* **90** 57 – 66

Elmayergi, H and Scharer, J.M. (1973) Physiological studies on *Aspergillus niger* fermentation with polymer additive. *Journal of General and Applied Microbiology* **19**, 385 – 392

Fagan, G. L., Kepner, R. E., Webb, A. D. (1981) Production of linalool, *cis-* and *trans*-nerolidol, and *trans, trans*-farnesol by *Saccharomyces fermentation* growing as a film on simulated wine. *Vitis – Journal of Grapevine Research.* **20** 36 – 42

Feofilova. E. P., Ivashechkin. A. A., Alekhin. A. I., Sergeeva. Y. E. (2012) Fungal spores: dormancy, germination, chemical composition and role in biotechnology (review). *Applied Biochemistry and Microbiology* **48** (1) 1 – 11

Fernandes, L., Araujo, M. A. M., Amaral, A., Reis, V. C. B., Martins, N. F., Felipe, M.S. (2005) Cell signalling pathways in *Paracoccidioides brasiliensis-* inferred from comparisons with other fungi. *Genetics and Molecular Research.* **4** 216 – 231

Francko, D. A., Taylor, S. R., Thomas, B. J., McIntosh, D. (1990) Effect of low-dose ultrasonic treatment on physiological variables in *Anabaenaflos-aquae* and *Selenastrum capricornutum. Biotechnology Letters.* **12** (3) 219 – 224

Francko, D.A. Al-Hamdani, S., Joo, G. J (1994) Enhancement of nitrogen fixation in *Anabaena flosaquae* (Cyanobacteria) via low-dose ultrasonic treatment. *Journal of Applied Phycology* **6** (5) 455 – 458

Fuqua. C., Greenberg. E.P. (1998) Self-perception in bacteria: quorum sensing with acylated homoserine lactones. *Current Opinion in Microbiology.* **1** (2) 183 – 189

Gbewonyo. K., Hunt. G., Buckland. B. (1992) Interactions of cell morphology and transport processes in the lovastatin fermentation. *Bioprocess Engineering.* **8** 1 – 7

Gerhardt P, Marquis R.E. (1989) regulation of prokaryotic development: structural and functional analysis of bacterial sporulation and germination *American Society of Microbiology* 43 – 63.

Gerlach, S. R., Siedenberg, D., Gerlach, D., Schugerl, K., Giuseppin, M. L. F., Hunik, J. (1998) Influence of reactor systems on the morphology of *Aspergillus awamori.* Application of neural network and cluster analysis for characterization of fungal morphology. *Process Biochemistry* **33** (6) 601 – 615

Ghojavand, H., Bonakdarpour, B., Heydarian, S, M., Hamedi, J. (2011) The inter-relationship between inoculum concentration, morphology, rheology and erythromycin productivity in submerged cultivation of *Saccharopolyspora erythraea. Brazilian Journal of Chemical Engineering.* **24**(4) 565 – 574

Gibson, J., Sood, A., Hogan, D. A. (2009) *Pseudomonas aeruginosa – Candida albicans* Interactions: localization and fungal toxicity of a phenazine derivative. *Applied and Environmental Microbiology.* **75**(2) 504 – 513

Gori, K., Knudsen, P. B., Nielsen, K. F., Arneborg, N., Jespersen, L. (2011) Alcohol-based quorum sensing plays a role in adhesion and sliding motility of the yeast *Debaryomyces hansenii. FEMS Yeast Research.* **11** 643 – 652

Goswami. S., Vidyarthi. A.S., Bhunia. B., Mandal. T. (2012) A review on lovastatin and its production. *Journal of Biochemical Technology.* **4** (1), 581 – 587

Gougouli. M., Koutsoumanis. K. P. (2012) Modelling germination of fungal spores at constant and fluctuating temperature conditions. *International Journal of Food Microbiology.* **152** 153 – 161

Gougouli. M., Koutsoumanis. K. P. (2013) Relation between germination and mycelium growth of individual fungal spores. *International Journal of Food Microbiology.* **161** 231 – 239

Granshaw, T., Tsukamoto, M., Brody, S. (2003) Circadian rhythms in *Neurospora crassa:* farnesol or geraniol allow expression of rhythmicity in the otherwise arrhythmic strains Frq_{10}, wc-1 and wc-2. *Journal of Biological Rhythms* **18** 287 – 296

Hajjaj. H., Niederberger. P., Duboc. P. (2001) Lovastatin biosynthesis by *Aspergillus terreus* in a chemically defined medium. *Applied and Environmental Microbiology.* **67** (6) 2596 – 2602

Hassouni, H., Ismaili – Alaoui, M., Lamrani, K., Gaime – Perraud, I., Augur, C., Roussos, S. (2007) Comparative spore germination of filamentous fungi on solid state fermentation under different culture conditions. *Micologia Aplicada International.* **19**(1) 7 – 14

Hazen. K.C., Cutler. J.E, (1979) Autoregulation of germ tube formation in *Candida albicans. Infection and Immunity.* **24** (3) 661 – 666

Henriques, A.O. and Moran, C.P. Jr (2007) Structure, assembly and function of spore surface layers. *Annual Review of Microbiology.* **51** 555-588.

Hentzer, M., Wu, H., Andersen, J. B., Riedel, S., Rasmussen, T. B., Bagge, N., Kumar, N., Schembri, M. A., Song, Z., Kristoffersen, P., Manefield, M., Costerton, J. W., Molin, S., Eberl, L., Steinberg, P., Kjelleberg, S., Hoiby, N., and Givskov, M. (2003) Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors. *The EMBO Journal.* **22** (15) 3803 – 3815

Hermersdorfer, H., Leuchtenberger, A., Wardsack, C. Ruttloff, H. (1987) Influence of culture conditions on mycelial structure and polygalacturonase synthesis of *Aspergillus niger. Journal of Basic Microbiology.* **27** (6)

Higashiyama, K., Murakami, K., Tsujimura, H., Matsumoto, N., Fujikawa, S. (1999) Effects of dissolved oxygen on the morphology of an arachidonic acid production by *Mortiella alpine* IS-4. *Biotechnology and Bioengineering.* **63** 442 – 448

Holden, M. T. G., Chhabra, S. R., de Nys, R., Bainton, N. J., Hill, P. J., Manefield, M., Kumar. N., Labatte. M., England, D., Rice, S., Givskov, M., Salmon, G. P. C., Stewart, G. S. A. B., Bycroft, B. W., Kjelleberg, S., Williams, P. (1999) Quorum-sensing cross talk: isolation and chemical characterization of cyclic dipeptides from *Pseudomonas aeruginosa* and other Gram-negative bacteria. *Molecular Microbiology* **33** (6), 1254 – 1266

Hornby, J. M., Jacobitz-Kizzier, S. M., McNeel, D. J., Jensen, E. C., Treves, D. S., Nickerson, K. W. (2004) Inoculum size effect in dimorphic fungi: extracellular control of yeast-mycelium dimorphism in *Ceratocystis ulmi.* *Applied and Environmental Microbiology* 70 (3) 1356 – 1359

Hornby. J. M., Jensen. E. C., Lisec. A. D., Tasto. J.J., Jahnke. B., Shoemaker. R., Dussault. P., Nickerson. K. W (2001) Quorum sensing in the dimorphic fungus *Candida albicans* is mediated by farnesol. *Applied and Environmental Microbiology.* **67** (7) 2982 – 2992

J.H. Burnett and A.P.J. Trinci, (eds.), Fungal Walls and Hyphal Growth. Cambridge Univ. Press. London, New York, Melbourne, 1979, (pp. 1-25).

Jabra-Rizk, M. A., Shirtliff, M., James. C., Meiller, T. (2006) Effect of farnesol on *Candida dubliniensis* biofilm formation and fluconazole resistance. *FEMS Yeast Research.* **6** 1063 – 1073

Jaivel. N. and Marimuthu. P. (2010) Strain Improvement of *Aspergillus terreus* for increased lovastatin production. *International Journal of Engineering and Science and Technology.* **2** (7), 2612 – 2615

Jimenez – Tobon, G. A., Penninckx, M. J., Lejeune, R. (1997) The relationship between pellet size and production of Mn(II) peroxidase by *Phanerochaete chrysosporium* in submerged culture. *Enzyme and Microbial Technology.* **21** (7) 537 – 542

Jin, B., Leeuwen, H., Patel, B., (1999) Mycelial morphology and fungal protein production from starch processing wastewater in submerged cultures of *Aspergillus oryzae. Process Biotechnology* **34** (4) 335 – 340

Jones, P., Shahab, B. A., Trinci, A. P.J., Moore, D. (1988) Effect of polymeric additives, notably Junlon and Hostacerin, on growth of some basidiomycetes in submerged culture. *Transactions of the British Mycological Society.* **90**, 577 – 583

Kalia, C, V., Kumar, P. (2015) Potential applications of quorum sensing inhibitors in diverse fields. *Quorum Sensing Vs Quorum Quenching: A Battle with no end in Sight.* 359 – 370

Kanemitsu M Y, Jiang W, Eckhart W. (1998) Cdc2-mediated phosphorylation of the gap junction protein, connexin43, during mitosis*. Cell Growth and Differentiation.* **9** 13–21

Kinley, R. D., de Nys, R., Vucko, M. J., Machado, L., and Tomkins, N. W. (2016) The red macroalgae *Asparagopsis taxiformis* is a potent natural antimethanogenic that reduces methane production during *in vitro* fermentation with rumen fluid. *Animal Production Science.* **56** (3)

Kozakiewicz, Z. (1989) *Aspergillus* species on the stored products. *Mycological Papers.* **161** 1 – 188

Kruppa. M. (2009) Quorum sensing and *Candida albicans. Mycoses.* **52** (1) 1-10

Kugler, S., Sebghati, T. S., Eissenberg, L. G., Goldman, W. E. (2000) Phenotypic variation and intracellular parasitism by *Histoplasma capsulatum. Proceedings of the National Academy of Sciences of the United States of America.* **97** (16) 8794 – 8798

Kumar, S., Kolodkin-Gal, I., Engelberg-Kulka, H. (2013). Novel quorum sensing peptides mediating interspecies bacterial cell death. American Academy of Microbiology. **4** 1 – 12

Kumar. M.S., Jana. S.K., Senthil. V., Shashanka. V. Kumar. S.V., Sadhukhan. A.K (2000) Repeated fed batch process for improving lovastatin production. *Process Biochemistry.* **36** 363 – 368

Lai. L. S. T., Pan. C. C., Tzeng. B. K. (2003) The Influence of medium design on lovastatin production and pellet formation with high-producing mutant of *Aspergillus terreus* in submerged cultures. *Process Biochemistry*. **38**, 1317 – 1326

Li, L., Hooi, D., Chhabra, S. R., Pritchard, D., and Shaw, P. E. (2004) Bacterial N-acyl homoserine lactone-induced apoptosis in breast carcinoma cells correlated with down-modulation of STAT3. *Nature.* **23** 4894 – 4902

Lin, L., Wu, J., Ho, K., Qi, S. (2001) Ultrasound-induced physiological effects and secondary metabolite (saponin) production in *Panax ginseng* cell cultures. *Ultrasound in Medicine and Biology* **27** (8) 1147 – 1152

Lingappa K., Vivek Babu C.S., Siddalingeshwar K.G and Pramod T. (2004) Isolation, screening and rapid confirmation of lovastatin producing strains of *Aspergillus terreus, Indian Journal of Microbiology.,* **44** (2), 133-135

Lingappa, B. T., Prasad, M., Lingappa, Y., Hunt, D. F., Biemann, K. (1969) Phenethyl alcohol and tryptophol: auto antibiotics produced by the fungus *Candida albicans. Science* **163** 192 – 194

Liu, H., He, Y., Quan, X., Yan, Y., Kong, X., Lia, A. (2005) Enhancement of organic pollutant biodegradation in a biological activated carbon membrane reactor. *Process Biochemistry* **40** (2005) 3002 – 3007

Liu, P., Luo, L., Guo, J., Liu, H., Wang, B., Deng, B., Long, C., and Cheng, Y. (2009) Farnesol induces apoptosis and oxidative stress in the fungal pathogen *Penicillium expansum. Journal Mycologia.* **102**(2) 311 – 318

Lorek, J., Poggeler, S., Weide, M. R., Breves, R., Bockmuhl, D. P. (2008) Influence of farnesol on the morphogenesis of *Aspergillus niger. Journal of Basic Microbiology* **48** 99 – 103

Machida, M., and Gomi, K. (2010) *Aspergillus*: molecular biology and genomics. *Biotechnology Journal.* **5** (3) 336 – 337

Madhani, H. D. (2011) Quorum sensing in fungi: Q&A *Public Library of Science: Pathogens* **7** (10)

Majumdar, S., Mondal, S. (2015) Perspectives on quorum sensing in fungi. *International Journal of Modern Biology and Medicine.* **6**(3) 170 – 180

March, J. C., Bentley, W. E., (2004) Quorum sensing and bacterial cross-talk in biotechnology. *Current Opinion in Biotechnology* **15** 495 – 502

Martins, M., Henriques, M., Azeredo, J., Rocha, S. M., Coimbra, M. A., Oliveira, R. (2007) Morphogenesis control in *Candida albicans* and *Candida dubliniensis* through signalling molecules produced by planktonic and biofilm cells. *Eukaryotic Cell.* **6** 2429 – 2436

Matsuura, K., Hirotsune, M., Nunokawa, Y., Satoh, M., Honda, K. (1994) Acceleration of cell growth and ester formation by ultrasonic wave irradiation. *Journal of Fermentation and Bioengineering* **77** (1) 36 – 40

McIntyre, M., McNeil, B. (1997) Dissolved carbon dioxide effects on morphology, growth, and citrate production in *Aspergillus niger* A60. *Enzyme Microbiology and Technology*. **20** 135 - 142

McIntyre, M., McNeil, B. (1997) Effect of carbon dioxide on morphology and product synthesis in chemostat cultures of *Aspergillus niger* A60. *Enzyme Microbiology and Technology*. **21** 479 - 483

McIntyre, M., McNeil, B. (1998) Morphogenetic and biochemical effects of dissolved carbon dioxide on filamentous fungi in submerged cultivation. *Applied Microbiology and Biotechnology*. **50** (3) 291 – 298

McKenney, P.T., Driks, A. and Eichenberger, P. (2013) The *Bacillus subtilis* endospore: assembly and functions of the multi-layered coat. *Nature Reviews Microbiology*. **11,** 33-44.

Metz. B., Kossen. N.W.F. (1977) The growth of moulds in the form of pellets a literature review. *Biotechnology and Bioengineering*. **14** 781 – 799

Miles, E. and Trinci, A. P. J. (1983) Effect of pH and temperature on morphology of batch and chemostat cultures of *Penicillium chrysogenum. Transactions of the British Mycological Society*. **81** 193 – 200

Miller, M. B., Bassler. B. L. (2001) Quorum sensing in bacteria. *Annual Review of Microbiology.* **55** 165 – 199

Nasution, O., Srinivasa, K., Kim, M., Kim, Y., Kim, W., Jeong, W., Choi, W. (2008) Hydrogen peroxide induces hyphal differentiation in *Candida albicans. Eukaryotic Cell* **7** (11) 2008 – 2011

Nealson. K. H., Platt. T., Hastings. J. W (1970) Cellular control of the synthesis and activity of the bacterial luminescent system. *Journal of Bacteriology*. **104** (1) 313 – 322

Nickerson, K. W., Atkin, A. L., Hornby, J. M. (2006) Quorum sensing in dimorphic fungi: farnesol and beyond. *Applied and Environmental Microbiology* **72** (6) 3805 – 3813

Nielsen, J., Johansen, C. L., Jacobsen, M., Krabben, P., Villadsen, J. (1995) Pellet formation and fragmentation in submerged cultures of *Penicillium chrysogenum* and its relation to penicillin production. *Biotechnology Progress* **11** (1) 93 – 98

Nielsen. J., Krabben. P. (1995) Hyphal growth and fragmentation of *Penicillium chrysogenum* in submerged cultures. *Biotechnology and Bioengineering.* **46** 588 – 598

Nikerson. K.W. Atkin. A. L., Hornby. J.M. (2006) Quorum sensing in dimorphic fungi: farnesol and beyond. *Applied Environmental Microbiology.* **72** 3805 – 3813

Nishio K, Ishida T, Arioka H, Kurokawa H, Fukuoka K, Nomoto T, Fukumoto H, Yokote H, Saijo N. (1996) Antitumor effects of butyrolactone I, a selective cdc2 kinase inhibitor, on human lung cancer cell lines. *Anticancer Research.* **16** 3387–3395.

Ochi, K. (1987). Metabolic initiation of differentiation and secondary metabolism by *Streptomyces griseus*: significance of the stringent response (ppGpp) and GTP content in relation to A factor. *Journal of Bacteriology* **168** (8) 3608 – 3616

Ojha, R., Manzoor, N., Khan, L. A. (2009) Ascorbic acid modulates pathogenicity markers of *Candida albicans. International Journal of Microbial Research* **1** (1) 19 -24

Paidhungat, M., Setlow, B., Daniels, W. B., Hoover. D., Papafragkou, E., Setlow, P. (2002) Mechanisms of induction of germination of *Bacillus subtilis*

spores by high pressure. Applied and Environmental Microbiology. **68**(6) 3172 – 3175

Papagiani, M., Mattey, M. (2006) Morphological development of *Aspergillus niger* in submerged citric acid fermentation as a function of the spore inoculum level. Application of neural network and cluster analysis for characterization of mycelial morphology. *Microbial Cell Factories.* **5** (3)

Papagiani, M., Moo-Young. M., (2002) Protease secretion in glucoamylase producer *Aspergillus niger* cultures: fungal morphology and inoculum effects. *Process Biochemistry* **37** (11) 1271 – 1278

Papagianni, M. (2004) Fungal morphology and metabolite production in submerged mycelial processes. *Biotechnology Advances.* **22** 189 - 259

Papagianni. M., Mattey. M. (2006) Morphological development of *Aspergillus niger* in submerged citric acid fermentation as a function of the spore inoculum level. Application of neural network and cluster analysis for characterization of mycelial morphology. *Microbial Cell Factories*. **5** (3)

Park, A. H., Sugiyama, M., Harashima, S., Kim, Y. H. (2012) Creation of an ethanol-tolerant yeast strain by genome reconstruction based on chromosome splitting technology. *Journal of Microbiology and Biotechnology.* **22**(2) 184 – 189

Park, E. Y., Koike, Y., Cai, H. J., Fujikawa, S. (2001) Morphological diversity of *Mortierella alpina:* effect of consumed carbon to nitrogen ratio in flask culture*. Biotechnology and Bioprocess Engineering.* **6** (3) 161 – 166

Paul, N. A., de Nys, R., Steinberg, P. D. (2006) Chemical defence against bacteria in the red alga *Asparagopsis armata*: linking structure with function. *Marine Ecology Progress Series.* **306** 87 – 101

Paul. C.G., Thomas. C.R. (1996) A structured model for hyphal differentiation and penicillin production using *Penicillium chrysogenum. Biotechnology and Bioengineering.* **51** 558 – 572

Pawlak, M., Bizukojc, M., and Ledakowicz, S. (2012) Impact of bioreactor scale on lovastatin biosynthesis by *Aspergillus terreus* ATCC 20542 in a batch culture. *Chemical and Process Engineering.* **33** (1) 71 – 84

Pazouki, M., Panda, T. (2000) Understanding the morphology of fungi. *Bioprocess and Biosystems Engineering.* **22** (2) 127 - 143.

Porcel, E. R., Casas Lopez, J. L., Vilches Ferron, M. A., Sanchez Perez, J. A., Garcia Sanchez, J. L., Chisti, Y. (2006) Effects of the sporulation conditions on the lovastatin production by *Aspergillus terreus. Bioprocess and Biosystems Engineering.* **29** 1 – 5

Quinones, B., Dulla, G., Lindow, S. E. (2004) Quorum sensing regulates exopolysaccharide production, motility and virulence in *Pseudomonas syringae. The American Phytopathological Society.* **18** (7) 682- 693

Rahim. M.H., Hasan. H., Montoya. A., Abbas. A. (2015) Lovastatin and (+)-geodine production by *Aspergillus terreus* from crude glycerol. *Engineering in Life Sciences.* **15**, 220 – 228

Raina, S. 2008. *The effects of gamma butyrolactone as quorum sensing molecule on secondary metabolite production in filamentous fungi*. PhD printed, University of Westminster.

Raina, S., De Vizio, D., Palonen, E. K., Odell, M., Brandt, A. M., Soini, J. T., Keshavarz, T. (2012). Is quorum sensing involved in lovastatin production in the filamentous fungus *Aspergillus terreus*? *Process Biochemistry* **47** (2012) 843 – 852

Ramage. G., Saville. S.P., Wickes. B.L., Lopez-Ribot. J.L., (2002) Inhibition of *Candida albicans* biofilm formation by farnesol, a quorum-sensing molecule. *Applied and Environmental Microbiology.* **68** (11) 5459 – 5463

Randhawa, M. A., Aljabre, S. H. M (2006). Dimethyl sulfoxide (DMSO) has an additive effect and alters minimal inhibitory concentrations of antifungal drugs. *Journal of Rawalpindi Medical College* **11** (2) 54 – 60

Rasch, M., Buch, C., Austin, B., Slierendrecht, W. J., Ekmann, K. S., Larsen, J. L., Johansen, C., Riedel, K., Eberl, L., Givskov, M., Gram, L. (2004) An inhibitor of bacterial quorum sensing reduces mortalities caused by *Vibriosis* in rainbow trout (*Oncorhynchus mykiss*, Walbaum)*. Systematic and Applied Microbiology.* **27** (3) 350 – 359

Raudabaugh, D. B., Miller, A. N. (2015) Effect of trans, trans-farnesol on *Pseudogymnoascus destructans* and several closely related species. *Mycopathologia.* **180** 325 - 332

Redfield, R. J. (2002) Is quorum sensing a side effect of diffusion sensing? *Trends in Microbiology.* **10** (8) 365 – 370

Rodriguez-Tudela, J. L., Cuenca-Estrella, M., Diaz-Guerra, T. M. (2001) Standardization of antifungal susceptibility variables for a semi-automated methodology. *Journal of Clinical Microbiology* **39** (7) 2513 – 2517

Rugsaseel, S., Morikawa, S., Kirimura, K., and Usami, S. (1995) Stimulation of citric acid production in *Aspergillus niger* by addition of viscous substances in shake culture. *Applied Microbiology and Biotechnology.* **42** (6) 839 – 843

Ryoo, D. (1999) Fungal fractal morphology of pellet formation in *Aspergillus niger. Biotechnology Techniques.* **13** (1) 33 – 36

Sainz Herrán, N., Casas López, J.L., Sánchez Pérez, J.A. and Chisti, Y. (2008) Effects of ultrasound on culture of Aspergillus terreus. *Journal of Chemical Technology and Biotechnology* **83,** 593 – 600.

Sainz Herrán, N., Casas López, J.L., Sánchez Pérez, J.A. and Chisti, Y. (2010) Influence of ultrasound amplitude and duty cycle on fungal morphology and broth rheology of Aspergillus terreus. *World Journal of Microbial Biotechnology* **26,** 1409 – 1418.

Sardi, J.C.O., Gullo, F.P., Pitangui, N.S., Fusco-Almeida, A.M., Mendes-Giannini, M.J.S. (2014) *In vitro* Antifungal susceptibility of *Candida albicans* isolates from patients with chronic periodontitis and diabetes. *Clinical Microbiology.* **2** (1).

Sato. T., Watanabe. T., Mikami. T., Matsumoto. T., (2004) Farnesol, a morphogenetic autoregulatory substance in the dimorphic fungus *Candida albicans,* inhibits hyphae growth through suppression of a mitogen-activated protein kinase cascade. *Biological and Pharmaceutical Bulletin.* **27** (5) 751 – 752

Schimmel, T. G., Parsons, S. J (1999) High purity, high yield procedure for butyrolactone I production from *Aspergillus terreus. Biotechnology Techniques* **13** 379 – 384

Schimmel. T.G., Coffman. A.D., Parsons. S.J. (1998) Effect of Butyrolactone I on the producing fungus, *Aspergillus terreus. Applied and Environmental Microbiology.* **64** (10) 3707 – 3712

Schlafer, O., Sievers, M., Klotzbucher, H., and Onyeche, T. I. (2000) Improvement of biological activity by low energy ultrasound assisted bioreactors. *Ultrasonics.* **38** 711 – 716

Schugerl, K., Gerlach, S. R., Siedenber, D. (1998) Influence of process parameters on the morphology and enzyme production of *Aspergilli. Advances in Biochemical Engineering and Biotechnology.* **60** 195 – 266

Segev E, Smith Y, Ben – Yehuda S, (2012) RNA Dynamics in aging bacterial spores. *Cell* **148,** 139 – 149

Segev, E., Rosenberg, A., Mamou, G., Sinai, L., Ben-Yehuda, S. (2013) Molecular kinetics of reviving bacterial spores. *Journal of Bacteriology.* **195**(9) 1875 – 1882

Semighini, C. P., Hornby, J. M., Dumitru, R., Nickerson, K. W., Harris, S. D (2006) Farnesol-induced apoptosis in *Aspergillus nidulans* reveals a possible mechanism for antagonistic interactions between fungi. *Molecular Microbiology.* **59** 753 – 764

Seong, K. Y., Zhao, X., Xu, J. (2009) Conidial germination in the filamentous fungus *Fusarium graminearum*. *Fungal Genetics and Biology.* **45**(4) 389 – 399

Seong. K.Y., Zhao. X., Xu. J. R., Guldener. U., Kistler. H.C. (2008) Conidial germination in the filamentous fungus *Fusarium graminearum*. *Fungal genetics and Biology.* **45**, 389 – 399

Setlow P, (2006) Spores of *Bacillus subtilis:* their resistance to radiation, heat and chemicals *Journal of Applied Microbiology.* ***101,*** 514 – 525.

Setlow, B., Peng, L., Loshon, C. A., Li, Y. Q., Christie, G., and Setlow, P. (2008) Characterization of the germination of *Bacillus megaterium* spores lacking enzymes that degrade the spore cortex. *Journal of Applied Microbiology.* 1-11

Setlow, P. (2013) Summer meeting 2013 – When the sleepers wake: the germination of spores of *Bacillus* species *Journal of Applied Microbiology.* **115** 1251 – 1268

Setlow, P. (2014) Germination of Spores of *Bascillus* Species: What We Know and Do Not Know. *Journal of Bacteriology* ***196,*** (7), 1297 – 1305.

Sharma, R., Jangid, K. (2015) Fungal quorum sensing inhibitors. *Quorum sensing Vs Quorum quenching: A battle with no end in sight.* 237 – 257

Sinha, J., Bae, J., Park, J., Kim, K., Song, C., and Yun, J. (2001) Changes in morphology of *Paecilomyces japonica* and their effect on broth rheology during production of exo-biopolymers. *Applied Microbiology and Biotechnology.* **56** 88 – 92

Steel R., Martin S.M., Lentz C.P (1954). A standard inoculum for citric acid production in submerged cultures. *Canadian Journal of Microbiology.* **1** 150-157

Swift, S., Downie, J. A., Whitehead, N. A., Barnard, A. M., Salmond, G. P., Williams, P. (2001) Quorum sensing as a population-density-dependent determinant of bacterial physiology. *Advances in Microbial Physiology* **45** 199 – 270

Taff, H. T., Mitchell, K. F., Edward, J. A., Andes, D. R. (2013) Mechanisms of *Candida* biofilm drug resistance. *Future Microbiology.* **8**(10)

Tucker. K.G., Thomas. C.R, (1994) Inoculum effects on fungal morphology: shake flasks vs. agitated bioreactors. *Biotechnology Techniques.* **8** 153 – 156

Tucker. K.G., Thomas. C.R., (1992) Mycelial morphology: the effect of spore inoculum level. *Biotechnology Letters.* **14** 1071 – 1074

Van Hauwenhuyse, F., Fiori, A., Van Dijck, P. (2014) Ascorbic acid inhibition of *Candida albicans* Hsp90-mediated morphogenesis occurs via the transcriptional regulator upc2. Eukaryotic Cell **13** (10) 1278 - 1289

Van Suijdam J.C., Kossen N.W.F., Paul P.G., (1980). An inoculum technique for the production of fungal pellet. *European Journal of Applied Microbial Biotechnology.* **10** 11-221

Van Suijdam, J, C., Metz, B. (1981) Influence of engineering variables upon the morphology of filamentous moulds. *Biotechnology and Bioengineering.* **23**(1)

Vardar, F., Lilly, M. D. (1982) Effect of cycling dissolved oxygen concentrations on product formation in penicillin fermentations. *European Journal of Microbial Biotechnology.* **14** 203 – 211

Wainwright, M. P., Trinci, A. P. J., Moore, D. (1993) Aggregation of spores and biomass of *Phanerochaete chrysosporium* in liquid culture and the effect of anionic polymers on this process. *Mycological Research* **97** (7) 801 – 806

Wang, L., He, Y., Gao, Y., Wu, J. E., Dong, Y., He, C., Wang, S. X., Weng, L., Xu, J., Tay, L., Fang, R. X., Zhang, L. (2004) A bacterial cell-cell communication signal with cross-kingdom structural analogues. *Molecular Microbiology.* **51** (3)

Weinrauch, Y., Penchev, R., Dubnau, E., Smith, I., Dabnau, D. (1990) A *Bacillus subtilis* regulatory gene product for genetic competence and sporulation resembles sensor protein members of the bacterial two-component signal-transduction systems. *Genes and Development.* **4** 860 – 872

Winzer, K., Hardie, K. R., Williams, P. (2002) Bacterial cell-to-cell communication: sorry, can't talk now – gone to lunch! *Current Opinion in Microbiology.* **5** (2) 216 – 222

Wongsuk, T., Pumeesat, P., and Luplertop, N. (2016) Fungal quorum sensing molecules: Role in fungal morphogenesis and pathogenicity. *Journal of Basic Microbiology.* **56**, 440 – 44

Wongwicharn, A., McNeil, B., and Harvey, L. M. (1999) Effect of oxygen enrichment on morphology, growth and heterologous protein production in chemostat cultures of *Aspergillus niger* B1-D. *Biotechnology and Bioengineering.* **65** (4) 416 – 624

Wosten, H. A. B., Moukha, S. M., Sietsma, J. H., Wessels, J. G. H. (1991) Localization of growth and secretion of proteins in *Aspergillus niger. Journal of General Microbiology* **137** 2017 – 2023

Wu, J. and Lin, L. (2002) Elicitor-like effects of low-energy ultrasound on plant (*Panax ginseng)* cells: induction of plant defence responses and secondary metabolite production. *Applied Microbiology and Biotechnology* **59** (1) 51 -57

Yang, D., and Hwang, L. S. (2006) Study on the conversion of three natural statins from lactone forms to their corresponding hydroxy acid forms and their determination in Pu-Erh tea. *Journal of Chromatography A.* **1119** (2006) 277 – 284

Yi, X., Setlow, P. (2010) Studies of the commitment step in the germination of spores of *Bacillus* species. *Journal of Bacteriology.* **192** (13) 3424 – 3433

Yu, R.C., Pesce, C. G., Coleman-Lerner, A., Lok, L., Pincus, D., Serra, E., Holl, M., Benjamin, K., Gordon, A., Brent, R. (2008) Negative feedback that improves transmission in yeast signalling. *Nature* **456** 744 – 761

Zhu, H. U. A., Thuruthyil, S. J., Willcox, M. D. P. (2002) Determination of quorum-sensing signal molecules and virulence factors of *Pseudomonas aeruginosa* isolates from contact lens-induced microbial keratitis. *Journal of Medical Microbiology* **51** (12) 1063 – 1070

Znidarsic, P., Komel, R., Pavko, A. (1998) Studies of a pelleted growth form of *Rhizopus nigricans* as a biocatalyst for progesterone 11 alpha-hydroxylation. *Journal of Biotechnology* **60** (3) 207 – 216

8. APPENDIX

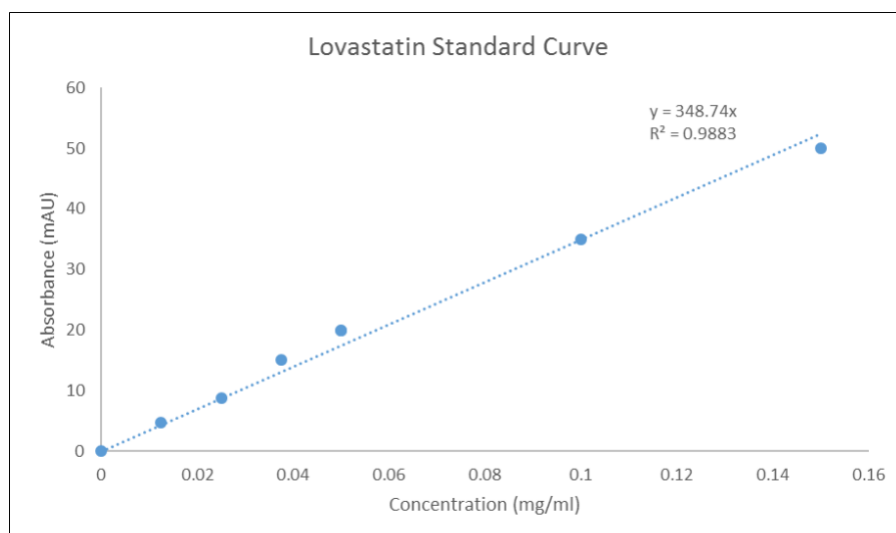## 8.1 Lovastatin Standard Curve

Figure 8.1: *Lovastatin standard curve. In order to determine the unknown lovastatin concentrations in samples obtained from the liquid cultures, a standard curve of known lovastatin concentrations was plotted against HPLC peak height absorbance (mAu). The resulting equation of the trend line was used to determine the unknown concentrations by rearranging and solving for x, where x is lovastatin concentration and y is the peak absorbance. Values were obtained in duplicate, R_2 value is specified. Each lovastatin assay accompanies a separate standard curve.*
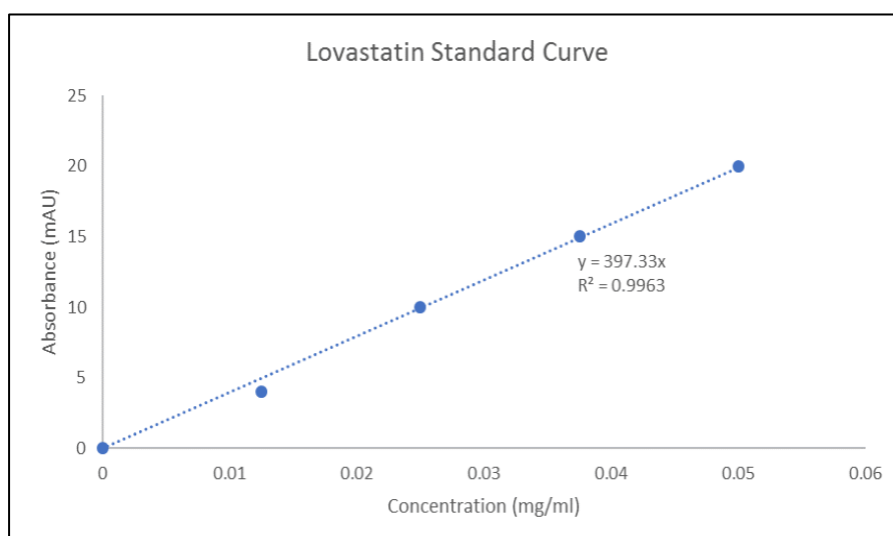

Figure 8.2 – *Lovastatin standard curve*
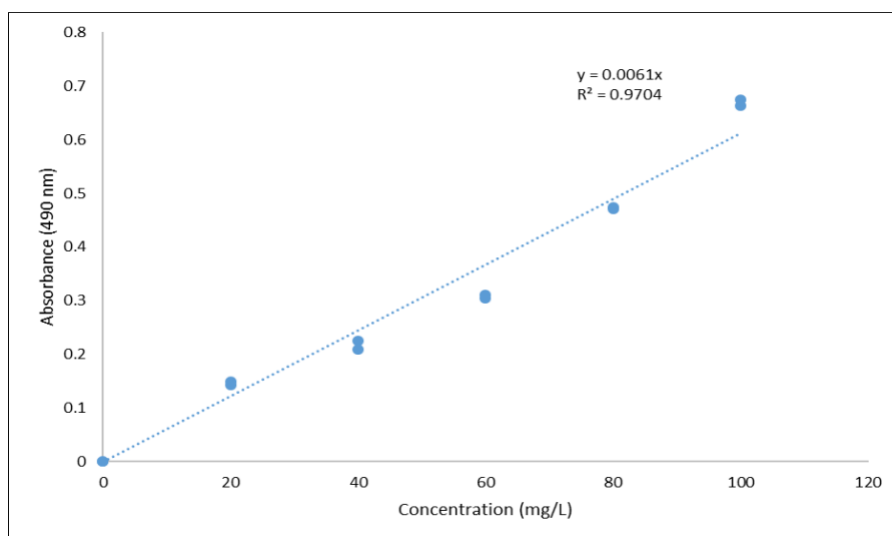
8.2 Carbohydrate Standard Curve

Figure 8.3: *Carbohydrate standard curve. In order to determine the unknown carbohydrate concentrations in the samples obtained from the cultures, a standard curve of known glucose concentrations was plotted against absorbance. The resulting equation of the trend line is used to determine the unknown concentrations by rearranging and solving for x, where x is concentration and y is absorbance. Values were obtained in duplicate, R_2 is specified. Each carbohydrate assay has a separate standard curve.*
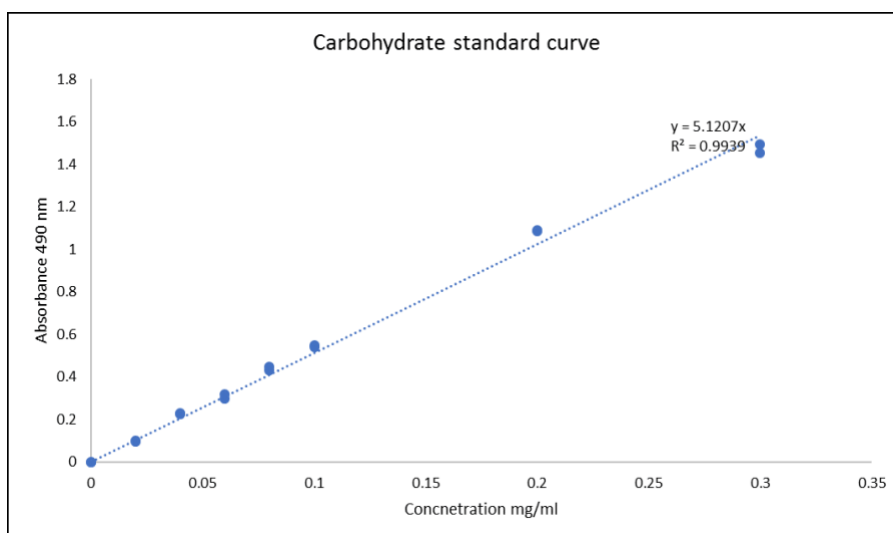

Figure 8.4 – *Carbohydrate standard curve*

8.3 Comparison of Microscope Analysis Software

Morphological analysis was based oy hyphal tip numbers and pellet diameters. "NIS Elements" software, supplied by Nikon was first used as a manual method for counting hyphal tip numbers and measurement of pellet diameter.

A more automated count and measure system was searched for, as manual methods were time consuming. Cellsens, by Olympus was trialled for analysis of pellet morphology.

8.4 Microscope Software Analysis

Imaging of pellets was captured using a Nikon Ci eclipse light microscope, the images were analysed using two different software. The first was via Nikon NIS Elements. This package was equipped with count and measure capabilities, of which required the user to manually click on objects and it would take record of numbers. This was a very time consuming method and required many hours and precise and accurate work by the user. The software was also able to measure lengths, and so was used to determine diameters of pellets. This was also a manual process, requiring the user to manually select points to be measured. The limitations faced by this software were that it was more susceptible to human error and was very time consuming to use for the large sample numbers to be analysed.

The second software was the Olympus Cellsens. This software was able to calculate numbers based on the colour present. The software was more

automated, and could, once programmed, count objects based on their colour, and various parameters could also be set for the program to include or exclude when counting. The system could also be set up to run automatically once images were uploaded to the software. The main downfall was that from image to image variation between colours meant that each time the software would have to be reprogrammed and shown which objects were of interest. This meant that the process was more time consuming than manually counting tips. Pellet diameters were also measured in the same manner as that of the NIS Elements software. The Nikon system was deemed preferable, and therefore used throughout these studies.

The two software packages used for counting and measurements were compared by analysing the same data for hyphal tip numbers and measurement of pellet diameters. The main requirement was for an accurate and automated system that allowed for the automatic count of hyphal tip numbers and diameter measurements of the pellets. It was found that the Olympus Cellsens software, although automated, was not as accurate. It caused the user to go back and double check the work the software had done, this was more time consuming than manually counting tip numbers. As for each image analysed the information given had to be altered to suit even the slightest differences in the colour or quality of the image.

It was therefore concluded that it was more accurate and reliable to count hyphal tip numbers manually and was done so using the Nikon NIS elements software.