Preliminary Investigation of Growth and Antimicrobial Production by *Streptomyces polyantibioticus*: From Shake Flask to Stirred Tank Bioreactor

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Thesis presented in partial fulfilment of the requirements for the degree of

MASTER OF SCIENCE IN ENGINEERING

(120 credits)

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FEBRUARY 2016

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February 2016

Acknowledgements

Numerous parties have contributed to the compilation of this report and the work it represents. My heartfelt appreciation goes out to them all.

Particular thanks goes to:

- South African Research Chairs Initiative (SARChI) of the Department of Science and Technology and CeBER for financial support.
- My supervisors Dr C Fenner and Professor STL Harrison for their invaluable guidance and support.
- Dr P Meyers and Dr M le Roes-Hill for providing the project framework on which this work was built and the relevant microbial cultures.
- CeBER staff and students for all their assistance, encouragement and fellowship inside and out of the laboratory. A special mention to:
  - Miss S Rademeyer for all her technical assistance and support in the laboratory.
  - Dr. M Johnstone-Robertson sharing her expert knowledge and technical expertise on the subject matter.
  - Dr S Jones and Miss K King for their assistance with editing the document
- My parents for all the love and support.
- Last but not least, God Almighty through whom all things are possible.

Abstract

Resistance to antibiotics by microbial pathogens continues to be a major global health problem. Treatment of bacterial infections is becoming increasingly complex and expensive. Tuberculosis (TB), caused by *Mycobacterium tuberculosis* infection, is affected by antibiotic resistance. In South Africa, the Western Province is the worst affected, with an increasing incidence of both multi-drug resistant (MDR) and extensively drug resistant (XDR) strains of *M. tuberculosis*. Both resistant forms of TB increase the length of treatment to almost 24 months and cost by as much as 1400 times that of regular anti-tubercular chemotherapy. A potential solution to this problem is the discovery of new drugs, which can be obtained from natural sources. Actinomycetes are good sources of these drugs, with over 45% of current medicines derived from these bacteria. The actinobacterium *Streptomyces polyantibioticus* SPR^T (SPR^T) was locally isolated and first described by Le Roes (2006). It has been shown to produce bioactive molecules active against a range of bacteria, including compounds (drugs) that have anti-tubercular properties. One of the anti-tubercular molecules was identified as 2,5-diphenyloxazole (DPO). DPO is currently used as a component of scintillation fluid for its luminescent properties and is synthesised chemically in industry. SPR^T is the only reported biological source of DPO, it is however not yet produced commercially via a biological route. The present study was performed to inform future process development of DPO production from SPR^T.

An investigation into the growth and production of antimicrobial compounds from submerged cultures of SPR^T in shake flasks, and scale-up of the process into a laboratory stirred tank bioreactor (STR) was done in the present study. The work focused on obtaining growth kinetics and suitable operating conditions for cultivation. Characterisation of the growth profile of SPR^T and determination of the kinetic growth parameters was carried out. Additionally, the antimicrobial production phases, and factors influencing their production was investigated. It was determined that the most reliable method of measuring biomass concentration was by dry cell gravimetric measurement of whole shake flasks following vacuum filtration, as it best suited the non-homogenous filamentous nature of SPR^T.

The SPR^T culture was grown in shake flasks using Hacène's medium, at 28 °C with agitation and had a growth phase of 12-15 hours at a maximum specific growth rate of 0.260 h^{-1}, achieving a maximum biomass concentration of 3.30 ± 0.070 g/L. SPR^T produced three sets of

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metabolites, including 2,5-diphenyloxazole (DPO), when ethyl acetate culture extracts were tested on the developed TLC and HPLC protocols. Bacterial overlays and bioautographic tests showed that the produced molecules displayed antimicrobial activity against *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923) and *Mycobacterium aurum* A+. The production of DPO and the other antimicrobials was facultatively growth associated. The maximum DPO concentration achieved was 286 µg/L.

Foaming was a major challenge for cultivation of SPR^T in the STR. A number of strategies for the alleviation of foaming were employed. These included the addition of antifoam, a mechanical foam breaker and pH control. Upon stable cultivation of SPR^T in the STR, a comparable maximum specific growth rate and biomass concentration to shake flask cultures were achieved (0.266 ± 0.002 h-1 and 3.53 g/L respectively). Antimicrobial production was observed in negligible amounts.

Shake flask experiments in which the initial concentration of the glucose in the medium was increased two- and four- fold did not result in further biomass production, suggesting a different limiting nutrient. The cultures continued to utilise glucose at the same rate after the maximum biomass concentration was achieved, but this did not result in an increase in antimicrobial production. A change in culture temperature from 28 °C to either 20, 30, 37 or 45°C did not substantially affect the overall production of antimicrobials. The level of each bioactive metabolite was uniquely affected at each temperature resulting in different ratios occurring.

This study has provided kinetic data pertaining to the growth and antimicrobial production patterns of SPR^T at lab scale (shake flasks and stirred tank bioreactors). It has also highlighted challenges that should be addressed prior to further scale-up of the process. This information can be used as a reference point for additional optimisation studies aimed at improving yields of DPO and other bioactive molecules.

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Nomenclature and Abbreviations

7H9	-	Middlebrook 7H9 medium
AA	-	Acetic acid
AIDS	-	Acquired immune deficiency syndrome
CDW	-	Cell dry weight
COV	-	Coefficient of variance
CWW	-	Cell wet weight
DO	-	Dissolved Oxygen
DPO	-	2,5-diphenyloxazole
e.g.	-	*exempli gratia*, 'for the sake of example' (for example)
glu	-	Glucose
gly	-	Glycerol
H5	-	Hacène's medium (same as HM)
H10	-	Modified Hacène's medium, contains 10 g/L glucose instead of 5 g/L
H20	-	Modified Hacène's medium, contains 20 g/L glucose instead of 5 g/L
HCl	-	Hydrochloric acid
HGLY	-	Modified Hacène's medium, contains additional 5 g/L glycerol
HIV	-	Human immunodeficiency virus
HM	-	Hacène's Medium
HPLC	-	High performance liquid chromatography
ISP	-	International *Streptomyces* Project
KOH	-	Potassium hydroxide
MDR-TB	-	Multi-drug resistant tuberculosis
MeOH	-	Methanol
MS	-	Mass spectrometry
MTT	-	Thiazolyl blue tetrazolium bromide
OD	-	Optical density
PMV	-	Packed mycelial volume
R_f	-	Retention factor
rpm	-	Revolutions per minute
SAFD	-	Stirring as foam disruption
SD	-	Standard deviation
SEM	-	Standard error of the mean

SF	-	Shake flask
SPR^T	-	*Streptomyces polyantibioticus* SPR^T
STR	-	Stirred tank bioreactor
TB	-	Tuberculosis
Temp	-	Temperature
TLC	-	Thin layer chromatography
TSB	-	Tryptone soy broth medium
UV	-	Ultraviolet
WHO	-	World Health Organization
XDR-TB	-	Extensively drug-resistant
YEME	-	Yeast extract malt extract medium

Symbols and Units

%	-	Percentage
>	-	greater than' or 'more than'
°C	-	Degrees Celsius
h	-	Hours
M	-	Molar
mg	-	Milligrams
min	-	Minutes
mL	-	Millilitres
mm	-	Millimetres
µ	-	Growth rate [h^{-1}]
μ_{max}	-	Maximum specific growth rate [h^{-1}]
µg	-	Micrograms
µL	-	Microliters
v/v	-	Volume per unit volume
vvm	-	Volume of air per volume of medium per minute
X	-	Biomass concentration [g/L]
$Y_{x/s}$	-	Biomass yield [g_x/g_s]

Chapter 1: Introduction

1.1 Background and Study Scope

Antibiotics have revolutionised contemporary medicine allowing fatal diseases to be treated. There is, however, a growing concern about antibiotic resistant strains of microbes. An ever increasing need for novel antibiotics with unique modes of action exists, as currently used antibiotics become more and more ineffective (Okudoh and Wallis 2007).

One area of impact of antibiotic resistance is in tuberculosis. Africa is currently facing the worst tuberculosis (TB) epidemic, with the rise in antibiotic resistance of great concern. Resistance to TB has resulted due to misuse of antibiotics by both patients and doctors. This has led to the emergence of multi-drug resistant strains of *Mycobacterium tuberculosis*. It is of immediate importance in South Africa, among the top five countries in the world, with respect to multi-drug resistant TB strains. South Africa is plagued with both high TB incidence rates and high rates of co-infection with Human immunodeficiency virus (HIV). New drugs are required to combat this and are typically sought from natural sources.

Molecular diversity underpins biological diversity, thus for an increased chance of discovering novel antibiotics, bioprospecting for antibiotics requires selective isolation of novel microorganisms. There is an increased chance of finding novel taxa when searching in extreme and or neglected habitats. A number of screening steps are required to identify bioactive molecules of interest and characterise their structural chemistry, mode of action and potency (Goodfellow and Fiedler 2010). Candidate molecules and their producing microorganisms can then be engineered into a bioprocess.

During routine isolation and screening at the Department of Molecular and Cell Biology at the University of Cape Town, a novel strain of *Streptomyces* was isolated. This bacterium, *Streptomyces polyantibioticus* SPR^T (SPR^T), was found to produce multiple bioactive metabolites with antimicrobial activity against common bacterial pathogens, both gram negative and gram positive. Amongst these metabolites, 2,5-diphenyloxazole (DPO) was produced, which has anti-tubercular properties (Le Roes 2006). This compound/drug is currently only produced by chemical synthesis through a production process involving multiple step reaction schemes and harsh reaction conditions (Giddens *et al*. 2005; Wan *et al*. 2010; Xu

et al. 2013). The preliminary study by Le Roes (2006) thus presented the only known biological source of DPO; providing a source of the molecule which is produced relatively simply under less harsh conditions and is potentially cheaper.

To facilitate production of DPO or similar compounds, it may be necessary to modify the cell genetics and optimise cultivation (fermentation) conditions for higher product yields (Liu *et al*. 2013). Growth kinetics and operating conditions for cultivation are considered in the present study, with taxonomic studies, bacterial isolation and genetic manipulation lying outside the scope.

The present study was conducted to provide detailed information on the growth of SPR^T and its production of antimicrobial molecules; specifically, to understand the growth patterns of SPR^T in laboratory scale bioreactors (shake flasks and stirred-tank bioreactors). The study aims to inform the future development of a scalable and commercially viable industrial system for the production of antimicrobials from SPR^T.

1.2 Thesis Structure

This thesis comprises six chapters. In Chapter 1, an overview of the study is provided, giving context towards the objectives and research approach. The literature is reviewed in Chapter 2 outlining the history and need for antibiotics. A description of actinomycetes follows including physiological and phylogenetic information, as well as their ability to produce antibiotics. Furthermore what is known of SPR^T physiology and antibiotic producing ability is presented. Additionally the bioprocess challenges associated with filamentous culture, especially those of *Streptomycetes,* which require consideration are outlined. Analysis of the gaps in the literature is used to establish the research objectives and key questions, these conclude the chapter. In Chapter 3, the materials and methods used in this study are presented. Further, the development of the analytical procedures for SPR^T biomass and antimicrobial determination necessary for this study are presented in Chapter 4. The results from shake flask and lab scale bioreactor experiments detailing growth profiles and associated growth rates and yields in each set-up are found in Chapter 5. The effects of altering the initial carbon concentration on SPR^T growth is investigated. The antimicrobial metabolite production profiles in both shake flask and stirred

tank bioreactors are shown, followed by a general discussion concluding the chapter. The conclusions and recommendations for further work are presented in Chapter 6.

Chapter 2: Literature Review

2.1 Antibiotics and antibiotic resistance

Ancient societies as old as 2000 years made use of traditional medicines comprised of plant and fungal extracts with antimicrobial properties (Lindblad 2008). Antibiotics began to appear in modern clinical medicine in the mid-20th century with the use of penicillins and gramicidin (Bush 2004). Today, there is a wide range of antibiotics in use with the major classes including cephalosporin, tetracyclins, ansamycins, glycopeptides and quinolones. Across these many types of current antibiotics, there are only a few mechanisms of action (Yoneyama and Katsumata 2006). These include inhibition of cell wall synthesis, nucleic acid synthesis, and protein synthesis (Piddock 1998). Due to antibiotic resistance, new drugs are required to make up for the ever increasing number of ineffective drugs to which resistance is shown. Pathogenic microorganisms evolve to become antibiotic resistant via natural mutations, many of which are plasmid borne and easily transmitted. Overuse of broad spectrum antibiotics and ignorance has led to negligent use of prescribed antibiotics by society, exacerbating the problem (Levy 1998; Powers 2004; Yoneyama and Katsumata 2006; D'Costa *et al*. 2011; Nathan and Cars 2014).

Antibiotic resistance is a contemporary issue. Key organisations such as the Centres for Disease Control (CDC), World Health Organisation (WHO), World Economic Fund (WEF) and World Health Assembly (WHA) place key focus on antibiotic resistance (Nathan and Cars 2014). Without urgent coordinated action, treatable common infections and minor injuries will once again become lethal. Resistance to first and second line drugs once effective against *Neisseria gonorrhoeae* (gonorrhoea), *Escherichia coli* (urinary tract, and intestinal infections) and *Staphylococcus aureus* (skin infections, common hospital pathogen) has become widespread. This is expected to lead to them becoming as notoriously problematic and lethal as antibiotic resistant strains of *Plasmodium falciparum* (Malaria) and *Mycobacterium tuberculosis* (Tuberculosis).

2.2 Tuberculosis and available antibiotics

Tuberculosis (TB), a droplet infection affecting the lungs, brain, intestines, kidneys and spine is caused by the bacillus *M. tuberculosis*. It is a prime example of a disease affected by antibiotic resistance. TB, the first infectious disease to be declared as a global emergency by the WHO, has high morbidity and mortality rates in developing countries (Zaman 2010). The TB threat is particularly relevant to Sub-Saharan Africa owing to the concomitant prevalence of (HIV-AIDS). In South Africa, the Western Province is the worst affected, with an increasing incidence of both multi-drug resistant (MDR) and extensively drug resistant (XDR) strains of *M. tuberculosis* (Gandhi *et al*. 2006; Andrews *et al*. 2007). Both resistant forms of TB increase the length of antitubercular chemotherapy to almost 24 months and cost by as much as 1400 times that of regular treatment (Bateman 2006).

Despite advances in TB diagnosis over the years, the current methods of treatment are little changed, with the newest approved TB drug, rifampicin, having been discovered almost fifty years ago. As a result, survival rates of most patients with MDR- or XDR-TB are declining. HIV co-infection (SA 520 cases per 100 000 people) exacerbates the TB problem, by interfering with initial diagnosis and the immune-compromised state complicating the TB infection (Andrews *et al*. 2007; WHO, 2015). The solution to combat these problems is novel TB drugs, with unique structures and unusual antimicrobial mechanisms of action to ensure long-lasting and effective antibiotics, since new antibiotics of the same classes as those already in use will likely be susceptible to cross resistance (File 1999). Ideally, the drugs should be capable of killing MDR- and XDR-TB, in a period shorter than the current six months required for treatment (van Wezel *et al*. 2006). A reduced number of pills required, reduced dosage frequency and no undesirable side effects would also help simplify treatments. Compatibility with HIV drugs and ability to kill latent TB (Goldfeld and Ellner 2007; King *et al*. 2010) are desirable for HIV co-infection and to reduce the prevalence and number of deaths caused by TB, in line with the mission and targets of the Global Plan to stop TB (http://www.stoptb.org/global/plan/) – an initiative of the WHO.

2.3 Drug discovery

Products found naturally in the environment have provided a rich source of multifarious chemical structures. New antibiotics can be derived from natural products. Natural products from microorganisms, particularly bacterial secondary metabolites, have shown potential with actinobacteria typically being a good source. It has been estimated that fewer than 3% of all antibiotics have been discovered; therefore, there is still a high chance of being able to isolate new antibiotic molecules from bacteria and, particularly, actinobacteria. Isolation of molecules with antimicrobial properties has historically come from terrestrial sources. These sources are widely studied mostly due to easy access. Despite past success, the larger pharmaceutical companies no longer prioritise natural product discovery and finding novel antibiotics is becoming less common, however the potential to find many novel antibiotics remains. It therefore falls to other players such as universities and small biotech companies to fill this gap (Nathan and Cars 2014). Improved high throughput screening methods with de-replication technologies, are being developed and reduce the occurrence of rediscovering known molecules and their producing microorganisms (Lam 2006; Goodfellow and Fiedler 2010). One must also be careful not to disregard a test isolate of species already isolated too early as antibiotic production is strain specific (Kokare 2008).

2.4 Actinomycetes

In 2009, the order *Actinomycetales* was one of five orders belonging to the class *Actinobacteria*, the others were *Acidimicrobiales, Bifidobacteriales, Coriobacteriales* and *Rubrobacterales*, (Zhi *et al*. 2009) and this has since been amended due to the increasing number of novel species being discovered. There are 13 sub-orders within the *Actinomycetales* which encompasses 42 families.

Bacteria belonging to the class *Actinobacteria* are Gram-positive with high (greater than 50%) guanine cytosine (G+C) content (Stackebrandt *et al*. 1997). The word actinomycetes is of Greek origin meaning “ray fungus” as they closely resemble fungi due to their filamentous nature (Lechevalier and Lechevalier 1981). Many are spore forming aerobes. They have a variety of life cycles. Spore-forming strains produce spores which may occur as conidia, illustrated in Figure 2.1 (A) and Figure 2.1 (B), or sporangia. Various strain specific spore

surface morphologies occur and can be differentiated by the use of an electron microscope as seen in Figure 1(B) (Lechevalier, 1989).

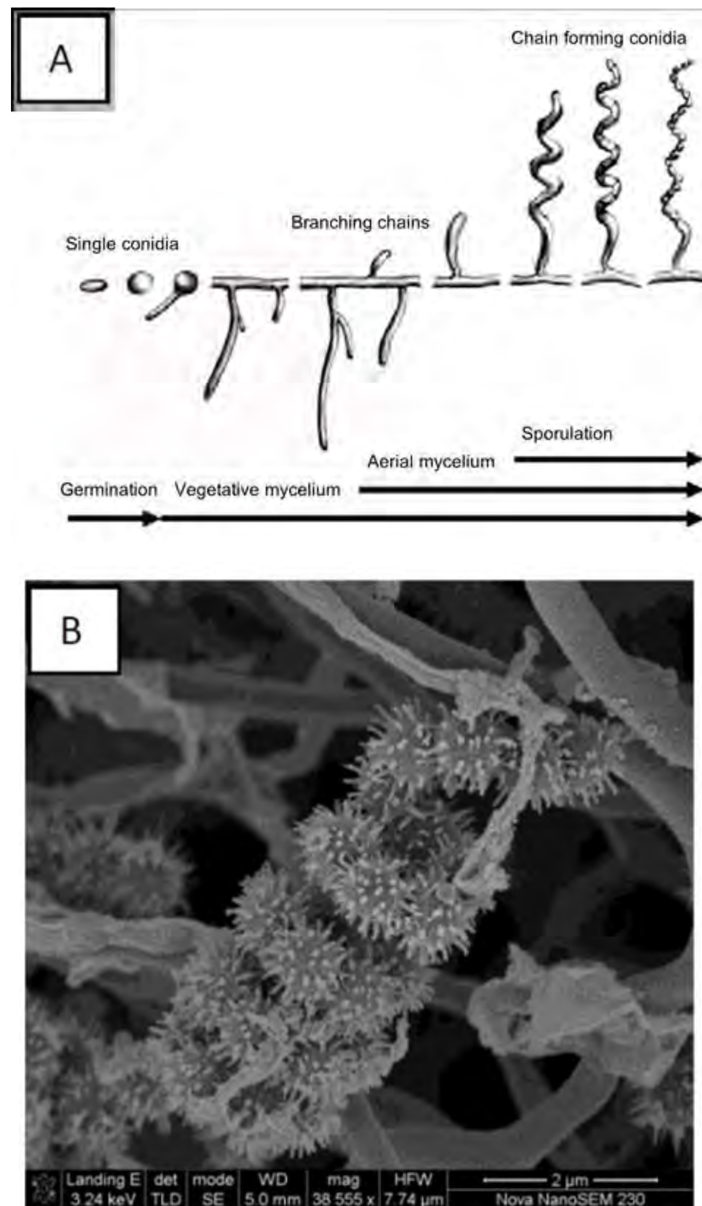

Figure 2.1 (A) Morphological features and typical developmental cycle of actinomycetes. [Adapted from Hempel (2012)] (B) Electron micrograph showing (spiny) spore surface ornamentation and (*Spirales*) spore chain morphology of an actinomycete isolate. [Sourced from Matongo (2011)]

Actinomycetes are typically saprophytic bacteria, residing in all ecological niches in both natural and man-made environments. These include locations such as compost, fresh and salt water, soils and some extreme environments (Kokare 2008). They sometimes associate with

plants and animals forming mutualistic relationships (Hasegawa *et al*. 2006). Environmental factors such as pH and temperature have a bearing on their role and distribution in their surroundings.

Generally, actinomycetes are mesophiles with optimal growth between 25-30°C. There are exceptions with facultative thermophiles, such as *Thermoactiomycetes* and *Saccharomonospora,* growing at temperatures above 40°C. These can be found in more uncommon environments such as the deep sea and in self-heating compost heaps (Kokare 2008). Actinomycetes play a vital role in the decomposition and recycling of organic matter, as they are able to decompose complex and recalcitrant polymers such as lignin, cellulose, lignocellulose, chitin, keratin, hemicellulose, fungal cell wall material and pectin (Kokare 2008). This can be attributed to their broad metabolic capabilities. Whilst actinomycetes are generally salt tolerant, this can be used to separate terrestrial strains from marine strains. Marine strains have an even higher salt tolerance. Deep sea actinomycetes are obligate in their requirement for sea water whereas terrestrial strains are only facultatively marine. Exploiting these properties and characteristics, and mimicking natural conditions in the laboratory allows actinomycetes to be selectively isolated from the environment (Goodfellow and Williams 1983; Kurtböke and Williams 1991). More importantly it is the recreation of the environment (Gavrish *et al*. 2008) from whence they were isolated which leads to higher success rates of producing desirable bioactive metabolites such as antibiotics.

2.5 Actinomycetes: a source of antibiotics and other useful compounds.

Actinomycetes, primarily streptomycete, are involved in antagonistic interactions in their environment. Rhizosphere *Streptomyces* suppress growth of fungal root pathogens and *Actinoplanes* regulate plant diseases (Kokare 2008). The ability of actinomycetes to produce bioactive compounds and other secondary metabolites under low nutrient conditions allows them to secure their survival in their niche by eradicating competitors (Maplestone *et al*. 1992). The systematic screening of actinomycetes for their antibiotics began with Selman Waksman in 1914 (Bush 2004). Today it is known that actinomycetes are one of the best sources of antimicrobial compounds, with the vast majority of the current antibiotics produced originating from actinomycetes (Marinelli and Marcone 2011). Antibiotics with a vast range of chemical structures and mechanisms of action are produced. Table 2.1 lists the major classes of

antibacterial (by chemical structure) on the mark*et al*ong with their cellular targets (Marinelli and Marcone 2011). In addition to antibiotics, biosurfactants, enzymes, enzyme regulators, plant growth promoting substances and immunomodifiers are produced (Kokare 2008). Eukaryotic sources are at this stage not a viable source of bioactive secondary metabolites as they have more complex, relatively less understood biosynthetic and associated regulation pathways (Lane and Moore 2011) and seldom reach market.

Furthermore it has been reported that the average actinomycete has the genetic potential to produce 20 or more secondary metabolites (Donadio *et al*. 2002; Baltz 2011) with streptomycetes having the genetic capacity for 40 distinct secondary metabolites (Yoon and Nodwell 2014). The difficulty is that the majority of these genes are expressed at low levels at laboratory scale (Yoon and Nodwell 2014). The use of molecular tools to activate these ‘cryptic’ genes can be tricky especially when the gene clusters are unknown. Some success has been reported from the use of crude methods, (e.g. co-culture, change in conditions – a ‘shock’ or addition of chemicals), to elicit a stress response to trigger antimicrobial production and or improve fermentation titres (Table 2.2) in conjuncture with recombinant DNA technologies (Wang *et al*. 2012; Bertrand *et al*. 2014; Bode *et al*. 2002; Yoon and Nodwell 2014; Zhu *et al*. 2014).

Table 2.1 Major classes of antibacterial antibiotics [adapted from Marinelli and Marcone 2011]

Chemical structure	***Mechanism of action***	***Cellular target***	***Drugs***	***Antimicrobial spectrum***	***Microbial producers***
Aminoglycosides	Inhibition of protein synthesis	30S ribosome	Gentamicin, Kanamycin, Tobramycin, Streptomycin	Aerobic Gram-positives and Gram-negatives. *M. tuberculosis*	*Streptomyce* s spp.(-mycins) *Microminospora* spp. (-micins)
Ansamycins	Inhibition of RNA synthesis	DNA-dependent RNA polymerase	Rifamycin, Rifampin Rifapentine	Gram-positives and Gram-negatives, M. tuberculosis	*Amycolatopsis mediterranei*
Garbapenems	Inhibition of peptidoglycan synthesis	Penicillin binding proteins	Thienamycin	Aerobic and anaerobic Gram-positives and Gram-negatives	*Streptomyces cattleya*
Cephalosporins	Inhibition of peptidoglycan synthesis	Penicillin binding proteins	Cefalexin, Cefaclor, Cefdinir, Cefepime	Aerobic and anaerobic Gram-positives and Gram-negatives	*Cephalosporium acremonium*
Glycopeptides	Inhibition of peptidoglycan synthesis (transglycosilation, transpeptidation)	Peptidoglycan units (terminal $_D$-Ala-$_D$-Ala dipeptide)	Vancomycin, Teicoplanln	Gram-positives	*Amycolatopsis orientalis* *Actinoplanes teichomyceticus*
Lipopeptides	Interference with membrane	Cell membrane	Daptomycin, Polymyxin B	Gram-positives (Daptomycin) and Gram-negatives (Polymyxin)	*Streptomyces roseoporus* *Bacillus polymyxa*
Macrolides	Inhibition of protein synthesis	50S ribosome	Erythromycin, Azythromycln	Aerobic and anaerobic Gram-positives and Gram-negatives	*Saccharopolyspora erythraea*
Penicillins	Inhibition of peptidoglycan synthesis	Penicillin-binding proteins	Amoxycilin, Ampicillin	Aerobic and anaerobic Gram-positives and Gram-negatives	*Penicillium notatum/chrysogenum*
Polypeptides	Interference with peptidoglycan synthesis	Bactoprenol	Bacitracin	Gram-positives	*Bacillus subtilis*
Tetracyclines	Inhibition of protein synthesis	30S ribosome	Aureomycin, Oxytetracycline	Aerobic Gram-positives and Gram-negatives	*Streptomyces aureofaciens* *Streptomyces rimosus*

Table 2.2 Examples of streptomycetes that had an increase in antimicrobial production after being subjected to change of conditions

Product	***Organism***	***Trigger***	***Reference(s)***
Jadomycin B	*Streptomyces venezuelae*	heat/ethanol shock*	(Doull *et al*. 1993) (Doull *et al*. 1994)
Validamycin A	*Streptomyces hygroscopicus*	heat/ethanol shock	(Liao *et al*. 2009) (Wei *et al*. 2012) (Zhou *et al*. 2012)
Manumycin	*Streptomyces parvulus*	hydrostatic pressure	(Bode *et al*. 2002)
Ectoine, 5-hydroxyectoine	*Streptomyces coelicolor*	salinity/heat shock	(Bursy *et al*. 2008)
Methylenomycin	*Streptomyces coelicolor*	alanine limitation/acid pH Shock	(Hayes *et al*. 1997)

* Pulse shock treatment during growth phase (1 h),

2.5.1 *Streptomyces polyantibioticus* SPR^T (SPR^T)

SPR^T was named for its antibiotic producing potential. It was isolated from the Umgeni River in KwaZulu-Natal and has brown substrate mycelium. Figure 2.2 shows filamentous nature of SPR^T with *Rectiflexibiles*-type spore chain morphology. SPR^T is non-pathogenic. Rigorous polyphasic taxonomic work was performed on SPR^T during characterisation (Le Roes-Hill and Meyers 2009). Growth occurs at 4 and 30°C and at pH 4.3. It is able to utilise a vast array of molecules as sole carbon and nitrogen sources. Carbon utilisable by SPR^T includes D(-) fructose, D(-) lactose, D(+) cellobiose, D(+) galactose, D(+) glucose, D(+) mannose, D(+) melibiose, D(+) xylose, *meso*-insositol, raffinose, salicin, sodium acetate (0.1%), sodium citrate (0.1%) and trehalose, whilst adonitol, D(-) mannitol, D(+) melezitose, inulin, L(+) arabinose, L(+) rhamnose, sucrose and xylitol are not. Utilisable nitrogen sources includes DL-α-amino-n-butyric acid, L-arginine, L-cysteine, L-histidine, L-hydroxyproline, L-phenylalanine, L-serine, L-threonine, L-valine and potassium nitrate. SPR^T showed weak utilisation of ribose and L-methionine (Le Roes-Hill and Meyers 2009).

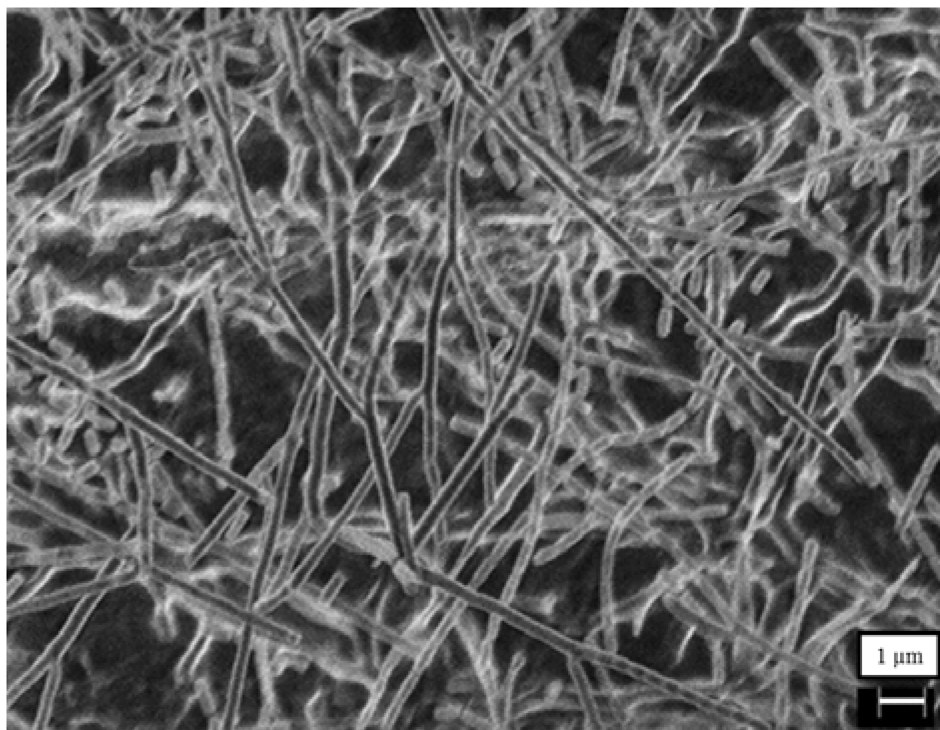

Figure 2.2 Cryo-scanning electron micrograph of strain SPR^T grown on Middlebrook 7H9-glucose agar at 30 °C for 19 days. [Adapted from Le Roes (2006)]

Limited research on antibiotic production of SPR^{T} has been done as preliminary screening. Molecules produced by SPR^{T} showed activity against all bacteria used in the test (Table 2.3), with stronger activity against Gram-positives. Activity against both Gram-positive and Gram-negative pathogens was to be expected because they have overlapping, target profiles. The stronger activity against Gram-positives is unusual as Gram-negatives are usually easier to kill due to their weaker cell wall structure (Le Roes 2006; Le Roes-Hill and Meyers 2009; Walsh and Wencewicz 2014).

Thin layer Chromatography coupled with Bioautography on culture extracts of SPR^{T} initially showed five different fractions exhibiting antimycobacterial properties. High Performance Liquid Chromatography, Nuclear Magnetic Resonance and Mass Spectral analysis confirmed one of those fractions to contain 2,5-diphenyloxazole (DPO). DPO (Figure 2.3) has uses in liquid scintillation spectrometry and is in preliminary development as an anti-TB drug. This is the only documented biological source of DPO; hence, it is of great interest. Two of the other fractions were designated EAM115B and EAM115P; these were deduced by Le Roes (2006) to be different configurations of the same antimicrobial compound. EAM115B/P was a pH indicator antibiotic: it is blue-purple in the presence of 6M HCl and luminous pink in the presence of 10M NaOH. Subsequent SPR^{T} culture extracts only showed three antimicrobial fractions, leading to the conclusion that SPR^{T} had either stopped or lost the ability to produce EAM115B/P (Le Roes 2006).

Table 2.3 The range of antibiotic activity exhibited by SPR^{T} [Adapted from Le Roes (2006)]

	Pathogen	Wall type	Disease
STRONG	*Bacillus coagulans* ATCC 7050^{T}	G +ve	GRAS
	Enterococcus faecium VanA*	G +ve	Meningitis, Endocarditis, Bacteremia
	Enterococcus phoeniculicola JLB-1^{T}	G +ve	Meningitis, Endocarditis, Bacteremia
	Micrococcus sp.*	G +ve	Meningitis, Endocarditis, Bacteremia
	Mycobacterium aurum A+	G +ve	---
	Streptococcus sp*	G +ve	Pneumonia, necrotizing fasciitis, arthritis
WEAK	*Citrobacter braaki* strain 90*	G -ve	UTI, meningitis
	Enterobacter cloacae strain 67*	G -ve	Septic arthritis, osteomyelitis, ophthalmic
	Escherichia coli ATCC 25922	G -ve	Gastroenteritis, cholecystitis, cholangitis
	Klebsiella oxytoca strain K52* [resistant to Augmentin and cefuroxime]	G -ve	Coloitis, sepsis

*clinical isolates, GRAS = generally regarded as safe

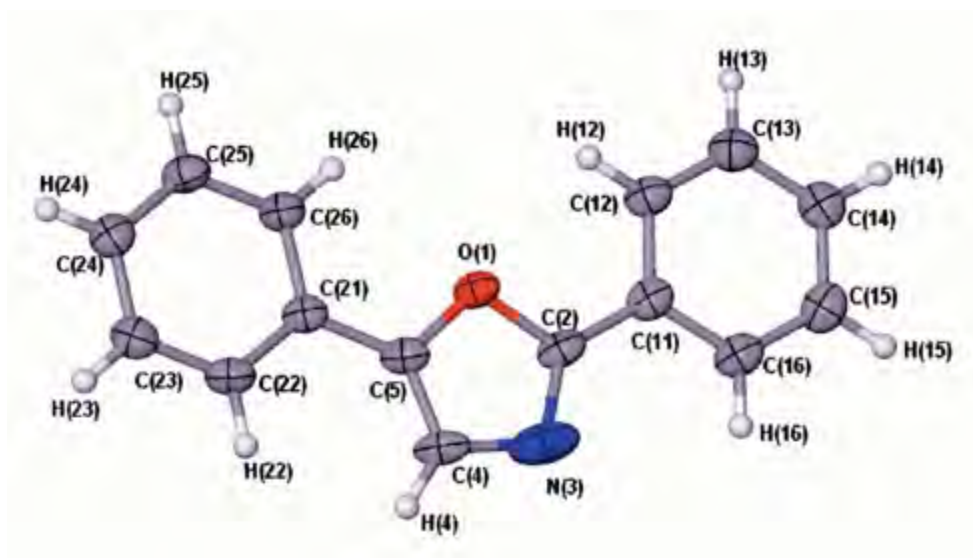

Figure 2.3 Structure of 2,5-diphenyloxazole produced by SPR^T as determined by X-ray crystallography [sourced from Le Roes (2006)]

2.6 Bioprocess Considerations

The production of antibiotics from actinomycete bacteria needs to be scaled-up and assessed for potential commercial scale production. To enable the efficient design and optimisation of industrial microbial cultivations, for controlled operation, knowledge of basic kinetic parameters such as the growth and product formation rates, yields of growth and product formation and an understanding of microbial physiology is necessary. This enables researchers to develop, optimise, model and scale (Appendix A) a bioprocess (Koutinas *et al*. 2003; Rahulan *et al*. 2012).

Living organisms have highly complex metabolic pathways which adapt to their environment. Their metabolic performance in a bioreactor depends on complex interactions of the various operating conditions. Whilst continuous steady state processes give the highest productivity, the complexity of microbial systems and process challenges, such as sterility, has resulted in most industrial processes being run in a batch or semi-batch manner. Systems in which secondary metabolites are harvested are particularly poorly suited to continuous culture as the low specific growth rate needed for product formation results in long (infinite) residence times, and thereby a two-stage continuous process. Secondary metabolites are regulated in a complex manner and typically require a change in conditions, usually those of a stressful nature, to be

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produced (e.g. change in phase from growth to stationary), making batch and fed-batch conditions more suitable for their production (Glazer and Nikaido 2007). The resultant environment of the microbial culture is dependent on both biological and operational parameters which are process specific.

Scaling of biological systems from laboratory scale to a viable commercial production system is a lot more challenging than chemical processes, as microbes are more sensitive to changes in environmental variables such as raw materials, pH, temperature, oxygen, ionic strength, mass transfer, circulation time, shear stress and hydrodynamic forces (Vanags *et al*. 1995; Silva *et al*. 2001; Koutinas *et al*. 2003; Rahulan *et al*. 2011). These affect biomass production, morphology and product formation, both in terms ofquality and quantity (Hsu and Wu 2002). The impact of a single parameter is often scale dependent (Rahulan *et al*. 2012).

In order to overcome the many issues surrounding optimisation and scale-up, it is imperative to identify the critical factor(s) affecting the bioprocess, both in terms of growth and product formation. Process conditions for the industrial biological production of antibiotics are mostly determined empirically and they mostly constitute proprietary knowledge (Glazer and Nikaido 2007). This makes development of novel systems challenging. Due to the very process specific interactions and uniqueness of various actinomycetes, there is no universally applicable strategy or set of process conditions for a successful scale-up (Junker *et al*. 2004; Xia *et al*. 2015).

Some of the major complications associated with cultivation of filamentous organisms, such as fungi and actinomycetes, include the viscous nature of the culture, unknown kinetics, and heat and mass transfer requirements. These factors often hinder optimisation and scale-up of industrial bioprocesses (Koutinas *et al*. 2003; Rahulan *et al*. 2012). Morphology and foaming, which are common challenges experienced in early stage development, of actinomycete culture are reviewed below.

2.6.1 Process challenges of actinomycete culture: Morphology

Actinomycetes present a range of possible morphologies in submerged culture, ranging from predominantly pelleted to predominantly mycelial (filamentous/dispersed) forms. Mycelial

cultures are characterised by hyphal geometry (length, diameter and branching frequency), whilst pellets and clumps are characterised by their geometry as well as their density (Olsvik and Kristiansen 1994), and often classified microscopically and macroscopically, respectively (Nielsen 1996). Whitaker (1992) presented an extensive list of the actinomycetes and the growth forms they present in shake flasks and stirred vessels. Some strains exclusively exhibited one morphological form over the other, whereas others could exhibit both, dependent on the prevailing conditions. The predominant morphology during cultivation is dependent on several factors, both biological and operational alike, such as the strain, inoculum concentration and quality, and agitation (van Wezel *et al*. 2006; Posch *et al*. 2013). A summary of these interactions is presented in Figure 2.4. Junker *et al*. (2004) listed the general effects of agitation, inoculum, media composition, media additives and fermentation parameters on morphology type. These are by no means universal due to multiple interacting factors often resulting in counter intuitive effects. For example, increasing agitation has the positive effect of increasing critical dissolved oxygen in mycelial cultures of *Streptomyces niveus* by opening the mycelial network (Wang and Fewkes 1977), but the increased turbulence causes increased shear, which can be detrimental to growth.

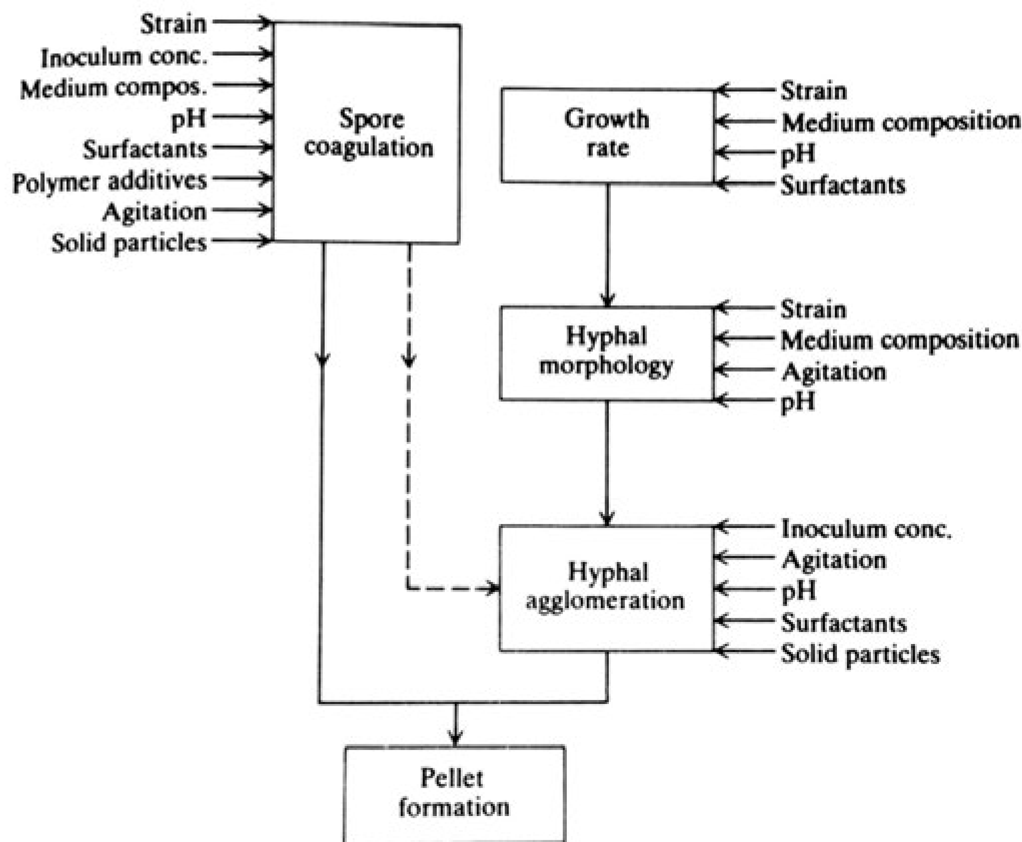

Figure 2.4 Summary of the numerous interconnecting factors that interact to determine pellet formation of filamentous organisms. [Adapted from (Bailey and Ollis 1986)].

Each growth form (pelleted vs dispersed) has unique rheology and kinetics (Mitard and Riba 1988). These will in turn have a great influence on reactor performance (Olsvik and Kristiansen 1994). Predominantly pelleted broths consist of discrete compound masses of hyphae, which typically display Newtonian behaviour (Heydarian *et al*. 2002; Kelly *et al*. 2006). Whilst this favours well mixed cultures, mass transfer to the centre of each pellet becomes limited, particularly as the pellets increase in size; this results in slower cultivation (Heydarian *et al*. 2002; Junker *et al*. 2004; Grimm *et al*. 2005). However, there are reports of cultures with elliptical 'doughnut' shaped pellets, which experience better mass transfer (Hamanaka *et al*. 2001). Growth of pellets are dependent on initial pellet size and may be described by the cube root law (Nielsen 1996):

$$m^{1/3} = k_p t + m_0^{1/3} \quad \text{(Equation 1)}$$

where *m* is the pellet mass and m_0 is the pellet mass at time t = 0. The parameter k_p can be specified as a function of the thickness and density of the pellet and the specific growth rate of the cells within the pellet (Nielsen 1996; Tough and Prosser 1996).

Cultures which are predominantly suspensions of filamentous mycelia are typically non-Newtonian and become more viscous than predominantly pelleted cultures, resulting in poor mixing and mass transfer (Wang and Fewkes 1977; Gbewonyo and Wang 1983; Liu and Yu 1993; Posch *et al*. 2013). The higher apparent viscosities in the mycelial cultures require higher energy inputs to maintain mixing and aeration (Liu and Yu 1993). Growth rates are governed by rate of tip extension and branching frequency of hyphal elements (Aynsley *et al*. 1990; Viniegra-González *et al*. 1993; Indermitte *et al*. 1994). With respect to process design and control, it is important to note that the morphologies and rheologies of actinomycetes change with time during cultivation, with past studies reporting all combinations of morphology-rheologies (Charles 1978; Warren *et al*. 1995; Heydarian *et al*. 2000; Heydarian *et al*. 2002). Thus, due to complex morphologies, the most accurate growth models need to be process specific.

Assuming medium composition and the producing organism remain constant for a process, the three major classes of controllable variables to influence culture morphology include inoculum type and concentration, addition of polymer additives to the medium and shear forces in the fermenter (Paul *et al*. 1999; Junker *et al*. 2004). A dispersed inoculum is favourable for industrial seed cultures to ensure providing maximum number of growth nuclei (van Wezel *et al*. 2006), homogeneity, and preventing issues related to clogging of peristaltic tubing and uniform pipetting (Junker *et al*. 2004). Strategies to break up clumped biomass in seed cultures include the use of a blender, baffled flasks or inclusion of glass beads. These, however, are potentially problematic in that purity of culture might be compromised by the use of blender or mixer, baffled flasks could cause foaming (outlined further in Section 2.6.2) and the effectiveness of glass beads is limited by shaking rate (high shake rate will cause beads to damage or break the shake flask and or the cells). The use of a spore inoculum is effective in providing homogenous seed cultures, but additional time during process start-up is required to allow for sporulation to occur (Junker *et al*. 2004).

Of the cultivation parameters which can be adjusted to influence the morphology of actinomycetes in submerged culture, agitation requires great attention. Primarily agitation during microbial cultivation is to provide improved mixing, heat, and mass transfer (Smith *et al*. 1990). The lower limit on agitation rates are set by mass transfer considerations (mainly of dissolved oxygen and carbon dioxide). However, the induced turbulence may alter microbial activity (Chisti 2002; Groslambert *et al*. 2002) and the upper limits are often dictated by shear-sensitivity of biomass (Blanch and Bhavaraju 1976).

2.6.2 Process challenges of actinomycete culture: Foaming in stirred tank bioreactors

Gas dispersion within the medium is required to provide gaseous exchange for microbes. Sparging at the bottom of the bioreactor provides air to the medium. The air bubbles travel through the medium and erupt from the liquid surface and exit through the outlet at the top of the vessel. Gas holdup is the volume of gas (air) held within the medium; whilst it is held up in the medium, gaseous exchange takes place through the gas-liquid and liquid-microbe interfaces. Foam is the distribution of a gas in a continuous liquid phase, with linking lamella, where the gas phase is the dominant phase. Foam is formed when the gas bubbles provided by sparging do not disengage from the media and are stabilised. Foam has a larger gas-to-liquid volume ratio (usually greater than 3) than regular gas holdup and the gas bubbles are not as spherical (Prins and van't Riet 1987; Riet and Tramper 1991; Vardar-Sukan 1991). Foaming is dynamic, especially in bioprocesses and can be classified as being stable, unstable or metastable, dependent on the bioprocess and the prevailing conditions (Junker 2007).

Foaming is a process challenge commonly occurring in stirred tank bioreactors. There are many contributing factors that lead to foam formation and govern its severity in bioreactors. These include gas bubbles (aeration and off gas evolution), surface active compounds, operating conditions and reactor vessel geometry (Delvigne and Lecomte 2010). Table 2.4 shows a summary of the impact that operating conditions have on foam formation. The major contributing surface active compounds are proteins, as medium components, microbial metabolites and expelled contents of lysed cells. Other surface active compounds include lipopolysaccharides (LPS), lipopeptides, pigments, salts, carbohydrates and alcohols, which are either components of the growth medium or metabolites of the microorganism. In addition

to the metabolic activity of microbes, their physical presence (and that of other solids) affects foam formation and stability (Junker 2007; Delvigne and Lecomte 2010).

Foam tolerance is process dependent. Typically excessive foaming is unacceptable; however, even mild transient foams can cause undesirable effects. During the microbial cultivation foaming can remove biomass from the liquid culture and associated bulk environment, resulting in lower productivity. Cells may also be trapped in the foam and experience nutrient limitation, forcing a change in microbial metabolism and cell death. Cells caught in foam can contribute to foam stability by excreting proteins or LPS and in extreme cases, lysing, further exacerbating the problem. Foaming may promote wall growth and biofilm formation, leading to culture non-homogeneity, which interferes with monitoring equipment and results in non-representative sampling (Vardar-Sukan 1991; Junker 2007; Delvigne and Lecomte 2010) and incorrect process adjustment based on the erroneous readings.

Foaming can also lead to challenges with liquid containment. Foam increases gas hold up, resulting in elevated liquid levels and upsetting bioreactor configurations based on working volume (Vardar-Sukan 1991), such as mixing patterns and medium addition in fed batch and continuous systems. Breach of liquid containment can be far reaching if medium components are corrosive and/or hazardous (Junker 2007).

Whilst the increased back pressure (owing to obstructed filters) and gas holdup caused by foaming actually favours mass transfer, cultivations in which foaming occurs, can often experience mass transfer problems. Addition of chemical defoamers can induce gas bubble coalescence and form thin layers around said bubbles adding another barrier to transfer (Pelton 2002). The increased gas retention time can also result in oxygen depleted bubbles and carbon dioxide accumulation (Junker 2007).

Besides manual foam observation, various detectors can be used to detect the foam levels and its stability. Conductivity or resistivity probes are most commonly used. However, these are often susceptible to fouling. Other detector technologies include capacitance, ultrasonic and radio frequency admittance systems. There are various chemical, physical and mechanical methods that can be used in foam mitigation. Often a combination of these are used in bioprocesses (Junker 2007; Delvigne and Lecomte 2010).

‘Antifoam’ is the general term used for chemical substances used in combating foam; though technically ‘antifoams’ are agents that prevent foam formation and are added to media prior to cultivation, whereas ‘defoamers’ are agents that break up already formed foam and are dosed as required (Delvigne and Lecomte 2010). Antifoams and defoamers are, however, fundamentally the same type of chemical (Pelton 2002). Commercial antifoams typically consist of oils, hydrophobic solid particles, long chain alcohols, fatty acids/esters, polyesters, or a combination there of (Delvigne and Lecomte 2010).

Table 2.4 Summary of the impact of bioprocess operating conditions on foam formation[a]

Condition	*General impact*	*Mechanism*	*Comments*	*Reference*
Gas flow rate	increases with higher flow rates	greater amount of bubbles erupting from surface	often foam maximum exists and then decreases	(Vardar-Sukan 1991; Van't Riet and Tramper 1991)
Superficial velocity	increases with higher velocities	greater speed of bubbles erupting from surface	if vvm held constant with scale-up, superficial velocity increases	(Pandit 1989)
Sparger orifice size	increases with smaller orifices	smaller bubbles form smaller foam cell structures which collapse more slowly	sintered spargers may produce high amounts of foam	(Chisti 1993)
Agitation rate	increases with higher rates	gas entrainment; cell lysis owing to high shear environment	depending on relative broth/impeller geometry, possibly can decrease foam by providing mechanical disturbance	(Hoeks *et al*. 1997; Hoeks *et al*. 2003; Pandit 1989)
Viscosity	increases as viscosity rises above initial water-like values	decreases film drainage which increases foam	system-dependent behaviour	(Prins and van't Riet 1987)
Temperature	decreases with higher temperature	decreases viscosity which increases liquid film drainage and reduces foam	foaming can increase when broth cooled awaiting harvest	(Gaden and Kevorkian 1956; Prins and van't Riet 1987; Vardar-Sukan 1991)
Broth pH	increases when near protein isoelectric point (pI)	proteins least soluble near pI	foaming can increase protein denaturation	(Vardar-Sukan 1991; Van't Riet and Tramper 1991)
Sterilisation	increases with longer sterilisation hold times, higher temperature, higher pre-sterilisation pH	Maillard reaction products formed from sterilising nitrogen sources and reducing sugars together	foam may decrease after aeration applied for a duration since Maillard reactions partially reversible	(Kotsaridu *et al*. 1983; Schügerl 1985.; Vardar-Sukan 1991)

[a] Adapted from Junker 2007 and reproduced with permission from John Wiley and Sons

"vvm" = $volume_{air}/volume_{culture}$ liquid"

A good antifoam should suppress foam, be quick acting, cheap, and only be required in miniscule amounts to limit interference in the overall bioprocess. However, antifoams do negatively affect the bioprocess by reducing mass transfer (additional barrier at gas-liquid interface), increasing the mixing power required (lowering gas entrainment), affecting the metabolic state of microbes, probe response (e.g. accumulation on membranes) as well as downstream processing. For these reasons, non-chemical methods are preferred.

Non-chemical methods, include mechanical foam scrapers or breakers, ultrasound, thermal and electrical methods, and adjustment of operating conditions. Ultrasound, thermal and electrical treatments are generally not used, primarily due to their adverse effects on the microbes. Aside from the mechanical breaker, stirring as foam disruption (SAFD) techniques have been developed in which the bioreactor's hydrodynamics are changed to limit foam formation. SAFD techniques work by increasing the liquid velocity at the liquid surface by changing impeller geometries and increasing agitation rate, such that foam is disrupted through shear stress and wave action (Delvigne and Lecomte 2010). Other operating conditions that can be changed include increasing the back pressure, decreasing agitation or decreasing aeration, or both, during the process (i.e. minimising the effects shown in Table 2.4). However, these adjustments can limit productivity of the organism or bioprocess (Vardar-Sukan 1991; Junker 2007).

2.7 Research Objective and Key Questions

The aim of this study was to build on work of Le Roes (2006) and investigate the growth of SPR^T and its production of DPO and other bioactive molecules in submerged shake flask cultivations, followed by scaling-up the process into a laboratory stirred tank bioreactor (STR). This was investigated by addressing the following objectives:

a. Develop experimental procedure(s) for characterisation and analysis of biomass and antimicrobial production profiles by SPR^T.
b. Determine the growth profile and growth rate of SPR^T under standard conditions and time of onset of antimicrobial production.
c. Investigate change in growth and antimicrobial production of SPR^T in response to modified glucose in the medium and a variation in temperature.

The key questions posed in conjunction with the objectives were:

I. What are the kinetic growth parameters (including time frame, growth phases, rates and yields) of SPR^T grown in Hacène's Medium in shake flasks (SF)?
II. How does SPR^T growth compare between the SF and STR? How does this compare to the end point data presented by Le Roes (2006)?
III. What is the relationship between biomass and antimicrobial production phases?

2.8 Research Approach

In order to achieve the above objectives and answer the key questions, methodology for reproducible measurements of filamentous biomass concentration and of DPO concentration were developed prior to carrying out standardised growth studies. Shake flask experiments grown under the same conditions as Le Roes (2006) were performed with additional measurements to establish growth and production profiles. These experiments were used to inform scale-up into the stirred tank bioreactor.

In order to investigate antimicrobial production, empirical experiments were carried out, testing both association with growth phase and response to stress. No genomic or metabolic flux analysis was carried out due to the absence of information of SPR^T metabolic pathways in the literature. DPO production by SPR^T was a key marker for antimicrobial experiments; this was based on it being the predominant identifiable bioactive molecule and its potential use as an anti-tuberculosis drug.

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Chapter 3. Materials and Methods

3.1 Materials

3.1.1 Microorganisms

Bacterial strains used in this study were obtained from Dr Paul Meyers of the Department of Molecular and Cell Biology at the University of Cape Town. *Streptomyces polyantibioticus* SPR^T (DSM 44925^T, NRRL B-24448^T) is a pigment producing, spore forming actinomycete which produces branching, vegetative mycelia (Le Roes-Hill and Meyers 2009) and was the focal strain of the present study. Other bacterial strains used in the present study included *Mycobacterium aurum* A+, *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923).

For long time storage, stock cultures were made from liquid cultures (1200 µl culture, 300 µl 80% glycerol) and frozen at -60 °C. Each bacterial strain was maintained as a pure culture, using standard agar streak plates. Gram stains and light microscopy [BX40, Olympus Optical Company Ltd, Japan] (Burke 1922; Cowan and Steel 1965) were utilised to check their purity.

3.1.2 Culture media

Standard culture media for bacterial cultivation was used in this study. Luria-Bertani (LB; 10 g/L tryptone, 5 g/L yeast extract and 5 g/L NaCl), double strength yeast-tryptone broth (2XYT; 16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl) and yeast extract-malt extract agar (ISP 2 or YEME; 4.0 g/L glucose, 10.0 g/L malt extract, 4.0 g/L yeast extract, pH 7.3) (Shirling and Gottlieb 1966) were used. Agar plates of Difco Middlebrook 7H9 media (7H9), prepared according to the manufacturer's specifications (4.7 g/L powder) and supplemented with separately autoclaved glucose at 1.8 g/L (i.e. 10 mM) (albumin and catalase omitted), were used as a sporulation medium for SPR^T. SPR^T spores were suspended in Tryptone Soy Broth (TSB; Merck, Modderfontein, RSA). Hacène's medium (HM; 5.0 g/L glucose, 4.0 g/L yeast extract powder, 10.0 g/L malt extract and 1.0 g/L sodium chloride, pH 7.4) (Hacène and Lefebvre, 1995) was used as production medium for SPR^T. Solid plates of the aforementioned media were made by adding 15 g/L of agar. All media and glassware were autoclaved at 121 °C for 20 minutes prior to use.

3.2 Cultivation methods

3.2.1 Pre-culture preparation

SPR^T was maintained on YEME, *M. aurum* on 2XYT and *E. coli* and *S. aureus* on LB (media described in Section 3.1.2). The actinomycetes and bacteria were incubated at 28 °C and 35 °C, respectively.

The SPR^T inoculum was typically prepared by aseptically removing spores from an agar plate, and re-suspending in 5 mL diluted TSB (1 in 5); the TSB helps counteract hydrophobicity of the spores by breaking surface tension. The spore suspension was used to inoculate 50 mL HM broth in a 500 mL Erlenmeyer flasks, closed with a cotton bung to facilitate mass transfer of gas. The flasks were incubated at 28 °C on an orbital shaker at 170 rpm.

3.2.2 Shake flasks

Bacterial cultures were grown in Erlenmeyer flasks containing culture medium, to a volume equivalent to 10 % of the flask volume. A 10 % (v/v) inoculum was used and flasks were incubated at 28 °C and 170 rpm. The entire contents of a single flask was sacrificed per time point sample. Temperature and pH were monitored in discrete samples using external probes.

3.2.3 Batch stirred tank bioreactor system

Batch experiments were performed in a 7-L New Brunswick reactor, connected to a New Brunswick BioFlo 110 controller (New Brunswick Scientific, USA). In the reactor system, utilising BioCommand software (New Brunswick Scientific, USA) pH, temperature, dissolved oxygen and agitation were measured and controlled by means of internal probes (pH: 405-DPAS-SC-K8S120; dissolved oxygen: InPro 6100/220/S/N Mettler Toledo, Ohio, USA) and feedback loops. The culture temperature was maintained by means of a fitted heating jacket and an internal cooling coil, through which ethylene glycol (4°C) was pumped. The pH was controlled by means of automated titration of 5M KOH and 5M HCl. The culture was aerated by sparging compressed air into the reactor after filter sterilisation, using a 0.2 µm Millipore

Millex® membrane filter (Merck, Modderfontein, RSA). The exhaust gas outlet was fitted with a condenser and two depth filters in series (one column filled with glass beads, followed by another column filled with glass wool). Culture homogeneity and mass transfer was ensured by agitation via a regulated magnetic drive, equipped with a single six-blade Rushton impeller, positioned one third (of culture volume) up from the bottom of the vessel, and four vertical baffles lining the inner reactor wall. To contain foaming, a mechanical foam breaker was fitted just above the surface of the liquid, and Antifoam 204, (Sigma Aldrich, Missouri, USA) was added drop-wise to the medium, as needed.

The inoculum train was as follows: a 5 mL, diluted TSB spore suspension, prepared as described in section 3.2.1 above, was used to inoculate a 500 mL Erlenmeyer flask containing 50 mL HM. This pre-inoculum culture was grown at 28 °C and 170 rpm for 48 hours. Ten millilitres of the pre-inoculum culture was used to inoculate three 1L Erlenmeyer flasks, each containing 100 mL HM. These flasks were incubated for 30 – 36 hours at 28°C and 170 rpm. The three cultures, combined, were used as the inoculum for the bioreactor experiments. The schematic in Figure 3.1 shows an overview of the protocol. The culture system parameters outlined in Table 3.1 were used unless otherwise stated.

Figure 3.1 The inoculum train used in the bioreactor studies

Table 3.1 System parameters for bioreactor experiments

Parameter	***Setting***
Organism	*Streptomyces polyantibioticus*
Medium	Hacène’s (HM)
Culture volume	3 L (7 L vessel)
Agitation	250 - 800 rpm (adjusted to maintain DO >40%)
Aeration	0.75 vvm
Temperature	28 °C
pH	7.4

- ‘rpm’ = revolutions per minute. ‘DO’ = dissolved oxygen, “vvm” = $volume_{air}/volume_{culture\ liquid}$

3.3 Extraction of bioactive compounds

Bioactive compounds were solvent extracted at room temperature from the SPR^T culture (shake flask /bioreactor) using a method adapted from Taddei *et al.* (2006). Whole culture samples (including cells and liquid medium) were extracted with an equivalent volume of ethyl acetate. The mixtures were vigorously hand-shaken for 5 minutes before being transferred to an orbital shaker where they were shaken at 90 rpm for 20 hours. Large culture-solvent volumes (>200 mL) were mixed using a magnetic stirrer. The mixtures were transferred into separating flasks and left to stand for a few minutes to allow for separation of aqueous and organic solvent layers. The organic layer was removed and retained, while the aqueous layer, containing cells, was discarded. Cultures were extracted twice (sequentially) and organic layers pooled. Organic layers were concentrated using a rotary evaporator.

3.4 Analytical procedures

3.4.1 Determination of biomass concentration

The cell dry weight (CDW) was determined gravimetrically. Culture samples were vacuum-filtered through 0.45 µm pre-weighed filter paper. The culture filtrate was collected and kept for subsequent glucose and pH analysis. The filter cake was dried to constant weight in an oven at 75 °C for 48 hours before being cooled in a desiccator. Gravimetric analyses were performed in triplicate.

3.4.2 Carbon utilisation and formation of organic acids determined by HPLC

Glucose and glycerol were measured using HPLC, (Finnigan Surveyor Plus, Thermo Scientific, Kempton Park, RSA) equipped with a Refractive Index (RI) detector (Finnigan Surveyor RI Plus, Thermo Scientific, Kempton Park, RSA), using a Biorad Aminex® HPX-87H organic acid column (7.8 mm x 300 mm, Biorad, Cape Town, RSA). Isocratic analyses was performed using 0.01M H_2SO_4, as the mobile phase, and an external column oven set to 40 °C. Samples were diluted 20 times in mobile phase before analysis, and the injection volume used was 10 µl. The flow rate used was 0.5 mL/min. Concentrations of glucose and glycerol in the samples were determined by comparing retention times and peak areas to that of a calibration curve (Appendix B) constructed from analyses of standard compounds.

3.4.3 Antimicrobial activity

3.4.3.1 Bacterial overlays for determination of antimicrobial activity

Standard bacterial overlays were used as confirmatory tests for known antibiotic activity. SPR^T was stab inoculated on agar plates of HM and 7H9, allowed to grow for 10 days at 30 °C, followed by overlaying with 6 mL sloppy– agar containing an actively growing culture of the test bacteria (*E. coli, S. aureus* and *M. aurum*) and further incubated for 24 hours (Sambrook *et al.* 1989). Zones of clearance around SPR^T were sought. Similarly, plate overlays with stab inoculations was replaced by filter paper discs (6 mm in diameter) soaked in crude antibiotic extract. The presence of clear zones were a positive indication that SPR^T produced bioactive molecule(s) with inhibitory activity against the test bacterium, with the zone size being indicative of inhibitory strength.

For anti-tubercular activity, the test bacterium used was *M. aurum*. It is a faster growing, non-pathogenic mycobacterium with a similar susceptibility profile to that of *M. tuberculosis* (Chung *et al*. 1995). To investigate a range in activity, *E. coli* and *S. aureus* were used as Gram-negative and Gram-positive controls, respectively.

3.4.3.2 Separation of active compounds using TLC and bio-autography

Thin Layer Chromatography (TLC) was performed as a quick, qualitative test for the presence of active compounds in the culture extracts. The crude “antibiotic” extract was spotted on silica TLC plates. Two sets of mobile phases were used to develop the plates including: a) ethyl

acetate and acetic acid (90:10 (v/v)) and b) ethyl acetate, hexane and acetic acid (90:9:1 (v/v)). The developed plates were visualised under UV light (254nm) and retention factor (R_f) compared to a standard, where R_f is the distance travelled by compound relative to the solvent front.

The culture extracts were also subjected to spot - test bioautography according to Betina (1973). Volumes of 5, 10, 20 and 25 µl of the extract were spotted on silica TLC plates. An actively growing culture of the test bacterium, diluted to an OD_{600} reading of 0.5, was evenly applied onto the prepared TLC plate using sterile non-absorbent cotton discs. The TLC plates were incubated in a humidified container for 24 hours; *E. coli* and *S. aureus* tests incubated at 30 °C and *M. aurum* incubated at 35 °C, temperature selected for optimal growth of each strain. Following incubation, a 0.25% solution of thiazolyl blue tetrazolium bromide (MTT) in phosphate buffer (MTT Sigma, M2128 ; 1.78 g/L Na_2HPO_4 , 8.50 g/L NaCl, pH 7.3) was evenly applied to the plates, returned to the container and further incubated for 3 hours (12 hours in the case of *M. aurum*). The colour change from yellow to violet, attributed to the reduction of MTT in the presence of actively respiring cells, was sought. Zones of no activity were attributed to the presence of antimicrobials. Bioautographic tests with solvent and commercial DPO served as negative and positive controls respectively.

3.4.3.3 Identification and quantification of active compounds using HPLC

The extracts of the SPR^T culture were analysed using HPLC, with UV detection at 303 nm (Finnigan Surveyor Plus, Thermo Scientific). Compounds were separated using a Phenomenex Luna® C18 (2) 5 µ column (250 mm x 4.6 mm), and a dual mobile phase system, with a linear gradient method, as shown in Table 3.2. Mobile phase A consisted of 5% (v/v) methanol and 1% (v/v) acetic acid, while mobile phase B comprised of 95% (v/v) methanol and 1% (v/v) acetic acid.

Table 3.2: HPLC mobile phase gradient profile used in SPR^T culture extracts, UV detection at 303nm

Time (min)	5% MeOH-1%AA	95% MeOH-1%AA	Flow rate (mL/min)
0.0	80.0	20.0	0.8
1.0	80.0	20.0	0.8
41.0	0.0	100.0	0.8
61.0	0.0	100.0	0.8
61.1	80.0	20.0	0.8
66.0	80.0	20.0	0.8

- "MeOH" = Methanol, "AA" = Acetic acid

3.4.4 Data analysis

Basic statistical analysis (mean, standard deviation and confidence intervals) were used in reporting data. T- and F-tests were used to show significant differences when comparing data sets. All analyses were performed in Microsoft Excel (2013).

3.5 Experimental plan

The objectives of this study were to provide biokinetic data for the growth of SPR^T and its production of DPO and other bioactive metabolites in liquid cell culture in shake flasks and laboratory stirred tank bioreactors. This was addressed by three sets of experiments.

In the first set of experiments, the methodology for reproducible measurements of filamentous biomass concentration and of DPO concentration was developed. Visual observations of SPR^T biomass in submerged culture were made by direct examination of the culture and the use of light microscopy. This informed the choice of parameter by which biomass concentration was measured. Optimisation of biomass measurement protocol was done by testing the effects of the sampling method, sample volume and sample preparation. The mobile phase composition and gradient profile for TLC and HPLC methods was investigated for the analysis of DPO and other bioactive molecules.

Having developed the experimental procedures necessary for characterisation and analysis of biomass and antimicrobial production by SPR^T, standardised growth studies in shake flasks were performed to establish production profiles. The shake flask system was used to inform scale-up into the stirred tank bioreactor. Experiments to ensure a stable stirred tank bioreactor run, with respect to excessive foaming, were performed by adjusting the operational parameters (aeration rate, agitation regime, inclusion of mechanical foam breaker, antifoam addition and pH control). The previously established shake flask system served as a reference for comparison with production profiles obtained from growth studies performed in the stirred tank bioreactor.

Further investigation of DPO and bioactive molecules by SPR^T was done in a third set of experiments by testing both the association with growth phase and response to stress. This included cultivations in which the starting glucose concentration in the growth medium and the cultivation temperatures were varied.

Chapter 4. Development of Methods

4.1 Determining biomass concentration of filamentous microorganisms

4.1.1 Direct methods for measuring biomass.

Traditional methods to determine the biomass content of microbial cultures include optical density (OD), packed mycelial volume (PMV) and gravimetric measurements; cell wet weight (CWW) and cell dry weight (CDW) (Posch *et al.* 2013). The OD of a culture is the degree to which it retards light shined through it, determined by the use of a spectrophotometer; the quick settling of cells results in inaccurate OD measurements. Furthermore, a homogenous suspension is required, where varying particle size due to aggregation and filament formation will affect absorbance. PMV is the ratio of biomass to liquid after being subjected to centrifugation; complex culture morphology, the impact of morphology on packing and low biomass density are limitations to this measurement method. CWW is determined by weighing filtered samples before drying; accurately accounting for the moisture content of each sample and keeping this consistent can be problematic. Again, the effect of morphology on packing must be recognised.

Streptomyces polyantibioticus SPR^T (SPR^T) has a non-homogenous filamentous nature (Figure 4.1), which may influence biomass measurement. Due to the limitations of the methods for quantifying biomass mentioned above, dry weight gravimetric measurement following centrifugation was used. In separate experiments performed under the same conditions, the cell dry weights of SPR^T culture samples were analysed using centrifugation in Eppendorf® tubes. This data is presented in Figure 4.2. Culture samples of 1 mL were centrifuged at 13 000 rpm for 15 min. The supernatant was discarded and cell mass washed with 500 µL distilled water and centrifuged at 13 000 rpm for a further 15 min. The supernatant was discarded and samples dried for 48 hours at 75 °C before being weighed.

As seen in Figure 4.2, CDW biomass data derived from centrifugation in Eppendorf® tubes was highly variable with readings for the same time point differing by up to 1.8 g/L. Very low concentrations, such as that of the inoculum, was over estimated. The water wash step method (Figure 4.2A) consistently gave larger variances than data derived without the wash step (Figure 4.2B), exhibiting average standard deviations (SD) of 0.51 and 0.43 g/L respectively (F-test, p=0.007).

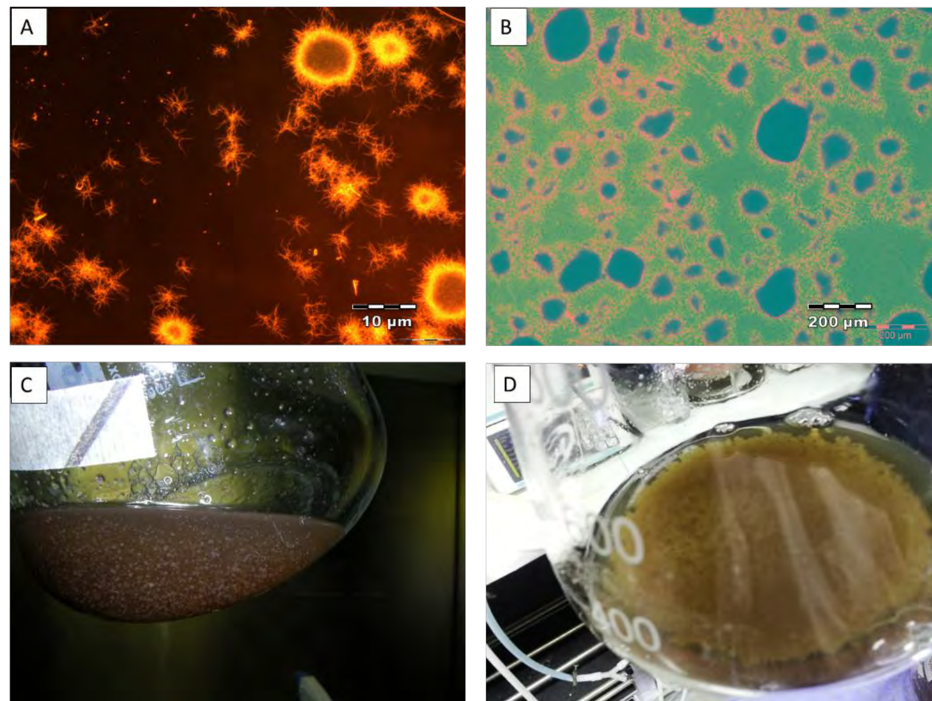


Figure 4.1 Images showing typical morphological forms of SPR^T in submerged batch culture. A and B were imaged using a light microscope, at 1000x magnification. (A) Presence of spores, free hyphal fragments, entangled filaments and pellets, (B) Pellets of varying size and density. C and D depict liquid cultures in shake flasks showing predominantly (C) pelleted and (D) filamentous forms.

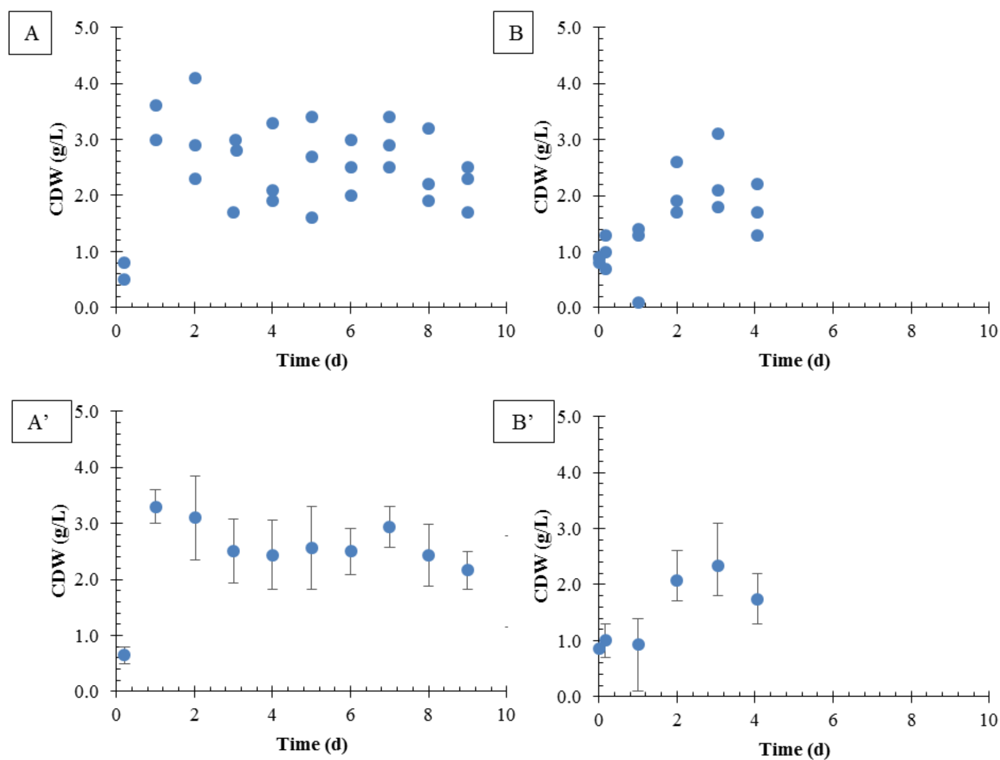
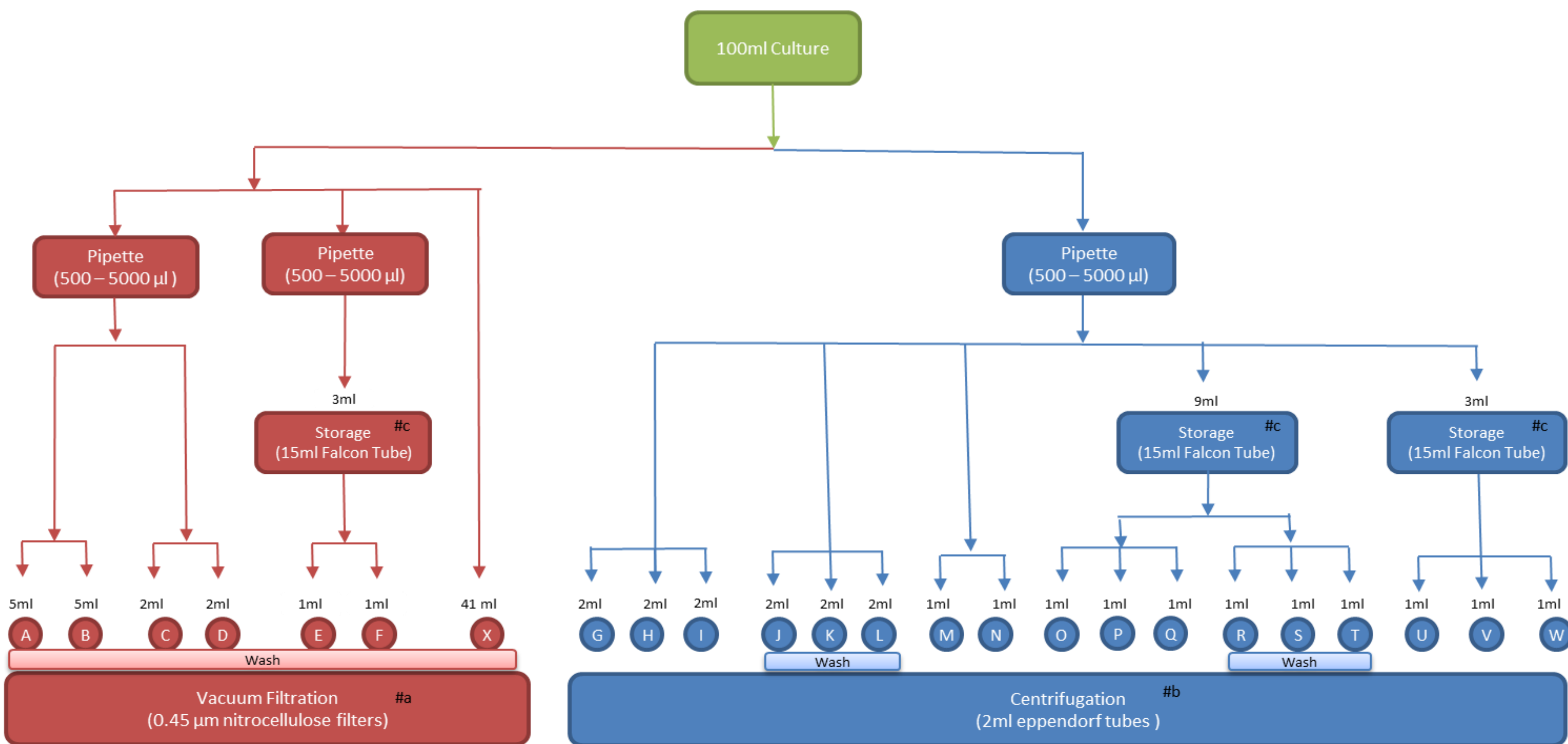


Figure 4.2 Dry weight gravimetric analysis of SPR biomass using centrifugation in Eppendorf® tubes (A) with a water wash step, (B) without a wash step. The corresponding graphs A' and B' show the same information averaged, with error bars showing standard deviation of the mean (n=3).

4.1.2 Optimisation of Gravimetric analysis: Vacuum Filtration vs Centrifugation

Different protocols were used to test for measurement accuracy as described by the schematic in Figure 4.3. Samples were subjected to either vacuum filtration using filter paper (A-F and X) or centrifugation in Eppendorf® tubes (G-W) at 13 000 rpm for 15 min at room temperature with varying sample volume, inclusion of intermediary wash step and volume pipetted. The 10 day old SPR culture was shaken to ensure even distribution of biomass in liquid medium. All vacuum filtered samples and centrifuged samples (K-M; S-U) included a water wash step. All samples were dried for 48 hours at 75 °C before being weighed to constant mass. The data generated from this experiment are presented in Figure 4.4 and Table 4.1.

A wide range of individual biomass concentrations (1.45 – 15.20 g/L), with discrepancies between readings taken from different protocols, as well as between technical repeats of the same protocol, was obtained with coefficients of variance (COV) ranging between 5.6 and 97.4% (Table 4.1). On average, samples that followed a filtration protocol (Figure 4.3 A-F and X) gave lower biomass readings, 2.45 fold lower than the centrifuged samples (Figure 4.3 G-W), with 2.59 and 6.36 g/L recorded on average for filtration and centrifugation, respectively. Since SPR^T biomass has a mixed morphology (Figure 4.1) with mycelia existing as both free mycelia and in compact pellets, larger sample volumes give a better overall representation of the flask contents. Therefore it was to be expected that across both the filtered and centrifuged samples, the smaller the sample volume the larger the discrepancies between repeats and resultant deviation. This was not true of all the results obtained in this experiment (Table 4.1) with the 2 mL filtered samples (C-D) having a SD of 1.50 g/L which is larger than the SD obtained for 1 mL samples (0.92 g/L). Similarly the 1 mL centrifuged samples had SDs that were both higher (R-T with SD of 4.16 g/L), and lower (0.70 g/L) than the 2 mL centrifuged samples (SDs of 2.16 and 0.36 g/L).



#a - Vacuum filtration: at room temperature; water wash of cells on filter paper and dried for 48 hours at 75 °C
#b - Centrifugation: 13 000rpm for 15 min at room temperature.; for wash step (J-L and R-T,) cell pellet re-suspended in water, centrifuged again as before and dried for 48 hours at 75 °C.
#c – Storage at 4 °C for 30min;

Figure 4.3 Sample preparation for biomass quantification by dry weight gravimetric analysis using vacuum filtration (A-F and X) and centrifugation (I-Y).

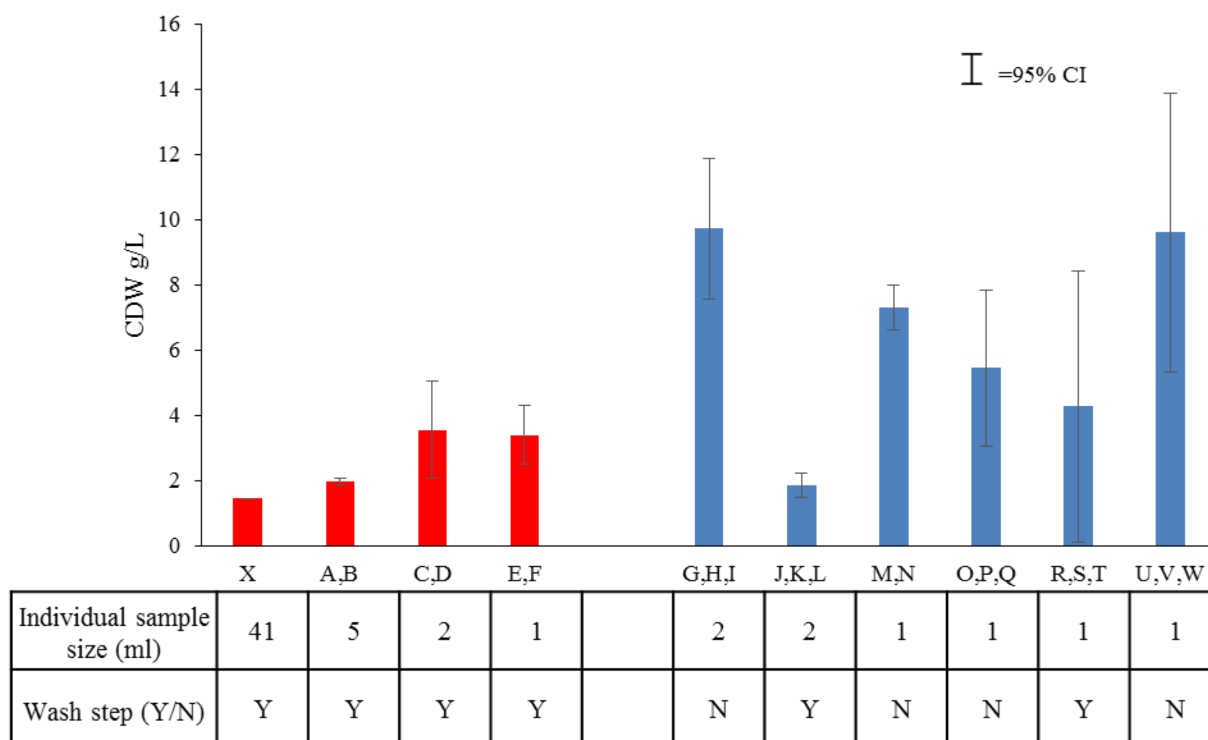


Figure 4.4 Biomass readings taken from the same shake flask culture of SPR^T using different gravimetric measurement methods (see Figure 4.1). Red bars represent vacuum-filtered samples through 0.45 µm nitrocellulose acetate filters, blue bars represent samples centrifuged in Eppendorf® tubes at 13 000 rpm for 15 minutes at room temperature. The average of technical repeats are shown, with error bars representing the 95% confidence interval of the mean.

Table 4.1 Standard Deviations (SD) and coefficients of variance (COV) in CDW measurements taken from the same shake flask culture of SPR^T (cf Figure 4.3 and Figure 4.4)

Sample	*Filtered / Centrifuged*	*Wash step (Y/N)*	*Sample size (mL)*	*CDW (g/L)*	*SD*	*COV (%)*
A,B	f	Y	5	1.97	0.11	5.6
C,D	f	Y	2	3.55	1.50	42.3
E,F	f	Y	1	3.40	0.92	27.0
G,H,I	c	N	2	9.72	2.16	22.2
J,K,L	c	Y	2	1.85	0.36	19.2
M,N	c	N	1	7.30	0.70	9.6
O,P,Q	c	N	1	5.45	2.39	43.9
R,S,T	c	Y	1	4.27	4.16	97.4
U,V,W	c	N	1	9.60	4.28	44.6

Large standard deviations and potential overestimation of the biomass concentration was likely caused by non-representative sampling of the culture. Other causes of errors could be attributed

to loss of biomass during various stages of the centrifugation protocols as SPR^T did not pellet well after centrifugation and biomass was possibly lost when decanting the supernatant. A longer centrifugation time might have helped to abate this problem but would have meant a lengthy, inconvenient protocol. Further loss of biomass to the sides of containers or of pipette tips during transferring of sample could also contribute to the error. Conversely, during vacuum filtration, there was no loss of biomass all biomass material was retained in the filter cake, due to the use of an absolute filter.

In addition to the causes of error cited above, the difference in biomass concentration between centrifuged samples, in the absence of washing, (G-I) and following a water wash (J-L), resulting in mean biomass readings of 9.72 g/L ± 2.16 and 1.85 g/L ± 0.36, respectively, could be attributed to biofilm formation by SPR^T. The water wash step of J-L ensures that extracellular polymeric substances (EPSs) were removed and not included in the gravimetric reading.

The true biomass concentration of the SPR^T shake flask culture was best approximated by sample X (1.45 g/L), as its large sample volume and minimisation of intermediary steps circumvent most of the problems outlined above. This value was consistent with concentrations from other shake flask experiments run under the same conditions (see Section 5, Figure 5.1). Vacuum filtered samples A-B (1.97 g/L ± 0.11) and centrifuged samples J-L (1.85 g/L ± 0.36) were closest in approaching this true value, with a smaller associated error as compared to the centrifugation protocols tested (Figure 4.4.) Protocols A-B and J-L would be sufficient to follow trends in biomass concentration, but may prove insufficient where more precise readings are needed for further calculations.

Figure 4.5 illustrates the improvement in the quality of gravimetrically-obtained biomass data (refer to Appendix C) when the above-mentioned sources of error were minimised by using vacuum filtration with a 5 mL sample volume (protocol A-B, Figure 4.3), rather than centrifugation of a 1 mL sample (Figure 4.2). The average COV of the data in each experiment varied with 17.7, 14.3 and 3.07% obtained for experiments A, B and C, respectively (Figure 4.5 A-C). The filtration method (Figure 4.1C) resulted in biomass data that gave the expected growth curve, whereas the centrifugation methods did not.

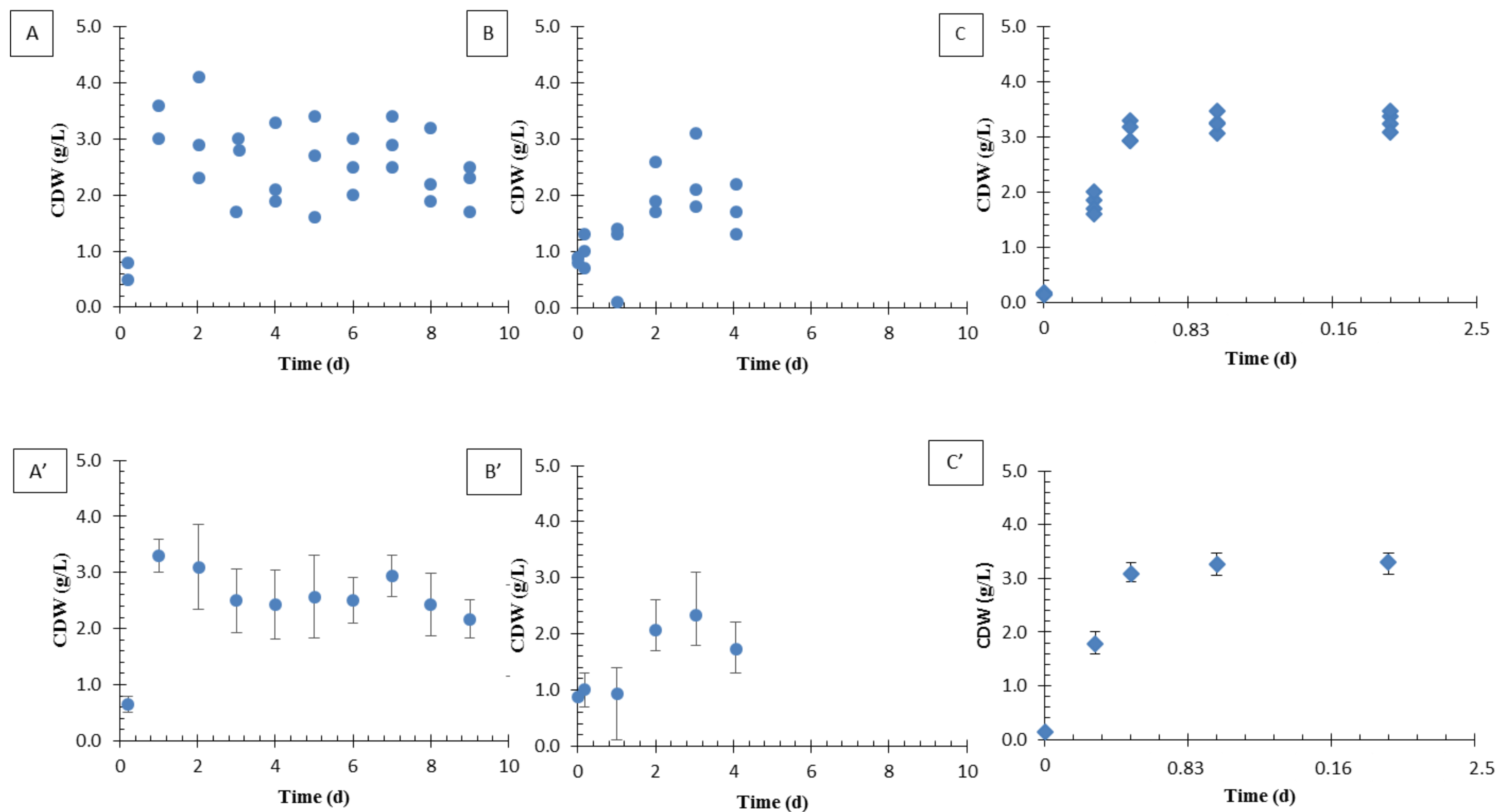


Figure 4.5 Dry weight gravimetric analysis of SPR^{T} biomass using (A) centrifugation of 1 mL in Eppendorf® tubes with a water wash step, (B) centrifugation of 1mL in Eppendorf® tubes without a wash step and (C) vacuum filtration of 5 mL with washing. The corresponding graphs A’, B’and C’ show averaged data, with error bars representing standard deviation of the mean (n=3).

4.1.3 Gravimetric analysis of SPR^T: Sampling bias of pipetting

Since SPR^T cultures are non-homogenous and settling of mycelial biomass occurs, it was postulated that pipetting in itself may have a certain inherent bias, in which the ratio of biomass to liquid medium in the pipetted sample is not equal to the same ratio in the bulk liquid. To test this, a shake flask growth experiment was performed in which each data point was quantified by four discrete samples of 5 mL each, taken from a single shake flask culture. These were then compared to readings taken from duplicate flasks from which the entire content of the flask was sacrificed per data point. CDW analysis was done by filtration with washing. The inoculum in all flasks was standardised. The results are displayed in Figure 4.6.

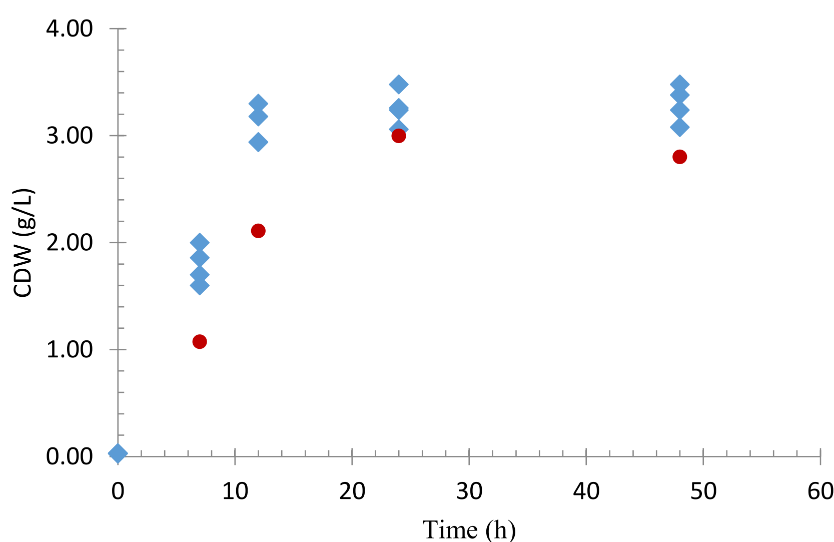


Figure 4.6 Comparison of SPR^T growth curve : Blue diamonds represent discrete 5 mL samples, red circles represent full contents of the shake flask sacrificed (25 mL) per data point.

The growth curve obtained from the discrete 5 mL samples exhibited a similar overall trend as the sacrificed flasks in which 25 mL culture was filtered as the sample. However, the pipetted samples consistently exhibited over-estimated CDWs (T-test, $p \leq 0.01$). The pipetted samples also displayed a trend, where at each time point, each pipetted sample would yield a CDW which was progressively lower than the previous one in terms of order of sampling. This suggested that the suction action of the pipettes did demonstrate some bias in which biomass was collected in preference to medium and hence, an elevated CDW was achieved. To obtain representative CDW readings, the entire contents of a single flask should be used to represent a data point, rather than discrete samples taken by pipetting.

4.1.4 Gravimetric analysis of SPR^T: Concluding remarks

For direct methods of measuring biomass concentration, determination of CDW by gravimetric analysis is best suited towards the morphological characteristics of SPR^T. Results generated by vacuum filtration protocols showed a smaller variance than those generated by centrifugation owing to poor pellet formation. Discrete samples obtained by pipetting consistently showed an inherent sampling bias which lead to over-estimated CDWs. To obtain true and accurate CDW gravimetrically, whole contents of the flask sacrificed per data point, coupled with vacuum filtration and washing, should be used. It is important to have a standardised inoculum across all flasks when using the sacrificial flask approach.

4.2 Chromatographic method development

The development of chromatographic methods used a one-factor-at-a-time approach in which a single factor was altered to fine tune the method in a step-wise fashion. This approach, whilst tedious and neglecting the interaction between different variables, was helpful in fine tuning the methods to improve detection and resolution of compounds. Analysis of antimicrobial compounds produced by SPR^T was done using chromatographic methods described by Le Roes (2006). The solvent extraction method used for obtaining antimicrobial compounds from SPR^T cultures was adapted from Taddei *et al*. (2006) and is described in Section 3.3. Further method development was done to validate the protocols and presence of antimicrobials. Crude ethyl acetate extracts of SPR^T cultures were filtered as used for analysis.

4.2.1 Characterisation of antimicrobials: TLC

Thin Layer Chromatography with Ultra Violet visualisation (TLC-UV) was used as quick tool to monitor the different groups of bioactive compounds present in the extracts. The aluminium backed silica plates were UV active, so compounds showed up as negative zones on chromatograms. A number of different solvent combinations were used as mobile phases. These are shown in Table 4.2. Mobile phase composition was varied according to polarity indices of the solvents. For semi quantitative analysis when comparing samples, the same volume was spotted on plates, darker spots were taken to be indicative of higher quantity.

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Table 4.2 Composition of mobile phases used in TLC to separate bioactive compounds produced by SPR[T].

Mobile Phase	Solvent (%)		
	A	B	C
1	Chloroform 100		
2	Chloroform 90	Acetic acid 10	
3	Chloroform 10	Acetic Acid 90	
4	Ethyl Acetate 90	Acetic Acid 10	
5	Ethyl Acetate 98	DMSO 2	
6	Ethyl Acetate 90	DMSO 10	
7	Ethyl Acetate 80	Methanol 20	
9	Ethyl Acetate 80	Methanol 18	Acetic acid 2
10	Ethyl Acetate 90	Methanol 9	Acetic Acid 1
11	Ethyl Acetate 9	Hexane 90	Acetic acid 1
12	Ethyl Acetate 90	Hexane 9	Acetic acid 1
13	Ethyl Acetate 45	Hexane 65	Acetic acid 0.1
14	Ethyl Acetate 9	Hexane 90	Acetic acid 1

The majority of mobile phases (1, 2, 4, 5, 7 10, 11 and 12) used resulted in separation of the extract into 3 spots on the TLC plates. Mobile phases 3, 6 and 14 resulted in separation of the extract into 2 spots and no separation was achieved with mobile phase 13. Plates developed with DMSO (5 and 6) were more prone to smearing and took up to 4 times longer to develop. In terms of distance of spots from each other, the baseline and mobile phase front (resolution), mobile phases 4 and 12 produced the best separation patterns and were used going forward (Section 5.5). No further separation was observed when 2 mobile phases were used in tandem in 2 dimensional TLC. It is also noteworthy that no other spots were detected when plates were stained with the universal stains, anisaldehyde/H_2SO_4, vanillin/H_2SO_4 and cerium (IV) ammonium sulphate.

4.2.2 Characterisation of antimicrobials: HPLC

High Performance Liquid Chromatography with Ultra Violet visualisation (HPLC-UV) was used to achieve greater separation of the antimicrobial extracts and to quantify compounds. HPLC with mass spectrometry (HPLC-MS) was used in an attempt to identify bioactive compounds. A wavelength scan of the antimicrobial extracts showed absorbance maxima at 223 and 303 nm; whilst a scan of the pure solvent resulted in peak absorbance at 236 nm (Figure 4.7). This helped inform the detection wavelength choice for HPLC-UV protocols investigated. Ethyl acetate is of medium polarity and favours extraction of polar and non-polar molecules alike hence the "crude" nature of the extracts. The marker molecule DPO is non-polar due to its ring structures (Figure 2.3) so reverse phase HPLC was deemed suitable for separation. Acid was included in the mobile phase to improve peak shape (reduce tailing) and assist with protonation in the ionisation step for MS. The various mobile phases, columns, flowrates and wavelengths investigated are shown in Table 4.3.

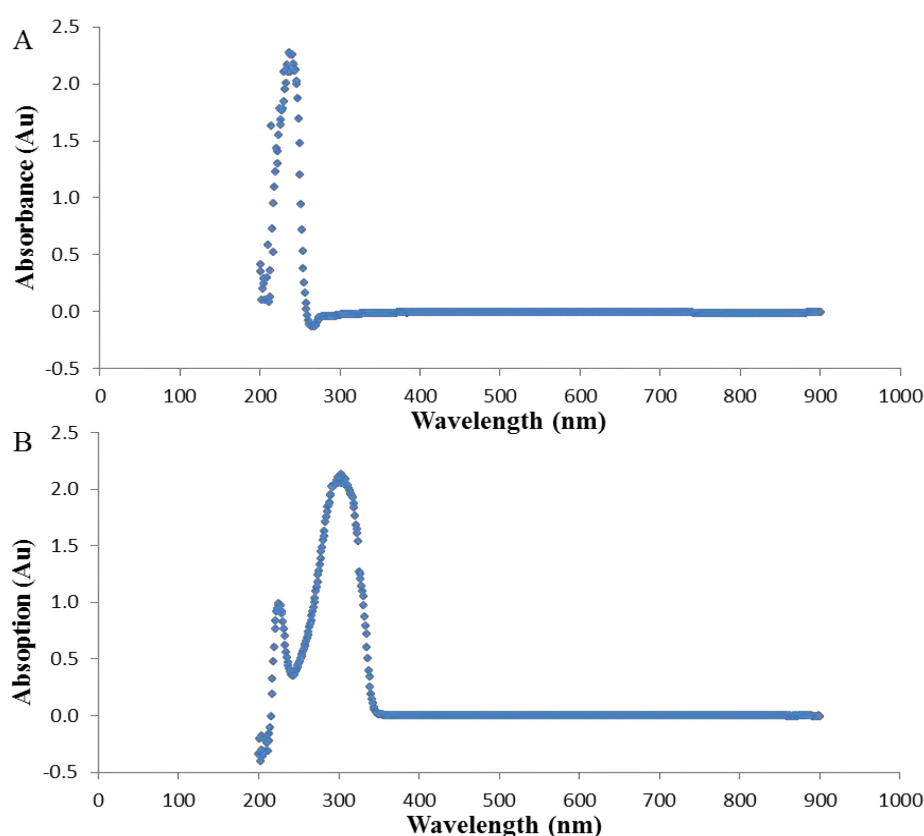

Figure 4.7 Wavelength scans of a) ethyl acetate (solvent blank) b) ethyl acetate extract of SPR^T culture (containing antimicrobials)

Table 4.3 HPLC conditions investigated for separation of bioactive compounds produced by SPR[T].

Method	Column	Time (min)	Solvent (%) A 5% MeOH 1% AA	Solvent (%) B 95% MeOH 1% AA	Flowrate (mL/min)	Wavelength(s) (nm)
1	C18 - Short*	0.0	0	100	0.5	216 ; 303
		22.0	0	100	0.5	
2	C18 - Short	0.0	100	0	0.5	216 ; 303
		22.0	100	0	0.5	
3	C18 - Short	0.0	50	50	0.5	303
		45.0	50	50	0.5	
4	C18 - Short ; C18 - long**	0.0	100	0	0.5	303
		2.0	100	0	0.5	
		30.0	0	100	0.5	
		32.0	100	0	0.5	
5	C18 - long	0.0	100	0	0.75	303
		2.0	100	0	0.75	
		30.0	0	100	0.75	
		40.0	100	0	0.75	
6	C18 - long	0.0	100	0	0.5	303
		2.0	100	0	0.5	
		30.0	0	100	0.5	
		38.0	0	100	0.5	
		40.0	100	0	0.5	
7	C18 - long	0.0	70	30	0.75	303
		2.0	70	30	0.75	
		30.0	0	100	0.75	
		30.1	0	100	0.75	
		46.0	0	100	0.75	
		46.1	70	30	0.75	
		50.0	70	30	0.75	
8	C18 - long	0.0	100	0	0.75	303
		2.0	100	0	0.75	
		2.1	80	20	0.75	
		7.1	80	20	0.75	
		7.2	60	40	0.75	
		12.2	60	40	0.75	
		12.3	40	60	0.75	
		17.3	40	60	0.75	
		17.4	20	80	0.75	
		22.4	20	80	0.75	
		22.5	10	90	0.75	
		27.5	10	90	0.75	
		27.6	0	100	0.75	
		44.0	0	100	0.75	
		44.1	100	0	0.75	
		50.0	100	0	0.75	

Method	Column	Time (min)	Solvent (%) A	Solvent (%) B	Flowrate (mL/min)	Wavelength(s) (nm)
			5% MeOH 1% AA	**95% MeOH 1% AA**		
9	C18 - long	0.0	100	0	0.8	303
		2.0	100	0	0.8	
		22.0	20	80	0.8	
		50.0	20	80	0.8	
		50.1	100	0	0.8	
		60.0	100	0	0.8	
10	C18 - long	0.0	100	0	1	303
		2.0	100	0	1	
		30.0	0	100	1	
		38.0	0	100	1	
		40.0	100	0	1	
		45.0	100	0	1	
11	C18 - long	0.0	100	0	1	303
		2.0	100	0	1	
		30.0	0	100	1	
		30.1	0	100	1	
		46.0	0	100	1	
		46.1	100	0	1	
		50.0	100	0	1	
12	C18 - long	0.0	100	0	1	303
		2.0	100	0	1	
		30.0	0	100	1	
		30.1	0	100	0.5	
		46.0	0	100	0.5	
		46.1	100	0	1	
		50.0	100	0	1	
13	C18 - long	0.0	80	20	0.8	303
		1.0	80	20	0.8	
		41.0	0	100	0.8	
		61.0	0	100	0.8	
		61.1	80	20	0.8	
		66.0	80	20	0.8	
			0.1% Formic Acid	**100% Acetonitrile**		
14	C18 - long	0.0	98	2	1	210 ; 254 ; 280 ; 303
		22.0	0	100	1	
		27.0	0	100	1	
		28.0	98	2	1	
		35.0	2	98	1	

* C18 – short = Phenominex luna c18 – 3 μ (2) MeOH – Methanol

** C18 – long = Phenomenex luna c18 – 5 μ (2) AA - Acetic Acid

Isocratic method (Methods 1 – 3 Table 4.3) did not result in compound separation, with the chromatograms showing just a single peak. Gradient elution was used in methods 4 – 14. Increasing the run time and column length whilst making adjustments to the flow rate, as well as the gradient elution profile resulted in noticeable separation of the antimicrobial extract. Further adjustments to the gradient profile, most notably an increase in the hold time, improved peak sharpness and resolution. Of the protocols investigated, the most suitable methods were methods 13 and 14 which were used going forward for HPLC-UV and HPLC-MS, respectively. HPLC-UV and HPLC-MS analyses were done at different facilities hence the change in LC protocols.

Separation of the antimicrobial extract by HPLC-UV produced several peaks (Figure 4.8), showing more efficient separation than TLC-UV (3 spots). Achieving a good baseline and resolution of peaks in HPLC-UV chromatograms was challenging. This was attributed to the crude nature of the extract. Identification of DPO peak was made by comparing the chromatogram of the antimicrobial extract with chromatograms generated by running a) the pure DPO compound and b) antimicrobial extract spiked with the pure DPO. The elution time for DPO was 19.1 minutes and 36.1 minutes for methods 13 and 14, respectively. Quantitative measurements were made by comparing peak areas with peak areas from a standard curve. Results showing production of antimicrobials by SPR^T are shown in the next Chapter (Section 5.6).

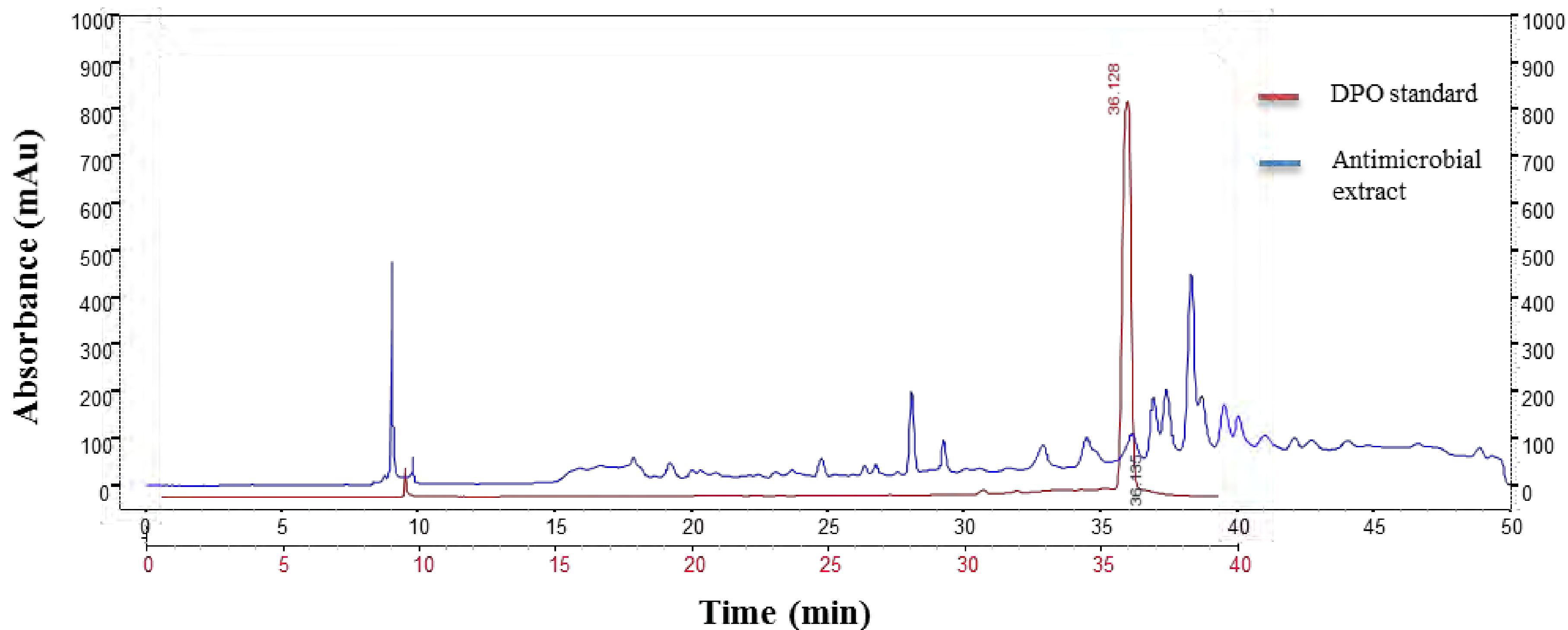

Figure 4.8 Typical HPLC-UV chromatogram obtained after separation of antimicrobial extract using a gradient method (refer to method 13, to **Table 4.3**). Chromatogram of DPO standard (red) shows position of peak corresponding to DPO, t=36.1. Antimicrobial extracts are from 10 day old batch shake flask cultivations of SPR^T grown in Hacène's medium at 28 °C and 170 rpm. The lower limit of quantification was 0.01 g/L for the DPO standard, this limit increased ten-fold in spent culture media..

Chapter 5. Results and Discussion

5.1 Introduction

In order to scale-up a bioprocess successfully, it is important to understand the critical parameters affecting the system to provide the required environment at the larger scale (Katzer *et al*. 2001). The metabolic performance of microorganisms in submerged culture is highly dependent on the culture environment (Rahulan *et al*. 2011).

The only published reports on the growth of *Streptomyces polyantibioticus* (SPR^T) and its antimicrobial production are by Le Roes (2006) and Le Roes-Hill and Meyers (2009). Here the description of the organism, its characterisation and production of bioactive compounds by 10-day old cultures of SPR^T including 2,5-diphenyloxazole (DPO) is provided. Of the bioactive compounds produced by SPR^T, DPO is the only identified compound. It is also produced in the highest quantities amongst the other antimicrobials (Le Roes 2006) and has shown potential for use as a new anti-tuberculosis drug. There is, however, an absence of bioprocess data, including kinetic data and data on response to process variables. In this study the submerged growth of SPR^T, under batch conditions in shake flasks and stirred tank bioreactors, was investigated using the methods outlined in Chapters 3 and 4 to ascertain the time-based profiles of growth and antimicrobial product formation, the response to process variables and the basic kinetic parameters of DPO production. This was investigated to create a basis from which to assess potential for scale-up as an antimicrobial production process.

5.2 Batch cultivation of SPR^T in shake flasks

The SPR^T growth profile was determined in its production medium, Hacène's medium, (HM; 5.0 g/L glucose, 4.0 g/L yeast extract powder, 10.0 g/L malt extract and 1.0 g/L sodium chloride, pH 7.4) (Hacène and Lefebvre 1995; Le Roes 2006) in shake flasks. The cultures were grown at a constant temperature of 28 °C, shaken at 170 rpm using a 10% (v/v) inoculum (Chapter 3.2.2). The full contents of one shake flask was sacrificed daily, per sample point, over a 10-day period. Biomass concentration and pH were measured throughout the experiment. The data are presented in Figures 5.1 and 5.2.

The SPR^T culture did not exhibit a lag phase, with growth phase and maximum biomass concentration achieved in less than 50 hours (Figure 5.1). The highest biomass concentration obtained was 3.30 g/L, while the lowest pH of 5.28 was recorded after 24 hours of cultivation. The cell dry weight (CDW) decreased gradually at a rate of 0.146 g/day for the remainder of the cultivation, whereas pH increased sharply over the next 24 hours to a pH value of 7.94. Complete glucose depletion was observed at 50 hours, but based on the glucose utilisation rate and reaching the maximum biomass concentration around 24 hours, complete glucose depletion is postulated to have occurred between 26-30 hours (after extrapolating consumption rate in Figure 5.1). Biomass production appeared to be limited by both pH and carbon availability.

The biomass data in Figure 5.1 does not provide a clear depiction of the growth phase, as it occurred between two sampling times. This necessitated further experimentation focussing on the growth phase. These growth experiments confirmed that the SPR^T culture grown under these conditions adapted quickly, with a lag phase of not more than 3 hours (Figure 5.2A). A maximum specific growth rate of 0.260 h^{-1} was determined from the logarithmic plot of biomass as a function of time (Figure 5.2B), assuming exponential growth, according to the equation:

$$\frac{dX}{dt} = \mu X \quad \text{(Equation 2)}$$

$$\ln X = \mu t \quad \text{(Equation 3)}$$

The exponential growth phase was supported between 3 and 9 hours, where after μ decreased with decreasing glucose availability.

Reproducibility of biomass measurements was described in Section 4.1. The reproducibility of the biomass growth was evaluated by performing the growth experiment in triplicate under identical conditions. The biomass profiles are displayed in Figure 5.3. The maximum biomass concentrations of 3.30, 3.00 and 3.01 g/L for cultures 1, 2 and 3, respectively, were obtained after 24 hours of cultivation. Again, assuming logarithmic growth (using Equations 2 and 3), maximum specific growth rates of 0.21, 0.26 and 0.20 h^{-1} were determined. The cultures also exhibited the same pH profiles, with a decrease in pH from pH 7.0 to below pH 6.0 coinciding with the formation of biomass (refer to Appendix D for summarised data).

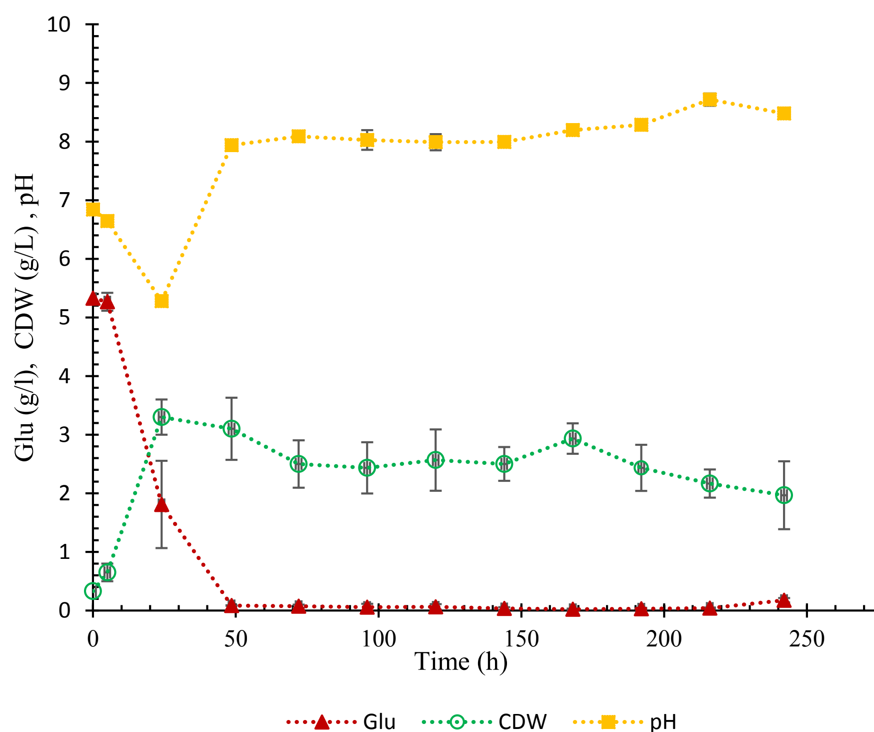

Figure 5.1 Batch cultivation of SPR[T] grown in shake-flasks containing Hacène 's medium, incubated at 28 °C and 170 rpm, displaying biomass production, glucose utilisation and the pH profile. Profiles shown represent average values obtained from triplicate experiments; error bars represent standard error of the mean.

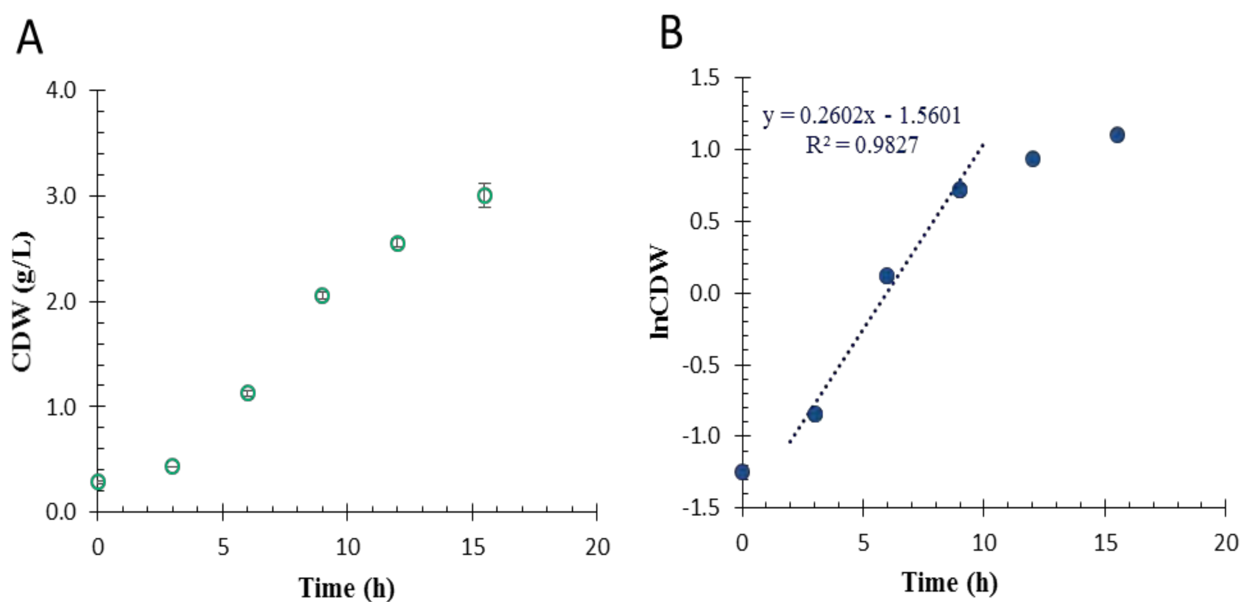

Figure 5.2 Batch cultivation of SPR[T] grown in shake-flasks containing Hacène 's medium, incubated at 28 °C and 170 rpm. Biomass production is displayed as cell dry weight (CDW) (A), with a natural log plot displaying a short lag phase (< 3 h) (B). Profiles shown represent average values obtained from duplicate experiments; error bars were calculated using standard deviation.

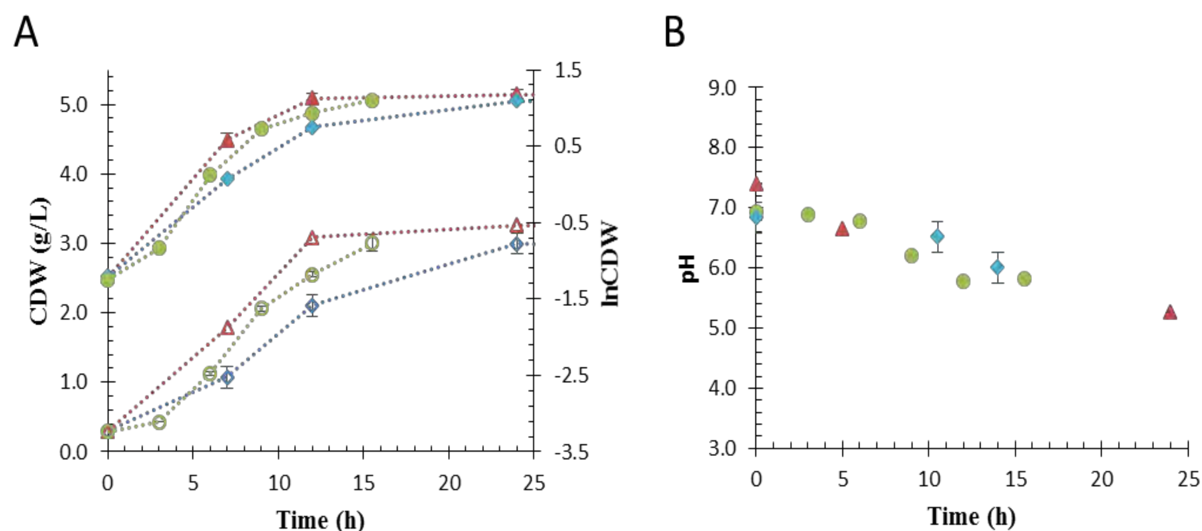

Figure 5.3 Batch cultivation of SPR^T grown in shake flasks showing A) biomass production (open markers = CDW, solid markers = natural log of CDW) and B) pH profile. Cultures were grown in Hacène's medium at 28 °C and 170 rpm. Data points shown represent the average obtained from triplicate experiments (whole flasks sacrificed); error bars represent one standard deviation from the mean (note some symbols are larger than error bars).

5.3 Effect of glucose supply on SPR^T cultures

Hacène's medium is comprised of glucose, malt extract, yeast extract and salt (NaCl) in concentrations of 5, 10, 4 and 1 g/L, respectively. The glucose is the most readily available carbon source for SPR^T to utilise, with more complex carbon available as oligomers of glucose and as peptides and proteins in malt and yeast extract. To test whether glucose availability in the medium was limiting biomass production, cultures grown in Hacène's medium with differing initial glucose concentrations were compared (Figure 5.4). Furthermore the effect of glycerol addition to the medium on biomass production was investigated. Samples were retained for DPO analysis, which is presented in Section 5.5.

Despite differing glucose and glycerol concentrations, each culture exhibited a similar growth profile (Figure 5.4A) and produced similar maximum biomass concentrations of 3.04, 2.90, 3.46 and 3.48 g/L for cultures H5 (5 g/L glucose), H10 (10 g/L glucose), H20 (20 g/L glucose) and HGLY (5 g/L glucose and glycerol) respectively. The amount of glucose consumed over time is shown in Figure 5.4B. Over the first 25 hours, the glucose utilisation rate was 0.25, 0.20, 0.30 and 0.16 $g_{glucose}$/h for H5, H10, H20 and HGLY. The biomass yield in this time was similar for cultures H5, H10 and H20 (0.49, 0.54 and 0.46 $g_{biomass}/g_{glucose}$) but was slightly

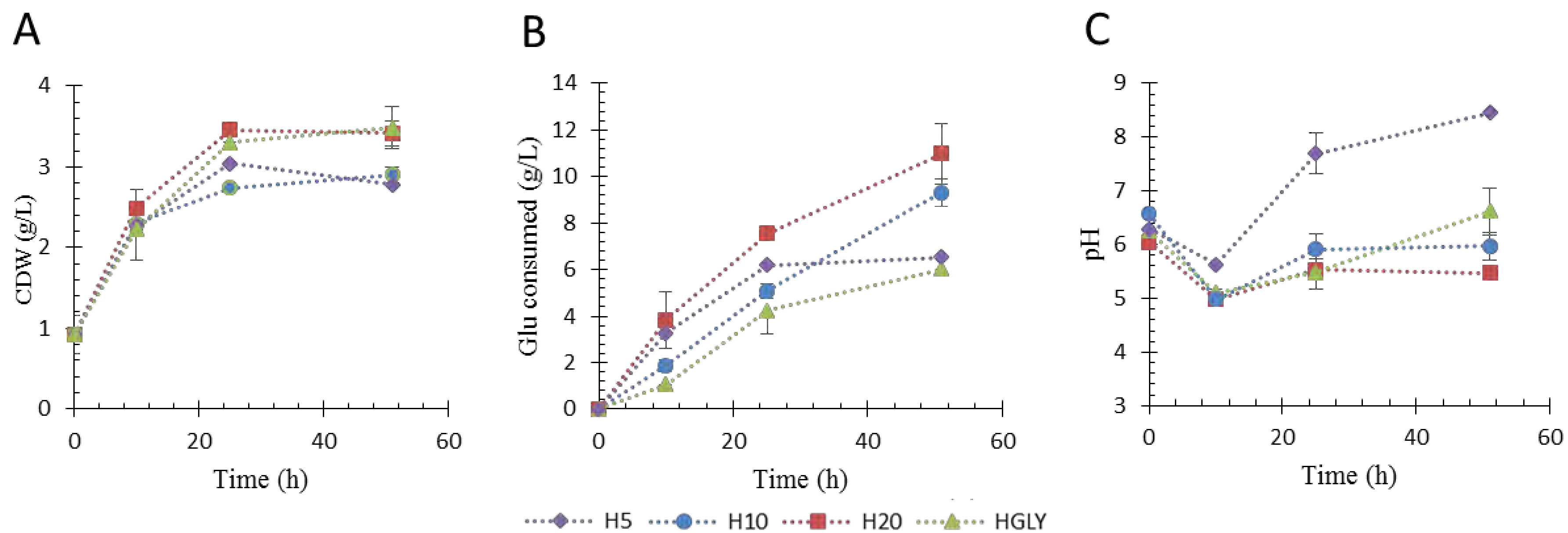

Figure 5.4 Batch cultivations of SPR^T grown in shake flasks containing Hacène's medium with 5 (H5), 10 (H10) and 20 (H20) g/L glucose, or 5 g/L glucose and 5 g/L glycerol (HGLY), incubated at 28 °C and 170 rpm. Biomass production (A), glucose consumption (B) and pH profiles (C) are shown, representing average values obtained from triplicate experiments. Error bars represent standard error of the mean.

elevated for the HGLY culture (0.78 $g_{biomass}/g_{glucose}$). After 51 hours, the glucose in H5 and HGLY cultures was completely exhausted, the H10 culture had 0.69 g/L remaining and the H20 culture still contained just over half (10.99 g/L) the initial starting glucose concentration. Cultures H10 and H20 continued to consume glucose at the same rate, even after having attained maximum biomass concentration. This only occurred at high glucose concentration and may be attributed to the production of an extracellular product such as extracellular polymeric substances (EPS) and volatile fatty acids (VFA's). Accumulation of EPS substances could also explain the increase in pH following the initial pH drop, caused by the metabolism of glucose in the medium. Whilst similar trends in pH were observed with an initial decrease over the first ten hours followed by an increase, the pH of cultures H10, H20 and HGLY only returned to the starting pH after 51 hours. Conversely, cultures grown in regular Hacène's (H5) experienced a sharper pH increase and were two pH units above the starting value after 51 hours (Figure 5.4C).

5.4 Batch cultivation of SPR^T in a lab scale STR

There are many reactor types, both conventional and unconventional in which microbial cultivation can occur. Considering the absence of literature on SPR^T cultivation in vessels other than a shake flask, reactor choice was decided by considering other streptomycete cultures. The two reactor types that are used for the cultivation of streptomycetes are stirred tank bioreactors (STR) and bubble columns. The most common bioreactors for commercial production of commodity products are mechanically agitated stirred tank reactors (Erickson 2011); notably, preferred industrial hosts *Streptomyces coelicolor* and *Streptomyces lividans* are grown in a STR (van Wezel *et al*. 2006). Batch cultivations in this study were thus performed in a lab scale STR's. This vessel was chosen based on the ease of measurements, close control over process parameters and comparatively good mixing leading to increased homogeneity.

Batch experiments with SPR^T in HM were performed in a 7-L New Brunswick bioreactor, described in Section 3.2.3, using a 3-litre working volume with the aim of up-scaling the shake flask process described in Section 5.3. The growth profile and basic kinetic parameters were determined for each run. The initial run is described in Section 5.4.1, where notably severe

foaming and wall growth was encountered. Section 5.4.2 describes the steps taken to overcome the foaming and displays the growth profiles of experiments leading to a foam free run.

5.4.1 Reference case batch STR cultivation: Experiment A

SPR^T inoculum was prepared in the same manner as the SF experiments, with an additional inoculum step included to raise biomass (Figure 5.5) as described in Section 3.2.3. The culture system parameters outlined in Table 5.1 were used. Biomass concentration (CDW), pH and dissolved oxygen concentration (DO) were measured throughout the experiment. Samples were also tested for DPO and other antimicrobials, as shown in Section 5.6. The experiment was terminated after 6 days; the time profiles of the first 25 hours of the culture are shown in Figure 5.6A.

Figure 5.5 Inoculum train used in bioreactor studies.

Table 5.1 System parameters used in initial unstable STR growth experiment

Parameter	***Setting***
Culture volume	3 L (unmaintained)
Agitation	400 rpm (constant)
Aeration, (air)	1 vvm
Temperature	28 °C (constant)
pH	7.4 (uncontrolled)
Medium	Hacène's (5.0 g/L glucose, 4.0 g/L yeast extract powder, 10.0 g/L malt extract and 1.0 g/L sodium chloride, pH 7.4)

The SPR^T culture exhibited no noticeable lag phase with an immediate exponential increase in cell dry weight and reduction in dissolved oxygen. Exponential growth continued for 12.5 hours with a specific growth rate of 0.246 h^{-1}. Growth slowed substantially as the culture depleted the glucose and entered stationary phase. A maximum cell dry weight of 6.24 g/L was obtained after 18 hours. Culture pH remained neutral for the first 8 hours and then began to steadily decline until reaching a minimum at pH 5.28 after 18 hours, after which it rose back to the starting pH. Glucose was largely utilised after 18.5 hours, with a constant residual concentration of <0.5 g/L remaining. Towards the end of the exponential growth period the culture began to foam, severely compromising the experiment. This resulted in clogged filters and excessive wall growth. After 20 hours of cultivation the outlet air filters were replaced, aeration and agitation were reduced to 0.5 vvm and 200 rpm, respectively, for the remainder of the experiment (124 hours), but did not result in the alleviation of foaming. The excessive foaming resulted in difficulties in containment and analysis; hence, early termination of the experiment was necessary.

5.4.2 Foam mitigation in STR: Experiments B – D

Two main strategies were employed to reduce the foaming in subsequent bioreactor cultivations. These were the addition of antifoam and the use of a mechanical foam breaker. Adjustments to the bioreactor set up and operating procedure were also made, as described in Table 5.2, below. Each experiment was performed in duplicate bioreactor cultivations, using Experiment A as the reference cultivation (Section 5.4.1).

The strategy adopted in Experiment B was a reduction in aeration rate from 1 to 0.75 vvm, a change in agitation regime to maintain a DO concentration above 40% and the inclusion of 0.075 % antifoam (Antifoam 204) in the medium. Additionally, water was sprayed directly (from above through a port on head plate) to suppress the formation of the foam head. In Experiment C, physical modifications were made to the bioreactor set up with depth filters fitted to the air outlet (as opposed to the absolute (membrane) filter used previously) and a mechanical foam breaker to the agitation shaft. The culture volume was also maintained by replacing volume lost due to sampling with an equivalent amount of water. In Experiment D, pH control was implemented with culture pH maintained at 6.5-7.6, using 5M HCl and 5M KOH. Time profiles showing the first 25 hours of cultivations are shown in Figure 5.6. The basic kinetic parameters are shown in Table 5.3.

Table 5.2 Experimental strategies implemented to curb excessive foaming

Expt.	*Aeration (vvm)*	*Agitation (rpm)*	*Culture volume (3L)*	*pH control*	*Antifoam in media*	*Additional antifoam (ml)*	*Water spray*	*Mechanical foam breaker*	*Foaming (h)**	*Air outlet Filters*
A	1.00	400 constant	unmaintained	-	0.000%	-	-	-	20	membrane
B	0.75	DO > 40%	unmaintained	-	0.075%	12	✓	-	13	membrane
C	0.75	DO > 40%	constant	-	0.100%	>17	-	✓	18	depth
D	0.75	DO > 40%	constant	✓	0.100%	5.5	-	✓	-	depth

* Time taken for experiment to foam excessively.

Table 5.3 Basic kinetic parameters for batch cultivations of SPR^T in Hacène's medium.

Expt	μ_{max} (h^{-1})		*max CDW* (g/L)		$Y_{x/s,\,max}$ ($g_{biomass}/g_{glucose}$)		*Productivity - 15 h* (g/L/h)		*Productivity - 240 h* (g/L/h)
SF	0.260		3.15		0.59		0.21		0.01
STR-A	0.246		6.24		1.02		0.42		-
STR-B	0.212	± 0.05	3.43	± 0.10	0.65		0.24		-
STR-C	0.250	± 0.02	3.51	± 0.22	0.64	± 0.04	0.23	± 0.02	-
STR-D	0.266		3.53		0.52	± 0.06	0.24		0.02

NB. Errors shown are standard errors of the mean, errors <0.005 not shown

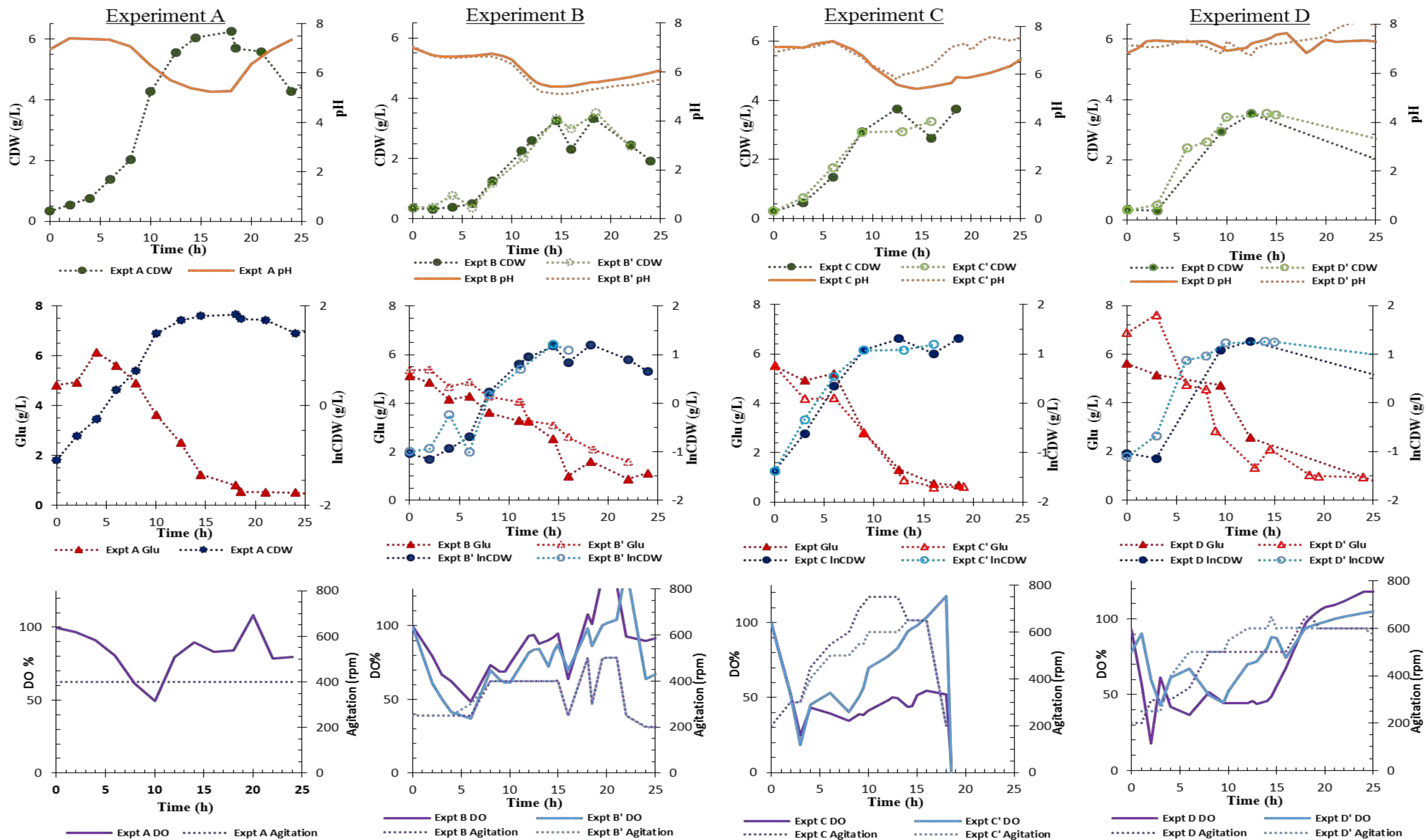

Figure 5.6 Time profiles of batch cultivations of SPR^T grown in a 7 L New Brunswick stir tank bioreactor (3 L working volume) containing Hacène's medium, incubated at 28 °C. The biomass, DO, residual glucose and pH profiles are shown for the first 25 hours of cultivation. Duplicate experiments are shown with open markers and dotted lines. Experiment C was terminated at 18 hours due to uncontrollable foaming and Experiment D was continued for further 10 days (data not shown – refer to Appendix E)

Excessive foaming and wall growth resulting in early termination occurred in Experiment A, B and C. Despite the foam mitigating strategies employed in Experiment B, the onset of excessive foaming occurred after 13 hours; 5 hours earlier than in Experiment A. Increasing the initial antifoam dosage from 0.075% to 0.1% and the mechanical modifications added in Experiment C did not alleviate foaming and excessive foaming occurred after 18 hours.

A stable run was achieved with pH control in Experiment D, with runs lasting the full duration of the 10-day experiment. Maintaining a pH between 6.5 - 7.6 in Experiment D contributed to a stable run by ensuring some of the surface active compounds (likely proteins) remained soluble in the media (Junker 2007). Antifoam addition to the starting medium, as well as drop-wise addition was needed during the experiment. Adjustments to the aeration, pH and agitation regimes reduced the total amount of antifoam needed to achieve a stable run (12ml in experiment B to 5.5 ml in experiment D). Samples were reserved for DPO analysis (Section 5.5).

Stable cultures (as demonstrated in Experiment D, figure 5.6) experienced a lag phase of under 3 hours, biomass increased exponentially with a μmax of 0.27 h^{-1} until 12 hours before the culture entered stationary phase. The biomass concentration peaked at 3.53 g/L after 14 hours, and glucose was largely depleted after 18.5 hours, with a residual glucose concentration of 1.04 g/L. For the first 16 hours, agitation was increased stepwise from 250 to 600 rpm to ensure a dissolved oxygen content of greater than 40%. Agitation was maintained until day 4, primarily to ensure that mild foaming could be controlled by the mechanical foam breaker. Agitation was then stepped down to 300 rpm for the remainder of the experiment (a further 6 days).

5.5 Production of DPO and other bioactive compounds by SPR^T

SPR^T cultures (from plates and shake flasks) were subjected to a series of antimicrobial activity tests to validate its capacity for bioactive compound production, including that of DPO, as first reported by Le Roes (2006). This was investigated by the use of agar overlays and spot test bioautography. Table 5.4 shows the results of these antimicrobial activity tests using *Mycobacterium aurum, Escherichia coli* and *Staphylococcus aureus* as test bacterium. SPR^T showed diverse bioactivity by yielding positive results against all test bacterium, which is consistent with activity reported by Le Roes (2006). SPR^T demonstrated its potential for

producing anti-TB antimicrobials by the positive activity against *M. aurum*, a bacterium with a similar susceptibility profile as *M. tuberculosis*. SPR^T also demonstrated that it could produce antimicrobials under both submerged and solid state growth conditions.

Table 5.4 Bioactivity tests carried out on compounds produced by SPR^T.

Source	Sloppy Agar overlays						Spot test Bioautography		
	M. aurum		*E. coli*		*S. aureus*		*M. aurum*	*E. coli*	*S. aureus*
	day 5	day 10	day 5	day 10	day 5	day 10	day 10	day 10	day 10
Stab innoculted SPR Agar plate	-	+++	-	++	-	++			
Extract of SPR Agar plate culture	-	-	-	-	-	+	+	-	-
Extract of SF - Mycelial Mass	+	++	-	+	-	+	++	+	+
Extract of SF - Liquid filtrate	-	+	-	+	-	+	+	+	-
Extract of SF - Whole culture	+	++	-	+	+	++	+++	++	+

- '+++' = Strongly positive; ' +' = weakly positive; '–' = negative
- All extracts were ethyl acetate based.

Ethyl acetate extracts from 10-day shake flask cultures were separated using thin layer chromatography (TLC). TLC chromatograms were visualised using ultraviolet light (TLC-UV) and bioautography (TLC-B). The use of general visualising agents, (anisaldehyde/H_2SO_4, vanillin/H_2SO_4 and cerium (IV) ammonium sulphate) was deemed unnecessary as described in the method development (Section 4.2.1). These extracts showed 3 distinct groups of spots with retention factors (R_f) of 0.42, 0.81, 0.94 when a mobile phase of ethyl acetate and acetic acid (90:10) was used. Spots had R_f of 0.79, 0.48 and 0.07 for mobile phase with ethyl acetate, hexane and acetic acid (90:9:1), an example of which is shown in Figure 5.7. The polarity of the spots can be induced from the spot R_f, with the more polar molecules (demarcated C, Figure 5.7) having low R_f and the more non polar molecules (demarcated A and B, Figure 5.7) having higher R_f. DPO was shown to be present in the extract by running the pure standard and spiked extract (Lanes 1 and 3, Figure 5.7) under the same conditions, confirming DPO production by SPR^T. It was noted that DPO in the extract had a slightly different R_f than the pure compound, this was likely due to some interaction(s) with other compounds in the extract (Snell *et al.* 1956). The presence of DPO in the samples was further confirmed by mass spectrometry as shown in Figure 5.8. This figure shows an ion extracted chromatogram peak at a mass charge (m/z) ratio of 222.0853 (the molecular weight of DPO) and mass spectral analysis at 19.1 min (the expected DPO elution time).DPO production by SPR^T in shake flask cultures was quantified using HPLC. The time based profiles are shown in Figure 5.9. DPO concentration

increased with increasing biomass concentration, and after 26 hours, 272 ± 40.45 µg per litre of SPR^T culture was achieved. Ethyl acetate extracts from the bioreactor samples exhibited the same TLC-UV spot patterns as extracts from the shake flask and agar cultures, but yielded weakly positive results (extracts needed to be concentrated >100x than those from the shake flasks). A summary of results are shown in Table 5.5. The DPO in the bioreactor extracts was not quantifiable by HPLC despite ‘spot A’ being present on TLC chromatograms.

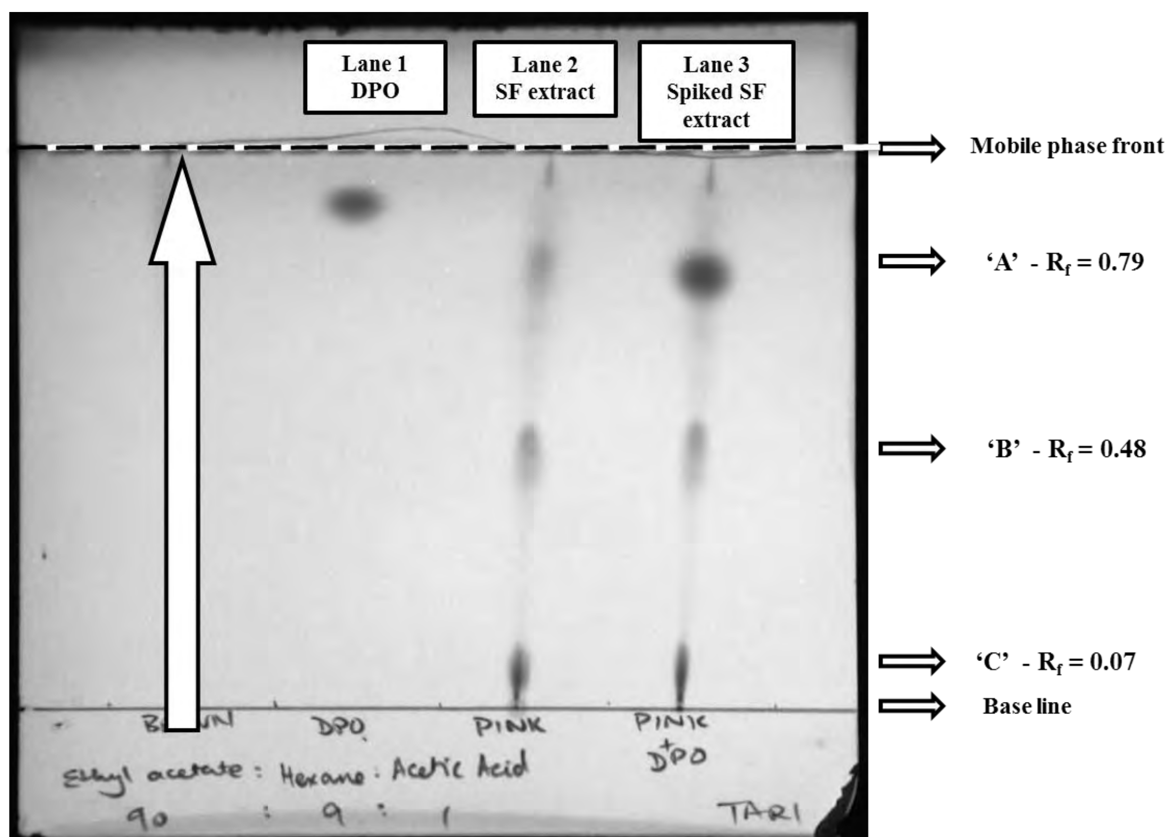

Figure 5.7 Thin layer chromatogram of SPR^T shake flask extracts developed using ethyl acetate, hexane and acetic acid (90:9:1 v/v) as mobile phase. Three lanes are shown, 1) DPO standard, 2) Shake flask extract (t=10d) and 3) Shake flask extract (t=10) spiked with DPO standard.

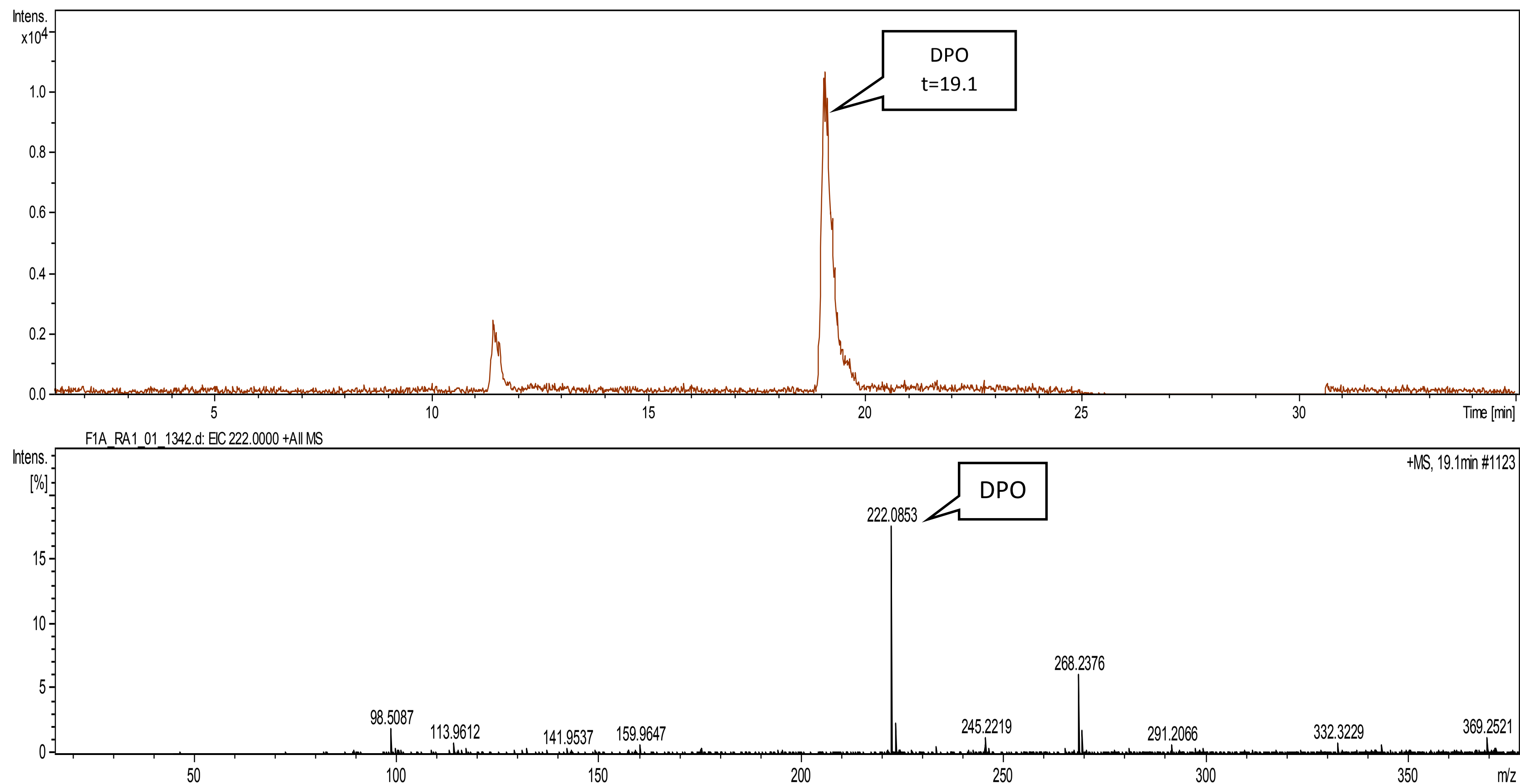

Figure 5.8 Ion extracted chromatogram and mass chromatogram of ethyl acetate extracted shake flask culture of SPR confirming production of DPO (Mr – 221).

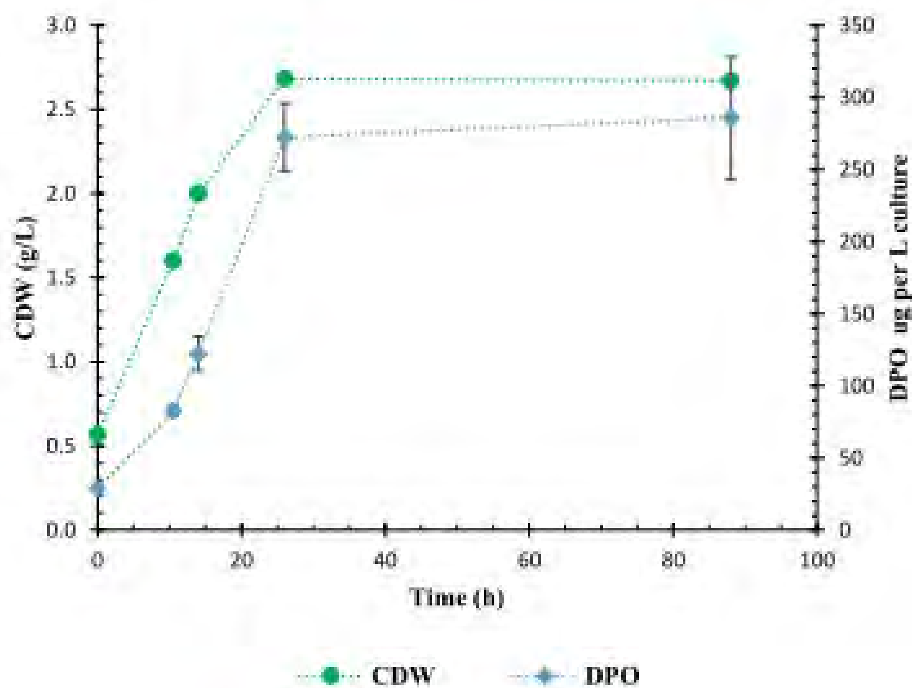

Figure 5.9 Profile of biomass and DPO production in shake flasks of SPR^{T} in HM, at 28 °C and 170 rpm

Table 5.5 TLC bands present in ethyl acetate extracts of SPR^{T} cultures.

		Shake Flask	*STR - Expt A*	*STR - Expt B*	*STR - Expt C*	*STR - Expt D*
'A' Rf = 0.79	day 1	✓	✓	✓	✓	*x*
	day 10	✓	*n/a*	*n/a*	*n/a*	✓
'B' Rf = 0.48	day 1	✓	✓	✓	✓	*x*
	day 10	✓	*n/a*	*n/a*	*n/a*	✓
'C' Rf = 0.07	day 1	✓	✓	✓	✓	*x*
	day 10	✓	*n/a*	*n/a*	*n/a*	✓

✓ = present, *x* = absent, n/a = no test – experiment terminated before day 10

5.5.1 Effect of initial glucose supply on the production of DPO and other bioactive compounds

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The medium was modified to increase glucose concentration and explore the addition of glycerol to look at the effect on DPO production. Shake flask cultures grown in Hacène's medium with differing initial glucose concentrations (from Section 5.3) were extracted with ethyl acetate and compared. with TLC chromatograms of cultures grown in modified media (H10, H20 and HGLY) did not show any spots. TLC chromatograms of cultures grown in conventional Hacène's medium (H5) had the same spot patterns as above (Figure 5.7).

5.5.2 Effect of temperature on the production of DPO and other bioactive compounds

To assess the effect of temperature on the production of DPO and other bioactive compounds, SPR^T was grown in a stirred tank bioreactor to mid exponential growth phase (t = 15 h). This culture was aliquoted and transferred to shake flask cultures aseptically. The cultures were incubated at a range of temperatures (20, 30, 37 and 45 °C). Two flasks were sacrificed at each time point (0, 3, 6 and 10 days following transfer) for biomass and bioactive compound analysis. Figure 5.10 shows the change in biomass over the time (refer to Appendix F for raw data). After 3 days the cultures incubated at 30 and 37 °C had biomass concentrations of 5.37 ± 0.001 g/L and 5.23 ± 0.003 g/L, this was higher than cultures incubated at 20 and 45 °C which had 3.74 ± 0.300 g/L and 3.86 ± 0.000 g/L, respectively.

Ethyl acetate extracts of the aforementioned shake flask cultures contained three groups of bioactive compounds, across all temperatures, when separated by TLC-UV. Figure 5.11 shows the separation of these compounds into 3 bands A, B and C (R_f 0.94, 0.81 and 0.42 respectively). Results were interpreted semi quantitatively by assigning a score to spot densities. Negative results were given a score of 0 and positive scores graded at either 30, 60 or 90. Spot intensity in relation to the temperature at which SPR^T was grown are shown in Figure 5.12 across the time profile of the cultures. Antimicrobials were produced to a maximum between days 0 and 3 following transition in temperature after the 15 h biomass cultivation in STR. Variations in the relative ratios of the antimicrobial metabolites as a function of temperature were observed. Compound A, containing DPO, was favoured at 20 and 30 °C, whereas compound B and C were favoured at 45 and 37 °C, respectively. In all cases, titres remained very low and not quantifiable by HPLC.

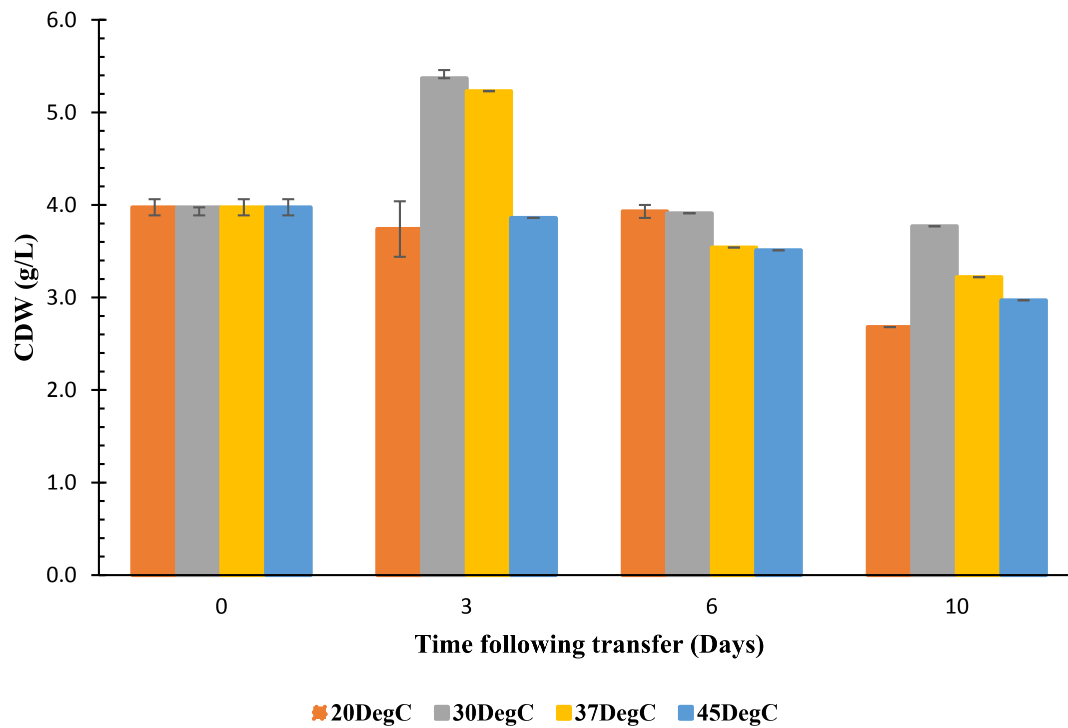

Figure 5.10 Time based profile of SPR^T cultures in shake flask cultivated in Hacène's medium and shaken at 170 rpm, following transfer from exponential phase STR culture. Error bars represent standard error of the mean (n=2).

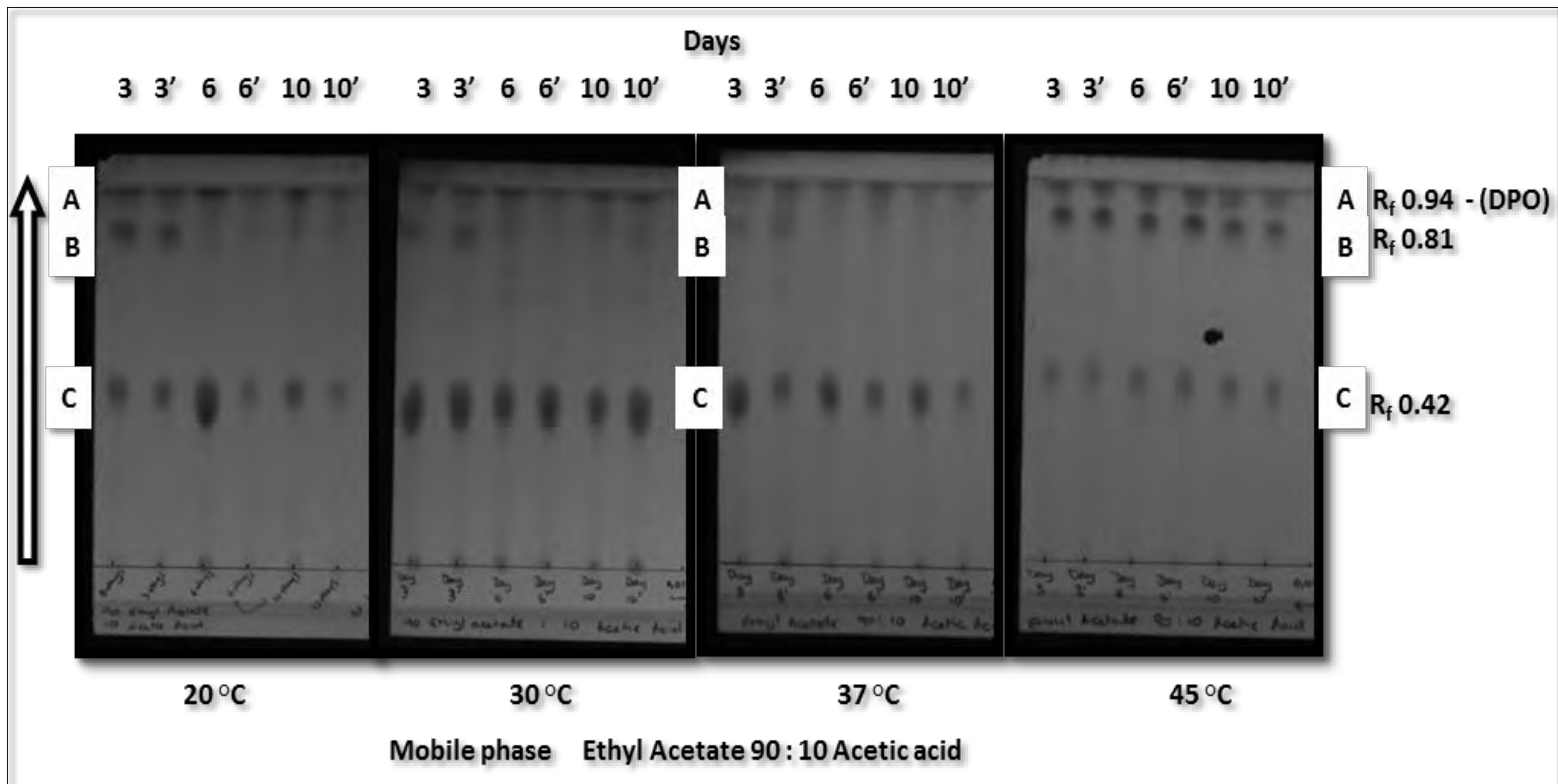

Figure 5.11 TLC of SPR^T shake flask extracts developed using ethyl acetate and acetic acid (90:10 v/v) as mobile phase. Extracts are from cultures grown at 20, 30, 37 and 45°C. Lanes show number of incubation days (3, 6 and 10) and repeats (3’, 6’ and 10’) following transfer from exponential phase STR culture before extraction. DPO is found in band ‘A’ at R_f 0.94.

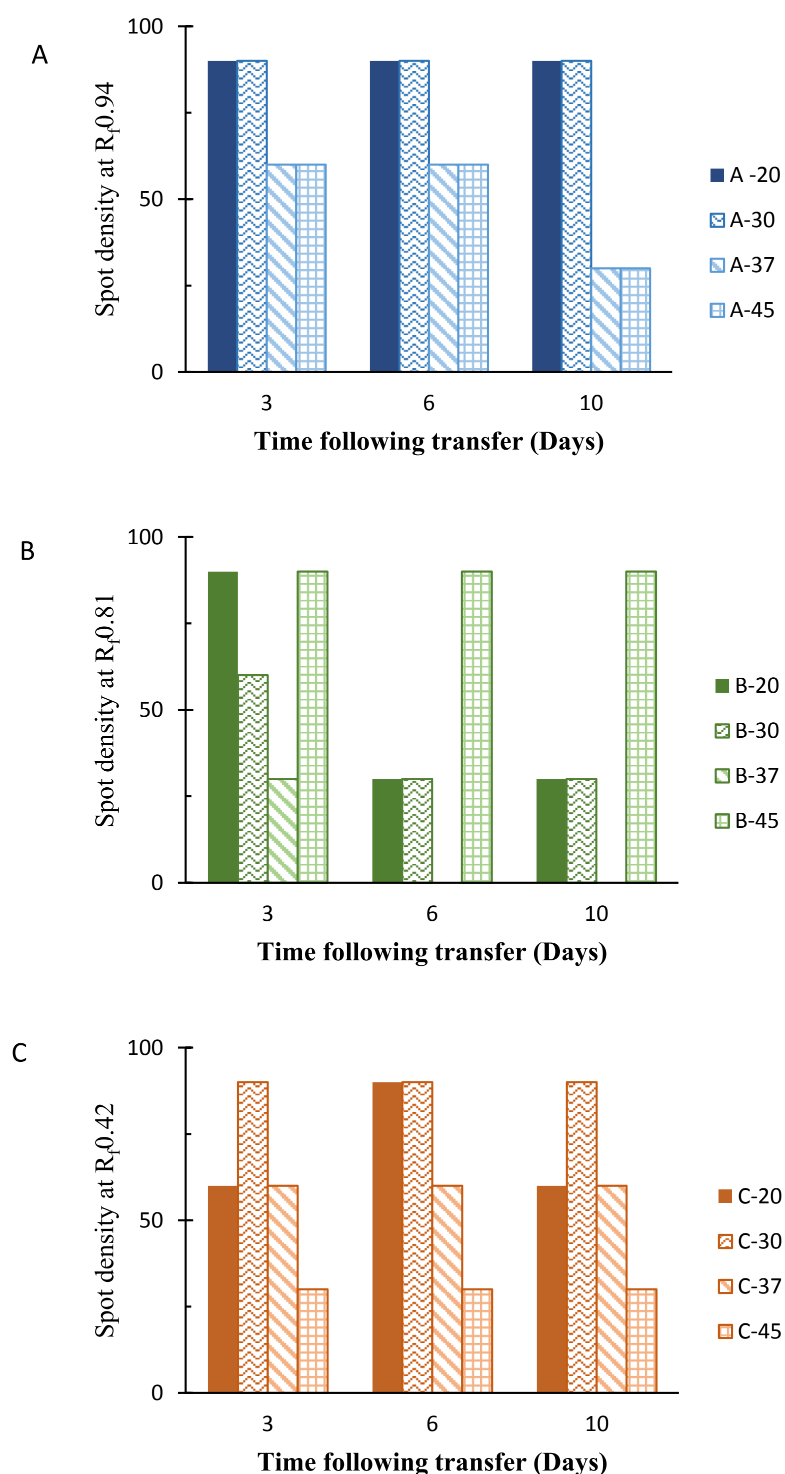

Figure 5.12 Semi-quantitative interpretation of TLC results (Figure 5.11) for the effect of temperature on bioactive products, A (DPO), B and C, produced by shake flask cultures of SPR^T following transfer from exponential phase STR culture. Weakly, moderately and strongly positive results were graded 30, 60 and 90 respectively. Negative results are scored as 0.

5.6 General discussion

5.6.1 SPR^T growth and production of antimicrobials in shake flasks: a reference case

SPR^T is a filamentous microbe which grows by tip extension of its hyphae. This differs from growth of other bacteria and fungi which grow by fission and budding, respectively. When cultivated in liquid culture, filamentous microbes, such as SPR^T, exhibit morphologies ranging from homogenous, dispersed mycelia to discrete compact pellets (Tough and Prosser 1996). This can often lead to difficulties in measurement of biomass concentration and result in variable growth. Shake flask cultures used in the present study consistently grew at a maximum specific rate of 0.260 h^{-1}, achieving maximum biomass concentrations of 3.10 ± 0.098 g/L. This maximum biomass concentration was not limited by glucose availability because it was reached prior to glucose depletion (Figure 5.4). It is recommended that the potential of other limiting nutrients or growth factors be explored.

Antimicrobial activity was confirmed towards *M. aurum, E. coli* and *S. aureus* using bacterial overlay plates. TLC results from shake flasks confirmed the production of DPO (contained in spot A, Figure 5.7) and 2 other un-identified groups of antimicrobials (spots B and C in Figure 5.7). Spots A and B were fairly non polar, based on their proximity to the solvent front (high R_f), a property typical of Type II aromatic polyketide antibiotics. Spot C contained molecules significantly more polar than A and B because they adhered strongly to the TLC plate (low R_f). From this one can infer that Spot C likely contained polar compound(s) such as aldehydes, ketones, carboxylic acids, amines and or alcohols.

DPO production was growth associated, increasing as the biomass concentration increased (Figure 5.9). It was, however, only produced at low levels in this study, with a maximum of 286 μg/L (0.11 $\mu g/mg_{cells}$), which is significantly lower than the 24 400 μg/L previously reported by Le Roes (2006) at 10 days. No improvement in the DPO content, or that of other antimicrobials, was seen by increasing the stationary phase time of the culture. This is unusual considering that antimicrobials are typically reported as secondary metabolites (Bibb 2005; Zhu *et al*. 2014). Growth-associated production of antimicrobials, whilst unusual, is not unprecedented with similar growth associated production of undecylprodigiosin (5-60 $\mu g/mg_{cells}$) by *Streptomyces coelicolor* reported by Hobbs *et al*. (1990). Results from the present study showed growth associated production to lower levels plateauing at day 3.

Similarly, Hobbs *et al* (1990) also showed that this growth association was not constitutive, since no production was observed in altered media. The low titres could be explained by genetic instability (loss in virility), a down regulation of the responsible genes (Hranueli *et al*. 1995; Yagüe *et al*. 2012) or some combination. Domesticated laboratory strains typically experience a reduction in production titres due to a lack of selective pressure. Non defined media, like Hacène's used in the present study, have variable constituents and even small changes to media contents can affect antimicrobial production (Iwai and Omura 1982) Whilst not absolute, correlation has been made between mycelial morphology and secondary metabolite production (Yagüe *et al*. 2012). However, in this case this phenomenon does not account for differences in DPO production observed in this study and those by Le Roes (2006) as morphology type was maintained.

5.6.2 SPR^T growth and production of antimicrobials in a stirred tank bioreactor: shake flask parallels and challenges of scale-up.

Both the shake flask and bioreactor cultures exhibited non-homogenous pelleted morphologies. Biomass concentration profiles as a function of time over a ten day cultivation period showed SPR^T cultures exhibited exponential growth, stationary and death phases. Shake flask (SF) and stirred tank bioreactor (STR) cultures exhibited similar growth profiles and basic growth kinetics. The lag phase lasted not more than 3 hours in both the SF and STRs. During the growth phase, which lasted for 15 and 12 hours, biomass increased exponentially at a rate of 0.260 h^{-1} and 0.266 ± 0.002 h^{-1} for SF and stable STR cultures (Expt D), respectively. The maximum biomass achieved was 3.10 ± 0.098 g/L and 3.53 ± 0.000 g/L.

Whilst similar growth, morphology and yields were achieved in both reference shake flask experiments and stable stirred tank bioreactors, only negligible amounts of DPO and other antimicrobials were produced in the STR experiments. This was true across all bioreactor experiments, regardless of whether excessive foaming was experienced. The differences in the growth environment in the STR as compared to the SF was the likely cause for the low titres of antimicrobial compounds. This also suggested that a trigger is needed for production of antimicrobials (see section 5.6.3). The major differences between shake flasks and stirred tank bioreactors are agitation and aeration, which may have contributed to the lack of antimicrobials being produced. High agitation rates required to mix the culture and mitigate foaming provide

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a high shear environment. Additionally the antifoam included in the bioreactor runs B – D added an additional barrier to mass transfer of nutrients and gases.

Excessive foaming occurred in stirred tank bioreactor experiments A – C (Section 5.4), forcing experimental runs to end prematurely. The foaming resulted in a non-homogenous system. Liquid was trapped in the foam layer whereas the biomass partitioned to the bulk media resulting in a more concentrated microbial suspension in the bulk medium. This was evident in Experiment A where the biomass concentration of the bulk liquid reached 6.24 g/L, which is almost double the biomass yield experienced in all the other shake flask and stirred tank bioreactor experiments (Table 5.3). Furthermore, biomass was deposited on the walls, impellers and probes. The resultant biofilms clogged air filters and interfered with probes, thus increasing the back-pressure. Inoperable conditions were created where sampling became non-representative due to non-uniform biomass distribution. This is evident in the rapid fall and erratic biomass concentrations recorded post commencement of excessive foam (Figure 5.6). Measurements of dissolved oxygen and pH became less reliable due to compromised monitoring equipment (fouling of probes) and liquid containment is an issue due to rising foam and liquid levels. Not only were conditions inoperable, they also created conditions of nutrient limitation for the culture. High biomass in the bulk medium quickly deplete nutrients, whilst biomass present as wall growth has limited access to the nutrients in the liquid medium (Junker 2007). Conditions of nutrient limitation are typically conducive to production of secondary metabolites, including antimicrobials (Coze *et al*. 2013); however, all stirred tank bioreactor experiments showed negligible amounts of DPO and other bioactive metabolites (Section 5.5). TLC-UV results of the undiluted crude extracts tested negative and weakly positive after being concentrated 100-fold.

Since the factors affecting foam formation (discussed in Section 2.7) are many and often complexly interdependent, the foam mitigation strategy employed (Experiments B-D, section 5.4) was multifaceted. Careful control of the pH, aeration rate, agitation rate, antifoam addition and pH control was used to create a stable run (Experiment D). Decreasing the aeration rate decreased the amount of gas bubbles erupting from the liquid surface contributing to foam build up (Van't Riet and Tramper 1991; Vardar-Sukan, 1991).

Agitation control was tricky, with the agitation rate having convoluted consequences. Increasing agitation rate, increased foaming, due to higher gas entrainment. However, high agitation was needed during the growth phase in order to provide sufficient mass transfer and to maintain the DO content above 40%. Using a stepped agitation protocol to maintain the DO concentration ensured the agitation was not higher than needed. Conversely, agitation can be used to mitigate foam. In the present study it was observed that very high agitation rates (>600rpm) decreased foaming, due to mechanical disturbance. Stirring as a form of foam disruption (SAFD) was further aided by inclusion of a mechanical foam breaker to the agitation shaft. Use of high agitation rates was avoided since this creates an environment of high shear, which adversely affects the metabolism of microbes, especially filamentous microbes. High shear stress also promotes lysis which releases cell contents, increasing the foaming potential of the culture, by increasing the concentration of surface active compounds (mostly proteins) in the medium.

5.6.3 Stimulating production of antimicrobials

Single parameter alterations to growth conditions have been shown to elicit responses resulting in increased yields of secondary metabolites, as well as to stimulate production of 'cryptic' metabolites previously not produced (Bode *et al*. 2002; Zhu *et al*. 2014; Bertrand *et al*. 2014; Yoon and Nodwell 2014; Imai *et al*. 2015). In order to explore increasing levels of bioactive metabolites obtained by SPR^T cultures, experiments were undertaken in which the starting glucose concentration in the production medium and the cultivation temperatures were varied. This was to be investigated by either increasing the machinery (biomass) available, increasing the inputs (feeding more substrate) or activating production (stress response) or some combination of the above (Zhu *et al*. 2014; Yoon and Nodwell 2014).

The shake flask growth profiles (Section 5.2) showed a simultaneous increase in biomass concentration and decrease in glucose concentration with time, with cessation of growth coinciding with glucose depletion to less than 1 g/L. Yeast extract and malt extract provide alternative organic carbon sources in the form of larger reducing sugars (mostly maltose) and some proteins (Heron 1966). Malt extract in Hacène's medium (10 g/L) provides an additional ~12 g/L of glucose equivalents (Sigma reports malt extract at ~60% maltose). However, despite most streptomycetes being able to metabolise maltose (van Wezel *et al*. 1997), no further

increase in biomass was observed over 240 h. This suggested that glucose may be growth limiting for SPR^T. To investigate this, the starting glucose concentration was increased 2- and 4- fold with the aim of achieving higher biomass concentrations and higher titres of bioactive metabolites. The results reported in Figure 5.4 showed that, despite increasing the initial glucose concentration in the medium, the maximum biomass concentration obtained was largely unchanged at 3.04, 2.90 ± 0.10 and 3.46 g/L dry mass achieved from 5, 10 and 20 g/L glucose, respectively. Similarly, no further increase in biomass was attained nor diauxic growth observed in cultures grown with 5 g/L glucose and 5 g/L glycerol in the medium for which a maximum biomass concentration of 3.48 ± 0.26 g/L was obtained. For the first 23 h $Y_{x/s, glucose}$ is 0.49 , 0.54 and 0.46 g_x/g_s for H5, H10 and H20 cultures. Between 23 h and 51 h there is no increase in the biomass concentration, yet the utilisation rate of the residual glucose in the cultures H10 and H20 remained unchanged. This indicated that there was a change in metabolism where other additional product(s) were being produced in place of biomass production, this was only observed in cultures with excess glucose concentration.

Furthermore, the culture extracts tested negative for bioactive products in the altered medium despite the additional carbon being available. These findings indicate that, on increasing carbon availability, biomass formation ceased in the presence of residual glucose, i.e carbon was no longer growth limiting. Growth limitation may have resulted from insufficient nitrogen, some trace element or the initial pH decrease experienced by the cultures. The lack of DPO and other bioactive molecules in the cultures provide evidence supporting glucose related secondary metabolite regulation.

To investigate triggering increased bioactive metabolite titres as a stress response, SPR^T was subjected to temperature fluctuations following cultivation (Section 5.6.1). In all cases titres produced remained very low and not quantifiable by HPLC. However TLC results did show that there were slight variations in the relative ratios of the antimicrobial metabolites as a function of temperature. Compound A, containing DPO, was favoured at 20 and 30 °C, whereas compound B and C were favoured at 45 and 37 °C, respectively (Figure 5.14). Antimicrobials were produced to a maximum between days 0 and 3 following transition in temperature after the 15 h biomass cultivation. Regardless of temperature, the bioactive metabolites detected either remained at a similar level or decreased between day 3 and 10. Antimicrobials were produced only in the shake flasks. The highest temperature (45°C) gave the highest total

antimicrobial concentration. This improved performance at higher temperature is consistent with Yoon and Nodwell (2014) who reported an increase in jadomycin B production by *Streptomyces venezuela*, following a temperature shift from 27 to 42°C.

Results from the above mentioned production stimulation experiments conducted in the present study showed that increasing the starting glucose concentrations can globally down-regulate antimicrobial production, possibly through some mechanism of carbon catabolite repression or the Crabtree effect. They also show differential activation of antimicrobial production via some temperature dependent mechanism. Further, the physio-chemical conditions present in the bioreactor environment appeared to inhibit antimicrobial production. These suggest that there are multiple levels of regulation dependent on different external stimuli. To fully understand this, further investigation of the internal metabolic pathways for production of antimicrobials in SPR^T is required.

Chapter 6. Conclusions and Recommendations

6.1 Conclusions

Resistance to antibiotics by microbial pathogens continues to be a major global health problem and the discovery of novel antimicrobial molecules is important for disease control. The actinobacterium, *Streptomyces polyantibioticus* SPR^T (SPR^T) has been shown to produce bioactive molecules active against a range of bacteria. One such antimicrobial is 2,5-diphenyloxazole (DPO), which has anti-tubercular properties (Le Roes 2006). Thus, SPR^T has the potential to assist in abating antibiotic resistance by providing new antimicrobials. SPR^T is the first reported biological source of DPO, providing an alternate to chemical synthesis, which involves multiple-step reaction schemes and harsh reaction conditions (Giddens *et al*. 2005; Wan *et al*. 2010; Xu *et al*. 2013).

This study was done to investigate the growth of SPR^T and the production of DPO and other bioactive molecules to provide biokinetic data. Further, the performance of SPR^T in stirred tank bioreactors was investigated as a first step towards scale-up of production. Characterisation of the growth profile, determination of the kinetic growth parameters of SPR^T and bioactive compound production phases, as well as investigation of factors influencing antimicrobial production were carried out.

This study confirmed the production of multiple antimicrobial metabolites including that of DPO, albeit at a lower level, which is consistent with results by Le Roes (2006). The study provided additional process information including growth kinetics, and the effects of temperature and initial glucose concentration on both biomass and antimicrobial production. To achieve these outcomes, method development was required for reproducible determination of filamentous biomass concentration using gravimetric methods and quantitative determination of the DPO using HPLC. This study also outlined some process challenges associated with cultivation of filamentous bacteria in the stirred tank bioreactor. Limitations in sampling and measurements were addressed.

From the experimental work done in the present study the following conclusions could be made with regards to the dissertation objectives:

Development of experimental procedure(s) that allowed characterisation and analysis of biomass and antimicrobial production profiles of SPR^T.

In order to achieve an acceptable level of reproducibility, optimisation of sampling approaches were required (Section 4.1). The most reliable method of biomass determination was by dry weight gravimetric measurement of the full contents of shake flasks following vacuum filtration. This was due to the filamentous nature of SPR^T biomass in submerged culture and poor pellet formation when subjected to centrifugation. TLC following solvent extraction of SPR^T culture was a useful tool for quick analysis of antimicrobials produced. Good separation of the antimicrobials into 3 spots, with minimal smearing, was achieved with 2 mobile phases: (i) ethyl acetate and acetic acid, 9:10 and (ii) ethyl acetate, hexane and acetic acid, 90:9:1. A reverse-phase HPLC method using 23-95% methanol with 1% acetic acid at 0.8 mL/min on a 40 min linear gradient with a 10 minute hold time was developed, which gave good resolution of DPO and other unidentified antimicrobials.

Determination of the growth profile and maximum specific growth rate of SPR^T under standard conditions and time of onset of antimicrobial production.

Cultivation of SPR^T in Hacène's medium in shake flasks at 28 °C with agitation over 10 days yielded reproducible cell culture. The growth phase occurred over 12-15 hours at a maximum specific growth rate of 0.260 h^{-1}, achieving a maximum biomass concentration of 3.30 ± 0.070 g/L. Under conditions investigated, SPR^T produced 3 sets of antimicrobials, including DPO, displaying activity against both gram negative (*E. coli*) and gram positive (*S. aureus*) bacteria. Antitubercular bioactivity was inferred from antimycobacterial activity displayed against *M. aurum.* The production of DPO and the other antimicrobials is facultatively growth associated. The DPO titre of 286 µg/L was significantly lower than the 24 400 µg/L previously reported by Le Roes (2006).

Aerated stirred tank bioreactor growth of SPR^T posed many challenges, the most prominent challenge being foaming. The addition of antifoam, foam breakers and pH control was necessary to alleviate foaming. Cultivation of SPR^T in Hacène's medium in aerated stirred tank bioreactors at 28 °C with aforementioned foam mitigation considerations over 10 days yielded reproducible cell culture with a maximum specific growth rate (0.266 ± 0.002 h^{-1}), maximum CDW (3.53 g/L) and productivity (0.52 ±0.06 g_x/g_s) comparable to that of shake flask

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cultivations. Production of DPO and other antimicrobials was not quantifiable by HPLC but were more than 100x lower than that produced by the shake flask cultures. The conditions used in the present stirred tank bioreactor experiments were not ideal for bioactive metabolite production by SPR^T.

Investigation of the change in growth and antimicrobial production of SPR^T in response to modified glucose concentration in the medium and variation in temperature

Increasing the initial concentration of the glucose in the medium, did not result in further biomass production, suggesting the presence of a different limiting substrate. A change in culture temperature from 28 °C to either 20, 30, 37 or 45°C did not substantially affect overall production of antimicrobials. The level of each bioactive metabolite was affected differently at each temperature, resulting in different ratios of bioactive metabolites. Increasing the initial concentration of the glucose in the medium resulted in negligible amounts of antimicrobials being produced.

6.2 Recommendations for further work

The work performed in this study has provided information for the growth of SPR^T and its production of antimicrobial molecules. This was achieved by gaining an understanding of the growth patterns of SPR^T at laboratory scale (shake flasks and stirred tank bioreactors). From the findings, the following recommendations were made:

- Studies into the selection and use of different bioreactors for growth of SPR^T should be undertaken to overcome issues of foaming experienced with the STR. Bubble columns and solid state technologies such as a fluidised bed and membrane bioreactors should be investigated. Alternatively, a STR with customised dimensions, impellor type (number and position) could manipulate fluid dynamics to provide sufficient mass transfer and culture homogeneity, whilst providing a low shear environment.

- Analysis of the critical factor impeding antimicrobial production and design of a process decoupling biomass and antimicrobial production phases to circumvent it should be done.

- Extensive media studies should be performed to find suitable alternatives to the current laboratory medium (Hacène's) that would favour antimicrobial production whilst keeping costs low.

- Identification of the antimicrobial compounds and determination of their production triggers will aid research to improve product formation.

- A better understanding of the metabolic pathways for antimicrobial agents and their induction trigger(s) is needed to enable more directed research to improve product formation. It will also allow monitoring of the effect of selective pressure(s) on responsible genes to keep the strain virile.

- Antimicrobial activity and chemical entity tests should be used jointly in such studies, with ongoing assessment of antimicrobial activity spectrum of each compound.

- Bioprospecting for novel compounds in niche environments should continue and contemporary techniques (e.g metagenomics) should be used to exploit the vast potential of antimicrobial compounds that nature has to offer.

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Appendix

A – Example process parameters used in actinomycete cultures.

Process parameters used in actinomycete systems are process specific due to strain sensitivities to prevailing conditions and thus are not universally applicable (Junker *et al*. 2004; Xia *et al*. 2015). However in early phase process design when limited information is known about the system, such as in the current study, a look at other systems can be used as a starting point, thereafter process optimisation can take place. Information on industrial conditions used are mostly proprietary (Glazer and Nikaido 2007); however, shake flask and bioreactor process conditions can be gleaned from academic research studies (Table A1 and A2).

NB. "vvm" = $volume_{air}/volume_{culture}$ liquid", "DO" = dissolved oxygen. "h" = hours, "rpm" = revolutions per minute.

Table A1 Example of process parameters used for metabolite production from actinobacterial cultures: shake flasks.

Actinobacterium	*Product(s)*	*Vessel*	*Vessel Volume (mL)*	*Culture Volume (mL)*	*Temp (°C)*	*pH*	*Duration (h)*	*Agitation (rpm)*	*Reference*
Actinoplanes teichomyceticus	teicoplanin	shake flask	500	50	30	7	48	250	(Jung *et al*. 2008)
		baffled flask	500	100	30	7	36	250	
		baffled flask	500	50	34	7	120	250	
		shake flask	250	25	28	7	144	220	(Jin *et al*. 2002)
Streptomyces felleus YJ1	unnamed	shake flask	250	75	28	7	96	180	(Song *et al*. 2012)
Streptomyces gedanensis	leucine amino peptidase	shake flask	250	100	30	7	120	200	(Rahulan *et al*. 2011)
Streptomyces griseus	grisorixin	shake flask	500	100	27	7	168	100	(Gachon *et al*. 1975)
Streptomyces lydicus	natamycin	shake flask	250	75	30	7	120	200	(Atta and Radwan 2012)
Streptomyces Mobareensis	capsaicin acylase	shake flask	-	-	30	7	144	180	(Crognale *et al*. 2008.)
Streptomyces psammoticus	laccasse	shake flask	-	-	30	7.5	96	150	(Niladevi and Prema 2008)
Streptomyces sindenensis	actinomycin-D	shake flask	1000	200	28	7	168	200	(Praveen *et al*. 2008)
Streptomyces sp.	4- hydroxy nitrobenzene	shake flask	-	-	37.5	7	360	-	(Sathi *et al*. 2001)

Table A2 Example of process parameters used for metabolite production from actinobacterial cultures: bioreactors.

Actinobacterium	*Product(s)*	*Vessel Description*	*Vessel Volume (L)*	*Culture Volume (L)*	*Aeration (vvm)*	*Temp (°C)*	*pH*	*pH control*	*Duration (h)*	*Agitation (rpm)*	*Reference*
Actinoplanes teichomyceticus	teicoplanin	stirred tank bioreactor	-	3	1	30	7		167-181	-	(Heydorn *et al*. 1999)
		jar fermenter	5	3	1	28	7		168	150	(Lee *et al*. 2003)
		fermentor	7000	5	-	28	7		120		(Jin *et al*. 2002)
		stirred tank bioreactor	-	0.5-2	1	30	7	□	35-45	600	(Vara *et al*. 2002)
		fermentor	7	4	1vvm, DO above 20%	34	7		-	500-900	(Jung *et al*. 2008)
Streptomyces atroolivaceus	leinamycin, sanglifehrins	jar fermenter	6	3	1	28	7		-	400	(Kara *et al*. 1989)
		fermentor	2000	1000	-	22	7		72	100	
Streptomyces chrestomyceticus	toyocamycin	fermentor	120	68	-	28, 36	7.5	□	-		(Flickinger *et al*. 1990)
Streptomyces clavuligerus	clavulanic acid	stirred tank bioreactor	42	20-35	0.15-1.5	30	7	□	36	200-900	(Roubos *et al*. 2001)
Streptomyces coelicolor A3(2)	actinorhodin, undecylprodigiosin	fermentor	3	1 or 1.8	0.5	30	7		70	325-1300	(Wentzel *et al*. 2012)
		stirred tank bioreactor	42	20-35	0.15-1.5vvm, 50% DO maintained	30	7	□	85 - 115	500	(van Wezel *et al*. 2006)
Streptomyces fradiae	neomycin	jar fermenter	5	1.8	0.56	30	7		~85	200 - 800	(Ohta *et al*. 1995)
		air lift bioreactor	3	1.8	1.1-1.7	30	7		~85		

Streptomyces fradiae T1558	tylosin	jar fermenter	3000	1500	1	30	7		250	250-900	(Tamura *et al*. 1997)
		air lift bioreactor	-	-	2.5	30	7		250		
Streptomyces gedanensis	leucine amino peptidase	stirred tank bioreactor	0.75	0.5	0.1 -0.5	30	7	□	120	100-500	(Rahulan *et al*. 2011)
			6.6	5	1	30	7	□	120	300	
Streptomyces lividansTK24	human tumour necrosis factor-alpha	stirred tank bioreactor	5	3	0.66	30	7	□	55	400	(D'Huys *et al*. 2011)
		stirred tank bioreactor	6	3.6	1.8	30	7.2		65	400	(Lule *et al*. 2012)
		stirred tank bioreactor	6	3.6	0.56	30	7.2		65	400	
Streptomyces lividans	tyrosinase	stirred tank bioreactor	5	4	-		6.7	□	30	-	(van Wezel *et al*. 2006)
Streptomyces mobareensis	capsaicin acylase	stirred tank bioreactor	-	-	1	30	7		168	450	(Crognale *et al*. n.d.)
		air lift bioreactor	-	-	1	30	7		168	-	
Streptomyces sindenensis	actinomycin-D	stirred tank bioreactor	-	-	1	28	7		-	600	(Praveen *et al*. 2008)
Streptomyces sp.	4- hydroxy nitrobenzene		-	-	-	37.5	7		360	-	(Sathi *et al*. 2001)
Streptomyces toxytrincini	lipstatin	fermentor	1000	200	0.4	28-30	7		96-120	300	(Weibel *et al*. 1987)
Streptomyces tsukubaensis	tacrolimus (fujimycin) FK - 506	fermentor	2000	1 600	1	30	6.8		96	170	(Kino *et al*. 1987)

APPENDIX A – REFERENCES.

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B – Standard curve HPLC glucose measurements.

Biorad Aminex® HPX-87H organic acid column (7.8 mm x 300 mm, Biorad, Cape Town, RSA) was used for the quantification of glucose and organic acids. Isocratic analyses was performed using 0.01M H_2SO_4, as the mobile phase, and an external column oven set to 40 °C. Injection volume used was 10 µl. The flow rate used was 0.5 mL/min.

Glu (g/L)	peak retention time (min)	peak area
0	9.382	546
0	9.381	611
0.1	9.39	172353
0.1	9.388	211618
0.2	9.386	451316
0.2	9.385	492090
0.5	9.383	1048289
0.5	9.382	943361
1	9.385	1984191
1	9.383	1965752
2	9.387	4122677
2	9.385	4189369
4	9.392	10103255
4	9.39	8063567
5	9.387	10062652
5	9.392	10225337
6	9.387	16021532
6	9.39	11945317
8	9.382	15935128
8	9.39	11957819
10	9.388	19965048

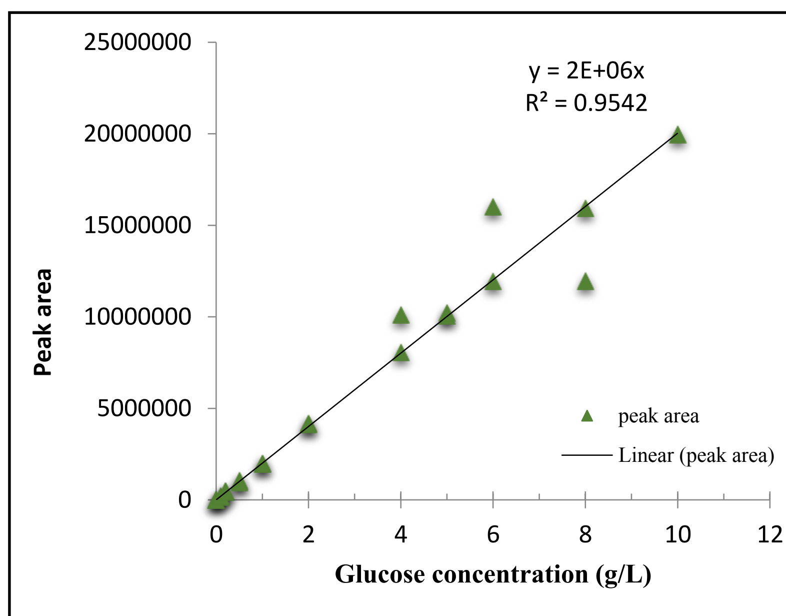

Figure B1 Example standard curve used for glucose measurements. (Fresh standards run alongside each experiment).

C - Raw data for biomass measurements in Chapter 4

Experiment A			
Time (h)	CDW (g/L)	Average (g/L)	SD
5	0.50	0.65	0.15
5	0.80		
24	3.00	3.30	0.30
24	3.60		
48.5	4.10	3.10	0.75
48.5	2.30		
48.5	2.90		
72	1.70	2.50	0.57
72	3.00		
72	2.80		
96	2.10	2.43	0.62
96	1.90		
96	3.30		
120	2.70	2.57	0.74
120	3.40		
120	1.60		
144	3.00	2.50	0.41
144	2.00		
144	2.50		
168	2.90	2.93	0.37
168	2.50		
168	3.40		
192	3.20	2.43	0.56
192	1.90		
192	2.20		
216	2.50	2.17	0.34
216	1.70		
216	2.30		
242	1.90	1.97	0.82
242	1.00		
242	3.00		

Experiment B			
Time (h)	CDW (g/L)	Average (g/L)	SD
0	0.9	0.87	0.05
0	0.8		
0	0.9		
4	1	1.00	0.24
4	0.7		
4	1.3		
24	0.1	0.93	0.59
24	1.4		
24	1.3		
48	1.7	2.07	0.39
48	2.6		
48	1.9		
73	2.1	2.33	0.56
73	1.8		
73	3.1		
97.5	1.7	1.73	0.37
97.5	2.2		
97.5	1.3		

Experiment C				
Time (h)	CDW (g/L)	Average (g/L)	SD	Comparison flask (g/L)
0	0.15	0.15	0.01	0.14
0	0.15			
0	0.14			
0	0.17			
0	0.15			
7	1.86	1.79	0.15	1.07
7	2.00			
7	1.70			
7	1.60			
12	3.30	3.09	0.16	2.11
12	3.18			
12	2.94			
12	2.94			
24	3.26	3.26	0.15	3.00
24	3.06			
24	3.24			
24	3.48			
48	3.38	3.30	0.15	2.80
48	3.48			
48	3.24			
48	3.08			
435	2.84	2.64	0.21	2.09
435	2.66			
435	2.70			
435	2.34			

D - Raw data for SPR^T time based profiles Chapter 5

Summarised data for time profile of batch cultivations of SPR^T grown Shake flasks containing Hacène's medium, incubated at 28 °C, 170 rpm. The biomass, pH and residual glucose profiles are shown. (n=3)

Time (h)	CDW (g/L)			lnCDW			pH			Residual Glucose (g/L)		
		SD	SEM		SD	SEM		SD	SEM		SD	SEM
0	0.330			-1.109	0.366		6.840			5.327		
5	0.650	0.212	0.150	-0.431	0.332	0.235	6.643	0.015	0.009	5.267	0.265	0.153
24	3.300	0.424	0.300	1.194	0.129	0.091	5.277	0.021	0.012	1.810	1.289	0.744
48.5	3.100	0.917	0.529	1.131	0.291	0.168	7.940	0.087	0.050	0.086	0.006	0.003
72	2.500	0.700	0.404	0.916	0.310	0.179	8.090	0.157	0.091	0.073	0.050	0.029
96	2.433	0.757	0.437	0.889	0.294	0.170	8.027	0.292	0.169	0.058	0.100	0.058
120	2.567	0.907	0.524	0.943	0.386	0.223	7.990	0.238	0.137	0.062	0.081	0.047
144	2.500	0.500	0.289	0.916	0.203	0.117	7.993	0.120	0.069	0.034	0.042	0.024
168	2.933	0.451	0.260	1.076	0.154	0.089	8.197	0.076	0.044	0.021	0.018	0.011
192	2.433	0.681	0.393	0.889	0.269	0.155	8.287	0.040	0.023	0.029	0.032	0.018
216	2.167	0.416	0.240	0.773	0.203	0.117	8.717	0.181	0.105	0.042	0.017	0.010
242	1.967	1.002	0.578	0.676	0.552	0.319	8.480	0.042	0.024	0.176	0.065	0.038

E - Raw data for batch Stirred tank reactor runs Chapter 5

Time profile of batch cultivations of SPR^T grown in a 7 L New Brunswick stir tank bioreactor (3 L working volume) containing Hacène's medium, incubated at 28 °C. The biomass, DO, residual glucose and pH profiles are shown.

NB. Agitation, DO, pH and Temperature were continuously measured online, only a selection of data is shown.

Experiment A									
Time (h)	CDW (g/L)	lnCDW (g/L)	Glu (g/L)	Agitation (rpm)		Time (h)	DO (%)	pH	Temp (°C)
0.0	0.33	-1.09	4.82	400		0	99.61	6.95	28
2.0	0.54	-0.62	4.94	400		2	96.82	7.41	28
4.0	0.76	-0.27	6.14	400		4	91.23	7.37	28
6.0	1.37	0.31	5.61	400		6	80.40	7.36	28
8.0	2.03	0.71	4.90	400		8	61.61	7.08	28
10.0	4.27	1.45	3.64	400		10	49.56	6.32	28
12.5	5.55	1.71	2.51	400		12	79.50	5.73	28
14.5	6.04	1.80	1.22	400		14	89.56	5.40	28
18.0	6.24	1.83	0.80	400		16	82.95	5.25	28
18.5	5.69	1.74	0.54	400		18	84.11	5.28	28
21.0	5.58	1.72	0.53	400		20	108.41	6.36	28
24.0	4.28	1.45	0.50	400		22	78.76	6.94	28
26.0	4.52	1.51	0.42	400		24	79.60	7.36	28

Experiment B					Experiment B'		
Time (h)	CDW (g/L)	lnCDW (g/L)	Glu (g/L)		CDW (g/L)	lnCDW (g/L)	Glu (g/L)
0	0.355	-1.037	5.143		0.371	-0.990	5.369
2	0.317	-1.149	4.855		0.393	-0.934	5.389
4	0.395	-0.929	4.149		0.787	-0.239	4.686
6	0.500	-0.693	4.298		0.366	-1.006	4.881
8	1.253	0.225	3.609		1.180	0.166	4.276
11	2.250	0.811	3.283		2.024	0.705	4.067
12	2.590	0.952	3.253		-	-	3.278
14.5	3.252	1.179	2.529		3.330	1.203	3.122
16	2.314	0.839	1.009		2.985	1.094	2.619
18.25	3.333	1.204	1.612		3.520	1.258	2.101
22	2.453	0.897	0.885		2.400	0.875	1.600
24	1.931	0.658	1.118		1.645	0.498	1.143

Experiment B						Experiment B'			
Time (h)	Agitation (rpm)	DO (%)	pH	Temp (°C)		Agitation (rpm)	DO (%)	pH	Temp (°C)
0.0	250.12	99.69	6.99	28.05		249.70	99.29	7.04	28.01
2.0	250.23	79.30	6.67	28.00		250.06	60.88	6.65	28.00
4.0	249.62	62.53	6.63	28.00		249.68	41.57	6.57	28.00
6.0	250.13	48.47	6.65	28.00		299.67	37.11	6.61	28.00
8.0	400.12	73.27	6.73	28.01		400.05	70.07	6.63	28.00
10.0	399.89	74.16	6.52	28.00		400.03	61.55	6.33	28.00
12.0	399.45	93.39	5.73	28.01		399.94	81.80	5.52	28.01
14.0	400.07	90.19	5.40	28.00		399.58	72.52	5.12	28.00
16.0	249.97	63.96	5.44	27.99		249.95	70.07	5.13	27.99
18.0	499.99	107.64	5.57	28.00		500.06	98.13	5.28	28.00
20.0	500.53	132.40	5.65	27.99		500.76	101.51	5.39	28.00
22.0	250.11	92.97	5.79	27.99		250.05	139.47	5.47	27.98
24.0	200.48	89.92	5.97	28.00		198.80	64.14	5.60	28.00
26.0	200.20	93.05	6.17	28.00		199.59	70.52	5.78	28.00
32.5	199.94	93.74	6.86	27.99		199.46	93.84	6.49	27.99
72.5	199.75	116.57	8.03	28.00		199.89	117.84	7.56	28.00
92.0	200.70	117.53	7.95	28.00		200.28	118.82	7.51	28.00
116.5	200.09	116.87	7.85	28.00		200.11	119.44	7.45	28.00
121.0	200.38	117.30	7.83	28.00		199.56	118.59	7.43	28.00
144.0	199.82	116.87	7.74	28.00		200.49	119.53	7.37	28.00
168.0	200.41	117.53	7.74	28.00		200.56	120.60	7.31	27.99
192.0	200.11	117.53	7.85	28.00		199.93	121.18	7.23	27.99

Experiment C					Experiment C'			
Time (h)	CDW (g/L)	lnCDW (g/L)	Glu (g/L)		Time (h)	CDW (g/L)	lnCDW (g/L)	Glu (g/L)
0.0	0.25	-1.37	5.52		0.00	0.25	-1.37	5.52
3.0	0.54	-0.62	4.93		3.00	0.72	-0.33	4.18
6.0	1.41	0.35	5.21		6.00	1.73	0.55	4.22
9.0	2.94	1.08	2.80		9.00	2.92	1.07	2.82
12.5	3.71	1.31	1.32		13.00	2.95	1.08	0.90
16.0	2.73	1.00	0.75		16.00	3.29	1.19	0.61
18.5	3.71	1.31	0.68		19.00	-	-	0.62

Experiment C						Experiment C'			
Time (h)	Agitation (rpm)	DO (%)	pH	Temp (°C)		Agitation (rpm)	DO (%)	pH	Temp (°C)
0	200.49	100.10	7.14	27.82		199.37	99.83	6.92	27.85
2	301.67	51.71	7.14	27.99		299.96	51.11	7.08	27.98
3	300.21	24.98	7.11	28.00		300.04	18.44	7.14	28.06
4	450.48	43.48	7.26	28.01		402.06	45.33	7.14	28.00
6	546.91	39.42	7.38	28.00		498.95	53.39	7.40	27.95
8	600.36	34.50	7.04	28.00		500.15	40.65	6.90	27.92
9	699.89	39.17	6.78	28.01		549.99	50.86	6.71	27.98
9.5	720.25	38.28	6.55	28.02		549.75	56.78	6.44	28.03
10	749.82	41.66	6.31	28.00		600.04	69.81	6.39	27.91
12	750.16	48.36	5.73	28.00		599.89	77.83	5.94	27.99
12.5	749.71	50.07	5.57	28.00		600.17	80.06	5.81	27.99
13	749.90	49.61	5.51	27.99		599.62	82.85	5.97	28.08
14	650.12	43.94	5.42	27.99		650.28	94.51	6.07	28.02
14.5	650.37	44.58	5.41	28.00		649.71	96.36	6.13	28.02
15	649.79	51.46	5.43	28.01		650.24	98.33	6.21	27.99
16	650.05	54.81	5.50	28.01		649.91	103.73	6.38	28.00
18	199.98	52.39	5.64	27.99		199.66	117.83	7.13	27.99
18.5	0.00	5.17	5.90	26.16		0.00	-0.56	7.21	27.90

Experiment D								
Time (h)	CDW (g/L)	lnCDW (g/L)	Glu (g/L)	Time (h)	Agitation (rpm)	DO (%)	pH	Temp (°C)
0.0	0.35	-1.04	5.62	0.0	200.00	92.53	6.81	28.05
3.0	0.32	-1.15	5.14	3.0	299.36	61.25	7.33	28.00
9.5	2.94	1.08	4.73	6.0	350.07	36.44	7.29	28.01
12.5	3.53	1.26	2.57	9.0	499.46	47.42	7.13	28.00
26.0	-	-	0.67	12.0	500.09	44.57	7.04	28.00
31.5	1.25	0.23	-	15.0	499.94	55.59	7.58	27.99
192.0	1.28	0.25	-	18.0	649.92	96.94	6.83	28.00
				21.0	599.55	108.99	7.27	27.99
				24.0	599.87	118.07	7.35	28.00
				32.5	499.83	119.72	7.23	28.00
				72.5	499.96	104.12	7.58	28.00
				92.0	500.10	104.00	6.65	28.00
				116.5	499.98	112.50	6.54	28.00
				121.0	500.32	113.66	6.52	28.00
				144.0	499.97	111.63	6.58	28.00
				168.0	500.18	116.30	6.96	28.01
				192.0	499.67	114.98	6.96	28.01

Experiment D'						
Time (h)	CDW (g/L)	Glu (g/L)	Agitation (rpm)	DO (%)	pH	Temp (°C)
0	0.33	6.88	600.15	78.35	7.14	24.34
3	0.51	7.62	249.94	42.92	7.06	27.91
6	2.4	4.74	500.83	66.86	7.33	28
8	2.6	4.56	499.91	49.88	7.08	28
9	3.41	2.84	499.88	46.3	6.9	28
13	3.53	1.34	600.02	72.06	7.03	28
15	3.5	2.1	600.18	87.53	7.19	27.99
18.5	-	1.04	600.71	95.11	7.38	28
19.5	-	0.98	600.35	97.23	7.47	28
26	2.64	0.93	550.16	105.61	7.64	27.99
72.5	-	0.98	599.85	115.55	8.54	28
92	2.47	0.99	599.86	113.24	8.56	28
116.5	1.74	1.02	300.11	107.62	8.76	28
121	1.22	0.98	300.22	109	8.83	27.99
168	1.23	1.06	299.86	110.89	8.85	28
192	0.04	1.01	299.87	107.54	8.7	28
202	1.3	1.11	297.83	108.54	8.63	28
242	0.97	0.84	300.31	106.82	7.35	28

F – Temperature study raw data Chapter 5

Time profile of batch cultivations of SPR^T grown in a 7 L New Brunswick stir tank bioreactor (3 L working volume) containing Hacène's medium, prior to transfer to shake flasks at different temperatures. The summary data for biomass, DO, residual glucose and pH profiles are shown for reactor data.

NB. Agitation, DO, pH and Temperature were continuously measured online, only a selection of data is shown.

Reactor data:

Time (h)	CDW (g/L)	lnCDW (g/L)	Glu (g/L)	Agitation (rpm)	DO (%)	pH	Temp (°C)
0.0	0.37	-0.99	5.62	199.90	96.94	7.08	27.80
2.0	-	-	-	299.68	25.77	6.91	27.99
3.0	0.39	-0.93	4.48	299.85	50.41	6.97	28.00
4.0	-	-	-	349.92	43.66	6.97	28.00
6.0	-	-	-	399.96	35.98	6.99	28.03
8.0	-	-	-	550.04	58.81	6.37	28.03
9.0	-	-	-	550.54	52.33	5.93	28.05
9.5	2.77	1.02	3.24	550.62	52.67	5.64	28.01
10.0	-	-	-	550.04	53.52	5.38	28.02
12.0	-	-	-	549.96	53.93	4.93	28.01
12.5	-	-	-	550.01	55.76	4.93	28.00
13.0	3.67	1.30	1.45	550.26	57.18	4.95	27.97
15.0	3.97	1.38	-	549.84	61.11	5.10	28.00

Shake Flask Data following transfer from STR, (n=2):

	CDW (g/L)							
Time (d)	20 °C		30 °C		37 °C		45 °C	
		SEM		SEM		SEM		SEM
0	3.97	0.087	3.97	0.087	3.97	0.087	3.97	0.087
3	3.74	0.300	5.37	0.001	5.23	0.003	3.86	0.000
6	3.93	0.070	3.91	0.000	3.54	0.002	3.51	0.001
10	2.68	0.000	3.77	0.002	3.22	0.001	2.97	0.000

G – Assessment of Ethics in Research Projects form

EBE Faculty: Assessment of Ethics in Research Projects (Rev2)

Any person planning to undertake research in the Faculty of Engineering and the Built Environment at the University of Cape Town is required to complete this form before collecting or analysing data. When completed it should be submitted to the supervisor (where applicable) and from there to the Head of Department. If any of the questions below have been answered YES, and the applicant is NOT a fourth year student, the Head should forward this form for approval by the Faculty EIR committee: submit to Ms Zulpha Geyer (Zulpha.Geyer@uct.ac.za; Chem Eng Building, Ph 021 650 4791).
NB: A copy of this signed form must be included with the thesis/dissertation/report when it is submitted for examination

This form must only be completed once the most recent revision EBE EiR Handbook has been read.

Name of Principal Researcher/Student: **Tarisayi Matongo** Department: **Chemical Engineering**

Preferred email address of the applicant:

If a Student: Degree : EMO24CHE01 MSc in Engineering specialising in Bioprocess Engineering
Supervisor: Prof. STL Harrison

If a Research Contract indicate source of funding/sponsorship:

Research Project Title: ~~INVESTIGATING ANTIBIOTICS OF *S. polyantibioticus* & *S. speibonae* AND THEIR PRODUCTION IN LABSCALE BIOREACTORS~~
Investigation of growth and antimicrobial production by Streptomyces polyantibioticus : from shake flask to stirred tank bioreactor

Overview of ethics issues in your research project:

Question 1: Is there a possibility that your research could cause harm to a third party (i.e. a person not involved in your project)?	YES	(NO)
Question 2: Is your research making use of human subjects as sources of data? If your answer is YES, please complete Addendum 2.	YES	(NO)
Question 3: Does your research involve the participation of or provision of services to communities? If your answer is YES, please complete Addendum 3.	YES	(NO)
Question 4: If your research is sponsored, is there any potential for conflicts of interest? If your answer is YES, please complete Addendum 4.	YES	(NO)

If you have answered YES to any of the above questions, please append a copy of your research proposal, as well as any interview schedules or questionnaires (Addendum 1) and please complete further addenda as appropriate. Ensure that you refer to the EiR Handbook to assist you in completing the documentation requirements for this form.

I hereby undertake to carry out my research in such a way that

- there is no apparent legal objection to the nature or the method of research; and
- the research will not compromise staff or students or the other responsibilities of the University;
- the stated objective will be achieved, and the findings will have a high degree of validity;
- limitations and alternative interpretations will be considered;
- the findings could be subject to peer review and publicly available; and
- I will comply with the conventions of copyright and avoid any practice that would constitute plagiarism.

Signed by:

	Full name and signature	Date
Principal Researcher/Student:	Tarisayi Martin Matongo Signed	29.01.2013
This application is approved by:		
Supervisor (if applicable):	Prof. STL Harrison Signed	1/2/2013
HOD (or delegated nominee): *Final authority for all assessments with NO to all questions and for all undergraduate research.*	Signed	12/2/2016
Chair : Faculty EIR Committee		