

# Bacterial Production of Antimicrobial Biosurfactants

*by*

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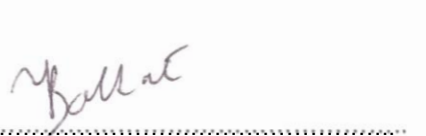
STELLENBOSCH


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# Declaration

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I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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# Abstract

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Surfactants are compounds that reduce interfacial surface tension, resulting in detergency, emulsifying, foaming and dispersing properties. Surfactants produced via biochemical processes (biosurfactants) form a niche market with their low toxicity, biodegradability and high specificity attributes. Biosurfactants have recently received considerable attention owing to their potential as biomedical molecules. In this study a knowledge base was established for the development of a process which produces biosurfactants for use as antimicrobial agents. Specifically, rhamnolipid biosurfactants were produced from *Pseudomonas aeruginosa* and tested for antimicrobial activity against target organisms.

Accurate and reproducible analyses for the quantification of rhamnolipids and antimicrobial activity were developed. The amount of rhamnolipid was determined indirectly by measuring the rhamnose concentration. A novel HPLC method as well as an orcinol colorimetric method were developed for rhamnose measurement. In order to obtain accuracy with the orcinol method it was found that samples must be extracted at least three times prior to the analysis. An examination of literature on rhamnolipid production showed that many studies used colorimetric methods without extraction. Antibacterial activity was quantified by zone clearing around wells of supernatant in soft agar containing the target organism *Mycobacterium aurum*. This target organism is especially important in a South African context, since it is used to indicate possible susceptibility of tuberculosis to antibiotics. This method was developed for antibacterial testing, after a standard disk diffusion method proved to be ineffective. Antifungal activity of rhamnolipids was evaluated against the fungus *Botrytis cinerea*, by growing a lawn of fungus on a plate and adding rhamnolipid.

The factors influencing rhamnolipid production were studied by growing different *Pseudomonas aeruginosa* strains from the ATCC culture collection, namely ATCC 9027 and ATCC 27853 as well as a locally isolated strain under different media conditions. The initial focus was on production of biosurfactants in media containing glucose as substrate. Alkanes were subsequently investigated as an alternative substrate, since they are readily available in South Africa as byproducts from the petrochemical industry. The rhamnolipids produced from the culture collection strains were evaluated for their antibacterial activity against *Mycobacterium aurum*.

A number of key factors were identified which were important for the development of a rhamnolipid production process. Of critical importance were the media conditions. Good production was achieved on glucose media containing a phosphate limitation, pH buffering around neutral pH and a high carbon concentration (2 % carbon). When *Pseudomonas aeruginosa* ATCC 9027 was cultured on this medium (a minimal salts phosphate limited medium with a Tris buffer), it produced 1.31 g/l rhamnose, equivalent to

4.0 g/l rhamnolipid. This rhamnolipid concentration is 2.7-fold higher than that of 1.47 g/l reported in the literature with the same strain (cultured on a different phosphate limited medium)

The particular strain also proved to be a factor which influenced the yield of rhamnolipids. A rhamnose concentration of 0.43 g/l was obtained with *Pseudomonas aeruginosa* ATCC 27853 grown on MSM+Tris medium, compared to 1.31 g/l produced by *Pseudomonas aeruginosa* ATCC 9027 on the same medium.

The most promising strain and medium, *Pseudomonas aeruginosa* ATCC 9027 and MSM+Tris medium, were evaluated under controlled conditions in an instrumented bioreactor. Nearly double the rate of growth and production were obtained in the bioreactor, indicating that production time can be shortened considerably under controlled conditions. However, when compared to shake flask studies, only a 4 % increase in growth and a 5 % increase in rhamnolipid production were achieved in the bioreactor, indicating that the yield was limited by the media components or process conditions.

With media containing hexadecane as sole carbon source, negligible rhamnolipid production was achieved. Slow growth was observed and the stationary phase had not been reached even after 2 weeks of growth. It was shown that in glucose media rhamnolipid production only commenced in the stationary phase. Since the stationary phase was not reached during growth on hexadecane, rhamnolipids, which are known to increase the availability of alkanes through emulsification and solubilisation, could not be produced. A strategy was devised to accelerate growth on alkane media. A dual substrate medium containing both glucose and hexadecane was investigated. It was hypothesised that growth would be promoted by glucose leading to rhamnolipid production, which would then increase the uptake of hexadecane. Rhamnolipid was produced in the dual substrate experiments, but the hexadecane uptake was still poor. This was suggested to be due to the exposure of the cells to glucose in the inoculum or test flask, which hampered the ability of the cells to utilise hexadecane. It was reasoned that the ability to utilise hexadecane was determined by the cell hydrophobicity, which was influenced by the exposure to hydrophilic or hydrophobic substrates.

Rhamnolipids from *Pseudomonas aeruginosa* ATCC 9027 and ATCC 27853 were shown to have antibacterial activity against *Mycobacterium aurum*. The largest zone of clearing of 45 mm was obtained with 4 g/l rhamnolipid from *Pseudomonas aeruginosa* ATCC 9027. The activity was shown to be directly related to the rhamnolipid concentration, highlighting the importance of maximising the biosurfactant yield when developing a process for the production of rhamnolipids as antimicrobial agents. Antifungal activity tests against *Botrytis cinerea* were inconclusive. Future studies should expand the antimicrobial application of rhamnolipids by testing their activity against a larger range of target organisms.

In order to maximise the rhamnolipid yield in future studies, a fed batch process is proposed which would increase the cell density thereby increasing rhamnolipid production and prolonging the stationary phase, which was found to be the phase associated with rhamnolipid production. Different feeding strategies should be investigated, depending on the kinetics of substrate consumption. It is desirable to feed the smallest volume of substrate that is necessary with a high concentration in order to keep the dilution rate low and maximise the product concentration. A factorial design is recommended for this purpose.

Further studies with alkanes as carbon source should be conducted using strains that have been maintained and cultured on media containing alkanes as sole carbon source. Alternative biosurfactant producing strains should also be investigated, which have higher natural cell hydrophobicities.

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# Uittreksel

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Surfaktante is verbindings wat oppervlakspanning aan die skeidingsvlak verminder, met gevolglike emulgerende, skuimende en verspreidende eienskappe. Surfaktante wat via biochemiese prosesse (biosurfaktante) geproduseer word, vorm 'n nismark vanweë hul lae toksisiteit, biodegradeerbaarheid en hoogs spesifieke eienskappe. Biosurfaktante kry deesdae heelwat aandag vanweë hul potensiaal as biomediese molekules. Hierdie navorsingstudie stel 'n basis van kennis daar vir die ontwikkeling van 'n proses om biosurfaktante as antimikrobiese middels te vervaardig. In besonder, ramnolipied-biosurfaktante is geproduseer uit *Pseudomonas aeruginosa* en getoets vir antimikrobiese aktiwiteit op teiken-organismes.

Akkurate en reproduseerbare analitiese metodes vir die kwantifisering van ramnolipiede en antimikrobiese aktiwiteit is ontwikkel. Die hoeveelheid ramnolipied is indirek bepaal deur bepaling van die ramnose konsentrasie. 'n Nuwe hoëdruk vloeistofchromatografie (HDVC) metode, asook 'n orsinol kolorimetriese metode, is vir die bepaling van ramnose ontwikkel. Daar is vasgestel dat akkuraatheid met laasgenoemde metode slegs verkry kon word deur die monster ten minste drie keer voor analise te ekstraheer. Raadpleging van literatuur oor ramnolipied produksie het getoon dat daar by heelwat navorsingstudies kolorimetriese metodes sonder ekstraksie gebruik is. Antibakteriële aktiwiteit is gekwantifiseer deur meting van die inhibisiesones om die holtes gevul met supernatant, in sagte agar bevattende die teikenorganisme, *Mycobacterium aurum*. Hierdie teikenorganisme is veral belangrik in 'n Suid-Afrikaanse konteks, aangesien dit gebruik word om moontlike vatbaarheid van tuberkulose vir antibiotika aan te dui. Hierdie metode is ontwikkel vir antibakteriële toetse, nadat daar gevind is dat 'n standaard skyfie-diffusiemetode nie doeltreffend was nie. Die teenswam-aktiwiteit van ramnolipiede is teenoor die swam *Botrytis cinerea* geëvalueer, deur 'n funguslaag op 'n plaat te kweek en die ramnolipied by te voeg.

Die faktore wat ramnolipiedproduksie beïnvloed is bestudeer deur verskillende *Pseudomonas aeruginosa* stamme van die ATCC kultuurversameling, naamlik ATCC 9027 en ATCC 27853, sowel as plaaslik geïsoleerde stamme onder verskillende mediatoestande, te kweek. Die aanvanklike fokus was op produksie van biosurfaktante op media bevattende glukose as substraat. Alkane is daarna ondersoek as 'n alternatiewe substraat, aangesien hulle geredelik beskikbaar is in Suid-Afrika as neweprodukte van die petrochemiese industrie. Die ramnolipiede, geproduseer deur die kultuurversamelingstamme, is vir hul antibakteriële aktiwiteit op *Mycobacterium aurum* geëvalueer.

'n Aantal sleutelfaktore, wat belangrik is vir die ontwikkeling van 'n ramnolipied produksieproses, is geïdentifiseer. Mediatoestand was van kritiese belang. Beduidende produksie is bereik op glukose media met 'n fosfaatbeperking, pH buffer en 'n hoë koolstofkonsentrasie (2 % koolstof). Toe *Pseudomonas aeruginosa* ATCC 9027 op

hierdie medium ('n minimale soute-fosfaatbeperkende metdium met 'n Tris-buffer) gekweek is, het dit 1.31 g/l ramnose, ekwivalent aan 4 g/l ramnolipied, opgelewer. Hierdie ramnolipiedkonsentrasie is 2.7-voudig hoër as die 1.47 g/l wat in die literatuur vermeld word vir dieselfde stam (gekweek op 'n verskillende fosfaatbeperkende medium).

Dit het ook geblyk dat hierdie spesifieke stam 'n faktor is wat die opbrengs van ramnolipiede beïnvloed het. 'n Ramnosekonsentrasie van 0.43 g/l is verkry met *Pseudomonas aeruginosa* ATCC 17852, gekweek op MSM+Tris medium, vergeleke met 1.31 g/l gekweek met *Pseudomonas aeruginosa* ATCC 9027 op dieselfde medium.

Die mees belowende stam en medium, *Pseudomonas aeruginosa* ATCC 9027 en MSM+Tris medium, is onder beheerde toestande in 'n bioreaktor toegerus met metingsinstrumente, geëvalueer. Feitlik dubbel die groeitempo en produksie is in die bioreaktor behaal, wat daarop dui dat die produksietyd aansienlik verkort kan word onder beheerde toestande. Wanneer dit egter met skud-fles navorsingstudies vergelyk is, is slegs 'n 4 % toename in groei en 'n 5 % toename in ramnolipied produksie in die bioreaktor bereik, wat daarop dui dat die opbrengs beperk is deur die samestelling van die media of die proestoestande.

By media waar heksadeksaan as enkele koolstofbron gebruik is, is negeerbare ramnolipiedproduksie bereik. Stadige groei is waargeneem en die stasionêre fase is selfs nie na 2 weke bereik nie. Daar is aangetoon dat ramnolipiedproduksie in glukose medium slegs in die stasionêre fase begin. Aangesien die stasionêre fase nie tydens groei op heksadeksaan bereik kon word nie, kon ramnolipiede, met hul bekende funksie om die beskikbaarheid van alkane deur emulsifisering en solubilisering te verhoog, nie geproduseer word nie. 'n Strategie is ontwikkel om die groei op alkaanmedia te versnel. 'n Dubbel-substraat medium, wat beide glukose en heksadeksaan bevat, is ondersoek. Daar is gepostuleer dat groei ondersteun sou word deur glukose, met verhoogde ramnolipied produksie, wat dan die opname van heksadeksaan sou verbeter. Ramnolipied is in die dubbel-substraat medium geproduseer, maar die heksadeksaan opname was nog onbeduidend. Daar is voorgestel dat dit toegeskryf kon word aan die blootstelling van die selle aan glukose in die inokulum of toetsfles, wat die vermoë van die selle om heksadeksaan te verbruik, belemmer het. Verder is daar redeneer dat die vermoë om heksadeksaan te gebruik deur hidrofobositeit van die sel bepaal word, beïnvloed deur die blootstelling aan hidrofiele of hidrofobe substrate.

Daar is aangetoon dat ramnolipiede van *Pseudomonas aeruginosa* ATCC 9027 en ATCC 27853 antibakteriële aktiwiteit het op *Mycobacterium aurum*. Die grootste inhibisiesone van 45 mm is met 4 g/l ramnolipied van *Pseudomonas aeruginosa* ATCC 9027 verkry. Dit is aangetoon dat die aktiwiteit direk verwant is aan die ramnolipiedkonsentrasie, wat die belangrikheid daarvan beklemtoon om die biosurfaktant opbrengs te optimaliseer wanneer daar 'n proses ontwikkel word vir die produksie van ramnolipiede as antimikrobiese middels. Teenswam-aktiwiteitstoets op *Botrytis cinerea* was nie oortuigend nie. Toekomstige navorsingstudies behoort die antimikrobiese

toepassing van ramnolipiede uit te brei deur hul aktiwiteit op 'n groter teikenreeks organismes te toets.

Ten einde die maksimum ramnolipied opbrengs te verseker in toekomstige navorsingstudies, word 'n voedingslot-proses voorgestel wat die seldigtheid sal verhoog en die ramnolipied produksie en stasionêre fase, vasgestel om die fase te wees wat met ramnolipiedproduksie geassosieer kan word, te verleng. Verskillende voedingstrategieë behoort ondersoek te word, afhangende van die kinetika van substraatverbruik. Dit is wenslik om die kleinste volume substraat wat nodig is met 'n hoë konsentrasie te voed ten einde die verdunningstempo laag te hou en die maksimum produksiekonsentrasie te bereik. 'n Faktoriale ontwerp word vir hierdie doel aanbeveel.

Verdere navorsingstudies met alkane as koolstofbron behoort gedoen te word deur gebruik te maak van stamme onderhou en gekweek in media wat alkane as enigste koolstofbron bevat. Alternatiewe biosurfaktant-produuserende stamme, met hoër natuurlike sel hidrofobisiteit, behoort ook ondersoek te word.



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# Glossary and nomenclature

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2bf	Local strain of <i>Pseudomonas aeruginosa</i>
Aerobic	An aerobic organism or aerobe is an organism that can survive and grow in an oxygenated environment
ATCC 27853	Strain of <i>Pseudomonas aeruginosa</i>
ATCC 9027	Strain of <i>Pseudomonas aeruginosa</i>
CaCl <sub>2</sub> ·4H <sub>2</sub> O	Calcium chloride tetrahydrate
CMC	Critical micelle concentration
CuSO <sub>4</sub> ·5H <sub>2</sub> O	Copper sulphate quinhydrate
DNS	Dinitrosalicylic acid
FeSO <sub>4</sub> ·7H <sub>2</sub> O	Ferrous sulphate heptahydrate
g/l	Gram per litre
h	hour
H <sub>3</sub> BO <sub>3</sub>	Boric acid
HPLC	High pressure liquid chromatography
K <sub>2</sub> HPO <sub>4</sub>	Dibasic potassium phosphate
KCl	Potassium chloride
KH <sub>2</sub> PO <sub>4</sub>	Monobasic potassium dihydrogen phosphate
l	Litre
MEL	Mannosylerythritol lipids
MgSO <sub>4</sub>	Magnesium sulphate
MgSO <sub>4</sub> ·7H <sub>2</sub> O	Magnesium sulphate heptahydrate
MIC	Minimum inhibitory concentration
ml	Millilitre
µl	Microlitre
mm	millimetre
mM	millimolar
mN/m	Milli Newton per meter
MnSO <sub>4</sub> ·H <sub>2</sub> O	Hydrous manganese sulphate
MoNa <sub>2</sub> O <sub>4</sub> ·2H <sub>2</sub> O	Molybdenum sodium oxide dihydrate
MSM	Minimal salts medium
MSM+PO <sub>4</sub>	Minimal salts medium containing phosphate buffer
MSM+Tris	Minimal salts medium containing tris buffer
Na EDTA	Sodium edetate
Na <sub>2</sub> HPO <sub>4</sub>	Dibasic sodium phosphate
NaNO <sub>3</sub>	Sodium nitrate
ND	Not done
NH <sub>4</sub> Cl	Ammonium chloride
NH <sub>4</sub> NO <sub>3</sub>	Ammonium nitrate
nm	Nanometre
PPGASM	Proteose peptone-glucose-ammonium salts medium
ppm	parts per million
<i>Pseudomonas aeruginosa</i>	A gram-negative, aerobic, rod-shaped bacterium
rpm	Revolutions per minute
vvm	Volume per volume per minuter
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	Zinc sulphate heptahydrate

# 1 Literature review

---

## 1.1 Introduction

In 1949, Jarvis and Johnson reported that they had isolated a crystalline glycolipid from *Pseudomonas aeruginosa* which showed antibiotic activity against tuberculosis in mice (Jarvis and Johnson 1949). At that time, little was known of biosurfactants and the immense possibilities of these compounds. Nearly six decades later, in a world where green technology has become a cornerstone of the search for sustainable development, biological compounds are being recognized increasingly as an alternative to synthetic ones because of characteristics such as low toxicity, biodegradability and mild process conditions. Biological surfactants, or biosurfactants, are an example of such compounds. They are produced by micro-organisms to fulfil various natural functions. They have a unique chemical structure, which consists of a hydrophobic and hydrophilic moiety, giving them detergency as well as emulsifying, foaming and dispersing traits. They are basically detergents that dissolve water-insoluble compounds by reducing surface tension and forming micro-emulsions at the interfaces. These properties have prompted applications amongst others in the food, pharmaceutical, environmental and cosmetic industries.

This study aimed to set the foundation for the development of a process for biosurfactant production, focusing on the application of biosurfactants as antimicrobial agents. Important factors in the research were the selection of a biosurfactant producer, the selection of a substrate and process conditions and the evaluation of antimicrobial activity. The literature study gave the necessary background to aid in the selection of these process components.

## 1.2 Classification of biosurfactants

Biosurfactants are classified according to their chemical structure, dictated by the different molecules that form the hydrophobic and hydrophilic moieties. The hydrophilic moiety may consist of amino acids, peptide anions or cations, mono-, di-, or polysaccharides. The hydrophobic moiety can consist of unsaturated, saturated or fatty acids (Desai and Banat 1997). Generally four different types of biosurfactants are differentiated between, namely glycolipids, lipopeptides, phospholipids and polymeric biosurfactants.

### 1.2.1.1 Glycolipids

Glycolipids consist of carbohydrates in combination with long-chain aliphatic acids or hydroxyaliphatic acids. These are further separated into rhamnolipids, trehalolipids and sophorolipids (Desai and Banat 1997). The production of rhamnolipids will form a focus of this study.

Probably the most well known of the glycolipids are rhamnolipids, which have rhamnose sugars as a hydrophilic moiety and fatty acids as a hydrophobic moiety. Up to 28 homologues have been detected by new analytical technology (Benincasa *et al.* 2004). Four of these are the most predominant (Figure 1), which are usually designated as R1, R2, R3 and R4 (R represents rhamnolipid). These rhamnolipids differ in the amount of sugar (rhamnose) and fatty acid chains present, with one or two rhamnose and fatty acid chains (C8 – C12) being predominant (Lang and Wullbrandt 1999).

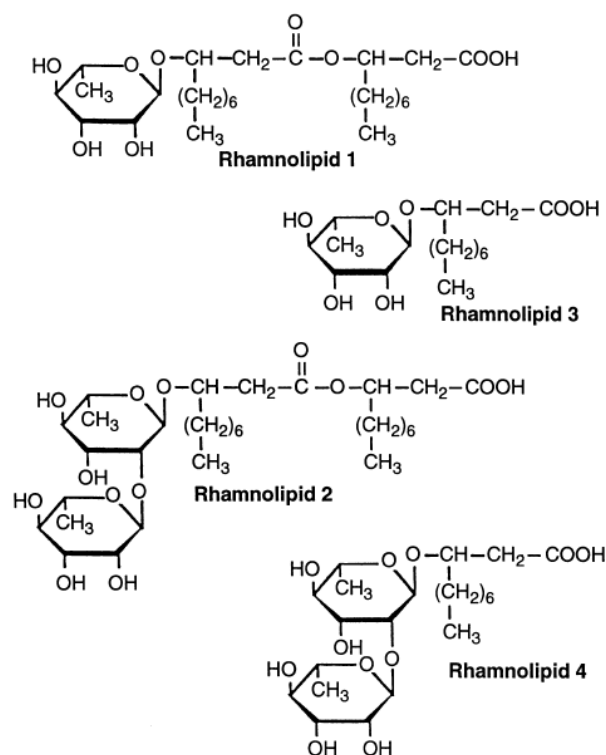


Figure 1: The four principal rhamnolipids (Reproduced from Lang *et al.* 1999)

Other glycolipids include trehalolipids, which are normally associated with the rhodococci. These organisms produce trehalose mycolates, which consist of trehalose (a disaccharide of two glucose molecules) connected to long-chain fatty acids. The chain lengths of the trehalolipids differ greatly between organisms, with lengths between C20 and C90 (Lang and Philp 1998).

Sophorolipids are glycolipids consisting of a dimeric sophorose linked to a long-chain hydroxyl fatty acid. They are primarily produced by yeasts such as *Candida bombicola*. The other high yielding glycolipid from yeasts are mannosylerythritol lipids (MEL), which are receiving much attention owing to their biomedical applications as antibacterial compounds and growth inhibition against human leukemia cells (Kitamoto *et al.* 2002). Other lesser known glycolipids that have been reported are cellobiose lipids and oligosaccharide lipids (Desai and Banat 1997).

### 1.2.1.2 Lipopeptides, phospholipids and polymeric biosurfactants

Lipopeptides consist of fatty acids attached to a chain of amino acids. These biosurfactants are produced from a variety of organisms such as *Pseudomonas*, *Bacillus* and *Streptomyces*

species (Van Hamme *et al.* 2006). One of the biosurfactants with the strongest surface active properties is the cyclic lipopeptide surfactin produced by *Bacillus subtilis* (Desai and Banat 1997).

Phospholipids are components of microbial membranes. They consist of a phosphate group and fatty acids and are produced in large amounts (40 – 80 % w/w) by many bacteria and yeasts such as *Aspergillus sp.*, *Thiobacillus thiooxidans* and *Arthrobacter sp.* during growth on hydrophobic substrates (Desai and Banat 1997).

Polymeric biosurfactants consist of polysaccharides, proteins, lipopolysaccharides and lipoproteins. These are complex molecules and a detailed discussion of their structures falls outside the scope of this study. Most polymeric biosurfactants, such as emulsan, has a backbone of three or four repeating sugars with fatty acids attached to the sugars (Rosenberg and Ron 1997).

### **1.3 Properties and applications**

#### **1.3.1 Properties**

Biosurfactants have very strong surface active properties. They lower the surface and interfacial tension between gases, liquids and solids. Biosurfactants lower the surface tension of water from 72 mN/m to values ranging between 25 and 38 mN/m (Desai and Banat 1997). The efficiency of the biosurfactant surface tension reduction ability is expressed by the critical micelle concentration (CMC). The CMC is the concentration where the reduction in surface tension reaches a level at which supramolecular micelles and vesicles start to form. Any increase in biosurfactant concentration will not reduce the surface tension any further, because the biosurfactant is saturated in the aqueous phase. Biosurfactants have about a 10- to 40-fold lower CMC than synthetic surfactants (Lang and Philp 1998). They are thus more efficient than synthetic surfactants, because smaller concentrations are needed to disperse solutions with insoluble phases. Biosurfactant CMC values range from 1 to 200 mg/l (Van Hamme *et al.* 2006). Small concentrations are therefore needed to effectively lower surface and interfacial tensions.

More recently, biosurfactants have been recognized as molecules with biological active properties. They have shown biocidal activity against viruses, yeasts, fungi and bacteria, which is expressed by the zone of inhibition or minimal inhibitory concentration (MIC). Biosurfactants also have other useful biomedical properties which will be discussed below.

#### **1.3.2 Applications**

The properties mentioned above can be exploited in a wide range of applications, which have been extensively reviewed, especially biodegradation applications. In a much cited review, Desai and Banat (1997) give examples of biosurfactants which have been used in oil recovery, remediation of hydrocarbon, heavy metals and crude-oil contaminated soils, as emulsifiers in the food industry and even in skin moisturising.

The focus of the study presented here is the use of biosurfactants as antimicrobial agents. Bioremediation will be discussed briefly while biomedical applications will be examined extensively. For detail on other applications the reader is referred to the many reviews on the subject (Banat *et al.* 2000, Kitamoto *et al.* 2002, Singh *et al.* 2007).

### **1.3.2.1 Bioremediation applications**

Biosurfactants have been widely used in bioremediation, owing to their biocompatibility, low toxicity and ability to disperse a wide variety of hydrophobic pollutants (Banat *et al.* 2000). A well known example of where biosurfactants were used in bioremediation was the Exxon Valdez oil spill. Rhamnolipids emulsified the hydrocarbons in the spillage, which enhanced biodegradation by micro-organisms in the seawater (Harvey *et al.* 1990). The addition of rhamnolipids led to 2 to 3 times the removal of oil than that obtained by pressurized water at high temperature.

In a comprehensive review, Mulligan (2005) discusses many examples of where biosurfactants were used in soil and water treatment, focusing on rhamnolipids and the lipopeptide surfactin, which are the most widely applied. However, as she points out, full scale tests are still required in order to predict the influence of the soil components on the extent of dispersion.

### **1.3.3 Biomedical applications**

Falagas and Bliziotis (2007) found clinical isolates of micro-organisms that were resistant to all available antibiotics. They concluded that intense research is necessary into the development of new antibiotics. Biosurfactants present such an opportunity to be developed as novel antibiotics. Biosurfactant antimicrobial activity has been reported against bacteria, fungi, algae and viruses and the first biosurfactants have reached the stage of development where they are now produced as commercial antibiotics. Micafungin, a derivative of one of the lipopeptides isolated from *Coleophoma impetri*, has been approved by the US Food and Drug Administration as well as the European Union (Hino *et al.* 2001); ([www.wikipedia.org](http://www.wikipedia.org) 2008). This new antifungal agent has the potential to contribute significantly in the fight against hospital-acquired infections against which no conventional antimicrobial agent is effective.

Many other biosurfactants have shown antimicrobial activity, of which the most notable are rhamnolipids and lipopeptides, but efforts are not far advanced in terms of their development for commercial use. One of the reasons for the lack of development is the high production costs of biosurfactants (Mukherjee *et al.* 2006). The high production costs are compensated for to an extent when biosurfactants are used as pharmaceutical compounds, which are high cost low volume molecules. However, process optimisation at the biochemical and engineering level is necessary for the production of biosurfactants to be viable (Cameotra and Makkar 2004). Another obstacle in the way of the development of biosurfactants as pharmaceuticals is the lack of research into their toxicity towards human cells (Singh and Cameotra 2004).

The focus of the study presented here is the use of biosurfactants as antimicrobial agents against organisms important in a South African context. For this purpose two target organisms were chosen. *Mycobacterium aurum* has particular significance in South Africa, because this target organism is used to indicate possible sensitivity for tuberculosis. Secondly, the fungus *Botrytis cinerea* is a particularly virulent spoilage agent on fruit and grape vine leaves, adversely affecting the lucrative South African wine and fruit industry.

In order to illustrate the enormous potential of biosurfactants as biomedical compounds, the antimicrobial activity of rhamnolipids and lipopeptides will be elaborated on and other biomedical applications will be discussed briefly.

### **1.3.3.1 Antimicrobial activity of rhamnolipids**

The various authors discussed below have researched the antimicrobial activity of rhamnolipids, usually from locally isolated strains. *Ps. aeruginosa* AT10, isolated from soybean oil refinery wastes in Cuba, produced seven rhamnolipids which showed excellent antifungal properties and showed relatively good activity against gram-positive bacteria (Abalos *et al.* 2001). The seven rhamnolipids were a mixture of R1, R2 and R3 containing different fatty acid chain lengths (section 1.2.1.1).

Benincasa *et al.* (2004) reported very good activity against *Bacillus subtilis* (MIC of 8 µg/ml) for their mixture of six rhamnolipid homologues. The mixture also performed well against gram-positive bacteria and fungi, but differed in MIC values from other rhamnolipid antimicrobial studies. Abalos *et al.* (2001) reported a low MIC of 32 µg/ml against *Escherichia coli*, whereas Benincasa *et al.* (2004) reported poor activity (MIC of 250 µg/ml). Although Abalos *et al.* (2001) did not obtain activity against gram-negative bacteria, rhamnolipids (R1 and R2) from *Ps. aeruginosa* 47T2 did (Haba *et al.* 2003). However, the antifungal activity of R1 and R2 compared poorly with the activity of the other rhamnolipid mixtures mentioned above. These differences demonstrate the possible impact the composition of the rhamnolipid mixture has on the antimicrobial activity.

### **1.3.3.2 Antimicrobial activity of lipopeptides**

A few studies have focused on the antimicrobial activity of lipopeptides. Bechard *et al.* (1998) isolated a lipopeptide antibiotic from *Bacillus subtilis*. It showed antimicrobial activity against fungi and bacteria, especially gram-negative bacteria. Yakimov *et al.* (1995) compared the antimicrobial activity of the lipopeptide surfactin against the lipopeptide lichenysin A that they isolated from *Bacillus licheniformis* BAS50. Surfactin performed better than lichenysin against all the target organisms, gram-positive as well as gram-negative bacteria.

### **1.3.3.3 Mechanisms of biosurfactant antimicrobial activity**

Another advantage of using biosurfactants as antimicrobial agents is the wide range of organisms they are active against, as can be seen from the examples mentioned above. It is generally accepted that biosurfactants act on the integrity of cell membranes, which leads to cell lysis (Cameotra and Makkar 2004). However, the ways in which the biosurfactants affect the

membrane integrity differ. The lipopeptide, iturin A, is thought to disrupt plasma membranes of yeast cells by the accumulation of intramembranous particles in the cells and by increasing the electrical conductance of the membrane (Thimon *et al.* 1995). The lipopeptide, surfactin, has been shown to increase membrane permeability through interaction with cell membrane phospholipids (Carrillo *et al.* 2003). A similar mechanism has been proposed for rhamnolipid glycolipids, which are thought to act on the lipid part of cell membranes or outer proteins, causing structural fluctuations in the membranes (Sotirova *et al.* 2007). Rhamnolipid antifungal activity has been attributed to zoospore lysis (Stanghellini and Miller 1997).

#### **1.3.3.4 Other biomedical applications**

Besides their antibiotic activity, lipopeptide biosurfactants have a diverse range of therapeutic applications as antiviral, antimycoplasmic and antitumor agents, as inhibitors of fibrin clot formation and as hypocholesterolemic agents (Peypoux *et al.* 1999).

In a much cited study by Isoda *et al.* (1997) it was found that six microbial extracellular glycolipids induced cell differentiation instead of cell proliferation in the human promyelocytic leukemia cell line HL60. These glycolipids were MEL-A, MEL-B, polyol lipid, sophorose lipid and succinoyl trehalose lipids STL-1 and STL-3. Kitamoto *et al.* (2002) have shown that mannosylerythritol lipids can be used as affinity ligands for human immunoglobulin.

Another medical use of biosurfactants is their ability to act as anti-adhesive agents in biofilm formation of pathogens. As anti-adhesives, biosurfactants can, for example, prevent colonisation of bacteria on voice prostheses (Rodrigues 2005) and urethral catheters (Mireles *et al.* 2001).

### **1.3.4 Measurement of biosurfactant characteristics**

#### **1.3.4.1 Quantification of rhamnolipid concentration**

Biosurfactants have a wide variety of structures (section 1.2), which means that no standard method can be applied to determine the concentration of all biosurfactants directly. One of the reasons that biosurfactant production from *Pseudomonas aeruginosa* is so well studied is because its exclusive production of rhamnolipids makes direct concentration measurement possible. A wide variety of tests exist to quantify rhamnolipids, mostly by quantifying the rhamnose (sugar) moiety. A study of literature (Table 1) revealed only colorimetric methods for direct rhamnose quantification and no reports could be found on chromatographic methods such as HPLC used for this purpose, although HPLC is commonly used to quantify reducing sugars such as glucose. HPLC was used in literature (Table 1) for the characterisation of the rhamnolipid molecules (together with gas chromatography and mass spectroscopy), but the entire molecule was analysed with expensive reverse phase columns and was not used for bulk concentration measurements.

Table 1 lists rhamnolipid analyses from literature together with the type of sample used in each analysis. All methods used were colorimetric methods (orcinol analysis, anthrone analysis and



acid hydrolysis). These methods involve hydrolysis of the rhamnolipid molecule to separate the rhamnose sugars and a reaction of the sugars with a reagent which produces colour. The preparation of the samples for the analyses differs between the studies. Some of the studies first extracted samples twice or three times before doing the analysis (7 out of 11 studies in Table 1) and some analysed the supernatant directly. It is not known if this extraction step is necessary for accurate determination of the rhamnose concentration.

#### **1.3.4.2 Surface activity**

Most biosurfactants are quantified indirectly by their surface activity. Every biosurfactant and mixture thereof has a specific physiochemical activity characterising it. At a certain concentration (CMC) the biosurfactant reaches a minimum surface tension, which does not decrease any further. The concentration of biosurfactant can be expressed as the dilution factor ( $\text{CMC}^{-1}$ ) necessary to reach the critical micelle concentration (Mulligan *et al.* 1989, Rodrigues 2005). The use of the CMC dilution factor as a measure of biosurfactant concentration is very time consuming and cannot be used to compare biosurfactant production from different studies directly, because the CMC of each biosurfactant is different from another.

There are a few potential problems with this method, because the surface tension is very sensitive to the specific conditions in the medium. The pH of the solution can greatly influence the surface tension measurements (Zhang and Miller 1992). In literature most studies have measured the surface tension and determined the CMC with supernatant samples taken directly from the culture broth (Table 1), which would lead to error because of pH changes in the growth cycle. Another possible issue is the effect of other surface active molecules in the medium such as alkanes.



**Table 1: Summary of analytical methods used in rhamnolipid production studies. The sample used in each analysis is given in each block.**

Reference	Acid hydrolysis method (Colorimetric)	Orcinol assay (Colorimetric)	Anthrone method (Colorimetric)	Surface tension
(Matsufuji <i>et al.</i> 1997)	ND*	Extracted sample purified by TLC	ND	ND
(Tang <i>et al.</i> 2007)	Supernatant	ND	ND	Sample not specified
(Rhaman <i>et al.</i> 2002)	Supernatant	ND	ND	Culture broth
(Déziel <i>et al.</i> 1996)	ND	Extracted supernatant	ND	Supernatant
(Raza <i>et al.</i> 2006a)	ND	Extracted supernatant	ND	Supernatant
(Zhang and Miller 1992)	Extracted supernatant	ND	ND	Extracted rhamnolipid
(Koch <i>et al.</i> 1991)	ND	Extracted supernatant	ND	ND
(Sim <i>et al.</i> 1997)	ND	ND	Extracted culture broth	Purified rhamnolipid
(Wei <i>et al.</i> 2005)	ND	Culture broth	ND	Supernatant
(Syldatk <i>et al.</i> 1985b)	ND	ND	Supernatant	Supernatant
(Guerra-Santos <i>et al.</i> 1984)	ND	Extracted supernatant	ND	Supernatant

\*ND – Not done

### 1.3.4.3 Quantification of antimicrobial activity

Several methods exist for antimicrobial susceptibility testing depending on the focus of the study. Quality control for antibiotic drug testing is extremely rigorous and standard protocols exist for their evaluation (Jorgensen and Turnidge 2003). However, in biosurfactant antimicrobial activity research many different methods are applied, which means that studies are not always directly comparable. In order to be reproducible and comparable, approximately the same concentration of biosurfactant and target organisms should be used for susceptibility testing (Jorgensen and Turnidge 2003). The amount of agar in plate tests also has to be consistent or false-positive results for resistance (excessively small zones) and false-positive results for susceptibility (excessively large zones) may be obtained.

The purity of the solution also differs between studies. Mostly the supernatant from the culture is tested directly for antimicrobial activity. It is therefore possible that other components could be in part responsible for the antimicrobial activity. In a study by Perneel *et al.* (2008) rhamnolipids and phenazines were purified from *Pseudomonas aeruginosa* culture and tested against fungi (*Pythium* sp.). They found that the rhamnolipids and phenazines have a synergistic effect and do not achieve as high activity separately as collectively.

## 1.4 Biosurfactant producing micro-organisms

Biosurfactants are frequently classified according to the organism which produces them. Rhamnolipids are extracted from *Pseudomonas aeruginosa*, the lipopeptides surfactin and iturin from *Bacillus subtilis* and trehalolipids from *Rhodococcus erythropolis*. However, many reports exist of novel biosurfactants produced by well known biosurfactant producing strains, for example the case where bacillocyin, plipstatin and surfactin were produced by the same strain of *Bacillus subtilis* (Roongsawang *et al.* 2002). Novel biosurfactants have also been extracted from relatively unknown producers, for example the novel lipopeptides found in cultures of *Streptomyces tendae* (Richter *et al.* 1998). Even though many biosurfactants have been identified, production is usually limited to shake flask studies, whereas larger scale production studies are limited to a few genera such as *Bacillus*, *Pseudomonas* and *Candida* (Mukherjee *et al.* 2006).

One of the foci of this study will be the production of rhamnolipids from *Pseudomonas aeruginosa*. Its production is well documented and it has many applications as was mentioned in section 1.3 (Chen *et al.* 2007, Gruber *et al.* 1993, Haba *et al.* 2000, Hisatsuka *et al.* 1971, Wei *et al.* 2005).

The type of rhamnolipid produced by *Pseudomonas aeruginosa* differs between strains. Mono-rhamnolipid (R1), which contains one rhamnose molecule attached to a lipid chain of two fatty acids, is produced by *Ps. aeruginosa* ATCC 9027 (Zhang and Miller 1994). Other strains usually produce a mixture of different rhamnolipids, one strain produced all

four principal rhamnolipids (R1,R2,R3 and R4) (Syldatk *et al.* 1985a) and others mixtures of six or seven rhamnolipid homologues from one strain (Abalos *et al.* 2001, Benincasa *et al.* 2004). However, mono-rhamnolipid and di-rhamnolipid (R1 and R2) are the most prevalent (Maier and Soberón-Chávez 2000).

Although only wild type strains will be used in this study for biosurfactant production, an area for future work is the use of genetically modified strains. Mukherjee *et al.* (2006) name several examples of where recombinant organisms have greatly increased the production of biosurfactants or rendered them nonpathogenic. A case where genetic manipulation was beneficial is the genetic engineering of the surfactin pathways in *Bacillus subtilis* which led to the creation of a novel lipohexapeptide. This molecule had altered antimicrobial properties, such as reduced toxicity against eukaryotic cells and enhanced lysis of pathogens (Symmank *et al.* 2002). The random mutagenesis of *Bacillus licheniformis* KGL11 led to 12 times the biosurfactant production than the wild type strain (Lin *et al.* 1998). Enhanced production of biosurfactants can lead to reduced process costs which will contribute to the commercialisation of biosurfactants (Mukherjee *et al.* 2006).

#### 1.4.1 Physiological roles of biosurfactants

The source of the different properties of biosurfactants is often related to the physiological role of the specific biosurfactant in the micro-organism. The physiological role of the specific biosurfactant determines its role in the metabolism of the micro-organism, of which the unique metabolic pathways and regulation mechanisms form part. However, a diversity of roles have been elucidated for biosurfactants, because of their variety in structure, and many biosurfactants have not been studied in detail (Van Hamme *et al.* 2006).

A widely accepted role of biosurfactants is the enhancement of the uptake of insoluble substrates (Hisatsuka *et al.* 1971, Koch *et al.* 1991, Zhang and Miller 1992). Three methods of uptake of hydrocarbons are mentioned in literature, i.e. the direct interfacial uptake of the hydrocarbon by hydrophobic cell membranes, interfacial uptake enhanced by emulsification by biosurfactants and solubilisation (micellar transfer) of hydrocarbons by biosurfactants (Bouchez-Naitali *et al.* 1999). Two of these methods are directly influenced by biosurfactants (emulsification and solubilisation). The direct uptake of hydrocarbons is enhanced in some cases by biosurfactants (Noordman and Janssen 2002). For example, the exogenous addition of rhamnolipid to *Pseudomonas aeruginosa* culture was shown to increase growth on hydrocarbons through an increase in cell membrane hydrophobicity (Zhang and Miller 1992). The increased hydrophobicity has been attributed to the loss of lipopolysaccharide from the cell membrane, which is influenced by the rhamnolipid concentration (Al-Tahhan *et al.* 2000).

The mechanism of uptake of hydrocarbons by *Pseudomonas aeruginosa* remains a topic of discussion in literature. It was explained above that direct uptake is thought to occur because of the increase in hydrophobicity coinciding with enhanced growth. Other

researchers have reported that the predominant mechanism is the solubilisation of hydrocarbons in micellar structures (Bouchez-Naitali *et al.* 1999). A possible explanation for this apparent incongruity is that *Pseudomonas aeruginosa* strains have different degrees of hydrophobicity of their cell membranes, and thus some are naturally superior in degrading hydrocarbons through direct uptake (Herman *et al.* 1997, Zhang and Miller 1995). For example, *Ps. aeruginosa* ATCC 27853 and ATCC 15442 have been found to have naturally hydrophobic membranes (Zhang and Miller 1994).

Another important possible role of biosurfactants is their role as antagonistic molecules against other organisms (Ron and Rosenberg 2001). Rhamnolipids, for example, have considerable antimicrobial activity as was detailed in section 1.3.3.1. It is likely that rhamnolipids are produced for this function, and not solely to enhance the availability of insoluble substrates, since rhamnolipids are produced from hydrophilic substrates as well as hydrophobic substrates (Soberón-Chávez *et al.* 2005).

Other roles for biosurfactants have been proposed. A few examples are motility, biofilm formation, quorum sensing, cellular differentiation, avoidance of toxic elements and compounds, storage of carbon and energy molecules and a protective mechanism against high ionic strength (Van Hamme *et al.* 2006). Although these roles play an important part in the understanding of why biosurfactants are produced and how to enhance their production, it falls outside the scope of this study to provide an in depth discussion.

#### 1.4.2 Biosurfactant location in cell culture

The physiological role of the biosurfactant will also determine its location in the cell culture. The location of the product is a critical parameter which will influence the complexity of downstream processing directly, and consequently, the overall production cost.

Rodrigues (2005) found that biosurfactants in cultures of four strains of *Lactobacillus* were extracellular as well as membrane attached. Centrifuged and washed cells were stirred for 24 hours in phosphate buffered saline, which caused surface tension to decrease by 17 to 21.5 mN/m, indicating the presence of released biosurfactants. Another example of cell bound biosurfactants is the formation of glycolipids from rhodococci (Lang and Philp 1998).

Rhamnolipids are formed in the periplasm of cells and excreted as extracellular products (Ochsner *et al.* 1995), but it is not known whether some attach to the cell membrane. An investigation of the analytical methods of 11 studies done on rhamnolipid production (Table 1) has shown that not one study took the location of rhamnolipid into account. Two of the eleven studies (Sim *et al.* 1997, Wei *et al.* 2005) measured rhamnolipid concentration in the culture broth whereas the rest removed the cells and only analysed the supernatant.

## 1.5 Biosurfactant synthesis

### 1.5.1 Biosynthetic pathways

Biosurfactants consist of a hydrophobic (type of fatty acid) and hydrophilic (usually polysaccharide or peptide) moiety. Depending on the carbon source used (hydrophilic or hydrophobic), the metabolic pathways used for biosurfactant production will differ. The superiority of one substrate over another will in turn depend on the organism and its specific biosynthetic pathways. Kitamoto *et al.* (2002) found that knowledge is still lacking of the precise influence on metabolism by different carbon sources. Other factors than the metabolic pathways will also play a role, such as the natural ability of the specific strain to take up hydrophobic substrates (see section 1.4.1).

Generally the metabolism of hydrocarbons such as alkanes to biosurfactants will consist of the alkane being oxidised via alkanols to mono-alkanoic acids (fatty acids), which normally make up the hydrophobic moiety of the biosurfactant. The fatty acid can also be further metabolised via  $\beta$ -oxidation to yield acetyl co-enzyme A, a central reactive intermediate. Acetyl CoA is converted further in the tricarboxylic acid cycle to sugars and amino acids, part of which forms the hydrophilic moiety of the biosurfactant (Hommel and Ratledge 1993).

Hydrophilic substrates such as glucose are easily converted into the hydrophilic moiety of the biosurfactant. However, a longer route is necessary to form the hydrophobic moiety, which would be the reverse of the route explained above. Sugars are converted to acetyl CoA, which is metabolised via  $\beta$ -oxidation to fatty acids.

### 1.5.2 Associated growth phases

In order to select best process conditions for biosurfactant production it is not only important to understand biosynthetic pathways, but also the associated growth phase of the biosurfactant. For example, if the biosurfactant is excreted primarily in the exponential phase, conditions must encourage an extension of this phase for maximum production.

Rhamnolipids are generally accepted to be secondary metabolites. Many researchers have found that rhamnolipid production began in the stationary phase, initiated by the exhaustion of a nutrient (Lang and Wullbrandt 1999, Maier and Soberón-Chávez 2000, Sim *et al.* 1997). Other researchers found that rhamnolipids were produced during the whole cell growth cycle, but that production increased during the stationary phase (Benincasa *et al.* 2002, Wei *et al.* 2005). Evidence on genetic level is in favour of rhamnolipids being a secondary metabolite. Transcription from some of the genes responsible for rhamnolipid synthesis (*rhlAB* promoter) is controlled by quorum sensing (Pearson *et al.* 1997, Soberón-Chávez *et al.* 2005). Quorum sensing is a cell-density dependent mechanism that initiates production of some secondary metabolites during the stationary phase.

Production of the other well known biosurfactant, surfactin, has been associated with the exponential growth phase. In a comprehensive review on the biosynthesis of surfactin, the authors state that surfactin production is induced by actively growing cells with a postexponential synthesis (Peypoux *et al.* 1999). They speculate that the competition with cellular growth is probably one of the reasons that surfactin yields are much smaller than for glycolipids. This statement is supported by the demonstration of a relationship between isocitrate dehydrogenase (a Krebs cycle enzyme) activity and surfactin production (Roubin *et al.* 1989). In other words, a decrease in the oxidative process correlated with an increase in surfactin concentration.

Generally, biosurfactants can be produced in any growth phase. Emulsan, a polymeric biosurfactant, accumulates on cell surfaces during the exponential phase and is released into the medium in the stationary phase (Goldman *et al.* 1982). Mannosylerythritol lipids are produced in the stationary phase (Kitamoto *et al.* 2002).

## **1.6 Process conditions**

The optimal medium composition and process conditions will be different for each organism and strain. The choice of medium composition will also possibly be influenced by economical factors. Since this study focuses on the production of rhamnolipids from *Pseudomonas aeruginosa*, this section of the literature study will be limited to the process conditions for that organism and biosurfactant.

### **1.6.1 Medium components**

#### **1.6.1.1 Carbon source requirements**

One of the criteria for the selection of the carbon source is the economical impact. The production costs of biosurfactant processes must be minimised in order for these processes to become commercially viable (Mukherjee *et al.* 2006). One solution is to minimize the substrate costs by using renewable sources. A variety of waste oils from food industry and other agro-based raw materials have been used as alternative and cheap substrates (Haba *et al.* 2000, Raza *et al.* 2006a). An exciting feedstock opportunity is presented in South Africa by the large quantities of linear alkanes currently produced as by-products in the petrochemical industry, specifically gas to liquid processes. The advantages of using alkanes as feedstock are numerous. They are low-priced and high in purity and as hydrophobic substrates they can possibly enhance the production of biosurfactants, whose natural role, amongst other things, is the uptake of hydrocarbons (Van Hamme *et al.* 2006).

The suitability of alkanes in terms of rhamnolipid metabolism has been discussed (section 1.5.1). It would seem that alkanes would be an ideal substrate for rhamnolipid production. Nevertheless, production studies from literature report a wide variety of substrates. Table 2 summarises media used in nine rhamnolipid production studies. Hydrophilic as well as

hydrophobic substrates were used. Matsufuji *et al.* (1997) found that ethanol, a hydrophilic substrate, was superior to propanol, butanol, glucose, sucrose, maltose, paraffin, glycerol and rape seed oil. Santa-Anna *et al.* (2002) obtained 530 % increase in production when glycerol was used instead of hexadecane. Others found that a hydrophobic substrate is the best for production. Wei *et al.* (2005) showed that olive oil led to the best biosurfactant production, as compared to sunflower oil, grape seed oil, glucose, glycerol, diesel and kerosene. Some *Pseudomonas aeruginosa* strains are naturally more suited for the uptake of hydrophobic substrates (section 1.4.1).

Even though *Pseudomonas aeruginosa* can be grown on hydrophilic and hydrophobic substrates, very few studies have tried a combination of both. Raza *et al.* (2006b) tested a setup where glucose used as carbon source together with different hydrophobic substrates. Growth was promoted by glucose in the exponential phase leading to a shortened lag phase and increased cell concentrations. The increased cell growth led to higher rhamnolipid production from the hydrophobic substrates in the stationary phase.

Wei *et al.* (2005) found that rhamnolipid production increased with increasing carbon concentration. It is widely accepted that a high carbon to nitrogen ratio (C/N) is necessary for biosurfactant overproduction (Lang and Wullbrandt 1999). An exception is glucose, which can inhibit growth and product formation at high concentrations due to the formation of acidic metabolites (Chen *et al.* 2007). Glycerol also has been shown to severely inhibit growth at concentrations above 4% (Wei *et al.* 2005).

The substrate not only has an influence on the amount of biosurfactant produced, but also on the variety of biosurfactants. Perfumo *et al.* (2006) found that the composition of the rhamnolipid mixture produced by *Ps. aeruginosa* AP02-1 varied with different carbon sources. In the glucose- and soybean oil-containing cultures, the main rhamnolipid produced was mono-rhamnolipid R1. In the hexadecane- and glycerol containing cultures, R1 and R2 were formed.



**Table 2: List of media used in the production of rhamnolipids from *Pseudomonas aeruginosa*. Total carbon, nitrogen and phosphate were calculated from components with carbon to nitrogen (C/N) and carbon to phosphate (C/P) ratios.**

Reference	Carbon source [g/l]	Nitrogen inorganic [g/l]	Nitrogen organic [g/l]	Phosphate sources[g/l]	Other [g/l]	Trace element solution [g/l]	Total C [%]	Total N [%]	Total P [%]	C/N	C/P
(Sim <i>et al.</i> 1997)	Canola oil:60	NaNO <sub>3</sub> :15	yeast extract: 0.5	KH <sub>2</sub> PO <sub>4</sub> : 3.4 K <sub>2</sub> HPO <sub>4</sub> : 4.4	KCl:1.1 NaCl: 1.1 FeSO <sub>4</sub> .7H <sub>2</sub> O: 0.00028 MgSO <sub>4</sub> .7H <sub>2</sub> O:0.5	ZnSO <sub>4</sub> .7H <sub>2</sub> O: 0.29 CaCl <sub>2</sub> .4H <sub>2</sub> O: 0.24 CuSO <sub>4</sub> .5H <sub>2</sub> O: 0.25 MnSO <sub>4</sub> .H <sub>2</sub> O: 0.17	4.70	0.25	0.16	18.63	30.18
(Zhang and Miller 1992)	Glucose: 5 (Tris-HCl:14.54)*	NH <sub>4</sub> Cl: 1	Peptone:10 Tris-HCl:14.54		KCl: 1.5 MgSO <sub>4</sub> :0.4		0.78	0.35		2.23	
(Matsufuji <i>et al.</i> 1997)	Ethanol:30	none	yeast extract: 5 soy flour:5	K <sub>2</sub> HPO <sub>4</sub> :1	MgSO <sub>4</sub> .7H <sub>2</sub> O:0.5		1.56	0.10	0.02	15.64	87.98
(Haba <i>et al.</i> 2000)	Olive oil, sunflower oil (1:1) :40	NaNO <sub>3</sub> :4	yeast extract: 0.02	KH <sub>2</sub> PO <sub>4</sub> :2 K <sub>2</sub> HPO <sub>4</sub> :4	KCl:0.2 MgSO <sub>4</sub> .7H <sub>2</sub> O:1 CaCl <sub>2</sub> :0.02 FeSO <sub>4</sub> .7H <sub>2</sub> O: 0.024	H <sub>3</sub> BO <sub>3</sub> : 0.26 CuSO <sub>4</sub> .5H <sub>2</sub> O:0.5 MnSO <sub>4</sub> .H <sub>2</sub> O:0.5, MoNa <sub>2</sub> O <sub>4</sub> .2H <sub>2</sub> O: 0.06 ZnSO <sub>4</sub> .7H <sub>2</sub> O:0.7	3.03	0.07	0.12	45.90	26.00
(Wei <i>et al.</i> 2005)	olive oil:100	NH <sub>4</sub> NO <sub>3</sub> :4		KH <sub>2</sub> PO <sub>4</sub> :4.08 Na <sub>2</sub> HPO <sub>4</sub> :5.68	CaCl <sub>2</sub> : 7.8E-4 MgSO <sub>4</sub> .7H <sub>2</sub> O: 0.197 Na EDTA: 1.17E-3 FeSO <sub>4</sub> .7H <sub>2</sub> O: 5.6E-4		7.49	0.14	0.22	53.47	34.54
(Raza <i>et al.</i> 2006a)	waste frying oils:20 and 10 after consumed	NaNO <sub>3</sub> :2.0		KH <sub>2</sub> PO <sub>4</sub> :0.7 Na <sub>2</sub> HPO <sub>4</sub> :0.9	MgSO <sub>4</sub> .7H <sub>2</sub> O: 0.4 CaCl <sub>2</sub> .2H <sub>2</sub> O:0.001 FeSO <sub>4</sub> .7H <sub>2</sub> O: 0.001		1.52	0.03	0.04	46.04	42.65



Reference	Carbon source [g/l]	Nitrogen inorganic [g/l]	Nitrogen organic [g/l]	Phosphate sources[g/l]	Other [g/l]	Trace element solution [g/l]	Total C [%]	Total N [%]	Total P [%]	C/N	C/P
(Chen <i>et al.</i> 2007)	glucose:60	NH <sub>4</sub> NO <sub>3</sub> :4		KH <sub>2</sub> PO <sub>4</sub> :4.08 Na <sub>2</sub> HPO <sub>4</sub> : 4.26	CaCl <sub>2</sub> : 3.9E-4 MgSO <sub>4</sub> ·7H <sub>2</sub> O: 0.191 Na EDTA: 1.17E-3 FeSO <sub>4</sub> ·7H <sub>2</sub> O: 6.7E-4		2.40	0.14	0.19	17.14	12.92
(Santa-Anna <i>et al.</i> 2002)	glycerol:30	NaNO <sub>3</sub> : 1.0		KH <sub>2</sub> PO <sub>4</sub> :3.0	MgSO <sub>4</sub> ·7H <sub>2</sub> O:0.2		1.17	0.02	0.07	71.27	17.19
(Santos <i>et al.</i> 2002)	glycerol:30	NaNO <sub>3</sub> : 1.78		KH <sub>2</sub> PO <sub>4</sub> :3.0 K <sub>2</sub> HPO <sub>4</sub> :7	MgSO <sub>4</sub> ·7H <sub>2</sub> O:0.2		1.17	0.03	0.19	40.00	6.10

\* Tris-HCl added only once to medium, but has carbon and nitrogen components

### 1.6.1.2 Nutrient requirements

Many authors report that the best nitrogen source for rhamnolipid production is nitrate (Haba *et al.* 2000, Santa-Anna *et al.* 2002). Nitrate was also used by most of the studies in Table 2 as inorganic nitrogen source. Other reports claim that rhamnolipid production is enhanced by nitrate, glutamate, and aspartate (Mulligan and Gibbs 1989). High concentrations of divalent cations, especially iron, are inhibitory (Lang and Wullbrandt 1999).

Little information is available on the influence of phosphate sources. Phosphates are usually included in a buffer solution for pH maintenance purposes (Table 2), which has been shown to be necessary for good rhamnolipid production (Chen *et al.* 2007).

### 1.6.1.3 Selection of limiting nutrient

An important factor to consider with the production of secondary metabolite biosurfactants, such as rhamnolipids, is the question of the limiting nutrient. A small percentage of literature found on rhamnolipid production investigates the nutrient limitation responsible for the onset of rhamnolipid production. Only two of the studies in Table 3, i.e. Raza *et al.* (2006a) and Sim *et al.* (1997), have mentioned what the nutrient limitation was in their processes and in both cases it was nitrogen. Mulligan and Gibbs (1989) showed that the activity of the enzymes involved in nitrogen metabolism decreased with the consumption of nitrogen sources, whereupon the organism switched to glucose metabolism and rhamnolipid production increased. Rhamnolipid production can therefore be triggered by the exhaustion of nitrogen, but other limitations could have the same effect.

Fewer studies have been carried out on other limiting nutrients. Mulligan *et al.* (1989) have shown that biosurfactant production coincides with a shift in phosphate metabolism. In particular they found that biosurfactant production started when phosphate was depleted, alkaline phosphatase activity was induced and transhydrogenase activity decreased.

## 1.6.2 Operating conditions

Literature sources report a wide variety of process conditions (temperature, pH, agitation and incubation time) for rhamnolipid production. In the eight studies summarised in Table 3 relatively long incubation times (6 to 12 days) were used. In the same studies temperatures between 28 °C and 37 °C were reported. The highlighted blocks are values that have been optimised in the appropriate study. Wei *et al.* (2005) measured rhamnolipid production between 25 °C and 47 °C and found that the optimum temperature was between 30 °C and 37 °C. Chen *et al.* (2007) reported 37 °C as the optimum temperature (tested between 30 °C to 42 °C).

The reported pH was all in the neutral range. A pH lower than 6.5 or higher than 7.5 could lead to significantly lower production along with lower cell growth rates (Chen *et al.* 2007).

Agitation rates for shake flask experiments reported in Table 3 range from 120 rpm to 220 rpm. Wei *et al.* (2005) tested agitation rates between 50 rpm and 250 rpm and found that the best

production was achieved at 250 rpm. However, they used 200 rpm in their experiments, because 250 rpm led to excessive foaming and only a slight increase in production. The agitation rate for the bioreactor study (Chen *et al.* 2007) is not reported, since reactor conditions are discussed in section 0.

**Table 3: List of process conditions reported in literature for the production of rhamnolipid from *Pseudomonas aeruginosa*. Highlighted blocks are parameters that have been optimised to an extent.**

Reference	Type of fermentation	Temperature [° C]	pH	Agitation	Incubation time	Nutrient limitation	Max Biosurf prod [g/L]
(Sim <i>et al.</i> 1997)	4 l flasks	30	6.5	200 rpm	9 days	Nitrogen	24.3
(Matsufuji <i>et al.</i> 1997)	Shake flasks	28	6.8	220 rpm	7 days	Not specified	3.7
(Haba <i>et al.</i> 2000)	Baffled shake flasks	30	7.2	150 rpm	Not specified	Not specified	2.7
(Wei <i>et al.</i> 2005)	Shake flasks	30 - 37	6.8	200 rpm	7 to 12 days	Not specified	3.6
(Raza <i>et al.</i> 2006a)	Shake flasks	37	6.7	150 rpm	7 days	Nitrogen	9.3
(Chen <i>et al.</i> 2007)	5 l Fermenter, fed batch	37	7 initial; controlled at 6.8	N/A	N/A	Not specified	6.06
(Santa-Anna <i>et al.</i> 2002)	Shake flasks	30	7	120 rpm	7 days	Not specified	3.16
(Santos <i>et al.</i> 2002)	Shake flasks	30		170 rpm	6 days	Not specified	3.34

## 1.7 Process operation

In this section, examples from literature of different process operations will be given for the production of rhamnolipids from *Pseudomonas aeruginosa*, which is what this study will focus on.

### 1.7.1 Mode of operation

Not many bioreactor studies on rhamnolipid production could be found in literature, with most being restricted to shake flasks (listed in Table 3). High production of rhamnolipid of 8.5 g/l and 12.8 g/l were obtained during batch culture on n-alkanes and n-paraffin respectively (Table 4) (Itoh *et al.* 1971, Syldatk *et al.* 1985b). Few shake flask studies could be found with hydrocarbons as carbon source, and in those cases rhamnolipid yields were small (0.7 g/l to 1.3 g/l) compared to the bioreactor studies in Table 4 (Wei *et al.* 2005). It is possible that the increased oxygen transfer in bioreactors is necessary for high rhamnolipid production, since the uptake of hydrocarbons is an energy dependant process (Beal and Betts 2000) and the required oxygen transfer rate is frequently higher for hydrocarbon bioprocesses (Clarke *et al.* 2006). The oxygen transfer would be especially important at such high concentrations of hydrocarbons (8 % – 10 %). High carbon concentrations have been shown to promote production of rhamnolipids (section 1.6.1.1).

In the studies that compared batch to fed batch fermentations (Benincasa *et al.* 2002, Chen *et al.* 2007), fed batch fermentation led to higher rhamnolipid production. Benincasa *et al.* (2002) added 1 % carbon source and 2g/l NaNO<sub>3</sub> after 48h, which increased rhamnolipid production from 8.5 g/l to 15.9 g/l with 70 % total conversion of the substrate. Chen *et al.* (2007) obtained 0.76 g/l more rhamnolipid in a fed-batch setup (where glucose was not inhibitory) as compared to batch fermentation. However, when comparing batch and fed-batch studies in general (Table 4), the range of rhamnolipid concentrations for batch and fed-batch fermentations is too wide to state conclusively that fed-batch will lead to better production.

### 1.7.2 Fermentation operating conditions

The temperature and pH used in the bioreactor studies listed in Table 4 are similar to those used in shake flask studies (Table 3). The optimal temperature for shake flask studies seemed to be between 30 °C and 37 °C, which is the temperature range for the bioreactor studies. The pH used in bioreactor studies is more or less neutral, with most of the studies controlling the pH at 6.8. The incubation times for the bioreactor studies ranged between 2.3 and 7.5 days, which is shorter than the shake flask studies (6 to 12 days). The increased oxygen transfer possibly accelerated growth.

Biosurfactant fermentations usually lead to heavy foaming, which can hinder transport of oxygen into medium and require large amounts of antifoam agents which also decrease oxygen transfer. Chen *et al.* (2007) found that an increase from 250 rpm to 500 rpm led to excessive foaming and decrease in productivity. However, they did not report on the effect of the aeration

rate, but used a constant rate of 1 vvm (volume per volume per minute). Itoh *et al.* (1971) also used an aeration rate of 1 vvm and Syldatk *et al.* (1985b) a slightly lower rate of 0.75 vvm. Benincasa *et al.* (2002) used an aeration rate of 2.5 vvm and a high agitation rate of 500 rpm. When they increased the agitation rate from 500 rpm to 800 rpm their substrate conversion rate increased from 14 % to 33 %. They concluded that a higher oxygen transfer rate leads to higher rhamnolipid production. A high rate was possible in this study, because no problems with foaming were reported. However, aeration conditions will have to be monitored carefully to ensure a high enough oxygen transfer rate as well as keeping foaming under control.

Table 4: Fermentor studies from literature on the production of rhamnolipid from *Pseudomonas aeruginosa*.

Reference	Fermentor size	Temperature [ C]	Initial pH	pH control	Agitation	Aeration	Volume of culture	Incubation time	Carbon source	Percentage carbon source	Solution added (fed batch)	Max Biosurf prod [g/l]	Specific formation
(Itoh <i>et al.</i> 1971)	30 l (batch)	30	6-6.5		450 rpm	1 vvm	15 l	55 h	n-paraffin (C12-C14)	10 %		8.5	
(Syldatk <i>et al.</i> 1985b)	20 l (batch)	30	6.8	10 % NH <sub>4</sub> OH in growth phase and 10 % NaOH later	1000 rpm	0.75 vvm		180 h	n-alkanes	8 %		12.8	630 mg/g
(Chen <i>et al.</i> 2007)	5 l (batch)	37	7	6.8	500 rpm	1 vvm	2 l	144 h	glucose	4 %		5.3	2.2 mg/g
	5 l (fed batch)	37	7	6.8 by Controlled addition of glucose	250 rpm	1 vvm	800 ml, final 2 l		glucose	1 %	6 % carbon source added	6.06	2.3 mg/g
	5 l ( fed batch)	37	7	6.8 by Controlled by NaOH and HCl	250 rpm	1 vvm	800 ml, final 2 l		glucose	1 %	6 % carbon source added at 400 ml per day	3.04	1.3 mg/g
(Benincasa <i>et al.</i> 2002)	2 l (batch)	30	6.8	6.8 by NaOH and HCl (both 2N)	500 and 800 rpm	2.5 vvm	1.2 l	80 h	soapstock	2.5 %		8.5	

Reference	Fermentor size	Temperature [ C]	Initial pH	pH control	Agitation	Aeration	Volume of culture	Incubation time	Carbon source	Percentage carbon source	Solution added (fed batch)	Max Biosurf prod [g/l]	Specific formation
	2 l (fed batch)	30	6.8	6.8 by NaOH and HCl (both 2N)	800 rpm	2.5 vvm	1.2 l	84 h	soapstock	2.5 %	50 ml of aqueous solution with 1 % soapstock and 2g/l NaNO <sub>3</sub> after 48h	15.9	

## 1.8 Hypotheses

Based on the study of the literature sources above, the following hypotheses were constructed.

### 1. Development of analyses

- HPLC can be used to accurately measure rhamnose sugar concentrations.
- The orcinol method with sample extraction can be used to measure rhamnose sugar concentrations.
- The minimum surface tension and CMC can be used to characterise biosurfactant solutions.
- The disk diffusion method or soft agar overlay can be used for antibacterial testing of rhamnolipids.
- The disk diffusion method can be used for antifungal activity testing of rhamnolipids.

### 2. Antimicrobial activity of rhamnolipids

- Rhamnolipids from *Ps. aeruginosa* ATCC 9027 and ATCC 27853 have antibacterial activity against *Mycobacterium aurum* and antifungal activity against *Botrytis cinerea*. The antimicrobial activity is directly related to the rhamnolipid concentration.

### 3. Selection of media

- Rhamnolipid production is promoted by selecting media that fulfill the following requirements.
  - A nitrogen or phosphate limitation
  - Adequate pH buffering around neutral pH
  - High concentration of carbon source (larger than 2%)
- Rhamnolipid production increases with increasing substrate concentration.

### 4. Affinity of strains for hydrophilic and hydrophobic carbon sources media

- *Pseudomonas aeruginosa* strains which naturally have a more hydrophobic membrane will be better at utilising hydrophobic substrates. The same is true for hydrophilic substrates. Strain ATCC 9027 is reported to have a more hydrophilic membrane and will therefore be better suited for hydrophilic substrates such as glucose. Strain ATCC 27853 has a more hydrophobic membrane and will be the superior utiliser of hydrophobic substrates such as alkanes. Strain 2bf, which was isolated from oil contaminated soil, will be better adapted for the utilisation of alkanes.

### 5. Associated growth phase of rhamnolipids

- Rhamnolipids are associated with the stationary phase of growth. Their production is triggered by the exhaustion of a nutrient (phosphate or nitrogen).



**6. Methods for enhancing growth on hydrocarbon media**

A dual substrate system containing glucose and hexadecane as carbon sources would enhance the growth of *Pseudomonas aeruginosa* on hexadecane similar to that observed with exogenous rhamnolipid addition to hydrocarbon cultures, through rhamnolipid production via glucose utilisation.

**7. Location of rhamnolipids**

- Rhamnolipids are products which are found predominantly in the extracellular fluid.

**8. Bioreactor fermentations**

- Shorter cell and rhamnolipid production times can be achieved under controlled conditions in a bioreactor when compared to those in shake flasks.
- Higher cell and rhamnolipid concentrations can be achieved under controlled conditions in a bioreactor when compared to those in shake flasks.

## 2 Materials and methods

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### 2.1 *Micro-organisms*

Three *Pseudomonas aeruginosa* strains were investigated for biosurfactant production (Table 5). A novel organism of South African origin *Ps. aeruginosa* 2bf was isolated from oil contaminated local soil and kindly provided by Prof S Reid (Department of Molecular and Cell Biology at the University of Cape Town). Two culture collection strains, *Ps. aeruginosa* ATCC 9027 and ATCC 27853, were obtained from the American Type Culture Collection (Rockville, Md.).

Although many *Ps. aeruginosa* strains have been used for rhamnolipid production, very few studies could be found that utilised a culture collection strain, nearly all strains being of a local origin. *Ps. aeruginosa* ATCC 9027 was used in this research, because it is a culture collection strain that has been shown to produce mono-rhamnolipid (one rhamnose molecule linked to a chain of two fatty acid molecules) (Zhang and Miller 1994).

As was detailed in section 1.4.1, different *Ps. aeruginosa* strains have different affinity for hydrophilic and hydrophobic substrates. *Ps. aeruginosa* ATCC 9027 has a naturally hydrophilic cell membrane and is a slow degrader of alkanes (Zhang and Miller 1995). This strain would therefore be more suited for studies on hydrophilic substrates such as glucose. However, in the study presented here, the possibility of alkanes as substrate was also investigated. *Pseudomonas aeruginosa* ATCC 27853 was therefore chosen, because it has been shown to be a fast hydrocarbon degrader (Zhang and Miller 1995). No studies could be found that investigated whether this strain produces rhamnolipids.

The antimicrobial activity of rhamnolipids formed part of this study. *Mycobacterium aurum* and *Botrytis cinerea* were used as target organisms and were kindly provided by Prof S Reid (Department of Molecular and Cell Biology at the University of Cape Town).

### 2.2 *Culture maintenance and storage*

For long term storage, cultures were stored in 30 % (v/v) glycerol at -20 °C. Cultures were recovered from the frozen stocks by transferring a loopfull to 25ml of nutrient broth and incubating for 48 hours at the temperature listed in Table 5 for the respective organisms. Plates were prepared from the broth and subcultures made for two to three passages. Finally the cultures were transferred to slants, which were stored in the refrigerator for up to 3 months.

For glucose experiments Nutrient agar was used as solid media. For alkane based experiments MSM+PO<sub>4</sub> medium (Table 10) supplemented with 1 % hexadecane and 1.5 % agar was used.

**Table 5: List of organisms and temperature of incubation.**

Organism	Temperature [°C]
<i>Pseudomonas aeruginosa</i> 2bf	30
<i>Pseudomonas aeruginosa</i> ATCC 9027	37
<i>Pseudomonas aeruginosa</i> ATCC 27853	37
<i>Mycobacterium aurum</i>	37
<i>Botrytis cinerea</i>	28

## 2.3 Culture media

### 2.3.1 Media for *Pseudomonas aeruginosa* culture

Two different media were selected from literature for *Pseudomonas aeruginosa* biosurfactant production studies as a basis for media studies. The first was a minimal salts medium (MSM), which was optimised for rhamnolipid production according to literature (Sim *et al.* 1997). This medium satisfies many of the conditions reported for good rhamnolipid production (see section 1.6.1). It has a large carbon to nitrogen (C/N) ratio, contains sodium nitrate as the inorganic nitrogen source as well as small amounts of sulphates and iron and is limited in nitrogen. The MSM medium was modified from literature to contain 1.2 % carbon (equivalent to 3 % glucose) instead of 6 % carbon source. The literature medium used vegetable oils as carbon source whereas glucose was primarily used in this study as carbon source. The nitrogen and phosphate concentrations were adjusted in the same ratios as in the original medium to maintain the same C/N and C/P ratios. The modified MSM medium components are listed in Table 6.

**Table 6: Minimal salts medium (MSM) composition**

Component	Concentration [g/l]
Glucose or Hexadecane	30
NaNO <sub>3</sub>	14.15
yeast extract	3.6
KCl	0.5
NaCl	1.1
FeSO <sub>4</sub> .7H <sub>2</sub> O	1.1
MgSO <sub>4</sub> .7H <sub>2</sub> O	0.00028
	0.5

In experiments conducted with one carbon substrate hexadecane or glucose was used in the MSM medium. The alkane based media contained the same concentration of components as their glucose counterparts, except that the concentration hexadecane is adapted so that the C/N and C/P ratios remained the same. It was decided to adjust the carbon concentration rather than the nitrogen and phosphate concentrations, in order to establish a basis for comparison between glucose and alkane based experiments. The

hexadecane was filtered through a sterile 0.22 µm Millex GP syringe filter (Millipore) and added aseptically after sterilisation to the cooled medium.

Dual substrate experiments were also conducted in MSM medium with glucose and hexadecane as carbon sources. These experiments contained the same amount of carbon as sole substrate experiments. Dual substrate flasks were inoculated from cultures containing glucose as sole carbon source. The different dual substrate systems with the percentage of each substrate present is listed in Table 7.

**Table 7: Different dual substrate setups used with concentrations of glucose and hexadecane and the label used in results section.**

Label	Description	Amount of glucose	Amount of hexadecane
1/4	Quarter of carbon is glucose	0.75 %	1.06 %
2/4	Half of carbon is glucose	1.5 %	0.707 %
2/4 Sequential	Half of carbon is glucose with hexadecane added upon consumption of glucose	1.5 %	0.707 %

In order to prevent a precipitate from forming in the MSM medium, sulphates ( $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  and  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ) and yeast extract were autoclaved separately from other components and combined aseptically after cooling. The MSM medium contained a trace element solution (Table 8), of which 5 ml was added to each litre of MSM medium. The trace element solution was prepared separately and filter sterilised through a sterile 0.22 µm Millex GP syringe filter (Millipore), since autoclaving would cause degradation of heat labile compounds .

**Table 8: MSM medium trace element solution composition**

Component	Concentration [g/l]
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.29
$\text{CaCl}_2 \cdot 4\text{H}_2\text{O}$	0.24
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.25
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	0.17

The second medium used as a basis in media selection experiments was proteose peptone-glucose-ammonium salts medium (PPGASM), which is a phosphate deficient medium that has been used successfully in the production of rhamnolipids from *Ps. aeruginosa* ATCC 9027 (Al-Tahhan *et al.* 2000, Mulligan and Gibbs 1989, Zhang and Miller 1992). This medium was shown to be limited in phosphate. The basic composition of this medium is listed in Table 9.

**Table 9: Proteose peptone-glucose-ammonium salts medium (PPGASM) composition**

Component	Concentration [g/l]
Glucose	5
NH <sub>4</sub> Cl	1
Peptone	10
KCl	1.5
MgSO <sub>4</sub>	0.4

The MSM and PPGASM media were each examined with two different buffers, a phosphate buffer and a tris (tris(hydroxymethyl)aminomethane) buffer. Tris buffer is an amine based buffer that contains no phosphate. The buffer concentrations and the derived media are listed in Table 10. The final pH of media containing a phosphate buffer was approximately 6.6. The pH of all media containing tris buffer was adjusted to 7.2 with concentrated HCl (buffering range of pH 7 – 9). The pH of tris buffer is very sensitive to temperature, so all pH measurements and pH adjustments were done at incubation temperature in tris buffer containing media.

**Table 10: Derived media using different buffer combinations**

Basic medium	Buffer [g/l]	Derived medium
MSM (Table 6)	KH <sub>2</sub> PO <sub>4</sub> : 0.87 K <sub>2</sub> HPO <sub>4</sub> : 1.124	MSM+PO4
	Tris-HCl:14.54	MSM+Tris
PPGASM (Table 9)	KH <sub>2</sub> PO <sub>4</sub> : 0.87 K <sub>2</sub> HPO <sub>4</sub> : 1.124	PPGASM+PO4
	Tris-HCl:14.54	PPGASM+Tris

Table 11 shows a list of all the media used in biosurfactant production studies with their carbon to nitrogen (C/N) and carbon to phosphate (C/P) ratios. PPGASM media notably have much smaller C/N ratios. MSM medium containing no buffer was used in some experiments in addition to the buffer containing media.

**Table 11: Carbon to nitrogen and carbon to phosphate ratios for media used in *Pseudomonas aeruginosa* growth and biosurfactant production studies**

Medium	Total Carbon [%]	Total Nitrogen [%]	Total Phosphate [%]	C/N	C/P
MSM	1.2	0.06	0	18.64	
MSM+PO4	1.2	0.06	0.04	18.64	30.17
MSM+Tris	1.78	0.2	0	9.03	
PPGASM+PO4	0.2	0.18	0.04	1.11	5.03
PPGASM+Tris	0.78	0.35	0	2.23	

### 2.3.2 Inoculum media

Two different inoculum media were used. MSM+PO<sub>4</sub> medium was used to inoculate all MSM based media (Table 6). MSM based media containing no phosphate were also inoculated with MSM+PO<sub>4</sub> medium, so that traces of phosphate were present in the test culture. All PPGASM based media (Table 11) were inoculated with Kay's minimal medium, since this was the inoculum used for all PPGASM test cultures in literature (Mulligan and Gibbs 1989, Zhang and Miller 1992). The phosphates in Kay's minimal medium were autoclaved separately and added to the other components aseptically to prevent a precipitate from forming.

**Table 12: Kay's minimal medium composition**

Component	Concentration [g/l]
NH <sub>4</sub> H <sub>2</sub> PO <sub>4</sub>	3
K <sub>2</sub> HPO <sub>4</sub>	2
Glucose	2
FeSO <sub>4</sub>	0.0005
MgSO <sub>4</sub> .SO <sub>4</sub>	1

### 2.3.3 Media for *Mycobacterium aurum* and *Botrytis cinerea*

*Mycobacterium aurum* was grown in Nutrient broth or in the case of solid media, Nutrient agar (both Biolab).

*Botrytis cinerea* was grown on Potato dextrose broth and Potato dextrose agar (PDA) (both Biolab).

## 2.4 *Pseudomonas aeruginosa* liquid culture conditions

### 2.4.1 Inoculum development

Two different inoculum media were used as was described in section 2.3.2. MSM+PO<sub>4</sub> medium was used to inoculate all MSM based media (Table 6) and Kay's minimal medium was used to inoculate all PPGASM based media. All inocula were made in 250 ml flasks containing 50 ml medium and incubated at 35 °C and 200 rpm for 24 h, except where mentioned otherwise. An inoculum size of 10% (v/v) was transferred to test flasks, when MSM+PO<sub>4</sub> was used as inoculum medium. For PPGASM media inoculation 1 % (v/v) of Kay's minimal medium was used, following the procedure outlined in literature (Mulligan and Gibbs 1989, Zhang and Miller 1992).

For glucose based experiments glucose was the sole carbon source in the inoculum and cultures were transferred to the inoculum from nutrient agar solid media. For alkane experiments hexadecane was the sole carbon source in the inoculum medium and cultures were transferred from alkane solid media (section 2.2), except where mentioned otherwise.

### 2.4.2 Test flask setup and operating conditions

Test cultures were carried out in duplicate with cultures drawn from the same inoculum flask for each duplicate set of test flasks. Shake flask experiments were conducted in 500 ml flasks containing a total of 150 ml liquid medium (including inoculum). Flasks were incubated at 35 °C with shaking at 200 rpm in an orbital shaker (Labcon). Glucose media were inoculated with cultures containing glucose as sole carbon source. Hexadecane test flasks were inoculated with cultures containing hexadecane as sole carbon source (see previous section).

Different conditions were used for the experiments done on the development of analytical methods. In these experiments *Ps. aeruginosa* ATCC 9027 was cultured following the protocol for mono-rhamnolipid production as described by Zhang and Miller (1992). The inoculum was transferred to 200 ml of PPGASM+Tris medium in 500 ml flasks. Duplicate flasks were incubated at 37 °C with shaking at 220 rpm.

### 2.4.3 Bioreactor setup and operating conditions

Fermentation was conducted in a 7.5 l Bioflo 110 modular benchtop fermentor with a working volume of 2l (Figure 2). The reactor was operated with one impeller, because of the low working volume. The following operating conditions were selected initially.

- 0.8 vvm air flowrate.
- Agitation controlled to maintain a 20 % dissolved oxygen level (between 300 rpm and 700 rpm). The dissolved oxygen was measured with a Mettler-Toledo oxygen probe.
- Temperature controlled at 35 °C.
- pH controlled at 6.8 with addition of 2M NaOH.

The inoculum was prepared as described in 2.4.1 for MSM media, except that a culture volume of 200 ml was used in a 3 l flask. The flask had a bottom exit tube port which was clamped off and protected with foil until inoculation.

The fermentation was conducted with MSM+Tris medium and prepared as described in section 2.3.1. A volume of 1.8 l MSM+Tris medium was made, which amounted to a total volume of 2 l in the reactor after inoculation.





**Figure 2: Equipment setup for bioreactor experiments.**

The detailed procedure for sterilisation prior to the fermentation is outlined below. The procedure is outlined in detail, because a rigorous approach was found to be necessary for successful and uncontaminated reactor runs.

- Before each run the dissolved oxygen probe (Mettler-Toledo) was inspected for damage and the membrane replaced according to the instructions in the leaflet.
- The bioreactor was cleaned with hot water and soap and all surfaces wiped with ethanol.
- All o-rings and threads were lubricated with grease before insertions. All adapters were only finger tightened.
- The glass vessel was lowered carefully with the opening of the baffles over the cooling coils. The bolts were tightened so that even pressure was exerted on the rubber bumpers.
- The reactor was filled with 3 l of tap water for sterilisation. All ports immersed in water were clamped off with the rest left open. No probes were inserted.
- The bearing housing cap was lubricated and the cap placed loosely over the motor.
- The assembly was autoclaved for 25 minutes.



After sterilisation the medium was prepared and the following preparations were done prior to medium sterilisation.

- The reactor was emptied and the medium added. All bolts and adapters were tightened as described above.
- The pH probe was inspected for damage and carefully inserted. No interference was present between probes and baffles and cooling coils.
- The sampling tube assembly was connected to the addition port and the sampling bottle tightened loosely.
- The dissolved oxygen probe (Mettler-Toledo) was inserted.
- Filters for the exhaust port and sparger were inspected and tested for blockage. These filters were installed on the respective ports.
- Two solutions for addition through the pump module (2 % Antifoam A and 2M NaOH) were attached to the addition ports with enough silicon tubing to reach the pump module.
- The air feed was connected and the system checked for leaks with soapy water.
- The pH probe was calibrated using pH 4 and pH 7 buffer solutions (Metrohm).
- All air and water lines and probes were disconnected.
- All other tubes were clamped off except the exhaust port filter.
- All filters and probes were wrapped with non-absorbant cotton wool and a protective cap of aluminium foil.
- The assembly together with the addition bottles were autoclaved for 30 minutes.

After sterilisation of the medium the system was reconnected as follows.

- All probes, air lines and water feed lines were connected.
- Glycerin was added to the thermowell and the temperature probe was inserted.
- The exhaust condenser was fitted with a cooling water loop on a separate pump system.
- The heat jacket was wrapped around the reactor and plugged in.
- The dissolved oxygen probe was polarised by plugging it into the controller and leaving on for 6 hours. This measure is necessary for all polarographic probes.
- The temperature control loop was set up at 35 °C and the medium was allowed to reach the temperature setpoint.
- pH calibration was confirmed by taking a sample aseptically and measuring pH with an external pH meter (Metrohm). The zero of the controller was adjusted accordingly. This was done after the medium had reached 35 °C, because tris buffer is sensitive to temperature.
- The dissolved oxygen probe was calibrated (after polarisation) by sparging with nitrogen until raw input stabilised and sparging with oxygen afterwards. Both were done at the flowrate used in the fermentation.

- The inoculum was added by connecting the bottom feed port of the inoculum flask with the addition port tube of the reactor. This was done quickly and tubes were clamped off and immersed in ethanol afterwards for aseptic transfer of culture.

## 2.5 Analytical methods

### 2.5.1 Cell concentration

Cell counts with a Helber bacterial counting chamber (Hawksley) were used initially as a method for determining cell concentration. Two or three layers of cells were visible which made precise counting very difficult and led to large errors in data. For the results reported later, optical density was used instead as an indirect measurement of cell concentration. Optical density was measured using a spectrophotometer at 620 nm (Varian). The samples for spectrophotometer measurements were prepared by the following method:

- A 1 ml culture sample was pipetted into a microcentrifuge tube and the cells separated by centrifugation (Eppendorf Minispin Plus, 10 000 rpm for 10 min).
- The cell pellet was resuspended in 1 ml physiological saline (0.85 % (m/v) aqueous NaCl).
- The tube was vortexed until the solids were resuspended.
- The tube was centrifuged for 10 min at 10 000 rpm, resuspended and vortexed as in the previous two steps.
- Optical density readings were taken against a blank of physiological saline, in 1.5 ml glass cuvettes (Hellma).

### 2.5.2 Glucose concentration

Glucose concentration was measured by dinitrosalicylic acid (DNS) analysis (Miller 1959), with modifications to concentrations and cooling method (A. Lind, personal communication). In this method the presence of reducing sugars is indicated by an orange colour as a result of the reduction of 3,5-dinitrosalicylic acid to 3-amino-5-nitrosalicylic acid. The components necessary to prepare the reagent are shown in the table below.

**Table 13: Composition of dinitrosalicylic acid reagent**

Compound	Amount [g]
3,5-dinitrosalicylic acid	5.30
Rochelle salts	153.00
Sodium hydroxide	9.90
Sodium metabisulphite	4.15
Phenol	3.80

The procedure for the analysis was as follows.

- All of the components listed in Table 13 (except the phenol) were dissolved in 708 ml distilled water.
- Phenol was melted at medium heat in a microwave and added to the solution.
- The solution was stored in a dark cupboard, because of light sensitivity.
- For each analysis supernatant from the culture was taken and diluted to contain a maximum of 2 g/l glucose.
- 200 µl of the diluted solution was put into a test tube and 600 µl of DNS reagent added.
- A test tube containing 200 µl distilled water and 600 µl DNS reagent was also made for each run to serve as the zero for the spectrophotometer.
- All tubes were placed in boiling water on a hotplate for 5 minutes.
- After 5 minutes tubes were removed and placed in ice water for 5 minutes.
- 8.2 ml of distilled water was added to each test tube.
- The optical density was measured on a spectrophotometer (Varian) at 523 nm wavelength in 1.5 ml glass cuvettes (Hellma). A zero reference point was established with the zero solution.
- A standard curve was constructed from glucose concentrations between 0 and 1 g/l. The glucose samples were prepared by adding the same components as for the culture samples. The concentrations of culture samples were determined from the obtained standard curve.

The standard curve used in this study is included in Appendix B. A regression coefficient ( $R^2$ ) of 0.999 was obtained.

### 2.5.3 Biosurfactant concentration

#### 2.5.3.1 HPLC analysis

High performance liquid chromatography (HPLC) was used to measure rhamnolipid concentrations. The rhamnolipid molecules were hydrolysed to release the rhamnose molecules, which were analysed by HPLC. Two different hydrolysis methods were investigated. The first hydrolysis method was adapted from the hydrolysis step of the orcinol method (Koch *et al.* 1991).

- 100 µl of culture supernatant was transferred to a microcentrifuge tube and 900 µl of a 53 % sulphuric acid solution was added.
- The tubes were inserted into a floating rack and were kept in boiling water on a hotplate for 30 min.

The second hydrolysis method is a general method for the rupture of oligosaccharides (Sluiter *et al.* 2005).

- 115 µl of a 53 % sulphuric acid was added to 2 ml of supernatant in a bijou bottle.
- The solution was autoclaved at 121 °C for 30 min and allowed to cool to room temperature.

The hydrolysed sample was treated to remove proteins, since proteins present in the supernatant can damage the HPLC column (E. Viljoen, personal communication).

- 1 ml of hydrolysed sample was centrifuged at 14 000 rpm (Eppendorf Minispin Plus) for 5 minutes.
- 31.5 µl of a 60 % perchloric acid solution was added to 900 µl of the supernatant.
- The sample was incubated on ice for 10 minutes.
- 49.5 µl of a 7M KOH solution was added to the sample.
- The sample was incubated on ice for 10 minutes.
- The sample was centrifuged at 14 000 rpm for 5 minutes.
- Each sample was filtered using a 0.22 µm syringe filter (Millipore).

The samples and L-rhamnose standards were filtered through a 0.22 µm syringe filter (Millipore) prior to injection in the column. Settings for the column are listed in Table 14 . Standards containing L-rhamnose and glucose with concentrations between 100 and 5000 ppm were injected to construct a standard curve.

**Table 14: HPLC settings**

Detector:	Shodex RI-101 Refractive Index detector
Column:	Rad Aminex HPX-87H Ion Exclusion Column
Column dimensions:	300 mm x 7.8 mm
Mobile phase:	5mM H <sub>2</sub> SO <sub>4</sub>
Flow rate of mobile phase:	0.6 ml/min

### 2.5.3.2 Colorimetric orcinol analysis

Samples were extracted before reaction with orcinol reagent by the following procedure.

- 500 µl of culture broth was centrifuged and 400 µl of supernatant removed.
- 750 µl diethyl ether was added.
- The mixture was vortexed for 3 min.
- The organic top layer was removed carefully with a micropipette (Socorex) and transferred to a microcentrifuge tube, without removing any of the aqueous phase.
- The solvent addition and extraction were repeated twice.
- Ether fractions were pooled and left to evaporate in air for 8 hours.

- pH 8 phosphate buffer was used to redissolve the precipitate left in the microcentrifuge tubes. The amount of buffer added was adjusted according to the dilution desired (400 µl of buffer added for an original sample volume of 400 µl would equal a 1x dilution).

Extracted samples were analysed for rhamnose concentration by the orcinol method (Koch *et al.* 1991).

- The orcinol reagent was prepared by adding concentrated sulphuric acid (98 % w/w) and 0.19 % orcinol (3,5-dihydroxytoluene) to distilled water. The final concentration of acid was 53 % w/w. For a total of 20 ml reagent, 12 ml distilled water and 8 ml sulphuric acid were added.
- 900 µl of the orcinol reagent was added to 100 µl of sample.
- Blanks of 100 µl of pH 8 phosphate buffer were also treated with orcinol reagent.
- Standard solutions of L-rhamnose between 0 and 100 mg/l were prepared, also in a pH 8 phosphate buffer.
- The sample tubes (samples, blanks and standards) were inserted in a floating rack and placed in boiling water on a hotplate for 20 min.
- The solutions were left in a dark cupboard for 35 min to cool to room temperature.
- The optical density was measured with a spectrophotometer (Varian) at 421 nm, after a zero point was established with the blanks.
- A standard curve was drawn up from the L-rhamnose solutions and the concentrations of rhamnose in the samples calculated accordingly. A standard curve was constructed for each batch of samples, because fresh reagent was used in each batch of analyses. Examples of standard curves are included in Appendix B.

## 2.5.4 Biosurfactant surface activity

### 2.5.4.1 Surface tension

The tensiometer (du Noüy ring type) used for surface tension measurements consisted of a platinum ring which was suspended from a precision balance connected to a scale measuring the force exerted (Newton meter). Figure 3 shows a graphical representation of the equipment setup. The surface tension was determined by the following method:

- A sample of 6 ml was obtained for each surface tension measurement and poured into a small container or beaker.
- Samples were raised with the tensiometer sample carrier platform until the ring was fully submerged in the liquid.
- The platform was then lowered and the ring was raised by increasing the tension in the balance. This was done simultaneously to ensure that the precision balance remained at the same horizontal angle.

- The liquid film beneath the ring was stretched until a maximum force was reached and the ring had detached from the liquid. The force measured at this point was recorded.
- The tensiometer was calibrated with distilled water, which has a known surface tension. The calibration was done by first setting the force scale to zero when the balance is aligned horizontally and then measuring the maximum force exerted by distilled water, as described in the previous steps. The relationship between the force measurement and the surface tension of distilled water (72 mN/m) was used to determine the surface tension of all other samples.

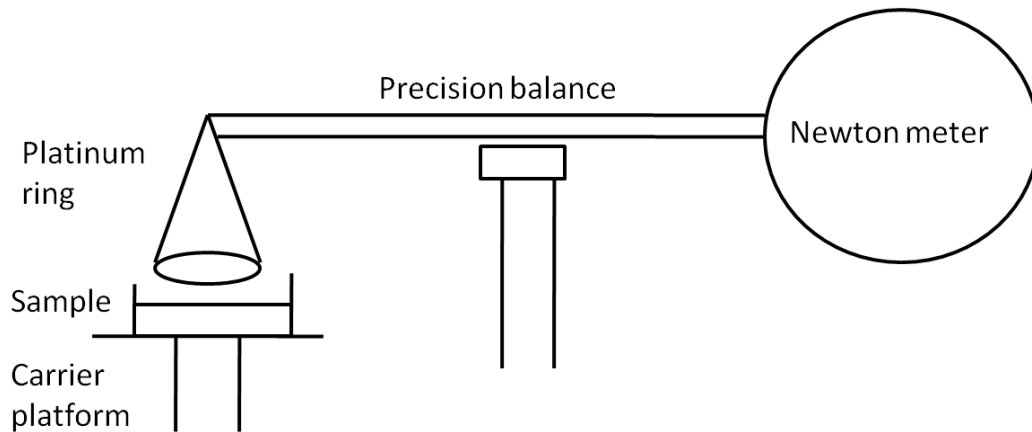


Figure 3: Diagram of tensiometer setup for surface tension measurements.

#### 2.5.4.2 Critical micelle concentration

In order to determine the critical micelle concentration (CMC), supernatant from the culture broth was serially diluted with phosphate buffer (pH 7). The surface tensions of the serial dilutions were determined. The log of the concentration was plotted against the surface tension and the point where the surface tension increased abruptly was registered as the critical micelle concentration (Cooper *et al.* 1978).

#### 2.5.5 Antimicrobial activity

##### 2.5.5.1 Determination of antibacterial activity by disk diffusion method

The standard disk diffusion method (Jorgensen and Turnidge 2003) was conducted by growing an even lawn of target organism on hard agar and placing disks impregnated with antimicrobial solution on the surface.

- *Mycobacterium aurum* was transferred from solid media (Nutrient agar) to 50 ml nutrient broth and incubated for 48h at 37 °C and 200 rpm.
- The culture was diluted to obtain an optical density in the range of 0.08 and 0.1 as measured by a spectrophotometer (Varian) at 621 nm.
- A further dilution of 1:10 was made of this solution.

- Plates of Mueller Hinton agar were evenly streaked in three different directions with a sterile cotton swab dipped in the bacterial suspension.
- The biosurfactant containing supernatant was filter sterilised with a 0.22 µm syringe filter (Millipore).
- Sterile round filter paper disks (5 mm diameter) were dipped in the filtered solution and placed evenly on the agar surface (2 per plate) with a pair of sterile forceps.
- A disk that had been immersed in sterile physiological saline (0.85 % w/w NaCl) was also placed on each plate as a control.
- The plates were incubated at 37 °C. The widths of inhibition zones, including the disk diameter, were measured to the nearest whole millimeter after 48h.

#### **2.5.5.2 Determination of antibacterial activity by soft agar overlay method**

The soft agar overlay method was modified from the method of (du-Toit and Rautenbach 2000).

- *Mycobacterium aurum* was cultured on solid nutrient agar plates and incubated for 48h at 37 °C.
- Colonies were then suspended by loop transfer directly in sterile physiological saline (0.85 % w/w NaCl) to obtain an optical density of 0.3 as measured by a spectrophotometer (Varian) at 621 nm.
- The cut off back ends of sterile blue pipette tips were evenly placed vertically in Petri dishes (three per plate) such that the lid of the Petri dish could still fit comfortably over the tips.
- 2 ml of the bacterial suspension was added to 10 ml of sterile soft nutrient agar (nutrient broth with 0.75 % agar) and dispersed by using a vortex.
- This solution was carefully poured into the Petri dish containing the positioned tips.
- The biosurfactant containing supernatant was filter sterilised with a 0.22 µm syringe filter (Millipore).
- 40 µl of the filtered solution was pipetted into two of the wells and 40 µl of sterile physiological saline (0.85 % w/w NaCl) to the third (control).
- The plates were incubated upright at 37 °C for 48 h.
- The inhibition zone widths were measured as the difference between the total inhibition zones and the well diameter.

#### **2.5.5.3 Determination of antifungal activity**

In order to determine the activity against the fungus *Botrytis cinerea* a different procedure was followed than in the antibacterial analysis, because the fungus is cultured differently (S. Reid, personal communication).

- Mycelia from the growth on solid media were spotted onto a plate of PDA.
- A hole was punched with the back of a sterile blue pipette tip away from the mycelial plugs.

- The biosurfactant containing supernatant was filter sterilised with a 0.22 µm syringe filter (Millipore).
- 40 µl of filtered solution was pipetted in the hole.
- The plate was incubated at 28 °C.
- The diameter of the inhibition zone was measured after 48 hours or more if growth had not reached the hole.

### 2.5.6 Nitrate and phosphate concentrations

Nitrate and phosphate concentrations were measured with an ionchromatograph (Dionex Series 4500i) equipped with a conductivity detector. Supernatant (volume of 10 ml) was filtered through a 0.22 µm syringe filter (Millipore) and injected into the column. The following settings were used.

**Table 15: Settings for Dionex ionchromatograph used for nitrate and phosphate analyses.**

Column:	Dionex IonPac AS4A-SC 4 mm
Guard column:	Dionex IonPac AG4A-SC 4 mm
Eluant:	1.80mM Na <sub>2</sub> CO <sub>3</sub> , 1.70mM NaHCO <sub>3</sub>
Eluant flow rate:	1.0 ml/min
Suppressor:	Anion MicroMembrane
Regenerant:	25mM H <sub>2</sub> SO <sub>4</sub>
Regenerant flow rate:	3ml/min



## 3 Results and discussion

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### 3.1 Development of analytical methodology for biosurfactant analyses

Two methods to quantify of biosurfactant measurement were investigated, i.e. the measurements of rhamnose concentration and surface tension activity. The concentration of rhamnose was determined by the orcinol analysis (a colorimetric method), and HPLC.

Assays for antimicrobial activity against bacteria and fungi were established. Activity against the bacterium *Mycobacterium aurum* was tested by using two different methods, i.e. the standard disk diffusion method and the soft agar overlay method (section 3.1.2). Activity was also tested against the fungus *Botrytis cinerea* using the disk diffusion method (section 3.1.2).

The biosurfactant analyses were developed using supernatant from *Ps. aeruginosa* ATCC 9027. Since the exact structure of the biosurfactant produced from *Ps. aeruginosa* ATCC 9027, as well as the conditions for its production have been documented in literature, this organism provided a useful bench mark for developing analyses. This strain produces a mono-rhamnolipid (rhamnolipid with one rhamnose molecule attached to a lipid molecule) exclusively, in contrast to other *Pseudomonas* sp. which normally produce mono- and di-rhamnolipid in varying proportions (section 1.4). Mono-rhamnolipid was produced under the culture and process conditions described in section 2.4.2.

#### 3.1.1 Development and modification of analytical procedures

##### 3.1.1.1 Direct measurement of rhamnose concentration with HPLC

The methods for direct measurements of biosurfactant concentrations were compared in the literature review (section 1.3.4.1). No reports could be found of HPLC used for the direct measurement of rhamnose concentrations, even though HPLC is commonly used for monomeric sugar measurements such as glucose concentrations. The advantages of using the HPLC method to quantify rhamnose are the accuracy of the results, the distinction between rhamnose and other reducing sugars and the absence of an extraction step. A calibration curve was established successfully on the HPLC for L-rhamnose on the Aminex column (section 2.5.3.1). Small concentrations of glucose were included in the standards to identify any residual glucose in the medium.

Prior to the injection in the column, the culture sample first had to be hydrolysed to rupture the link in the rhamnose-alkylic chain of the rhamnolipid molecule. No detailed protocol could be found in literature for this specific hydrolysis. Two hydrolysis methods used for other purposes were tested. One method was the acid hydrolysis step from the orcinol (colorimetric) method. The same concentration sulphuric acid and heating time was used

as described for this method (section 2.5.3.1). No noticeable peak for rhamnose was detected with this hydrolysis method. Also, the relatively large amount of acid added to the sample resulted in an acid peak outside the measuring range of the HPLC, which resulted in a pressure drop in the column when the sample was injected (chromatogram included in Figure A1, Appendix A).

Another hydrolysis method was tested, which was a general protocol for the hydrolysis of oligosaccharides. This method involved autoclaving solutions with dilute sulphuric acid (section 2.5.3.1). Clear peaks for rhamnose and glucose were obtained with this method. An average of 203.85 mg/l rhamnose was obtained for the duplicate flasks (Table 16). A very small peak corresponding to 32 ppm glucose was also detected (Figure A2, Appendix A). This indicated that the culture had used up practically all of the carbon by 60 h.

**Table 16: Rhamnose concentration as measured by HPLC in hydrolysed supernatant of *Pseudomonas aeruginosa* ATCC 9027**

	Rhamnose concentration [mg/l]		
	Sample 1	Sample 2	Average
Flask a	227.5	205.5	216.5±11
Flask b	200.7	181.7	191.2±9.5

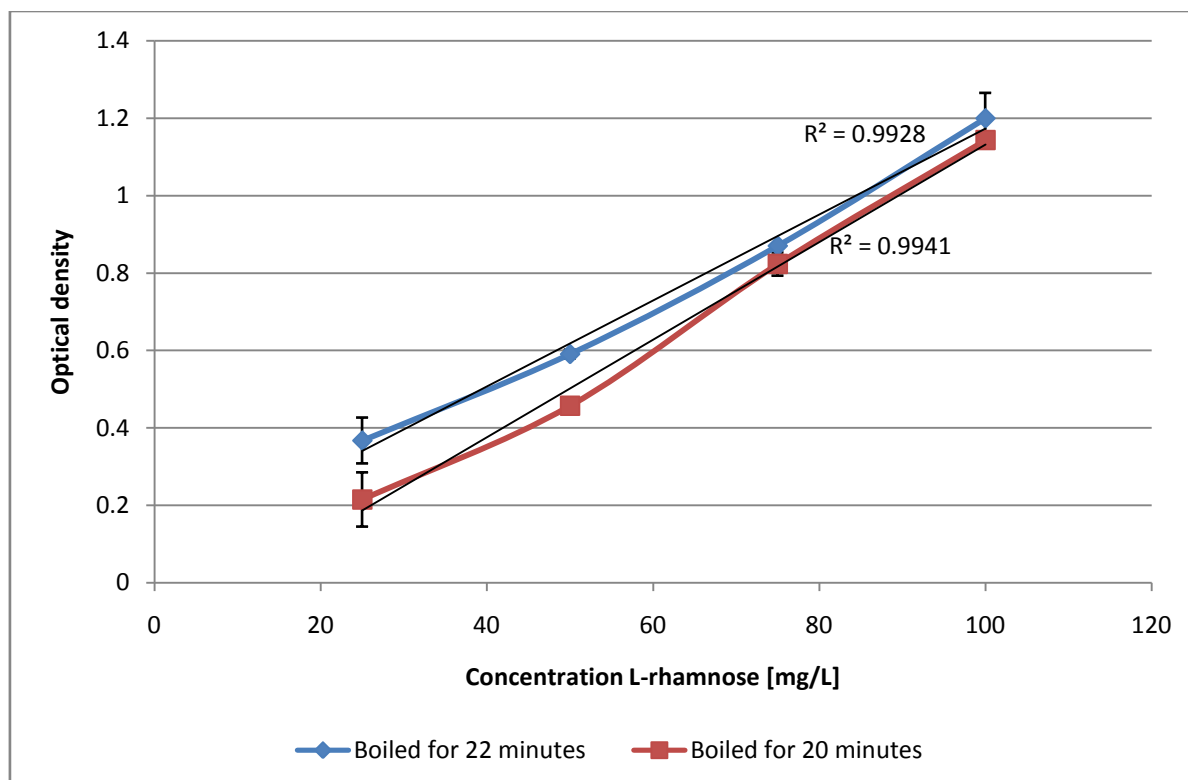
Characterisation of monorhamnolipids from *Ps. aeruginosa* ATCC 9027 resulted in an average molecular weight of 504 g/mol being assigned (Zhang and Miller 1994). With the molecular weight of rhamnolipid known the ratio of rhamnose weight to rhamnolipid weight could be calculated. Subsequently the average rhamnolipid concentration could be determined from the rhamnose concentration to be 625.9 mg/l.

### **3.1.1.2 Direct measurement of rhamnose concentration with the orcinol method**

An alternative method for the direct measurement of rhamnose concentration was established, because the HPLC became unavailable for further experiments. Colorimetric methods are the most common methods for measuring rhamnose concentrations (section 1.3.4.1). The orcinol method was chosen, because of the availability of equipment and chemicals. The protocols for the orcinol method differed greatly between reports. It was thus necessary to establish a protocol and determine the accuracy. The orcinol method involves boiling samples in a solution of sulphuric acid and orcinol reagent and measuring the optical density (section 2.5.3.2). The sensitivity of the spectrophotometer readings was determined as well as the influence of the purity of the sample (extracted or not) on the measurements.

Standard curves were established successfully using standard L-rhamnose solutions (Figure 4). The regression coefficient value ( $R^2$ ) obtained was 0.994, which showed that a linear relationship existed between the rhamnose concentrations and optical density. The orcinol method can therefore be used to determine rhamnose concentrations.

The sensitivity of the optical density measurements was investigated to ensure the accuracy of the method. The effect of the boiling time of the samples with orcinol-sulphuric acid reagent was investigated by boiling duplicate standard L-rhamnose solutions for 20 minutes and 22 minutes and subsequently determining the optical density. The analysis method is sensitive to a difference in reaction time, as seen in Figure 4. An average difference of 0.1 optical density was obtained for the 2 minute difference in boiling time. It is therefore important for reproducibility that exactly the same boiling time is used for all measurements or that a standard curve is constructed for each batch. In the following experiments of this study, a standard curve was constructed for each batch of samples.

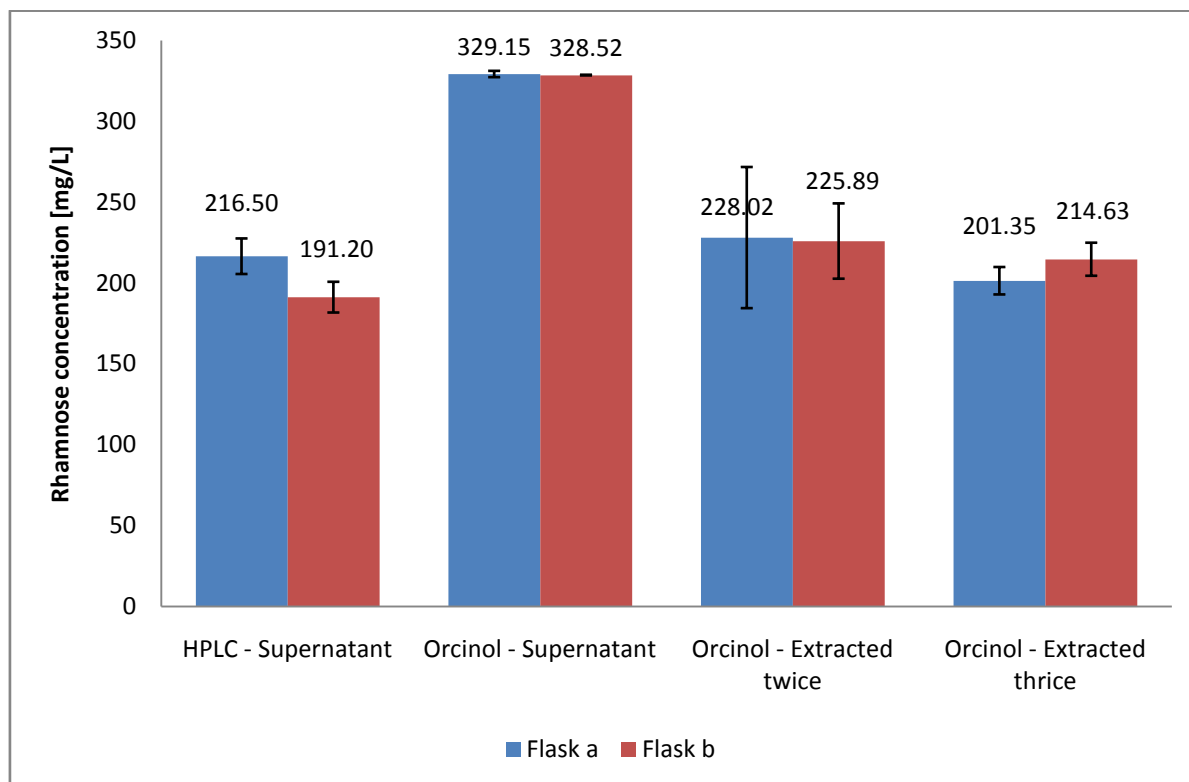


**Figure 4: Standards curves for L-rhamnose after reaction with orcinol reagent for 20 and 22 minutes.**

The sensitivity of the optical density to the cooling time (after boiling) was tested by taking a 100 mg/l L-rhamnose solution and measuring the optical density immediately, after 10 min, after 30 min, after 2 hours and after 1 day. It was found that the optical density relative to the blanks did not fluctuate with more than 0.8 %, even after a day. The time interval taken between boiling and measuring the optical density therefore did not influence the rhamnose concentration.

To evaluate the importance of the extraction step, the use of a benchmark for the rhamnose concentration was necessary. The concentration as determined by the HPLC was proven to be accurate (section 3.1.3.3) and was thus used as the benchmark. First the supernatant was analysed by the orcinol analysis without extraction. The procedure was then repeated after a double and a triple extraction of the supernatant. In each case,

duplicate samples (analysed in two separate batches) from duplicate flasks (4 in total) were analysed. The concentrations obtained by analysing the different solutions (supernatant, twice extracted and extracted three times) are presented in Figure 5 together with the results from HPLC analysis.



**Figure 5: Results from rhamnose analysis of supernatant by HPLC and orcinol analysis. Orcinol analysis was tested on supernatant and twice and three times extracted supernatants from *Pseudomonas aeruginosa* ATCC 9027.**

When compared to the results from HPLC, the measurement of the rhamnose in the supernatant alone by the orcinol method is clearly inaccurate. Other components such as pigments in the media possibly interfered with the spectrophotometer readings, which results in the rhamnose concentration being overestimated by approximately 125 mg/l rhamnose. A small error in the concentration measurement of the rhamnose moiety will translate into a large error in the biosurfactant concentration when multiplied by the dilution factor and the rhamnose to rhamnolipid ratio. In the literature review (section 1.3.4.1) colorimetric methods used for rhamnolipid analysis were listed together with the sample preparation method (Table 1). Four of the eleven studies in Table 1 analysed the supernatant or culture broth directly. The results showed in Figure 5 suggest that the rhamnolipid concentrations reported in these literature studies might be exaggerated.

The results from the orcinol analysis of the samples which were extracted twice and those extracted three times fall within the range of error. However, the samples extracted three times gave a standard deviation of 9.3 mg/l as compared to a standard deviation of 33.5 for

the samples extracted only twice. Rhamnolipid concentration reported in the rest of the results was consistently from samples extracted three times to ensure maximum accuracy.

### 3.1.1.3 Indirect measurement of rhamnose concentration with surface tension analysis

Supernatant from the same culture of *Ps. aeruginosa* ATCC 9027 as above was analysed for its surface activity. Duplicate serial dilutions were made of supernatant from only one of the culture flasks, since the goal was to determine the accuracy of the dilutions and the surface tension measurements and not the variation between flasks. The surface tension was measured (section 2.5.4.1) of the diluted samples. Figure 6 shows a plot of the surface tension against rhamnolipid concentration, where the error bars represent the two separate dilution series. The concentration of rhamnolipid in each diluted sample was calculated by using the concentration determined by HPLC analysis (see section 3.1.1.1). Measurements were also done in duplicate, but the error is not shown since the instrument gave very consistent readings.

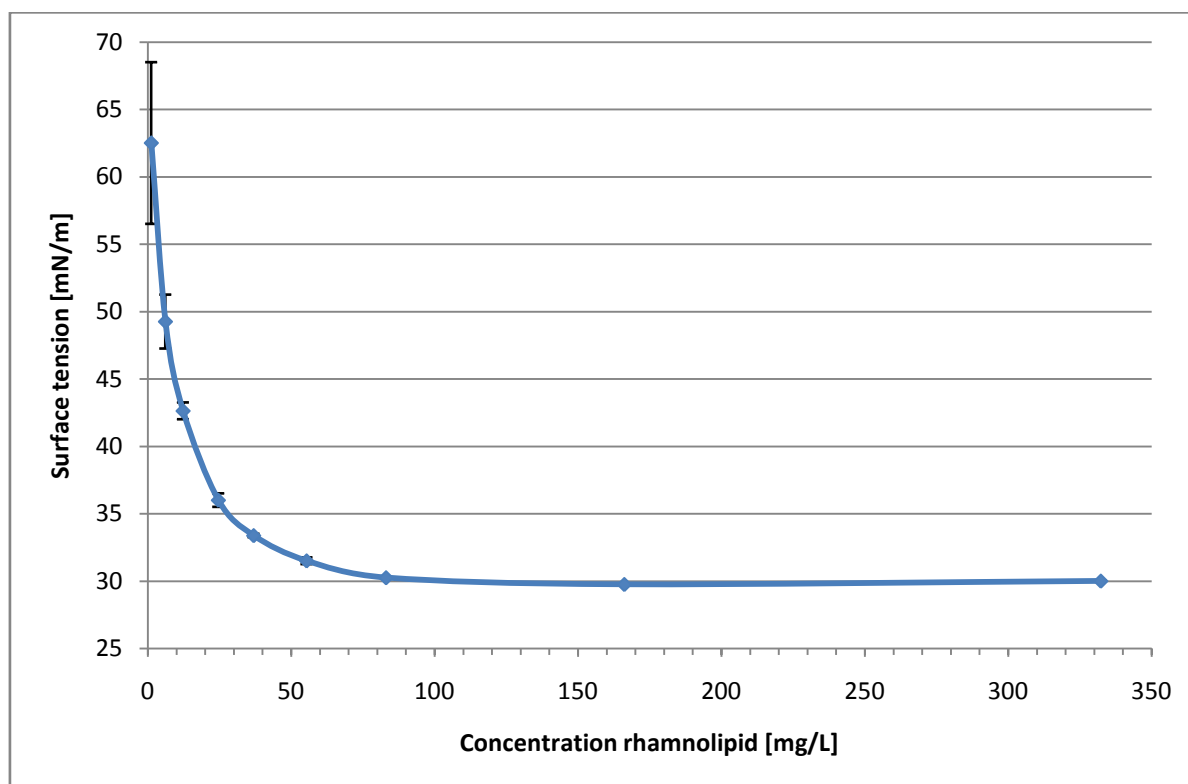


Figure 6: Surface tension plot of serial dilutions of supernatant from *Pseudomonas aeruginosa* ATCC 9027.

The surface tension decreased with increasing concentration of rhamnolipid until the minimum surface tension was reached, where micelle formation starts. The minimum surface tension achieved was 29.5 mN/m. The reported minimum surface tension of monorhamnolipids from *Ps. aeruginosa* ATCC 9027 is 29 mN/m (Zhang and Miller 1992). The analysis was therefore taken to be accurate and reliable.

The assumption made in the previous section that the rhamnolipid concentration measured by the HPLC was accurate was confirmed by the surface tension results. The surface tension measured for a concentration of 1 mg/l rhamnolipid (calculated from HPLC results) was 62.5 mN/m with an error of 6 mN/m. This is close to the surface tension of distilled water (72 mN/m) which would represent a sample containing no rhamnolipid. No accounts could be found in rhamnolipid production studies of HPLC being used for rhamnolipid measurement, although it was proven in this study that it is a rapid and accurate method.

The error due to the serial dilutions was smaller than 0.13 mN/m for large concentrations of rhamnolipid (>36 mg/l), but increased dramatically for the small concentrations (< 7 mg/l) to a maximum error of 6 mN/m. This indicates that care should be taken when dilutions are made for the determination of the critical micelle concentration (CMC) described below.

Although the CMC was not used in this study as a measure of concentration, as is sometimes reported in literature (section 1.3.4.2), the CMC of the mono-rhamnolipid solution was determined to confirm that it was the same rhamnolipid composition obtained from *Ps. aeruginosa* ATCC 9027 as reported by Zhang and Miller (1994). Every surfactant solution has a CMC which characterises it. The CMC was determined from a semilog plot of the surface tension of the dilution series (Figure 7). Concentrations below the CMC form an oblique line on this plot which intersects at with the horizontal line of the minimum surface tension. The CMC was determined to be 49.4 mg/l. This is slightly larger than 40 mg/l reported by Zhang and Miller (1992) and the 30 mg/l reported by Clifford *et al.* (2007), both for rhamnolipids from *Ps. aeruginosa* ATCC 9027. An error smaller than 10 mg/l may be considered negligible, since some error is bound to be introduced when diluting samples and from the original rhamnolipid concentration estimation. The biosurfactant used in this section for the development of analyses was therefore confirmed to be mono-rhamnolipid.

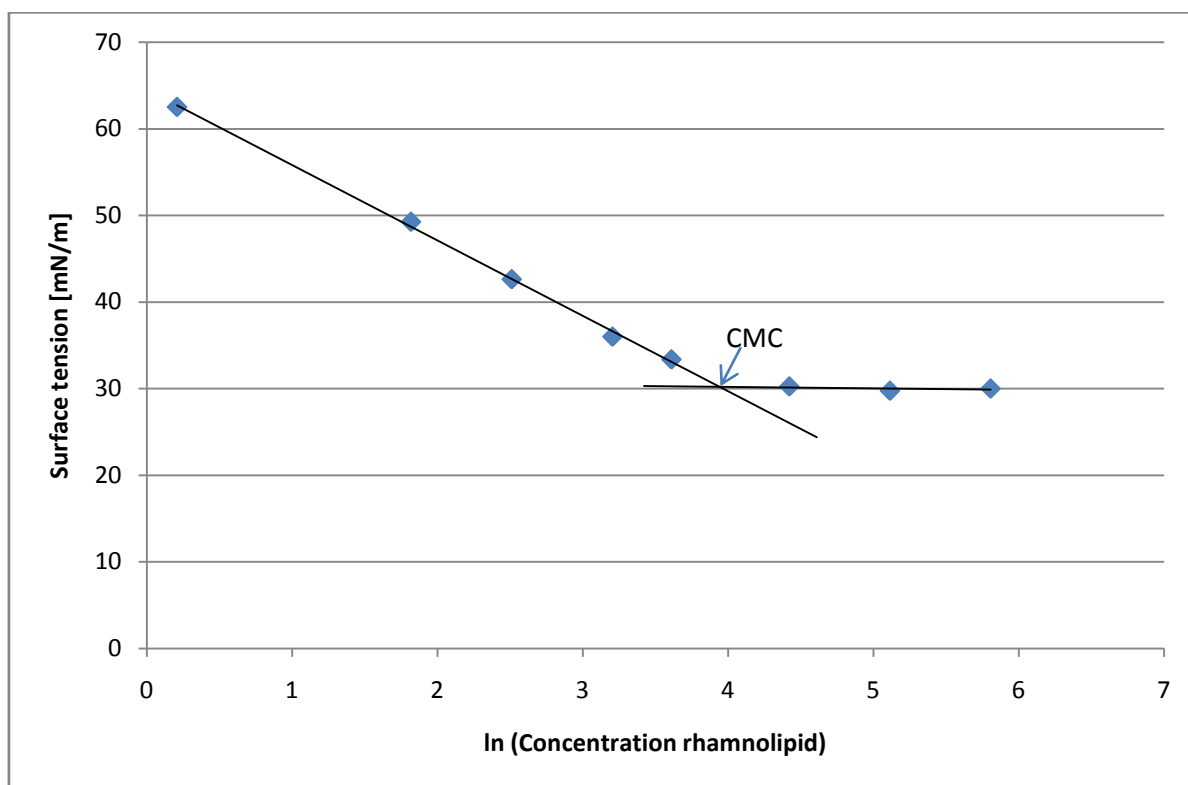


Figure 7: Determination of the CMC of monorhamnolipid solution from a dilution series of supernatant from *Pseudomonas aeruginosa* ATCC 9027.

### 3.1.2 Development of method for quantification of antimicrobial activity

Two different methods were evaluated for antibacterial testing, i.e. the disk diffusion method and soft agar overlay method (sections 2.5.5.1 and 2.5.5.2). The antimicrobial activity of mono-rhamnolipid against *Mycobacterium aurum* was evaluated for both methods. No inhibition was observed against *Mycobacterium aurum* using the standard disk diffusion method (Figure 8). No inhibition zones were observed around the filter paper disks, suggesting that either the mono-rhamnolipid was not active against the organism or the analysis was not successful.

Another susceptibility method was evaluated to establish whether mono-rhamnolipid was active against *Mycobacterium aurum*. The use of the soft agar overlay method led to a clear zone of inhibition of 2 cm (Figure 9). Mono-rhamnolipid therefore has activity against *Mycobacterium aurum*. The soft agar overlay method was chosen for use in later experiments. Although the disk diffusion method is a standard in antibiotic testing (Jorgensen and Turnidge 2003), this method was not effective in this study for the testing of biosurfactant antimicrobial activity.



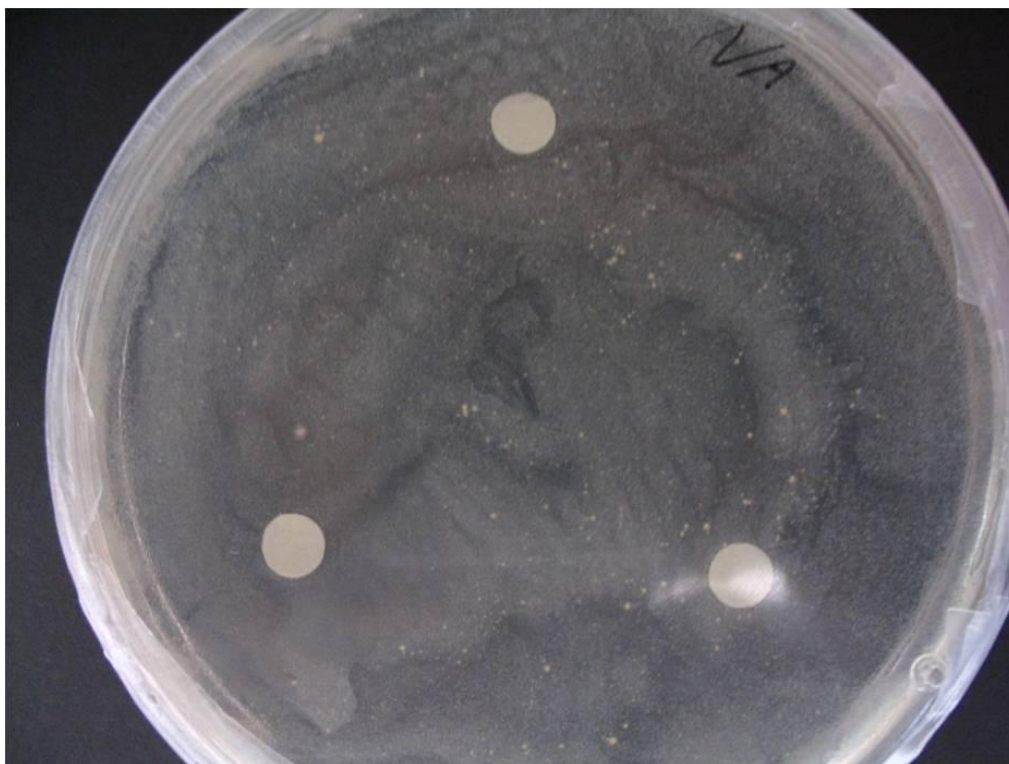


Figure 8: Standard disk diffusion method tested for mono-rhamnolipid activity against *Mycobacterium aurum*.

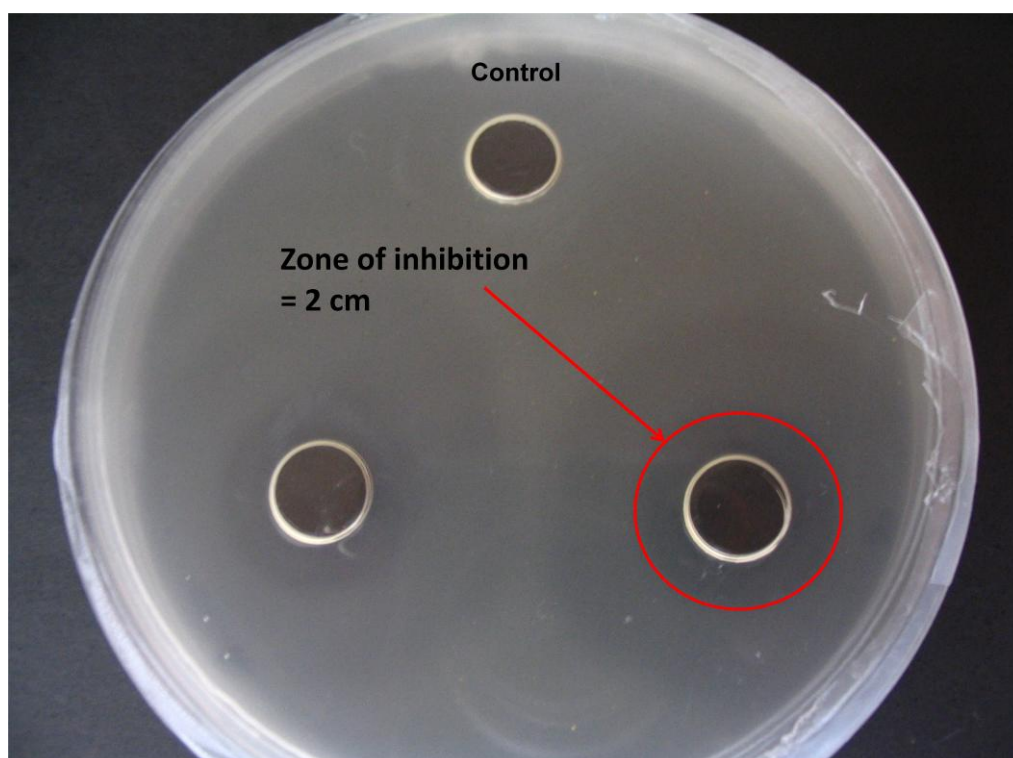


Figure 9: Soft agar overlay method tested for mono-rhamnolipid activity against *Mycobacterium aurum*.



Antimicrobial activity was also tested against the fungus *Botrytis cinerea* (section 2.5.5.3). Difficulties were experienced with growing the fungus. Growth could not be obtained with the disk diffusion method (Espinel-Ingroff *et al.* 2007), where the fungus was suspended and spread on a plate. Good growth was obtained when mycelial plugs were placed in the centre of the plate. However, when the antimicrobial susceptibility method was followed, where the mono-rhamnolipid solution was pipetted into wells, no zones of clearing were observed, with the fungus covering the entire plate. Mono-rhamnolipid therefore did not show fungicidal activity against *Botrytis cinerea*.

An alternative parameter could be used to evaluate the activity, i.e. the extent of growth inhibition. Perneel *et al.* (2008) used this approach when they tested the antimicrobial activity of rhamnolipids against *Pythium myriotylum*. They modified PDA with rhamnolipid concentrations ranging from 0.025 to 2.5 mg/l and inoculated the fungus in the middle of the plate. The difference between the mycelial growth on this plate and a control plate of normal PDA was used to indicate the activity. Such a measure of growth inhibition could be evaluated in future work to test for activity of rhamnolipids against fungi.

## 3.2 Evaluation of glucose media for growth and biosurfactant production

### 3.2.1 Investigation of media requirements for biosurfactant production from *Pseudomonas aeruginosa* ATCC 27853

#### 3.2.1.1 Media selection

*Ps. aeruginosa* ATCC 27853 was grown in five different MSM and PPGASM based media in shake flask culture (Table 11). The focus of these experiments was primarily to determine the nutrient requirements in a medium for good production of rhamnolipids. Additional experiments were also performed to evaluate the media buffering requirements by comparing a well buffered medium (MSM+Tris) with a non-buffered medium (MSM).

A key requirement for good rhamnolipid production is the presence of a nutrient limitation (section 1.6.1.3). The effect of nitrogen limitation on growth and biosurfactant production was studied by using a medium reported to be nitrogen limiting by Sim *et al.* (1997) (MSM+PO<sub>4</sub>). The effect of phosphate limitation was studied replacing the phosphate buffer (i.e. removing the phosphate source) with a tris buffer (MSM+Tris). During comparison of these media, similar buffering capacities were maintained with either the phosphate or tris buffers. The effect of nitrogen and phosphate limitation was similarly examined in low carbon media (PPGASM+PO<sub>4</sub> and PPGASM+Tris respectively) with this organism.

The influence of nutrient limitation was likewise examined using *Ps. aeruginosa* ATCC 9027 in MSM+PO<sub>4</sub> and MSM+Tris media. Finally, *Pseudomonas aeruginosa* 2bf was evaluated on MSM+PO<sub>4</sub>.

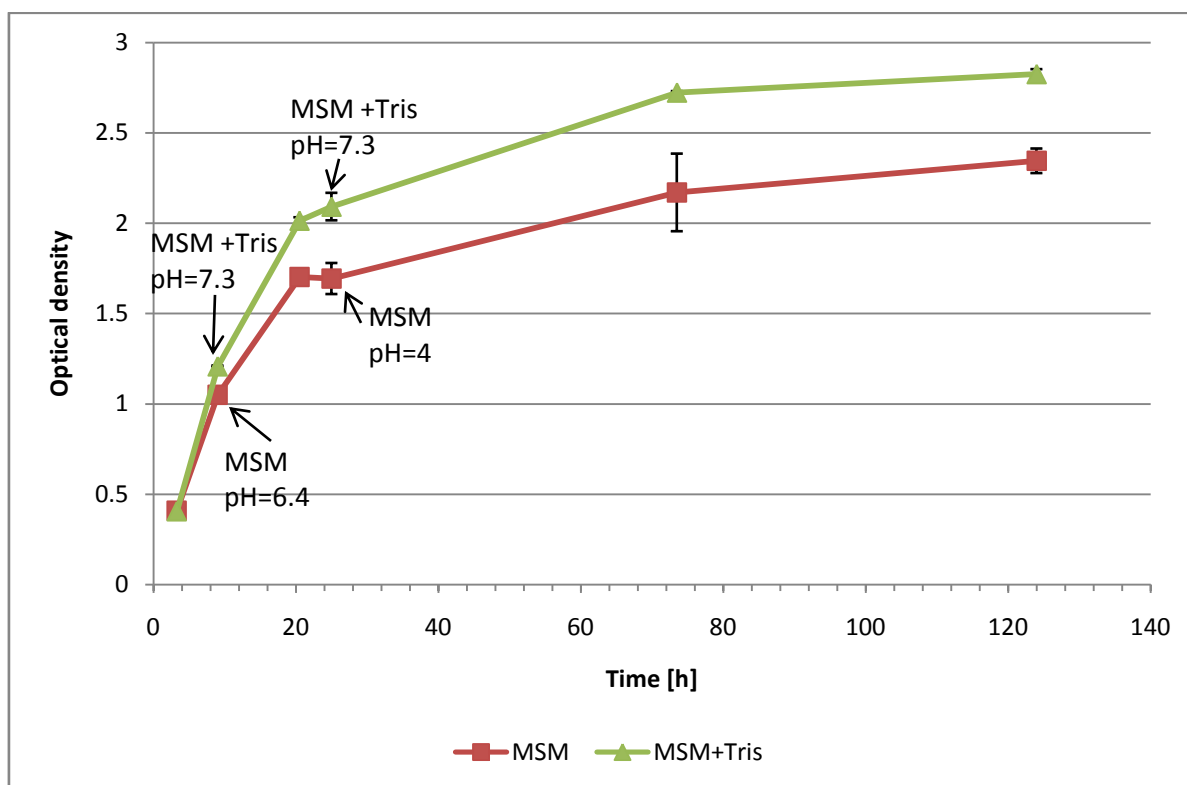
The assumption of nitrogen limitation as reported by Sim *et al.* (1997) and phosphate limitation (due to removal of the phosphate in the medium) could not be validated in this experiment, since these analyses required large volumes, the removal of which could hamper shake flask studies. However, in the later bioreactor culture where larger samples could be removed without affecting the outcome of the experiment, the assumption of phosphate limitation was confirmed (section 3.7.2).

#### 3.2.1.2 Effect of pH buffering on growth and biosurfactant production

The influence of pH buffering on growth and rhamnolipid production of *Ps. aeruginosa* ATCC 27853 was investigated. For this purpose, MSM medium, which contains no buffer, and MSM+Tris medium containing tris buffer were used. Both of these media had similar nutrient contents and were deficient in phosphate, but MSM+Tris medium contained a buffer.

After 28 hours of incubation, the pH in the MSM medium had dropped to 4 (Figure 10), which was expected for a medium without a buffering capacity. At the same time the tris

buffer in this MSM+Tris medium maintained the pH at a neutral level. Although growth had increased in this medium after the pH had dropped to 4, it was lower than growth on MSM+Tris medium, falling outside the range of error (Figure 10).



**Figure 10: Comparison of *Pseudomonas aeruginosa* ATCC 27853 growth on glucose medium containing no buffer (MSM) and glucose medium containing tris buffer (MSM+Tris).**

A significant difference was found in the rhamnolipid production between buffered and non-buffered media. The MSM medium production was much poorer than that in the MSM+Tris medium (Figure 11), with close to an order of magnitude difference. The acidic environment of the MSM medium is known to be detrimental to cell metabolism. Studies have shown that a low pH has a great influence on the amount of rhamnolipid produced (Chen *et al.* 2007, Das and Mukherjee 2005).

It can also be seen from Figure 10 and Figure 11 that rhamnolipid production in *Ps. aeruginosa* ATCC 27853 is associated with the stationary phase. Negligible production was obtained in the MSM+Tris medium up to 28 hours, when growth was still in the exponential phase. Production dramatically increased to 429 mg/l at 124 hours when the stationary phase was reached.

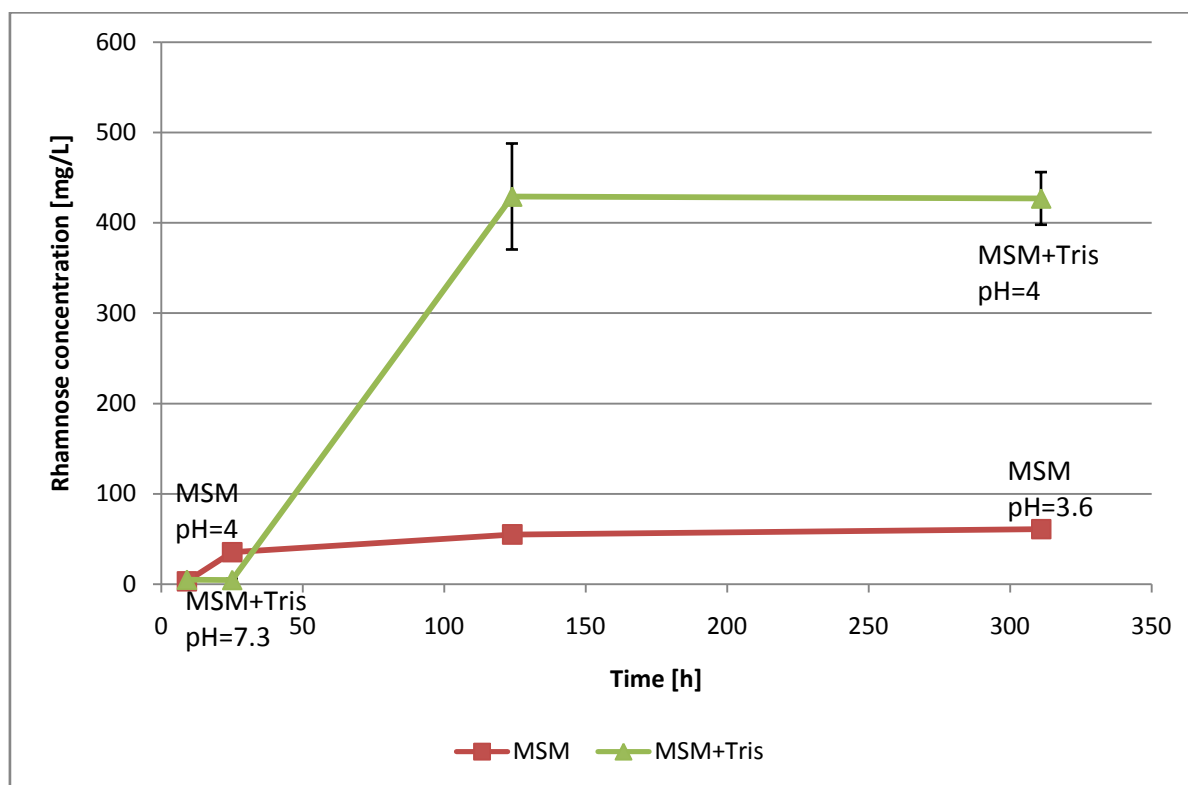


Figure 11: Comparison of *Pseudomonas aeruginosa* ATCC 27853 biosurfactant production on glucose medium containing no buffer (MSM) and glucose medium containing tris buffer (MSM+Tris).

### 3.2.1.3 Effect of limiting nutrient on growth and biosurfactant production

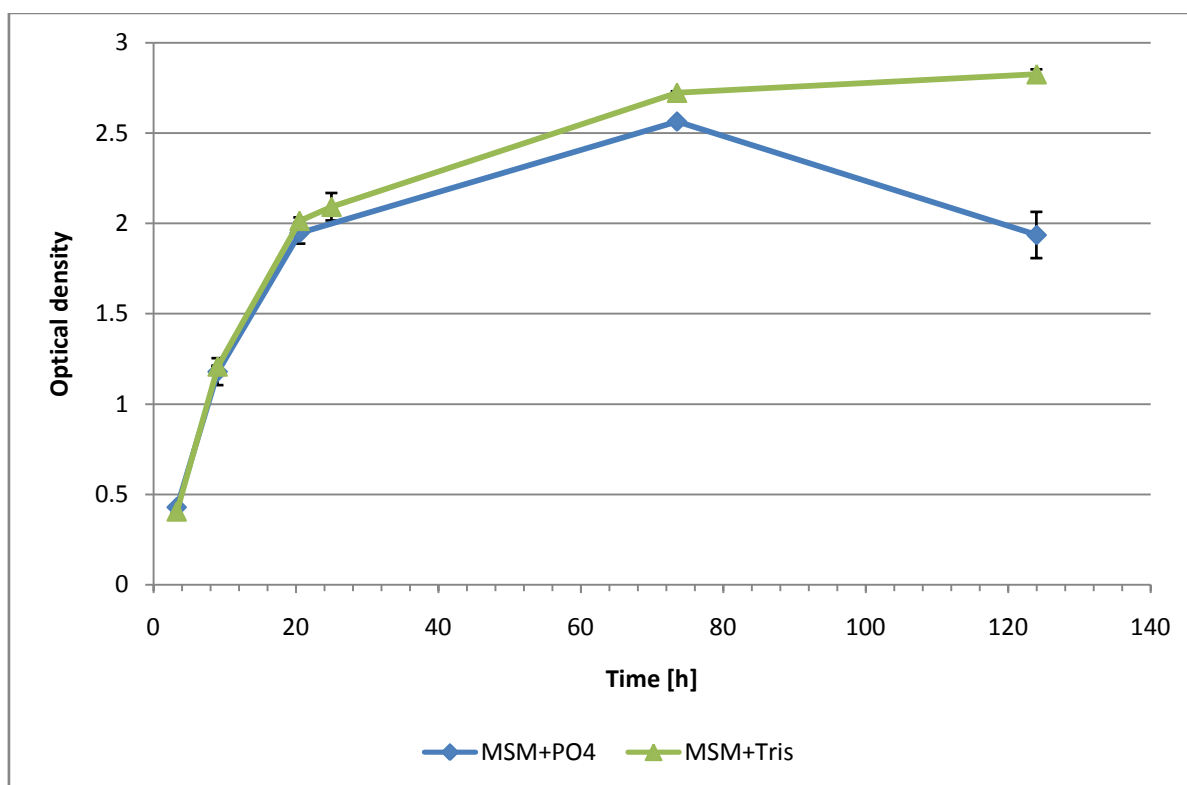
Rhamnolipid production has been shown to be triggered by the exhaustion of a nutrient. Many studies have reported rhamnolipid production with the exhaustion of nitrogen (Benincasa *et al.* 2002, Raza *et al.* 2006a, Syldatk *et al.* 1985a). Studies done under phosphate limited conditions are not nearly as prevalent as production done under nitrogen limitation, but have been reported (Mulligan *et al.* 1989). The effect of a phosphate limitation was investigated by evaluating media containing only trace amounts of phosphate (<0.004 %) transferred from the inoculum. Since pH maintenance was found to be critical, a tris buffer was used, which contains no phosphate. This medium (MSM+Tris) was compared to a medium containing phosphate buffer (MSM+PO<sub>4</sub>), which was reported to be limited in nitrogen (Sim *et al.* 1997).

The pH of the two media during growth was monitored to establish if the buffering capacities were similar. Both media had an approximately neutral pH up to 28 hours, with the pH dropping to acidic levels (about 4) only at the end of the run at 311 hours (Table 17).

**Table 17: Comparison of pH during growth of *Pseudomonas aeruginosa* ATCC 27853 on MSM+PO4 and MSM+Tris media.**

Medium	Time [h]	pH	Error
MSM+PO4	9	7.2	0.1
	28	6.9	0.1
	311	4.6	0
MSM+Tris	9	7.3	0
	28	7.3	0
	311	4.0	0

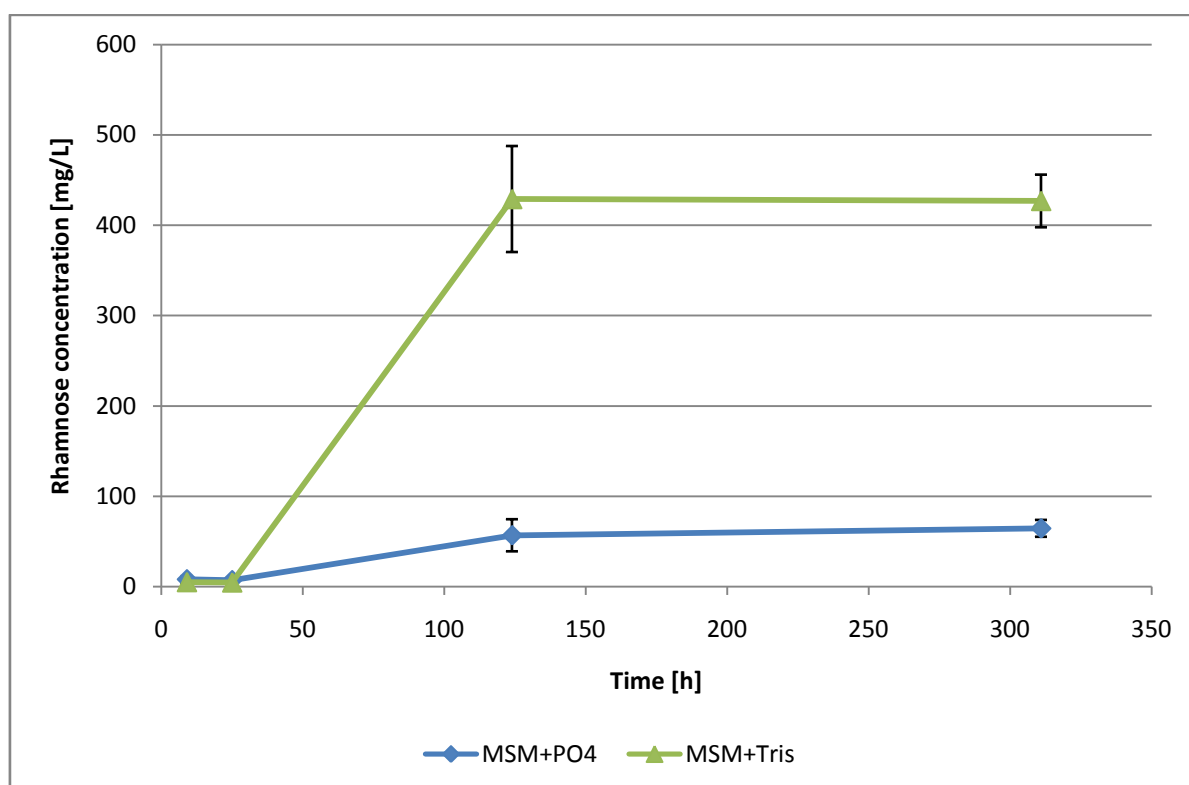
Thus with the similar buffering capacities established, the growth in the two media was compared. The best growth was obtained in the MSM+Tris medium with a maximum optical density of 2.83 (Figure 12). However, growth in the MSM+PO4 medium was very similar up to 74 hours, falling within the range of error. After 74 hours growth decreased in this medium with an optical density of 1.93. Thus, the phosphate deficiency in the MSM+Tris medium did not lead to a significant difference in growth.



**Figure 12: Comparison of *Pseudomonas aeruginosa* ATCC 27853 growth on phosphate containing MSM medium (MSM+PO4) and tris buffer containing medium (MSM+Tris).**

Production was much better in the MSM+Tris medium (Figure 13), increasing to 427 mg/l rhamnase after 124 hours. In the MSM+PO4 medium a maximum of 64.5 mg/l rhamnase was observed in the cell culture. This constitutes almost an order of magnitude difference in production levels. The only difference between the two media was the buffer present.

Tris buffer is an amine based buffer which contains carbon and nitrogen. It is very unlikely that the nutrients in the buffer could have been responsible for the order of magnitude difference in production. Since the MSM+Tris medium only contained traces of phosphate from the inoculum, it was assumed that this medium is limited in phosphate, which initiated the stationary phase. The phosphate concentrations were not measured in this experiment to test this assumption, but were measured and the assumption confirmed in the bioreactor experiment presented in section 3.7. The MSM+PO<sub>4</sub> medium is supposedly limited in nitrogen (Sim *et al.* 1997) and this limitation was proven many times to lead to good production (section 1.6.1.3). Future experiments will investigate nitrogen limitation and compare it with phosphate limitation.



**Figure 13: Comparison of *Pseudomonas aeruginosa* ATCC 27853 biosurfactant production on phosphate containing MSM medium (MSM+PO<sub>4</sub>) and tris buffer containing medium (MSM+Tris).**

The surface tension results supported the direct concentration measurements. Surface tension of the MSM+PO<sub>4</sub> medium dropped to 35.5 mN/m after 311 hours from 52.5 mN/m after 28 hours when nearly no rhamnose was measured in the cell culture (Table 18). The surface tension of the MSM+Tris medium was slightly lower after 311 hours, at 33.3 mN/m, but the rhamnose concentration measured was significantly higher as seen in Figure 13. It is therefore likely that the minimum surface tension was reached at 33.3 mN/m.

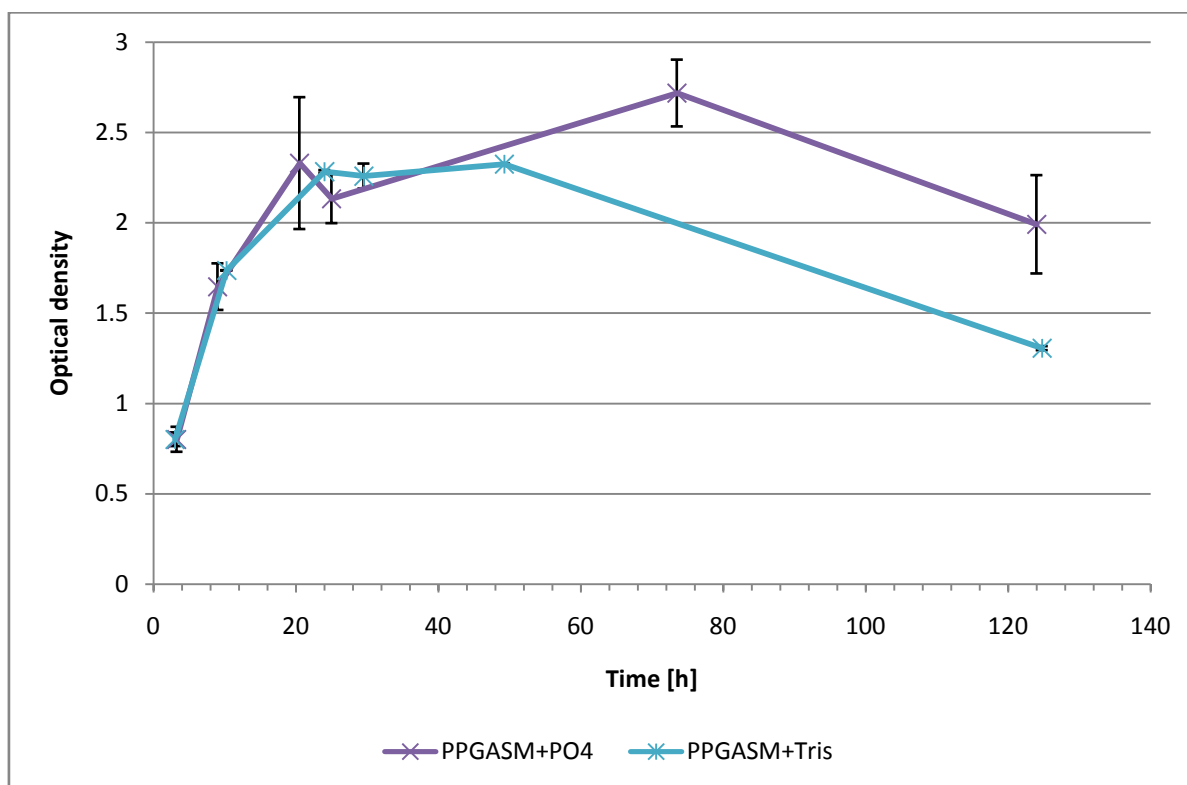
**Table 18: Surface tension of *Pseudomonas aeruginosa* ATCC 27853 cultures in MSM+PO4 and MSM+Tris media.**

Medium	Time [h]	Surface tension [mN/m]	Error
MSM+PO4	9	58.5	6.4
	28	52.5	0.6
	311	35.5	1.3
MSM+Tris	9	62.0	1.3
	28	44.0	0.4
	311	33.3	0.1

#### **3.2.1.4 Evaluation of low carbon containing media for growth and biosurfactant production**

Tests were done to investigate other medium factors influencing rhamnolipid production. For this purpose a medium with a completely different composition to MSM was evaluated, namely PPGASM medium. Notably, this medium had a low glucose concentration (0.5 %). Phosphate and tris buffer was added to this medium as for MSM medium. Since the phosphate deficient MSM+Tris medium led to good rhamnolipid production, it was tested to see whether the same would be observed for the PPGASM+Tris medium.

The PPGAS+PO4 medium supported supported better growth than the PPGASM+Tris buffer (Figure 12). Growth was the same (within the range of error) up to 25 hours, but in the PPGASM+PO4 medium growth reached a maximum optical density of 2 at 124 hours whereas growth in the PPGASM+Tris medium had decreased to a optical density of 1.3 at the same time. However, in the MSM medium experiments, MSM+Tris medium led to better growth than MSM+PO4 (Figure 12).



**Figure 14: Comparison of *Pseudomonas aeruginosa* ATCC 27853 growth on PPGASM medium containing phosphate buffer (PPGASM+PO4) and PPGASM medium containing tris buffer (PPGASM+Tris).**

Poor rhamnolipid production was obtained on both PPGASM media (Figure 15). Although slightly better production was obtained on PPGASM+Tris medium (43 mg/l) than PPGASM+PO4 (14 mg/l), these levels are far less than the order of magnitude difference obtained for the same experiment on MSM medium. The specific yield of rhamnose per gram of glucose was still much lower for the PPGASM+Tris (8.6 g/g) medium as compared to 14.23 g/g for MSM+Tris. The PPGASM+Tris medium has a very small C/N ratio of 2.2 and contains only 0.5 % glucose compared to a C/N ratio of 9 and glucose concentration of 3% for the MSM+Tris medium. Literature has shown that high carbon concentrations are necessary for overproduction of rhamnolipid (section 1.6.1.1). The MSM media also adheres to other nutrient requirements reported to lead to good rhamnolipid production, namely that it contains nitrate as nitrogen source and low concentrations of divalent ions and iron.



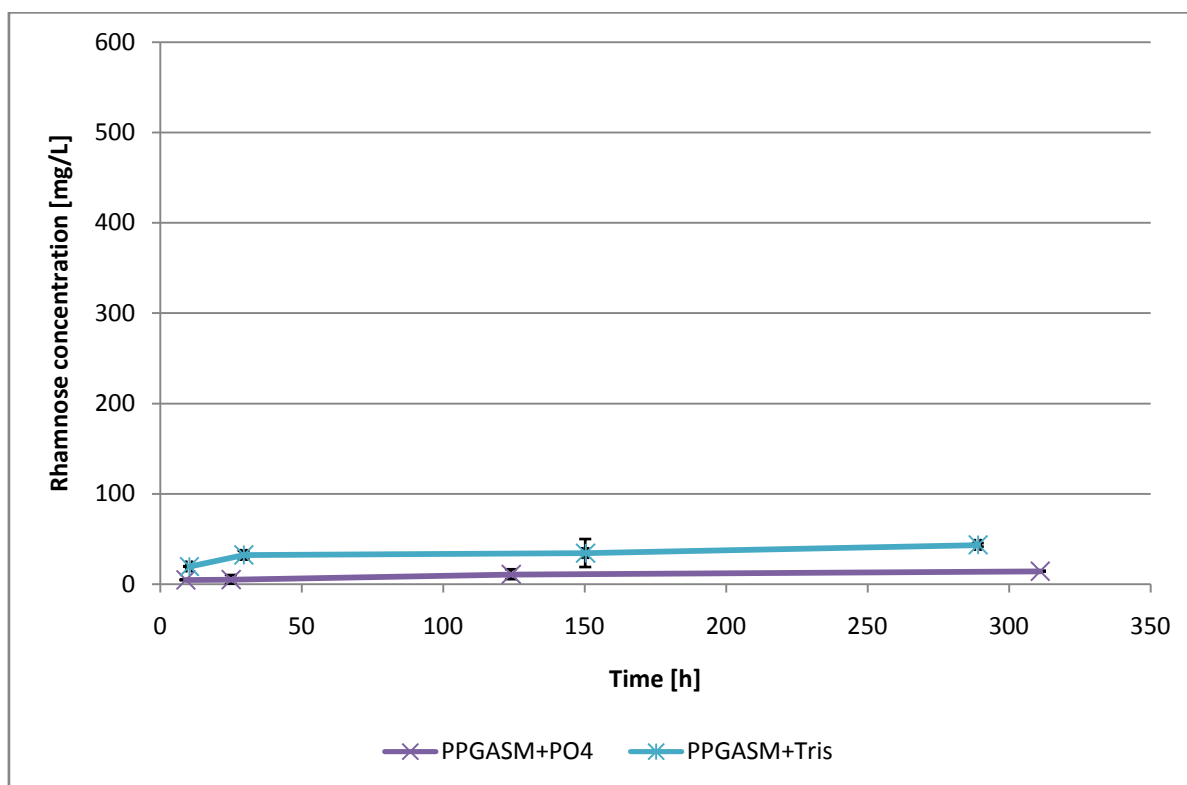
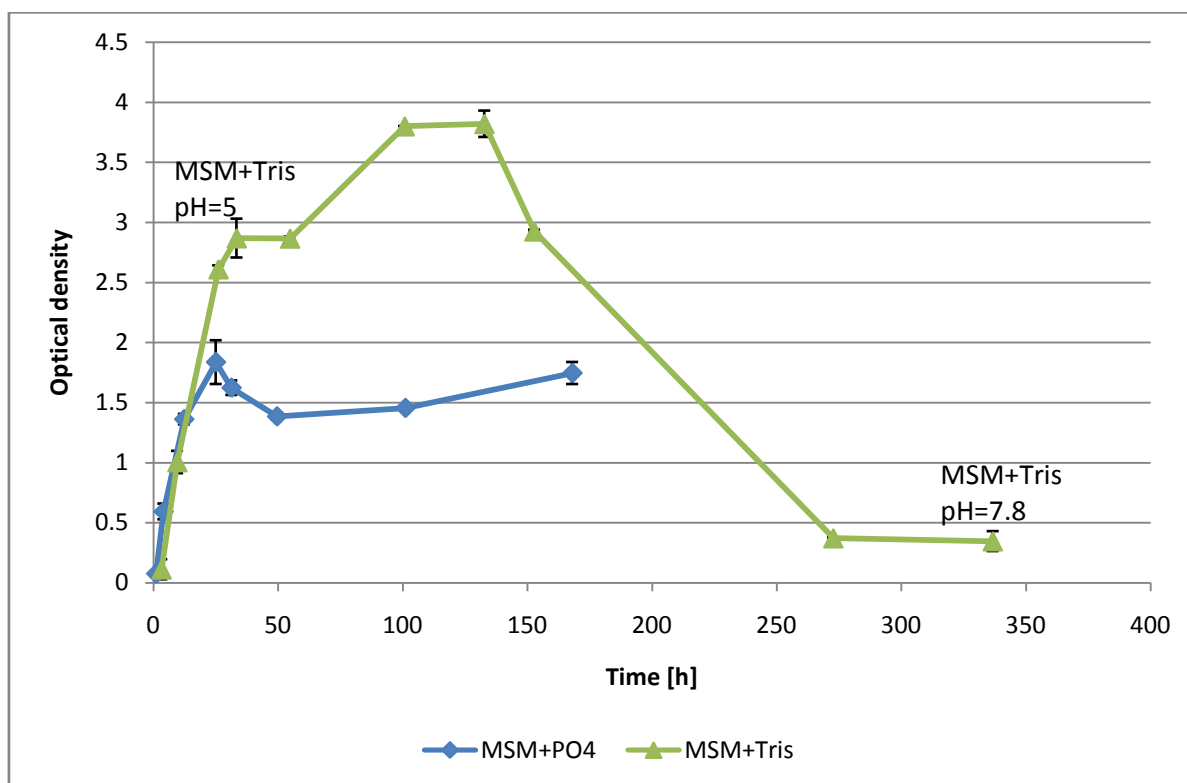


Figure 15: Comparison of *Pseudomonas aeruginosa* ATCC 27853 biosurfactant production on PPGASM medium containing phosphate buffer (PPGASM+PO4) and PPGASM medium containing tris buffer (PPGASM+Tris).

### 3.2.2 Effect of nutrient limitation on growth and biosurfactant production of *Pseudomonas aeruginosa* ATCC 9027

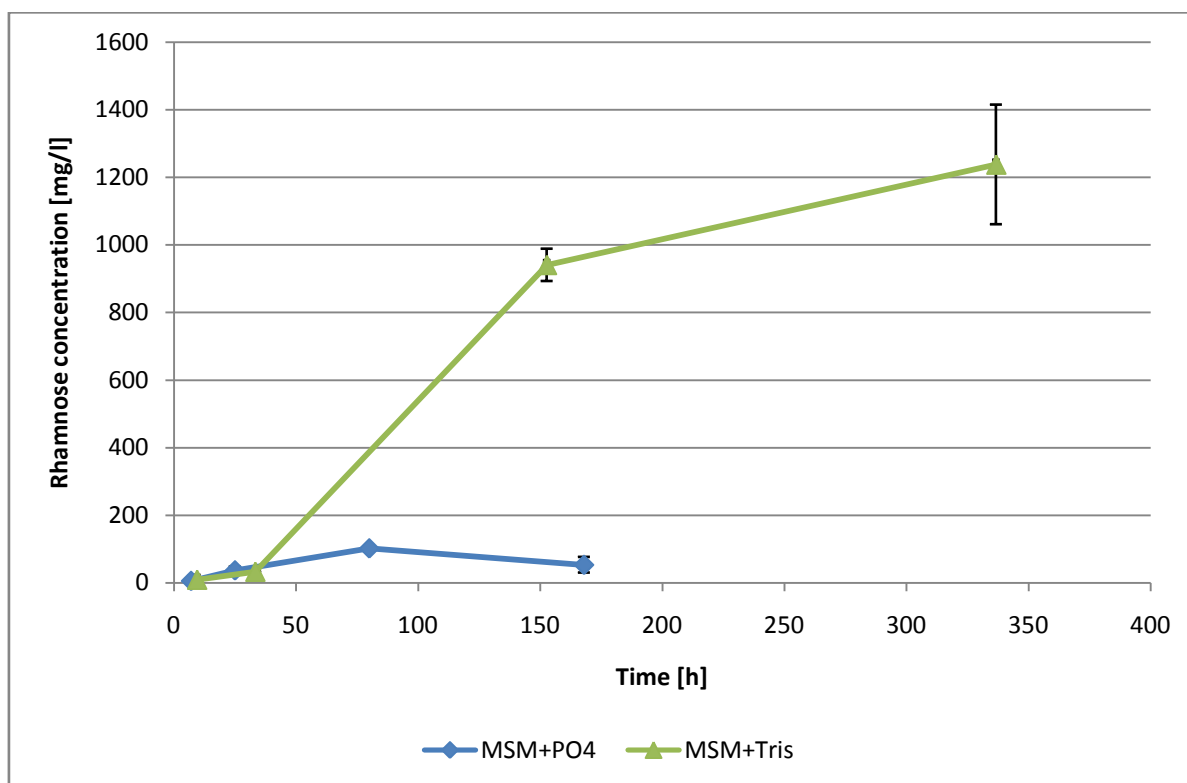
In the previous section the phosphate deficient MSM+Tris medium was found to be the superior medium for rhamnolipid production compared to phosphate containing MSM+PO4 medium in cultures of *Ps. aeruginosa* ATCC 27853. This finding was tested on a different strain, *Ps. aeruginosa* ATCC 9027, which is a strain naturally adapted for the utilisation of glucose (section 2.1).

The MSM+Tris medium led to much better results than MSM+PO4 for *Pseudomonas aeruginosa* ATCC 9027. As seen in Figure 16 growth was significantly higher on MSM+Tris with a maximum optical density of 3.8 as compared to an optical density of 1.83 for MSM+PO4 medium.



**Figure 16: Comparison of *Pseudomonas aeruginosa* 9027 growth on MSM medium containing phosphate buffer (MSM+PO4) and MSM medium containing tris buffer (MSM+Tris).**

As with *Ps. aeruginosa* ATCC 27853 production of rhamnolipid was significantly better in MSM+Tris medium than in MSM+PO4 medium. Production in MSM+Tris medium increased dramatically during the stationary phase, up to 1312 mg/l rhamnolipid at 337 hours (Figure 17). Production of rhamnolipid was clearly associated with the stationary phase, as was found for *Ps. aeruginosa* ATCC 27853. This finding is supported by literature (section 1.5.2). The rate of production decreased slightly after 130h when cell growth decreased. On MSM+PO4 medium the rhamnolipid concentration reached a maximum of 102 mg/l at 80 hours and then dropped to 53 by 168 hours.



**Figure 17: Comparison of *Pseudomonas aeruginosa* 9027 biosurfactant production on MSM medium containing phosphate buffer (MSM+PO<sub>4</sub>) and MSM medium containing tris buffer (MSM+Tris).**

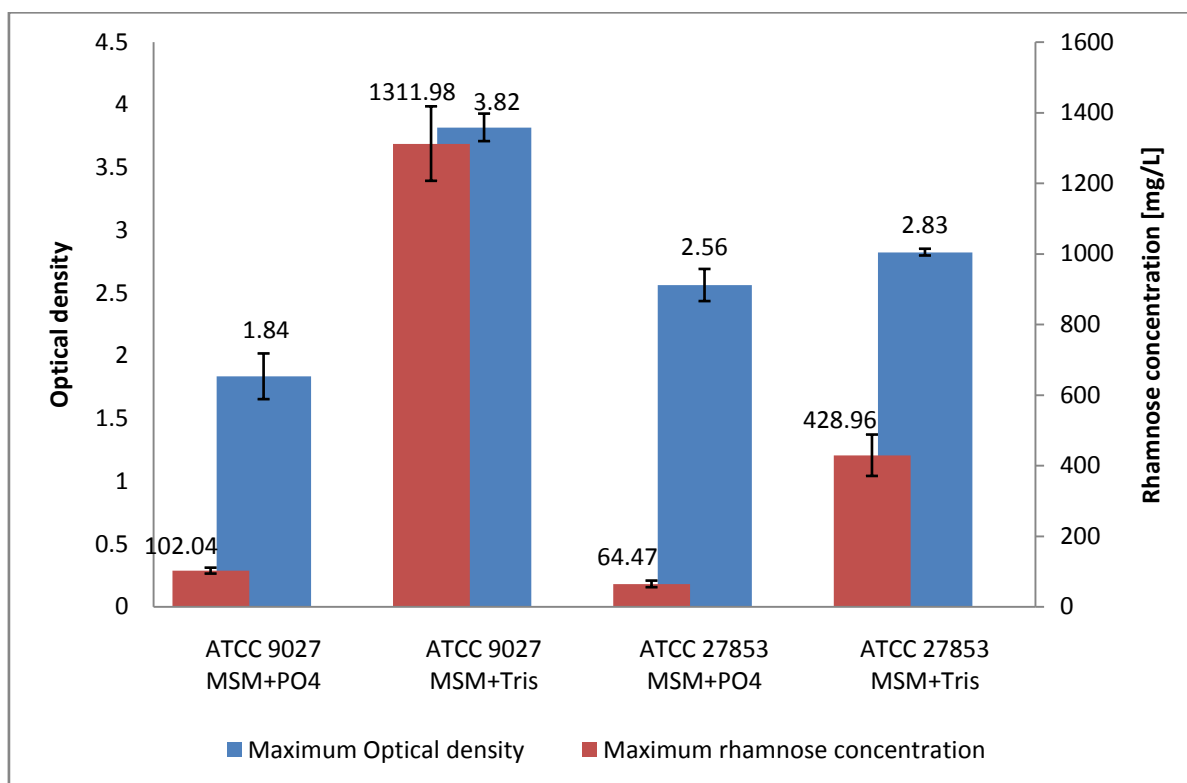
The pH also decreased rapidly in the MSM+Tris medium with a pH of 5 measured at 33 hours due to the rapid growth (Figure 16). If this strain were grown in MSM+Tris medium, pH control would be necessary for bioreactor studies, which could possibly lead to improved production. In section 3.2.1.3 it was shown that pH maintenance was important for good production by ATCC 27853.

Rhamnolipid from *Ps. aeruginosa* ATCC 9027 has been shown to be mono-rhamnolipid with an average molecular weight of 504 g/mol (Zhang and Miller 1994). A rhamnose concentration of 1312 mg/l as was produced on MSM+Tris medium thus equals a rhamnolipid concentration of 4028 mg/l. The research group of Mulligan *et al.* (1989) cultured the same strain on phosphate limited PPGASM+Tris medium and obtained rhamnolipid at a concentration of 30 times the CMC. If the CMC is taken as 49 mg/l (determined in section 3.1.1.3), their production equaled a rhamnolipid concentration of 1470 mg/l. Consequently, the rhamnolipid production by *Ps. aeruginosa* ATCC 9027 in the study presented here was 2.7 fold higher than that obtained in the literature. Production studies done with other strains of *Pseudomonas aeruginosa* (Table 3) obtained rhamnolipid concentrations between 2.7 g/l and 24.7 g/l. The latter value, as reported by Sim *et al.* (1997), was the exception and the maximum reported concentration from the other studies in Table 3 was 9.3 g/l. Production on hydrophilic substrates was lower (between 3.16 g/l and 6.06 g/l) than hydrophobic substrates. The study presented here therefore achieved average production. It is possible that higher levels of production can be achieved with a process developed for a hydrophobic substrate..

### 3.3 Comparison between *Pseudomonas aeruginosa* strains grown in glucose media

#### 3.3.1 Comparison of growth and biosurfactant production between culture collection strains

From the results above of the two culture collection strains grown on different media the performance of the two strains can be compared. The strain that performed the best would be selected for further production and kinetic studies in an instrumented bioreactor.



**Figure 18: Comparison between culture collection strains *Pseudomonas aeruginosa* ATCC 9027 and ATCC 27853 in terms of growth and biosurfactant production. Cultures were grown on both MSM+PO4 and MSM+Tris media containing glucose as carbon source.**

The performance in terms of growth and rhamnolipid production of the two strains are compared in Figure 18. On MSM+PO4 medium *Ps. aeruginosa* ATCC 27853 achieved better growth than ATCC 9027. However, in this medium ATCC 9027 showed superior rhamnolipid production. In the phosphate limited MSM+Tris medium, which led to high levels of production for both strains, ATCC 9027 produced nearly three times more rhamnolipid than ATCC 27853. In the literature review section it was explained that some *Pseudomonas aeruginosa* strains are better adapted to the uptake of hydrophilic substrates, because of a naturally hydrophilic membrane. *Pseudomonas aeruginosa* ATCC 9027 has been shown to have a more naturally hydrophilic membrane than ATCC 27853 (Zhang and Miller 1994).

As was detailed in section 2.1, *Ps. aeruginosa* ATCC 9027 produces mono-rhamnolipid containing one rhamnose molecule attached to a lipid molecule. The types of rhamnolipid that *Ps. aeruginosa* ATCC 27853 produce are not known. It is likely that it produces mono- as well as dirhamnolipid (two rhamnose molecules), which would lead to a lower rhamnose to rhamnolipid ratio than mono-rhamnolipid. Therefore, for the same amount of rhamnose measured, ATCC 9027 would yield a larger concentration of rhamnolipid than ATCC 27853. This suggests that the increase in rhamnolipid in *Ps. aeruginosa* ATCC 9027 is even greater than that which could be accounted for by comparing rhamnose concentration alone.

### 3.3.2 Associated growth phase of culture collection strains

Rhamnolipids are generally accepted to be secondary metabolites, with production starting at the onset of the stationary phase (Soberón-Chávez *et al.* 2005) (section 1.5.2). Other researchers have found that rhamnolipids were produced during the whole cell growth cycle, but that production increased during the stationary phase (Benincasa *et al.* 2002), (Wei *et al.* 2005). In the experiments reported above, production of rhamnolipids by *Ps. aeruginosa* ATCC 27853 and ATCC 9027 was associated with the stationary phase. Growing both on MSM+PO<sub>4</sub> medium and MSM+Tris medium (Figure 13 and Figure 17), rhamnolipid concentration did not reach above 15 mg/l after approximately 20 hours, when growth was in mid-exponential or early stationary phases. Afterwards production increased substantially in each case.

The production of secondary metabolites can often be enhanced by the use of a fed batch process. In such a process additional substrate is fed at a predetermined rate to the culture. This usually leads to enhanced cell growth and the maintenance of the specific production rate, which leads to higher yields of product.

### 3.3.3 Comparison of growth and biosurfactant production between local and culture collection strains

Experiments were done with a locally isolated strain, *Ps. aeruginosa* 2bf, to explore the possibility of developing a process using South African resources. *Ps. aeruginosa* 2bf was grown on MSM+PO<sub>4</sub> medium with 3 % glucose as carbon source, from an inoculum of the same composition. This strain was originally isolated from an oil contaminated site and therefore is likely to be better adapted for the uptake of hydrophobic substrates. Poor cell growth was observed compared to the culture collection strains with a maximum OD of only 0.6 at 12 hours and decreased cell concentrations afterwards (Figure 19). Rhamnolipid production was poor up to 76 hours with a rhamnose concentration of only 9.09 mg/l. At the end of the run at 168 hours a higher concentration was measured of 46.37 mg/l, which was comparable with the production of ATCC 27853 and much less than the production of ATCC 9027 (Figure 20). Although the concentration of rhamnose increased at the end of the run, it was assumed that it had more or less reached its maximum, since the rate of cell

growth was negligible at the end of the run (optical density of 0.13). Further research will be required on this strain to determine the reasons for its poor growth.

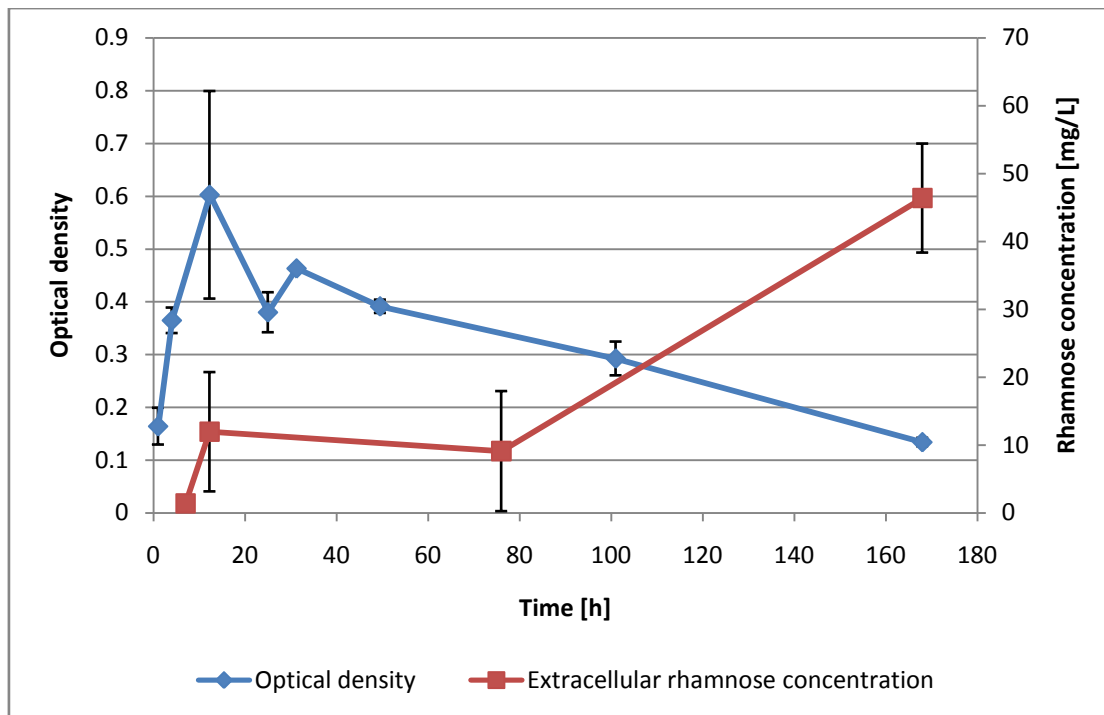


Figure 19: *Pseudomonas aeruginosa* 2bf growth and biosurfactant production on MSM medium with phosphate buffer (MSM+PO<sub>4</sub>) containing glucose as carbon source.

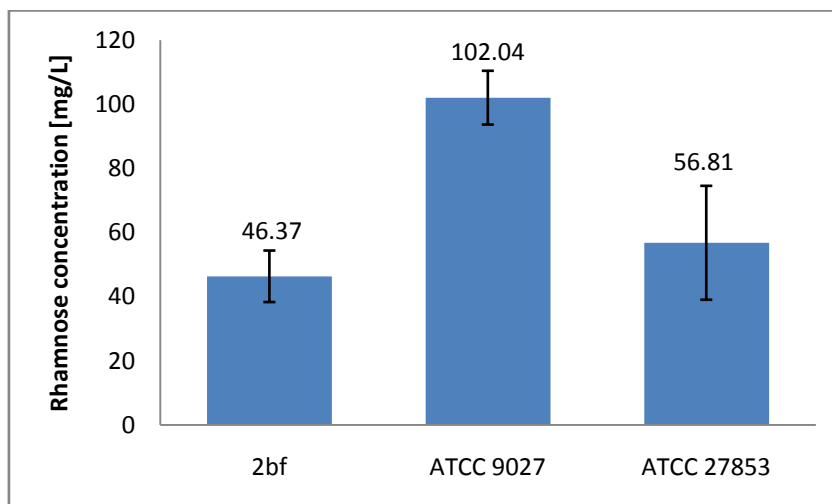


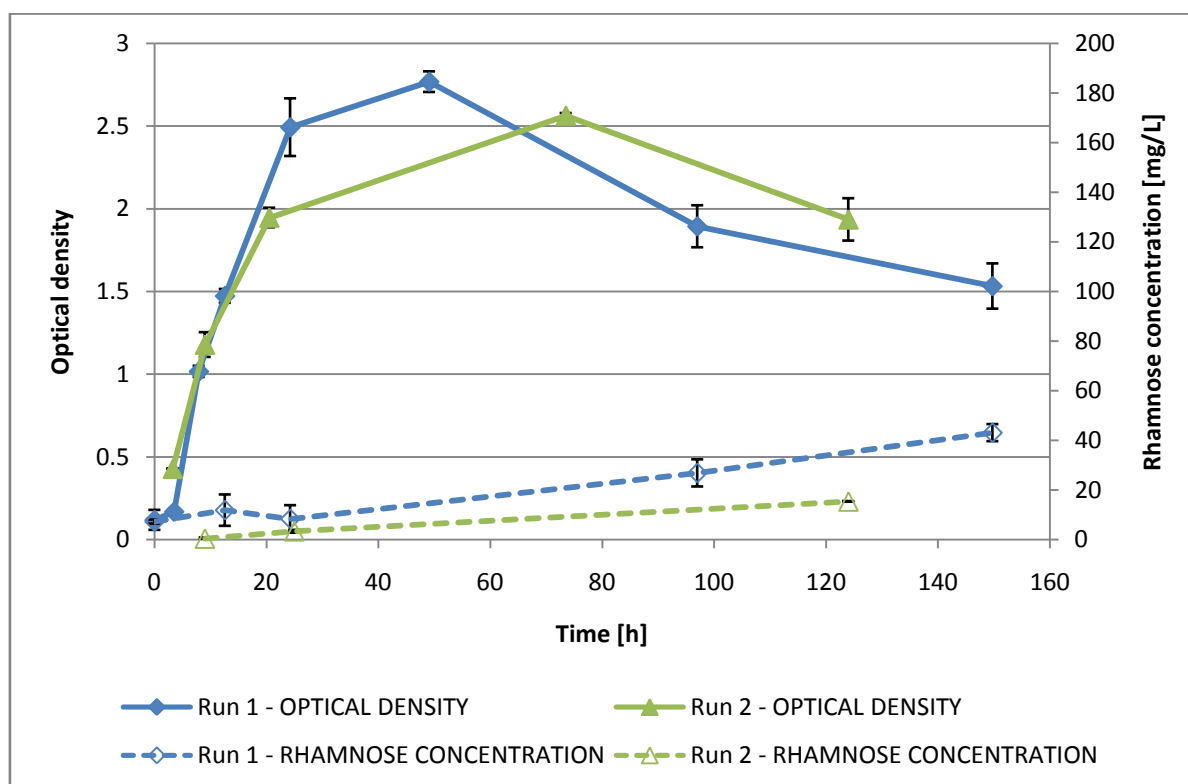
Figure 20: Comparison between culture collection strains *Pseudomonas aeruginosa* ATCC 9027 and ATCC 27853 and locally isolated strain *Pseudomonas aeruginosa* 2bf in terms of biosurfactant production. Cultures were grown in MSM+PO<sub>4</sub> medium with 3 % glucose as carbon source.

### 3.4 Reproducibility of experiments

All shake flask experiments were performed with duplicate flasks with single samples taken from each flask. The error between samples from the duplicate flasks is presented on each graph of this results section.

In order to test the reproducibility of experiments, the run was repeated in which *Ps. aeruginosa* ATCC 27853 was grown on MSM+PO<sub>4</sub> medium containing 3 % glucose as carbon source, where the optical density and rhamnose concentration were monitored (Figure 12 and Figure 13). As seen in Figure 21 data points of the growth curves lie very close to each other, especially in the exponential phase. A maximum error of 10 % was calculated for the growth curves, but this was based on interpolation of the curves, since data points were not aligned in time.

Some differences were observed in the rhamnose concentrations measured, with a difference of approximately 20 mg/l at 124 hours. This value was based on interpolation of the data. Although an error of 20 mg/l seems large on this scale of rhamnose production, it is small compared to the amounts of rhamnose produced in the high production experiments. For example, an error of 20 mg/l would be equal to only 4.7 % of 429 mg/l rhamnose, as produced on MSM+Tris medium. These results thus show that experiments can be reproduced within a reasonable range of error.



**Figure 21: Comparison of *Pseudomonas aeruginosa* ATCC 27853 growth and biosurfactant production between duplicate experiments grown on MSM+PO<sub>4</sub> medium containing 3 % glucose as carbon source.**

### 3.5 Evaluation of alkanes as carbon source

#### 3.5.1 Preliminary assessment of biosurfactant production on alkane media using *Pseudomonas aeruginosa* ATCC 9027

In the glucose experiments above, it was found that the phosphate limited MSM+Tris medium and the strain *Ps. aeruginosa* ATCC 9027 gave the best production. The best strain from the glucose experiments was tested for growth on alkanes, by growing in MSM+Tris medium containing 1.415 % hexadecane as sole carbon source. The percentage hexadecane was calculated so that the media contained the same amount of carbon as media with 3 % glucose (section 2.3.1).

Growth increased very slowly as compared with growth on glucose, with an optical density of 1.49 reached by 337 hours with growth still increasing (Figure 22). At this point the run was terminated, because of time constraints. Negligible amounts of rhamnolipid were produced, with a maximum of only 15 mg/l produced. The results on glucose were dramatically different, which reached a rhamnose concentration of 1311 mg/l (not shown on graph owing to scale), which was nearly a hundred fold more.

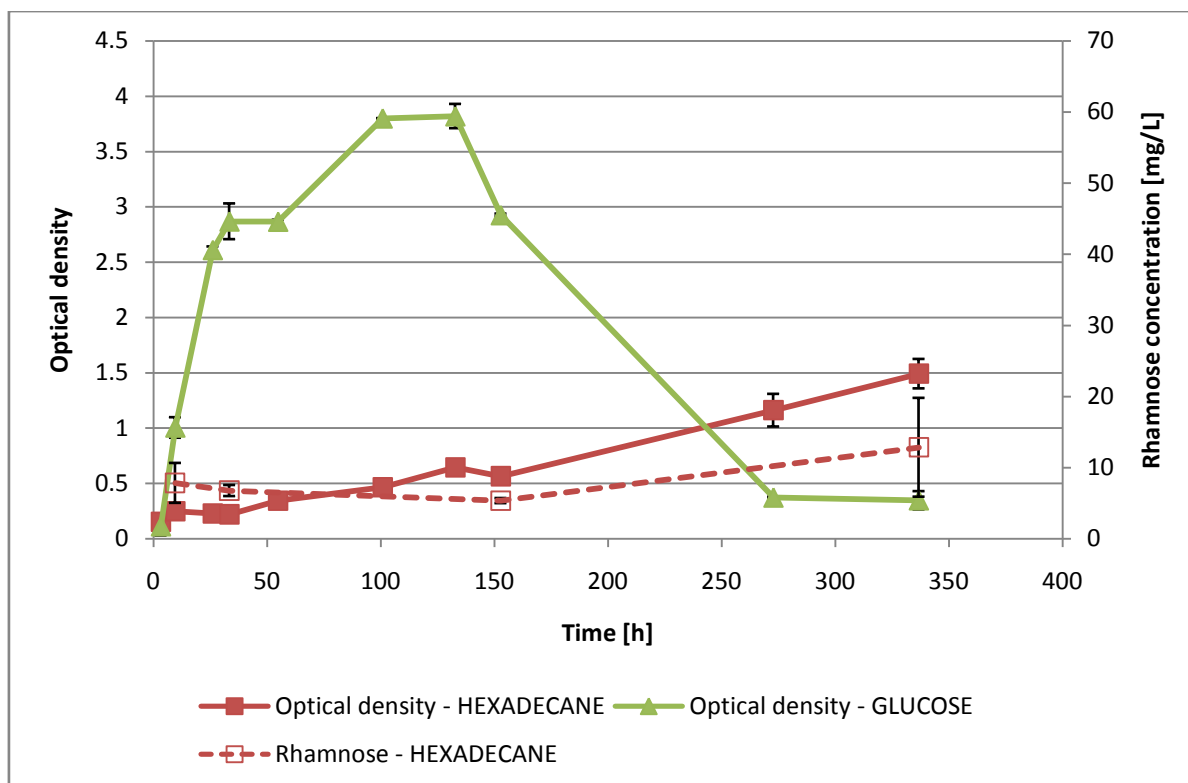


Figure 22: Comparison of *Pseudomonas aeruginosa* ATCC 9027 growth on MSM+Tris medium with hexadecane as carbon source. The biosurfactant production in hexadecane medium is included.



### 3.5.2 Comparison between different *Pseudomonas aeruginosa* strains grown on alkane containing media

Since good growth could not be obtained on MSM+Tris medium with hexadecane as the carbon source, *Ps. aeruginosa* ATCC 9027 was grown on MSM+PO<sub>4</sub> medium with hexadecane as the carbon source, to test whether the increased phosphate levels might improve growth. When the growth of *Ps. aeruginosa* ATCC 9027 on MSM+PO<sub>4</sub> medium is compared with that on MSM+Tris medium over 150 hours, it can be seen that growth was initially poorer on MSM+Tris medium, but showed better growth at the end of 150 hours (Figure 23). However, in both media the growth increased very slowly and negligible rhamnolipid production was observed.

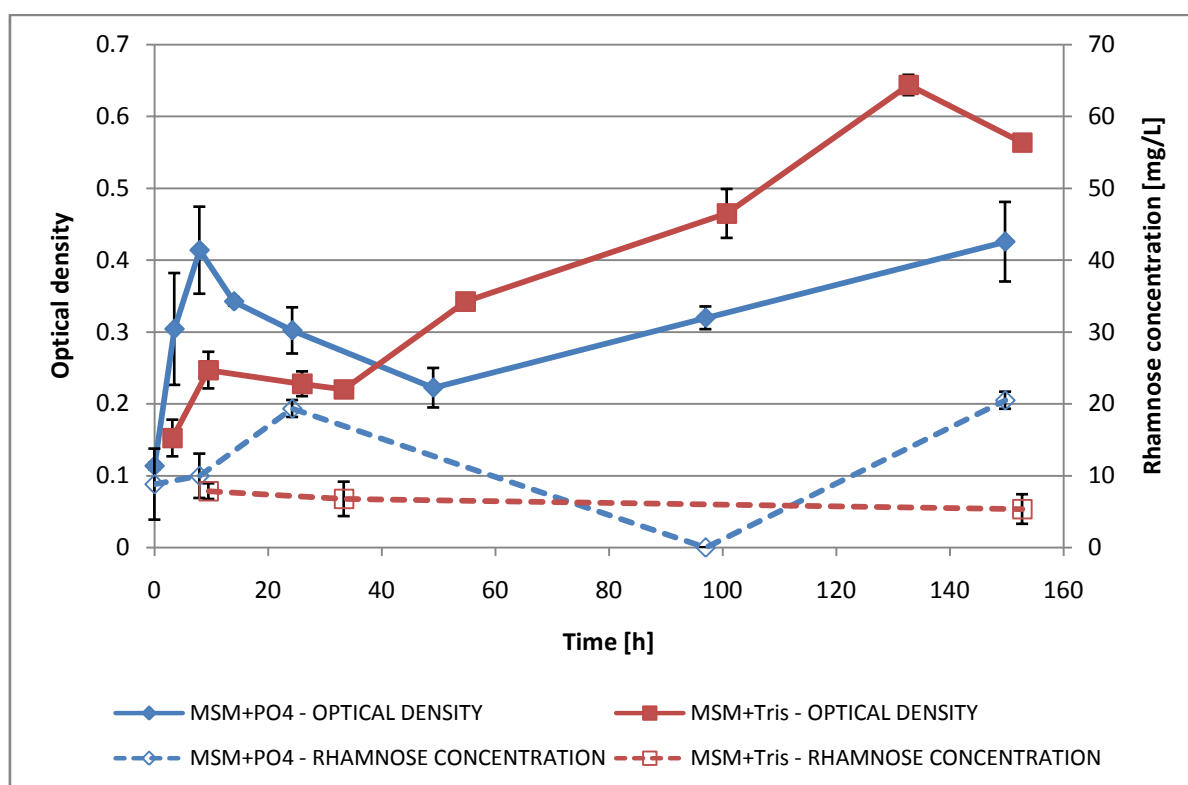


Figure 23: Comparison of *Pseudomonas aeruginosa* ATCC 9027 growth and biosurfactant production on MSM+PO<sub>4</sub> and MSM+Tris media with hexadecane as carbon source.

Zhang and Miller (1994) investigated the difference in natural hydrophobicity of the membranes of different *Pseudomonas aeruginosa* strains. They found that ATCC 9027 has a very hydrophilic membrane, with only 27 % of the cells attached to hexadecane when grown on octadecane. In a later paper they showed that ATCC 9027 had the slowest rate of degradation on octadecane between six *Pseudomonas aeruginosa* strains (Zhang and Miller 1995). ATCC 9027 is therefore not naturally suited for growth on alkanes. *Pseudomonas aeruginosa* ATCC 27853 was shown to have a more hydrophobic membrane with 55 % of the cells attaching to octadecane (Zhang and Miller 1994). The latter strain should therefore be more suited for growth on alkanes as sole carbon source. This strain was also grown on MSM+PO<sub>4</sub> medium containing alkanes as sole carbon source.

Slightly better growth was obtained for ATCC 27853 on the MSM+PO<sub>4</sub> medium with hexadecane as carbon source (Figure 24) at 150 hours. Growth improved after 24 hours, and increased slowly up to a maximum optical density of 0.76 after 150 hours. It is probable that growth would improve further if the run time was extended, as was shown for ATCC 9027 in Figure 22. Al-Tahhan *et al.* (2000) grew ATCC 9027 and ATCC 27853 on media containing 1 % hexadecane and reported similar growth over a long period (400 hours).

Tests were done to see whether the local strain *Ps. aeruginosa* 2bf was better suited for growth on alkanes as carbon source, since it was originally isolated from oil contaminated soil. Growth was initially slightly better than that of ATCC 9027, but had decreased greatly by 150 hours (Figure 25). No tendency was indicated to show that growth would increase over time as was the case with the culture collection strains. Negligible amounts of rhamnolipid were produced.

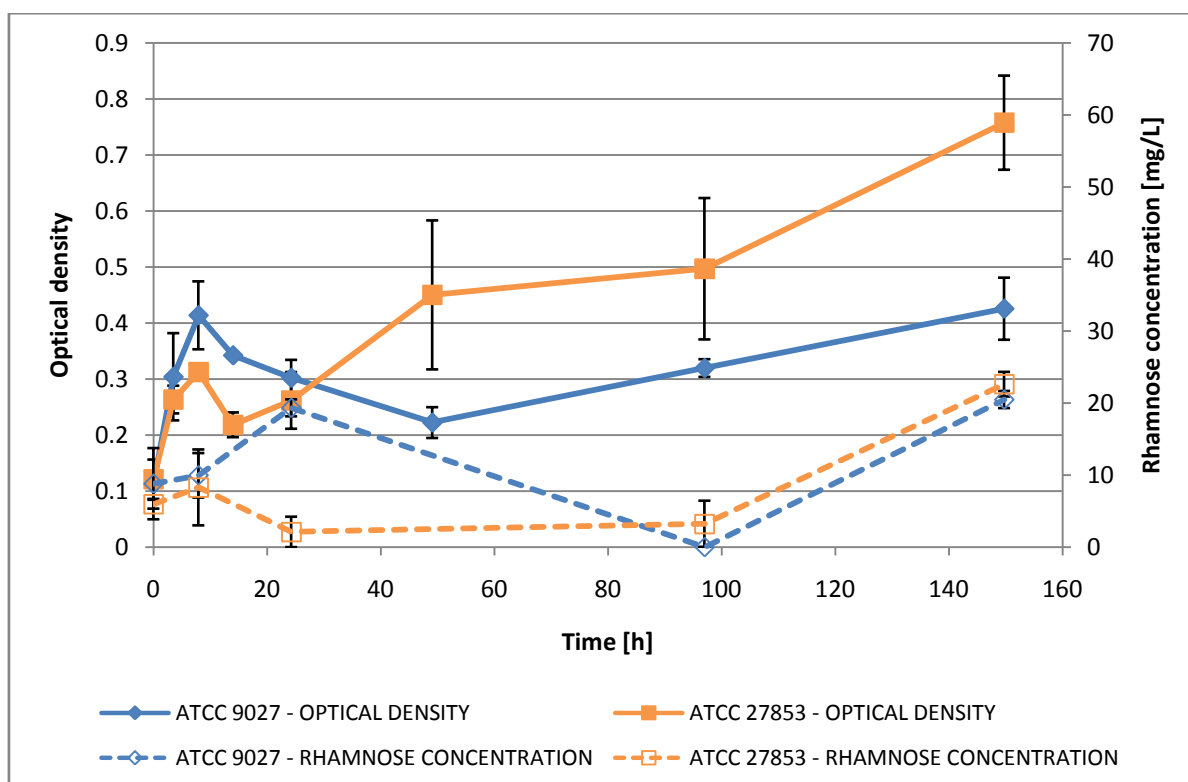
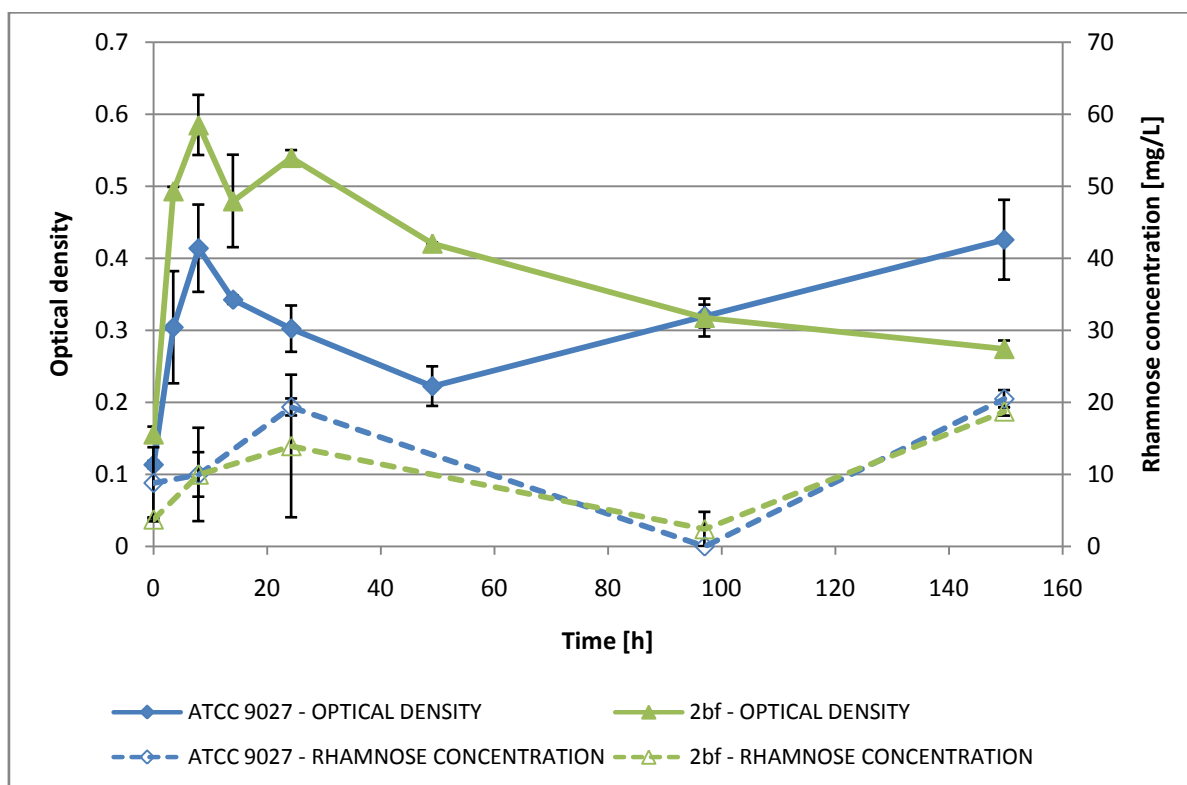


Figure 24: Comparison of *Pseudomonas aeruginosa* ATCC 27853 growth and biosurfactant production on MSM+PO<sub>4</sub> medium with hexadecane as carbon source.



**Figure 25: Comparison of *Pseudomonas aeruginosa* 2bf growth and biosurfactant production on MSM+PO<sub>4</sub> medium with hexadecane as carbon source.**

Thus, for all strains and media tested, rhamnolipid production on hexadecane was negligible. Rhamnolipid production commenced in the early stationary phase for the glucose experiments (section 3.2.1.3 and 3.7.2). In the alkane experiments growth had not reached the start of the stationary phase by the time the experiments were terminated. A strategy for increasing growth is therefore necessary to improve rhamnolipid production on alkanes. If rhamnolipid is only produced when the stationary phase is reached the organism cannot enhance the uptake of hydrocarbon through emulsification or solubilisation. The only method of alkane uptake that would occur according to literature, is the direct interfacial uptake of the hydrocarbon by hydrophobic cell membranes (Bouchez-Naitali *et al.* 1999). The hydrophobicity of the cell membrane depends on the strain used. Future work should investigate growth of more naturally hydrophobic strains on alkanes.

### 3.6 Strategy for enhancing growth and biosurfactant production on alkane media

#### 3.6.1 Dual substrate media

In the literature review (section 1.4.1) it was mentioned that the addition of rhamnolipids exogenously can improve the growth on hydrophobic substrates. However, Zhang and Miller (1994) found that a smaller amount of rhamnolipid (0.6 mM) inhibited growth for 100 hours on octadecane while a large amount (6 mM) substantially enhanced growth. If such a large amount of rhamnolipid, which equals approximately 3.2 g/l, is to be added the extraction and purification of such a large amount of would be costly and time consuming. Since even larger volumes would be necessary for production in a bioreactor, an alternative route was investigated. With a dual substrate system of glucose and alkanes it is possible that rhamnolipids will be produced by using glucose as carbon source, and the rhamnolipid thus produced would serve to solubilise the alkane substrate.

*Ps. aeruginosa* ATCC 9027 and ATCC 27853 were grown in shake flasks containing MSM+Tris medium with glucose and hexadecane as carbon sources. Different dual substrate systems were investigated containing different ratios of glucose to hexadecane. These experiments were compared to the sole substrate experiments. All the different combinations of substrates mentioned in this section are summarised in Table 19. All media contained the same amount of carbon. Dual substrate flasks were inoculated from cultures containing glucose as sole carbon source.

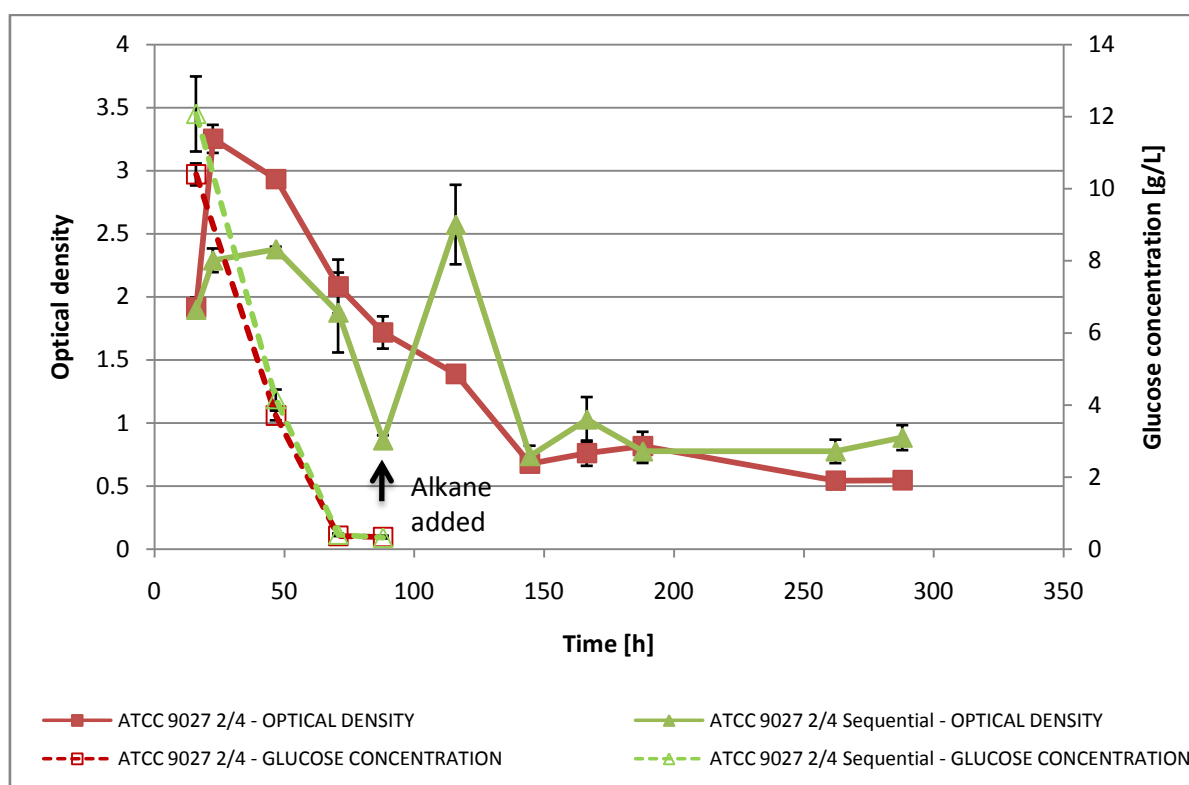
**Table 19: Percentage carbon as glucose and hexadecane in carbon supplied for shake flask experiments.**

% Carbon in carbon source (w/w)		Label
As hexadecane	As glucose	
100	0	0/4
75	25	1/4
50	50	2/4
0	100	4/4

#### 3.6.2 Comparison between sequential and non-sequential addition of alkane substrate

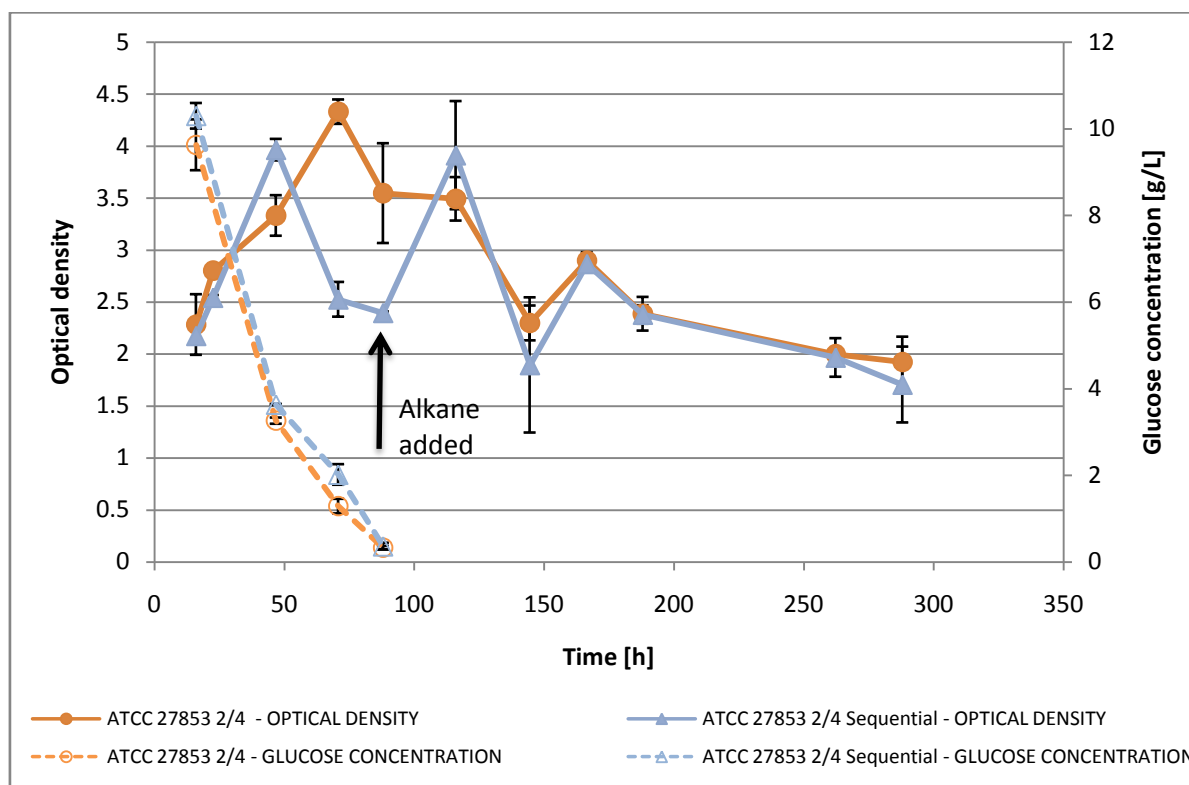
In one set of dual substrate experiments both substrates were added at the beginning and in another set hexadecane was added after glucose was consumed (sequential). With a sequential addition of hexadecane, where glucose was the sole substrate before addition, the reaction of the organism to the hexadecane in the system could be observed. Both experiments contained 50 % of the carbon as glucose (2/4 system), but in the one experiment 50 % of the carbon as hexadecane was added sequentially (2/4 Sequential). Two strains were used, *Ps. aeruginosa* ATCC 9027 and ATCC 27853. .

If the growth and glucose consumption of *Ps. aeruginosa* ATCC 9027 on the 2/4 and 2/4 sequential systems are studied in Figure 26, some differences between the flasks can be observed. The non-sequential (2/4) flasks showed slightly higher growth at the beginning (43 % higher growth at 23 hours), even though glucose consumption was the same at 48 hours. It is possible that the increased growth in the 2/4 non-sequential flasks was due to limited utilisation of alkane, since the 2/4 Sequential flasks contained only glucose up to 88 hours. However, the absorbance could have been affected by the presence of alkanes, leading to a false positive result. Alkane was added at 88 hours and subsequently growth increased by 33 %. At this point, all glucose was consumed in the flasks. However, after 145 hours growth decreased to the same level as the 2/4 flasks.



**Figure 26: Comparison of *Pseudomonas aeruginosa* ATCC 9027 cell growth and glucose consumption on MSM+Tris media containing 1.5 % glucose and 0.707 % hexadecane with different times of hexadecane addition. In one setup the hexadecane was added at the start of the run (2/4) and in another after glucose was consumed (2/4 Sequential).**

Similar observations regarding the growth were made for *Ps. aeruginosa* ATCC 27853 (Figure 27). Higher growth (71 %) was measured at 71 hours for the non-sequential flasks, with growth increasing by 63 % in the sequential flasks upon addition of hexadecane at 88 hours. By 88 hours all glucose was consumed, thus the increase in growth could not be due to glucose utilisation.



**Figure 27: Comparison of *Pseudomonas aeruginosa* ATCC 27853 cell growth and glucose consumption on MSM+Tris media containing 1.5 % glucose and 0.707 % hexadecane with different times of hexadecane addition. In one setup the hexadecane was added at the start of the run (2/4) and in another after glucose was consumed (2/4 Sequential).**

No significant difference was found between rhamnolipid production for non-sequential (2/4) and sequential (2/4 Sequential) feeding of hexadecane to the medium. As seen in Figure 28 and Figure 29, rhamnolipid production fell within the range of error for sequential and non-sequential feeding of hexadecane. For both strains increased cell growth when hexadecane was added did not lead to increased rhamnolipid production. This could indicate that glucose was used exclusively for rhamnolipid production.

In the next sections only the non-sequential 2/4 experiments will be compared to previous data, because of the negligible difference in the production of rhamnolipid between sequential and non-sequential setups.

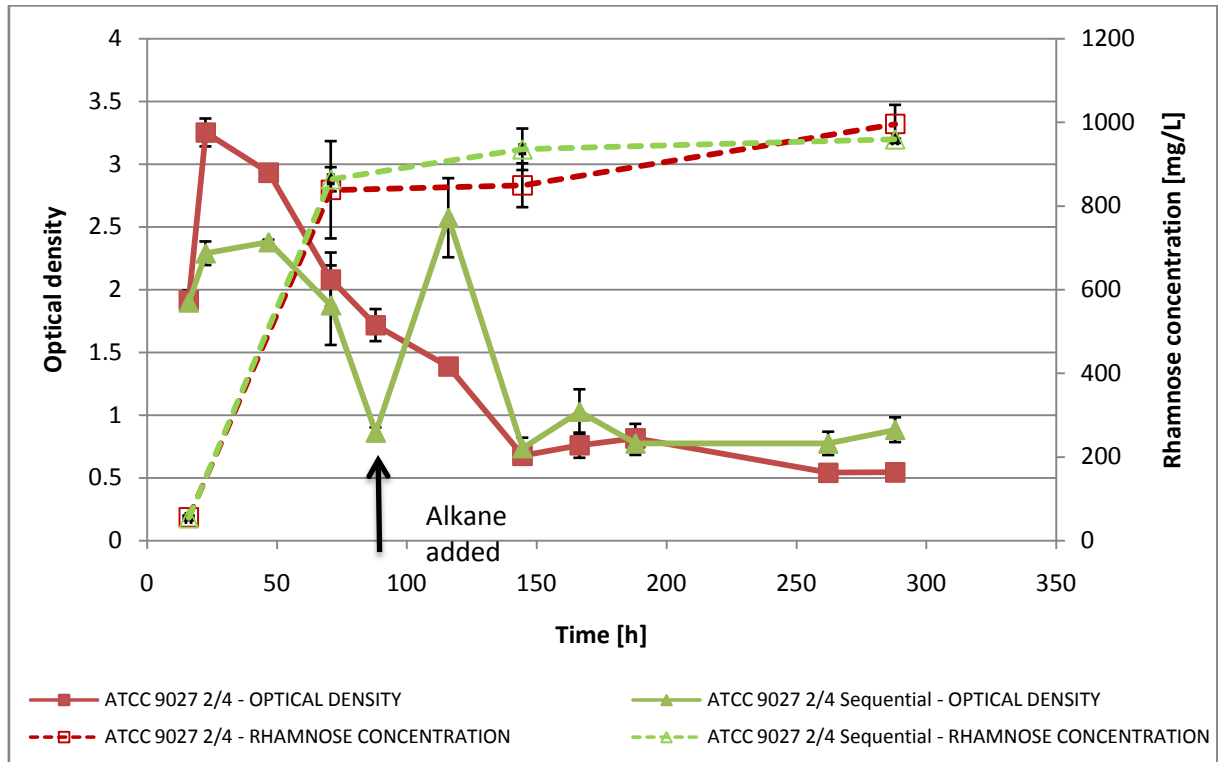


Figure 28: Comparison of *Pseudomonas aeruginosa* ATCC 9027 cell growth and biosurfactant production on MSM+Tris media containing 1.5 % glucose and 0.707 % hexadecane with different times of hexadecane addition. Hexadecane was added at the start of the run (2/4) or after glucose consumption (2/4 Sequential).

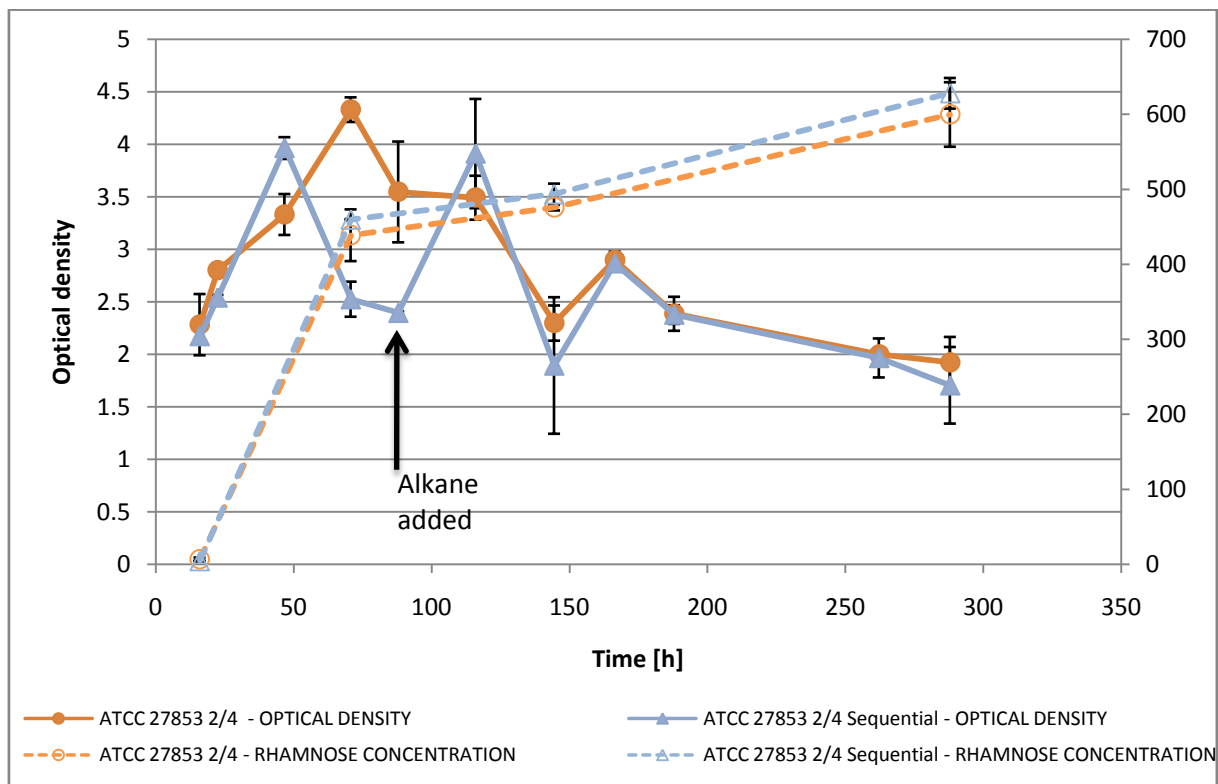
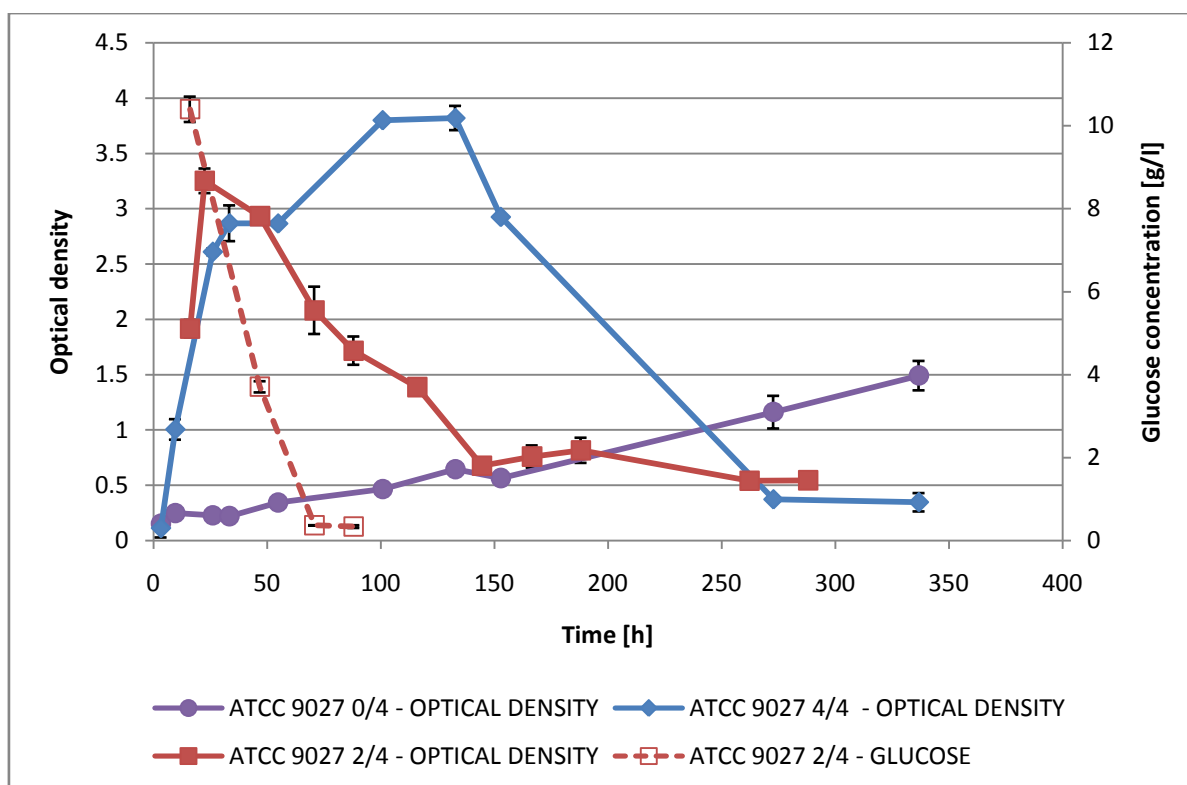


Figure 29: Comparison of *Pseudomonas aeruginosa* ATCC 27853 cell growth and rhamnolipid production on MSM+Tris media containing 1.5 % glucose and 0.707 % hexadecane with different times of hexadecane addition. In one setup the hexadecane was added at the start of the run (2/4) and in another after glucose was consumed (2/4 Sequential).

### 3.6.3 Comparison between single carbon source and dual carbon source for ATCC 9027

The 2/4 dual substrate system achieved similar growth to the glucose 4/4 system up to 47 hours (Figure 30). Both systems were inoculated from cultures which contained glucose as sole carbon source. After 47 hours growth in the 2/4 flasks decreased considerably, coinciding with the depletion of glucose. The 4/4 sole substrate system showed the same abrupt decrease in cell concentration after 133 hours. Although glucose concentration was not monitored for the 4/4 sole substrate system, it was assumed that the glucose concentration was depleted at 133 hours, which caused the cell concentration to decrease. However, the 2/4 and 4/4 flasks contained the same amount of carbon. Therefore, only glucose was used effectively for growth in the dual substrate system.

The improved growth of the 2/4 dual substrate flasks over the 0/4 hexadecane flasks (Figure 30) was therefore due to the glucose present in the dual substrate flasks. Although the dual substrate system showed an improvement over growth on alkane as sole substrate (0/4), the goal of developing a system where alkanes are used effectively was not achieved.



**Figure 30: Comparison of *Pseudomonas aeruginosa* ATCC 9027 growth on MSM+Tris media containing glucose (4/4) and hexadecane (0/4) as sole carbon sources and on a dual substrate system of glucose and hexadecane (2/4). Glucose consumption of the 2/4 dual substrate system is also shown.**

Rhamnose production was slightly lower in the 2/4 dual substrate system than in the 4/4 system, with 17 % less rhamnose measured in the 2/4 flasks at around 150 hours. It was assumed that all glucose was consumed in the 4/4 flasks at 337 hours (Figure 30), and thus



the rhamnose yield in terms of glucose utilised could be calculated. As was proven in section 3.6.2, hexadecane was not utilised for rhamnolipid production and therefore could not contribute to the yield in the 2/4 flasks. The yields for the 2/4 and 4/4 systems were compared (Figure 32). The dual substrate 2/4 yield was nearly 150 % higher than the 4/4 system yield. It is more probable that a large quantity of glucose was used in the 4/4 glucose system for cell maintenance during the stationary phase. Less glucose was thus available for rhamnolipid production. The trend shown in literature is that rhamnolipid production increases with an increasing amount of carbon (Wei *et al.* 2005). However, no reports were found comparing substrate yields for different concentrations.

The fact that much higher yields were obtained for less glucose added initially has implications for process development. A fed batch system is proposed where different concentrations of initial substrate should be tested which would maximise the rhamnolipid yield without too much substrate being used for cell maintenance. A good initial concentration for such tests would be 1.5 % as used in the experiments above. The next step would be the addition of substrate to boost cell concentrations for enhanced production of rhamnolipid. Different feeding strategies will have to be tested to find the optimal fed batch setup for maximum rhamnolipid production.

The rate of substrate feeding will depend on the kinetics of substrate consumption, which must be investigated in future work. It is desirable to feed the smallest necessary volume of substrate with a high concentration in order to keep the dilution rate low and maximise the product concentration.

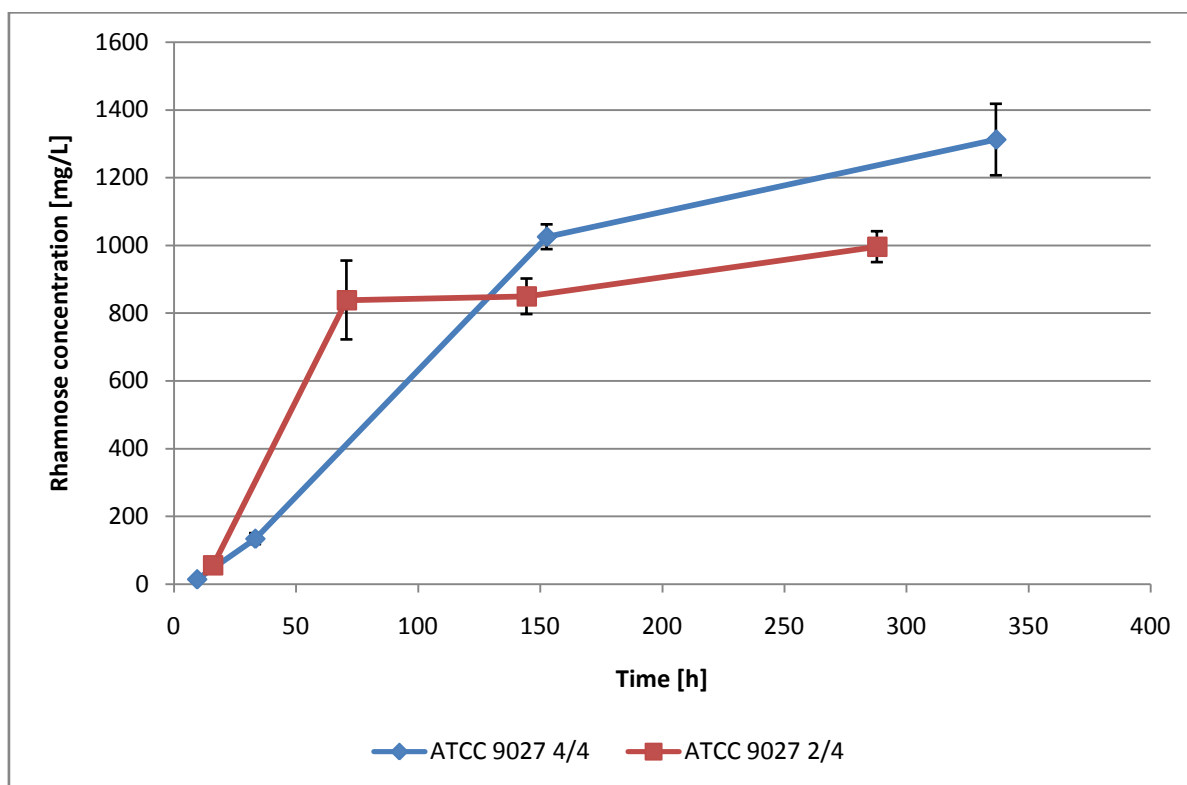


Figure 31: Comparison of rhamnolipid production of *Pseudomonas aeruginosa* ATCC 9027 between media containing glucose as sole carbon source (4/4) and media containing 50% of carbon as glucose and 50% as hexadecane (2/4).

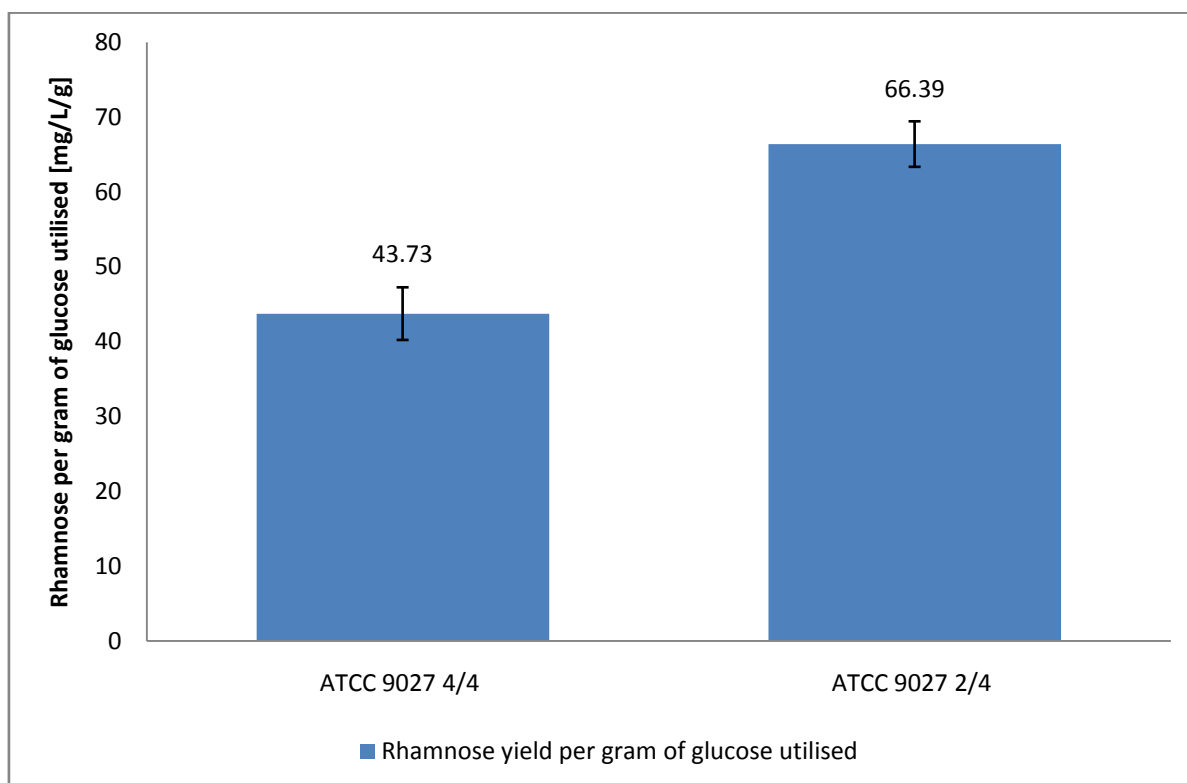


Figure 32: Comparison of specific rhamnolipid production from *Pseudomonas aeruginosa* ATCC 9027 grown on MSM+Tris media containing glucose as carbon source (4/4) and containing both glucose and hexadecane as carbon source (2/4). Specific production of rhamnolipid expressed as the total rhamnose production per gram of glucose utilised.

### 3.6.4 Comparison between single carbon source and dual carbon source for ATCC 27853

*Ps. aeruginosa* ATCC 27853 was cultured on a 1/4 system (where 25 % of the carbon was glucose and the rest hexadecane) in addition to the 2/4 and 4/4 systems. The glucose was consumed at 47 hours for the 1/4 system as compared to 88 hours for the 2/4 system (Figure 33). Growth in the 1/4 medium decreased when glucose was consumed and was significantly lower (up to optical density of 2.3) than the 2/4 system up to 88 hours. This would suggest that hexadecane was not used for growth, since all systems contained the same amount of carbon. However, growth eventually recovered in the 1/4 system and increased to similar levels as the 2/4 system, well after all glucose was consumed.

Hexadecane was not used for rhamnolipid production, since the 1/4 dual substrate system produced about half the amount of rhamnose than the 2/4 system (Figure 34), with both systems containing the same amount of carbon. If the glucose yields of the dual substrate systems (1/4 and 2/4) are compared, it can be seen that the yields are similar (13 % difference), within the range of error (Figure 35). The production of rhamnolipids was thus dependant on the amount of glucose available.

Rhamnose productions in the 2/4 and 4/4 systems were very similar with a 10 % difference in production between 2/4 at 145 hours and 4/4 at 124 hours (Figure 34). At 288 hours production was 28 % greater in the 2/4 system. The 4/4 system contained double the amount of glucose compared to the 2/4 system. Thus, at glucose concentrations higher than approximately 1.5 % (2/4 system), rhamnolipid production reaches a plateau, with the remaining glucose possibly being used for cell maintenance as was shown for ATCC 9027 in the previous section.

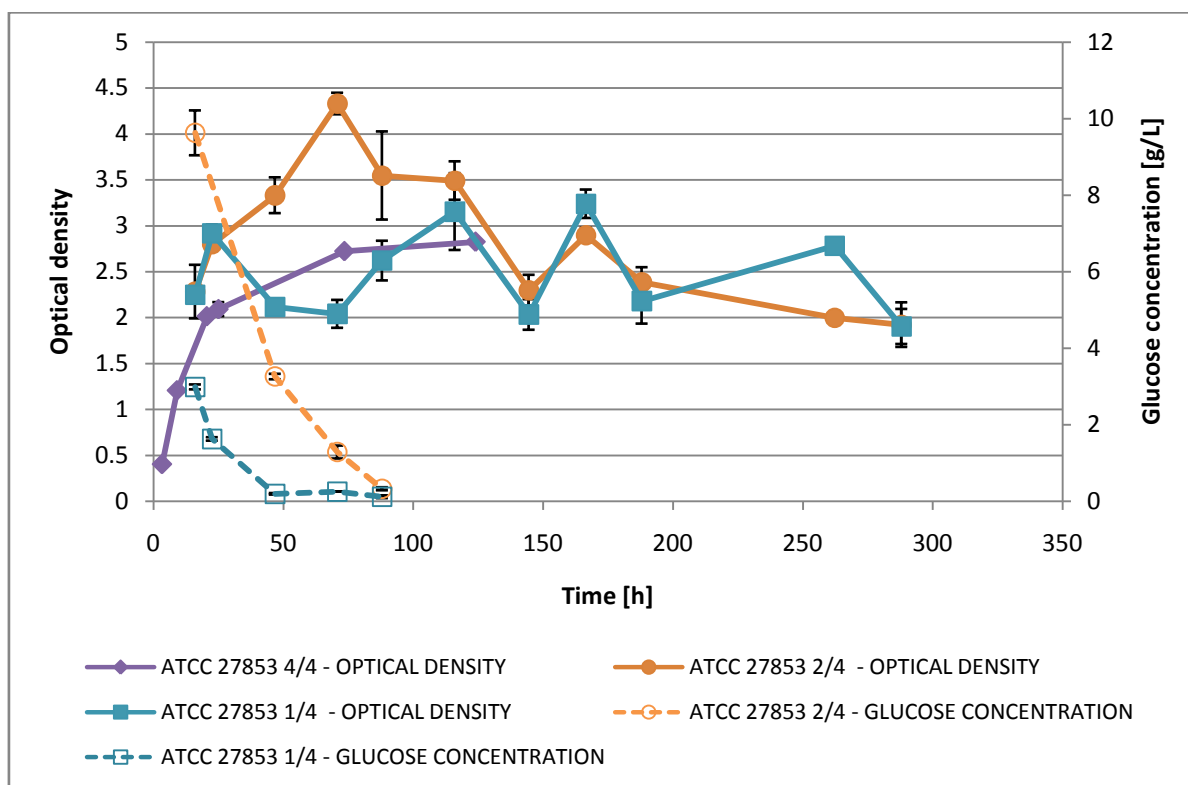


Figure 33: Comparison of *Pseudomonas aeruginosa* ATCC 27853 growth on MSM+Tris media with 25 % of carbon as glucose (1/4), 50 % of carbon as glucose (2/4) and 100 % of carbon as glucose (4/4). Glucose consumptions of 1/4 and 2/4 systems are included.

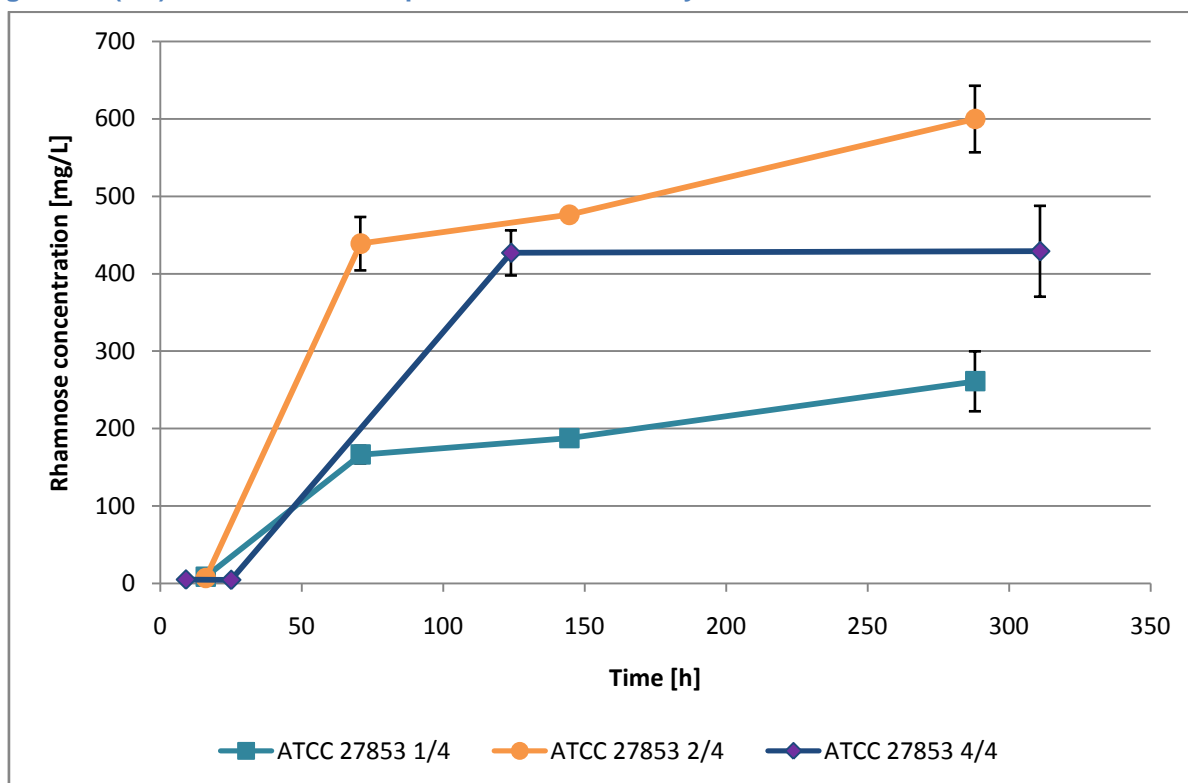
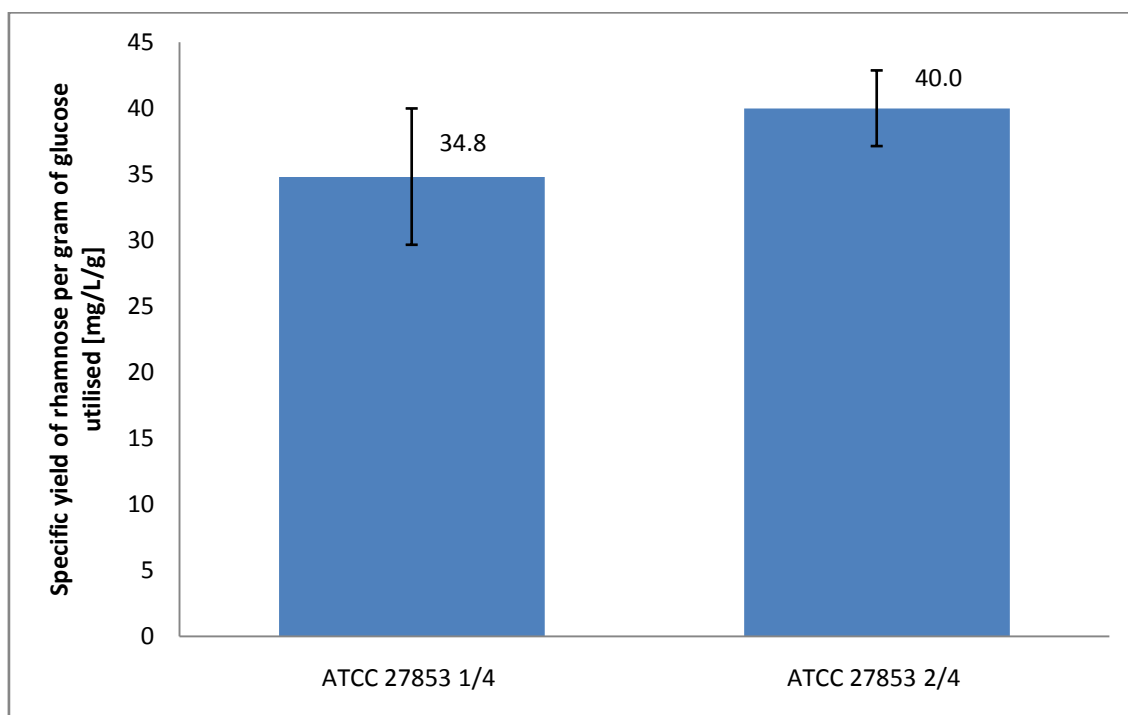


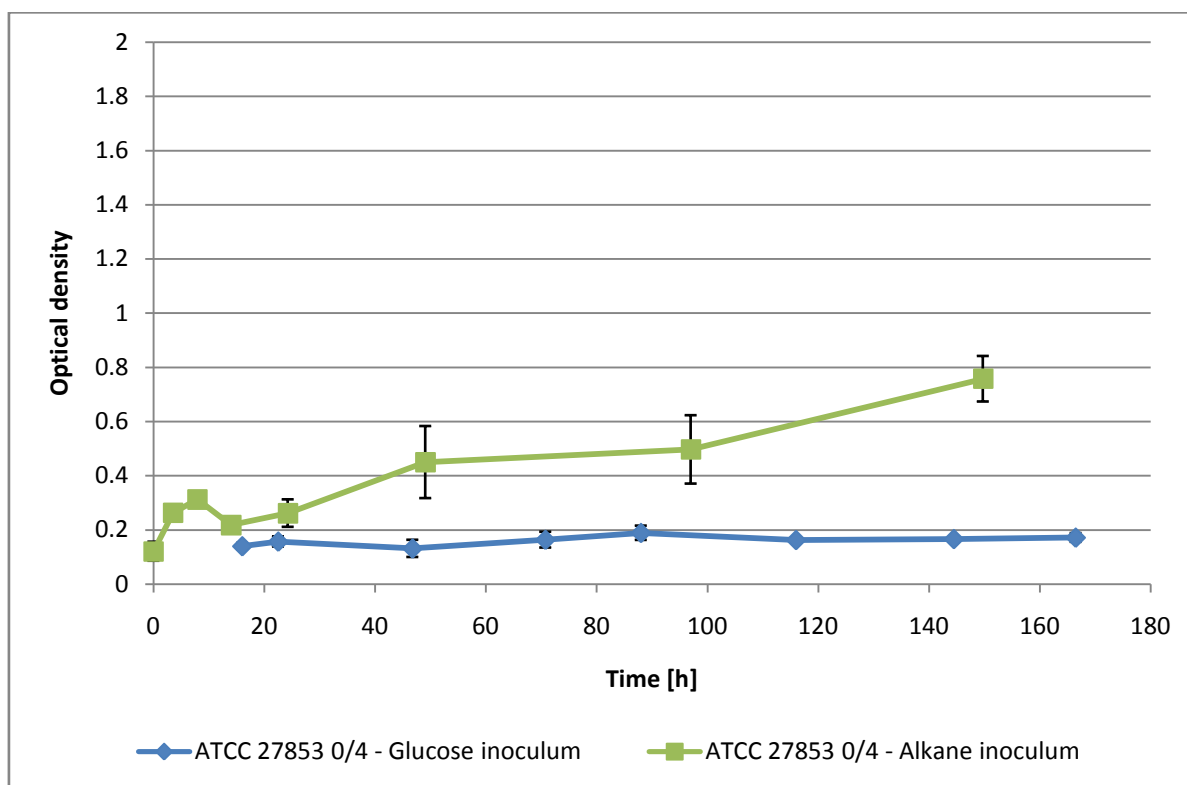
Figure 34: Comparison of rhamnolipid production of *Pseudomonas aeruginosa* ATCC 27853 on media containing glucose as sole carbon source (4/4) and dual substrate media containing 50 % of carbon as glucose (2/4) and 25 % of carbon as glucose (1/4).



**Figure 35: Comparison of specific production of rhamnolipid from *Pseudomonas aeruginosa* ATCC 27853 on MSM+Tris media containing 25 % of carbon as glucose (1/4) and 50 % of carbon as glucose (2/4). Specific production of rhamnolipid expressed as the total rhamnolipid production per gram of glucose utilised.**

In order to develop possible explanations for the poor uptake of alkanes, growth on alkane media was investigated further. As was reported previously, slow growth was observed over an extended period for all cultures using hexadecane as the sole carbon source (section 3.5). All these cultures were maintained and inoculated from media containing hexadecane as sole carbon source. However, when *Ps. aeruginosa* ATCC 27853 was grown on 0/4 hexadecane medium from an inoculum containing glucose as sole carbon source, the organism did not demonstrate the same slow growth over a long period as when an alkane inoculum was used (Figure 36). Negligible growth was observed throughout the run. An explanation may be that the cell membrane adapted itself to the glucose medium and became too hydrophilic to take up alkane by direct contact.

In all dual substrate experiments inocula containing glucose as sole carbon source were used. It is thus possible that in the dual substrate systems the cell membranes could perhaps not recover their hydrophobicity.



**Figure 36: Comparison of *Pseudomonas aeruginosa* ATCC 27853 growth on hexadecane media (0/4 system) inoculated from glucose based media and alkane based media.**

In literature, the presence of rhamnolipids have been shown to greatly increase the hydrophobicity of *Pseudomonas aeruginosa*, even when inoculated from media containing glucose as sole carbon source (Al-Tahhan *et al.* 2000). However, in these experiments rhamnolipid was added exogenously to test flasks containing hexadecane as sole carbon source. In the same study, rhamnolipid was added to flasks containing glucose as sole carbon source in which the hydrophobicity of the cell membranes increased and decreased again very rapidly. It is possible that in the dual substrate experiments presented here, the presence of glucose thus influenced the cell hydrophobicity, which influences the ability of the cell to take up alkanes directly. Future work on the dual substrate system should involve maintaining the strains on media with alkanes as the sole carbon source and inoculating exclusively from alkane media. Strains could also be investigated which are naturally better adapted to using hydrophobic substrates. Another option is adding biosurfactants exogenously to alkane containing media. This setup would be ideally suited to a recycle process, where product from one batch fermentation is fed to the start up stage of another batch fermentation.

### **3.7 Evaluation of *Pseudomonas aeruginosa* ATCC 9027 biosurfactant production in an instrumented bioreactor on glucose media**

*Ps. aeruginosa* ATCC 9027 was grown in an instrumented bioreactor in MSM+Tris medium containing 3 % glucose as carbon source. The minimum working volume of 2 l of medium was used, because the results obtained from this experiment will be used in fed batch studies, where a larger volume will be needed. Temperature was controlled at 35 °C. Aeration was initially set at 0.8 vvm and the agitation was controlled so that the dissolved oxygen level was around 20 %. An inoculum of 200 ml was grown in MSM+PO<sub>4</sub> medium in a 2l shake flask.

#### **3.7.1 Comparison of growth and biosurfactant production between bioreactor and shake flask batch studies**

The growth and production profiles were very similar to those obtained in the same medium in shake flasks (Figure 37). A maximum optical density of 3.8 was reached in the shake flasks, whereas a very similar maximum of 3.94 was reached in the bioreactor. Maximum rhamnose concentrations of 1312 mg/l and 1386 mg/l were reached in the shake flasks and bioreactor respectively. Rhamnolipid production in the reactor also coincided with the levelling of the cell concentration and onset of the stationary phase (Figure 39). However, a major difference was the rate of growth and rhamnolipid production. Figure 38 shows the average rate of growth for both systems at the end of the exponential phase and the average rate of production of rhamnolipid by the end of the stationary phase. More than double the rate of growth was observed during the exponential phase in the bioreactor. The rate of rhamnolipid production was 62% less in the shake flasks than in the bioreactor. It was probably the superior aeration in the bioreactor which accelerated the metabolic processes.

The maximum growth and rhamnolipid production is therefore not limited by oxygen transfer, but probably to the medium and operating conditions. Future studies should focus on increasing production by improvement of the medium, but also by alternative process operations. Rhamnolipid have been shown to be a secondary metabolite in this study, thus a fed-batch operation would be a promising avenue to explore in future work. Through fed-batch operation higher cell densities can be achieved, possibly leading to higher rhamnolipid concentrations (Chen *et al.* 2007).

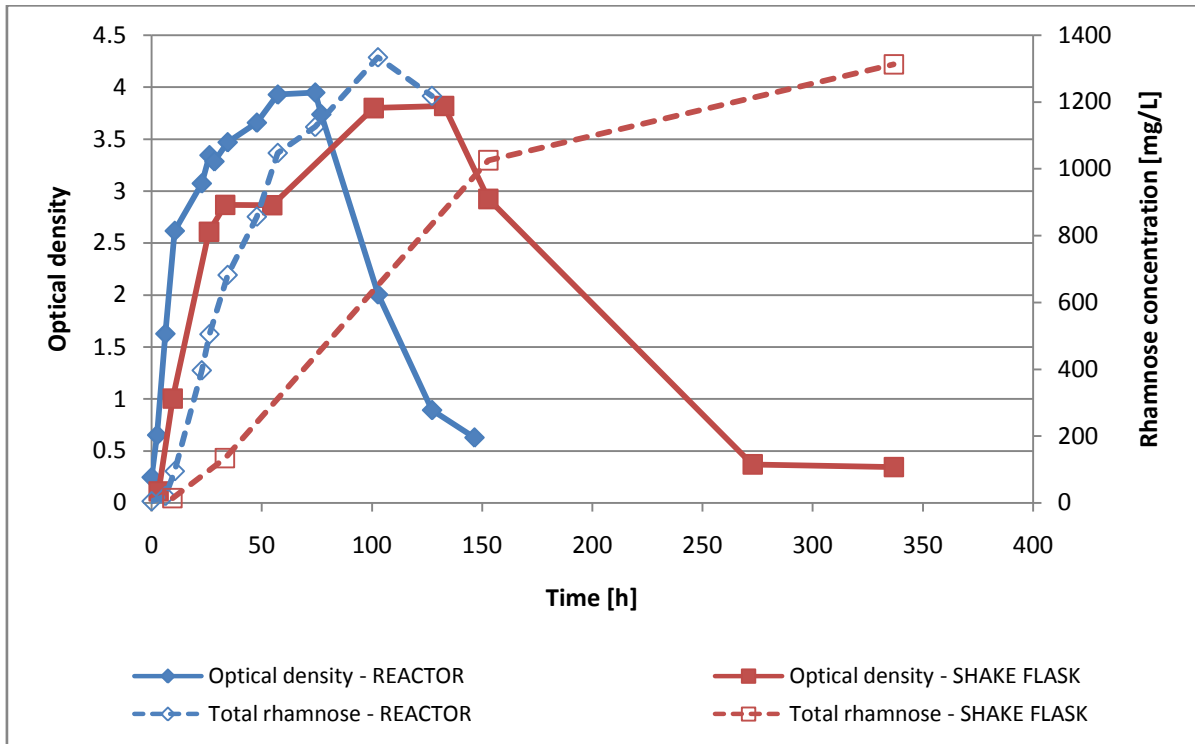


Figure 37: Comparison of growth and biosurfactant production of *Pseudomonas aeruginosa* ATCC 9027 in shake flasks and bioreactor. The medium was MSM+Tris containing 3 % glucose as carbon source. Temperature was controlled at 35 °C. Dissolved oxygen in the bioreactor was controlled at 20 %.

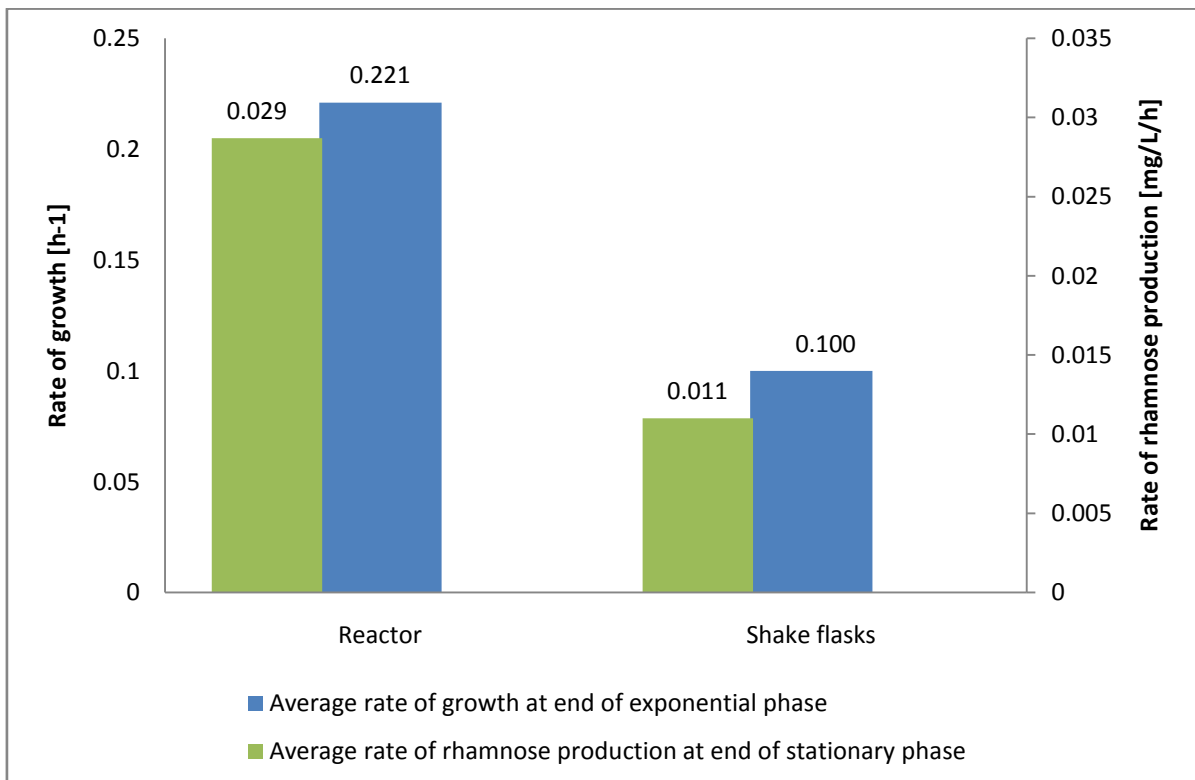
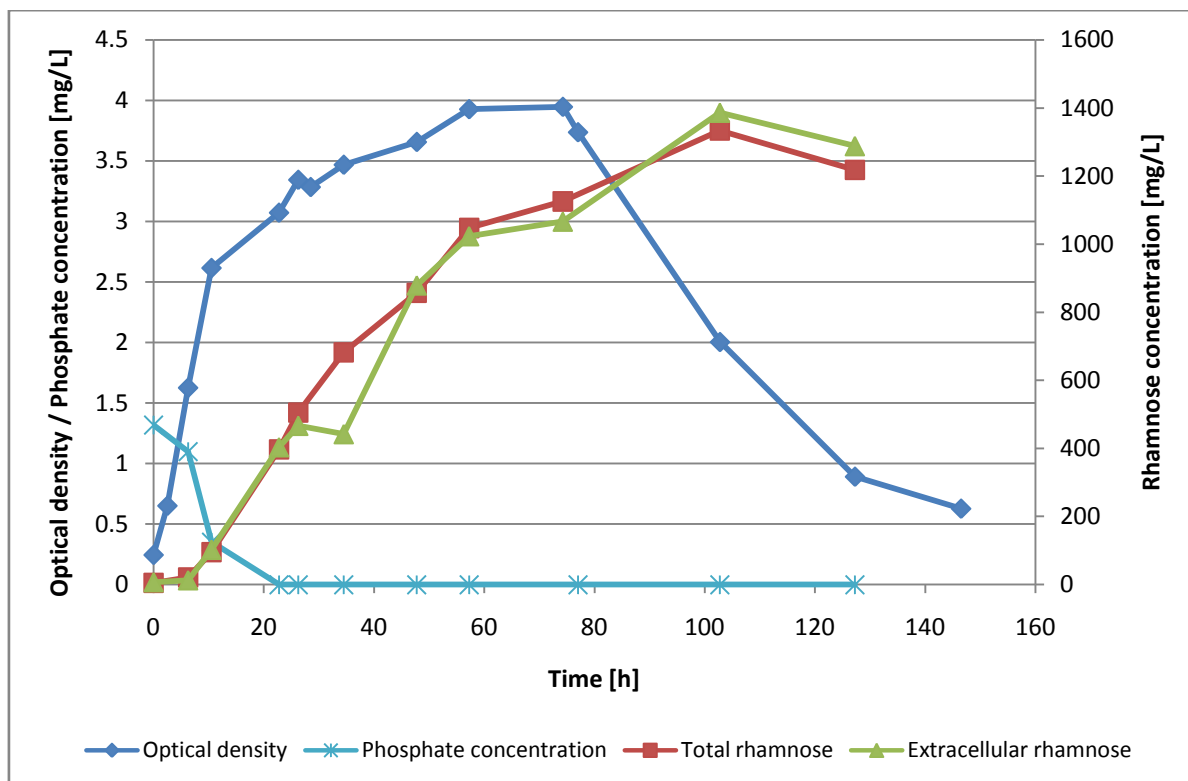


Figure 38: Comparison of the rate of growth and biosurfactant production of *Pseudomonas aeruginosa* ATCC 9027 in shake flasks and a bioreactor. The medium was MSM+Tris containing 3 % glucose as carbon source. Temperature was controlled at 35 °C. Dissolved oxygen in the bioreactor was controlled at 20 %.

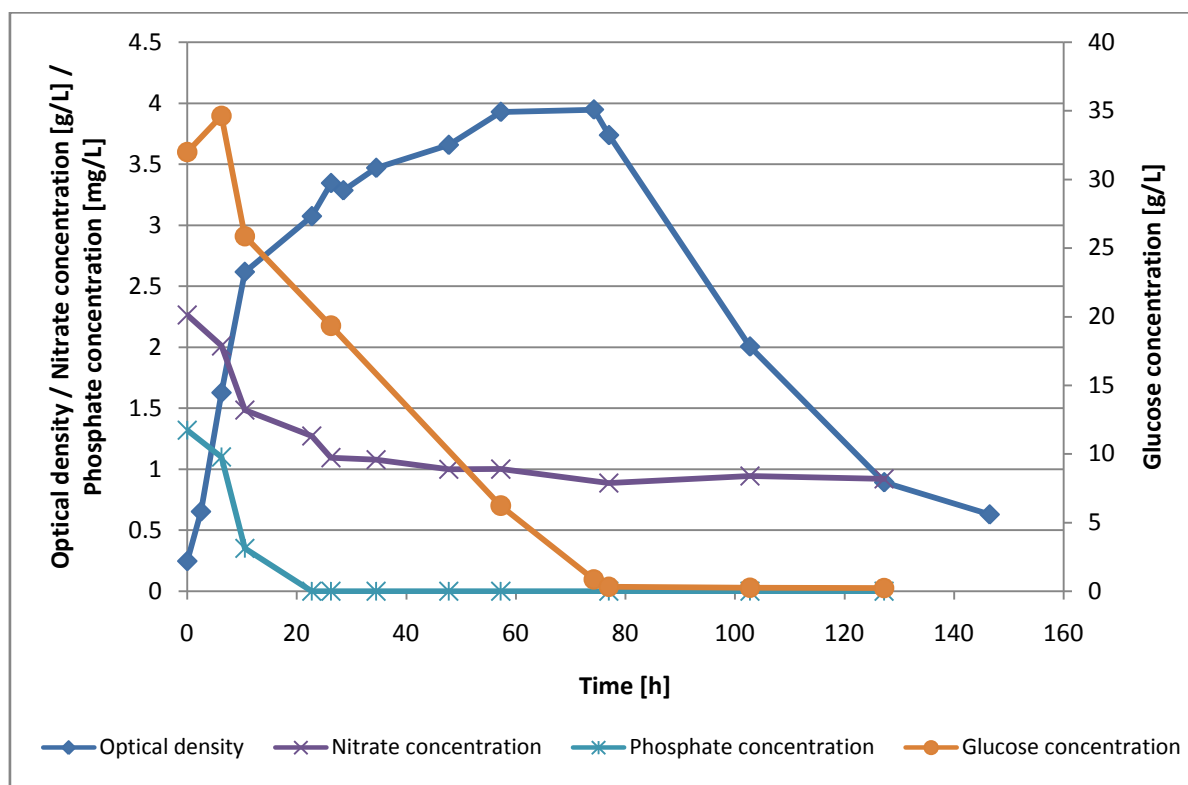


### 3.7.2 Nutrient limitation in bioreactor fermentation of *Pseudomonas aeruginosa* ATCC 9027

The phosphate concentration was monitored (Figure 39) to determine whether a phosphate limitation was present in the MSM+Tris medium. The initial concentration of phosphate in the medium was 1.32 mg/l, which was transferred from the inoculum (10 % v/v). Phosphate was depleted by 23 hours, which coincided with the start of the stationary phase. The rhamnose concentration in the cell culture was only 21 mg/l by 6 hours in mid exponential phase. By 23 hours at the onset of the stationary phase this had increased to 403 mg/l.



**Figure 39:** Batch fermentation of *Pseudomonas aeruginosa* ATCC 9027 in a 7.5 l bioreactor with a working volume of 2 l, temperature of 35 °C and dissolved oxygen at 20 %. Trends of growth, total and extracellular biosurfactant and phosphate concentration for fermentation in MSM+Tris medium containing 3 % glucose as carbon source.



**Figure 40: Batch fermentation of *Pseudomonas aeruginosa* ATCC 9027 in a 7.5 l bioreactor with a working volume of 2 l, temperature of 35 °C and dissolved oxygen at 20 %. Trends of growth, phosphate and nitrate consumptions as well as substrate (glucose) consumption for fermentation in MSM+Tris medium with glucose as carbon source.**

The nitrate and glucose concentrations were monitored to determine if they could also be limiting factors. The concentration of nitrogen dropped rapidly in the exponential phase, but only decreased slowly in the stationary phase to a minimum of 1 g/l (Figure 40). Nitrogen was thus not the limiting nutrient in the medium. Glucose was exhausted by 77 hours which caused cell growth to enter the death phase (Figure 40). Cell concentration dropped rapidly from an optical density of 3.94 at 74 hours to 0.628 at 147 hours. Thus, phosphate was the limiting nutrient and caused the large production in rhamnolipids. This confirmed the assumption made in section 3.2, that the MSM+Tris medium was phosphate limiting and therefore led to good rhamnolipid production.

The MSM+Tris medium had the same composition as the MSM+PO<sub>4</sub> medium, except that the tris buffer was substituted with a phosphate buffer (MSM+PO<sub>4</sub>). The MSM+PO<sub>4</sub> medium was adapted from literature, where it was claimed that it is limited in nitrogen (Sim *et al.* 1997) (see section 1.6.1.1 in the literature review). Results in the MSM+PO<sub>4</sub> showed poorer growth with an earlier onset of the stationary phase, thus it could not have been nitrogen limited and it was definitely not phosphate (nearly ten times the amount of MSM+Tris) or glucose limited as supported by the results in Figure 40. Another factor thus caused growth to slow down in that medium. Future work should adjust the MSM+PO<sub>4</sub> medium to be nitrogen limited and compare the phosphate and nitrogen limitations in terms of biosurfactant production.

### 3.7.3 pH monitoring of bioreactor fermentation of *Pseudomonas aeruginosa* ATCC 9027

The pH of the medium before autoclaving was 7.28. After autoclaving it increased to 7.47. The pH started to drop after the medium was inoculated and continued to decrease rapidly in the exponential phase (Figure 41). The pH was controlled to above 6.8 with 2M sodium hydroxide after around 6 hours. Studies done on *Pseudomonas aeruginosa* batch fermentation have found that 6.8 is the optimum pH for rhamnolipid production (Chen *et al.* 2007). The buffering capacity of tris buffer is slightly higher (7-9 pH) and therefore the initial pH was not adjusted to such a low value. A steady increase in pH was also observed probably due to deamination (Figure 41). The same phenomenon was observed in the shake flask study (Figure 16).

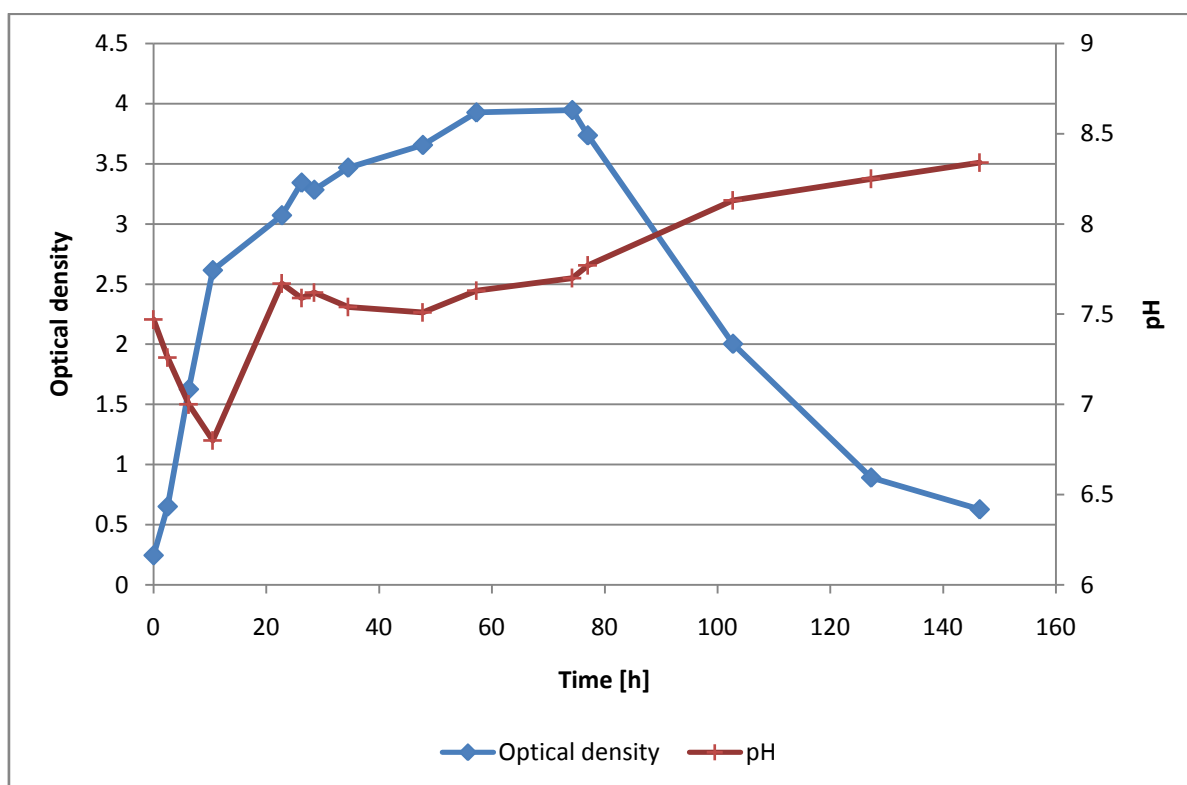


Figure 41: Growth and pH measurements for *Pseudomonas aeruginosa* ATCC 9027 grown in MSM+Tris medium with 3% glucose as carbon source. Batch fermentation was done in a bioreactor with 2 l working volume, temperature of 35 °C and dissolved oxygen at 20 %.

### 3.7.4 Control of aeration and foaming of bioreactor fermentation of *Pseudomonas aeruginosa* ATCC 9027

Antifoam of 0.025 % v/v was added to the medium at the start of the experiment, because excessive foaming occurred in a trial run. Up to 10 hours foaming was under control and the aeration was kept at 0.8 vvm and the agitation was controlled so that the dissolved oxygen level was around 20 %. This led to the agitation rate oscillating at approximately 400 rpm. At 10 hours when significant amounts of rhamnolipid were being produced, foaming increased very rapidly. Antifoam in a concentration of 2 % was added to dissipate the foam, but was not very effective. The entire 100 ml of the solution was added by

35 hours. By manipulating the aeration and agitation rate it was found that foaming was mainly influenced by the aeration and not as much by the agitation rate. Aeration was lowered to 0.2 vvm and the dissolved oxygen level was kept at 20 % by higher agitation of approximately 600 rpm. By 48 hours this setup was also not effective in controlling foaming and an antifoam solution of 10 % was added aseptically. This solution was much more effective in the control of foaming and no excessive foaming occurred for the rest of the experiment with the dissolved oxygen kept at 20 %.

In future bioreactor studies it will be important to monitor the amount of foaming. While it was shown that a high concentration of antifoam solution (10%) is necessary to control foaming, it is not desirable to add too much antifoam solution, as this can influence oxygen transfer and complicate downstream processing. In the bioreactor study presented here, there was found that the aeration rate has a considerable influence on the amount of foaming. Aeration must be kept to a minimum and agitation set at a higher level thereby ensuring proper oxygen transfer and control of foaming. A balance must be found between the control of foaming, cell densities and oxygen transfer.

### 3.8 Location of rhamnolipid in cultures

Some extracellular biosurfactants attach to the cell membrane and are not recovered if cells are separated before product recovery (section 1.4.2). An investigation was done to see if this was the case for rhamnolipids. Rhamnolipid was extracted from both the supernatant and the culture broth of *Ps. aeruginosa* ATCC 9027 and ATCC 27853. The extracellular and total rhamnose were measured at the end of successful rhamnose production experiments, which were the experiments done on MSM+Tris medium containing 3 % glucose as carbon source for both strains.

The ratio of total to extracellular rhamnose is given in Table 20 for both strains. Both ratios are close to 1 within the range of error, which signifies that the amounts of rhamnolipid extracted from cell culture and from the supernatant were equal. However, two data points were not sufficient from which to draw any conclusions. In the bioreactor fermentation done with *Ps. aeruginosa* ATCC 9027 (section 3.7), rhamnose production could be monitored for the entire growth cycle in both the supernatant and the cell culture. The production curve is reproduced below. It can be seen that the total amount of rhamnose was equal or very close to equal to the extracellular rhamnose measured in the supernatant at 10 different points in time. The exception occurred at 35 hours, where a difference of 240 mg/l rhamnose was observed. However, since no abrupt change was observed in the system at this point, the exception was considered an outlier. Rhamnolipid is therefore not cell bound, but is present in the extracellular liquid. The extreme outlier does raise questions as to the reproducibility of experiments. However, one outlier in 22 similar analyses confirms rather than contests reproducibility.

The location of the product is an important consideration in the design of the downstream process. These results have shown that the location of the product is primarily extracellular. Downstream processing would therefore require initial separation of the cells, followed by extraction and purification steps.

**Table 20: Ratio of total rhamnolipid to extracellular rhamnolipid in cultures of two different *Pseudomonas aeruginosa* strains grown on MSM+Tris medium with glucose as carbon source.**

Organism	Medium	Ratio total / extracellular rhamnose	Error
ATCC 27853	MSM+Tris	0.91	0.11
ATCC 9027	MSM+Tris	1.06	0.17

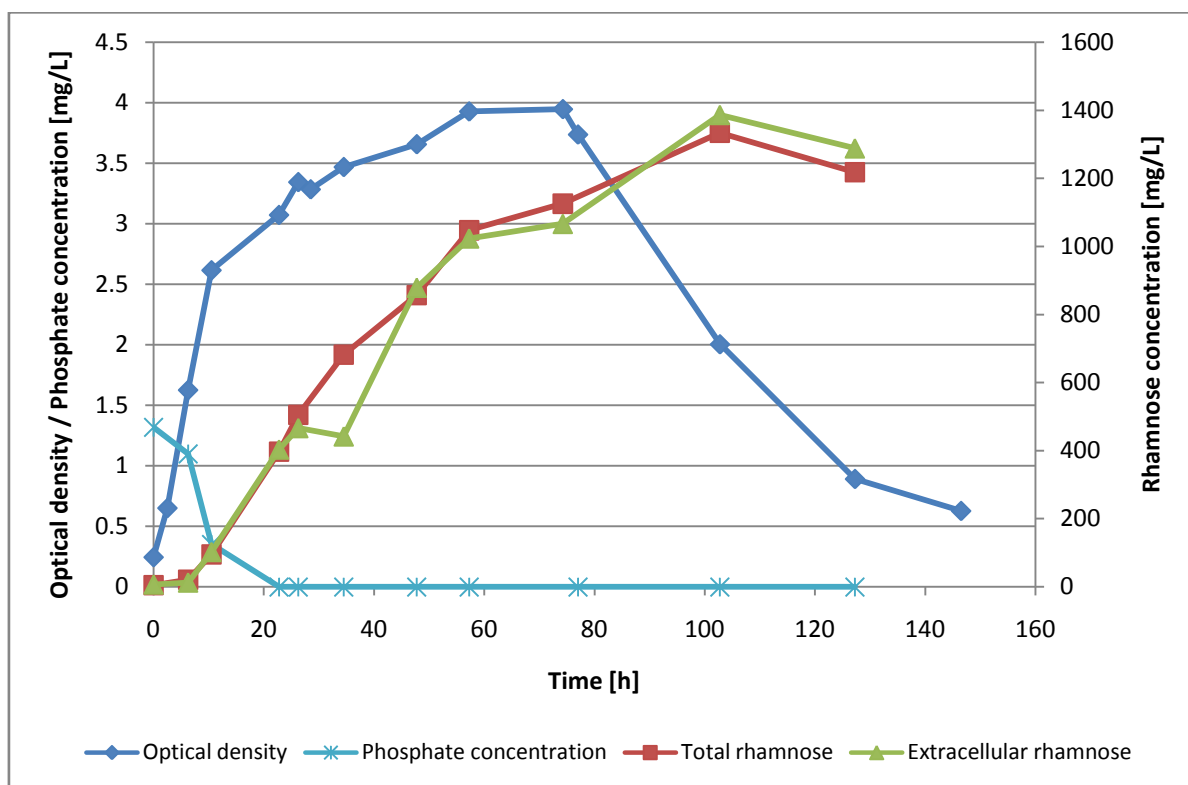


Figure 39: Batch fermentation of *Pseudomonas aeruginosa* ATCC 9027 in a 7.5 l bioreactor with a working volume of 2 l, temperature of 35 °C and dissolved oxygen at 20 %. Trends of growth, total and extracellular biosurfactant and phosphate concentration for fermentation in MSM+Tris medium containing 3 % glucose as carbon source.

### 3.9 Evaluation of antimicrobial activity of rhamnolipids from *Pseudomonas aeruginosa* ATCC 27853 and ATCC 9027 against *Mycobacterium aurum*

One of the focus points of this research is the evaluation of the antimicrobial activity of biosurfactants against microorganisms important in the South African context. The rhamnolipids produced by *Ps. aeruginosa* ATCC 9027 and ATCC 27853 were tested for activity against *Mycobacterium aurum*, an organism which is used to indicate susceptibility of tuberculosis to antibiotics. Supernatant from reactor and shake flask experiments of *Ps. aeruginosa* ATCC 9027 were tested for antimicrobial activity. For ATCC 27853 only supernatant from one shake flask experiment was tested. The media used in these experiments are listed in Table 21 with the zones of inhibition obtained. All tests were done within a week from harvesting the supernatant.

The rhamnolipid concentration could only be determined for ATCC 9027 supernatant, since the structure of its rhamnolipid is known (mono-rhamnolipid). For ATCC 27853 only the rhamnose concentration is given. Table 21 lists the average zones of inhibition for the different cultures. Mono-rhamnolipid was shown to have antimicrobial activity against *Mycobacterium aurum* in section 3.1.2, which is confirmed by these results (Figure 42). Rhamnolipids from *Ps. aeruginosa* ATCC 27853 also showed activity against *Mycobacterium aurum* (Figure 43).

Rhamnolipids from ATCC 9027 showed more activity per rhamnose content than rhamnolipids from ATCC 27853 (Figure 44). This is probably in part due to the different rhamnose to rhamnolipid ratio between the two strains. ATCC 27853 produces an unknown combination of dirhamnolipid and mono-rhamnolipid and therefore probably has a lower rhamnolipid for the same amount of rhamnose than ATCC 9027. Therefore, no definitive conclusion could be drawn as to which rhamnolipids were the most effective. Literature has shown that some rhamnolipids are more effective against certain organisms than other rhamnolipid mixtures (section 1.3.3.1).

**Table 21: Antimicrobial activity against *Mycobacterium aurum* of undiluted supernatant from *Pseudomonas aeruginosa* cultures. Activity expressed as average zone of inhibition.**

Strain	Medium	Reactor/ Shake flasks	Sample time [h]	Concentration rhamnose [mg/L]	Concentration rhamnolipid [mg/L]	Average zone [mm]	Error [mm]
ATCC 9027	MSM+Tris 4/4	Reactor	22.75	403.4	1238.4	21	0.5
			127.25	1288.28	3955.0	45	2
ATCC 9027	MSM+Tris 2/4	Shake flasks	288	995.82	3057.2	31	0
ATCC 27853	MSM+Tris 2/4	Shake flasks	288	599.69		17.5	0.5

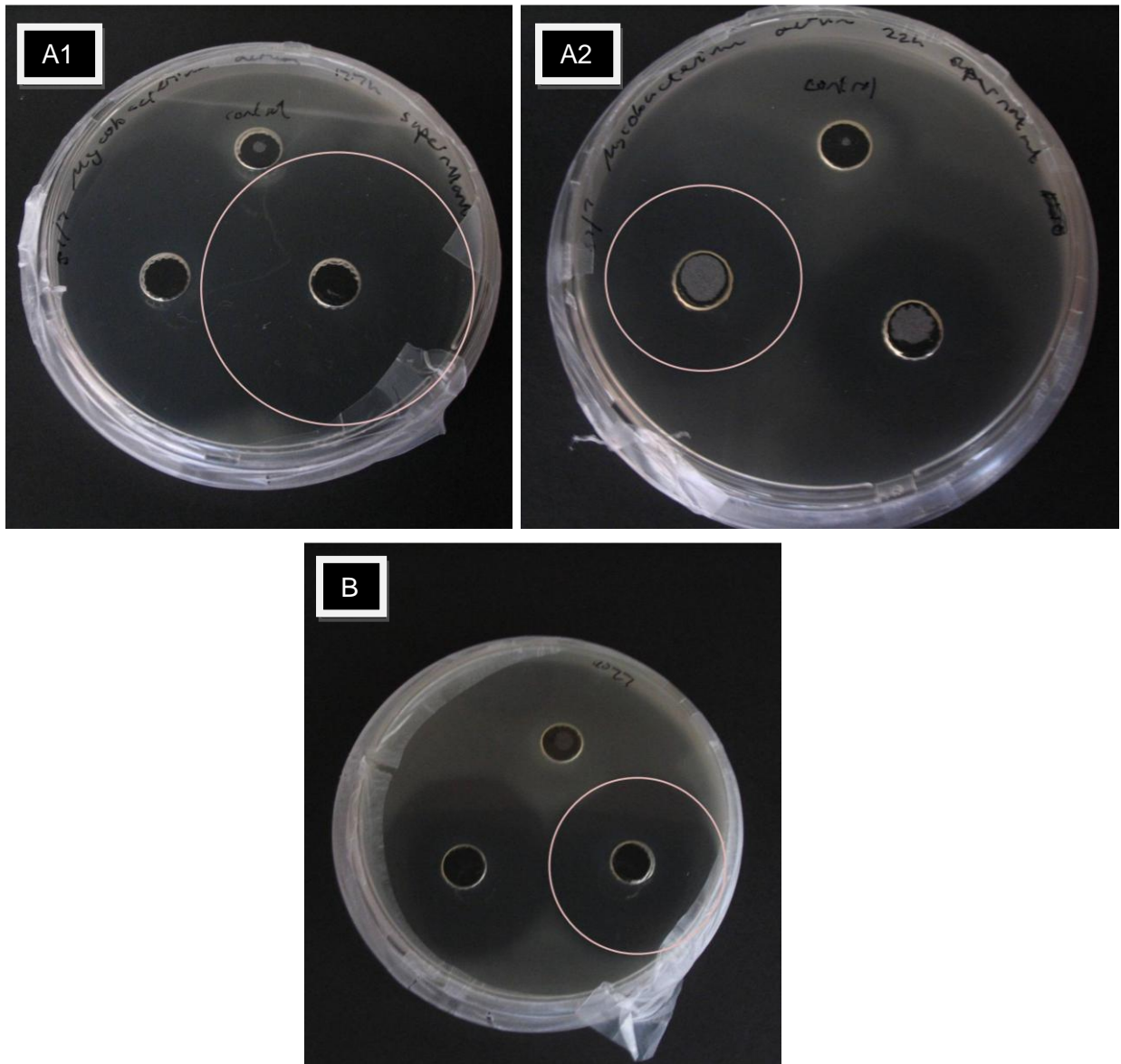


Figure 42: *Pseudomonas aeruginosa* ATCC 9027 supernatant antimicrobial activity against *Mycobacterium aurum*. Top well is the control in each case. Bottom wells are duplicate antimicrobial tests. A1 – Supernatant taken at 127 hours from reactor culture. A2 – Supernatant taken at 23 hours from reactor culture. B – Supernatant taken at 288 h from shake flask culture.



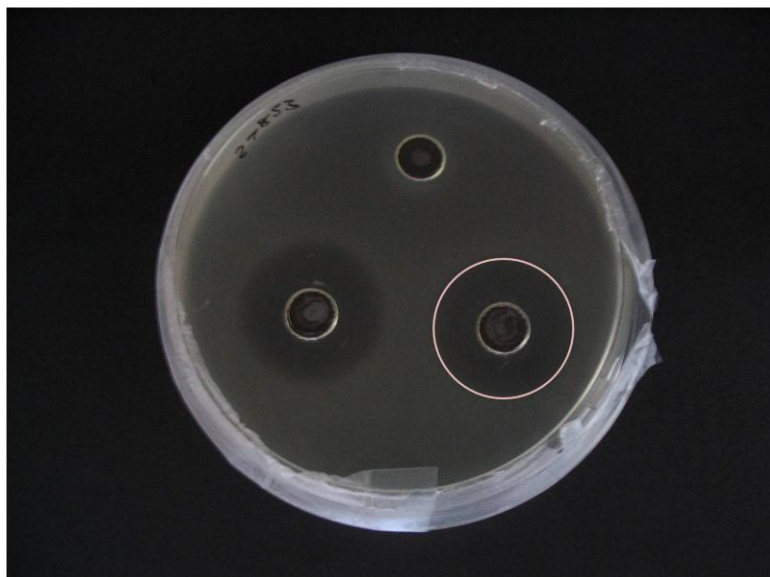


Figure 43: *Pseudomonas aeruginosa* ATCC 27853 supernatant antimicrobial activity against *Mycobacterium aurum*. The top well is the control. Bottom wells are duplicate antimicrobial tests.

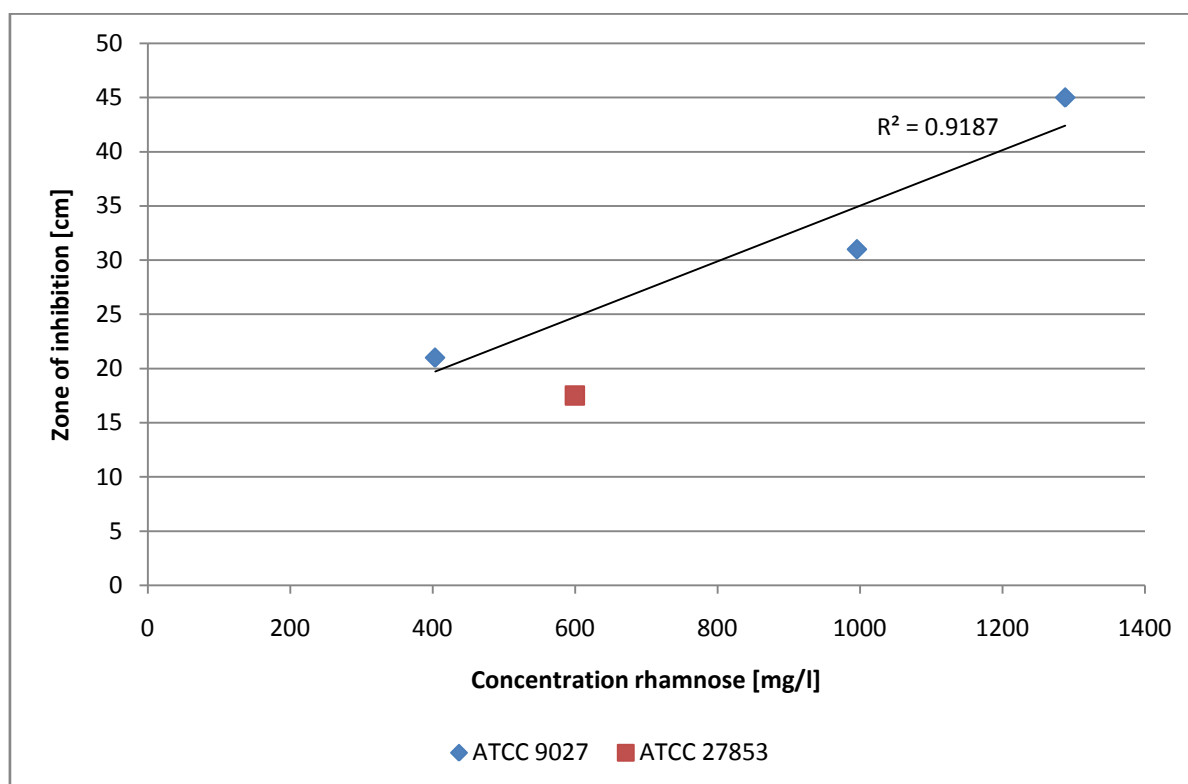


Figure 44: Relationship between rhamnolipids from *Pseudomonas aeruginosa* ATCC 9027 and ATCC 27853 and their antimicrobial activity expressed as a zone of inhibition.

The zones of inhibition for *Ps. aeruginosa* ATCC 9027 supernatant were plotted against the concentration rhamnolipid to determine their relationship. As seen in Figure 44 a linear trendline was drawn which gave a regression value of 0.918. It would seem that the concentration rhamnolipid is directly related to the antimicrobial activity. In view of the few points, further data would be needed to confirm this.

The possibility was tested that perhaps the rhamnolipid was not solely responsible for the antimicrobial activity. Perneel *et al.* (2008) found that rhamnolipids and phenazines acted synergistically in the control of *Pythium* sp. Supernatant from *Ps. aeruginosa* ATCC 9027 culture was extracted three times with diethyl ether and subsequently tested for antimicrobial activity. As seen in Table 22 the zones of inhibition were within the range of error for the extracted and non-extracted supernatant. This preliminary experiment could indicate that only rhamnolipids and not phenazines have activity against bacteria, but the purity of the solutions would have to be established to confirm it. In future work this experiment can be repeated against a fungal species, since phenazines have been found to be especially effective against zoospores (Perneel *et al.* 2008).

**Table 22: Comparison of antimicrobial activity against *Mycobacterium aurum* of supernatant and extracted supernatant taken from *Pseudomonas aeruginosa* ATCC 9027 culture grown in MSM+Tris 2/4 medium.**

	Zone 1 [mm]	Zone 2 [mm]	Average zone [mm]	Error [mm]
Supernatant	31	31	31	0
Extracted supernatant	31	36	33.5	2.5

The stability of mono-rhamnolipid antimicrobial activity was also tested. Supernatant from *Ps. aeruginosa* ATCC 9027 reactor run at 22.75 hours (Table 21) was left in the fridge for 2 months and subsequently tested for activity against *Mycobacterium aurum*. A decrease in activity could be observed as shown in Table 23. Future work should investigate the activity over time, as this would be an important factor in the commercialisation of rhamnolipids as antibiotics.

**Table 23: Stability of mono-rhamnolipid activity against *Mycobacterium aurum*. Supernatant from *Pseudomonas aeruginosa* ATCC 9027 reactor culture was tested after a week and after 2 months.**

	Zone 1 [mm]	Zone 2 [mm]	Average zone [mm]	Error [mm]
1 week old supernatant	20.5	21.5	21	0.5
2 months old supernatant	13	13	13	0

## 4 Conclusions

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Based on the results and discussion above, the following conclusions were drawn.

- **HPLC is a rapid and accurate method for the direct analysis of rhamnose concentration**

Literature research on rhamnolipid studies did not reveal evidence of HPLC methodology for rhamnose measurements. However, in this study, evidence for the accuracy of this method was demonstrated. The method further has the added benefit of not requiring extraction of samples prior to analysis.

- **The orcinol colorimetric method is an accurate method for the direct analysis of rhamnose concentration provided that complete extraction of the rhamnose is effected.**

Direct analysis of rhamnose containing supernatants by the orcinol method led to over-estimation of concentrations. Extraction, in this case with diethyl ether, prior to the colorimetric test, improved the accuracy. Samples that were extracted twice were accurate, but within a large standard deviation. Samples that were extracted three times gave accurate results and small error margins, comparable with HPLC results.

- **The minimum surface tension and CMC can be used to characterise rhamnolipid solutions.**

The tensioactive properties differ between biosurfactant solutions and in this case strongly suggested that the rhamnolipids produced by *Ps. aeruginosa* ATCC 9027 are mono-rhamnolipids.

- **The soft agar overlay method is superior to the disk diffusion method for antibacterial activity testing of rhamnolipid.**

Clear, duplicate inhibition zones were obtained with the soft agar overlay method, while no activity could be observed by using the filter discs.

- **Mono-rhamnolipid possibly does not have antimicrobial activity against *Botrytis cinerea*.**

No zones of clearing were observed when rhamnolipid solution was pipetted in wells of plates with fungal growth.

- **Rhamnolipids from *Pseudomonas aeruginosa* ATCC 9027 as well as ATCC 27853 have activity against *Mycobacterium aurum*.**

Rhamnolipids were shown to be active compounds with antimicrobial activity and have a potential profitable market as novel antimicrobial agents. Mono-rhamnolipid from ATCC 9027 at a concentration of 4 g/l (1.3 g/l rhamnose) led to a zone of inhibition of 45 mm. This rhamnolipid concentration is 2.7 fold greater than the maximum reported in the literature for this strain. Rhamnolipid from ATCC 27853 dual substrate cultures with a rhamnose concentration of 0.6 g/l rhamnose (rhamnolipid concentration could not be determined since the mono/di-rhamnolipid

ratio was unknown) led to a zone of inhibition of 17.5 mm. The antimicrobial activity was shown to be linearly related to the rhamnolipid concentration, and thus one of the goals of a process for the production of rhamnolipids as antimicrobial agents should be to maximise rhamnolipid production. Mono-rhamnolipid from *Ps. aeruginosa* ATCC 9027 achieved the highest production in MSM+Tris medium containing glucose as carbon source and therefore shows the most promise for use in an antimicrobial rhamnolipid production process.

- **The antibacterial activity of mono-rhamnolipid is not stable over time.**  
Activity of mono-rhamnolipid against *Mycobacterium aurum* decreased by 38% over a period of two months.
- **pH buffering in a medium is necessary for good rhamnolipid production.**  
Significantly lower production was obtained for *Ps. aeruginosa* ATCC 27853 when grown on a medium containing no buffer than on a medium containing tris buffer with a similar nutrient content. The pH in the medium with no buffer dropped to 4 after 28 hours, whereas the buffered medium had a neutral pH at that time.
- **A medium that is limited in phosphate, has pH buffering around neutral pH and has a high carbon concentration promotes rhamnolipid production.**  
Rhamnolipid production started upon the exhaustion of phosphate in MSM+Tris medium. The same medium with ten times the level of phosphate gave negligible production. MSM+Tris medium, which was limited in phosphate, led to good rhamnolipid production, while in phosphate limited PPGASM+Tris medium it did not. The low amount of carbon in the PPGASM medium was detrimental to rhamnolipid production. Phosphate limited MSM medium containing no buffer also led to poor production of rhamnolipid, which highlighted the importance of pH buffering.
- ***Pseudomonas aeruginosa* ATCC 9027 has better growth and biosurfactant production than ATCC 27853 on glucose media.**  
On MSM+Tris medium containing glucose as carbon source, strain ATCC 9027 produced 1.31 g/l rhamnolipid whereas strain ATCC 27853 produced 0.43 g/l rhamnolipid. Some strains are better adapted than others for rhamnolipid production on hydrophilic substrates such as glucose.
- **Rhamnolipid production starts with the onset of the stationary phase.**  
In cultures of both *Ps. aeruginosa* ATCC 9027 and ATCC 27853 on MSM+Tris medium, high production of rhamnolipid was obtained only after the start of the stationary phase.
- ***Pseudomonas aeruginosa* ATCC 9027 and ATCC 27853 have poor growth and biosurfactant production on media containing alkane as sole carbon source.**  
Slow growth over a long period was observed for these strains. The stationary phase was not reached where rhamnolipid is produced and therefore no emulsification or solubilisation of the substrate could occur to facilitate the uptake of the hexadecane.

- **A local strain *Pseudomonas aeruginosa* 2bf requires further investigation if to be used for rhamnolipid production studies.**

Poor growth was obtained for this strain on both glucose and alkane media cell growth decreasing after a short period of incubation. Low concentrations of rhamnolipid were produced accordingly.

- **A dual substrate system of glucose and hexadecane cultured from a glucose inoculum is not effective for increasing growth of *Pseudomonas aeruginosa*.**

In the dual substrate system only glucose, and not hexadecane, was used effectively for growth and rhamnolipid production and not hexadecane. Hexadecane was only used intermittently for growth and not at all for rhamnolipid production. In all dual substrate experiments inocula containing glucose as sole carbon source were used. However, a glucose inoculum was shown to adversely effect growth in test cultures containing hexadecane as the sole carbon source. It is thus possible that the organism adjusted its cell hydrophobicity in the hydrophilic glucose medium and could not recover its hydrophobicity to effectively take up alkanes.

- **Rhamnolipid yields do not increase with increasing substrate concentration in *Pseudomonas aeruginosa* ATCC 9027.**

A 150 % higher substrate yield of rhamnolipid was found for 1.5 % glucose medium than for 3 % glucose medium. At glucose concentrations higher than approximately 1.5 % rhamnolipid production reaches a plateau with the remaining glucose being used for cell maintenance.

- **Rhamnolipids are located primarily extracellularly with little or no rhamnolipid attached to the cell membranes.**

The location of the product is an important consideration in the design of the downstream process. These results have shown that the location of the product is primarily extracellular. Downstream processing would therefore be less complex, not requiring cell disruption, nor the purification of product from a complex mix of intracellular metabolites.

- **Production time can be shortened considerably in an instrumented bioreactor.**

Nearly double the rate of growth and biosurfactant production were observed for *Ps. aeruginosa* ATCC 9027 in the bioreactor than for shake flask studies.

# 5 Recommendations

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Based on the results and conclusions presented above, the following recommendations are made.

## 1. The development of rhamnolipids as antimicrobial molecules

- The range of target organisms should be extended for antimicrobial activity studies of rhamnolipids including gram-positive as well as gram-negative bacteria.
- The soft agar overlay method should be used as described in this study for the determination of antibacterial activity.
- Antifungal activity of rhamnolipids must be investigated further, and a reliable analytical method must be developed to evaluate the antifungal activity.
- The stability of rhamnolipid antimicrobial activity over time should be investigated further. The stability is an important parameter for possible future commercial development of rhamnolipids as antimicrobial agents.
- The contribution of the cytotoxic phenazines to the antimicrobial activity must be established.
- A process focusing on the production of rhamnolipids as antimicrobial agents should be developed. The production yield must be maximised since the activity was shown to be directly related to the concentration. Higher yields would also improve the economics of the process, which is important for commercialisation of the product.

## 2. Rhamnolipid concentration analyses

- For the direct measurement of rhamnose concentrations HPLC was shown to be a rapid and accurate method which does not require prior extraction of rhamnolipids. This method should be used, if available. However, rhamnolipids must be hydrolysed to release rhamnose molecules. This can be achieved with pressurised acid hydrolysis at a high temperature, but not at normal atmospheric pressure.
- If an HPLC is not available or its use for analysis of bulk samples would be too costly, the orcinol colorimetric method can be used for determining rhamnose concentrations. If the orcinol method is used, samples should be extracted at least three times with an appropriate solvent (such as diethyl ether), to ensure accuracy of the method.

## 3. Rhamnolipid production in bioreactor studies

- Purification of the product should be done after removal of the cells. Rhamnolipid was shown to be located in the supernatant with little or no rhamnolipid attached to the cells.
- It is important to control the amount of foaming in the bioreactor with antifoam solution, while ensuring proper oxygen transfer. Aeration, which was found to

directly influence the amount of foaming, must be kept to a minimum, which was found to directly influence the amount of foaming, and agitation must be set at a higher level to maintain an adequate dissolved oxygen level (around 20%). A balance must be found between the control of foaming, cell densities and oxygen transfer.

- The pH in the medium should be controlled around neutral pH, as pH maintenance was shown to be critical for good rhamnolipid production.

#### **4. Improvement of growth and biosurfactant production on alkane media**

- Biosurfactant producing strains must be maintained and inoculated from media containing alkanes as sole carbon source.
- The number of biosurfactant producing strains should be extended. Strains should be selected that naturally have more hydrophobic membranes and are therefore more suited for alkane utilisation. Ideally, a local strain should be isolated and developed.
- Rhamnolipid can be added exogenously to alkane media to enhance cell hydrophobicity and therefore uptake of alkanes. A recycle process will be ideally suited for such a setup, where product is separated and added to the start-up of the next batch.
- Different chain lengths of alkanes can be tested as substrate.

#### **5. Maximising the yield of rhamnolipid from *Pseudomonas aeruginosa* cultures:**

- Initially, a batch process with glucose as substrate should be developed as a basis. Different concentrations of glucose should be added at start up to establish a process where good rhamnolipid production is achieved without excessive glucose being used for cell maintenance. Subsequently, a fed batch process operation should be investigated where carbon is fed upon commencement of the stationary phase. Since a phosphate limitation was shown to promote rhamnolipid production, carbon feeding should occur in the presence of a phosphate limitation.
- The optimal feed rate should be investigated for rhamnolipid production in a fed batch process operation. The addition of substrate will increase cell concentrations for enhanced production of rhamnolipid. Different feeding strategies should be investigated for maintaining the specific production rate. It is desirable to feed the smallest volume of substrate that is necessary with a high concentration in order to keep the dilution rate low and maximise the product concentration.
- A factorial design done on different substrate concentrations could be of great value in the optimisation of a fed batch process.
- Investigations should be done to determine the media and process conditions that will give optimum production in batch fermentations. A media with a nutrient limitation other than phosphate, for example nitrogen, should also be tested and compared to the production data obtained from the phosphate limited media used in this study for biosurfactant production.



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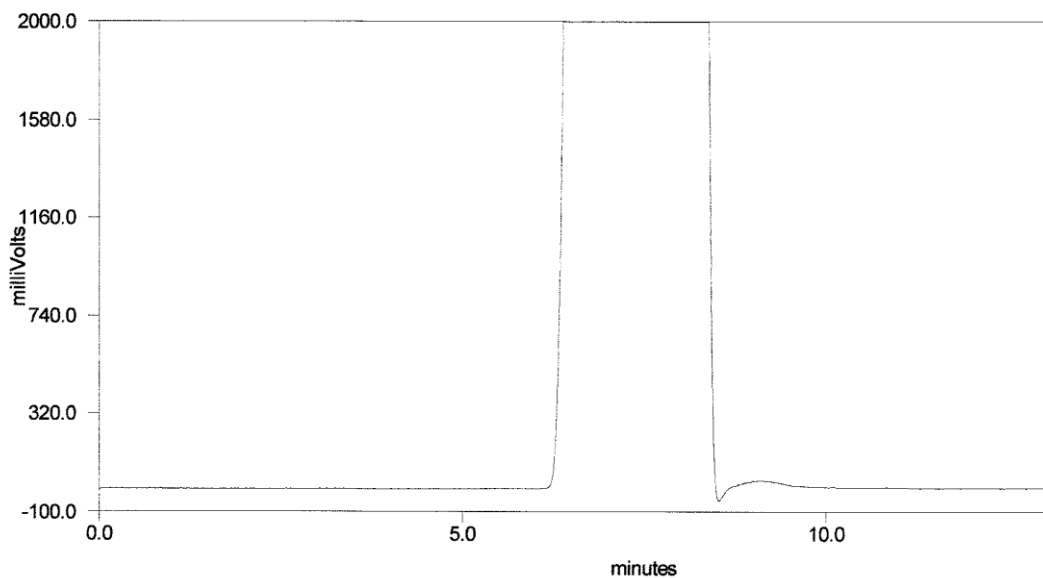
# Appendix A

## HPLC chromatograms

```
*****
*
*   TIME : 7:05 am   DELTA CHROMATOGRAPHY DATA SYSTEM   DATE :25/01/2008 *
*                   EXTERNAL STANDARDS REPORT           *
*****
```

```
SAMPLE NAME :                               CHROMATOGRAM FILE : 2401FB1
SAMPLE TYPE : SAMP                           CHROMATOGRAM SOURCE : ACQUIRE
SAMPLE AMOUNT      1.00                       METHOD NAME       : FRANCIS
DILUTE           : 1.00                       INJ              : 1 OF 1
```

Chromatogram 2401FB1 recorded at 6:47 am on 25/01/2008  
 Captured by analyst Hanlie using method : FRANCIS



PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
	2		Glukose				
	1		Rhamnose				
TOTALS :						0	

**Figure A1: Attempted measurement of rhamnose concentration by HPLC after attempted hydrolysis with 53% (m/v) sulphuric acid.**

```

*****
*
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*   EXTERNAL STANDARDS REPORT
*
*****

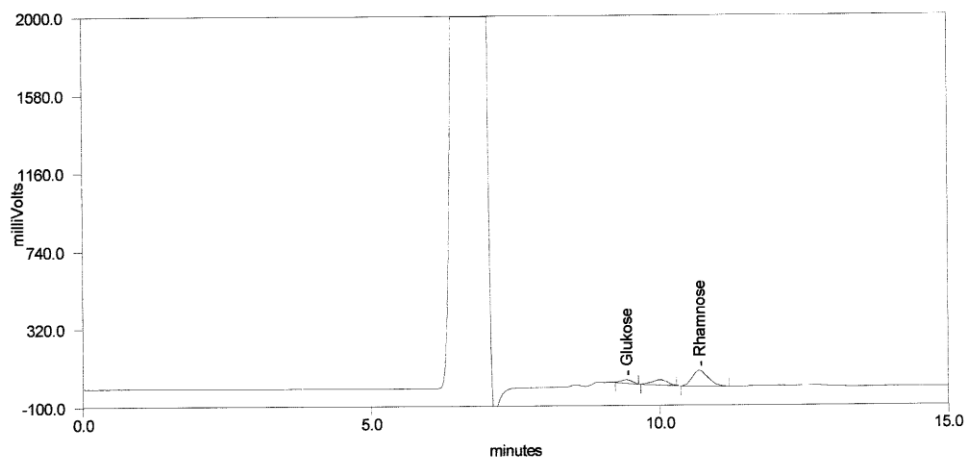
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```

SAMPLE NAME : FRANCIS           CHROMATOGRAM FILE : 2401FB6
SAMPLE TYPE : SAMP             CHROMATOGRAM SOURCE : FILE
SAMPLE AMOUNT : 1.00          METHOD NAME : FRANCIS
DILUTE : 1.00                 INJ : 1 OF 1

```

Chromatogram 2401FB6 recorded at 8:44 am on 25/01/2008  
 Captured by analyst Hanlie using method : FRANCIS



PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
1	2	MM	Glukose	9.42	369.00	266357	31.7
3	1	MM	Rhamnose	10.69	-24.40	1658730	216.5
TOTALS :						1925087	

**Figure A2: Successful measurement of rhamnose by HPLC after pressure hydrolysis of sample with dilute sulphuric acid.**

## Appendix B

Standard curves.

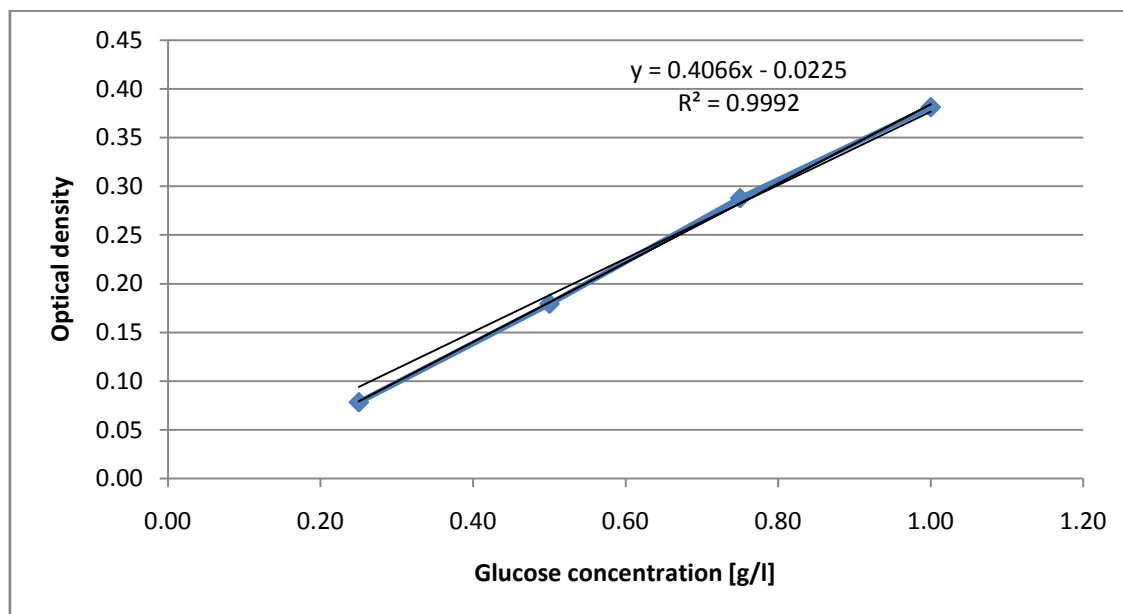


Figure B1: Example of a glucose standard curve used as reference when conducting DNS glucose analysis.

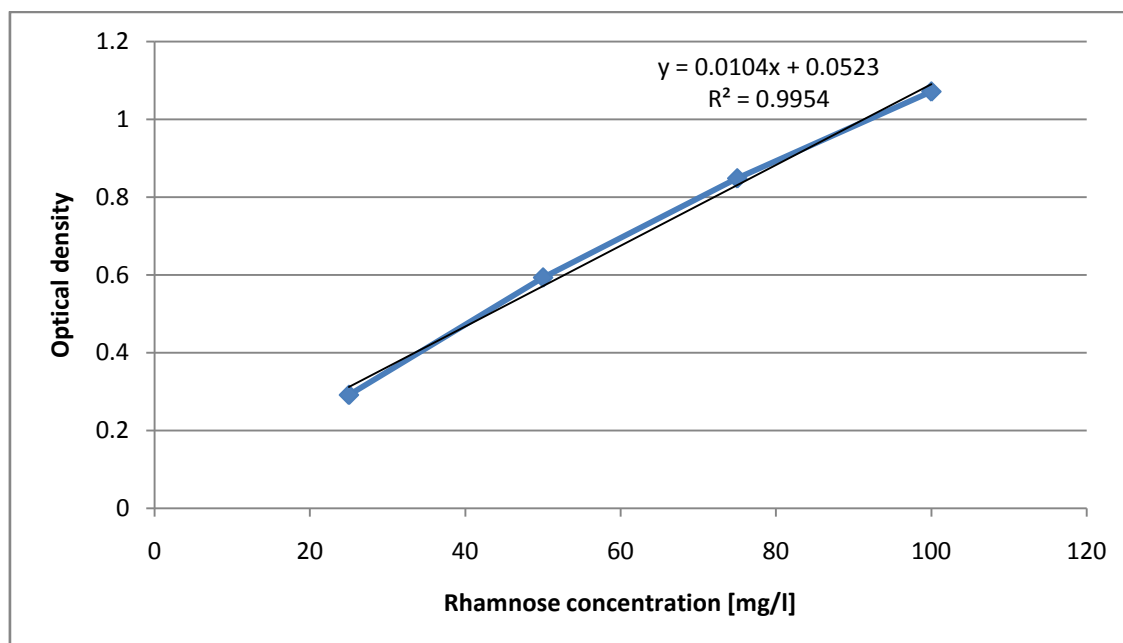


Figure B2: First example of an L-rhamnose standard curve as used in the orcinol analysis.



Figure B3: Second example of an L-rhamnose standard curve as used in the orcinol analysis.