CONVERSION OF HYDROCARBONS TO BIOSURFACTANTS

An insight into the bioprocess optimisation of biosurfactant production using alkanes as inducers

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Masters Thesis (120 credit)
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2009

Declaration

"I know the meaning of plagiarism and declare that all the work in the document, save for that which is properly acknowledged, is my own"

“I have little patience with scientists who take a board of wood, look for its thinnest part, and drill a great number of holes where drilling is easy.”

(A. Einstein)

Dedication

TO

My wife

Dr Hélène MUGABEKAZI BAMARA

Our children

JMJ G. MUGABA GWIZA

C-R R. BAGABE GWIRA

M-A. K. NIKEZA TUNGA

M-R. I NIBEZA TURWA

M-M. I NIBYIZA TUZA

Abstract

Surfactants are chemical compounds that are able to alter interfacial properties, particularly surface tension. When they are biologically produced, the term biosurfactant is used. One of the most important groups of biosurfactants is a family of chemical compounds known as glycolipids, whose structure consists of a sugar group and a lipid tail. Glycolipids are subdivided into three main groups: rhamnolipids, sophorolipids and trehalolipids, named following their sugar moieties, respectively rhamnose, trehalose and sophorose. Biosurfactants exhibit attractive advantages over chemical surfactants. Examples of these are biodegradability, low toxicity, and effectiveness at extreme temperature, pH and salinity.

The objective of the present research project was, first, to investigate the potential of liquid aliphatic hydrocarbons to induce biosurfactant production by the bacterium Ps. aeruginosa 2Bf isolated based on its ability to metabolise alkanes. The second objective was to optimise biosurfactant production using alkanes as sole carbon and energy source, through optimising the mixing & aeration conditions, media conditions as well as provision of alkane, in a stirred tank batch reactor system. The final objective was to describe the biosurfactant formed.

Experiments were organised in three major series: the exploratory shake flask based experiments, the bioreactor-based experiments to optimise biosurfactant production and characterise biokinetics and performance, and the biosurfactant characterisation experiments.

Following review of a number of methods, microbial cell counts were selected as the most reproducible measure of biomass formation in the presence of alkanes. The presence of biosurfactant was quantified functionally in terms of the emulsification index and alteration of surface tension.

Using a shake flask-based study, nitrogen source was investigated in terms of biomass and biosurfactant synthesis. Four pre-selected nitrogen sources were tested in order to select the best for bioreactor based study. These nitrogen sources consisted of specific combinations of three nitrogen compounds, NH_4NO_3, $NaNO_3$ and $(NH_4)_2SO_4$. During the study, long chain liquid n-alkanes were used as sole carbon source and the C/N ratio maintained at the value of 18.6 in mass terms. Results confirmed that both a combination of NO_3^- and NH_4^+ ions or a nitrogen source composed solely of NH_4^+ ions were suitable for biomass growth and biosurfactant production. $(NH_4)SO_4$ was used as the N-source of choice in the remainder of the study.

While the C_{14}-C_{17} alkanes cut was the carbon source of interest in the study, two pure alkanes, n-C_{12} and n-C_{16} were tested and compared to the C_{14}-C_{17} blend. The C_{14}-C_{17} fraction, sourced as an industrial byproduct, compared favourably as a carbon source with respect to hexadecane and dodecane.

Biosurfactant production was not observed in Ps. aeruginosa 2Bf cultures where glucose was the sole carbon source and the bacteria were not previously exposed to linear alkanes. Using a mixed carbon source of glucose and alkane, or on pre-exposure of the bacteria to alkane, biosurfactant production was induced. Induction was optimised where alkane was the sole carbon source over a period of four sub-culture steps.

In the quantitative optimisation of biosurfactant production through the bioreactor based study, mixing and aeration were optimised; agitation and aeration proved to be equally important, the first at intermediate rates, the second at lower rates. Their interaction, when maximum biomass was used as the variable for response, was found to be important for agitation rates up to 500 rpm. Beyond this range of agitation speed, the interaction between aeration and agitation became negligible. In the case of E_{index} as the variable for response, similar results were obtained with regard to the impact of the interaction between aeration and agitation on the process. It was significant from lower to intermediate agitation rates, and negligible from intermediate to higher rates of agitation. Lower aeration rate was found to enhance the oxygen utilisation rate, while mass transfer was relatively favoured by high aeration rate.

Regarding the emulsification power of the product, quantitative tests were carried out on culture suspension, supernatant prepared by centrifugation and supernatant prepared by centrifugation and filtration at 0.22µm pore size filters. Results showed that some emulsification effect was lost through centrifugation and filtration. This loss of emulsification effect was more pronounced in the filtration case, thus showing that some biosurfactant was removed along some other material or substance through sticking on filter paper.

Foam control was required, and two mechanical foam breakers were compared to anti-foam reagent. It was experimentally established that mechanical foam breakers are preferable to chemical anti-foam reagents. On comparing the two different mechanical foam breakers, the modified two blade paddle with three slits, FB-2, performed better than the simple two blade paddle foam breaker, FB-1. Further investigations showed that the interaction between type of foam control and agitation rate was negligible throughout the process.

The Biosurfactant was characterised at the structural level and the antibiotic potential of Ps. aeruginosa 2Bf's biosurfactant was analysed. In addition to the thin layer chromatography, three different spectroscopic methods (mass, infrared & nuclear magnetic resonance) were used to study the chemical structure of the biosurfactant produced. Up to six rhamnolipid structures were tentatively identified with spectrometric analysis whereas only four to five structures could be detected with thin layer chromatography. Possession of an anti-microbial activity by the rhamnolipids produced was confirmed with the B. subtilis inhibition test.

"When a man blames others for his failures,
it's a good idea to credit others with his successes "
(Howard W. Newton)

Acknowledgement

At the end of this work, I sincerely express my gratitude and heartfelt thanks to the following people whose contribution has been determinant and essential to the success of the whole project:

- Professor STL Harrison of the Chemical Engineering Department of the University of Cape Town, for having accepted to supervise this work and to provide her guidance and support to make the work accomplishable;

- Professor S Reid of the Department of Molecular & Cell Biology of the University of Cape Town for willingly having accepted to co-supervise this work despite her responsibilities and limited time;

- Dr KG Clarke from the Process Engineering Unit of the Stellenbosch University for having accepted to co-supervise the work; especially for her invaluable contribution in defining the direction, the scope & the context of this project;

- Reverend Sister L-F. Theresa Del Fajela, firstly for her prayers, secondly for her assistance and advice on how to present a large number of results in an intelligible way;

- Dr Hélène Mugabekazi Bamara, for her meaningful support in the many difficulties of different natures encountered during this project;

- The Chemical Engineering administration and staff for their support and care without which this project would not have known an end;

- Janine Buxey and Janine Willey of the Faculty of Engineering & The Built Environment's Administration for their very kind welcome and assistance I always received whenever I approached them ;

- The Chemical Engineering academic staff for their contribution to my real ascension knowledge wise;

- SASOL Ltd, South Africa, for supplying the hydrocarbons;

- Peter Clair Williams and Madelyn-Robertson for having been kind enough to introduce me to the different corners of bioprocessing and other chemical engineering labs and activities during my first days at UCT;

- Jeeten Nathoo & family for having created a real family environment for me during my whole stay in South Africa;

- Zani Kumalo and Martin Pangapanga for their company and encouragements;

Finally, my thanks and sincere gratitude are expressed to the following moral persons who sponsored this project: The Government of Rwanda through World Bank Project - HRDP, The Department of Chemical Engineering of UCT

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Abbreviations

Abs.: absorbance
AF: chemical anti-foam reagent
alkn: alkane
BSM: basal salt growth medium
CC: cell count
C_{14}-C_{17}: alkane cut utilised as the substrate
CMC: critical micelle concentration
cond.: conditions
CMC: critical micelles concentration
CTAB: cetyl trimethylammonium bromide
eq.: equation
DW-cyclh: dry weight measurement with cyclohexane washing
DW-cyclh: dry weight measurement with hexane washing
EI: electron ionisation
FB-1: two-blade paddle mechanical foam breaker without slits
FB-2: two-blade paddle mechanical foam breaker with two three slits
FT: Fourier transform
GC: gas chromatograph
glc: glucose
h: hour
HLB: hydrophilic-lipophilic balance
HPLC: high performance liquid chromatograph
IR: infra-red
LC: liquid chromatograph
MS: mass spectrometer
MST: minimal surface tension
MTBE: tertiary butyl methyl ether
NMR : Nuclear magnetic resonance
O/W: oil in water
Opt.: optimum
PBS: physiological buffered saline
PCR: polymerase chain reaction
PHAs: poly-hydroxyalkanoates
RF: radio frequency
s: second
T: absolute temperature
THF: tetra-hydro-furan
TLC: thin layer chromatography

UCT: University of Cape Town

vs: versus

W/O: water in oil

Nomenclature

Symbols	Units
α : molecular fraction entering liquid surface	dimensionless
ε: entropy	Jour
έ: gas hold up	dimensionless
φ: Harkins-Brown's factor	dimensionless
Φ: vapour pressure	dynes.cm^{-2}
μ: growth rate	h^{-1}
μ_{max}: maximum growth rate	h^{-1}
n : equilibrium number of molecules per unit area	cm^{-2}
ρ_l: liquid density	kg.m^{-3}
τ : mean residence time	μs
dH: heat of vaporization	J.mol^{-1}
μ_p : chemical potential	J.mol^{-1}
r_t: tube tip radius	mm
a: surface area	m^2
a: specific surface area	m^2.m^{-3}
C*: oxygen concentration at saturation	mg.l^{-1}
C_{crit}: critical oxygen concentration	mg.l^{-1}
C_L: oxygen concentration in the liquid	mg.l^{-1}
C_s: substrate concentration	g.l^{-1}
c_x: biomass concentration	g.l^{-1}
DO: dissolved oxygen	%
$E_{Centr. + filt.}$: emulsification index for centrifuged and filtered samples	%
$E_{Centr.}$: emulsification index for centrifuged samples	%
E_{index}: emulsification index	%
$E_{Uncentr.}$: emulsification index for uncentrifuged samples	%
F: total Helmholtz free energy	J.mol^{-1}
f: vertical pull (force)	mN
g: gravity force	N
K : desorption constant of water at room temperature	cm^{-2}.sec^{-1}
k_L: mass transfer coefficient	cm h^{-1}
m: mass	kg
n: number of moles	unit less
OD: optical density	unit less
OTR: oxygen transfer rate	mg.l^{-1}.s^{-1}

OUR: oxygen utilisation rate	$mg.l^{-1}.s^{-1}$
P: pressure	Pa
q : heat of vaporization	$calories.mole^{-1}$
q_p: specific product formation rate	$mol\ .mol^{-1}.h^{-1}$
q_s: specific substrate utilisation rate	$mol\ .mol^{-1}.h^{-1}$
R: platinum ring radius	mm
r: wire radius	mm
r_r: microbial respiration rate	$mmol.s^{-1}$
r_1, r_2 : radii of curvature of the liquid surface	mm
R_f : retardation factor	dimensionless
r_s: substrate consumption rate	$mg.s^{-1}$
r_x: microbial growth rate	(cell number).h^{-1} or $g.h^{-1}$
S: entropy	$J.mol^{-1}$
SP-OUR: specific oxygen utilisation rate	$mg.g^{-1}.s^{-1}$
ST: surface tension	$mN.cm^{-1}$
T : absolute temperature	K
V : volume	l
X/Xo: ratio *[cell number-initial cell number]*	dimensionless
X: biomass	g
$Y_{x/s}$: yield of biomass on substrate	$g.g^{-1}$

CHAPTER I: GENERAL INTRODUCTION

1.1 Biosurfactants

Surfactants, a term derived from *surf*ace *act*ive ag*ent,* are substances that can alter the interfacial surface's behavior (Thanomsub *et al.* 2006). Surfactants are chemical entities consisting of two groups of opposite affinity towards water molecules (Wu & Ju 1998). The most common hydrophilic moieties for surfactants are carboxylates, sulphates, sulphonates and phosphates, while the hydrophobic part is generally a lipid chain of diverse length. Surfactants are classified scientifically according to the nature of their hydrophilic groups and overall electrical charge into 6 main groups: anionic, nonionic, cationic, amphoteric, weakly acidic and weakly basic surfactants.

Such a molecular structure confers to surfactants the property of reducing surface and interfacial tension between aqueous and oil phases (Karanth & Veenanadig 2004; Bordas & Lafrance 2001).

Surfactants are produced either chemically or biologically through different metabolic processes. In the latter case they are referred to as *bio-surfactants* (Christova *et al.* 2004). Biosurfactants are produced by many bacterial and yeast species growing on a diversity of substrates (Al-Araji, Rahman & Salleh 2007), including toxic materials such as linear, branched or cyclic and chlorinated hydrocarbons (Kunihiro *et al.* 2005; Barathi & Vasudevan 2001). Once synthesised through a series of metabolic processes, biosurfactants can be released in the growth medium as extracellular products, thus requiring further separation and purification. Due to their biological origin, biosurfactants are often more specific and environmentally friendly than the ordinary surfactants.

Biosurfactants have a range of different chemical structures, all consisting of a hydrophilic and hydrophobic component (Yin *et al.* 2009). The most extensively studied are low molecular weight glycolipids whose hydrophilic component is a sugar group while the hydrophobic component is a lipid chain often consisting of one or two decanoic acids. The most effective glycolipids are rhamnolipids whose chemical structure consists of the six carbon rhamnose sugar and generally one or two decanoic acid chains. Rhamnolipids are known to be produced by many *Pseudomonas species* as a mixture of similar compounds, only distinct either from

the number of rhamnose or decanoic acid group acid groups (Reiling *et al.* 1986), or from the presence or lack of unsaturated chains in their structure.

The present study investigates biosurfactant production by the bacterial strain *Pseudomonas aeruginosa 2Bf* using medium and long chain liquid n-alkanes as sole carbon source.

Hydrocarbons in general and alkanes in particular, are susceptible to yield more diverse valuable products than carbohydrates due to their broader range of chain lengths (Rehm & Reiff 1981). *Ps. aeruginosa 2Bf* was previously isolated from an alkane contaminated site at an oil refinery and is fully described by Ghilamicael (2003).

The use of alkane medium is postulated to enhance biosurfactant production since these may lead to alkane dispersion by reducing surface tension between oil and aqueous phases (Van Hamme & Ward 2001; Vasileva-Tonkova *et al.* 2008), and thus facilitate the alkane take up by microorganisms (Beal & Betts 2000). The modes of alkane take up differ from one organism to another but a certain degree of alkane solubilisation is always important for their easy utilisation by microorganisms (Beal & Betts 2000). The cell contact with hydrophobic compounds such as alkanes is also an important step in the alkane utilisation, and microbial species demonstrate various strategies of interaction with hydrophobic substrates (Vasileva-Tonkova *et al.* 2008), as detailed in Section 2.7.

Alkanes are cheap substrates of equivalent value to some carbohydrate feedstocks, and they can be obtained as industrial wastes (Van Hamme & Ward 2001). Considering their high level of toxicity, their utilisation as carbon and energy source for biosurfactant production renders their use attractive as a bioremediation tool. However, alkane systems pose difficulties in bioprocessing, as outlined in the following sections.

1.2 Major challenges associated with alkane bioprocesses

The use of hydrocarbons as substrates is associated with a number of challenges. These are related to their poor solubility and dispersion in aqueous media, toxicity, lack of structural oxygen and, to a lesser extent, their flammability. When

biosurfactants are produced to aid alkane dispersion, resultant foaming becomes a serious challenge for the process.

1.2.1 Immiscibility

Liquid alkanes are among the most insoluble liquid compounds in water. At 25 ºC and 1atm, for instance, the n-C_9 and n-C_{10} solubilities in water are 0.22 ppm and 0.052 ppm respectively (McAuliffe 1969). When they are used as carbon sources for aqueous bioprocesses, their solubilisation is one of the major challenges. Alkane immiscibility is directly proportional to the carbon number present in their molecular structure (Goswani & Singh 1991).

1.2.2 Oxygen requirement

Another common challenge encountered in alkane aerobic bioprocesses is the high oxygen demand associated with the lack of oxygen atoms in hydrocarbon molecular structure (Clarke *et al.* 2005). In principle, all aerobic bioprocesses require sufficient oxygen for oxidation purposes, and oxygen can be availed by gas liquid mass transfer. Contrary to carbohydrates which have an oxygen content exceeding 50% by mass, hydrocarbons offer no oxygen at all, making the oxygen demand to be met by gas liquid mass transfer particularly high and the knowledge of oxygen transfer rate very important in bioprocess studies (Bailley & Ollis 1986).

1.2.3 Foam control

A foam is created when a gas is intimately mixed with a liquid containing a surface active agent. It appears as two-phase system in which gas bubbles are separated by a network of very thin liquid films. In many laboratory and industrial scale bioprocesses, foaming occurs as an undesirable phenomenon. In aerated systems, foam control is one of the main process challenges. In bioprocesses involving bacterial cells, for instance, rhamnolipid biosurfactants create severe foaming when produced under aerobic conditions by *Pseudomonas aeruginosa* (Chayabutra & Ju 2001). Foaming is detrimental to bioprocesses since it prevents optimal homogeneous culture conditions, causes fouling, restricts process control and may aggravate contamination. It is one of the main difficulties encountered in the operation of commercial bioreactors (Wilson 1989).

1.3 Project's rationale

Alkanes accumulate either as byproducts or wastes from different industrial processes. They also present a direct threat to neighboring ecosystems when released, since they are strong environmental pollutants. On the other hand, alkanes provide a potential raw material for bioconversion. As optimum productivity from carbon based resources is important for sustainable bioprocessing, researchers and business entrepreneurs need efficient collaboration in investigating new and efficient ways to maximise such resource productivity while minimizing environmental burden. In the present context, it is opportune to metabolise environmental pollutants such as alkanes as raw materials for the synthesis of valuables products via bioprocesses. The present work is part of a broader research project, initiated by the Department of Chemical Engineering and the Department of Molecular & Cell Biology at UCT. In the first phase bacterial microorganisms, isolated from SASOL (Sasolburg) and CHEVRON (Milnerton, Cape Town) sites, were found to be able to utilise alkanes and produce bioemulsifiers. The best bioemulsifier producer was identified as *Ps. aeruginosa*, and formed the subject of this study. The present research investigates the potential of *Ps. aeruginosa* to produce biosurfactants when using alkane substrates for induction.

1.4 Thesis structure

The present work is organised in eight chapters, as follows: general introduction; literature review; key questions, hypotheses and objectives; materials and methods; results and discussion provided as three chapters; and general conclusions and recommendations. At the end of each chapter, at the exception of Chapters 1 and 3, a partial conclusion is drawn. The final chapter draws these together to provide the integrated conclusion and recommendations. The thesis is completed with a list of the references consulted and appendices detailing essential experimental protocols and data.

Chapter 1 introduces the research project. It is divided into the following four subchapters: biosurfactant, process challenges, project's rationale, and thesis structure.

Chapter 2 provides the literature survey exploring current knowledge on biosurfactants, and various related areas, such as colloid and surface science. It brings the research topic into the global context of science and engineering research interests with regard to the use of biological ways of production as a more environmentally friendly alternative to the classic chemical synthesis of useful products. The microbial surfactants and their role in biological systems, their potential industrial applications, their chemical characterisation as well as their interaction with the growth media are discussed in this chapter. The rhamnolipids, which are a sub-class of glycolipid biosurfactants and the biosurfactants of interest in this research, are given a particular attention. The genetics of their production by *Ps. aeruginosa strains* is briefly described. Further, the bioreactor systems including microbial growth and kinetics, mass transfer in biological systems and mixing in bioreactors and bioreactor equipment are briefly described. In general, the chapter summarises the important concepts, definitions and theories in relation with the nature and objectives of the present work. For convenience, the literature survey concerning non-*UV-VIS* spectroscopic analysis was included in chapter seven which deals with the biosurfactant's structural and antimicrobial activity study.

Based on the literature understanding, Chapter 3 is concerned with the scope of the project, hypotheses, objectives, and research design.

Chapter 4 reviews materials and methods to be used in the research work. Materials and equipment to be used in this work are described first. Second, is a review of methods selected from literature, and modified where appropriate, which were proposed for the analyses required in this work. Finally, the statistical design of experimnts and factor analysis concepts are briefly described.

Chapter 5 presents and discusses results obtained during the qualitative and exploratory shake flask based phase. In this Chapter, a comparison of four analytical methods to monitor biomass growth is provided and the one which provided best quality of results was chosen to be used in the experiments. Details on the nitrogen source selection based on both microbial growth and emulsification index are provided. This chapter also presents the results obtained for biosurfactant induction tests, with *Ps. aeruginosa 2Bf* grown in the absence or presence of the alkane substrate. Induction of biosurfactant synthesis was quantified by the means of measuring the emulsification index in each case.

In Chapter 6, the quantitative optimisation of the bioreactor based study is presented and kinetic data analysed. Single parameter variation during culture growth was monitored for biomass formation, pH, emulsification index, surface tension, dissolved oxygen, alkane utilisation and viscosity. After the single parameter variation results, Chapter 6 presents the results obtained during the reactor study and different aeration, agitation and pH conditions, as well as the results of the foam control study. Further, results obtained during the reactor mixing, aeration and mass transfer are presented. Mass transfer was studied by monitoring the mass transfer parameters k_La and OTR, and oxygen utilisation parameters OUR and specific OUR, at three different phases of growth. The third part of the chapter concentrates on alkane utilisation by *Ps. aeruginosa 2Bf* during growth and the influence of substrate concentration on both growth and biosurfactant production. Results on foam control and its optimization are also presented.

In Chapter 7, the chemical structure and antibiotic activity of the biosurfactant produced was studied. The chapter provides a review of the TLC technique and three non-*UV-VIS* spectrometric techniques used for this purpose: NMR, MS and IR.

In Chapter 8, the findings of the thesis are integrated into the general conclusions and recommendations. The sub-chapter of *general conclusions* is subdivided into the following main sections:

- reminder of the work context;
- verification of hypotheses;
- assessment of the objectives' realisation;

The chapter is closed by recommendations drawn from findings and challenges encountered in the course of the work.

CHAPTER II: LITERATURE REVIEW

2.1 Introduction

In liquid phases, surfactants are located at the hydrophilic-hydrophobic interface with hydrophilic groups oriented towards the water molecules. Alternatively, they arrange as micelles. Surfactants are particularly useful in processes where interfacial tension requires alteration (Wu *et al*. 2007; Maier & Soberon-Chavez 2000). They are as foaming agents, emulsifiers and dispersants, assisting in suspending a gas, immiscible liquid or solid in water or other liquid (Ron & Rosenberg 2002; Kitamoto *et al.* 2002). No known surfactant can perform all these surface activities in a satisfactory way, hence the choice of surfactant used always depends on the purpose. Considering emulsification, as an example, selection of surfactant system depends on the materials in place and the properties desired in the end product. An emulsion can be oil droplets suspended in water, water suspended in a continuous oil phase, or a mixed emulsion.

The literature review provides the information necessary to the good understanding of the research project, which is directed to the biological conversion of hydrocarbon wastes released by oil and gas processing industry into valuable products, such as biosurfactants, using a batch reactor system. It provides a comprehensive introductory knowledge of the colloidal systems in general, nature of surfactants and biosurfactants, biosurfactant industrial applications, hydrocarbon substrate, kinetics, mass transfer, etc.

Microbial surfactants are given particular attention in this review, since they are more environmentally friendly and more specific than the normal surfactants. Their characterisation, the microorganisms by which they are produced, the microbial growth and kinetics, the bioreactor systems, as well as the statistical design of experiments are reviewed in the chapter.

2.2 Colloidal systems

Colloid systems are those in which one or more of the component phases has at least one dimension within the nanometer to micrometer range. In other words, they are systems containing large molecules or small droplets or particles or both (Shaw 1980). They are sometimes referred to as *micro-heterogeneous* systems (Hiemenz & Rajago 1997).

The factors which contribute most to the characteristics of the colloidal systems are: particle size, particle shape and flexibility, surface properties (including electrical), particle-particle interactions and particle solvent interactions (Hiemenz & Rajago 1997).

Colloidal systems are generally classified in three groups (Trapnell 1955; Hiemenz & Rajago 1997):

- *Colloidal dispersions*, which are thermodynamically unstable due to a high surface-free energy and are irreversible systems as they are not easily reconstituted after phase separation;
- *True solutions of macromolecular material*, natural or synthetic, which are thermodynamically stable and reversible as they are easily reconstituted after separation of solute from solvent;
- *Association colloids*, also called *colloidal electrolytes*, which are thermodynamically stable.

Most colloids are systems in which one phase is dispersed in the second phase also called the dispersion medium. In a colloidal dispersion, particles are sufficiently large for definite surfaces of separation to exist between the particles and the dispersion medium (Russel *et al.* 1989). Simple colloidal dispersions are two phase systems. The physical nature of dispersion depends on respective roles of its constituent phases. For example, an-oil in water (O/W) emulsion and water in oil (W/O) emulsion could have almost the same overall composition, and physical properties which are considerably different. Table 2.1 shows common examples of the types of colloidal dispersions (Russel *et al.* 1989; Trapnell 1955).

Table 2.1: Different types of colloidal dispersions

Dispersed phase	**Dispersion medium**	**Name**	**Examples**
Liquid	Gas	Liquid aerosol	fog, liquid sprays
Solid	Gas	Solid aerosol	Smoke, dust
Gas	Liquid	Foam	foam on detergent solutions, fire-extinguisher foam
Liquid	Liquid	Emulsions	milk, mayonnaise
Solid	Liquid	Sol, colloidal suspension; paste (high solid concentration)	Au sol, AgI sol; tooth paste
Gas	Solid	Solid foam	Expanded polystyrene
Liquid	Solid	Solid suspension	Opal, pearl
Solid	Solid	Solid suspension	Pigmented plastics

The terms *lyophilic* (liquid-loving) and *lyophobic* (liquid-hating) are frequently used to describe the tendency of a surface or a functional group to become wetted or solvated. When the liquid medium is aqueous, the terms *hydrophilic* and *hydrophobic* apply.

2.3 Liquid-gas and liquid-liquid interfaces

The phenomena of surface and interfacial tension can be explained in terms of short-range forces of attraction that exist between molecules and are responsible for the existence of the liquid state. The molecules within the bulk of a liquid are, on average, subjected to equal forces of attraction in all directions, whereas those at a liquid-air interface, for example, experience unbalanced attractive forces resulting in a net inward pull (Shaw 1993).

2.3.1 Surface tension

In the absence of external influences, liquid drops adopt a perfectly spherical shape (Davies & Rideal 1961). This is due to attractive and repulsive forces between the molecules constituting the liquid. Thus the cohesion between liquid molecules must surpass their tendency to separate under the influence of thermal motion (Clint 1992). Such a net attraction of neighbouring atoms is more or less completely realized in the bulk of the liquid phase, whereas atoms or molecules at the surface are attracted less completely than they would have been in the bulk. As a result, their energy is greater and since the free energy of a system tends to the minimum, the surface of such a pure phase tends to contract spontaneously.

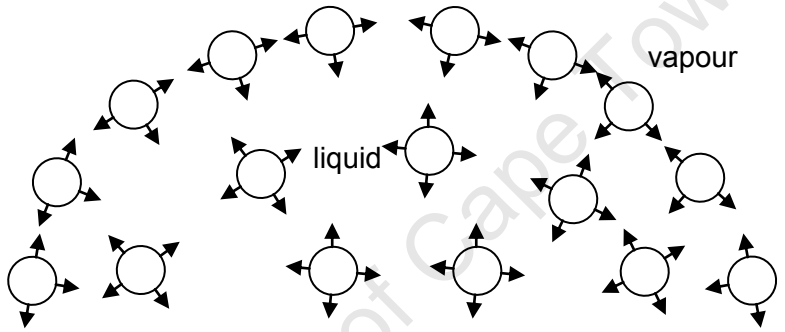


Figure 2.1: Attractive forces (arrows) between molecules (spheres) at the surface and in the interiour of a liquid

If "ST" is the force per cm tending to contract a liquid surface, and ε is entropy, T absolute temperature, P pressure, V volume, A surface area, μ_p chemical potential and n number of moles, then:

$$dF = -\varepsilon\, dT - PdV + (ST).dA + \mu_p\, dn \qquad \text{(eq. 2.1)}$$

where F is the total Helmholtz free energy of the system (Porter 1994). At constant temperature and volume for a given number of moles of system, this relationship reduces to:

$$ST \equiv \left(\frac{\delta F}{\delta A} \right)_{T,v,n} \qquad \text{(eq. 2.2)}$$

Under these conditions a spontaneous contraction of the surface area (-δA) will decrease F (δ F negative), provided ST is positive. Since the surface of a stable liquid phase always tends to decrease in area, ST is always positive. It is called the *surface tension*. Table 2.2 gives the standard surface tension values of the common liquids against air.

Table 2.2: Standard Surface Tensions of pure liquids against air (Porter 1994)

Compound	**ST (dynes.cm^{-1} or mN.m^{-1}), at :**				
	20 ºC	**24 ºC**	**25 ºC**	**30 ºC**	**40 ºC**
Water	72.94		72.13		
Methanol	22.50				
Ethanol	22.39			20	
n-octanol	27.53				
Methylene iodide	67				
Dimethyl sulfoxide	43.54				
Glycerine		62.6			
Propylene carbonate	41.1				
Propionic acid	26.69				
Benzene	28.88			27.56	
Toluene	28.52				
Dimethyl aniline	36.56				
1-methyl naphtalene	38.7				
Heptane	20.14				
Octane	21.62				
Nonane	22.85				
Perfluoro pentane	9.89				
Perfluoroheptane	13.19				

The surface tension of a liquid is commonly defined as the force acting at right angles to any line of unit length on the liquid surface (Hunter 1993). This definition is however misleading, although appropriate in the case of liquid films such as foams, since there is no elastic skin or tangential force as such at the surface of a pure liquid (Danielli *et al.* 1964). It would be more correct to define surface tension and surface free energy as the work required to increase the area of a surface isothermally and reversibly by unit amount.

2.3.2 Surface kinetics

On the molecular scale, the liquid surface is in a state of constant motion, where surface molecules are continually being replaced by others (Sherwood 1937). From the kinetic theory of gases, the number of molecules of vapour (at a vapour pressure φ dynes cm^{-2}) striking a unit area of surface per second is directly proportional to the molecular weight:

$$n_v = \varphi.(\pi\, m\, k\, T)^{-1/2} \qquad \text{(eq.2.3)}$$

where n_v is the number of vapour molecules and m the weight of one molecule (Valentin 1967). The entity kT is expressed in ergs, in this case. For instance, in the case of saturated water vapour at 20 **ºC,** the formula gives 8.5×10^{21} as the number of water molecules striking $1cm^2$ of surface each second. Assuming that a fraction α of the molecules enters the liquid surface implies that every second $8.5 \times 10^{21} \times \alpha$ molecules are condensing per cm^2. At equilibrium, this is equal to the rate of evaporation of water (Clint 1992). The value of α was experimentally found to be between 0.034 and 1 for water (Clint 1992).

The mean residence time in the surface (average time for which a given molecule stays in the surface) for these molecules, τ, can be expressed by the following relationship:

$$\tau = \frac{n}{\left(-\frac{dn}{dt}\right)_{desorption}} \qquad \text{(eq.2.4)}$$

where n is the equilibrium number of molecules present per cm^{-2} of surface, and

$\left(-\frac{dn}{dt}\right)_{desorption}$, is the rate of desorption (and adsorption).

In the present case, by taking n = 10^{15}, from the dimensions of the water molecules) and $\left(-\frac{dn}{dt}\right)_{desorption}$ = 2.9x10^{20}, the mean residence time in the surface, τ is found to be 3.4 μs. This is a very short life for a molecule before it evaporates, which implies an extremely agitated system. Such a rapid exchange is valid only for molecular exchange at equilibrium. If actual evaporation rates are measured, they are much lower than those calculated in the above way, since stagnant layers of vapour and cooling of the water phase both considerably retard any overall mass-transfer process.

2.3.3 Interfacial tension

When oil and water are in contact, the interface between the two phases has a contractile tendency, ST(o) usually expressed in dynes.cm^{-1}. ST(o) is called interfacial tension between oil and water (or any two liquid phases). In this sens, the surface tension of any liquid phase can be understood as the interfacial tension between the liquid and air. For the total free energy of the system, ST(o) is included in equation 2.2. For butanol against water for example, ST(o) is 1.8 dyne.cm^{-1}. This low value is characteristic of oils containing polar groups. This shows that the molecules of butanol (ST= 24) must concentrate at the oil-water interface, where the repulsion between the packed and oriented molecules (of alcohol) offsets the usual contractile tendency of an interface. Interfacial packing occurs as the hydroxyl heads of butanol molecules can escape from oil into water, while chains remain in oil, making the process result in a state of low standard free energy. In the same way, for nitrobenzene (ST= 43.9) against water ST(o) = 25.1 dyne.cm^{-1}. The difference indicates that considerable orientation of the dipolar molecules occurs at the interface. For hydrocarbon oils in general, ST(o) ≈ 50 dynes.cm^{-1}. Table 2.3 gives some accurately known values of interfacial tension for organic liquids in contact with water. Methods to measure surface and interfacial tension are reviewed in Sections 4.5.5 and 4.5.6.

Table 2.3: Standard Interfacial Tension values between water and pure liquids (Trapnell 1955)

Compound	ST(o) [dynes.cm^{-1} or mN.m^{-1}], at :		
	20 ºC	25 ºC	30 ºC
n-hexane	51.00		
n-octane	50.80		
Carbon disulphide	48.00		
Carbon tetrachloride	45.10	43.70	
Benzene	35.00	34.71	
Nitrobenzene	26.00		
Ethyl ether	10.70		
n-octanol	8.50		
Aniline	5.85		
Isobutanol	2.10		
n-butanol	1.60	1.80	
Bromobenzene		38.10	
n-hexanol		6.80	
n-pentanol		4.40	
Ethyl acetate			2.90

2.3.4 Emulsions and foams

2.3.4.1 Emulsions and micro-emulsions

Surface adsorption leads to important physical changes to liquid surface proprties, particularly by decreasing the surface tension (Russel *et al.* 1989). In a mixture of immiscible liquids, the presence of a surfactant reduces the surface tension at the interface (the interfacial tension) by adsorbing at the interface.

In this way, organic substances that are nearly insoluble in water may be dissolved in aqueous solution of surfactants (water phase containing a surfactant) to increased concentration. In this case, the surfactant solution must contain micelles and the solutions formed are clear and stable. On the other hand, even water can be dissolved in surfactant solutions of apolar solvents (hydrocarbon phase containing a surfactant), and the surfactant solution must contain reverse micelles (Hunter 1993).

These phenomena are known as *solubilisation or emulsification*. The formation of a stable solution is due to the fact that organic liquids dissolve in the micelles (Niven 1955). Under certain conditions, a large amount of an apolar compound can dissolve in water in the presence of a surfactant and vice versa. The resulting solutions are optically clear, and indefinitely stable. They are known as O/W microemulsions and W/O microemulsions respectively. To invert a microemulsion from O/W to W/O, the size of the hydrophilic group must be decreased and / or the size of the hydrophobic group must be increased (Porter 1994). Some possible methods, depending on the type of surfactants are (Clint 1992):

- *All surfactants*: (a) increase the chain length of the hydrophobic tail;
  (b) decrease the chain length of the oil;
  (c) add a cosurfactant with a very small head group.

- *Nonionic surfactants*: (a) increase the temperature (decrease solubility of EO chain);
  (b) decrease the number of EO units in the head group.

- *Ionic surfactants*: (a) add electrolyte;
  (b) change counterion to one that is less hydrated.

A more quantitative prediction of whether a W/O or O/W will be formed is provided by the HLB of the surfactant, determined from the chemical formula of the surfactant using empirically established *group numbers* (Clint 1992; Porter 1994). Table 2.4 summarises the classification of emulsifiers following the hydrophilic-lipophilic balance (HLB) scale.

Table 2.4: Classification of emulsifiers according to HLB (Porter 1994)

HLB values	**Application**
3 - 6	W/O emulsifier
7 - 9	Wetting agent
8 - 18	O/W emulsifier
12 - 15	Detergent
15 - 18	Solubilising agent

The HLB is not the only factor influencing the type of emulsion to be formed, since the relative phase volume of oil and water is even more important. Thus a more general and significant relation would be one that expressed the phase volume of oil, at which an emulsion inverts from O/W to W/O, as a function of the HLB of the surfactant.

2.3.4.2 Foams and foam destruction

Foams consist of a thermodynamically unstable two-phase system of gas bubbles in a liquid. The formation of foam from a bulk liquid involves the expansion of the surface area due to the work acting upon the system (Shaw 1992). As surface tension is the work involved in creating a new system, then the amount of new area formed (foam) will be inversely proportional to the surface tension. Thus reduction of surface tension by a surfactant enhances foam formation (McBain 1950; Danielli *et al.* 1964). The liquid in the lamellae drains into the junctions, and this drainage is not only due to gravity but also due to the pressure difference across a curved liquid surface (Porter 1994). The pressure difference has been quantified by Laplace as follows:

$$dP = \gamma\left(\frac{1}{r_1} + \frac{1}{r_2}\right) \qquad \text{(eq. 2.5)}$$

where P is the pressure, r_1 and r_2 , the radii of curvature of the liquid surface.

The pressure difference at the junctions of the cells must be greater than in the walls, but the actual pressure inside the bubble must be the same at the wall and the junction. Consequently, the pressure inside the junction must be less than in the walls. The liquid drains into the junctions between the cells leaving very thin lamellae (Figure 2.2).

The pressure difference is also proportional to the surface tension. The lower the surface tension, the lower is the pressure difference and the lower the drainage (i.e. more stable foam).

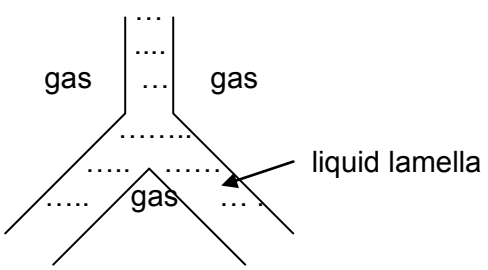


Figure 2.2: Gas bubble

Due to drainage of excess liquid, the bubbles, initially roughly spherical, are first distorted, and gradually become polyhedral cells with plateau borders along lines where usually three bubbles meet (Hunter 1993). Once bubbles are distorted in this way, capillary forces come into play, tending to draw liquid into the plateau border regions, thus providing an additional force causing thinning of the liquid lamellae. When foams are subject to disturbances in the air above them, film rupture can be more pronounced near the exposed upper surface. In such cases, the volume of foam decreases gradually and the liquid from the rupturing cells percolates through the network of plateau into the bulk liquid (Shaw 1992). When the liquid lamellae are inherently unstable, film rupture occurs in a random fashion throughout the whole foam, causing a gradual upwards shift in the cell size distribution. In this case, the volume of foam may decrease only very slowly but the foam becomes increasingly open and less dense. Foam study is also complicated by the fact that gases can easily permeate through the very thin liquid lamellae.

2.4 Microbial surfactants and their role

Microbial biosurfactants may be synthesized as extracellular products (Haferbur *et al.* 1986), able to emulsify hydrocarbons to facilitate their uptake (Janseen *et al.* 2002; Beal & Betts 2000), or as cell wall-associated products to facilitate the substrate penetration into the periplasmic cavity (Desai & Banat 1997). Hydrophobic substrates are generally better than the hydrophilic ones in augmenting biosurfactant production (Santa-Anna *et al.* 2002; Perfumo *et al.* 2006).

As detailed in Sections 2.6.2 and 2.6.3, due to the diverse structures and properties of biosurfactants, they offer potential applications in different industrial fields (Laha & Luthy 1991). Biosurfactants have preferred ecological acceptance since they are biodegradable and non-toxic to natural environment (Santa-Anna *et al.* 2002; Karsa & Porter 1995), but chemically prepared surface-active compounds are still the most used industrially. Examples of the industrial areas where surfactants are used are: Oil and gas, environmental remediation, food and cosmetic, pharmaceutical and agricultulture. More details on these applications are provided in Section 2.6.4.

The use of biosurfactants provides a promising approach to degradation of organic pollutants with very low solubility in water (Zhang & Miller 1995; Thaniyavarn *et al*. 2008; Parthasarathi & Sivakumaar 2009) such as linear or heterocyclic hydrocarbons (Chayabutra & Ju 2001; Cooper & Zajic 1980). Addition of biosurfactants into growth media has been considered as a potential strategy to enhance hydrocarbon degradation by microbial cells (Inakollu *et al.* 2004).

The role of biosurfactants in microbial culture and hence the reason for their production is still unclear (Jennings & Tanner 2000). One role commonly attributed to biosurfactants is the facilitation of microbial growth on hydrocarbons (Neu 1996; Beal & Betts 2000). Rosenberg (1986) questions the attribution of only one role to all biosurfactants, given their existing diversity of structures and functions. Rosenberg proposes a role in adhesion, the emulsification of water-insoluble compounds, deadhesion from interfaces, gliding and cell-cell interaction. Haferburg *et al.* (1986) also suggests a role of biosurfactants in gliding of bacteria and wetting of interfaces.

A biocidal activity of microbial surfactants has also been observed (Yussef *et al.* 2004). This was postulated to be closely related to the lipid moiety of the molecules, whose interaction with eucaryotic cells, for example, results into pyrogenicity, lethal toxicity, immunogenicity, mitogenicity and other molecular effects (Koch *et al.* 1991; Haba *et al*. 2002).

2.5 Interaction of biosurfactant with growth media

The interaction of biosufactants with growth media depends on whether they are cell wall associated or extracellular, and on the hydrophilic or hydrophobic nature of the media. Alkanes are taken up in different ways depending on the mixing conditions

and the type of microorganism (Vasileva-Tankova *et al.* 2008), and the primary product of their degradation depends on the position of the carbon atom(s) that is (are) oxidised. Alkane interaction with microbial cells follows the interaction mode between cells and hydrophobic media.

2.5.1 Case of cell wall associated biosurfactants

When biosurfactants are cell-wall associated, Figure 2.3, they are anchored with the hydrophobic part in the outer layers of the cell surface i.e. the cell wall in Gram-positive bacteria and the outer membrane in Gram-negative bacteria. They can expose either their hydrophilic or their hydrophobic component to the environment, depending on the hydrophobicity or the hydrophilicity of media. When growth media are hydrophilic, the cell will interact with a hydrophilic interface as shown in Figure 2.3a. In the case of hydrophobic media, biosurfactants will be oriented the other way around i.e. bound to the cell with the hydrophilic end and presenting the hydrophobic one to the environment, thus allowing the cell to interact with hydrophobic interfaces, as shown in figure 2.3d (Neu 1996).

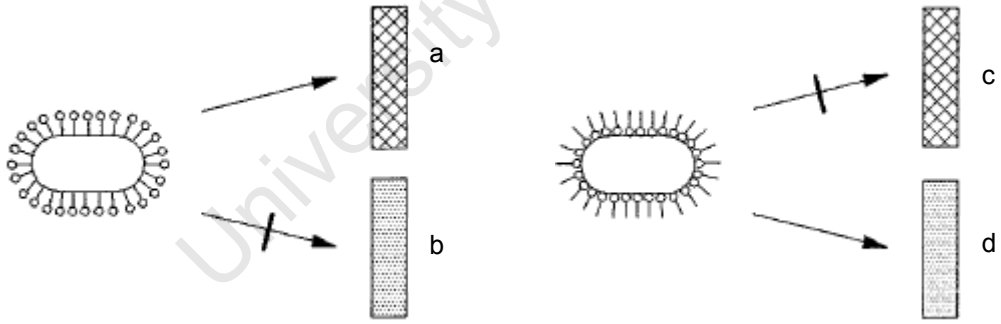


Figure 2.3: Interaction of cell wall associated biosurfactant with growth media. The hydrophobic component is represented by the strait line (tail) and the hydrophobic one by the spherical head. (a) & (c) orientation of microbial surfactant toward a hydrophilic surface; (b) & (d) orientation of microbial surfactant toward a hydrophobic surface (Neu 1996)

2.5.2 Case of extracellular biosurfactants

In the case of extracellular surfactants, the cell creates a conditioning film at the interface by excreting surfactants. On a hydrophobic interface, for example, this conditioning film will change the interface from hydrophobic to hydrophilic (Yussef *et al.* 2003). This results in a situation where only hydrophilic cells can interact with the interface, as illustrated in figure 2.4.

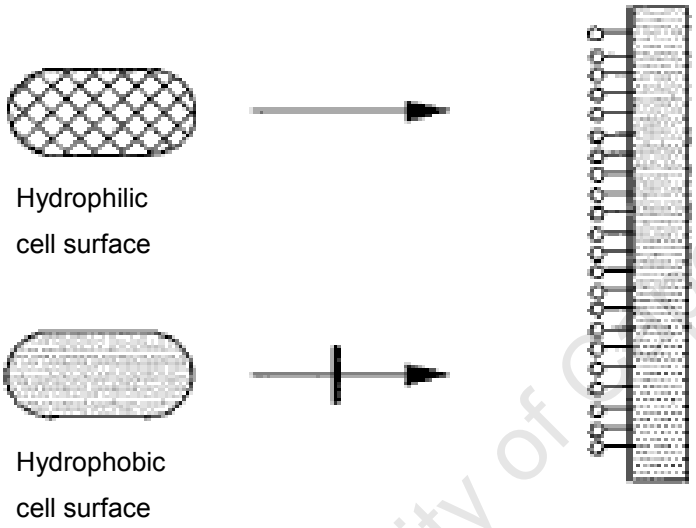


Figure 2.4: Orientation of the cells to the media after excreting biosurfactants. The released biosurfactants bound to the hydrophobic surface with their hydrophilic moieties, exposing the hydrophobic one to the cell (Neu 1996)

2.5.3 Cell deadhesion from media interfaces

Cell deadhesion from interfaces is also mediated by biosurfactant. This is made clear by considering the case of cell-bound surfactants, where, depending on the hydrophilic or hydrophobic properties of the interface, the bacteria leave a conditioning film with hydrophilic or hydrophobic properties, as shown on Figure 2.5 (Neu 1996).

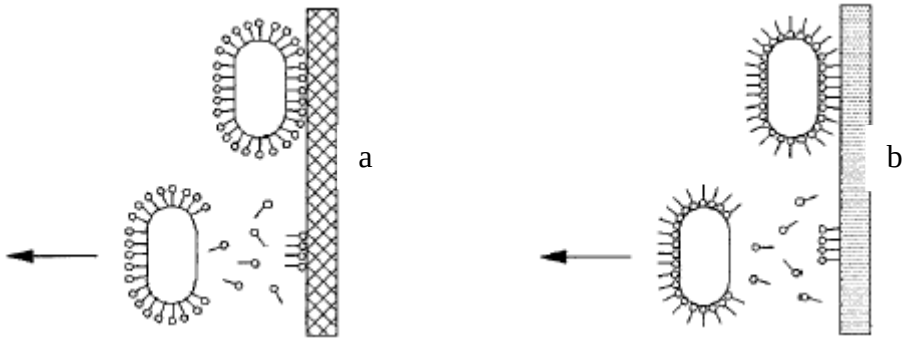


Figure 2.5: Deadhesion of cells from hydrophilic and hydrophobic media through *biosurfactant-media* interaction. The hydrophilic moiety of the biosurfactant is indicated by the spherical head and the hydrophobic moiety by the straight line. (a) Deadhesion of cells from a hydrophilic interface, by releasing a cell-bound biosurfactant; (b) Deadhesion of cells from a hydrophobic interface, by releasing a cell-bound biosurfactant (Neu 1996)

2.6 Biosurfactant characterisation

Biosurfactants vary in their chemical properties and molecular weight, but all present both hydrophilic and hydrophobic properties. Their chemical nature consists of many different structures, on which depend their physical and chemical properties as well as the industrial applications (Ron & Rosenberg 2001).

Like non-microbial surfactants, biosurfactant activity and micelles formation is concentration dependent. The lowest concentration at which surface activity can be observed is referred to as critical micelle concentration, CMC. In fact, at low concentration, surfactants exist as monomers. As their concentration increases, their molecules form micelles, and the smallest concentration on which micelles begin to be formed is called CMC (Chayabutra & Ju 2001). Surfactants with low CMC values are more valuable as they can be used in small amounts.

2.6.1 Chemical nature of biosurfactants

Like the chemically manufactured surfactants, biosurfactants exist in a wide range of structures, including peptides, fatty acids, phospholipids, glycolipids, lipopeptides, etc. In some cases, microorganisms produce surfactants, which are a combination of different chemical types, referred to as polymeric microbial surfactants or PMS (Desai & Banat 1997). High molecular weight biosurfactants are generally

polyanionic heteropolysaccharides containing both polysaccharides and proteins, whereas the more abundant low molecular weight biosurfactants are often glycolipids (Ron & Rosenberg 2001). It is important to note that intact microbial cells with high cell surface hydrophobicity are themselves surfactants (Ron & Rosenberg 2001).

2.6.2 Classification of biosurfactants

Surfactants are generally classified according to the nature of their hydrophilic part, as follows (Cullum 1994):

- anionic surfactants are those having a negative overall charge or with a hydrophilic part carrying a negative charge e.g. $RCOO^-$, $ROSO_3^-$
- nonionic surfactants are those in which the hydrophilic part is uncharged e.g. ethoxylated fatty alcohols, $RCON(C_2H_4OH)_2$
- cationic surfactants are those in which the hydrophilic part carries a positive charge e.g. alkyltrimethylammonium salts, alkyldimethylbenzylammonium salts
- amphoteric surfactants are those whose hydrophilic moiety carries both negative and positive charges e.g. alkylaminopropionates, alkylbetaines. In some cases, amphoteric surfactants have more than one charge of either sign, and can loose a charge by addition or removal of a proton.
- weakly acidic and weakly basic surfactants are the ones behaving as either weak acids or weak bases depending on their environment. The former are cationic only in acid solutions and nonionic in alkaline solutions, while the latter are anionic only in alkaline solutions and nonionic in acidic solutions.

For non obvious reasons, weakly acidic surfactants are commonly considered as anionic surfactants, but weakly basic surfactants are usually considered as nonionic (Cullum 1994). Following the above definitions, rhamnolipids are usually regarded as anionic.

Biosurfactants are also broadly categorised into six classes on the basis of both the chemical structure and the functional groups carried by their molecules. Examples of surfactants belonging to each of these classes are given in Table 2.5.

Table 2.5: Classification of biosurfactant on the basis of their chemical nature and functional groups

	Biosurfactants					
	Glycolipids	**Fatty acids**	**Phospholi -pids**	**Surface active antibiotics (mostly cyclic glycopeptid es)**	**Polymeric microbial surfactants**	**Particulate surfactants**
Examples	- Trehalose lipids - Sophorolipids - Rhamnolipids	- saturated fatty acids in the range of C_{12} to C_{14}; - complex fatty acids containing hydroxyl groups and alkyl branches	- N-phosphatidylethanolamine	- Gramicidin S - Polymixins - Surfactin - Antibiotic TA	- Emulsan - Polysaccharide liquid complexes	- Extracellular vesicles - Microbial cells with high cell surface hydrophobicity
References	Gautam & Tyagi (2006); Desa & Banat (1997); Kitamoto *et al.* (2002)	Kosaric (1993)	Gautam & Tyagi (2006)	Kosaric (1993); Gautam & Tyagi (2006)	Desai & Banat (1997); Kosaric (1993)	Karanth & Veenanadig (2004);

Examples of biosurfactants that have been reported to be produced by different bacterial organisms are given in Table 2.6. It is specified, in the Table, whether the biosurfactant is extracellular, intracellular or cell wall-bound.

Table 2.6: Examples of reported bacterial biosurfactant, main group of surfactants in which the biosurfactant is classified, producing microoraganism, main properties, location of the biosurfactant, and the typical reference

Biosurfactant	Structure	Microorganism	Main Property	Location	Author
Rhamnolipid	glycolipid structure consisting of the rhamnose sugar and hydroxydecanoic acid	*Ps. aeruginosa*	Good bioemulsifier and efficient surface and interfacial tension reducing agent	extracellular	Lang & Wullbrandt 1999 (cited in Ghilamicael 2003);Chayabutra & Ju 2001; Santa-Anna *et al.* 2002; Koch et al. 1991; Perfumo *et al.* 2006; Benincasa *et al.* 2002
Rhamnolipid	glycolipid structure consisting of the rhamnose sugar and hydroxydecanoic acid	*Renibacterium salmoninarum*	Good bioemulsifier and efficient surface and interfacial tension reducing agent	extracellular idem	Christova *et al.* 2004
Trehalolipid	glycolipid structure consisting of the trehalose sugar and dicarboxylic ester	*Rhodococcus erythropolis*	Lowering surface and interfacial tension ; make good emulsions when cells are grown on hydrocarbons	Cell bound	Desai & Banat 1997; Kretschmer *et al.* 1982 (cited in cited in Ghilamicael 2003)
Viscosin	lipopeptide	*Ps. aeruginosa*	Very efficient in lowering surface tension	extracellular	Neu *et al*. 1990; Neu & Poralla 1990
Peptidoglycolipid surfactants	Protein, carbohydrate and lipid components	*Ps. aeruginosa*	Good emulsifying agents, but not efficient in lowering surface and interfacial tension	extracellular	Ilori & Amund 2001
Saturated Fatty acids (C_{12} to C_{14})	Fatty acid biosurfactants	*Arthrobacter and Ps. aeruginosa*	surface active agents	Both extracellular and intracellular	Neu 1996; Kosaric 1993
Complex fatty acids containing hydroxyl groups and alkyl branches	Fatty acid biosurfactants	*Arthrobacter and Ps. aeruginosa*	surface active agents	Both extracellular and intracellular	Neu 1996; Kosaric 1993

2.6.3 Properties and applications

Some properties are specific to biosurfactants, but others are shared with chemically synthesized surfactants.

The main surfactant properties common to all surfactants are the following:

- changing surface and interfacial tension
- wetting and penetrating actions
- spreading
- hydrophobicity and hydrophilicity actions
- metal sequestration
- emulsification and emulsion stabilisation
- antimicrobial actions
- effectiveness at moderate temperature, pH, ionic strength, salinity, etc.

The following properties are characteristics specific to biosurfactants (Kosaric 2001):

- high specificity
- biodegradability
- low toxicity
- biocompatibility and digestibility (use in cosmetic, pharmaceuticals and as functional food additives)
- use in environmental control
- effectiveness at extreme temperature, pH, ionic strength, salinity, etc.

Due to the above properties, biosurfactants offer a broad range of potential applications, examples of which are given Table 2.7 (Ochsner *et al.*1995; Maier & Soberon-Chavez 2000; Palejwala & Desai 1989; Lafrance & Lapointe 1998; Young & Coons 1945; Gautam & Tyagi 2006; Neu 1996).

Table 2.7: Major industrial areas where biosurfactants are utilised, and examples of specific applications in the different respective areas

Major industrial areas of biosurfactant applications			
Bioremediation and Cleaning (Ochsner *et al.*1995; Maier & Soberon-Chavez 2000)	**Food and Beverage (Ochsner *et al.* 1995; Young & Coons 1945)**	**Agriculture (Ochsner *et al.* 1995 ; Palejwala & Desai 1989; Lafrance & Lapointe 1998)**	**Pharmaceutical (Neu 1996; Gautam & Tyagi 2006)**
- oil contaminated environmental media treatment - removing toxic materials (industrial wastes and by-products) from soil and water - descaling (wetting agents and corrosion inhibitors in acid cleaning of boiler tubes and heat exchangers) - detergents and sanitizers	- food processing plants (for cleaning sanitizing) - fruits & vegetables (removal of pesticides, etc.),bakery and ice cream (solubilisation of flavor oils, control of consistency, retard stailing, etc.)	- phosphate fertilizers (prevent caking during storage) - spray application (wetting, dispersing, suspending powdered pesticides and emulsification of pesticide solutions, etc.)	- antibiotic and anti fungal - emulsification - emulsion stabilization

2.7 Rhamnolipids

Rhamnolipids are a group of glycolipids containing generally one or two 3-hydroxy fatty acids linked to a mono- or di-rhamnose moiety (Déziel *et al.* 2000). They are reported to be among the most effective (Makkar & Cameotra 1998) and the best studied biosurfactants (Nelly *et al.* 2001). They are reportedly produced as complex mixtures by various species of the Pseudomonas genus, particularly *Pseudomonas aeruginosa* (Déziel *et al.* 2000; Reiling *et al.* 1986).

The structure of *Ps. aeruginosa* rhamnolipids consists of one or two L-rhamnose units and one or two units of ß-hydroxydecanoic acid, in most cases.

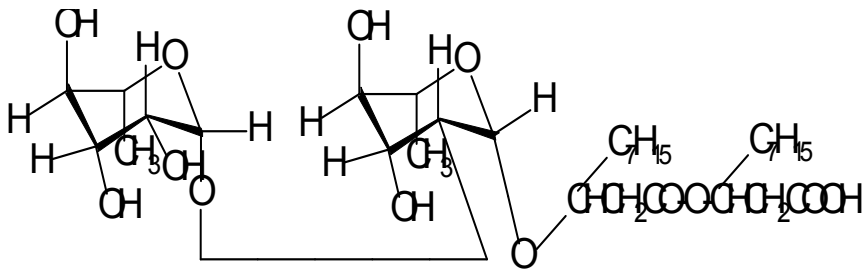


Figure 2.6: Example of a rhamnolipid molecule, with two rhamnose units and one decanoic acid unit

Their critical micelle concentration (CMC) in water can be as low as 10 to 20 mg.l^{-1}. The corresponding minimal surface tensions (MST) of 25 to 30 mN/m are among the lowest yet observed. These properties are very attractive, compared to those of synthetic surfactants, e.g. CMC of 2023-2890 mg/l and MST of 37 mN/m for sodium dodecyl sulfate, and CMC 590 mg/l and MST 47 mN/m for alkylate dodecylbenzene (Chayabutra & Ju 2001). In addition to extracellular rhamnolipids, *Ps. aeruginosa*, and fluorescent pseudomonads in general, are known to produce poly ß-hydroxyalkanoates acids (PHAs) as intracellular storage materials (Chayabutra & Ju 2001).

2.7.1 Rhamnolipid producing microorganisms

The *Pseudomonadaceae family* consists of gram-negative, polarly flagellated, straight and slightly curved rods shaped bacteria that grow aerobically and are not spore formers. They obtain their energy by aerobic respiration and, in some cases, by anaerobic respiration (nitrate respiration, denitrification). The pseudomonadaceae are dominantly chemo-organotrophs, though some are facultative chemolithotrophs (Stainer *et al.* 1976).

The genus *Pseudomonas* is the prototype of this family and is characterized, like other pseudomonads, by use of a wide range of substrates. They utilize a large number of hydrocarbons, heterocyclic and aromatic compounds that are not attacked by other bacteria. They generally metabolise sugars via the Entner-Doudoroff pathway (Schlegel & Schmidt 1990).

Some *Pseudomonas* species oxidise sugars incompletely and excrete sugar acids (gluconate, 2-oxogluconate). Pseudomonads are ubiquitous due to their simple requirements (Schlegel & Schmidt 1990). They are present in soil, water, sewage and air. When media containing mineral salts and organic acids or sugars are exposed to air, pseudomonads are usually the first colonisers. Many can be recognised by their production of water-soluble pigments (e.g. the blue-green derivative of phenazine, pyocynine, and the yellow-green fluorescing derivatives of pterin).

Examples of the most common pseudomonas species are presented in Table 2.8. The best biosurfactant producers isolated from Milnerton Caltex Refineries in Cape Town were found to be pseudomonaceae family (Mathopa 2004).

Table 2.8: Examples of species under the pseudomonaceae family (Schlegel & Schmidt 1990; Loewy & Siekevitz 1963)

Pseudomonas genus				***Xanthomonas genus***
Pseudomonas aeruginosa:	*Ps. fluorescens*	*Ps putida*	*Ps syringae*	
soil and aquatic species able to metabolise a very large number of organic compounds. Plant pathogen. May be opportunistic human pathogen.	soil and aquatic species able to oxidize a very large number of organic compounds.	soil and aquatic species that can oxidize a very large number of organic compounds.	many pseudomonas strains showing plant pathogenicity have been unified in the species *Ps. syringae.*	Xanthomonas genus represents all the yellow-pigmented plant pathogens of the Pseudomonadaceae family. The yellow pigment is a polyene compound containing bromine.

2.7.2 Genetics of rhamnolipid synthesis by *Ps. aeruginosa*

The metabolism of linear alkanes in *Ps. aeruginosa* cells is often accompanied by the synthesis of a mixture of two typical rhamnolipid molecules, rhamnosyl-ß-hydroxydecanoyl-ß-hydroxydecanoate and rhamnosyl-rhamnosyl-ß-hydroxydecanoyl-ß-hydroxydecanoate (Déziel *et al.* 2004). At the molecular level, rhamnolipid synthesis is regulated by a gene cluster, rhlABR which is responsible for the synthesis of RhlR regulatory protein and necessary rhamnose transfer enzymes (Desai & Banat 1997). Desai & Banat reported (1997) reported rhlABR and RhlR to

be transfer enzyme complexes, located in the cytoplasmic membrane, with 32.5-kDa RrlA protein harboring a putative signal sequence, while the 47-kDa RhlB protein is located in the periplasmic region and contains at least two putative membrane-spanning domains. The rhlR gene encodes a transcriptional activator, the 28-kDa RhlR protein belonging to the LuxR family, which positively regulates rhamnolipid biosynthesis (Desai & Banat 1997). Another regulatory gene, rhlI has been reported by Ochsner & Reiser (1995), and is located downstream of the rhlABR gene cluster (Figure 2.7). The synthesis of rhamnolipid is mediated by the rhlR-rhlI system involving often one autoinducer. According to Ochsner & Reiser (1995), the transcription of the rhlAB operon that encodes rhamnosyltransfertases is triggered by the binding of activated RhlR protein upstream of the rhlA promoter. Most of these enzymes involved in the regulation of rhamnolipid synthesis, are not constitutive but are produced on need. Rhamnolipid biosynthesis proceeds by two sequential rhamnosyl-transfer reactions, catalysed by the specific rhamnosyltransferases Rt1 and Rt2. The rhamnosyl donor is deoxy-thymidine-diphospho-$_L$-rhamnose, dTDP-$_L$-rhamnose (Déziel *et al.* 2004). Apart from the two rhamnolipid mentioned in this section, there are more than 28 other rhamnolipids that have been reported as resulting from from the metabolism of hydrocarbon chain lengths varying between C_{10} and C_{18} (Déziel *et al*. 1999). This indicates that Rt1 and Rt2 are not highly specific to rhamnosyl-ß-hydroxydecanoyl-ß-hydroxydecanoate and rhamnosyl-rhamnosyl-ß-hydroxydecanoyl-ß-hydroxydecanoate. The enzyme responsible for draining the fatty acid precursors of rhamnolipid from the general biosynthetic pathway is called RhlG and was reported to have significant sequence homology with numerous NADPH-dependent ketoacyl reductases (Sadovskaya *et al.* 1998; Compos-Garcìa *et al.* 1998). In *Pseudomonas aeruginosa* as well as in other gram-negative bacteria, $_L$-rhamnose is a common component of the LPS O-antigen (Sadovskaya *et al.* 1998). The genes “rml” have been described in many bacteria (Zhang *et al.* 1993) and are the ones encoding the enzymes involved in the synthesis of TDP-$_L$-rhamnose (Déziel *et al.* 1999). The regulation system for rhamnolipid synthesis is summarised in Figure 2.7.

The rhamnolipid synthesis pathway by *Ps. aeruginosa* strains is illustrated in Figure 2.8. It proceeds through the hydrocarbon chain oxidation in ß-position, chain shortening and acylation of the chain mediated by acyl-CoA, thus forming the hydroxy-alkanoyl-hydroxy–alkanoate. It is at this stage that the L-rhamnosyl unit of the rhamnolipid is transported in the form of dTDP-L-rhamnose by responsible

rhamnosyl transferases, and linked to the hydroxy-alkanoate moiety of the rhamnolipid (Desai & Banat 1997).

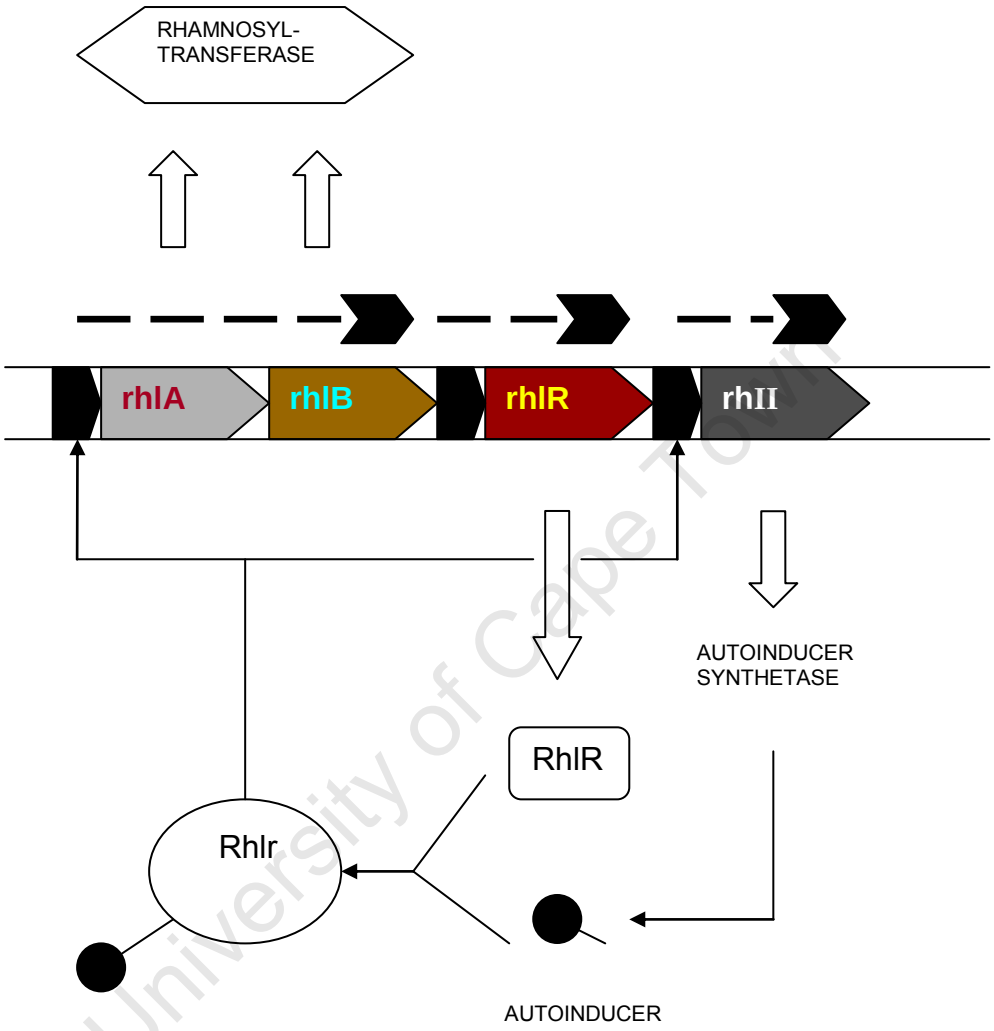


Figure 2.7: Model for the regulation of rhamnolipid synthesis in *Ps. aeruginosa* (Desai & Banat 1997)

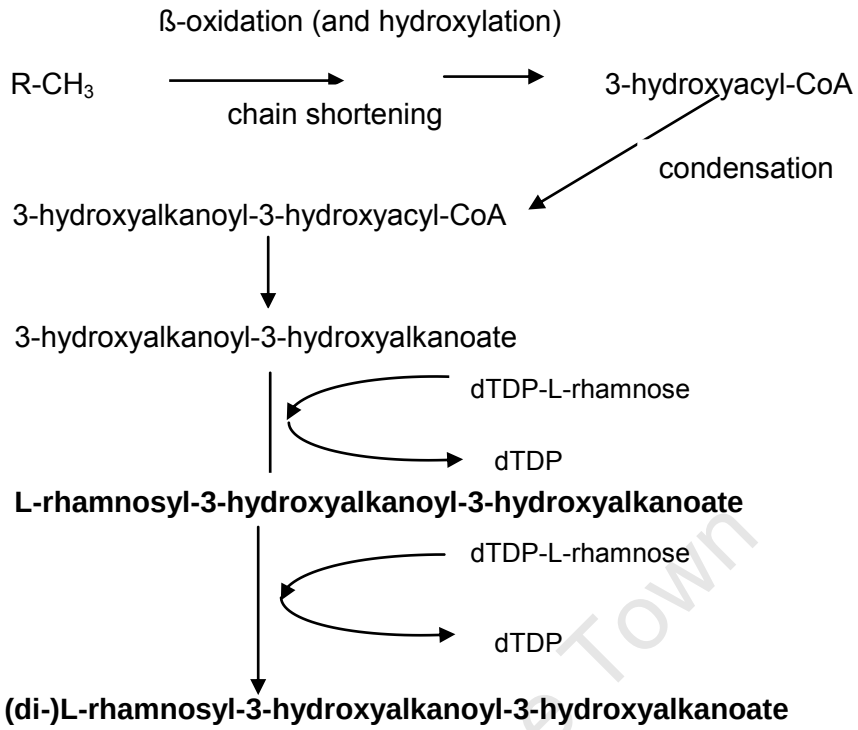


Figure 2.8: Main steps of the *Pseudomonas aeruginosa* rhamnolipids synthesis pathway (Déziel *et al.* 2004)

2.8 Overview of Mathopa and Ghilamicael's research projects

As indicated in Chapter 1, this study forms part of a larger project at UCT, following two sub-projects, initiated by the Department of Molecular & Cell Biology, that focussed on the screening of the best oil degrading strains microbial strains from several bacterial species isolated from oil contaminated sites, at the CHEVRON and and SASOL, Ltd S.A. refineries in South Africa. These projects were realised as the Masters projects of Ghilamicael (2003) and Mathopa (2004) under the supervision of Professor S. Reid. The fundamental objective of these two projects was to use hydrocarbon wastes, released in large amount by petrochemical industrial processes, as cheap raw materials for the production of valuable compounds. One of the known by-products of these petrochemical hydrocarbons is straight chain alkanes. Alkanes in these wastes might range from C_4 to C_{18}, and there are many microbial species that have been reported as being capable of degrading alkanes as carbon and energy source for their survival (Perfumo *et al.* 2006; Yakimov *et al.* 1998)

Ghilamicael (2003) investigated bacterial ability to produce bioemulsifiers on degrading oil substrates and sought promising new isolates characterised at the genus and species level. Mathopa (2004) investigated bacterial ability to produce PHAs by growing on oil substrates. Both PHAs and biosurfactants are industrially attractive products, the first offering applications in the manufacture of biodegradable plastics while the second are surface active agents that have a broad range of industrial applications, particularly in the areas of oil and gas industry, food & beverages, paper industry, detergent industry, agro-pharmaceuticul industry, biopesticide industry, etc.

2.8.1 Isolation of bacterial strains capable of efficient conversion of n-alkanes into value added products (Mathopa 2004)

The objective of this project was to isolate microorganisms capable of degrading the alkane fractions, C_{12}-C_{13} and C_{14}-C_{17}, and synthesising products with high commercial value, such as the biodegradable plastics of the class PHAs. It was anticipated that the discovery of such organisms would provide a promising approach to finding a sustainable solution to the plastic wastes pollution.

The bacterial strains used in this study were isolated from crude oil contaminated soil that was collected from CHEVRON Refineries at Milnerton, Cape Town. As crude oil had been leaking from the industrial vessels for some time, it was hypothesised that the soil around the refineries were inhabited predominantly by oil degrading organisms or by organisms that prefer the by-products of crude oil as their source of carbon.

During the bacterial collection, an approximately 5-10 cm opening was dug in the ground to avoid carrying most of the bacterial strains normally found on the soil surface rather than those growing on crude oil or by products of crude oil, such as alkanes. Other bacterial strains were also used in the study. *Pseudomonas oleovorans ATCC29347* and *Bacillus megatorium* (laboratory strain, UCT), which were used as positive controls since they are known to accumulate PHAs and have been shown to have the *phaC* gene (Sheu *et al.*, 2000, cited in Mathopa 2004). *Escherichia coli* JM109 strain served as a negative control because it does not accumulate PHAs in its cytoplasm. On solid medium *E Coli JM109* culture was maintained on 2xYT agar with the appropriate antibiotic selection where applicable.

For liquid medium, 2xYT broth or Luria broth (LB) with appropriate additives were used. The Environmental isolates were grown on two alkane fractions, C_{12}-C_{13} and C_{14}-C_{17}, provided by SASOL, Ltd SA, as sole carbon and energy sources, at a concentration of 1%.

When the environmental isolates were grown on a properly selected minimal salt medium (MSM), twenty three strains utilised the alkane fractions effectively. These strains were presumed different based on phenotypic characteristics. All the 23 strains were found to be gram negative and rod shaped, and one was gram positive and cocci. A Nile blue A staining technique was used as a preliminary step to identify which of the 23 strains were possible PHA accumulators. Twelve of the 23 strains gave a positive Nile Blue A test. But Nile Blue A stains any lipid compounds which may include other components of the cell membrane. So these 12 strains were just potential PHA accumulators and needed to be subjected to further more specific screening tests.

Preliminary biochemical tests were applied to the 12 isolates. These tests provided useful information on the probable genus to which the environmental isolates belonged. It was found that 83% of the strains were Pseudomonads, and it was concluded that the oil contaminated sites are predominantly inhabited by the *Pseudomonas* genus. One strain was identified as belonging to the *Klebsiella* genus. The 16S rDNA sequencing was applied to 5 strains, which were found to have the highest alkane degrading potential. The strain was found to possess the phaC gene fragment. The *phaC* gene encodes PHA synthase. Among the 5 isolates, 4 were Pseudomonads and one belonged to *Klebsiella* genus, when their sequence alignments were compared to the published sequences using the NCBI Database (www.ncbi.nlm.nih.gov/blast).

One strain which consistently provided good yields of PHA after Nile Blue A staining, was subjected to more rigorous DNA sequencing. It showed 99% identity to *Pseudomonas spp*. Over a region of 1302bp in 8 out of 10 strains detected by a BLAST search. Therefore, the strain was named *Pseudomonas aeruginosa MB2SA*. Most of the 10 top strains obtained after the BLAST search fell under the *Pseudomonas aeruginosa* species. While it is recognised that *Ps. aeroginosa* may represent an opportunistic human pathogen, such behaviour is not typical for environmental isolates.

From this stage the project concentrated on *Pseudomonas aeruginosa MB2SA*, studying its physiology as well as its growth on alkane fractions and different individual alkanes. The molecular analysis of the PCR product corresponding to the *phaC* gene, that is responsible for encoding PHA synthase, was also carried out.

2.8.2 Isolation and characterization of n-alkane utilizing bacteria, which produce bioemulsifiers (Ghilamicael 2003)

The fundamental objective of this project was to isolate and characterise novel South African bacterial strains, that are capable of efficient utilisation of the n-alkane fraction (C_{12}-C_{17}), which is a by-product of the SASOL, Ltd S.A., petrochemical industry, and produce biosurfactant materials.

During the bacterial isolation, soil samples were aseptically collected from CHEVRON Oil Refinery in Cape Town, South Africa, in 250 ml autoclaved bottles with screw tops (from Duran, Germany). Samples were taken in duplicate from the following three different sites: (a) CHEVRON Oil Refinery Company, around the vicinity of the crude oil tank 202; (b) CHEVRON Oil Refinery, near the receiving station where an oil leakage occurred on 7th May 2002; (c) CHEVRON Oil Refinery Company, near the Land Farm, which used to be a dumping site for hydrocarbon waste ten years ago. A proper MSM was selected and the oil was used as carbon source at the concentration of 1%.

After four sequential enrichment cultures supplemented with crude oil as the sole carbon source, various phenotypically distinct strains were isolated based on the shape, size, texture, color etc. of the colonies plated on nutrient agar. From the newly isolated strains, 18 phenotypically different strains were subjected to the screening for emulsifying potential, against a 1:1 kerosene aqueous phase mixture. Five strains, labelled 1Ab, 1Ba, 2Bf, 2Bf* and 2Bg gave a positive test with emulsification index values of 25%, 35%, 69%, 70% and 66% respectively. These strains were identified with standard biochemical tests and partial 16S rDNA PCR. The standard biochemical methods for preliminary classification helped to identify strains 2Bf, 2Bf* and 2Bg (Table 2.9) as belonging to the genus *Pseudomonas*. The study was continued 2Bf which showed slightly higher emulsifying capacity.

Table 2.9: Primary characterisation and identification of the three best bioemulsifier producing strains, as reported by Ghilamicael (2003)

	Strain		
	2Bf	**2Bf***	**2Bg**
Biochemical tests			
Gram stain	Gram negative	Gram negative	Gram negative
Shape	Rods	Rods	Rods
Motility	Motile	Motile	Motile
Catalase test	Catalase positive	Catalase positive	Catalase positive
Oxidase test	Oxidase positive	Oxidase positive	Oxidase positive
O-F test	Oxidation	Oxidation	Oxidation

The 16S rRNA sequences were used in the classification of microorganisms, since it is highly conserved in different species. By the means of the universal primers (Weisburg *et al.* 1991, cited in Ghilamicael 2003), a 1.5 kb rDNA fragment was amplified from the strain 2Bf by PCR. The PCR product was sequenced using F3 and R3 universal primers and was analysed with the BLAST search. This sequence analysis revealed 97% sequence identity to four strains of *Ps. aeruginosa* and 99% to the strain *Pseudomonas aeruginosa PAO1* 16S rRNA gene. Therefore, the strain was confirmed as *Ps. aeruginosa*.

Further research concentrated on the growth of *Pseudomonas aeruginosa* on alkane fractions and the production of bioemulsifier from alkane substrates. The Nile Blue A test and the molecular analysis of the PCR product corresponding to the *phaC* gene revealed that *Ps. aeruginosa 2Bf* accumulated PHAs.

2.8 Bioreactor systems

The purpose of a bioreactor is to create, control and contain a suitable environment for the growth of living cells (Asenjo & Merchuk 1995). This review of bioreactor systems is a brief description of the main characteristics of bioreactors. It is organised in three sections: microbial growth and kinetics, mass transfer in biological systems, and mixing in bioreactors and bioreactor equipment.

2.8.1 Microbial growth and kinetics

Microbial growth is often seen as the most fundamental response to the microbial physicochemical environment (Shuler & Kargi 1992). It is the result of the process in which microorganisms consume nutrients and increase in number and in size, through a large number of biochemical reactions designated by the general term of *cellular metabolism* (Blanch & Clark 1996). Through the metabolic process, formation of intracellular and extracellular compounds takes place, and their nature depends on the nutrient medium and the microorganism concerned.

In biokinetic studies, the rates of microbial growth, substrate utilisation and product formation are determined. The following parameters are particularly useful in a biokinetic study: specific substrate utilisation rate, q_S; specific product formation rate, q_P; biomass concentration, c_x and specific growth rate μ which corresponds to the microbial growth per unit biomass as expressed by Bailey & Ollis (1986):

$\mu = r_x / c_x$ (eq. 2.6)

and

$r_x = d\, c_x / dt$ (eq. 2.7), where $\mu = f$ (T, pH, C_s, etc.).

where the growth rate is controlled by the nutrient availability, the microbial kinetics can be described by the Monod equation.

This relates the specific growth rate μ to the concentration of the growth limiting substrate, according to Eq. 2.8, where k_s is the saturation constant and C_s the concentration of the growth limiting substrate:

$\mu = \mu_{max}\, c_s / k_s + c_s$ (eq. 2.8)

The biomass yield is the relationship between the growth rate and the substrate utilisation rates, as given in Eq. 2.9:

$Y_{x/s} = - r_x / r_s$ (eq. 2.9)

where $Y_{x/s}$ is the yield of biomass on substrate and r_s the substrate consumption rate; the negative sign indicates substrate disappearance (Bailey & Ollis 1986).

Another important kinetic parameter is the process productivity. While the yield specifies the amount of product obtained from the substrate, the productivity specifies the rate of product formation (Ratledge & Kristiansen 2006). The rate at which the cells synthesise a particular metabolite is directly given by the specific productivity.

2.8.2 Mass transfer in biological systems

In aerobic bacterial systems, oxygen is often the rate limiting substrate since it is sparingly soluble under culture conditions. The supply of oxygen proceeds through seven steps (Bailey & Ollis 1986): (1) diffusion of (oxygen) from bulk gas to the gas-liquid interface; (2) movement through the gas-liquid interface; (3) diffusion (of oxygen) through a relatively non-mixed liquid region adjacent to the gas bubble; (4) transport of oxygen through the well-mixed liquid to a relatively unmixed liquid region surrounding the cells; (5) diffusive transport through the second unmixed liquid region surrounding the cells; (6) transport from the liquid to the microbial floc, pellet, etc.; (7) diffusive transport into the pellet, etc.; (8) transport across the cell envelope; (9) transport from the cell envelope to the intracellular reaction site.

For most processes, one or more of the steps are in pseudo-steady state. Thus transport through the well-mixed liquid is normally very rapid in laboratory-scale bioreactors because of the reasonable assumption of homogeneity in the medium. Steps 5, 6 and 7 are relevant only for processes in which pellets or cell aggregates appear (Nielsen & Villadsen 1994). Transport resistance within the cell is usually neglected, because of the small size of most cells. The major resistances to oxygen transfer are considered to be the liquid film surrounding the air bubble or the liquid film around the microorganisms (Bailey & Ollis 1986). Oxygen solubility defines the driving force for transfer. It is dependent on both physical conditions and liquid composition, in any liquid or mixture of liquids.

The rate at which oxygen is transferred to the system, termed the oxygen transfer rate, is defined as:

$$OTR = k_L a.(C^* - C_L) \quad \text{(eq. 2.10)}$$

where k_La is the volumetric mass transfer coefficient, C* the oxygen concentration at saturation, C_L the oxygen concentration in the liquid phase, and *a* the specific interfacial surface area. The transfer of oxygen from the gaseous phase proceeds by molecular diffusion.

In bioreactors, the oxygen requirement depends on many factors, including: structural oxygen present in the substrate molecule, specific growth rate, biomass concentration, yield of cells on oxygen, oxygen supply from external sources, etc. The requirement of oxygen for microbial growth varies with cell number, cell activity and microbial nature. The oxygen utilisation rate is, in fact, the change or decrease of oxygen concentration, due to microbial activity, per unit time. It can be expressed as given in Eq. 2.11 and Eq. 2.12.

$$OUR = - dC/dt + k_La.(C^*-C_L) \quad \text{(eq. 2.11)}$$

Or

$$OUR = k_La.(C^*-C_L) - OTR \quad \text{(eq. 2.12)}$$

But when oxygen is the limiting substrate, there will be oxygen limitation, and the OTR will be equal to OUR:

$$OUR = OTR = k_La.(C^*-C_L) \quad \text{(eq. 2.13)}$$

Following Eq. 2.10, it is evident that the oxygen transfer rate is maximal when the dissolved oxygen concentration is zero, and it is necessary to define a concentration of oxygen such that the oxygen uptake is at its highest rate.

The *critical dissolved oxygen concentration*, $C_{crit.}$, is the lowest value of dissolved oxygen concentration at which the specific oxygen uptake is maximum. At a lower dissolved oxygen concentration, a decline in the microbial growth results. The dissolved oxygen concentration should be maintained above $C_{crit.,}$ in order to maintain a fully aerobic bioreactor at maximum productivity.

The term k_La represents the volumetric coefficient of oxygen transfer characterising the aeration capacity of the system. It consists of k_L and a. The first being the mass

transfer coefficient and the second is the specific surface area ($m^2.m^{-3}$) depending on the both the bubble diameter and the gas hold up. Gas hold up can be defined in two ways:

$\acute{\varepsilon} = V_g / V_L$ (eq. 2.14) or $\acute{\varepsilon} = V_g / (V_L + V_g)$ (eq. 2.15)

where ε represents the gas hold up, V_g is the volume of gas and V_L the volume of liquid. The major factors affecting k_La are: agitation, aeration, temperature, surface active agents and type of sparger (Aiba *et al.* 1965). In most stirred tank reactors, k_La value increases with superficial gas velocity until it reaches a certain critical value corresponding to the point of impeller flooding. By studying an aerated and agitated reactor system in which the liquid density, the surface tension of liquid and the ascending velocty of bbubble are assumed constant, it was established that k_La can be expressed as a function of the stirrer power input and the superficial gas velocity, as shown in Eq. 2.16 (Aiba *et al.* 1965).

$$k_L a = \omega\left(\frac{P_g}{V}\right)^{\alpha} v_s^{\beta} n^{\delta} \qquad \text{(eq. 2.16)}$$

where, $\left(\frac{P_g}{V}\right)$ is the power input per unit volume of liquid in aerated system ($hp.m^{-3}$); v_s is the nominal air velocity based on empty cross-sectional area of vessel ($m.h^{-1}$); n s the rotational speed of impeller (revolution per hour); and ω, α, β are empirical constants essentially depending on the nature of the liquid and gas phases, and on the type of impeller used for stirring up the system.

Oxygen transfer rate decreases with increasing medium viscosity, and the latter depends on the type of microbes present and their concentration.

2.8.3 Mixing in bioreactors and bioreactor equipment

Although some industrial processes are done as surface cultures (solid state bioprocesses) where the design criteria are very specific for the applied microorganisms, the large majority of industrial bioprocesses are based on

submerged cultures, where the stirred tank bioreactors are widely used (Shuler & Kargi 1992).

The stirred tank reactor is a typical example of a reactor with internal mechanical agitation. Stirred tank bioreactor systems are highly flexible and are capable of providing high k_La values for mass transfer (Shuler & Kargi 1992). They are typically cylindrical, with a slightly curved or almost flat bottom. The ratio between tank height and tank diameter, called *aspect ratio*, varies but it is typically 1:1, 2:1 or 3:1. In smaller tanks, temperature control is achieved by a heating/cooling jacket, whereas for larger tanks internal or external heat exchanger loops are necessary to maintain a constant temperature. In aerated processes, air is normally supplied through a sparger placed at the bottom of the tank below the lowest stirrer. Usually, the sparger is formed as part of a ring or a plate, or it may simply be an open tube end with only one orifice hole. Most stirred tanks are equipped with baffles to prevent the formation of a large central vortex due to the centrifugal forces introduced by the action of the stirrer.

Stirring is accomplished by one or more impellers mounted on a centrally placed impeller shaft (Blanch & Clark 1996; Nielsen & Villadsen 1994; Aiba *et al.* 1965). There exist many designs of impeller stirrers, but they can be roughly classified into two groups, *turbine* and *axial flow* impellers (Blanch & Clark 1996). The turbine impellers have vertically placed blades that rotate around its axis. Often the blades are placed equidistant from each other on a horizontal circular plate. An example of this type of impeller is the largely used Rushton turbine. The radial mixing pattern that results maximises dispersion. The axial mixing patterns achieved by paddles and axial flow impellers maximise suspension and homogeneity.

In bioreactor design, particular attention is given to the mixing systems, as homogeneity is sought for the multi-phase system (gas, liquid and the biotic solid phases). It is only by rigorous mixing that pseudohomogeneity can be obtained, characterised by small concentration gradients in the gas and liquid phases (Nielsen & Villadsen 1994). In this case, all the cells experience approximately the same environmental conditions independently of their position in the bioreactor. The ideal reactor state where, such mixing attains perfection is referred to as *maximal mixedness* (Nielsen & Villadsen 1994). Mixing is usually divided into two parts, macro- and micro-mixing. The first is the distribution of volume elements among one another on a scale that is very small compared with the total reactor volume, but still

large enough to contain thousands of cells and billions of substrate molecules, while the second is the exchange of material between these volume elements on the molecular level or the cell level (Nielsen & Villadsen 1994). Macromixing is consequently mainly due to convection and turbulence, while micromixing occurs by molecular diffusion. However, turbulence indirectly affects micromixing due to its influence on the contact areas between the volume elements.

Bioreactors are generally equipped with proper sparger system, agitators of different designs, baffles designed to fit a specific agitator performance, and other mechanical and monitoring devices.

2.9 Conclusion

Chapter 2 has provided the literature survey on colloidal systems and biosurfactants. In the review on colloidal systems, the main concepts covered were: phase dispersion and emulsion/microemulsion systems, liquid-gas and liquid-liquid interfaces, surface kinetics (surface tension, interfacial tension, etc.) chemical surfactants. In the review on biosurfactants, Chapter 2 has defined biosurfactants, and provided information on biosurfactant interaction with growth media, their route of production, their classification, applications, etc. A particular group of glycolipid biosurfactants, known as rhamnolipids, were given a particular attention, since they were hypothesised to be produced by *Ps. aeruginosa 2Bf*, the microbial of interest in this work. Biosurfactants are an important group of surfactants and colloidal systems, allowing phase dispersion, being environmentally friendly, consistent with bioprocesses, able to be produced from waste feedstocks, etc. *Pseudomonas* was identified as a key microbe associated with biosurfactant production, particularly rhamnolipid biosurfactants. Rhamnolipids, which are a sub-group of the glycolipid biosurfactants, were postulated to be among the most effective biosurfactants known todate, and are often produced as a mixture of similar extracellular compounds.

The literature review revealed that although the reason for biosurfactant production by microorganisms is still unclear, they are hypothesised to play an important role in the solubilisation of hydrocarbon substrates, when they are released in the growth media.

CHAPTER III: SCOPE, HYPOTHESES, OBJECTIVES AND RESEARCH DESIGN

3.1 Scope

Extracellular products other than glycolipids, e.g. glycoprotein emulsifiers can be produced (or co-produced) when *Ps. aeruginosa* is grown on alkanes (Goswami & Singh 1991). The type, quality and quantity of biosurfactant produced are influenced by the nature of the carbon substrate, the concentration of N, P, Mg, Fe and Mn ions in the medium (Karanth & Veenanadig 2004) and the culture conditions, such as pH, temperature, agitation and dilution rate in continuous culture. In this work, the nitrogen source is considered as key to biosurfactant production, and its choice as the first step forward.

The fundamental questions to be addressed in this research are related to: firstly, selecting the best nitrogen source for Ps. aeruginosa 2Bf growth and biosurfactant production; secondly, establishing whether linear liquid hydrocarbons have potential to induce the production of biosurfactant by Pseudomonas aeruginosa 2Bf; thirdly establishing the phase in which biosurfactant is produced; fourthly, identifying appropriate reactor conditions for bacterial growth on alkanes and biosurfactant production, fifthly comparing mechanical and chemical foam control; and sixthly, identifying the type of biosurfactant produced

3.2 Hypotheses

In order to set the goals of this project, the following hypotheses were postulated:

- it is possible to induce biosurfactant production by *Ps. aeruginosa 2Bf* with liquid n-alkanes as sole carbon source;

- the choice of nitrogen source is a determinant step for biosurfactant production by bacteria;

- the mechanical foam control is a better alternative to chemical foam control;

- agitation and aeration rates are among the most important factors affecting the growth of *Ps. aeruginosa 2Bf*

- the biosurfactants synthesised by *Ps. aeruginosa 2Bf* are rhamnolipids and possess an antibiotic activity.

3.3 Objectives

3.3.1 General objectives

The following objectives were established to test the hypotheses:

- Use a mixture of liquid alkanes to induce biosurfactant production by *Ps. aeruginosa 2Bf*, successfully test the emulsification effect and carry out the product's structural identification;

- Optimise the biosurfactant production conditions in a batch reactor system;

- Qualitatively investigate the performance of mechanical foam control versus chemical control.

3.3.2 Specific objectives:

- Propose the best way of monitoring growth of *Ps. aeruginosa* bacterial cells

- Establish the growth patterns of *Ps. aeruginosa 2Bf* growth on a mixture of liquid alkanes

- Monitor variation of essential parameters for bacterial growth and biosurfactant characterisation, namely: pH, viscosity, alkane depletion, emulsification effect, surface tension and dissolved oxygen variation

- Study the process in both shake flasks and bioreactor systems

- Monitor the main mass transfer parameters at least at three different times of the growth process in a three phases batch reactor system

- Propose the most probable type of biosurfactant produced by *Ps. aeruginosa 2Bf*

- Study the alkane utilisation during the process up to individual chain lengths level

- Use at least three different methods for the structural identification of the product.

In order to achieve these objectives, the research was organised in three sets of experiments:

- the exploratory shake flask study, in which growth meda and monitoring method were investigated, as well as the potential of alkanes to induce biosurfactant production;

- the quantitative bioreactor-based study of biomass growth and biosurfactant production, the impact of agitation rate, aeration rate and mass transfer on the system, as well as the impact of alkane utilisation and concentration.

- and the product characterisation study, aimed at identifying the type of biosurfactant produced by the bacterial cells, by usng linear hydrocarbons as inducers.

CHAPTER IV: REVIEW OF MATERIALS AND EXPERIMENTAL METHODS

4.1 Introduction

In this study, bacterial cells were used to produce glycolipid surfactants using alkane substrates. This chapter describes the major equipment and materials used in the research project. The protocols for bacterial growth and product formation are presented. It introduces the experimental approach used to select the medium used with respect to nitrogen source and carbon source as well as to induce biosurfactant production. Bioreactor conditions used are specified and parameters for optimisation noted. Methods of foam control are presented.

In this chapter, all analytical methods required are reviewed in order to select appropriate methods for a particular analysis. These methods selected from literature and modified where necessary are presented in detail, and those selected for routine analyses are indicated. Methods to characterise the biosurfactant are included. Detection limits, sensitivity and reproducibility are included as appropriate. In Section 4.5.10, the experimental design and statistical analysis is introduced.

4.2 Materials

4.2.1 Microorganism

The microorganism used in the present research is a Gram negative bacterium isolated from crude oil polluted soil samples in a previous study carried out by the Department of Molecular and Cell Biology of the University of Cape Town (Ghilamicael 2003; Mathopa 2004). The species was identified as belonging to the genus *Pseudomonas* by the means of standard biochemical tests. With 16S rDNA PCR identification, it showed 99% identity with the previously identified strain *Pseudomonas aerugionosa* PAO1 AE004844. It was confirmed as *Pseudomonas aeruginosa* and the strain named *Pseudomonas aerugionosa 2Bf* (Ghilamicael 2003). It is noted that *Ps. aeruginosa* may be both a opportunistic human pathogen presenting a risk to immuno-compromised people and a plant pathogen (Iglewski, 1996), however typically an environmental isolate such as this study is centred on is most likely not a pathogen. Figure 4.1 shows *Pseudomonas aerugionosa 2Bf* cells after gram staining.

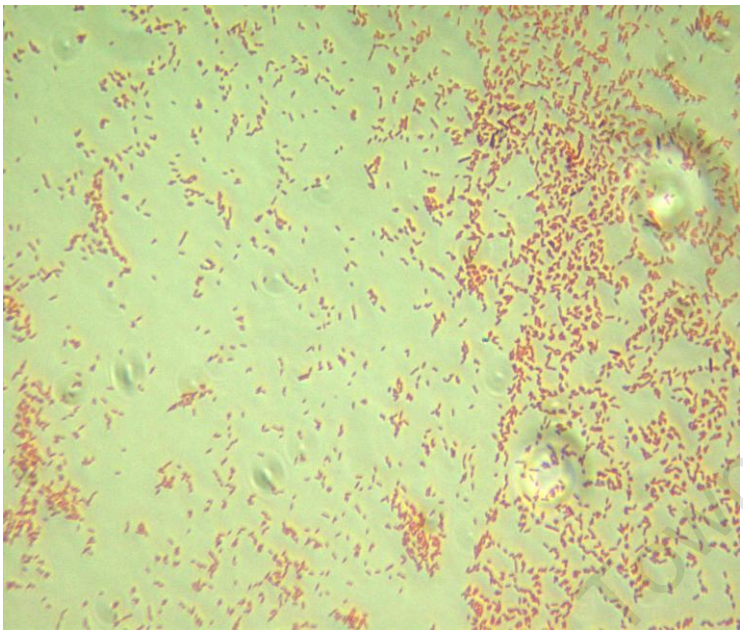


Figure 4.1: *Pseudomonas aeruginosa 2Bf* cells captured with the image Olympus Microscope, after the gram stain test

4.2.2 Carbon source for bacterial growth

Liquid medium containing alkanes as sole carbon source was used. The preliminary shake flask experiments were carried out with 99.8% GC-grade n-dodecane and 99.8% GC-grade n-hexadecane from Fluka, and hydrocarbon blends C_{14}-C_{17} from Sasol (BATCH-1 and BATCH-2). Only the C_{14}-C_{17} BATCH-2 blend was used for bioreactor studies. When the two hydrocarbon blends were analysed using gas chromatography, they presented different compositions, with BATCH-2 presenting higher purity. The alkane blend was filter-sterilised to avoid losses by evaporation at the elevated pressure and temperature used on heat sterilisation. The filter-sterilised alkane fraction and heat-sterilised culture medium were mixed at room temperature.

4.2.3 Nitrogen source for bacterial growth and product formation

Four combinations of three nitrogen sources were selected from literature for use in preliminary shake flask experiments. These nitrogen sources are detailed in Table 4.1.

Table 4.1: Mass composition of the four selected nitrogen sources

	NH_4NO_3 ($g.l^{-1}$)	$NaNO_3$ ($g.l^{-1}$)	$(NH_4)_2SO_4$ ($g.l^{-1}$)	Total Nitrogen (mass)	C/N Ratio (mass)
M1	2.0	3.3	0.0	1.24	19
M2	2.0	2.7	0.5	1.25	19
M3	0.0	0.0	5.9	1.25	19
M4	1.1	4.1	0.9	1.25	19

Throughout the experiments, the carbon to nitrogen ratio was maintained approximately constant at 19 for ease of comparison of these media.

From the results presented in Section 5.2, nitrogen source 3 was selected for the bioreactor study. The preference was based on the observed specific growth rate, maximum cell number and emulsification index. The stock culture and the inoculum media were prepared with nitrogen source 2.

4.2.4 Growth medium

The basal salt growth medium (BSM) used in this research contained (per litre): 3.000 g KH_2PO_4, 4.000 g K_2HPO_4, 0.200 g $MgSO_4.7H_2O$, 0.015 g $FeSO_4.7H_2O$, 0.010 g $CaCl_2$, 0.100 g NaCl, 0.030 g $FeCl_3.6H_2O$, 0.010 g $MnCl_2.4H_2O$. BSM was supplemented with 3 ml per litre of trace element solution, which contained per litre: 5.25 mg $ZnSO_4.7H_2O$, 200.00 mg $MnSO_4.4H_2O$, 70.50 mg $CuSO_4.5H_2O$, 15.00 mg $Na_2MoO_4.2H_2O$, 200.00 mg $CoCl_2.6H_2O$ and 15.00 mg H_3BO_3 (Chayabutra & Ju 2001; Christova *et al.* 2004; Desai & Banat 1997; Déziel *et al.* 1996; Ghilamicael 2003; Goswami & Singh 1991; Koch *et al.* 1991; Kosaric 2001; Nelly *et al.* 2002). To avoid the need for initial pH control, KH_2PO_4 and K_2HPO_4 were added at a ratio of 0.7937 to provide a buffering effect at pH 6.8. Unless otherwise specified, 3% w/v alkane was added. This was observed to reduce the pH slightly.

4.2.5 Bioreactor configuration

Biomass growth and product formation was conducted in the New Brunswick Bioflo 110 bioreactor with a volume of 7 litres and working volume of 5 litres. Two six bladed Rushton turbines were used to provide mixing and mass transfer in the culture. A three-slit, two-blade paddle mechanical foam-breaker was used for foam control. The geometry of the reactor set up is provided in Figure 4.2.

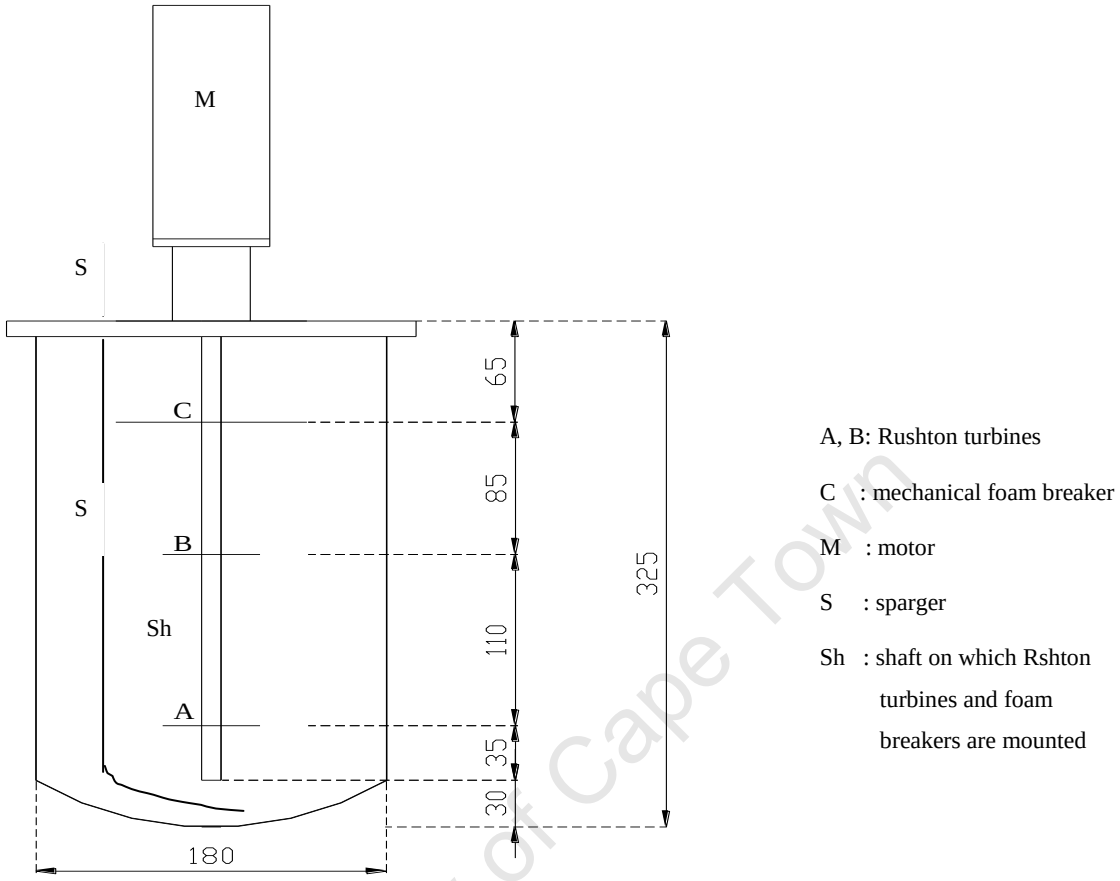


Figure 4.2: Geometry of the reactor used

As chemical antifoam agents interfere with surface active agents the biosurfactant, a mechanical foam breaker was installed. Two types of mechanical foam breakers, a conventional two-blade paddle and a two-blade paddle with 3 slits formerly proposed by Deshpande & Barigou (1999, 2000), were tested. In order to increase the mechanical resistance of the foam breaker, especially in the late exponential phase when the high biomass concentration increases the broth viscosity, a slight modification at the base of the blade was applied to the slit-two-blade paddle, also called comb paddle (Barigou 2001), prior to experimental testing. The resulting foam breaker is referred to as the *modified two-blade paddle with three slits* to distinguish between the type of paddle used in this work from that of Deshpande and Barigou (2000). The geometry of the foam breakers is shown in Figure 4.3. These foam breakers are mounted at the same impeller shaft as the Rushton turbines, rotating at the same speed, as shown in Figure 4.3.

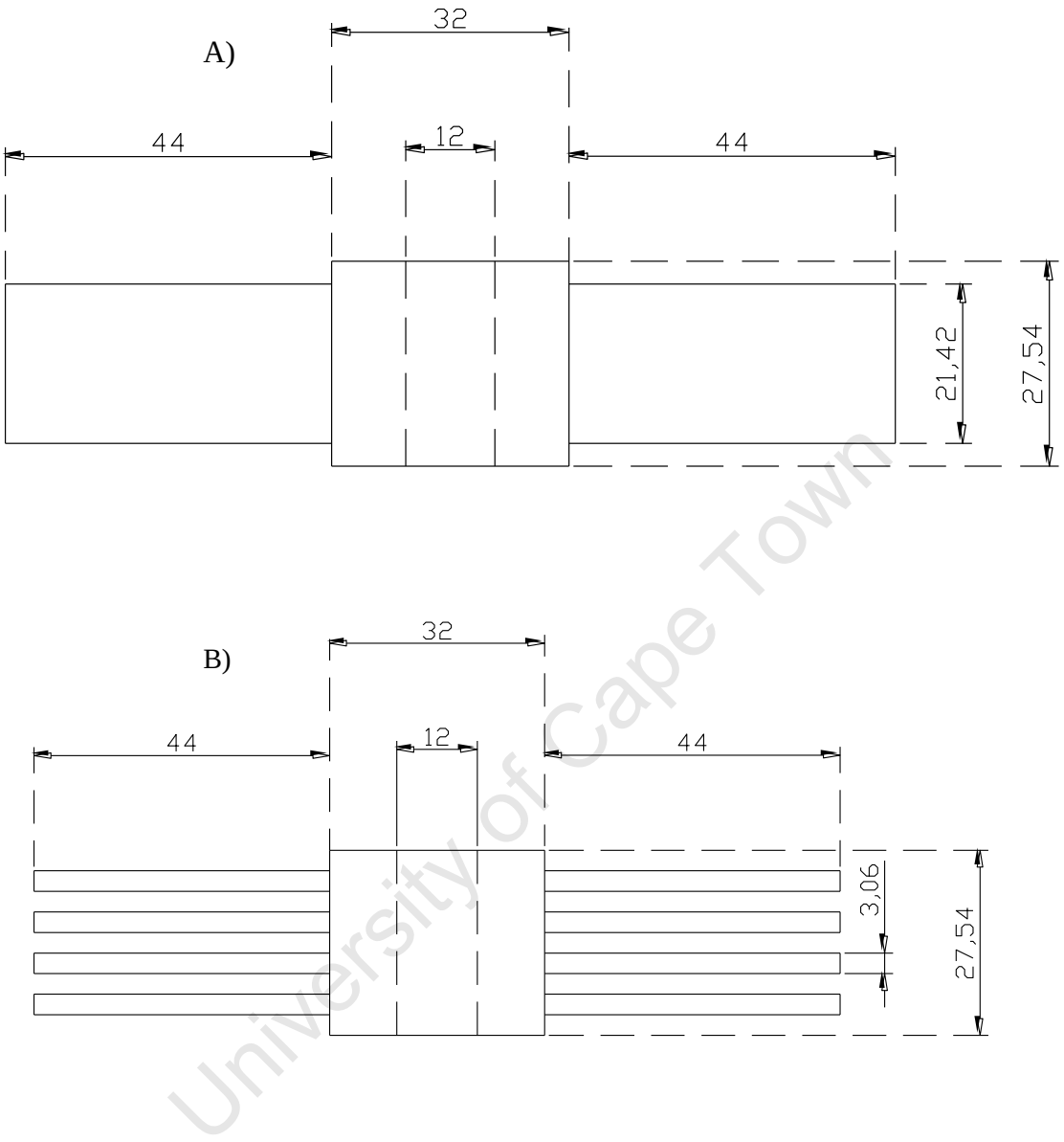


Figure 4.3: Foam breakers used: A) Conventional two-blade paddle; B) Modified Two-blade paddle with three slits

Dissolved oxygen in the bioreactor was monitored in terms of saturation percentage, by the means of a submerged polarographic oxygen electrode, Mettler Toledo/Ingold® Inpro® 6000 series with a 12mm T-96 membrane, coupled to a New Brunswick Bioflo 110 data analyser. The choice of a suitable sensor to be used in a series of experiments largely depends on the liquid environment and on the cost. Hydrocarbons quickly and irreversibly damage oxygen sensors with silicon membranes; hence a more resistant Teflon membrane was used in this study. Prior

to calibration and use, the probe was polarised for a minimum of six hours, by connecting it to the power source according to the manufacturer's instructions.

Temperature was controlled at the setpoint by the means of a heating jacket, a cooling coil, and a submerged temperature probe connected to the control unit. The temperature was maintained at 30 ºC.

A Mettler Toledo 405-D PAS-SC-K8S/425 pH electrode was submerged into the bioreactor to monitor the pH variation in the culture.

Air entering the bioreactor was passed through a pre-sterilised air filter. Further effluent gas was filtered. Sampling was done asceptically. The sampling port was located in the lower third of the liquid volume and the reactor was evenly mixed during sampling.

4.3 Experimental procedures for microbial production

4.3.1 Inoculum preparation

A two-stage inoculum was prepared as follows: a 200 ml pre-inoculum was prepared in a 2000 ml Erlenmeyer flask, inoculated with *Ps. aeruginosa* cells maintained on agar slants, and incubated for 24 hours at 30ºC on a shaking incubator at 315 rpm. This pre-culture was then used to prepare the inoculum of 200 ml pre-inoculum was prepared in a 2000 ml Erlenmeyer flask, using a 5% pre-inoculum culture volume. After 24 hours of incubation in the same conditions, the main culture was inoculated with 0.5% inoculum volume.

4.3.2 Shake flask experiments

Shake flask experiments were essentially qualitative and consisted of a series of tests intended to optimise the growth media, particularly with respect to the nitrogen source. Shake flask experiments were also used to compare the performance of the C_{14}-C_{17} blend with two other alkane carbon sources, n-hexadecane and n-dodecane.

Two litre Erlenmeyer flasks were used containing 200 ml growth medium. The medium, described in Section 4.2.4, was supplemented with 3% carbon source, inoculated with a 5% culture volume from the inoculum, and incubated for 47 hours at 315 rpm. Initial pH value was fixed at pH 6.80 $\pm$ 0.04. In the course of the process, 1

ml samples were withdrawn at specific time intervals for analysis. Sampling was done asceptically by the means of a 1 ml pipette, using pre-sterilised tips.

During the biosurfactant induction study, shake flask experiments were performed in the same way, but no sampling was done in the course of the process. The culture broth was harvested at the end of the process, centrifuged at 14'500 gravity. The supernatant was used to measure the surface tension.

4.3.3 Reactor based experiments

The reactor based experiments were essentially quantitative and were aimed at optimising the reactor conditions for *Pseudomonas aeruginosa 2Bf* growth and biosurfactant production. All the experiments were done in a batch reactor system using the vessel and instrumentation presented in Section 4.2.5.

A two-stage inoculum was employed for bioreactor studies, as in the case of shake flasks. This was described in Section 4.3.1, and used with the same volume ratio of inoculum:culture.

Standard reactor conditions were established. These conditions are given in Table 4.2 and were used as the base case in the study of pH, agitation and aeration effects.

Table 4.2: Standard reactor conditions

Agitation	Aeration	Alkane	Inoculum	Temperature	Foam control
500 rpm	0.4 vvm	0.3%	0.5% v/v	30 ºC	FB-2

On establishing the base case, operation in the presence of pH control at pH 6.6 and its absence was considered. To optimise reactor operation, the reactor was maintained at 30 ºC, and the reactor behaviour was investigated at aeration rates of 0.3 vvm, 0.4 vvm, 0.6 vvm and 0.8 vvm and an agitation speed of 480 rpm, 500 rpm, 600 rpm and 800 rpm. The high agitation speed contributed partly to the foam control. The medium initial pH was 6.80 $\pm$ 0.02 and decreased to 6.71$\pm$ 0.02 upon the alkane addition. No initial pH control by either acid or base was applied to the system. During the process, approximately 15 ml samples were aseptically drawn at specific time intervals through the pre-sterilised sampling port.

Where pH was controlled, aqueous solutions of NaOH 5N and HCl 5N were used to control the reactor pH. Their addition was regulated from the bioreactor control unit.

4.4 Analytical methods to monitor microbial system

Typically, multiple methods exist for the quantitative or qualitative analysis of a given parameter. A pre-selection of suitable methods is necessary before starting the experimental analysis based on the envisaged type of analysis, safety requirements, required accuracy, as well as accessibility of laboratory facilities. This was done by reviewing methods and, where needed, testing two or more methods. The selected method were used either as presented in the literature or modified first in order to suit the context of the work. This review and selection of methods, provided below, includes a detailed specification of methods used in this study.

4.4.1 Microbial growth

The following methods were reviewed to monitor cell growth: cell dry weight with hexane or cyclohexane washing to remove hydrocarbons, turbidity measurement and direct cell counts. These methods are detailed below. Following evaluation presented in Section 5.2, direct cell counting was selected as the preferred method for routine analysis. The main reasons for the rejection of the other methods were lack of reproducibility of results.

4.4.1.1. Cell dry weight with hexane washing

A 10ml sample was transferred to a centrifuge tube, the pH adjusted to 9, the sample extracted with 10ml hexane, and centrifuged at 14 500g for 15min. Cells were collected and washed with hexane to remove hydrophobic materials such as alkane substrates adhered to the cell surface, as these had been observed to cause errors in cell dry-weight measurement. The cells were resuspended in deionised water, filtered, dried at 110°C for 3h, and measured for the cell dry weight (Chayabutra & Ju 2001).

4.4.1.2 Cell dry weight with cyclohexane washing

A 4 ml aliquot of agitated sample was added into each of 3 test tubes. Aliquots of 2 ml cyclohexane and 400 µl of 5 M NaOH were added to each tube. The contents of the tube were mixed and filtered using pre-weighed filter papers. The supernatant was discarded and the cells remaining on the filter paper were washed three times. The first wash consisted of 4 ml distilled water, 2 ml of cyclohexane and 400 µl of 5 M NaOH. The second wash consisted of 6 ml distilled water and the third wash consisted of 20 ml distilled water. The filter papers were then placed in an oven at 80°C for 48 hours, and measured for cell dry weight (Williams 2005).

4.4.1.3 Turbidity measurement

A culture sample of 500 µl was added to 200 µl of cyclohexane and 100 µl of 5M NaOH in an Eppendorf microfuge tube. This was vortexed for 5 min, then centrifuged for 10 min at 1200 rpm. The supernatant was discarded and the pellet resuspended in 500µl of physiological buffered saline (0.9% NaCl) solution.

The absorbance of a well-agitated sample was determined at 620 nm using PBS as blank. In order to obtain good results, a prior determination of a dilution factor was necessary to obtain an absorbance below 0.8, as absorbance readings were linear with respect to cell concentration in the range of 0 to 0.8.

4.4.1.4 Direct cell count

Microbial growth was quantified by direct cell counting using the Olympus Optical Microscope No 4401709 Model Bx40F and the Helber Bacteria Single Round Cell Counting Chamber with Thoma Ruling (No Z30000). Prior to microscopic observation and counting, samples were suitably diluted to ensure the number of cells counted in the field laid in the region 20 to 45 cells.

4.4.2 Alkane utilisation

To study the rate of the substrate depletion, GC analysis was used. Sample preparation involved the extraction of the alkane substrate into the organic MTBE. This extraction approach is similar to the one used by Green *et al.* (2000). The method has been extensively investigated and was used by Williams (2005)

previously in the UCT laboratory when studying the bioconversion of alkanes into dicarboxylic acids.

A 500 µl aliquot of the sample was placed into an Eppendorf tube. As internal standard, n-dodecane, was dissolved in tertiary butyl methyl ether (MTBE) at a concentration of 30 $g.l^{-1}$. A 300 µl aliquot of this ether solution was added to the sample in order to extract the alkanes.

Following vortexing for 5 min and centrifuging for 10 min at 12'000 rpm, the top organic layer was separated. A 1 µl aliquot was injected into the GC. The alkane analysis by GC was carried out using a TRB-1 column and Helium grade 6.0 as the mobile phase. The detailed method is provided in Appendix II.

4.4.3 Approximation of K_La and OTR

There are many methods used in the determination of the oxygen transfer rate and volumetric mass transfer coefficient. The dynamic methods were used in this work. Two different methods can be distinguished among these, the gassing out, also known as the unsteady state method, and the steady state method (Shuler and Kargi 2002).

As the bioreactor process was carried out in the presence of actively respiring organisms, dynamic methods were used. Two different methods can be distinguished among these, the unsteady state gassing out methods based on solution dissolved oxygen concentrations, and the steady state methods based on material balances across the incoming and outgoing gas streams (Shuler & Kargi 2002). As accurately calibrated gas analysis equipment was not available, the gassing out method, requiring only a dissolved oxygen probe to monitor the change of oxygen concentration in the medium, was used. Although this method was suspected to have drawbacks associated with the use of a polarographic oxygen probe in a three-phase system (Hassan & Robinson 1977), it has been reported that the probe measures overall oxygen transfer from the gas phase to the liquid (Williams 2005). This overall transfer has two components: oxygen transferred from air directly to the aqueous phase and the component of oxygen transferred via alkane droplets as vectors. Therefore, the method is appropriate for determination of K_La and OTR in bioprocesses (Williams 2005).

In the gassing out method, the air flow rate was stopped temporarily allowing the oxygen concentration to fall in accordance with the oxygen utilisation rate. It was ensured, in this work, that oxygen was not lowered below 5 mg/l to avoid situations of growth inhibition. On re-instating the air flow, the value of K_La was derived from the slope of the graph where dC/dt is plotted against C. The result gives K_La x C*-OUR as the y-intercept, whereas K_La is directly given by the slope. The oxygen profile are provided in Figures 4.4 and 4.5.

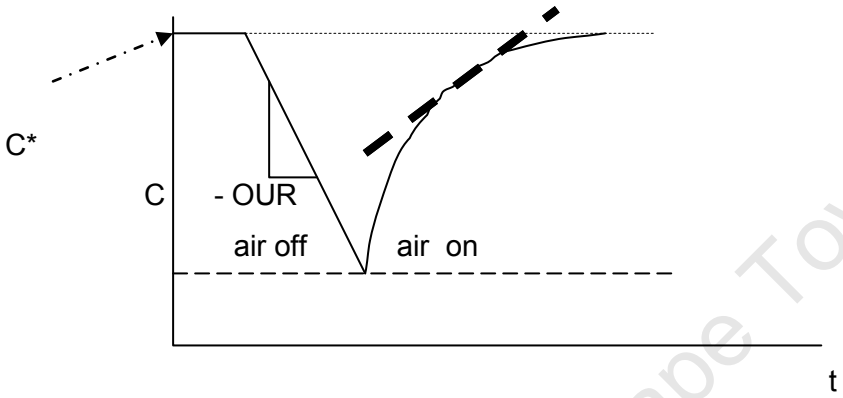


Figure 4.4: gassing out method

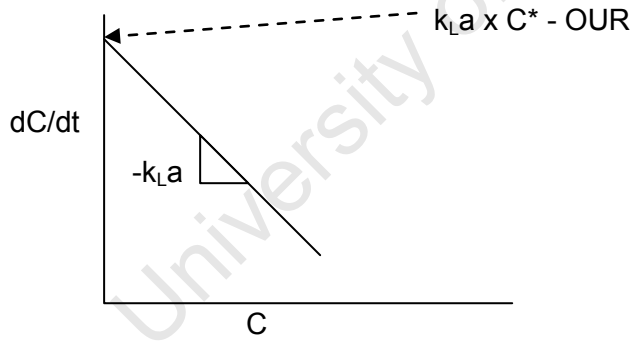


Figure 4.5: Example of the graphical determination of k_La

4.4.4 Measuring the broth viscosity

Dynamic or absolute viscosity of a fluid is the tangential force per unit area required to move one horizontal plane with respect to the other at unit velocity when maintained a unit distance apart by the fluid (McBain 1950; Danielli, Pankhurst & Riddiford 1964).

In this project, viscosity of the bacterial culture was analysed by the HAAKE rheometer Rheolab MC-1. Measurements are done by placing the sample in the gap

of the sensor system of the rheometer, and allowing rotation of the rotor at a preset speed. The sample exerts a resistance to this rotational movement, due to its viscosity, which becomes as a braking torque applied to the measuring shaft of the rheometer. The built-in computer calculates the relevant measuring values for viscosity (mPas), shear rate (s^{-1}) and shear stress (Pa). The temperature can also be calculated in ºC if a temperature sensor is attached. For viscosity measurements, a 20 ml sample was required.

4.5 Biosurfactant analysis

Biosurfactant analysis can be devided into two categories: methods directly aimed at characterising the product, and methods aimed at providing a quantitative estimate of biosurfactant production, monitored indirectly in terms of its surface tension lowering potential or its emulsification power.

Biosurfactant was also analysed for chemical structure determination and for antibiotic properties using the *B. subtilis* inhibition method described in section 4.5.9.

4.5.1 Spectrophotometric methods for rhamnose determination using Orcinol assays

Orcinol assay is based on reacting sugars with orcinol, through heating a mixed solution of a carbohydrate aqueous solution and the orcinol reagent, so that an absorbing complex is formed. This complex is detected using radiations corresponding to its highest absorption frequency. As sugar materials generally absorb in the visible region (some times in the ultraviolet), a UV-VIS instrument is satisfactory for analyses. The detailed protocol for the method is provided in Appendix IV. Orcinol method was reported to have been successfully used in the analysis of rhamnose in the visible region of radiation frequencies, precisely at the wave length of 421 nm (Koch *et al.* 1991; Tahzibi *et al.* 2004; Ghilamicael 2003). The orcinol reagent is prepared by dissolving orcinol to a total of 0.19% in a 53% sulphuric acid solution (Christova *et al.* 2004; Kosaric 1993). However, in this study, no absorption was observed at 421 nm with the UV-VIS spectrometer, and for this reason, the method was not used in the study.

4.5.2 High Performance Liquid Chromatography for analysis of rhamnolipids

HPLC is a chromatographic technique of separation proper for liquid, particularly non-volatile liquids. The sample is eluted with a mobile phase running through a chromatographic column, and samples are separated following the speed at which they are eluted, which depends much on their affinity with both the mobile phase. The detector to be used may depend on the nature of the compound being analysed and its absorption frequency. This method allows both qualitative and quantitative determination of the analyte, since accurately measured standards are used. In this work, a reverse-phase column (Supercosil LC-18) was used in the HPLC analysis of rhamnolipids. The mobile phase was the mixture 55:45 acetonitrile:THF, at a flow rate of 0.75 ml/min. A UV detector was used to detect the peaks at 225nm, determined to be optimal from the absorbance spectrum of rhamnolipid solutions in the mobile phase, as reported by (Chayabutra *et al.* 2001). Chayabutra *et al.* (2001), however, reported the HPLC method to be negatively affected by the interfering peaks from other compounds in some systems, thus being not very sensitive (Chayabutra, Wu & Ju 2001). In this study, an HPLC full scan of the sample revealed maximum absorption at around 210 nm. These results and the details on the sample preparation are provided in Appendix V. For this reason, this method was not selected to be used in the experimental analyses of biosurfactant in this project.

4.5.3 Anthrone method for determination of the rhamnose unit of rhamnolipids

The anthrone method is based on the reaction of anthrone reagent and sugar compounds, resulting in the formation of a complex absorbing a certain specific light frequency. This frequency is detected with UV-VIS spectrophotometric technique. The method was extensively described by Ashwell (1957). Anthrone reagent is prepared with concentrated strong acids, HCl, $HClO_4$, etc. for pentoses, and H_2SO_4 for hexoses. Details on the preparation of the anthrone reagent used for hexoses determination and the general method procedure are provided in Appendix IV.

In the case of rhamnolipid analysis, two methods, different from the sample preparation point of view, were successfully used by Wu & Ju (1998) and Chayabutra & Ju (2001). These methods, labelled (a) and (b), were used during the TLC study of the sugar moiety of the biosurfactant produced by *Ps. aeruginosa 2Bf* (Section 7.3.1). (a) A 10 ml sample was adjusted to pH 2.0 with 1N HCl and extracted with 20ml of

ethyl acetate at room temperature. The ethyl acetate extract (10 ml) is then dried at 40°C (Wu & Ju 1998). The residue was redissolved in a 0.05 M sodium bicarbonate solution and quantified for rhamnolipids as the rhamnose concentration, by the anthrone method; (b) A 10ml sample of the culture broth is transferred to a centrifuge tube, the pH adjusted to 9, the sample extracted with 10ml hexane, and centrifuged at 14'500g for 15min. Cells were collected and washed with distilled water. The aqueous supernatant was adjusted to pH 2, extracted with 10ml of ethyl acetate and centrifuged at 14'500g for 15 min to separate the two phases (Chayabutra & Ju 2001). The collected organic phase was vacuum-dried at 40°C, redissolved in 0.05M $NaHCO_3$, and analysed for the concentration of the sugar moiety of rhamnolipids (in g rhamnose/l) by the standard anthrone method.

4.5.4 Thin layer chromatography

4.5.4.1 Method using orcinol and anthrone solutions for revelation of rhamnolipids

Anthrone and orcinol reagents can be used as spray reagents for the TLC revelation of glycolipids in the form of their sugar moiety (Tuleva et al. 2002; Wu & Ju 1998).

In this method, the culture supernatant treated following methods 4.5.4a or 4.5.4b is applied to a silica gel thin layer plate and typical glycolipid spots are revealed as rhamnose after the orcinol-sulfuric staining (Tuleva *et al.* 2002) or by spraying the plates with anhtrone solution (Wu & Ju 1998; Chayabutra *et al.* 2001). These methods were not used in this study. The method given in Section 4.5.5.2 was preferred due to its specificity to glycolipids.

4.5.4.2 Thin layer chromatography for glycolipid determination

Analytical TLC made with silica gel plates was used. Two different systems were used in the study (Wu & Ju 1998; Chayabutra *et al.* 2001): Solvent (1) = mixture of $CHCl_3$, CH_3OH and Acetic acid at volume ratio 65:15:2, and solvent (2) = mixture of 2-propanol, NH_4O and H_2O at 6:2:1. The two solvents were used for the development of glycolipids and sugars, respectively. After ascending development of the plates, different spray reagents were used to reveal the functional groups. Glycolipids were identified with diphenylamine and sugars were detected with 4-methoxy-benzaldehyde.

4.5.5 Emulsification index measurement

Biosurfactant was also quantified by measuring its emulsifying capacity. This was done by mixing, in equal proportions, an aqueous phase consisting of the culture supernatant and kerosene in a 20 ml bottle. After shaking the mixture vigorously, the mixture was allowed to rest for at least one hour, before measuring the height of the emulsified portion. At the end, the emulsification index was established as the percentage of the emulsion layer relative to the total height of the mixture.

4.5.6 Surface tension measurement

4.5.6.1 The Drop-weight Method for surface tension measurement

In this method, the weight or volume of each liquid drop detaching from the tip of a vertical tube is determined by the surface tension of the liquid. Assuming that the drops are formed extremely slowly, they detach themselves completely from the tips when the gravitational pull just reaches the restraining force of surface tension.

$$M.g = V.\ \rho_l\ .\ g = 2\ .\ \pi.\ r.(ST) \qquad \text{(eq. 4.1)}$$

or

$$ST = M.g\ /\ 2\ .\ \pi.\ r = V.\ \rho_l\ .\ g\ /\ 2\ .\ \pi.\ r \qquad \text{(eq. 4.2)}$$

where g is the gravitational acceleration, M and V the mass and volume of each drop that falls from the tip, ρ_l the density of the liquid, and "r" the radius of the tip of the tube. These relations require correction, since the liquid forming the drop does not completely leave the tip (unless the tip is wiped with a tissue or let to dry) and since surface tension seldom acts exactly vertically. The correction factors are important in this method as drops are often as much as 40% smaller than predicted by the above equations. The correction factor φ was established by Harkins and Brown (Davies & Radieal 1961) as a function of "a" and the volume of the drop:

$$ST = \varphi V \rho_l g\ /\ 2\pi\ r \qquad \text{(eq. 4.3)}$$

4.5.6.2 The Wilhelmy Plate Method for surface tension measurement

The principle of the Whilhelmy method can be explained in the following way: suppose that a very thin plate is attached to an arm of a balance, or is suspended from a light beam attached to a torsion wire, the additional pull on the plate when it becomes partly immersed is equal to the product of the perimeter and the surface tension. To obtain accurate results, it is preferable not to detach the plate from the surface, but to keep the plate partly immersed and to correct the buoyancy (Harkins & Jordan 1930). The latter effect is very small if a thin mica plate can be used: convenient dimensions are 10 cm wide, 5 cm high and 0.02 cm thick. The method is valid only when the contact angle is zero, the plate being completely wetted by the liquid. To promote wetting, the mica plate may be roughened by rubbing with a very fine emery paper, moving this in small circles over the mica surface to make the roughening as uniform as possible in all directions. In general the meniscus takes a certain typical form showing liquid pull up. It is the weight of the liquid pulled up above the mean level of the liquid surface that is measured. This weight is [(S.T.). $\cos\theta$] per unit perimeter of the plate, or if θ=0, it is equal to S.T. This method was described by Harkins & Jordan (1930) and Findlay & Kitchner (1965), and was reviewed as a good method for surface tension studies. The method described in Section 4.5.6.3 is the most commonly used in the case of biosurfactant analysis, and was, therefore preferred over this one.

4.5.6.3 The ring detachment method

In principle, the surface tension determines the force required to detach a metal ring from the surface of a liquid. This can be used to measure the surface tension, with the wire ring connected either to one arm of a balance or to a light beam carried on a horizontal torsion wire whose constants are known (Findlay & Kitchner 1965). The simplest theoretical interpretation of the results equates the pull required to detach the ring from the surface to the total perimeter of the ring multiplied by the surface tension. The total perimeter is twice the linear perimeter, since the meniscus pulls on each side of the wire, as illustrated in Figure 4.7a. In this method description, R designates the ring radius and r the radius of the wire of which the ring is made.

Equating the vertical pull P on the ring, at the moment of the ring breaking away from the surface, to the radius R of the ring, the following is obtained:

$$P = 4\pi R(ST) \qquad \text{(eq. 4.4)}$$

or

$$ST = P / (4\pi R) \qquad \text{(eq. 4.5)}$$

Typically, a platinum ring readily cleaned by gentle flaming is used in the *du Noüy tensiometer*. Equations 4.4 and 4.5 assume that the surface tension acts vertically and that the contact angle is zero i.e. that the liquid wets completely the ring. Since the surface tension does not act exactly vertically in general due to the curvature phenomena, shown in Figure 2.1 (Section 2.3.1), a correlation factor *f* is necessary, and equation (5.5) becomes:

$$ST = (f.P)/(4\pi R) \qquad \text{(eq. 4.6)}$$

The *f* factors, which depends on the dimensions of the radius of the ring R, the radius of the wire used for the ring r, the density and surface tension of the liquid (Findlay & Kitchner), have been calculated and tabulated for different *R/r* and R^3/V, V being the volume of liquid raised (Harkins & Jordan 1930), as shown in Appendix I. In cases where two liquids are in contact, the ring method can also be applied to determining interfacial tension provided that the lower liquid preferentially wets the platinum ring. Water, with overlaying benzene or other oil, satisfies this condition, but if the ring is wetted by the upper liquid (e.g. water overlaying tetra-chloromethane), the method requires modification, possibly a stainless-steel ring coated with silicone to make it preferentially oil-wetted would be satisfactory.

This method, also known as *Du Nouy method* (Fiigure 4.6) was used to measure surface tension in the course of the present work. According to Findlay & Kitchener (1965), even for substances like biological fluids, colloidal solutions, etc., the surface tension can be quickly determined with sufficient accuracy by measuring the force required to detach a horizontal ring of platinum wire of radius R from the surface of the liquid.

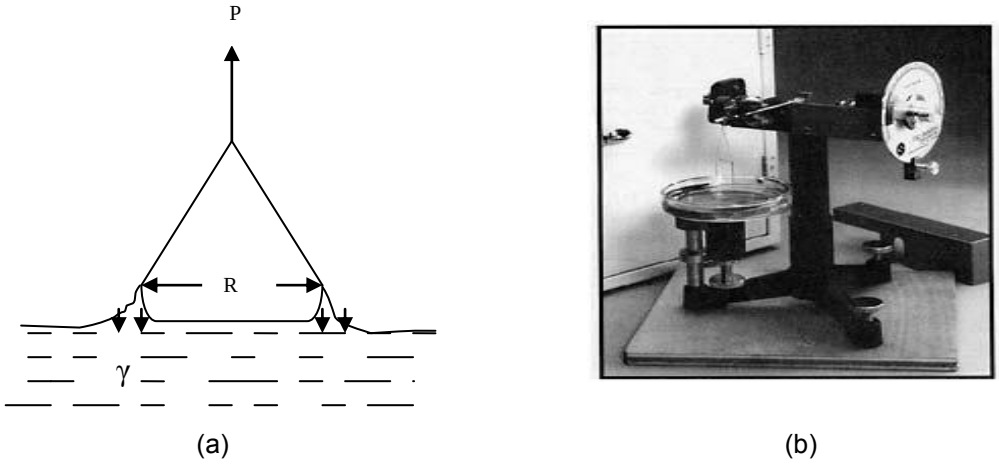


Figure 4.6: Surface tension measuring apparatus and ring contact with liquid: (a) Platinum ring interaction with a liquid surface; (b) Du Nouy tensiometer

In more precise terms, the pull must be multiplied by the correction factor, *f* (Young & Coons 1945). In order to obtain good results, a prior calibration of the tensiometer is always necessary. This was done by determining the torsion of the wire with different known weights. A calibration curve was obtained by plotting the mass of wire against the torque readings. The calibration curve for this method is given in Appendix I.

4.5.7 Non UV-VIS spectroscopic analysis

Non-*UV-VIS* analytical spectroscopic methods are useful for both identification and quantification of chemical compounds. Following separation from solution components, glycolipids can be identified by at least two of the following methods: nuclear magnetic resonance, mass spectrometry and infrared spectroscopy.

In this work, FT – infrared spectroscopy, NMR and MS following have been used to identify the chemicall nature of the product. Rhamnolipid separation was done according to Tahzibi *et al.* (2004). The supernatant was prepared from the culture sample by centrifugation. The supernatant was acidified to pH 2 and held at 4ºC prior to extraction with a 2:1 mixture of chloroform and methanol. The solvent was evaporated and the residue dissolved in 3 ml $NaHCO_3$ 0.1M. This solution was used for spectroscopic analysis. The detailed protocol of this separation method and a review of the three spectroscopic methods are provided in Appendix VI.

4.5.8 Erythrocytes haemolysis method

The erythrocyte haemolysis method is used to study the antimicrobial activity of biosurfactants (Dehgahan-Noudeh 2009), by testing the haemolytic activity of biosurfactants against sheep blood erythocytes. Blood agar plates containing 5% (v/v) sheep blood are used. A small diameter filter paper (6 mm for example) on which culture supernatant containing biosurfactant is layered, is placed onto the agar medium at the centre of the plate, and incubated at an adequate temperature for a sufficient time for growth to take place. Haemolitic activity is detected as the presence of a definite clear zone around a colony. The method has been successfully used by Tuleva *et al.* (2002) and Dehgahan-Noudeh (2009), but was not used in this study. The method presented in Section 4.5.7 was found to be equally efficient (Tuleva *et al.* 2002) and easier to operate. The detailed description of this method is provided in Appendix XI.

4.5.9 *Bacillus subtilis* inhibition method

The *Bacllus subtilis* method for determination of the antibiotic activity of biosurfactant is based on assessing the potential of the biosurfactant to inhibit *B. subtilis* growth (Tuleva *et al.* 2002). This method was used as follows: the pH of 20ml of the culture supernatant was adjusted to 6.6 - 6.8 and $ZnCl_2$ was added to a final concentration of 69 mM. The precipitated material was dissolved in 10ml of 0.1 M sodium phosphate buffer pH 6.5 and extracted five times with an equal volume of MTBE. The pooled organic phases were evaporated to dryness and the pellets were dissolved in 70 µl of methanol. Concentrated culture supernatant was spotted onto paper filter discs (6.0 mm Whatman discs). The filters were put onto the agar plates containing freshly grown *B. subtilis* cells. The *B. subtilis* plates were put at 37°C overnight. The zones of inhibition were a clear evidence of the antibiotic nature of the product. The agar plates contained CTAB 0.2 $mg.ml^{-1}$ and methylene blue 5 $\mu g.ml^{-1}$

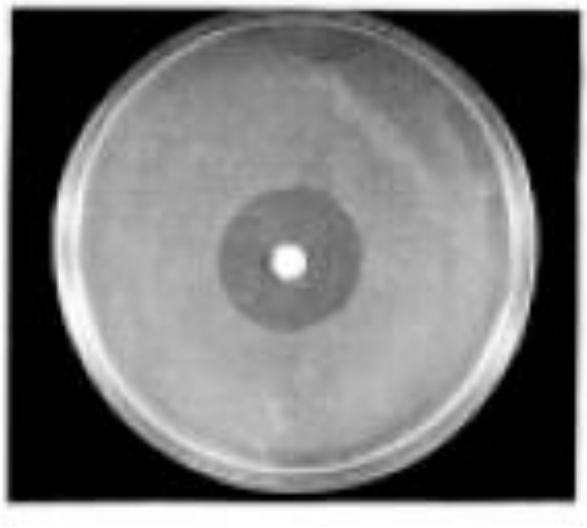


Figure 4.7: Example of *B. subtilis* inhibition by a concentrated culture supernatant (Tuleva *et al.* 2002)

4.5.10 Statistical design of experiments and factor analysis

Statistical design of experiments can be understood as the process of planning the experiments so that appropriate data susceptible to be analysed by statistical methods is collected (Montgomery 2005), resulting in valid and objective conclusions. When the problem involves data that are subject to experimental errors, statistical methods are the only objective approach to analysis (Montgomery & Runger 2007).

The three basic principles of experimental design are *randomisation*, *replication* and *blocking* (Das & Giri 1979). The first means that both the allocation of the experimental material and the order in which the individual runs are to be performed are randomly determined. A proper randomisation of an experiment also helps in averaging out the effects of extraneous factors that may be present. The second refers to an independent repeat of each factor combination. This makes it possible to obtain an estimate of the experimental error, which in turn becomes a basic unit of measurement for determining whether observed differences in data are really statistically different or not (Wardlaw 2000). The third principle, blocking, is a design technique used to improve the precision with which comparison among the factors of interest are made (Li 1964; Montgomery 2005). Often blocking is used to reduce or eliminate the variability transmitted from *nuisance factors*, which are factors that may influence the experimental response, but in which the experimenter is not directly interested.

When the effects of two or more factors in an experiment are to be analysed, it is most useful to use a particular type of the experimental design known as *factorial design*, where, in each complete trial or replication of the experiment, all possible combinations of the levels of the factors are investigated. The results from such combinations are usually understood through the analysis of variance or ANOVA.

Among the general situations of the factorial design, there are special cases that are of considerable interest because they are widely used in research works and, also, because they form the basis of other designs of notable practical interest.

The most important of such special situation is the case of *f* factors in which each factor is at just two levels (Hicks & Turner 1999). These levels might be quantitative such as two values of temperature, pressure or time; or they might be qualitative such as two machines, two operators, the "high" and the "low" levels of a factor, or perhaps the presence or absence of a factor (Hicks & Turner 1999; Montgomery 2005). A complete replicate of this design requires 2 X 2 X **. . .** X 2 = 2^f observations; it is called a 2^f *factorial design*. In the simplest design of the 2^f series, two factors are of interest and each factor is set at just two levels: 2 X 2 = 2^2 factorial design; the design is considered to be completely randomised.

Another interesting special case of factorial experimentation is the 3^f factorial design, which refers to situations where *f* factors of interest are considered each at three levels, meaning that there are 2 degrees of freedom between the levels of each of these factors. The first design in the 3^f series is the 3^2 factorial design, which considers two factors, each at three levels.

Apart from the full factorial designs where all the factors have the same number of levels, there also exist special factorial designs with mixed levels of factors, in which all the factors do not have the same number of levels (Draper & Stoneman 1968). An example of these special designs is the 2^1 X 3^1 factorial design, also denoted 2 X 3 (or 3 X 2) which considers two factors, one at two levels and the other at three levels. Mixed two and three level designs are similar to two-level fractional factorials, but they allow one factor to have three levels (low, middle and high) instead of just two levels. They are particularly useful when one of the factors under consideration may have its best sitting in the middle of the range of interest (Haaland 1989).

In this study, both the 3^f and 2^fX3^f factorial designs were used to investigate the significance and interaction of the effect of aeration rate, agitation rate and type of foam breaker on biomass growth and emulsification index. The Data analysis was done through analysis of variance for the different experimental data, as presented in Sections 6.3.3.

4.6 Experimental approach

Chapter 4 provided the review of potential analytical methods proposed for the experimental work carried out, from which a limited number were used during both the exploratory and the bioreactor-based experiments. Further, the microbial systems and bioreactor used have been fully described. The following experimental approach was adopted to achieve the objectives of this work:

(a) to proceed through a series of shake flask based experiments so as:

- to select the best method for biomass quantification;
- to study the growth media for *Ps. aeruginosa 2Bf*
- to investigate the potential of linear hydrocarbons to induce biosurfactant production;

(b) to use bioreactor-based experiments in order to:

- to optimise the reactor conditions for biosurfactant production;
- to study the reactor mixing, aeration and mass transfer;
- to comparatively study three ways of foam control;
- to investigate alkane utilisation during the process;
- to study the effect of alkane concentration on the process;

(c) to close the experimental work for this project by analysing the chemical nature of the product and testing it for antibiotic activity.

CHAPTER V: BACTERIAL GROWTH AND PRODUCTION OF BIOSURFACTANTS: SHAKE FLASK STUDY

5.1 Introduction

Prior to bioreactor experiments and product optimisation, exploratory experiments were carried out in order to generate information with regard to optimal conditions for microbial growth, microbial behaviour at different growth stages, microbial performance as a function of the substrate and growth medium, nature of the product, etc. This chapter presents and discusses results of these preliminary experiments, including method selection for growth monitoring, media selection, establishment of the growth curves of *Ps. aeruginosa 2Bf* and biosurfactant induction by alkanes.

5.2 Method selection for Ps. aeruginosa 2Bf growth monitoring

This sub-chapter discusses results from the preliminary experiments used to select the most appropriate analytical method for *Ps. aeruginosa* cells, from those identified as of potential in Section 4.4.1, where cell dry weight, microscopic cell count and optical density were tested (Chayabutra & Ju 2001; Williams 2005; Koch et al. 1991; Tahzibi, et al. 2004). However the potential interference of the alkanes phase was noted. It was proposed that washing the biomass with either cyclohexane or hexane could be used to remove the interference. All the four methods proposed were used to monitor the growth of *Ps. aeruginosa 2Bf*, and the data obtained were compared. In Figure 5.1, parity plots are presented to compare reproducibility of each method of biomass monitoring across four different media. The linear regression coefficients for the above results are provided in Table 5.1.

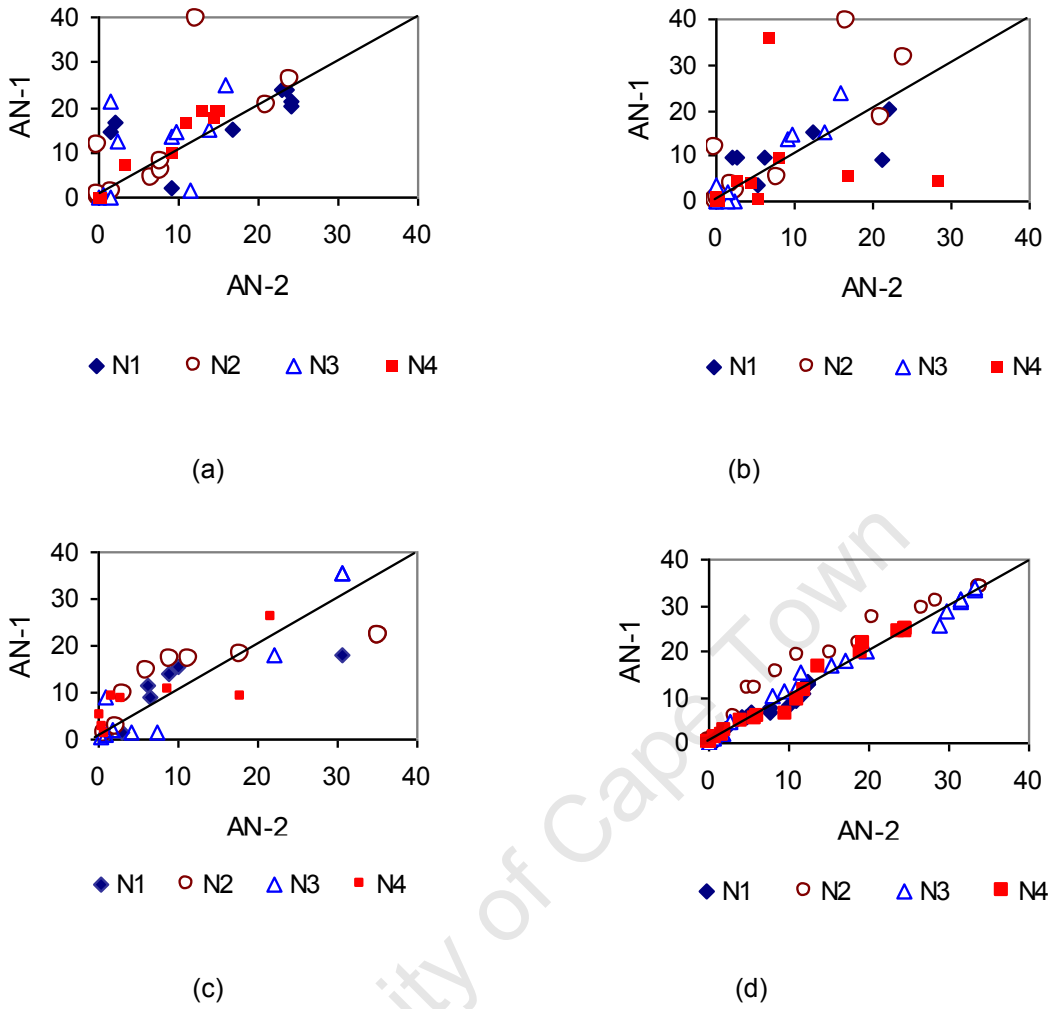


Figure 5.1: Comparison of reproducibility of the four methods of growth monitoring across four media (M1, M2, M3, M4): a) dry weight measurement following cyclohexane washing; b) dry weight measurement following hexane washing; c) optical density measurement; d) direct cell count.

Table 5.1: Linear regression coefficients describing the parity of repeat data collected for each growth monitoring method

Method *	M1	M2	M3	M4
DW-cyclh	0.582	0.730	0.163	0.975
DW-hex	0.655	0.857	0.784	-0.304
OD	0.735	0.638	0.820	0.361
CC	0.968	0.949	0.987	0.986

These results demonstrate high dispersion of data obtained with dry weight and optical density methods. The linear regression coefficient for the cell count method is the highest and good parity is shown (Montgomery 2005).

Characteristic coefficient for the second order polynomial trends between biomass measured and time for the data are shown in Figure 5.2. These coefficients are tabulated as shown in Table 5.2.

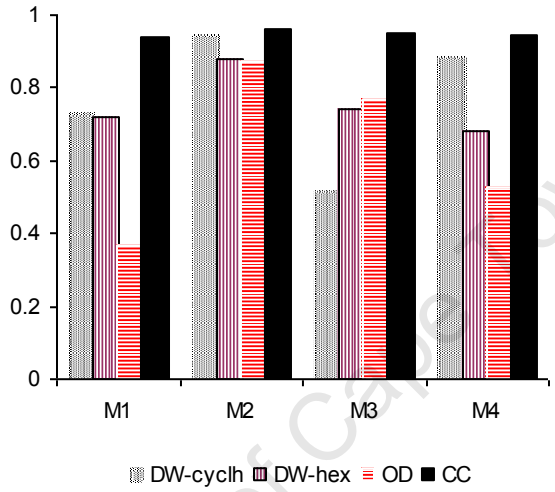


Figure 5.2: 2nd order polynomial trend of the results

Table 5.2: Linear regression coefficient for the polynomial trends of the graphs corresponding to the four media used, as illustrated in Figure 5.2.

Method	M1	M2	M3	M4
DW-cyclh	0.729	0.944	0.516	0.884
DW-hex	0.721	0.878	0.743	0.679
OD	0.370	0.876	0.769	0.529
CC	0.940	0.960	0.950	0.946

The coefficients in Table 5.2 were higher for the direct cell count method, meaning that data point values were less dispersed compared to those obtained with the other methods. This shows that measured values were closest to the sample mean value in this method (Montgomery & Runger 2007; Haaland 1989).

The standard deviations, the covariance and the statistical correlation of AN1 and AN2 are provided in Tables 5.3 and 5.4. For all the four media used, standard

deviations obtained with the direct cell count method were smaller than those with the other methods.

Table 5.3: Standard deviation values for the four methods

		STDEV		
Method	M1	M2	M3	M4
DW-cyclh	0.560	0.352	0.929	0.307
DW-hex	0.494	0.339	0.450	0.810
OD	0.663	0.627	0.516	1.030
CC	0.125	0.280	0.115	0.081

Table 5.4: Covariance (COV) and statistical correlation (COR) for each of the four methods used for monitoring growth

	COV AND COR							
	M1		M2		M3		M4	
Method	COV(AN1,AN2)	COR(AN1,AN2)	COV(AN1,AN2)	COR(AN1,AN2)	COV(AN1,AN2)	COR(AN1,AN2)	COV(AN1,AN2)	COR(AN1,AN2)
DW-cyclh	2.553	0.766	2.498	0.882	0.943	0.463	2.481	0.988
DW-hex	1.659	0.810	3.035	0.933	2.096	0.928	1.034	0.435
OD	3.217	0.858	2.029	0.799	4.167	0.906	2.492	0.603
CC	1.414	0.984	3.740	0.978	3.555	0.995	2.665	0.993

For all methods, positive values were obtained for covariance, meaning that analyses AN1 and AN2 were positively correlated and varied in a similar manner (Li 1964). However, covariance values depend on the units used for different parameters (Wardlaw 2000). Hence, the statistical correlation was used to compare the co variability of measurements (Li 1964; Grissom & Kim 2005) in each of the four analytical methods. In Table 5.4, it is seen that correlation values obtained with direct cell count were the closest to unity, thus suggesting a higher reproducibility for this method.

Based on the above results, direct cell count was chosen as the best method to monitor growth of *Ps. aeruginosa 2Bf*. The method was used as the standard method for biomass quantification in both the exploratory and the quantitative optimisation bioreactor based experiments.

5.3 Media selection

Growth media can be divided into two main groups, the defined and the complex media (Shuler & Kargi 2002). The former have a pre-determined chemical

composition, whereas the latter contain natural compounds whose exact chemical composition, in terms of the relative mass of individual constituents, is not specified. In this project, a basic defined complex media has been selected from the literature (Desai & Banat 1997; Ghilamicael 2003; Karanth *et al.* 2004; Nelly *et al.* 2002; Wu & Ju 1998). Refinement of this media to select the most suitable combination of nitrogen sources for biosurfactant production was required.

As indicated in Section 4.2.3, after a preliminary screening of the literature, four media were chosen, each corresponding to a particular combination of three nitrogen sources. A final screening, based on both cell growth and biosurfactant production potential, was used to select the best nitrogen source for use in the bioreactor study.

Prior to studying the nitrogen source, the C_{14}-C_{17} alkane blend was compared to two other carbon sources for *Ps. aeruginosa 2Bf* growth, n-dodecane and n-hexadecane.

5.3.1 Growth of *Ps. aeruginosa 2Bf* on the four selected media: effect of nitrogen source and alkane chain length

With all the four nitrogen sources, *Ps. aeruginosa 2Bf* showed very poor growth on n-dodecane. This was also verified by Mathopa (2003) during a screening of alkane utilizing bacteria. On solid media, no colony was observed in the first 12 days, while on liquid media a microbial cell concentration of $1x10^5$ cells per ml was attained on the 10^{th} day. *Ps. aeruginosa 2Bf* showed good growth on both n-hexadecane and C_{14}-C_{17} in liquid media, particularly in the case of M3 and M4 for n-hexadecane and M2, M3 & M4 for the C_{14}-C_{17} fraction. The growth curves for these experiments are presented in Figure 5.3.

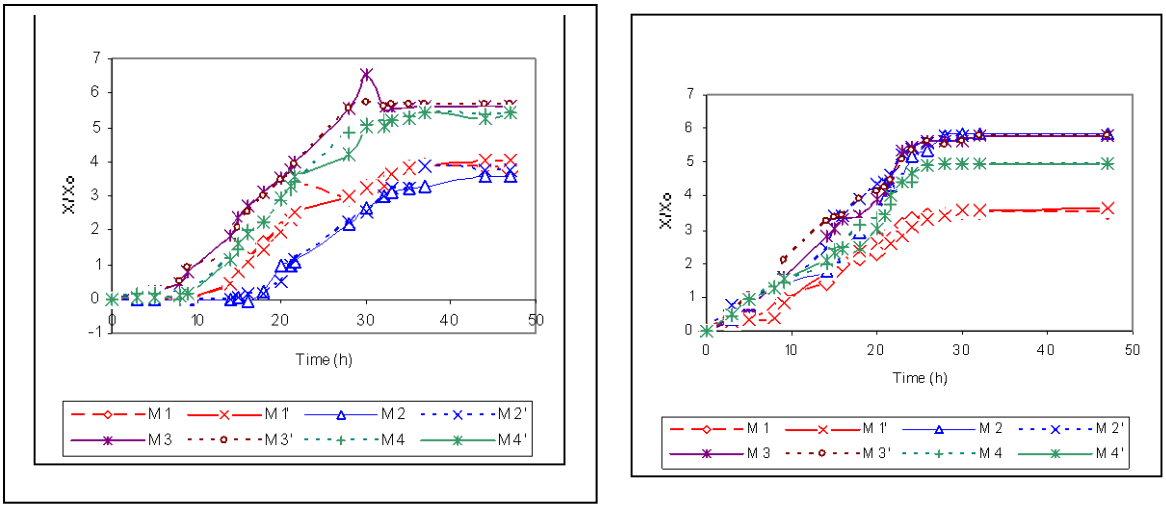


Figure 5.3: Growth of *Ps. aeruginosa* 2Bf on pure n-hexadecane (a) and C_{14}-C_{17} (b) using media M1, M2, M3 and M4 and represented as natural logarithms. Experiments were conducted in duplicate

In the case of hexadecane, the maximum cell number was less than 10^9 cell per ml and the lag phase varied between 12 and 18 hours for M1 and M2, while for M3 and M4, the lag phase varied between 8 and 10 hours.

For the C_{14}-C_{17} fraction, media M1 again showed poor growth. However, in all cases the lag phase was very much shorter (0 to 8 hours). The maximum cell number was larger in all cases except M1. The exponential phase showed a good linearity on the logarithmic scale (Montgomery & Runger 2007). The stationary phase was reached in less than 30 hours for all media. The maximum growth rates and maximum cell number obtained during these experiments are compared across nitrogen and carbon sources in Table 5.5.

Table 5. 5: Maximum growth rates and cell number attained by *Ps. aeruginosa* 2Bf cells on hexadecane and C_{14}-C_{17} fraction on media M1, M2, M3 and M4

	M1	**M2**	**M3**	**M3**	
C_{14}-C_{17}	0.17	0.27	0.22	0.21	μ_{max} (h^{-1})
Hexadecane	0.16	0.18	0.25	0.25	
C_{14}-C_{17}	1.50	270	210	34	X_{max} $(x10^9$ cells.$ml^{-1})$
Hexadecane	*0.63*	*0.41*	*26*	*15*	

From these results, the C_{14}-C_{17} cut was chosen as being a better substrate than n-hexadecane for *Ps. aeruginosa 2Bf*. Media M1, in which NH_4N_{O3} and NaN_{O3} were present at a total nitrogen concentration of 1.24 g/l and a molar C:N ratio of 1.49, was discarded based on poor performance. This was attributed to the lack of sulphate in the nitrogen source, which might have resulted in sulfur limitation during the process.

5.3.2 Media selection based on the emulsification index

Following selection of the C_{14}-C_{17} alkane fraction as carbon source, further selection of a good nitrogen source was undertaken, using the four preselected media (M1, M2, M3 & M4) and monitoring both microbial growth and biosurfactant production. As detailed in Section 5.2.1, the first test was to monitor the growth of *Ps. aeruginosa 2Bf*, while the second was to quantify the potential for oil in water emulsification index against a 1:1 mixture kerosene:supernatant (Makkar & Cameotra 1998; Vasileva-Tonkova *et al.* 2008). To achieve the research objectives, samples were measured in three different forms, to test whether the biosurfactant was located in the supernatant or associated with the biomass (Yin *et al.* 2008; Vasileva-Tonkova *et al.* 2008). The results obtained for culture suspension, supernatant prepared by centrifugation and supernatant prepared by centrifugation and filtration at 0.22µm pore size filters, were summarised in Table 5.6. M1 did not provide any significant emulsification and was eliminated at this stage. Emulsification was observed with M2, M3 and M4.

Table 5. 6: Growth parameters and emulsification index (aqueous-kerosene system) results as a function of medium type using the C_{14}-C_{17} fraction as carbon source

	M1	M2	M3	M4
μ_{max}	0.17 h^{-1}	0.27 h^{-1}	0.22 h^{-1}	0.21 h^{-1}
X_{max}	$1.30x10^{9}$	$2.40x10^{11}$	$2.12x10^{11}$	$3.25x10^{10}$
E_{index} for suspension	-	++	+++	++
E_{index} for supernatant	-	-	++	+++
E_{index} for filtered supernatant	- -	- -	-	-

Removal of biomass phase by centrifugation and filtration exerted a strong negative effect on the emulsification potential of the culture supernatant, which was more pronounced on filtration, as noticed in Table 5.7. This can be explained in two ways:

(a) either the produced biosurfactant possesses an important affinity with the bacterial outer membrane (Vasileva-Tonkova *et al.* 2008; Yin *et al.* 2008);

(b) or the cell envelope itself contribute to the emulsification activity (Neu 1996);

(c) or most emulsification effect (biosurfactant) was removed along some other substance through sticking to the filter paper.

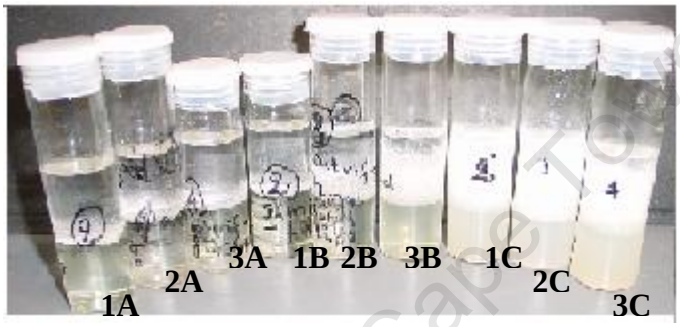


Figure 5.4: Emulsification power of *Ps. aeruginosa 2Bf*'s culture supernatant: C refers to the cell suspension, B to the centrifuged supernatant and A to the filtered & centrifuged supernatant

Table 5.7: Emulsification effect quantified as E_{index} for different sample treatments: A. filtered and centrifuged supernatant; B. centrifuged supernatant; C. cell suspension

	C			B			A		
	M2 (1C)	M3 (2C)	M4 (3C)	M2 (1B)	M3 (2B)	M4 (3B)	M2 (1A)	M3 2A)	M4 (3A)
E_{index}	60.78	63.73	58.63	1.51	41.18	57.25	2.00	7.20	7.60

Emulsification tests were applied to the selected media M2, M3 and M4. The values obtained for emulsification index are given in Table 5.7. These showed that the kinetics of bacterial growth and bioemulsifier production are not dependent on the nitrogen form alone, since different results were obtained with media M2 and M4, which were very similar from the nitrogen source point of view. From this observation, it was postulated that other mineral components of the nitrogen sources, such as sulphate and Na, also played an important role in the process. While E_{index} of the culture suspension and growth rate are high for M2, very little biosurfactant activity

was observed in the supernatant. Nitrogen source M3 and M4 performed best in terms of emulsification potential of the supernatant while maintaining good growth performance and overall emulsification potential. The absence of NO_3^- in M3 illustrated that its presence is not essential. M3 was chosen to be used in the quantitative bioreactor based study, as it presented the highest emulsification index, the maintenance of emulsification in the supernatant, a high specific growth rate (average 0.22 ± 0.0086) and a high final cell concentration ($2.1x10^{11}$ ± $1.41x10^{10}$ cell per ml).

5.4 Induction of biosurfactant production by alkanes

A series of experiments were performed to investigate biosurfactant induction potential of n-alkanes in comparison to a benchmark glucose based medium. Liquid n-alkanes were hypothesised to be good alkane inducers, since hydrocarbon were reported to induce biosurfactant production by bacterial species (Lee et al. 1998; Barathi & Vasudevan 2001). Biosurfactant induction was measured in the presence of glucose only, C_{14}-C_{17} alkane only and a blend containing equal amounts of glucose and C_{14}-C_{17} alkane in terms of carbon content. In each case, the carbon source was used to a total of 3% w/v in the growth media. M2 and M3 were used as growth media. M2 served as the stock culture medium throughout the experiment; hence it was necessary to verify whether it exerted any important influence on the biosurfactant induction process. Induction of biosurfactant synthesis was quantified by the determination of E_{index} in % (Vasileva-Tonkova *et al.* 2008)

From the 4th generation, only the M2-alkn tree was considered following the results of generation 1, 2 & 3. Then experiments were narrowed to pursue the exploration, with the M3-alkn of the group.

Table 5.8 reveals that from a same inoculation slant, induction due to alkane is close to 5 times higher than that due to glucose with M2. For the 2nd inoculation, the highest induction, at this stage, was obtained with pure alkane on M2, 64.71 %, though M3 was expected to be more influencial in the process, according to the results of the previous section. In fact, at this stage, microorganisms from M2 inoculum may still be adapting to M3. Pure glucose did not result in any biosurfactant synthesis.

1st and 2nd generation

Table 5.8: Biosurfactant induction results – 1st and 2nd generations: E_{index} is given in %; the slant was used to start two liquid cultures that will be used for further inoculation. One contained glucose only and the other contained alkane only

Slant–M2			
M2-gl :		M2-alkn :	
M2-glc	0.000	M2-glc	1.961
M3-glc	0.000	M3-glc	1.961
M2-glc+alkn	1.300	M2-glc+alkn	11.76
M3-glc+alkn	1.260	M3-glc+alkn	1.961
M2-alkn	14.000	M2-alkn	64.710
M3-alkn	14.000	M3-alkn	29.410

3rd generation

a) Parental medium: M2-glc

Table 5.9: Biosurfactant induction results provided as E_{index} (%) for the 3rd generation from M2-glucose as the parental medium

M2-glc		M3-glc		M2-glc+alkn		M3-glc+alkn		M2-alkn		M3-alkn	
M2-glc	0.000	M2-glc	0.000	M2-glc	0.000	M2-glc	0.000	M2-glc	0.000	M2-glc	0.000
M3-glc	0.000	M3-glc	0.000	M3-glc	0.000	M3-glc	0.000	M3-glc	0.000	M3-glc	0.000
M2-glc+alkn	0.700	M2-glc+alkn	1.310	M2-glc+alkn	1.300	M2-glc+alkn	1.230	M2-glc+alkn	2.405	M2-glc+alkn	2.340
M3-glc+alkn	0.630	M3-glc+alkn	1.251	M3-glc+alkn	1.200	M3-glc+alkn	1.400	M3-glc+alkn	2.230	M3-glc+alkn	2.340
M2-alkn	9.220	M2-alkn	7.830	M2-alkn	9.200	M2-alkn	7.698	M2-alkn	23.000	M2-alkn	23.230
M3-alkn	7.900	M3-alkn	9.400	M3-alkn	7.601	M3-alkn	9.575	M3-alkn	22.910	M3-alkn	23.700

b) Parental medium: M2-alkn

Table 5.10: Biosurfactant induction results provided as E_{index} (%) for the 3rd generation from M2-alkane as the parental medium

M2-glc		M3-glc		M2-glc+alkn		M3-glc+alkn		M2-alkn		M3-alkn	
M2-glc	0.000	M2-glc	0.000	M2-glc	0.000	M2-glc	0.000	M2-glc	14.000	M2-glc	13.320
M3-glc	0.000	M3-glc	0.000	M3-glc	0.000	M3-glc	0.000	M3-glc	13.020	M3-glc	13.400
M2-glc+alkn	1.100	M2-glc+alkn	0.670	M2-glc+alkn	1.432	M2-glc+alkn	1.435	M2-glc+alkn	22.430	M2-glc+alkn	21.340
M3-glc+alkn	0.963	M3-glc+alkn	0.900	M3-glc+alkn	1.300	M3-glc+alkn	1.435	M3-glc+alkn	21.400	M3-glc+alkn	21.340
M2-alkn	2.330	M2-alkn	2.000	M2-alkn	6.890	M2-alkn	7.993	M2-alkn	64.690	M2-alkn	60.990
M3-alkn	2.165	M3-alkn	2.000	M3-alkn	5.999	M3-alkn	10.110	M3-alkn	56.990	M3-alkn	65.220

4th generation

Table 5.11: Biosurfactant induction results provided as E_{index} (%) for the 4th generation

M2-glc		M3-glc		M2-glc+alkn		M3-glc+alkn		M2-alkn		M3-alkn	
M2-glc	0.000	M2-glc	0.000	M2-glc	0.000	M2-glc	0.000	M2-glc	13.470	M2-glc	13.330
M3-glc	0.000	M3-glc	0.000	M3-glc	0.000	M3-glc	0.000	M3-glc	13.200	M3-glc	13.230
M2-glc+alkn	1.410	M2-glc+alkn	0.900	M2-glc+alkn	2.431	M2-glc+alkn	1.304	M2-glc+alkn	19.110	M2-glc+alkn	19.100
M3-glc+alkn	1.410	M3-glc+alkn	1.443	M3-glc+alkn	2.425	M3-glc+alkn	1.401	M3-glc+alkn	19.100	M3-glc+alkn	19.210
M2-alkn	7.138	M2-alkn	7.000	M2-alkn	9.000	M2-alkn	7.033	M2-alkn	64.330	M2-alkn	64.390
M3-alkn	6.999	M3-alkn	7.065	M3-alkn	8.999	M3-alkn	9.040	M3-alkn	62.890	M3-alkn	65.040

5th generation

Table 5.12: Biosurfactant induction results provided as E_{index} (%) for the 5th generation

M2-glc		M3-glc		M2-glc+alkn		M3-glc+alkn		M2-alkn		M3-alkn	
M2-glc	0.000	M2-glc	0.000	M2-glc	0.000	M2-glc	0.000	M2-glc	13.040	M2-glc	13.020
M3-glc	0.000	M3-glc	0.000	M3-glc	0.000	M3-glc	0.000	M3-glc	13.040	M3-glc	13.030
M2-glc+alkn	1.999	M2-glc+alkn	1.000	M2-glc+alkn	1.621	M2-glc+alkn	2.032	M2-glc+alkn	19.110	M2-glc+alkn	19.520
M3-glc+alkn	1.999	M3-glc+alkn	1.107	M3-glc+alkn	1.622	M3-glc+alkn	2.103	M3-glc+alkn	19.130	M3-glc+alkn	19.440
M2-alkn	6.606	M2-alkn	7.11	M2-alkn	7.001	M2-alkn	7.001	M2-alkn	64.220	M2-alkn	64.810
M3-alkn	6.600	M3-alkn	6.931	M3-alkn	6.991	M3-alkn	7.126	M3-alkn	64.010	M3-alkn	64.800

6th generation

Table 5.13: Biosurfactant induction results provided as E_{index} (%) for the 6tth generation

M2-glc		M3-glc		M2-glc+alkn		M3-glc+alkn		M2-alkn		M3-alkn	
M2-glc	0.000	M2-glc	0.000	M2-glc	0.000	M2-glc	0.000	M2-glc	12.010	M2-glc	12.020
M3-glc	0.000	M3-glc	0.000	M3-glc	0.000	M3-glc	0.000	M3-glc	12.030	M3-glc	12.020
M2-glc+alkn	1.975	M2-glc+alkn	0.754	M2-glc+alkn	1.984	M2-glc+alkn	2.001	M2-glc+alkn	17.000	M2-glc+alkn	18.980
M3-glc+alkn	1.988	M3-glc+alkn	0.657	M3-glc+alkn	1.971	M3-glc+alkn	2.111	M3-glc+alkn	19.970	M3-glc+alkn	19.000
M2-alkn	6.193	M2-alkn	9.999	M2-alkn	9.800	M2-alkn	8.127	M2-alkn	64.120	M2-alkn	65.100
M3-alkn	6.766	M3-alkn	9.981	M3-alkn	9.800	M3-alkn	8.223	M3-alkn	65.130	M3-alkn	65.410

7th generation

Table 5.14: Biosurfactant induction results provided as E_{index} (%) for the 7th generation

M2-glc		M3-glc		M2-glc+alkn		M3-glc+alkn		M2-alkn		M3-alkn	
M2-glc	0.000	M2-glc	0.000	M2-glc	0.000	M2-glc	0.000	M2-glc	10.120	M2-glc	10.140
M3-glc	0.000	M3-glc	0.000	M3-glc	0.000	M3-glc	0.000	M3-glc	9.1400	M3-glc	10.450
M2-glc+alkn	0.023	M2-glc+alkn	0.322	M2-glc+alkn	2.222	M2-glc+alkn	2.281	M2-glc+alkn	15.900	M2-glc+alkn	16.100
M3-glc+alkn	0.230	M3-glc+alkn	0.301	M3-glc+alkn	2.021	M3-glc+alkn	2.222	M3-glc+alkn	15.300	M3-glc+alkn	16.320
M2-alkn	7.000	M2-alkn	6.670	M2-alkn	7.032	M2-alkn	8.102	M2-alkn	64.000	M2-alkn	64.320
M3-alkn	7.000	M3-alkn	6.897	M3-alkn	7.000	M3-alkn	8.332	M3-alkn	65.210	M3-alkn	65.210

8th generation

Table 5.15: Biosurfactant induction results provided as E_{index} (%) for the 8tth generation

M2-glc		M3-glc		M2-glc+alkn		M3-glc+alkn		M2-alkn		M3-alkn	
M2-glc	0.000	M2-glc	0.000	M2-glc	0.000	M2-glc	0.000	M2-glc	7.324	M2-glc	7.320
M3-glc	0.000	M3-glc	0.000	M3-glc	0.000	M3-glc	0.000	M3-glc	7.232	M3-glc	7.400
M2-glc+alkn	0.020	M2-glc+alkn	0.092	M2-glc+alkn	1.011	M2-glc+alkn	2.000	M2-glc+alkn	13.210	M2-glc+alkn	13.330
M3-glc+alkn	0.006	M3-glc+alkn	0.092	M3-glc+alkn	0.999	M3-glc+alkn	2.121	M3-glc+alkn	13.190	M3-glc+alkn	13.330
M2-alkn	1.092	M2-alkn	1.000	M2-alkn	1.034	M2-alkn	7.000	M2-alkn	64.970	M2-alkn	64.970
M3-alkn	1.034	M3-alkn	1.035	M3-alkn	1.034	M3-alkn	7.012	M3-alkn	65.670	M3-alkn	65.700

9th generation

Table 5.16: Biosurfactant induction results provided as E_{index} (%) for the 9th generation

M2-glc		M3-glc		M2-glc+alkn		M3-glc+alkn		M2-alkn		M3-alkn	
M2-glc	0.000	M2-glc	0.000	M2-glc	0.000	M2-glc	0.000	M2-glc	10.340	M2-glc	9.960
M3-glc	0.000	M3-glc	0.000	M3-glc	0.000	M3-glc	0.000	M3-glc	10.120	M3-glc	9.854
M2-glc+alkn	0.120	M2-glc+alkn	0.143	M2-glc+alkn	1.432	M2-glc+alkn	0.976	M2-glc+alkn	13.240	M2-glc+alkn	12.650
M3-glc+alkn	0.131	M3-glc+alkn	0.163	M3-glc+alkn	1.232	M3-glc+alkn	0.934	M3-glc+alkn	12.780	M3-glc+alkn	12.700
M2-alkn	6.990	M2-alkn	5.783	M2-alkn	6.431	M2-alkn	6.000	M2-alkn	64.990	M2-alkn	64.900
M3-alkn	6.954	M3-alkn	6.056	M3-alkn	5.888	M3-alkn	6.021	M3-alkn	65.360	M3-alkn	65.730

In the 3rd generation, cultures of the M2-glc tree (Table 5.9) still manifested poor emulsification with glucose, while alkanes produced a greater induction for both M3 and M2. The highest emulsification was produced in the presence of alkane as sole carbon source and was comparable for M2 (23.0 %) and M3 (23.7 %). Glucose produced detectable effects only when combined with the alkanes to form a mixed carbon source. However, the emulsification index remained low (2.3 %). In the 3rd generation of the M2-alkn tree (Table 5.10), biosurfactant production was not observed in media containing glucose and inoculated from glucose containing inocula. However, where an alkane inoculum was used with glucose as sole substrate, at this stage emulsification was observed. Very efficient biosurfactant production, with values of E_{index} in the range of 57 to 65 % was observed in alkane media inoculated from alkane based inocula.

At the fourth generation level (Table 5.11), a low production of biosurfactant (E_{index} 13 %) was again observed in glucose media inoculated with an inoculum grown on alkane. This remained less important than in the case of mixed carbon source medium or pure alkane medium. Induction effects in the presence of alkane and glucose were less important than those observed in the presence of alkane alone.

Clearly the best induction was obtained in alkane media (Lee *et al* 1998) where this has been used for the inoculum as well. In the fourth generation case, E_{index} of 63 to 65 % was obtained under these conditions, similar to the 3rd generation results. Similar results are presented for generations 5 to 9 in Table 5.12 to 5.16.

The alkane substrate has been shown to be necessary as an inducer of biosurfactant production by *Ps. aeruginosa 2Bf*. These findings agree with Lee *et al.* (1998) who reported the importance of alkane substrates in biosurfactant inducuction. *Ps. aeruginosa 2Bf*'s biosurfactant was not produced in glucose media in the absence of alkane unless induction occurred during the inoculum phase.

Following three to four generations of cultivations of *Ps. aeruginosa 2Bf* on the pure alkane carbon source, no further increase in induction level for biosurfactant was observed and these levels were maintained in the presence of alkane as sole carbon source.

5.5 Conclusion

In the 5th chapter, the method selection for monitoring growth of *Ps. aeruginosa 2Bf* bacterial cells was studied by testing four different methods, cell dry weight with cyclohexane washing, cell dry weight with hexane washing, optical density measurement and direct cell count. The last method was found to be most accurate.

Further, three carbon sources, dodecane, hexadecane and C_{14}-C_{17} were tested for bacterial growth enhancement. C_{14}-C_{17} was found to be the best.

Alkanes were confirmed to be good inducers for bacterial production of biosurfactants. When compared to glucose, alkanes were found to possess higher inducing capacity for biosurfactant production by *Ps. aeruginosa 2Bf*. In the absence of alkane, or previous exposure to it, biosurfactant production was induced. Nitrogen source composition was confirmed to play a key role in both the growth of bacterial cells and biosurfactant production. However, mineral constituents of the nitrogen sources were also found to influence the process of bacterial production of biosurfactant from alkanes.

Regarding the product's location, biosurfactants were essentially found in the supernatant, but some cell associated surface activity was also observed.

CHAPTER VI: BACTERIAL GROWTH AND PRODUCTION OF BIOSURFACTANT: QUANTITATIVE BIOREACTOR STUDY FOR OPTIMISATION

6.1 Introduction

In the fifth chapter, linear hydrocarbons were confirmed as inducers of biosurfactant production by *Ps. aeruginosa 2Bf*. In Chapter 6, biosurfactant production by *Ps. aeruginosa 2Bf* is further investigated through reactor studies to determine the extent of biosurfactant production, select preferred conditions for reactor operation and quantify growth kinetics. Chapter 6 is subdivided into the following sections:

- the base case reactor operations;
- mixing, aeration and mass transfer study; and
- alkane concentration and utilisation study.

The study is introduced by characterising growth and biosurfactant production under standard conditions of aeration, agitation and pH: 0.4 vvm, 500 rpm and uncontrolled pH. The reactor set up and the conditions described in Sections 4.2.5 and 4.3.3 were used. The following parameters were monitored at different growth phases: pH, DO, E_{index}, ST, alkane utilisation and, in some of the cases, viscosity. Surface tension and E_{index} were used as indicators of the level of biosurfactant production during the process.

Foam control was given particular attention. Since chemical anti-foams are surfactants by nature, the mechanical foam control was considered for a system that involved production of surfactants as the product of interest. The use of anti-foam was compared to two different mechanical foam breakers, in order to test their performance and find the best way of controlling foam formation during bacterial production of biosurfactant in batch reactor systems where liquid alkanes were used as both inducers and sole carbon source. The use of mechanical foam breakers was reported previously to be efficient in controlling foam formation (Takesono *et al.* 2002), and to have no adverse effect on mass transfer (Andou *et al.* 1997).

Mixing and aeration conditions as well as the oxygen mass transfer in the system were investigated in order to establish the optimum combination especially between agitation and aeration rates when *Ps. aeruginosa 2Bf* is used to produce biosurfactant in a batch reactor system.

Finally, the alkane or substrate depletion and utilisation during the process were investigated at the individual alkane level to check for any important selectivity during alkane usage by *Ps. aeruginosa 2Bf*.

6.2 Standard reactor conditions for biosurfactant production by *Ps. aeruginosa 2Bf* (base case)

The standard conditions selected for bioreactor operation were: aeration at 0.4 vvm, agitation at 500 rpm. The standard conditions were investigated both with pH control at pH 6.6 and without pH control, as detailed in Table 4.2. During the base case study, all growth and biosurfactant associated parameters considered in this work were measured.

6.2.1 Biomass formation

The results are presented for each parameter measured, providing comparison across pH regime, since culture pH has been reported to have an impact on the rhamnolipid production by *Ps. aeruginosa* (Chen *et al.* 2007). In all cases the biomass profile in terms of cell number is included as a reference point.

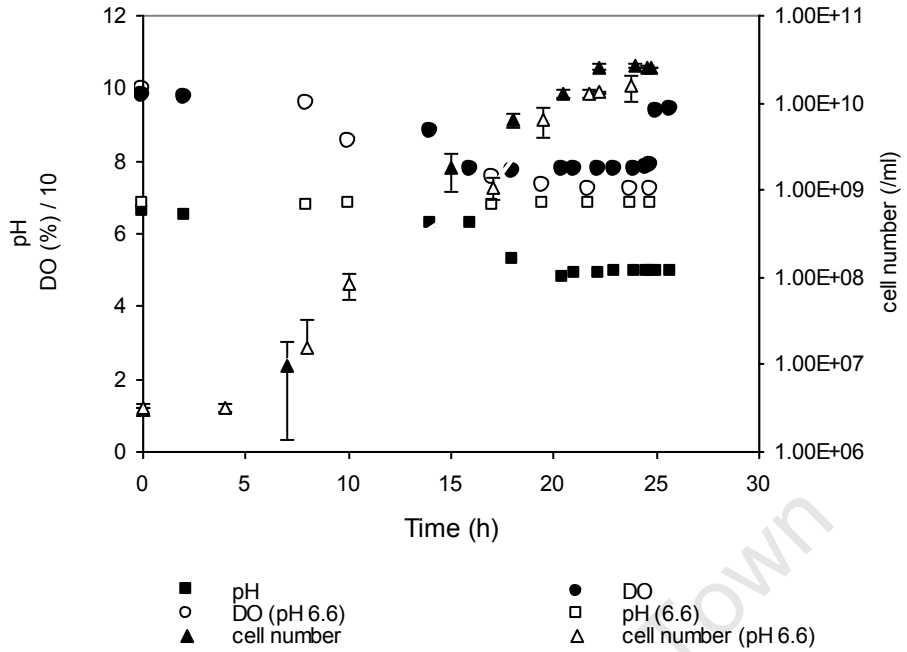


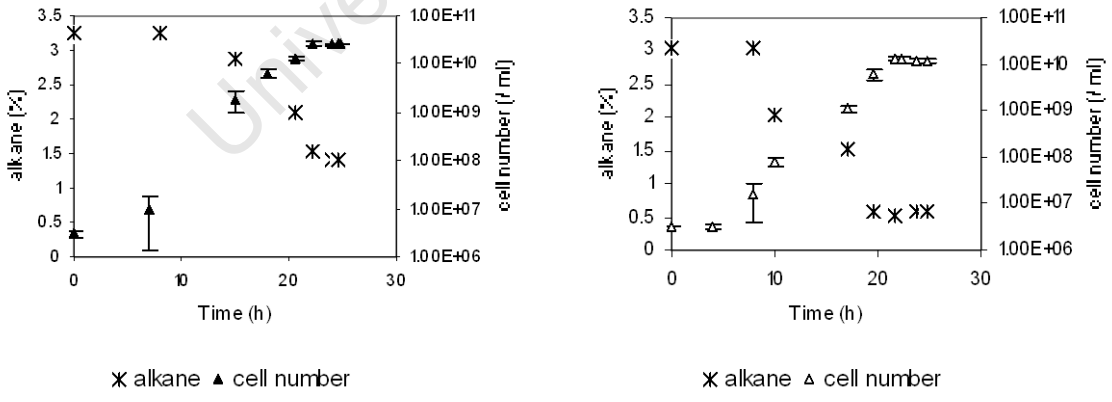
Figure 6.1: Biomass formation, pH and dissolved oxygen profiles under both uncontrolled (closed symbols) and pH conditions controlled at pH6.6 (open symbols)

Figure 6.1 shows similar biomass profiles for controlled and uncontrolled pH. A 0.5% inoculum was used. The initial cell number was approximately 3.21×10^6 cells per ml in both cases, and the exponential phase was observed between 4 hours and 7 hours in both cases. The duration of the exponential phase was approximately 13.5 h for uncontrolled conditions and 15.5 h for pH 6.6. The maximum cell numbers achieved were 2.6×10^{10} cells per ml and 1.30×10^{10} cells per ml respectively for uncontrolled and controlled pH conditions, while the specific growth rates calculated were 0.24 h^{-1} and 0.21 h^{-1} respectively. Under the non-controlled pH conditions, during the first sixteen hours corresponding to the exponential phase, the pH change was small, from pH 6.58 to pH 6.26. During the late exponential phase, the pH decreased significantly from pH 6.26 at 16 hours to pH 4.88 at 21 hours. The final pH value at 36 hours was 5.03. As the pH decrease can be linked to consumption of a non-limiting carbon source and production of acidic metabolites (Chen *et al.* 2007), these data suggest that the formation or export of an extracellular product in late exponential phase may have influenced pH variation.

As shown in Figure 6.1, in the case of uncontrolled pH, significant decline in the dissolved oxygen from 88.3% to 77.4% saturation was observed between 14 and 16 hours. The value of dissolved oxygen remained relatively constant up to the 25th

hour, after which there was a sharp increase to 94.2%. At 36 hours DO reached the value of 98.2 %, which was comparable with the initial DO of 97.9%. The DO decrease is most probably due to an augmentation of the oxygen demand by cells growing at high rate during the exponential phase (Shuler & Kargi 2002; Stainier *et al.* 1976; Nielsen & Villadsen 1994). However, some other factors such as hydrocarbon depletion, etc. may also have a significant impact on dissolved oxygen in the culture medium, since alkanes droplets can play a role in oxygen transportation (Williams 2005; Clarke *et al.* 2006; Rehm & Reiff 1981).

The control of pH at pH 6.6 resulted in DO decrease from 8 hours, while this happened from 14 hours in the case of no pH control. The evolution of the two DO trends looks very similar up to the stationary phase where the DO goes up under non controlled pH conditions while it remains at its lowest level at pH 6.6. The initial and highest value of DO in the system was set at ~ 100%, the lowest value of DO reached 77.25% with no pH control and 72.3% with pH control at 6.6. From these data, it could be assumed that no oxygen limitation occurred in the process. The stabilization of DO at lower values in early stationary phase may be due to oxygen consumption for product formation (Calik *et al.* 1997; Kuo *et al.* 2003). The sudden DO decrease in late exponential phase observed under non controlled pH conditions may have been caused by a shift in metabolism with oxygen consuming product. The time intervals for pH 6.6 did not allow this to be identified.



(a) (b)

Figure 6.2: Alkane utilisation and biomass production as a function of time under standard operating conditions (a) without pH control (b) pH = 6.6

The C_{14}-C_{17} alkane blend, previously identified as being a good substrate for many *Ps. aeruginosa* strains (Mathopa 2003), was used as the energy and sole carbon source, in order to induce biosurfactant synthesis by *Ps. aeruginosa 2Bf*. Figure 6.2 illustrates alkane depletion during the process. During the first eight hours, the alkane utilisation rate was low. From the 8th hour, the total alkane present in the growth medium decreased more rapidly till the 25th hour in the absence of pH control and the 20th hour in its presence. In the case of no pH control, alkane concentration decreased from 3.26% to 1.41% at 25 hours. In pH controlled case, the alkane concentration decreased to 0.53% at 20 hours, showing faster depletion. These trends confirm that the bacteria are able to metabolise the hydrocatbon substrate as carbon and energy source as reported for a range of bacterial strains (Chayabutra & Ju 2001; Janssen *et al.* 2002; Koch *et al.* 1991).

6.2.2 Biosurfactant production

Biosurfactant production was determined in terms of its impact, through measurement of the emulsification index and surface tension (Jennings & Tanner 2000; Perfumo *et al.* 2006).

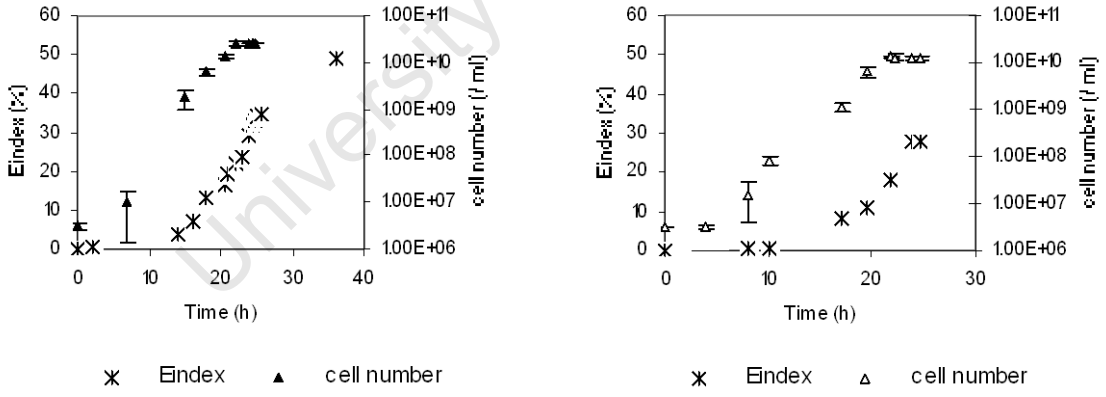


Figure 6.3: Comparison of emulsification effect and cell growth profile as a function of time under standard operating conditions (a) without pH control (b) pH = 6.6

In the bioreactor operated without pH control, emulsification was detected from the 2nd hour of the process. E_{index} remained low (< 3.6%) till the 14th hour from which it showed a rapid increase up to the stationary phase (35%) as seen in Figure 6.3a.

Thereafter, further increase at a lower rate was observed to 50% at 30 hours. With pH control, emulsification remained below detection limit up to ten hours, whereafter it increased to 23.75 hours (Figure 6.3b). The maximum value of E_{index} was 23.75%. On comparing E_{index} curves in Figure 6.3, it was noticed that the potential of *Ps. aeruginosa 2Bf* to produce emulsifying products was more pronounced where no pH control was applied to the system. A maximum E_{index} of 49% was reached without pH control and this was still increasing after 36 hours.

When the emulsified samples were left at room temperature, emulsions remained stable for a period up to three months.

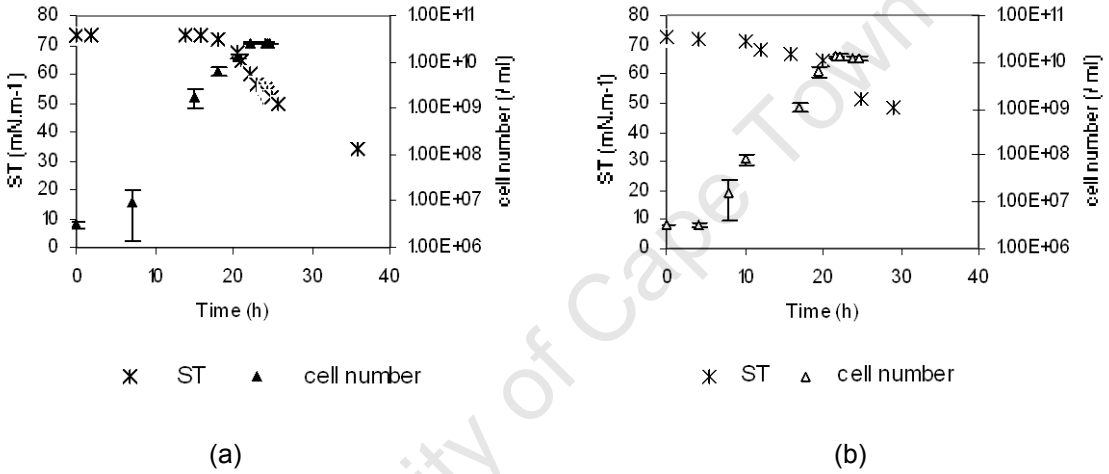


Figure 6.4: Surface tension and cell growth variation as a function of time under the standard reactor operating conditions (a) without pH control (b) pH = 6.6

Biosurfactant production was also monitored by measuring the surface tension of culture supernatant at different stages of the growth process (Maier & Soberon-Chavez 2000; Haferbur *et al.* 1986), as shown in Figure 6.4. Only surface active compounds are able to alter the air-liquid and liquid-liquid surface tension (Danielli 1964; Cullum 1994). The decrease in surface tension was noted from the late exponential phase in all cases.

In the absence of pH control, surface tension decreased from the value of 72.10 $mN.m^{-1}$ to 34.11 $mN.m^{-1}$ between 18 hours and 36 hours Fig. 6.4a, while it dropped from 71.3 to 48.28 between 10 and 29 hours at pH 6.6 Fig. 6.5b. These significant changes of surface tension, particularly in the first case, were attributed to the presence of a strong surface active substance in the supernatant (Beal & Betts 2000; Monteiro *et al.* 2007; Yin *et al.* 2009).

These observations agree with the emulsification results, and support the assumption that conditions of no pH control favour biosurfactant production over those of controlled pH at pH 6.6.

6.2.3 Broth viscosity

During growth and extracellular product synthesis, the culture composition becomes progressively modified due to many biochemical reactions taking place (Blanch & Clark 1997). The presence of cells, the consumption of substrates, the release of products and byproducts, etc. also contribute to this modification (Blanch & Clark; Shuichi *et al.* 1985). This can be associated with a noticeable change in physical characteristics of the systems such as viscosity. These in turn affect mass transfer and mixing in the reactor.

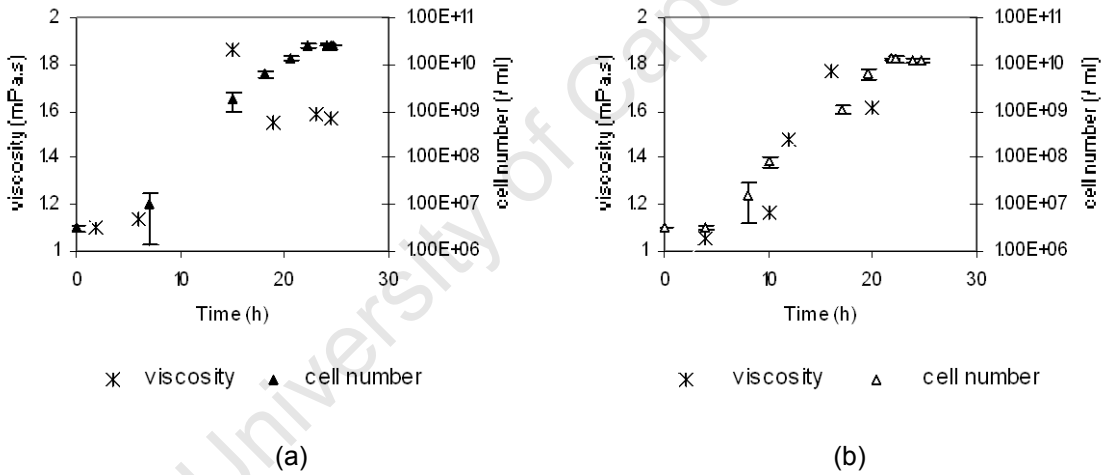


Figure 6.5: Comparison of broth viscosity and cell growth under standard reactor operating conditions (a) without pH control (b) pH = 6.6

In the absence of pH control (Figure 6.5a), an increase of viscosity from 1.1 mPa.S at 0 h to 1.4 mPa.s at 6 h and 1.86 mPa.s at 15 h was recorded. The viscosity decreased to 1.55 mPa.s at 19 hours, whereafter it remained constant.

With pH control at pH 6.6 (Figure 6.5(b)), viscosity increased from 0.86 mPa.s initially to its highest value of 1.78 mPa.s at 16 hours. The viscosity increase from 10 to 16 hours was more rapid than from 0 to 10 hours. In both cases, viscosity did not increase with biosurfactant production. The data suggested that viscosity increase is most likely more growth-dependent and associated with biomass increase.

6.3 Mixing, aeration and mass transfer study

Mixing, aeration and mass transfer play an important role in the biosurfactant production by bacterial organisms (Benincasa *et al.* 2002). In order to optimise reactor conditions, mixing and mass transfer were varied by varying agitation rate and aeration rate as described in Section 4.3.3. Four agitation rates and four aeration rates were considered during the study: 480 rpm, 500 rpm, 600 rpm and 800 rpm (at a constant aeration rate of 0.8 vvm), and 0.3 vvm, 0.4 vvm, 0.6 vvm and 0.8 vvm (at a constant agitation rate of 500 rpm) respectively. For each experiment, the following parameters were monitored throughout the process: media acidity or alkalinity in terms of pH, dissolved oxygen, cell number, emulsification index and the surface tension of culture supernatant. Initial alkane concentration was maintained at the value of 3%. To investigate the impact of agitation, aeration and their mutual interaction on growth, the 2^1X3^1 (or 2X3) factorial design was used as indicated in Section 4.5.10. Agitation was considered at three levels 210 rpm, 500 rpm and 800 rpm. The agitation levels of 500 rpm and 800 rpm were previously used by Benincasa *et al.* (2002), in studying *Ps. aeroginosa* in a bioreactor responding to the following characteristics: 2 l Biostat B reactor (B. Braun Biotech International) with a working volume of 1.2 l, operating with a foam recycling system. The reactor was aerated by a sparger at 3 l air/min. The culture medium was inoculated with a 24 h inoculum. Aeration levels were set at 0.3 vvm and 0.8 vvm, the first being the lowest used in this study and the second the highest. Finally, to investigate the mass transfer, the oxygen transfer rate (OTR), volumetric mass transfer coefficient (k_La) and the oxygen utilisation rate (OUR) were investigated under the standard conditions of agitation (500 rpm), and aeration levels of 0.4 vvm (with and without pH control) and 0.3 vvm.

6.3.1 Effect of agitation rate

As stated above, four different agitation rates were used in these experiments: 480 rpm, 500 rpm, 600 rpm and 800 rpm. In all cases, the aeration rate was maintained constant at 0.8 vvm.

Under the growth conditions of 480 rpm, 0.8 vvm and no pH control, the cell number increased from 5.05×10^6 cells per ml on inoculation to a maximum cell number of 1.98×10^{10} cells per ml. The exponential growth phase from 8 to 20 hours was

characterized by a specific growth rate of 0.249 h^{-1}. The pH and ST showed similar decreasing trends (Figure 6.6) from the 8th hour of the process. Between 8 and 24 hours, surface tension decreased from 71.3 $mN.m^{-1}$ to 36.7 $mN.m^{-1}$., and pH decreased from 7.13 to 3.67 in this time interval. E_{index} change remained below detection limits in the first 16 hours, increasing to 13.3% at 26 hours. The DO decreased from 8 hours to a minimum of 65.42% at 16 hours. On reaching late exponential phase, an increase in DO was observed. This shows that DO decrease was mainly associated to the metabolic activity of microbial cells (Calik *et al.* 1997).

Assuming that one type of compound was being produced by *Ps. aeruginosa 2Bf,* it can be postulated that its surface tension reducing capacity is expressed at lower concentrations than its emulsifying power.

From the 24th hour (stationary phase), the surface tension remained constant whereas emulsification index continued to increase even after 26 hours. From this observation, it can be hypothesised that more than one surface active compound was produced by *Ps. aeruginosa 2Bf* (Cameotra & Singh 2008; Benincasa *et al.* 2002; Koch *et al.* 1991), and that these have varying emulsifying or surface tension reducing strength.

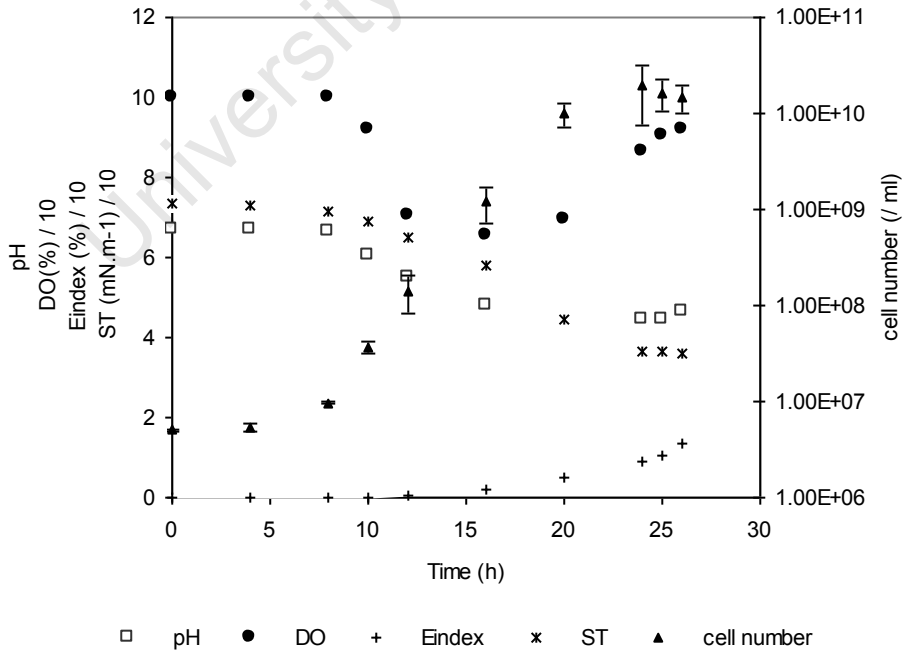


Figure 6.6: Monitoring of cell growth, pH, DO, E_{index} and surface tension, as a function of time, under the reactor operating conditions of 480 rpm, 0.8 vvm and uncontrolled pH

Under the conditions of 500 rpm, 0.8 vvm and no pH control, an initial concentration of $3.5x10^6$ cell per ml was used. As shown in Figure 6.7, the exponential phase continued till 21 hours at a maximum specific growth rate of 0.226 h^{-1}. The maximum cell number of $1.26x10^{10}$ cells per ml was attained. As illustrated in Figure 6.7, a rapid pH decrease, from pH 6.69 to pH 5.53, was noticed between 2 hours and 6 hours, wherafter it remained practically stable up to the 21st hour. At this stage, the pH value increased to the value of 5.64 at the 24th hour, which it maintained up to the end of the process. A small decrease in DO, from 99.97% to 97.2 was observed in the first 4 hours. From 4 hours, DO decreased more rapidly to reach the value of 69.2%, which it maintained up to 16 hours. From 16 hours, DO increased gradually and reached the value of 95.6 at 25 hours. This stabilization of DO at lower values during the exponential phase was attributed to a high cell activity and associated consumption of oxygen for cell growth (Chen *et al.* 2007). E_{index} remained below the limits of detection up to 6 hours. From 6 hours to 19 hours, it increased to 6.13%. Thereafter, as the culture entered late exponential phase, E_{index} increased more rapidly and reached the value of 18.8% at 27 hours. Surface tension showed a small decrease of only 4 units in the first 8 hours. Between 8 hours and 16 hours, ST decreased from 69.2 to 62.8 $mN.m^{-1}$, and reached its lowest value of 39 $mN.m^{-1}$ at 24 hours. The rapid decrease in surface tension in late exponential and stationary phases indicated a higher rate of biosurfactant production during these phases of growth (Chayabutra & Ju 2001; Christova *et al.* 2004; Koch *et al.* 1991).

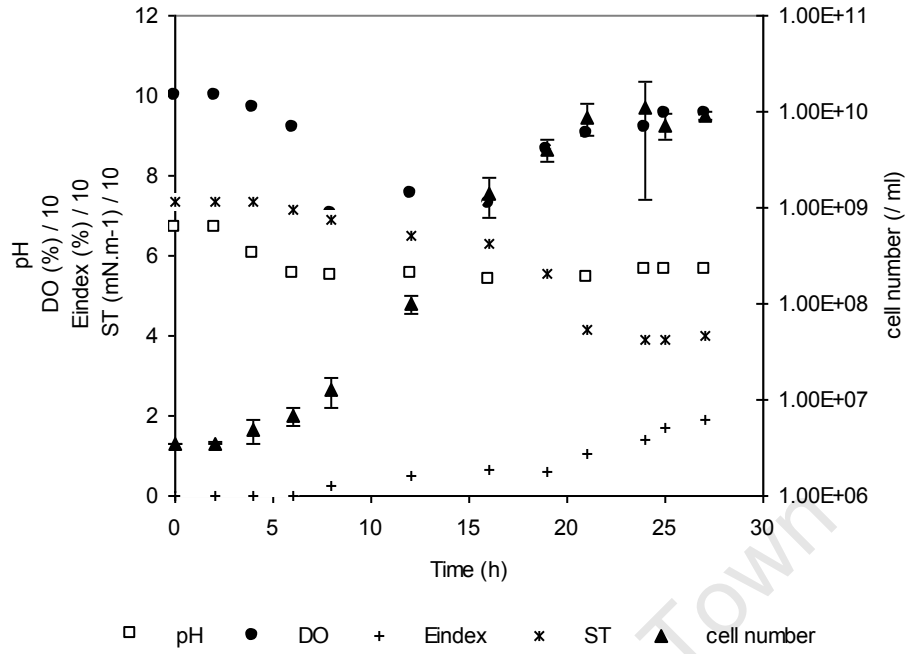


Figure 6.7: Monitoring of cell growth, pH, DO, E_{index} and surface tension, as a function of time, under the reactor operating conditions of 500 rpm, 0.8 vvm and uncontrolled pH

The results obtained when the reactor was operated under the conditions of 600 rpm, 0.8 vvm and uncontrolled pH, are illustrated in Figure 6.8. The initial cell concentration following inoculation was 2.14×10^6 cells per ml. The exponential phase continued till 20.5 hours at a maximum specific growth rate of 0.351 h^{-1}. The maximum number of cells attained was 1.23×10^{10} cells per ml. The pH decreased rapidly between 16 hour and 23 hours from pH 7.01 to pH 4.9. It was maintained at this value up to 27.5 hours. E_{index} increase was detected from 10 hours and increased gradually to 17.7% at 21 hours, then decreased to 14.8% at 27.5 hours. The DO decrease was very small up to 10 hours. From 10 hours to 18 hours, it decreased fro 97.1% to 72.2% and was stabilized at this value till the 21st hour, when it increased again to reach the value of 86.9% at 25.7 hours. ST decreased from the initial value of 73.5 $mN.m^{-1}$ to 65.5 $mN.m^{-1}$ in the initial 18 hours. Thereafter it decreased rapidly to 49 $mN.m^{-1}$ at 23 hours. It remained at this level up to the end of the process. The increase of E_{index} and the decrease of surface tension, pH & dissolved oxygen occurred mainly during the late exponential phase (Koch *et al.* 1991).

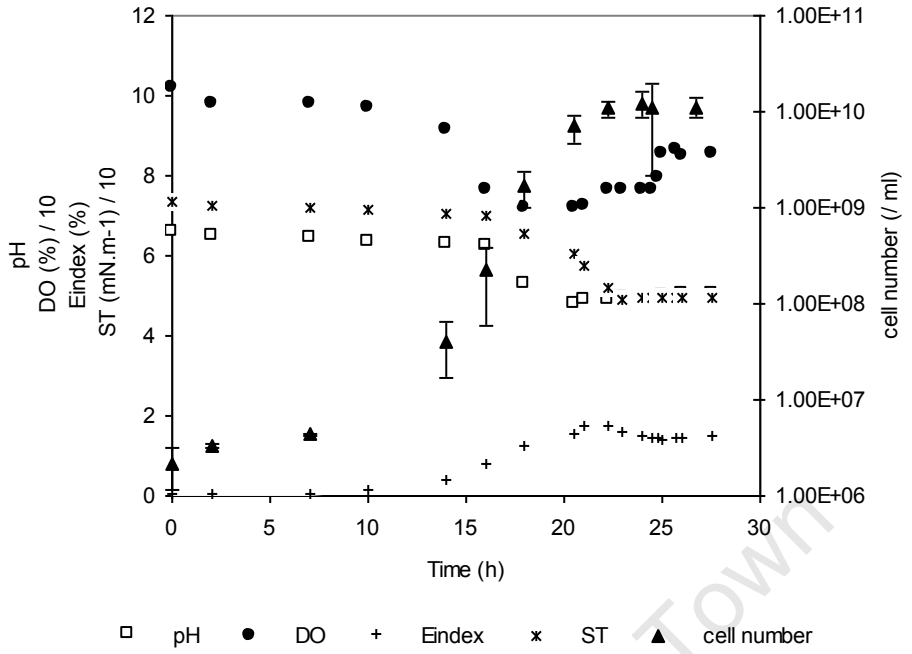


Figure 6.8: Monitoring of cell growth, pH, DO, E_{index} and surface tension, as a function of time, under the reactor operating conditions of 600 rpm, 0.8 vvm and uncontrolled pH

Under the operating conditions of 800 rpm, 0.8 vvm and uncontrolled pH, poor microbial growth was observed. The initial cell number on inoculation was 1.8×10^6 cell per ml. The exponential phase was complete by 19 hours. A maximum specific growth rate of 0.139 h^{-1} was calculated. A maximum cell number of 5.9×10^7 cells per ml was attained. The minimum pH observed in the whole process was 6.71. The DO decreased from 100% to 96.7% between 0 hours and 8.2 hours, and reached its lowest value, 81.1% at 17 hours. DO did not show any further decrease but increased between 25.5 hours and 27 hours from 81.1% to 84.7%. The significant decrease in ST, from 68.8 $mN.m^{-1}$ to 56.7 $mN.m^{-1}$, was observed between 17 hours and 20 hours. E_{index} remained below detection level up to the 15th hour, then increased to 4% at 27 hours. These results are shown in Figure 6.9. The poor biosurfactant production in these conditions resulted in poor alkane solubilisation (Zhang & Miller 1995; *Koch et al.* 1991), which in turn led to less reproducible results, as shown by the cell growth error bars in Figure 6.9. The main results obtained in this series of experiments performed to investigate the effect of agitation rate (Section 6.3.1), are summarised in Table 6.1.

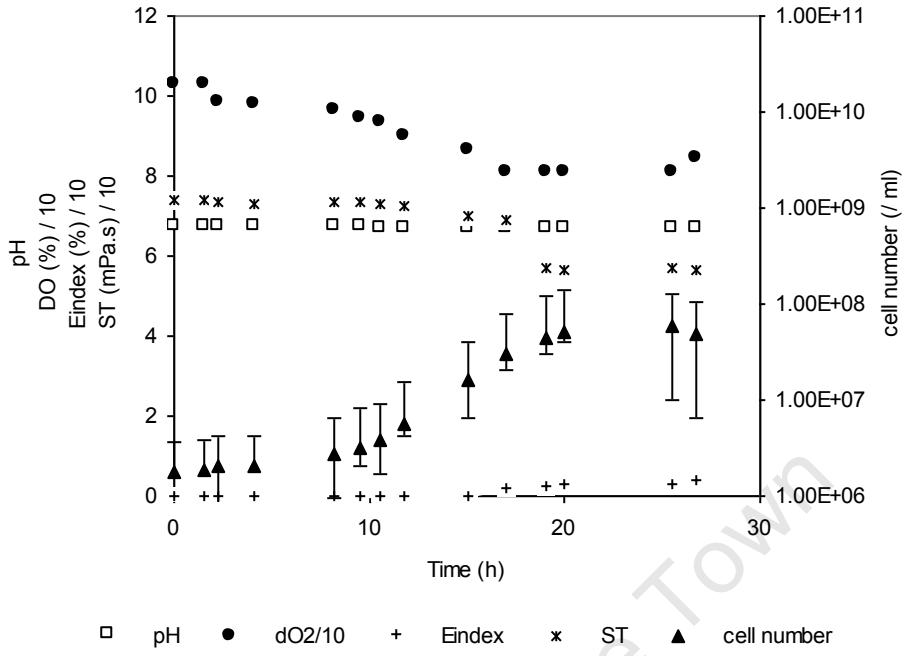


Figure 6.9: Monitoring of cell growth, pH, DO, E_{index} and surface tension, as a function of time, under the reactor operating conditions of 800 rpm, 0.8 vvm and uncontrolled pH

Table 6.1: Summary of the main results obtained by operating the reactor at a fixed aeration rate of 0.8 vvm and variable agitation rates: 480 rpm, 500 rpm, 600 rpm and 800 rpm

	480 rpm	**500 rpm**	**600 rpm**	**800 rpm**
Time elapsed at the end of exponential phase (h)	20	21	20.5	19
μ_{max} (h^{-1})	0.249	0.226	0.351	0.139
X_{max} ($cell.ml^{-1}$)	$1.98x10^{10}$	$1.26x10^{10}$	$1.23x10^{10}$	$5.9x10^{7}$
LN (X_{max}/X_0)	8.0887	8.0299	8.6556	3.4906
DO_{min} (%)	65.42	69.2	72.2	81.1
ΔpH_{max}	3.74	3.43	2.45	0.04
$E_{index\ (max)}$ (%)	13.3	18.8	17.72	4
Productivity on biomass ($cell.l^{-1}.h^{-1}$)	$1.65x10^{11}$	$9.00x10^{10}$	$1.03x10^{11}$	$4.63x10^{08}$
Productivity on E_{index} ($l^{-1}.h^{-1}$)*	102.31	139.26	128.87	29.91
ΔST_{max} ($mN.m^{-1}$)	37.4	34.3	24.5	17.7

*E_{index} is expressed in %

In order to identify and propose the best agitation rate with regard to both biomass growth and biosurfactant production, the information provided in Table 6.1 was used to establish a relative order of performance, by rating the different agitation rates used at a fixed aeration rate, as shown in Table 6.2.

Table 6.2: Order of the reactor performance under agitation rates of 480 rpm, 500 rpm, 600 rpm and 800 rpm, and a fixed aeration rate of 0.8 vvm

	480 rpm	**500 rpm**	**600 rpm**	**800 rpm**
Time elapsed at the end of exponential phase (h)	3rd	1st	2nd	4th
μ_{max} (s^{-1})	2nd	3rd	1st	4th
X_{max} ($cell.ml^{-1}$)	1st	2nd	3rd	4th
LN (X_{max}/X_0)	2nd	3rd	1st	4th
DO_{min} (%)	1st	2nd	3rd	4th
ΔpH_{max}	1st	2nd	3rd	4th
$E_{index\ (max)}$ (%)	3rd	1st	2nd	4th
Productivity on biomass ($cell.l^{-1}.h^{-1}$)	1st	3rd	2nd	4th
Productivity on E_{index} ($l^{-1}.h^{-1}$)	3rd	1st	2nd	4th
ΔST_{max} ($mN.m^{-1}$)	1st	2nd	3rd	4th

From the data in Table 6.2, it was noticed that agitation at 600 rpm presented highest μ_{max} and LN (X_{max}/X_0) at an equivalent maximum cell concentration to that achieved at 480 and 500 rpm. The results of DO_{min} showed a better performance of the reactor at agitation rates of 480 rpm and 500 rpm, indicating that the slower kinetics result in less oxygen depletion. On considering ST and E_{index} results, agitation rates of 500 and 600 rpm favoured emulsification while 480 and 500 rpm favoured reduction in surface tension. Productivity on emulsification was highest with the agitation rate of 500 rpm, while the highest productivity on biomass was obtained with the agitation rate of 480 rpm. Therefore, since biosurfactants are characterised by their surface tension reducing and emulsification power (Lee *et al.* 1998; Janssen *et al.* 2002; Neu 1996), 500 rpm can be proposed as preferable for biosurfactant production by *Ps. aeruginosa* 2Bf in a stirred tank batch reactor system, under hydrocarbon induction. Most importantly, hydrodynamic conditions resembling those achieved at 800 rpm in the benchtop bioreactor must be avoided for effective growth of *Ps. aeruginosa 2Bf* and its use for biosurfactant production.

6.3.2 Effect of aeration rates

The influence of aeration was studied at four different levels: 0.3 vvm, 0.4 vvm, 0.6 vvm and 0.8 vvm. The rotational speed of the Rushton turbines was kept constant at 500 rpm across this study. At each level, cell concentration, emulsification index, pH, dissolved oxygen and surface tension were monitored during the process. The data under these conditions are presented graphically and described. Thereafter, these are compared to propose optimum operating conditions.

Under the reactor operating conditions of 500 rpm, 0.3 vvm and uncontrolled pH, *Pseudomonas aeruginosa 2Bf* culture grew from an initial cell number of $2.0x10^6$ cells per ml. The exponential growth phase was characterised by a growth rate of 0.308 h^{-1}. The maximum cell number of $3.0x10^{10}$ cells per ml was achieved. The pH varied from pH 6.2 to 4.6 between 12 and 15 hours, and was relatively stable at this level until the 23rd hour, and showed an increasing trend from the 23rd hour. For E_{index} and DO, noticeable variation was observed from the 7th hour. The first showed a gradual increase from 0% to 39.1% at 27 hours, while the second decreased from 94.6% to 62% at 12 hours. It remained at this level and showed an increasing trend from the 19 hours. The ST showed a rapid decrease from 72 $mN.m^{-1}$ at 18 hours to 49.5 $mN.m^{-1}$ at 27 hours, suggesting a continuing decreasing trend. These data, illustrated in Figure 6.10, showed that more than one biosurfactant compound was being synthesised (Desai & Banat 1997; Koch *et al.* 1991), and that some of these which were more emulsifying than surface tension reducing agents were produced earlier than the others.

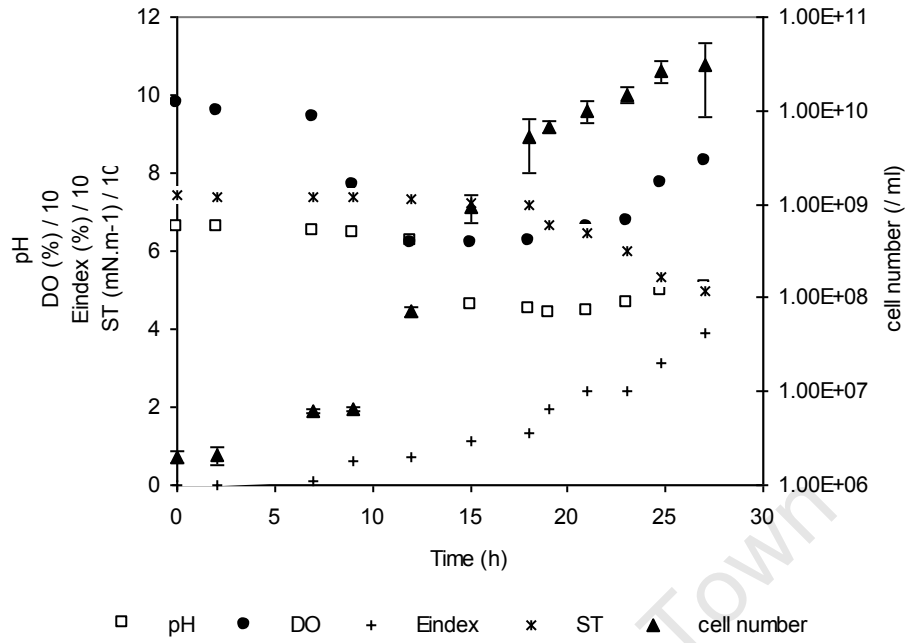


Figure 6.10: Monitoring of cell growth, pH, DO, E_{index} and surface tension, as a function of time, under the reactor operating conditions of 0.3 vvm, 500 rpm, and uncontrolled pH

The data resulting from the reactor operation at 0.4 vvm, 500 rpm and uncontrolled pH are shown graphically in Figure 6.11. The initial cell number was 3.08×10^6 cells per ml. The exponential growth phase, completed by 20.5 hours, was characterized by a growth rate of 0.238 h^{-1}, while the maximum cell number attained was 2.61×10^{10} cells per ml. The DO decreased from an initial value of 97.9% to 88.3% at 14 hours, reaching its lowest value of 73%, at 16 hours. The pH decreased from the 6.26 to 4.8 between 16 and 20.5 hours, and remained at this value up to the end of the experiment. The ST decreased rapidly from 73 $mN.m^{-1}$ to 4.99 $mN.m^{-1}$ between 18 hours and 26 hours. E_{index} increased from 3.6% to 34.8% between 14 hours and 26 hours, and was still showing an increasing trend.

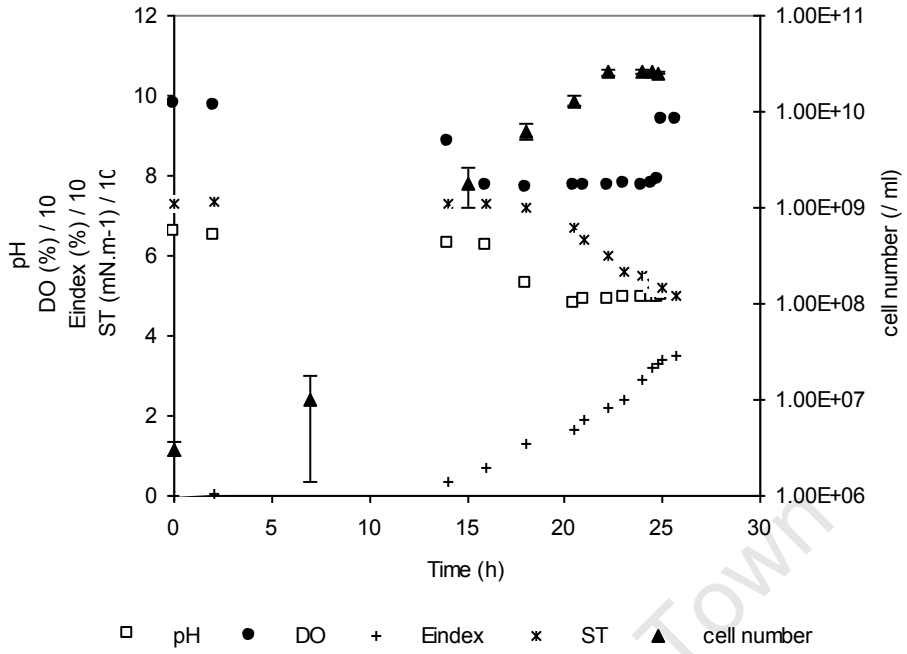


Figure 6.11: Monitoring of cell growth, pH, DO, E_{index} and surface tension, as a function of time, under the reactor operating conditions of 0.4 vvm, 500 rpm, and uncontrolled pH

Under the reactor operating conditions of 0.6 vvm, 500 rpm and uncontrolled pH, the cell number increased from an initial cell number of $3.2x10^6$ cells per ml. Exponential growth was observed from 6.4 hours to 21 hours. In this exponential phase which was characterised by a maximum specific growth rate of 0.210 h^{-1}, the cell number increased from $3.9x10^6$ cells per ml to $5.9x10^9$ cells per ml. The maximum cell number of 1.09 $x10^{10}$ cells per ml was attained. The pH decrease through the process was small, from pH 6.71 at 4 hours to 5.9 at 27 hours. From the initial value of 100%, DO decreased to 97.1% in the first 4 hours, and reached its lowest value of 82.2% at 21 hours. E_{index} remained below the detection limit till the 18th hour, thereafter it increased to 25% at 27 hours. The ST showed a small decrease from 72.9 $mN.m^{-1}$ to 64.4 $mN.m^{-1}$, between 4 hours and 15 hours. From 15 hours, it decreased rapidly to the value of 38.3 $mN.m^{-1}$ at 24 hours. ST remained unchanged up to the end of the experiment. These data are shown in Figure 6.12.

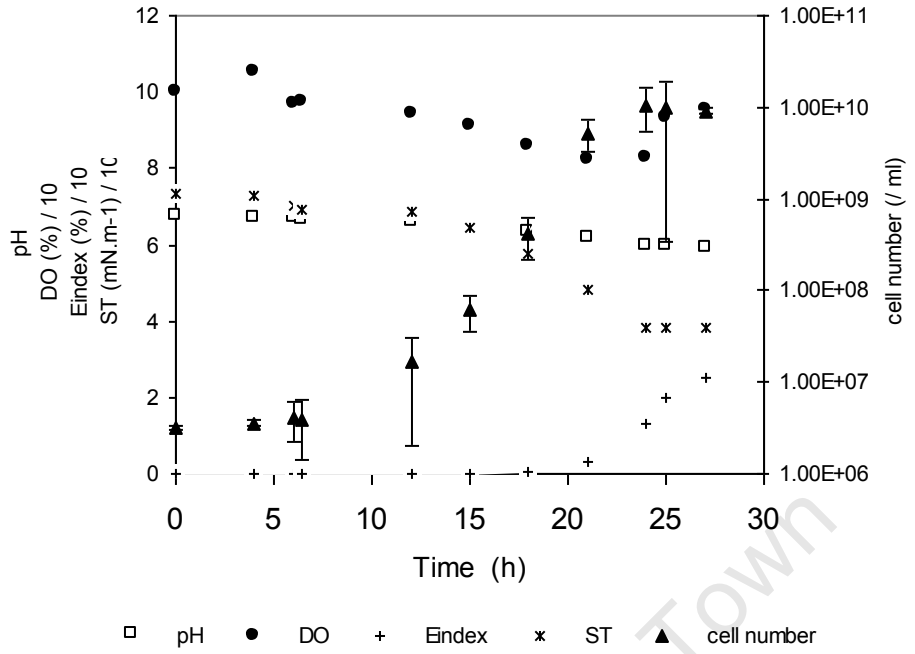


Figure 6.12: Monitoring of cell growth, pH, DO, E_{index} and surface tension, as a function of time, under the reactor operating conditions of 0.6 vvm, 500 rpm, and uncontrolled pH

The results corresponding to the reactor operation at 0.8 vvm, 500 rpm and uncontrolled pH are illustrated graphically in Figure 6.13. The initial cell number on inoculation was $3.5x10^6$ cells per ml. The exponential growth phase lasted until 19 hours and was characterized by a maximum specific growth rate of 0.236 h^{-1}. The maximum cell number of $1.08x10^{10}$ cells per ml was achieved. The pH showed a remarkable decrease, from pH 6.69 to 5.53, between 2 hours and 6 hours, and remained at this level for the rest of the experiment. The DO showed a similar trend in the first 6 hours, decreasing from 99.87% at 2 hours to 70.3% at 6 hours. The DO increased gradually from 16 hours, and reached the value of 95.4% at 25 hours. The E_{index} remained below detection level in the first 6 hours. From the 6th hour, it increased moderately to 6.1% at 19 hours. Between 19 hours and 27 hours, E_{index} increased more rapidly to the final value of 18.8%. The ST decreased after 4 hours. Between 4 hours and 16 hours, ST decreased from 73 $mN.m^{-1}$ to 63 $mN.m^{-1}$. Thereafter it decreased more rapidly and reached the value of 39 $mN.m^{-1}$ at 24 hours.

From these data, and as earlier suspected in the study of the effect of agitation rate, it can be hypothesised that more than one biosurfactant was being released into the growth medium (Cameotra & Singh 2008; Benincasa *et al.* 2002; Koch *et al.* 1991).

In such a case, these data suggest that either some biosurfactants were produced in early exponential phase while others were synthesized in late exponential phase, or the rate of biosurfactant production was higher in late exponential phase (Maier & Soberon-Chavez 2000; Christova *et al.* 2004).

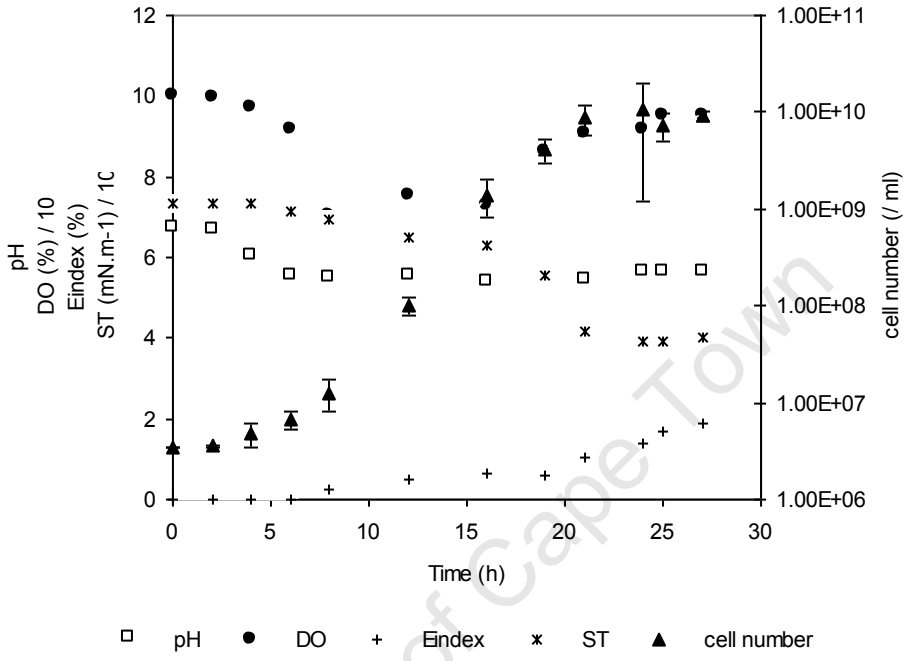


Figure 6.13: Monitoring of cell growth, pH, DO, E_{index} and surface tension, as a function of time, under the reactor operating conditions of 0.8 vvm, 500 rpm, and uncontrolled pH

The main results obtained in the study of the influence of aeration rate on the process were summarised in Table 6.3. Table 6.3 was used to generate a comparative table, in which the different aeration rates used were rated in performance relative to each other, as shown in Table 6.4.

Table 6.3: Summary of the main results obtained by operating the reactor at a fixed agitation rate of 500 rpm and variable aeration rates: 0.3 vvm, 0.4 vvm, 0.6 vvm and 0.8 vvm

	0.3 vvm	**0.4 vvm**	**0.6 vvm**	**0.8 vvm**
Time elapsed at end of exponential phase (h)	18 – 23*	22.2	21	21
μ_{max} (h^{-1})	0.308	0.238	0.210	0.236
X_{max} (cell.ml^{-1})	$3.0x10^{10}$	$2.61x10^{10}$	$1.09\ x10^{10}$	$1.08x10^{10}$
LN (X_{max}/X_0)	9.70	9.04	8.11	8.03
DO_{min} (%)	62	77.25	82.2	70.3
ΔpH_{max}	2.2	1.78	0.85	1.3
$E_{index\ (max)}$ (%)	39.1	34.8	25.12	18.8
Productivity on biomass (cell.l^{-1}.h^{-1})	$2.22x10^{11}$	$2.35x10^{11}$	$9.08x10^{10}$	$9.0x10^{10}$
Productivity on E_{index} (l^{-1}.h^{-1})	289.63	270.82	186.07	139.26
ΔST_{max} (mN.m^{-1})	24.9	23.3	35.3	34.3

* between 18 and 23

Table 6. 4: Order of the reactor performance under aeration rates of 0.3 vvm, 0.4 vvm, 0.6 vvm and 0.8 vvm, and a fixed agitation rate of 500 rpm

	0.3 vvm	**0.4 vvm**	**0.6 vvm**	**0.8 vvm**
Time elapsed at the end of exponential phase (h)	2^{nd}	1^{st}	*3^{rd}	*3^{rd}
μ_{max} (h^{-1})	1^{st}	2^{nd}	4^{th}	3^{rd}
X_{max} (cell.ml^{-1})	1^{st}	2^{nd}	3^{rd}	4^{th}
LN (X_{max}/X_0)	1^{st}	2^{nd}	3^{rd}	4^{th}
$E_{index\ (max)}$ (%)	1^{st}	2^{nd}	3^{rd}	4^{th}
Productivity on biomass (cell.l^{-1}.h^{-1})	2^{nd}	1^{st}	3^{rd}	4^{th}
Productivity on E_{index} (l^{-1}.h^{-1})	1^{st}	2^{nd}	3^{rd}	4^{th}
ΔST_{max} (mN.m^{-1})	3^{rd}	4^{th}	1^{st}	2^{nd}

* 0.6 vvm = 0.8 vvm

Aeration rate is one of the most important factors affecting the mass transfer in aerobic bioprocesses (Sivaprakasam *et al.* 2008; Benincasa *et al.* 2002). The data in Table 6.3 and Table 6.4 showed that the reactor behaviour is affected by changing the rate of aeration. Based on these data, the four aeration rates used can be rated in terms of biomass production in the following order of performance: 0.3 vvm > 0.4 vvm > 0.6 vvm > 0.8 vvm. Similarly emulsification is rated in the order: 0.3 vvm > 0.4

vvm > 0.6 vvm > 0.8 vvm, whereas for ΔST_{max}, aeration of 0.6 vvm provided the highest change in surface tension. Finalising a recommendation for the best aeration rate for biosurfactant production is complicated by the fact that biosurfactant production, seen both in terms of emulsification and surface tension, was not at its maximum on termination of the experiment.

6.3.3 Optimisation of important reactor parameters

Optimisation of reactor conditions was done using statistical analysis for a factorial experimental design where 2 factors were varied simultaneously each at a given number of levels, and measurements of the variable for response were carried out. This was done with the aim of identifying and proposing the best aeration and agitation rates, as well as the best way of controlling foam among three possibilities envisaged.

Although biosurfactant appeared to be produced from the mid exponential phase, it was assumed that the best conditions are those leading to high rates of production for both biosurfactant and biomass.

In this sub-chapter, additional data, not presented in previous sections, are presented as appropriate. These additional data correspond to agitation rates of 210 rpm at 0.3 vvm and 0.8 vvm, 800 rpm at 0.3 vvm, 600 rpm at 0.3 vvm. In order to investigate the significance of the different parameters at their respective levels, two response variables were investigated for agitation-aeration study, one using maximum level of biomass achieved as the variable for response, the other using the maximum emulsification index obtained achieved in the first 26 hours. In the case of foam control, which was essentially meant to mitigate or eliminate the adverse effect of foaming on the biomass growth (Wilson 1989; Chayabutra & Ju 2001), only one variable for response, the highest level of biomass production achieved, was used, and the maximum biomass attained was measured in each case, as shown in Table 6.7.

The experimental data corresponding to the reactor operating conditions of 210 rpm at 0.3 vvm, 210 rpm at 0.8 vvm, 600 rpm and 0.3 vvm, 600 rpm at 0.8 vvm and 800 rpm at 0.3 vvm, are provided as used in this section. The full data for biomass and E_{index} on these runs for 600 rpm at 0.3 vvm and 800 rpm at 0.3 vvm, are provided in Appendix X. For the 210 rpm at 0.3 vvm and 0.8 vvm, sampling was done at the only

points shown in this section. These data are illustrated in Table 6.5, Table 6.6 and Table 6.7.

Table 6.5: Data set used to generate the Analysis of Variance Table during the optimisation of reactor agitation and aeration, with respect to the maximum biomass produced during the process

	Agitation1	Agitation2	Agitation3
	210	500rpm	800rpm
Aeration1 0.3vvm	1.08E+08	3.62E+10	2.53E+09
	2.12E+08	2.80E+10	1.00E+08
Aeration2 0.8vvm	2.34E+09	8.85E+09	5.91E+07
	9.62E+08	7.50E+09	4.85E+07

Table 6. 6: Data set used to generate the Analysis of Variance Table during the optimisation of reactor agitation and aeration, with respect to the maximum emulsification index achieved at 26 h

	Agitation1 500rpm	Agitation2 600rpm	Agitation3 800rpm
Aeration1 0.3vvm	35.13	27.9	16
	36.01	27.5	14.3
Aeration2 0.8vvm	17.4	17.52	4
	16.9	17.52	4

Table 6. 7: Data set used to generate the Analysis of Variance Table during the optimisation of the foam control inside the reactor, with respect to the maximum biomass produced during the process

	AF	FB-1	FB-2
Agitation1 210 rpm	3.84E+07	2.22E+07	1.05E+08
	4.04E+07	6.75E+07	1.08E+08
	4.62E+07	8.38E+07	2.12E+08
Agitation2 500 rpm	9.61E+08	2.14E+08	3.62E+10
	7.12E+08	2.63E+09	2.80E+10
	7.00E+08	1.46E+09	3.67E+09
Agitation3 800 rpm	1.35E+08	2.48E+09	2.53E+09
	4.81E+08	9.60E+07	1.00E+08
	7.25E+08	9.98E+07	2.37E+09

6.3.3.1 Optimisation of aeration and agitation rates

In order to identify the most favourable aeration and agitation conditions for biomass and biosurfactant production, the study was carried out using two different variables for response, while varying the other factors at selected rates.

In the case where biomass was used as the variable for response, a two-factor factorial design was used at 2 levels of aeration rate and 3 levels of agitation rate, namely 0.3 vvm, 0.8 vvm and 210 rpm, 500 rpm and 800 rpm respectively. The analysis of variance summarised in Table 6.8 showed that *F(interaction)* = 30.376 was greater than *F(critical)* at 5%-confidence level = 5.143 This means that there was a significant influence of the interaction between agitation rate and aeration rate. It was further noticed that both agitation and aeration rate had a significant impact on the process since in both cases *F-value* is greater than *F-crit*. Interaction between the two factors was visualised by plotting the total responses obtained at each parameter combination. The lack of parallelism between trends, especially at lower agitation rates, indicated a significant interaction between factors (Montgomery & Runger 2007; Montgomery 2005), as illustrated in Figure 6.14. The data used to generate the analysis of variance Table for this study were provided in Table 6.5.

Table 6.8: Analysis of variance table for the optimisation study of biomass formation as a function of aeration-agitation

Anova: Two-Factor With Replication							
SUMMARY	210 rpm	500 rpm	800 rpm	Total			
0.3 vvm							
Count	2	2	2	6			
Sum	3.2E+08	6.42E+10	2.63E+09	6.72E+10			
Average	1.6E+08	3.21E+10	1.32E+09	1.12E+10			
Variance	5.41E+15	3.36E+19	2.95E+18	2.7E+20			
0.8 vvm							
Count	2	2	2	6			
Sum	3.3E+09	1.63E+10	1.08E+08	1.98E+10			
Average	1.65E+09	8.17E+09	53775000	3.29E+09			
Variance	9.49E+17	9.05E+17	5.57E+13	1.52E+19			
Total							
Count	4	4	4				
Sum	3.62E+09	8.05E+10	2.74E+09				
Average	9.06E+08	2.01E+10	6.84E+08				
Variance	1.06E+18	2.02E+20	1.51E+18				
ANOVA							
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*	
Aeration	1.87E+20	1	1.87E+20	29.22469	0.001655	5.987378	*significant (+)*
Agitation	9.98E+20	2	4.99E+20	77.87642	5.1E-05	5.143253	*significant (++)*
Interaction	3.89E+20	2	1.95E+20	30.37625	0.000726	5.143253	*significant (+)*
Within	3.84E+19	6	6.41E+18				
Total	1.61E+21	11					

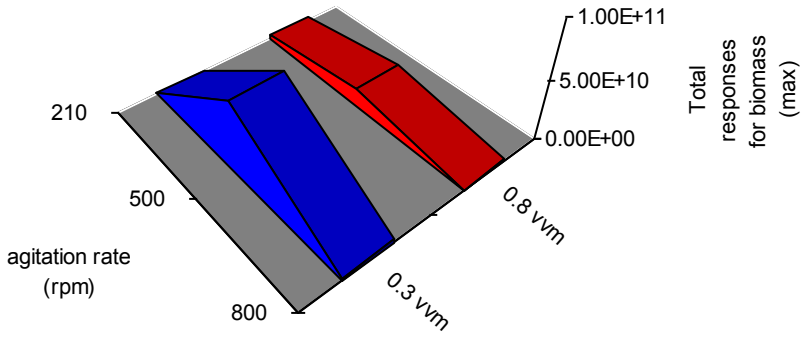


Figure 6.14: Total responses at each factor combination between aeration and agitation rates, with biomass as the variable for response

Figure 6.14 showed agreement with analysis in Table 6.8, confirming that aeration1 (0.3 vvm) favored growth more than aeration2 (0.8 vvm). From this plot, it was noticed that aeration2 (0.8 vvm) performed better at lower agitation rates, and, as the speed was increased, aeration1 started performing better. The plot revealed that at high agitation rate, the interaction component became negligible, and the dependence of growth on aeration rate lost significance, which was evidenced by the parallelism in the plot (Hicks & Turner 1999). It was further observed that in general, cell growth was enhanced by the agitation rate of 500 rpm. Overall, 0.3 vvm and 500 rpm gave best results.

The same investigation was repeated with the emulsification index as the variable for response, but this time the three levels of agitation rates were chosen among those used for the study of the effect of agitation and aeration rates on the reactor behavior. Agitation rates of 500 rpm, 600 rpm and 800 rpm were used, each time at two levels of aeration rates, 0.3 vvm and 0.8 vvm. There were labeled Agitation1, Agitation2, Agitation3 and Aeratio1 and Aeration2 respectively. The results obtained are detailed in Table 6.9 revealed that *F(interaction)* = 220.6319 was much greater than *F(critical)* at 5%-confidence level = 5.143253, thus indicating a significant interaction between agitation rate and aeration rate during biosurfactant production (Haaland 1989; Montgomery & Runger 2007; Wardlaw 2000). It was further noticed

that both agitation and aeration rate had a more significant impact on *biosurfactant production*, as reflected by an *F-value* that is greater than *F-crit*, in both cases. Interaction between the two factors was visualised by plotting the total responses obtained at each parameter combination. The lack of parallelism at the lower levels of agitation indicated a significant interaction between factors (Montgomery & Runger 2007; Montgomery 2005), as illustrated in Figure 6.15. The data used to generate the analysis of variance Table for this study were provided in Table 6.6.

Table 6.9: Analysis of variance table for the optimisation study of biosurfactant production, quantified by *kerosene-aqueous* system's emulsification index measurement, as a function of aeration-agitation

Anova: Two-Factor With Replication							
SUMMARY	500 rpm	600 rpm	800 rpm	Total			
0.3 vvm							
Count	2	2	2	6			
Sum	71.14	37.4	30.3	138.84			
Average	35.57	18.7	15.15	23.14			
Variance	0.3872	0.08	1.445	95.60588			
0.8 vvm							
Count	2	2	2	6			
Sum	34.3	35.04	8	77.34			
Average	17.15	17.52	4	12.89			
Variance	0.125	0	0	47.47164			
Total							
Count	4	4	4				
Sum	105.44	72.44	38.3				
Average	26.36	18.11	9.575				
Variance	113.2695	0.4908	41.9225				
ANOVA							
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*	
Aeration	315.1875	1	315.1875	928.2962	8.3E-08	5.987378	*significant ++++*
Agitation	563.5266	2	281.7633	829.8546	4.67E-08	5.143253	*significant ++++*
Interaction	149.8238	2	74.9119	220.6319	2.41E-06	5.143253	*significant +++*
Within	2.0372	6	0.339533				
Total	1030.575	11					

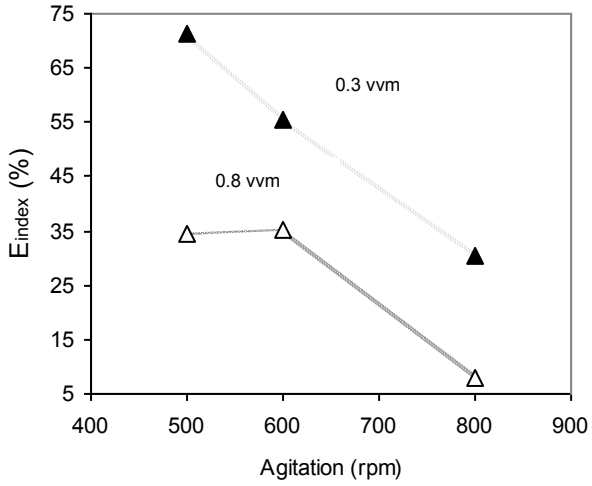


Figure 6.15: Total responses at each factor combination between aeration and agitation rates, with the E_{index} as the variable for response

Figure 6.15 shows that at all the levels of agitation rate, aeration1 (0.3 vvm) was more favorable to biosurfactant production than aeration2 (0.8 vvm). It was noticed that the significance of the interaction term reflected by the ANOVA Table applies to lower levels of agitation rates exclusively. This lack of interaction between the two variables measured was evidenced by the parallelism observed at higher rates of agitation (Hicks & Turner 1999). In general, biosurfactant production was enhanced by the 500 rpm. The aeration rate of 0.3 vvm and the agitation rate of 500 rpm provided best results. Lack of significant impact of *aeration-agitation* interaction at high agitation rates was observed for both biomass and emulsification index as variables for response. As reported by Sivapracasam *et al.* (2008), at a certain level of increasing aeration rates, the mass transfer coefficient ceases to be influenced by further increases. This also applies to agitation rates. Therefore, it can be hypothesised that at a certain level of increased aeration or agitation, biomass production ceases to be influenced by the interaction between the two parameters, but can still be influenced by either of the parameters individually.

6.3.3.2 Foam control optimisation

During foam control optimisation, the variable for response was the maximum biomass achieved during the process. A Two-factor factorial design at three levels was chosen as explained Section 4.5.10. The type of foam control and the agitation rate were chosen as the varying factors, while the aeration rate was maintained at

0.3 vvm during these experiments. The levels of agitation considered were 210 rpm labelled Agitation1, 500 rpm labelled Agitation2 and 800 rpm labelled 'Agitation3', while the types of foam control used were AF, FB-1 and FB-2, corresponding to the use of anti-foam, the two-blade paddle mechanical foam breaker and the modified two-blade paddle with three slits mechanical foam breaker respectively. The mechanical foam breakers were described in Section 4.2.5. Use of AF, which is generally a surfactant by chemical nature, needed to be avoided regardless its performance, because one of the main objectives of this research project was to produce surface active agents, and eventual occurrence of surfactant-biosurfactant interferences during the process could be anticipated.

The results obtained during this study were pulled into an ANOVA Table in order to assess the significance of the effect of different factors and their interaction on the process (Montgomery & Runger 2007; Montgomery 2005), as shown in Table 6.10. The data used to generate the analysis of variance Table for this study were provided in Table 6.7.

Table 6.10 revealed a relatively significant agitation influence on the system, while the interaction between foam control and agitation rate showed no significant impact. This translated into parallel trends on the plot of total responses against agitation rate, given in Figure 6.16. Table 6.10 showed that *F(interaction)* of 0.503 was smaller than the *F(critical)* at 5%-confidence level = 2.928. This means that the interaction term was not signifant in this case (Montgomery 2005; Hicks & Turner 1999). The same situation was observed in the case of the type of foam control, where the *F(value)* of 2.226, of the type of foam control, was smaller than the F(critical) at 5%-confidence level of 3.555.

Table 6.10: Analysis of variance table for the optimisation study of biomass formation as a function of *type of foam control-agitation*

Anova: Two-Factor With Replication							
SUMMARY	210 rpm	500 rpm	600 rpm	Total			
AF							
Count	3	3	3	9			
Sum	1.25E+08	2.37E+09	9.28E+08	3.43E+09			
Average	41666667	7.91E+08	3.09E+08	3.81E+08			
Variance	1.64E+13	2.17E+16	1.31E+17	1.46E+17			
FB-1							
Count	3	3	3	9			
Sum	3.65E+08	4.3E+09	2.68E+09	7.35E+09			
Average	1.22E+08	1.43E+09	8.93E+08	8.16E+08			
Variance	6.45E+15	1.46E+18	1.89E+18	1.17E+18			
FB-2							
Count	3	3	3	9			
Sum	2.82E+08	6.61E+09	5E+09	1.19E+10			
Average	93833336	2.2E+09	1.67E+09	1.32E+09			
Variance	2.29E+14	2.7E+18	1.85E+18	2.04E+18			
Total							
Count	9	9	9				
Sum	7.72E+08	1.33E+10	8.61E+09				
Average	85755556	1.48E+09	9.56E+08				
Variance	2.91E+15	1.42E+18	1.32E+18				
ANOVA							
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*	
Foam control	3.99E+18	2	1.99E+18	2.225963	0.136827	3.554557	*not significant (-)*
Agitation	8.88E+18	2	4.44E+18	4.960117	0.019239	3.554557	*significant (+)*
Interaction	1.8E+18	4	4.5E+17	0.503064	0.733963	2.927744	*not significant (- - -)*
Within	1.61E+19	18	8.95E+17				
Total	3.08E+19	26					

The total responses were plotted as a function of agitation rate as illustrated in Figure 6.16. The parallelism between the trends in Figure 6.16 confirmed a quasi-absence of a significant impact of the '*foam control type-agitation rate*' interaction on the variable for response (Hicks & Turner 1999; Montgomery & Runger 2007). From this observation, it can be hypothesised that the batch reactor process studied was affected by the type of foam breakers and agitation rates, individually, but their

interaction had no effect on the process. This means that it is possible to fix an optimum agitation rate, and study the performance of a certain number of mechanical foam breakers in a given process.

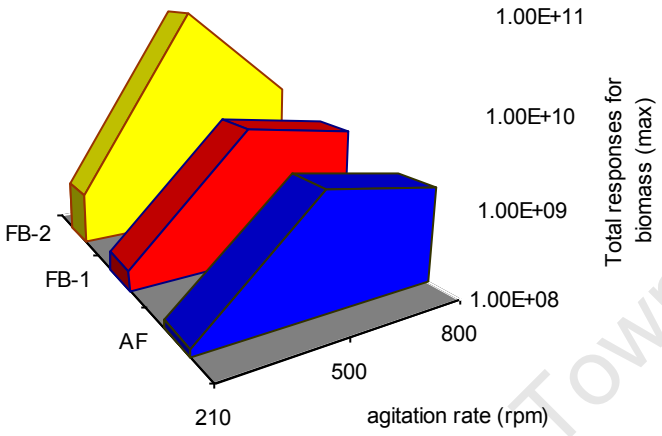


Figure 6.16: Total responses at each factor combination between Type of foam control and agitation rate, with the biomass as the variable for response

At all the levels of agitation, growth was higher with FB-2, followed by FB-1 and poor with AF. From low to intermediate agitation rate, growth increased with all the three types of foam control tested, while it decreased from intermediate to high agitation rates. For FB-1 and AF, the results obtained at intermediate and high agitation rates were comparable, while the intermediate agitation rate performed much better than the lower and the higher levels of agitation, with FB-2. The modified two blade paddle with 3-slit mechanical foam breaker was identified as offering the most efficient means to control foam formation during the production of biosurfactants in a stirred tank batch reactor system. This foam breaker, also described in Section 4.2.6.2 is illustrated in Figure 6.17. The effectiveness of the different types of foam control that were tested in this study was rated in the following descending order: FB-2, FB-1 and AF.

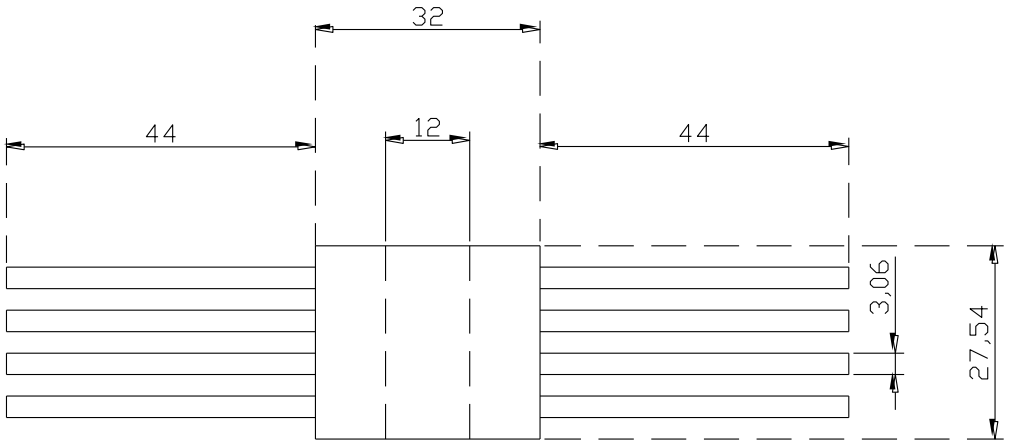


Figure 6.17: Modified Two-blade Paddle Mechanical foam breaker, proposed as the best means of controlling foam during biosurfactant production in a stirred tank batch reactor system

6.3.4 Mass transfer study

As reported in Chapter 1, the major challenges in the alkane bioprocesses are flammability, immiscibility with aqueous phases, inhibition of cell growth at certain hydrocarbon concentrations, insolubility (Rothen *et al.* 1998) and mass transfer limitations with regard to both oxygen and hydrocarbon substrate (Preusting *et al.* 1993; Schmid *et al*. 1998). It was also noted that hydrocarbon usage as substrate is associated with a high oxygen demand, relative to carbohydrates, owing to their oxidation state (Bailey & Ollis 1986). This makes oxygen supply an important challenge in all bioprocesses involving hydrocarbon substrates. In the case of hydrocarbon substrates, all oxygen is typically supplied from the gas phase, and its mass transfer depends strongly on reactor mixing, particularly aeration and agitation rates applied to the system. Upon a good mixing, both the gas phase and the hydrocarbon phase are dispersed to fine bubbles and oil droplets of various sizes that play an important role in the oxygen transportation from bulk to microorganisms.

In this section, mass transfer was studied based on the volumetric mass transfer coefficient (k_La), the transfer rate of oxygen to cells, OTR and the utilization rate of oxygen by cells, OUR. These parameters are dependent on the oxygen saturation and actual concentrations, as well as the applied aeration and agitation rates. Two different combinations of agitation and aeration rates were used: 0.4 vvm at 500 rpm

without pH control and 0.3 vvm at 500 rpm without pH control, respectively labelled BO2, and BOX.

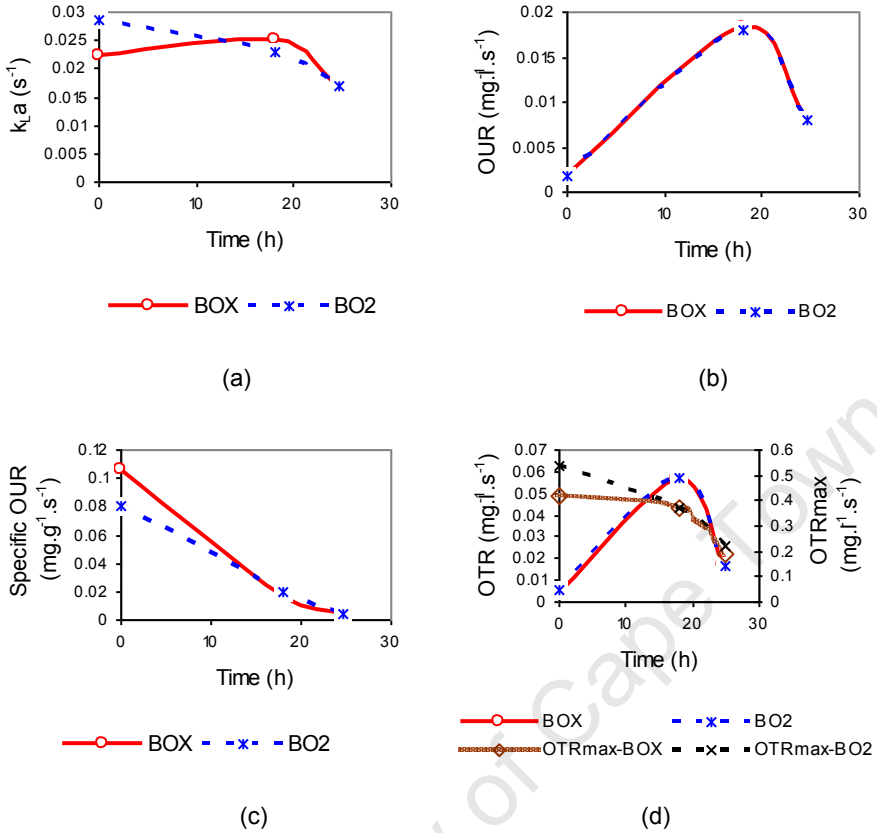


Figure 6.18: Important mass transfer parameters as determined for the multi-phase reactor system used in this work. (a) k_La as a function of time; (b) OUR as a function of time; (c) specific OUR expressed as a function of time; (d) OTR and OTR_{max} as a function of time. These data are provided for two sets of bioreactor conditions: BOX operated at an aeration rate of 0.3 vvm and and agitation rate of 500 rpm, and BO2 operated at 0.4 vvm and 500 rpm.

It was observed that for the aeration rate of 0.4 vvm, k_La decreased during growth. OUR and OTR increased with growth in accordance with the increasing biomass concentration until the 20th hour was approached and growth stabilised. The specific oxygen utilisation rate decreased throughout the process. The maximum OTR showed a decreasing trend during the process. This can be explained by both the decrease in k_La toward the end of the process and the change of the saturation oxygen concentration C* that results from the change of media composition following alkane depletion. The solubility of oxygen in the system may decrease in proportion with the rate of alkane consumption, since oxygen is more soluble in hydrocarbons than in water (Williams 2005, Clarke *et al.* 2006). In general, these results showed

similar trends for the parameters measured. Table 6.11 summarises data for oxygen concentration and biomass level that correspond to the above mass transfer parameters. The data corresponding to aeration of 0.3 vvm and 0.4 vvm were comparable, particularly for biomass. The saturation and dissolved oxygen concentrations decreased slightly more rapidly in the case of lower aeration rate.

Table 6.11: Summarised results for oxygen concentration at saturation, C*, dissolved oxygen, and biomass.

		BO2		BOX	
		0.4vvm, 500rpm, no pH control		0.3vvm, 500rpm, no pH control	
		C*	**C**	**C***	**C**
	initial stage	18.87	18.63	18.90	18.68
O2 con.	18h	16.42	13.12	14.95	11.96
(mg/l)	24.75h	12.78	11.36	11.37	9.94
	initial stage	0.022		0.017	
Biomass (g/l)	18h	0.923		1.095	
	24.75h	1.743		1.760	

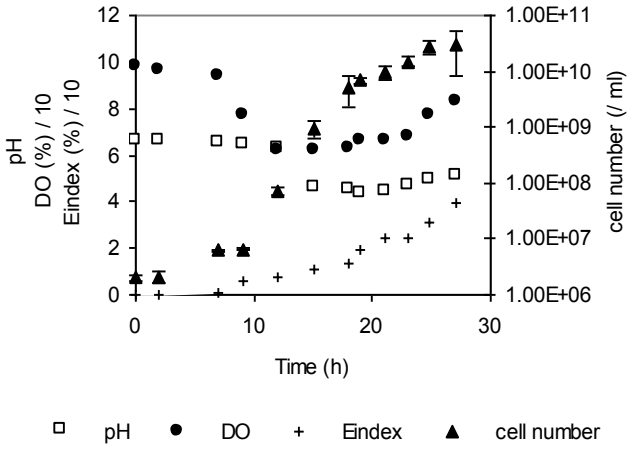
6.4 Alkane concentration and utilisation

6.4.1 Alkane concentration study

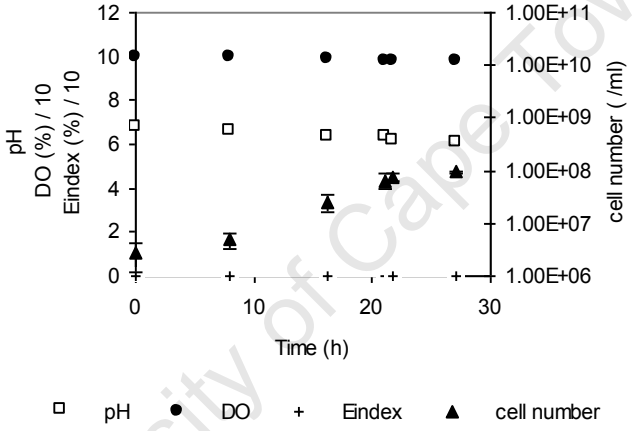
The concentration of alkane present plays an important role in the alkane uptake rate and the cells activity. Very low concentrations support low levels of growth, while very high concentrations may lead to growth inhibition, especially for substrates presenting a certain level of toxicity such as hydrocarbons.

In this section, the role of hydrocarbon substrate concentration was investigated by considering three concentrations: 3%, 10% and 20%. Agitation and aeration were maintained at 500 rpm and 0.3 vvm respectively. The medium pH, DO and E_{index} were measured during the process, and plotted against cell number. The data collected for cell growth, culture pH, E_{index} and dissolved oxygen with the addition of 3, 10 and 20% alkane as the C_{14}-C_{17} blend are shown in Figure 6.19. It was noted that the data in Figure 6.19a was also presented in Section 6.3.2.

a)



b)



c)

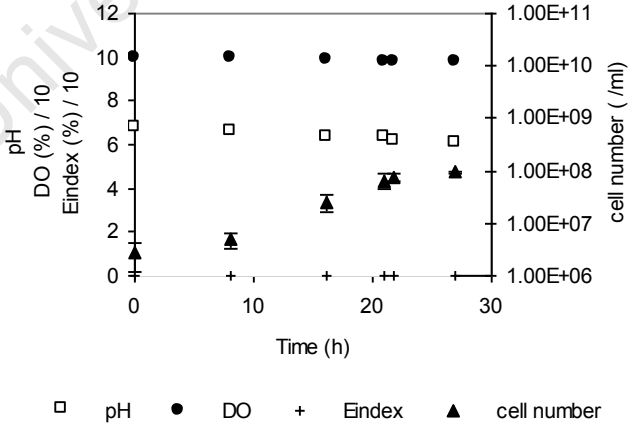


Figure 6.19: Effect of alkane (C_{14}-C_{17}) concentration on biomass formation and biosurfactant production on culture, at aeration rate of 0.3 vvm and agitation rate of 500 rpm; (a) 3% alkane; (b) 10% alkane; (c) 20% alkane

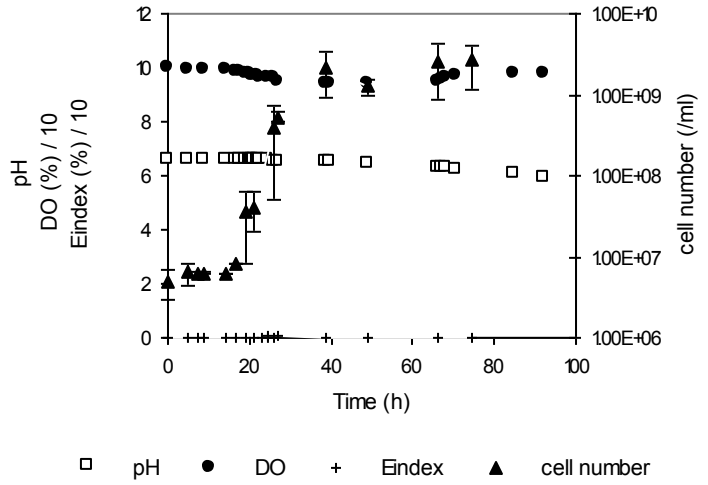
Figure 6.19a refers to the case of 3% alkane discussed in Section 6.3.2 as Figure 6.10. The exponential growth phase continued till 19 hours and was characterised by a growth rate of 0.308 h^{-1}. In this case, a maximum cell number of $3.0x10^{10}$ cells per ml was achieved in the first 27 hours of the process.

With the substrate concentration of 10%, the growth pattern showed little increase in cell number during the first 17 hours. The exponential phase was complete by 27 hours, and characterised by a growth rate of 0.17 h^{-1}. A maximum cell number of only $5.35x10^{8}$ cells per ml was achieved at 27 hours. The monitored parameters did not exhibit any changes to their initial level, with the exception of DO which showed a very slight decrease from 19 hours, as reflected in Figure 6.18b. These observations indicated an inhibition of biosurfactant production caused by high alkane concentrations in the growth media. The growth inhibition by high alkane concentration has been also observed by Ghilamicael (2003).

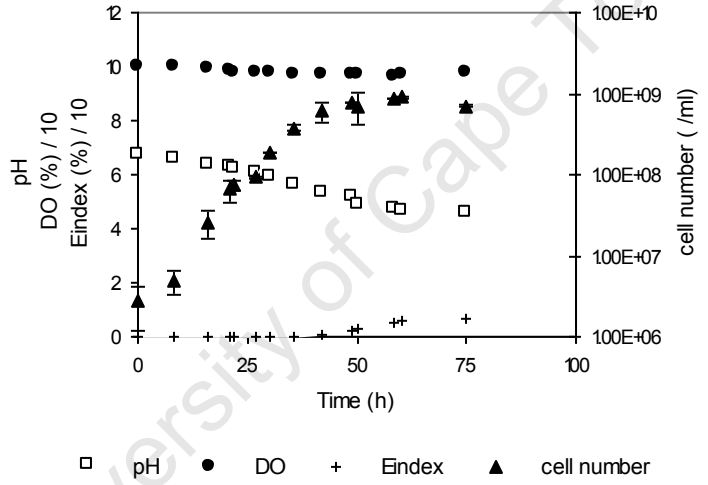
Under the conditions of a 20% alkane concentration (Figure 6.19c), the maximum cell number remained low, $9.30x10^{7}$ cells per ml. The exponential phase was characterised by a growth rate of 0.09 h^{-1}. A very small pH decrease, from pH 6.76 to 6.08 was observed after 10 hours, while other parameters remained stable at their initial respective levels. The poor biomass growth was attributed to the presence of high alkane concentrations. Using too high a substrate concentration may also have hindered good mixing in the system, and rendered the phase homogenisation very difficult. This, in turn, may have affected substrate uptake by microbial cells, thus leading to a growth inhibition (Goswami & Devendra 1990).

To identify if the bacteria could adapt to high alkane concentration, growth monitoring was extended over 74.5 hours, as shown in Figure 6.20. The exponential phase for the concentration of 20% alkane condition occurred between 8 and 35.5 hours, at a maximum specific growth rate of 0.07 h^{-1} and a maximum cell number of 8.80 $x10^{8}$ cells per ml achieved at 50 hours. In the case of 10% alkane, the maximum cell number attained reached 2.61 $x10^{9}$ cells per ml at 35 hours while the maximum specific growth rate was unchanged at 0.17 h^{-1}.

a)



b)



(b)

Figure 6.20: Effect of alkane (C_{14}-C_{17}) concentration on biomass formation and biosurfactant production on culture, at aeration rate of 0.3 vvm and agitation rate of 500 rpm, when the process monitoring was extended over 74.5 hours; (a) 10% alkane; (b) 20% alkane

Almost no pH change was observed in the case of 10% alkane concentration. At 20% alkane, the pH decreased from 22 hours to pH 4.73 at 50 hours at which time the emulsification index started to show an increasing trend, indicating that extracellular biosurfactant was produced during the stationary phase. The maximum value for E_{index} was only 6.41% and was reached after 74.75 hours. Dissolved oxygen did not show any considerable change in both cases; its lowest values

obtained were of 95.13% and 96.5% respectively for the 10% and 20% alkane concentrations.

High alkane concentrations are usually associated with poor phase miscibility. At low levels of miscibility large oil droplets may dominate (Goswami & Singh 1991). Following this, it can be deduced from the observations of these data, that, in addition to the influence of alkane concentration, the predominant mode of substrate uptake plays a role in biosurfactant production (Goswami & Singh 1991).

In general, it was noticed that biomass and growth rate decreased with increasing alkane concentration, and that biosurfactant production was affected by high alkane concentrations, probably through growth inhibition. During the experiments, reproducible measurements were more difficult due to poor phase homogeneity.

6.4.2 Alkane utilisation study

One of the objectives of the present research was to monitor the alkane depletion during the process, and to establish the rate of consumption of the alkane compounds contained in the substrate.

Analyses were done under two different aeration rates, with a substrate concentration of 3% and an agitation rate of 500 rpm. Aeration rates of 0.3 vvm and 0.4 vvm were used, the latter in both controlled and uncontrolled pH conditions, in order to investigate the alkane consumption in different conditions of aeration and pH. The results obtained were graphically presented in Figure 6.21 to 6.23.

Under aeration and agitation conditions of 0.3 vvm and 500 rpm respectively, alkane usage was detected from the 2nd hour of the process, as shown in Figure 6.21. Figure 6.21a and Figure 6.21b showed that, initially, tetradecane was consumed preferentially, while the consumption of pentadecane, hexadecane and heptadecane was detected only after nine hours. Heptadecane was quickly exhausted probably due to its low percentage in the substrate. Consumption of pentadecane was very limited from 21 hours on, while that of tetradecane and hexadecane was maintained at a high level up to the end of the process. The following important observations could be made:

- there was a preferential consumption of lower chain lengths during the initial stages of growth;

- tetradecane usage became limited when that of pentadecane started being remarkable, and was resumed at around 21 hours, when the latter stopped being appreciably consumed;
- there was a short period between 15 and 18 hours during which hexadecane was not consumed and only pentadecane was observed to be highly utilised;
- from 18 hours up to the end of the process, hexadecane and tetradecane were the most utilised alkanes.

Figure 6.21 does not show any stage where all alkanes were consumed at the same time, showing that at least one of the four alkanes contained in the substrate was preferred to others at each growth phase (Ghilamicael 2003; Mathopa 2004).

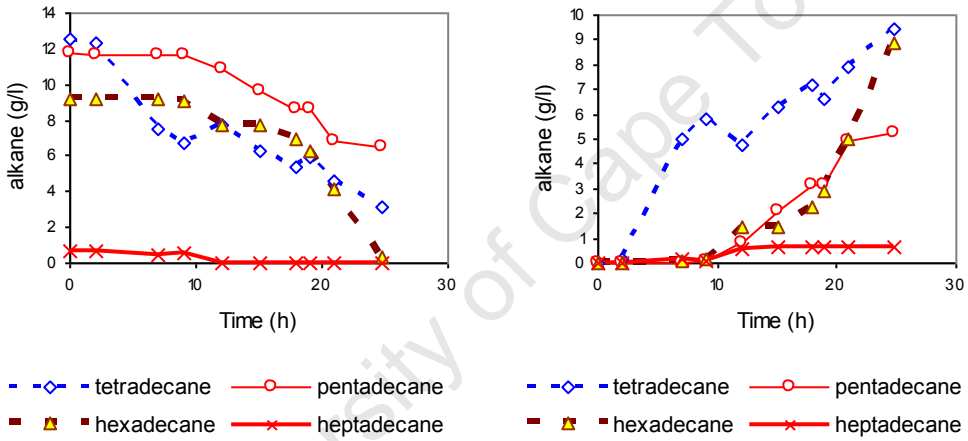
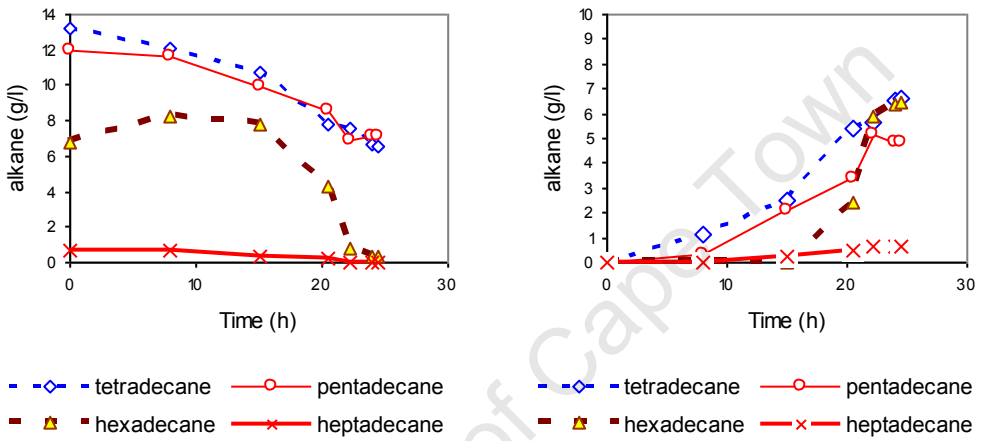


Figure 6.21: Alkane consumption during the process under the conditions of 0.3 vvm, 500 rpm, 0.3% alkane and uncontrolled pH; (a) monitoring data for the remaining alkane during the process plotted as a function of time; (b) monitoring of the amount of alkane utilised as a function of time

These results showed that longer hydrocarbon chains were mainly consumed from the mid exponential phase onward. Since biosurfactant was found to be produced during these late phases of bacterial growth, it was postulated that longer chains are likely to be stronger inducers of biosurfactant production by *Ps. aeruginosa 2Bf* (Ghilamicael 2003; Beal & Betts 2000). This is supported by the fact that no significant emulsification or surface activity was detected during the initial hours of growth, when tetradecane was preferentially utilised.

The results obtained with the aeration rate of 0.4 vvm are shown in Figure 6.22. Some similarities were singled out between Figure 6.21 and Figure 6.22. For instance, only tetradecane was used initially and after 20.5 hours hexadecane and tetradecane were preferred, while pentadecane usage was detected between 8 hours and 9 hours. The two important uncommon points between Figure 6.21 and Figure 6.22 are that in the latter case: (i) no consumption of hexadecane and heptadecane was detected before 15 hours; (ii) It was also noted that pentadecane utilisation curve showed no progress after 22.2 hours.



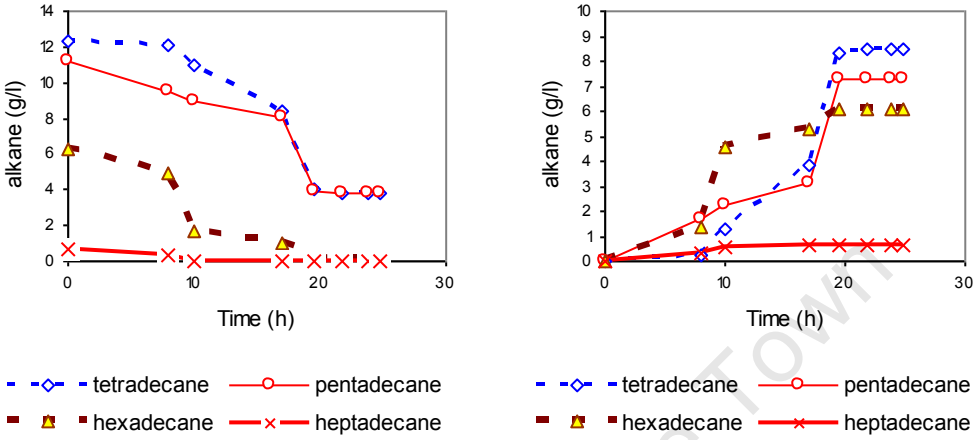
(a) (b)

Figure 6.22: Alkane consumption during the process under the conditions of 0.4 vvm, 500 rpm, 0.3% alkane and uncontrolled pH; (a) monitoring data for the remaining alkane during the process plotted as a function of time; (b) monitoring of the amount of alkane utilised as a function of time

Referring to Figure 6.11, where, similar to the present case, the reactor was operated under the conditions of 0.4 vvm, 500 rpm and no pH control, it was observed that emulsification increased after 14 hours and surface tension detection occurred after 15 hours. It is, therefore, possible to reiterate the hypothesis that biosurfactant production is more strongly induced by longer alkane chains and required for their efficient use (Ghilamicael 2003; Beal & Betts 2000).

Under the operating conditions of 0.4 vvm, 500 rpm, 3% alkane and pH 6.6 (Figure 6.23), teradecane consumption was detected only after 8 hours of the process whereas, in the non pH control conditions, this detection was noticed from the 2nd hour. By contrast to the previous cases, consumption of pentadecane, hexadecane and heptadecane, was observed from the very initial hours of the process. From the

8 hours tetradecane and pentadecane in Figure 6.23a and 6.23b adopted similar trends up to the end of the process. Between 10 hours and 16 hours, little consumption of hexadecane was recorded, while from 19.5 hours to the end of the process, cells used heptadecane preferentially.



(a) (b)

Figure 6.23: Alkane consumption during the process under the conditions of 0.4 vvm, 500 rpm, 0.3% alkane and controlled at pH 6.6; (a) monitoring data for the remaining alkane during the process plotted as a function of time; (b) monitoring of the amount of alkane utilised as a function of time

The results in Figures 6.3b and 6.4b, where similar conditions were used, showed that surface tension and emulsification index were detected from close to 10 hours, while Figure 6.23 showed a sharp variation of hexadecane trend between 8 hours and 10 hours. This is consistent with the observation made in the case of Figure 6.21 and Figure 6.22, that biosurfactant is likely to be more enhanced by longer alkane chains.

The main observations made in the first 25 hours were tabulated in order to visualise all the three sets of conditions used to investigate the alkane utilisation, as shown in Table 6.12. In all the cases, pentadecane was the least utilised and heptadecane the most utilised. Table also revealed that with the exception of tetradecane, pH control enhanced the individual alkane consumption rates in general, since the percentage of utilised alkane was higher in pH controlled conditions, as also reflected in Figure 6.2. Tetradecane was more consumed under the conditions of uncontrolled pH and low aeration rate. This showed that alkane was not consumed for biosurfactant production exclusively. Other products, such as pHAs (Kosaric 1993; Mathopa 2004;

Chayabutra & Ju 2001) were probably produced from alkane substrates, since production of biosurfcatant was not higher in pH controlled conditions, whereas the alkane consumption seemed to be is high in this case.

Table 6.12: Summarised results for individual alkanes present in the substrate. These results are given in terms of mass percentages utilised with reference to the initial content of the alkane in the substrate.

	Total % of alkane used in the first 24.75 hours of growth				
		C14	**C15**	**C16**	**C17**
0.3 vvm		75.16	44.34	96.84	99.78
0.4 vvm		50.11	40.56	95.3	96.89
0.4 vvm	pH = 6.6	68.10	65.49	97.60	99.95

6.5 Conclusion

The current study investigated the growth of *Ps. aeruginosa 2Bf* on n-alkanes in a batch reactor system. The potential of this bacterium to produce biosurfactant was investigated quantitatively. This was achieved by analysing the single parameter variation with the number of cells for the following seven parameters: pH, DO, alkane utilisation/depletion, E_{index}, ST, dynamic viscosity and biomass. According to the results discussed herein, viscosity was shown to be strongly influenced by the growth of biomass, while in general non-pH controlled conditions offered better performance than those of controlled pH.

Findings of Chapter 5 concerning the potential of liquid long chain alkanes to induce biosurfactant production were confirmed by results of the bioreactor-based study compiled in the present chapter. Reactor mixing and oxygen mass transfer were studied, where four levels of agitation and aeration as well as three levels of foam control were considered during the optimisation of mixing and aeration. Two combinations agitation and aeration were used for mass transfer study.

In the case of mixing and aeration study, agitation and aeration proved to be equally important. The influence of their interaction on the system was found to be more pronounced at lower agitation rates. Aeration and agitations rates of 0.3 vvm and 500 to 600 rpm were selected as preferred for the bench top reactor system. During the optimisation of foam control, type FB-1 and type FB-2 were found to be more

efficient than type AF. Apart from performing worse than the mechanical foam breakers, the latter has the disadvantage of being a chemical compound with surface activity, thus being likely to cause interference with the produced biosurfactant. The analysis of interaction between type of foam breaker and agitation rate led to the conclusion that the impact of the interaction of these two factors on the system is negligible. Foam breaker type FB-2 was confirmed to be more efficient than type FB-1.

Mass transfer was studied under the reactor aeration conditions of 0.3 vvm and 0.4 vvm. The results obtained showed similar trends in both cases, but it was noticed that mass transfer was enhanced in the case of higher aeration rate.

Regarding the impact of alkane concentration on growth and biosurfactant production, it was found that at lower concentration (3%), E_{index}, pH and DO vary on cell growth. High alkane concentrations adversely affected biomass growth and biosurfactant production. In the case of 10% alkane, no emulsification was detected until 74.75 hours, and with 20 % alkane, limited emulsification was detected from 50 hours onward. A maximum cell number of $3.0x10^{10}$ cells.ml^{-1} was achieved with the concentration of 3% alkane in the first 27 hours, while the maximum cell number obtained with the concentrations of 10% and 20 % alkane in the first 27 hours was $5.35x10^{8}$ cells per ml and $9.48x10^{7}$ cells per ml respectively.

It was further concluded that alkane utilisation was strongly dependent on aeration rate and on whether pH is controlled. With an aeration rate of 0.3 vvm and no pH control, only tetradecane was consumed from the 2nd hour, while alkanes of longer chain length were not consumed before ten hours at which stage biosurfactant activity was evident. This shows a preference for shorter chain length during the initial growth stage. When aeration was raised to 0.4 vvm and pH was not controlled, tetradecane was consumed at a considerable rate from time zero, pentadecane was consumed from eight hours, while the utilisation of hexadecane and heptadecane was not detectable before 15 hours. Where pH was controlled at pH 6.6, tetradecane was the least preferred alkane during the initial growth stages. Under these conditions, pentadecane and hexadecane were utilised from the very first hour of the process, heptadecane after two hours, and consumption of tetradecane was detectable only after height hours. These observations indicated that pH conditions influence the process of biosurfactant production (Ishigami *et al.* 1987).

CHAPTER VII: CHARACTERISATION OF THE BIOSURFACTANT

7.1 Introduction

In Chapters 5 and 6, evidence of the potential of hydrocarbons to induce surface active product synthesis by *Ps. aeruginosa 2Bf* was experimentally established. Chapter 7 concentrates on characterisation of the product. Structural analysis of the biosurfactant produced in this project was tentatively hypothesised by three spectrometric methods: mass spectrometry, nuclear magnetic resonance spectroscopy and infrared spectrometry. Biosurfactant was also tested for antibiotic activity against *B. subtilis* cells, since rhamnolipids have been reported to possess a strong biological activity. An antibiotic activity was reported by Tuleva *et al.* (2002), while Thanomsub *et al.* (2006) reported an anti-cancer activity. TLC tests were performed to confirm glycolipid presence in the synthesised compounds, prior to spectrophotometric analysis.

In the case of nuclear magnetic resonance, hydrogen and carbon NMR studies of the sample extracts were carried out. The results were not distinct enough to allow useful conclusions with regard to the expected product structure. Samples were subjected to the mass and FT-IR spectrophotometric analysis in order to gather sufficient information on the biosurfactant molecular structure. The Fourier transform infrared technique was used to identify the functional groups of different glycolipid molecules that were present in the product. During mass spectrometry study, samples were first scanned between m/z 100 and m/z 1000. The information obtained, supported by the available literature on the subject, provided enough information for molecular structural identification of the product.

7.2 Review of biosurfactant identification methods exploited in this work

7.2.1 Thin layer chromatography

Thin layer chromatography is a highly sensitive technique and simple to operate The separation by TLC is based on the differential migration of compounds through a stationary phase, carried by a mobile phase. The stationary phase is a thin layer (~ 100 – 200 µm), often made of silica gel, immobilised on a rectangular plate usually in aluminium or glass material; plastic plates are less commonly used (Rouessac & Rouessac 2007). To maintain the stationary phase on the support and to assure a

good cohesion of the particles, an inert binder like gypsum (or organic linker) is mixed into the stationary phase during the manufacture of the plate. A standard compound and the unknown are deposited on to the stationary phase at the base of the plate, and eluted with the capillary movement of the mobile phase across the plate. This is done in a chromatographic chamber.

Figure 7.1: example of a tlc plate with eluted samples

From the physical-chemico-point of view, TLC can be characterised by the following points (Touchstone & Dobbins 1983):

- TLC represents a three-phase system between which equilibria are established: solid (stationary), liquid (mobile) and vapour phases;
- The stationary phase is only partially equilibrated with the liquid phase before compound migration starts. Depending on the way separation is obtained, the mobile phase may or may not be in equilibrium with the vapour phase;
- The speed of migration of the solvent front is not constant. It follows a complex function in which the particle size of stationary phase plays a part. Migration can be described by a quadratic law:

$$x^2 = kt \qquad (6.1)$$

where x is the distance of the migration front, t the time and k constant.

- The flow rate of the mobile phase cannot be modified to improve the efficiency of separation. A possible remedy to this problem is the multi-development technique, by drying the plate before each new cycle of migration;

- The adsorption phenomenon of the stationary phase is substantially reduced once a large part of the adsorption sites are occupied. This creates an effect of elongation of spots. Consequently, the R_f (retardation factor) of a compound in the pure state is slightly different from that of the same compound present in the mixture.

7.2.2 Infrared Spectroscopy

The infrared region of electromagnetic spectrum of wave length ranges approximately between 1 μm and 1000 μm. The infrared spectral range is subdivided in three sub-regions, the near infrared (1 – 2.5 μm), the mid-infrared (2.5 – 50 μm) and the far infrared (beyond 50 μm). A vast majority of organic compounds absorb light in the mid-infrared, following transitions between different vibration states of a molecule. The observed transitions are, in general, from the ground vibrational state to the first excited state. The near-IR is poor in specific absorptions, but is used by quality control laboratories for quantitative applications (Akitt 1992; Pinchas & Laulicht 1971). The mid-IR provides more information upon structures of compounds and is used in identifying organic compounds for which it remains a form of functional group fingerprinting (Bible 1965). To conduct analysis, there exist a full range of instruments, from Fourier transform (FT) spectrophotometers to a multiplicity of analysers of dispersive or non-dispersive types, specialised in the measurement of pre-defined compounds (e.g. analysis of gases and vapours) or which allow continuous analyses on production lines. The Fourier transform infrared (FT-IR) spectrophotometry offers numerous possibilities for the treatment of spectra (Smith 1999) and has applications for the analysis of structured microsamples (infrared microanalysis).

Samples for infrared analysis can be solid, liquid or gas; they can be analysed in solution or following separation. With an appropriate technique, environments can range from *in situ* measurements to the analysis of pictogram quantities of material for forensic analysis. This versatility and power are unrivalled amongst other instrumental analytical techniques.

The IR technique uses NaCl plates, commonly referred to as *salt plates*, because NaCl materials are transparent to IR radiation. Apart from being expensive, the salt plates present the challenge of being very sensitive to water. Even the moisture on

fingers may dissolve the plates. IR measurement is applied to thin sample films. A Pasteur pipette is used to place a drop of liquid sample on salt plate. A second salt plate is put on top in such a way that the sample spreads into a thin film, before the plate is placed in the instrument for measurement. For solid samples, only one plate is needed. A small amount of solid sample in the range of 50 mg can be dissolved in a small amount of acetone or methylene chloride (about 0.5 to 1 ml). The plate is placed in the V-shaped sample holder inside the instrument, and a drop of this solution is put on the salt plate and allowed to dry to form a thin solid film for observation.

7.2.3 Nuclear Magnetic Resonance Spectroscopy

NMR is a powerful and theoretically complex analytical tool that allows the study of compounds in either solution or in the solid state. It is used for both quantitative and structural analysis (Wehrli 1988), especially the latter where it is efficient in providing structural information concerning molecular compounds (Akitt 1992; Bible 1965). When it is used as a complementary technique to methods of optical spectroscopy and mass spectrometry, NMR leads to precise information on the structural formula, stereochemistry and in some cases, the preferred conformation of molecules.

7.2.3.1 Atomic nucleus as a useful source of information concerning molecular structure

The nuclei of some natural isotopes of the majority of the elements possess intrinsic angular momentum or spin, of total magnitude $\frac{h}{2\pi}\left[I(I+1)\right]^{1/2}$, where h is the Planck's constant and I the nuclear spin quantum number. The latter can have either integral or half-integral values (0, ½, 1, 3/2 …) depending on the isotope (Cullum 1994). Several discrete values of angular momentum may, therefore, be observable and its magnitude is given by hm, where the quantum number m can take the values I, I -1, I -2,…, $-I$; in total, there are $2I+1$ equally spaced spin states of a nucleus with angular momentum quantum number I. A nucleus with spin is also associated with a magnetic moment μ. Its components associated with the different spin states can be defined as $m\mu/I$, so that μ also has $2I+1$.

Nucleons (proton or neutron) possess intrinsic spin in the same way as do electrons, though they can only pair with nucleons of the same type. Thus, in a nucleus of equal number of both neutrons and protons, all spins are paired and $I = 0$.

In the absence of an external magnetic field, all the spin states have the same potential energy. The situation is modified by the application of a magnetic field (Cullum 1994; Akitt 1992). The origin of the NMR technique lies in these energy differences.

7.2.3.2 NMR as an analytical tool in structural analysis of molecules

As a general rule, all nuclei with an odd atomic number (number of protons) or odd mass number (protons + neutrons) possess a permanent dipole moment, and, as a consequence, behave like tiny bar magnets, because such nuclei are not perfect spheres but either oblate or prolate spheroid (Akitt & Mann 2000; Cullum 1994).

When placed in a magnetic field, such nuclei absorb electromagnetic radiation in the radio-frequency (RF) region of the light spectrum. The exact frequency depends upon various factors, but the most important for the chemical analyst is that this frequency (also referred to as the frequency of resonance) varies with the chemical environment of the nucleus, and each nucleus in a molecule gives a characteristic signal (Bible 1967). NMR, therefore, makes it possible to *see* the individual atoms composing molecules (Field *et al.* 1998), and, hence, to determine the molecular structure (Akitt & Mann 2000).

Nuclei of the most commonly occurring isotopes are not NMR active (e.g. ^{12}C, ^{16}O, ^{32}S), hence specific NMR active isotopes must be selected for these elements. Another drawback associated with NMR is that the nuclei of most atoms of the periodic table with a dipole moment also have a nuclear quadrupole moment, making them hard (in many cases impossible) to detect by NMR.

The dominant hydrogen isotope, ^{1}H, is NMR-active. ^{1}H - NMR has been used in molecular structure elucidation from the time that NMR spectrometers first became commercially available in 1956 (Cullum 1994). In the early 1970s improvements in NMR technology and in computing allowed ^{13}C, the rarer isotope of carbon present at 1.1% natural isotopic abundance, to be detected with ease (Cullum 1994). ^{13}C-NMR

provided a rapid and unambiguous way of seeing the carbon skeleton of a molecule. ^{1}H and ^{13}C are the only two nuclei that are routinely used in the analysis of surfactant molecules by NMR (Cullum 1994).

During NMR study, chloroform is often used as NMR solvent. In order to obtain good results, the time of sample analysis may depend on both the resolution of the equipment and the product concentration.

7.2.4 Mass Spectrometry

Mass spectrometry (MS) differs from other common forms of organic spectral analysis in that the sample does not absorb radiation such as infrared, ultraviolet or radio waves from the electromagnetic spectrum. MS is a destructive method of analysis, that is highly sensitive and requires a smaller sample size than either IR or NMR in order to provide more information about the structure of the analyte. Mass spectrometers are often connected to a chromatograph (GC, LC, HPLC, etc.). The chromatograph separates mixtures into component molecules and introduces the components into the mass spectrometer. The mass spectrometer ionizes the sample molecules, then separates and detects the resulting ions (Middleditch 1979). The computer system controls the operation of the chromatograph and the MS, and provides data manipulation and storage during and after data collection. For volatile samples, gas chromatography (GC) is used for mixture separation, while for non volatile or thermally labile molecules, HPLC or just LC is used.

7.2.4.1 Sample ionisation

In order to be analysed, sample molecules must be ionised before detection. The most common ion source used is electron ionisation (EI), particularly used in GC/MS work. The EI source is often a small chamber of about one 1 ml in volume, in which sample molecules interact with a beam of highly energetic electrons that have typically been accelerated through a potential difference of 50-70 V across the volume of the ion source [50-70 eV (1eV = 23kcal)] (Cullum 1994). Surrounding the entire ion source is in some cases a collimating magnet, which causes the electrons in the beam to travel in a helical path (Middleditch 1979). This helical trajectory improves the probability of the electron and molecules to interact.

When such energetic electron approaches the electron density field of a molecule closely enough so that sufficient energy is transferred (quantum mechanically) to overcome the ionisation potential of the molecule, one electron is ejected from one of the bonding or nonbonding orbitals of the molecule. Ionisation energies for most organic compounds range from ~ 5-15 eV. The bond dissociation energy is even smaller. Hence this method of ionisation not only causes molecules to expel one or more electrons, but also provides enough energy for substantial fragmentation of the first-formed ion ($M^{+\bullet}$). Because of the excess energy present in 50-70 eV electrons, enough additional energy may be transferred to overcome the second, or even the third, ionisation potential of the molecule, leading to ions having +2 or +3 charges (Cullum 1994; Middleditch 1979).

7.2.4.2 MS as an analytical tool in structural analysis of molecules

As mentioned in Section 7.2.4.1, more than one electron can be ejected from the analyte, though the one electron ejection is far more frequent, and ions having charges of +2, +3, or even +4 are formed. In some specific cases more electrons can be ejected. Biopolymers such as peptides, for instance, may have charge states of +10 or more from protonation of basic sites on the molecule (Middleditch 1979). Since mass spectrometry actually measures the mass-to-charge ratio (m/z) of an ion but not its mass, an ion having a charge greater than +1 is found not at the m/z value corresponding to its mass (m), but rather at $m/2$, $m/3$ etc.

If m is not evenly divisible by the number of charges z, m/z will have a nonintegral value. For example, the double charged molecular ion M^{2+} of a compound having a molecular mass 179 is found at m/z 179/2 = 89.5 . Most compounds do not produce multiple charge molecular ions in EI, but they may be, and are often, formed in low abundance from small molecules that have few possible modes of fragmentation or from compounds with aromatic rings or large hetero-atoms such as Cl, Br or S (Cullum 1994; Smith & Busch 1998).

7.2.4.3 MS and biosurfactant analysis

Mass spectroscopy is a very sensitive technique that allows quantities as low as a few picograms to be analysed (Cullum 1994; Smith & Busch 1998). It is, however, the least specific of all spectroscopic techniques, as there may be many possible

empirical formulae corresponding to any particular peak in a low resolution mass spectrum (Smith 2004). This disadvantage is averted by either using very expensive and sophiscated MS techniques (Cullum 1994) or combining the MS with separation techniques such as GC or LC.

Areas of application of MS in the surfactant industry include: (a) characterisation of new synthetic materials; (b) raw materials analysis; (c) whole product analysis; (d) surfactant chain-length distribution determination; (e) analysis of factory emissions and trace contaminants; (f) determination of trace levels of surfactant-related materials in environmental samples; (g) determination of contaminants in products.

7.3 Analysis of the surface active product of *Ps. aeruginosa 2Bf*

Rhamnolipids have been identified through the literature (Section 2.7) as the most likely surface active agents produced by *Ps. aeruginosa*. In this final component of thethesis, chemical analysis is undertaken to confirm whether the surface active agent produced by this specific *Ps. aeruginosa* strain is indeed a rhamnolipid.

7.3.1 Thin layer chromatography

Thin layer chromatography was used for the identification of glycolipids in product. It was a qualitative study, aimed at verifying the presence or absence of rhamnolipids in the product. In this analysis, samples were prepared in different ways depending on whether the rhamnose component or the glycolipid as a chemical entity was being targeted. In the case of rhamnose component, the sample was treated according to methods 4.5.3a and 4.5.3b (Section 4.5.3). Method 4.5.3a (Wu & Ju 1998) refers to the treatment of the sample with concentrated HCl to pH 2, followed by extraction with ethyl acetate, and redissolving the product in sodium bicarbonate after evaporating the solvent (ethyl acetate). The second method (b) is the treatment of the sample with both strong base and strong acid, and extraction with hexane. The pH 9 of culture sample is brought to the value of pH 9 and extracted with hexane, before being treated as described in Section 4.5.3 (a). In the case of glycolipid determination, samples were treated according to Chayabutra *et al.* (2001) and Tahzibi *et al.* (2004). The sample was prepared as stated in Section 4.5.7, by acidifying the supernatant to pH 2, extracting with a 2:1 mixture of chloroform and methanol, and dissolving the residue in sodium bicarbonate after evaporating the

solvent (Tahzibi *et al.* 2001). After this treatment, the resulting solution was spotted onto the TLC plates, which were developed with Solvent (1) described in Section 4.5.4.2, a mixture of $CHCl_3$, CH_3OH and acetic acid in a volume ratio 65:15:2. This treatment was reported to be specific to glycolipid (Chayabutra *et al.* 2001), as indicated in Section 4.5.4.2. Owing to the highly reactive nature of the compounds used for glycolipid detection e.g. diphenylamine, the following precautions needed to be taken: very gently spray the reagent used to reveal the product onto the plate, and repeat the exercise until readable plates are obtained.

Figure 7.2 shows the results of TLC after revelation of the products. Plates A and C were developed for detection of sugars and plate B for the detection of glycolipids. The aqueous phase that resulted from the preparation described in Section 4.5.3 (a) was spotted onto plate C and those from the preparations described in Sections 4.5.3 (b) and 4.5.4.2 (solvent-2), onto plate A. Both plates were intended to reveal rhamnose. Plate B was used to detect glycolipids following the method described in Section 4.5.4.2, specific to glycolipids. On plate A, only an extract from M3 and the rhamnose standard were used, while on plate B both M3 and M4 were used, M3 corresponding to B-3 and M4 to B-2, while B-1 corresponded to the mixture M1-M2. On plate C, C-3 corresponds to the rhamnose standard, C-2 to the mixture M1-M2, while C-1 corresponds to the mixture M3-M4.

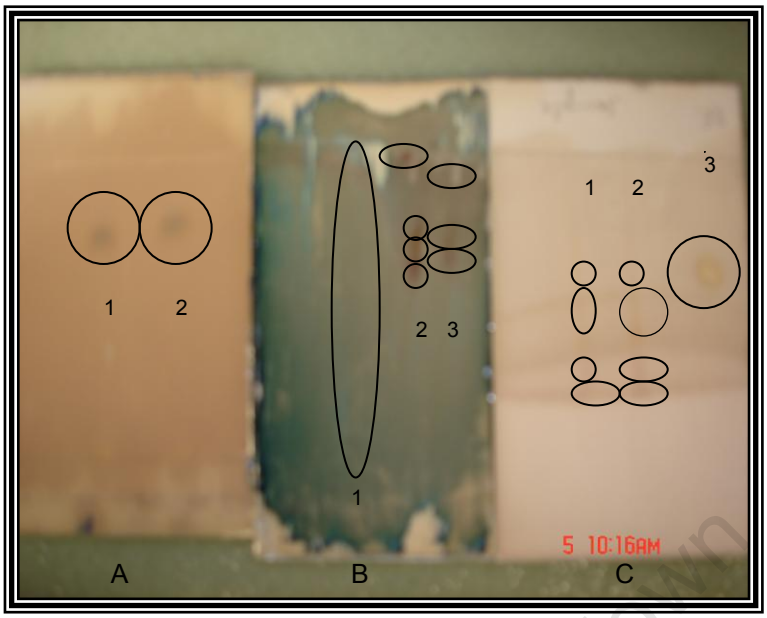


Figure 7.2: TLC plates after development and revelation of biosurfactant molecules: (A) extracts treated with the 4.5.3b and 4.5.4.2 (solvent-2) method: rhamnose standard (A-2) and M3 (A-1); (B) glycolipids from the 4.5.4.2 method applied to samples of M3 (B-3), M4 (B-2) and mixture M1-M2 (B-1); (C) rhamnose from the 4.5.3a method applied to samples from rhamnose standard solution (C-3), mixture M1-M2 and mixture M3-M4 (C-1)

The results revealed that both C-1 and C-2 contained one compound of same R_f as rhamnose (C-3), and three other compounds which were not identified, but found in each case with the same migration speed. These results supported the presence of rhamnose in the product.

As indicated above, plate A in Figure 7.2, corresponds to the identification of the sugar moiety of the product against pure L-rhamnose standard (A-2). Plate A and Plate C differ from the sample treatment method only. Plate A showed only one type of sugar molecule as shown in Figure 7.2 (A), with practically the same migration speed as the L-rhamnose standard.

Plate B was used for a TLC identification specific to glycolipids, as indicated in Section 4.5.4.2. As no rhamnolipid standard was available, positive revelation was assumed to indicate glycolipid presence, as previously reported for this method (Chayabutra *et al.* 2001). Extracts from M3, M4 and M1/M2 correspond to B-3, B-2 and B-1 respectively. Although the plate was damaged by the solvents used in the process of revelation, the revealed spots could be distinguished, and the number of

similar or non-similar glycolipid compounds present in the samples could be established. The results revealed four different compounds in B-2 corresponding to M4 sample and three in B-3 corresponding to M3 sample (Figure 7.2). As these spots were not very distinct, presence of additional compounds could be suspected. On B-2, the spot found towards the upper limit of the solvent migration level mark, cannot be confirmed as a glycolipid molecule owing to its migration speed. Extracts from M1 and M2 (B-1) did not show any glycolipid presence. This might have been due to insufficient product in samples or may indicate the absence of glycolipid in the extracts. Further characterisation was carried out with spectrophotometric techniques and the results are discussed in the following section.

7.3.2 NMR Spectrometry

NMR results did not suggest very meaningful information with regard to the product hypothesised. Both ^{1}H and ^{13}C NMR showed little little success. Most of the important structural hydrogen atoms were not detected by ^{1}H -NMR. Some CH_2- and CH_3- hydrogen atoms were detected in a few cases, but did not provide enough information for spectral analysis. The most expected shifts that would characterise glycolipids, e.g. those corresponding to the carbonyl or esters groups did not appear distinctly. Some hydrogens could be related to the sugar head of the glycolipid (Wei *et al.* 2005), but no other neighbouring hydrogen was noticed in such cases. Even in the case of ^{13}C-NMR, no evidence of carbonyl groups or ester carbons was clearly established, as almost all the carbons detected seemed to be of the saturated hydrocarbon skeleton. However, spectrograph integrals could reveal chain length of 8 to 14 carbons, with the assumption that no single-lipid chain glycolipid was present in the extract i.e. all the glycolipid molecules were assumed to have at least carboxylic acid in their structure. Analysis to date suggested a possible presence of rhamnolipids with C_{12}, C_{10} and possibly C_8 chain length, with predominance of C_{10} and C_{12}. The possible reason for such limited detection by NMR could be that the instrument used had a low resolution which required either a large amount of pure compound in the sample, or a relatively long time of sample analysis. These were not satisfied in the present case.

7.3.3 Mass Spectrometry

Table 7.1 provides the main rhamnolipid fragments obtained during MS study and compares these to those obtained by other researchers who worked on the same subject. Sample spectrogram from which these data were collected are provided in Appendix VI. Only a few samples were scanned below 400 m/z and no specific scan in the range of 300 to 400 m/z was done. While the main fragments of the molecules corresponding to rhamnolipid structures were well identified based on their m/z values, the smaller ones (below 400 m/z) were identified as resulting from further fragmentations of the pseudomolecules or main fragments, whenever they were found to be supporting the hypothesised structure. In fact, small fragments are often common to more than one pseudomolecular ions.

Up to 6 different compounds were tentatively confirmed to be present in the majority of samples analysed. Four of these were likely to be in the form of two structural isomers. One of two such isomers is always assumed to be largely more abundant than the other (Kosaric 1993; Syldatk *et al.* 1985). Carboxylic acids of different chain lengths were contained in the rhamnolipids identified as follows: at least one C_{10} was found in 4 of the six structures, at least one C_{12} found in 4 of the six structures, while C_8 appeared in only one compound.

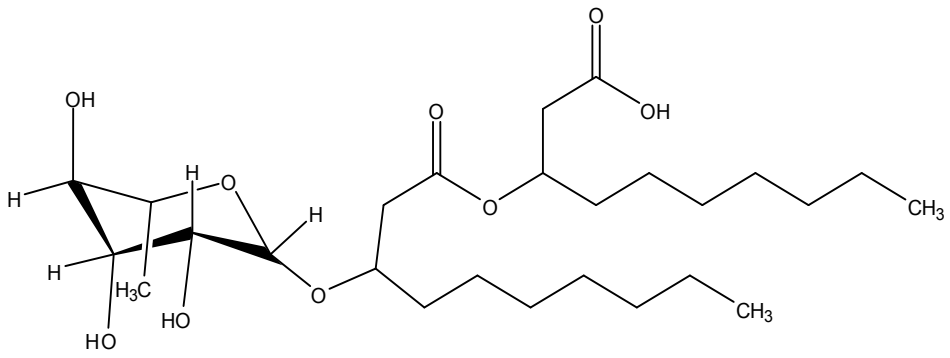
Table 7.1 shows eleven structures that were all proposed as being potentially present, but only the six showed in *bold*, were consistently observed in samples. The structural formulas for the rhamnolipid compounds, given in Table 7.1, are provided in Figure 7.3.

Table 7.1: Comparative table compiling MS results of biosurfactant structural analysis from different authors

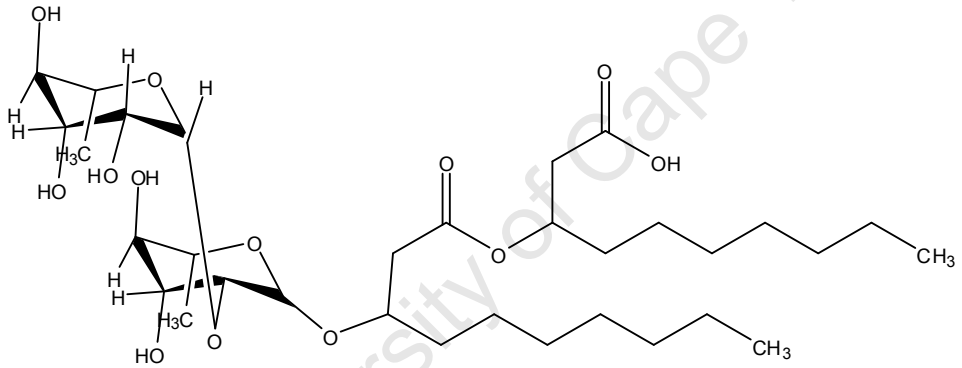
		Monteiro *et al.* (2007);	**Haba *et al.* (2003); Heyd *et al.* (2008)**	**Present Work**
	Hypothesised Compound	**Pseudomolecular ion (m/z)**	**Pseudomolecular ion (m/z)**	**Pseudomolecular ion (m/z)**
A	**Rha-C10-C10**	**504** ***103, 119, 163, 333***	**503** ***103, 119, 163, 333, 339 [339, 333, 169]***	**503** ***102, 119, 335, 340***
B	**Rha-Rha-C10-C10**	**650** ***145, 163, 169, 205, 246, 479***	**649** ***163, 169, 311, 339, 479 [479, 339, 169]***	**650, 651 & 648** ***146, 167, 340***
C	**Rha-Rha-C12-C12:1 (-C12:1-C12)**		**703** ***197, 507 [505, 393, 197, 195]***	**704** ***195, 197, 505, 507***
D	**Rha-Rha-C8-C12:1 (-C12:1-C8)**		**647** ***195, 452 [505*, 451#, 337, 195, 141]***	**648** ***195, 143, 451#, 452****
E	**Rha-C10-C12# (-C12-C10)***	**531** ***103, 163, 169, 197, 333#, 361****	**531** ***103, 119, 163, 169, 197, 311, 333, 361, 367 [367, 361*, 333#, 197, 195, 169]***	**531** ***102, 119, 167, 197, 335***
F	**Rha-C10-C12:1 (-C12:1-C10)**	**529** ***103, 119, 169, 187, 333***	**529** ***103, 119, 163, 169, 311, 333, 365 [365, 359*, 333#, 195, 169]***	**529** ***102, 119, 335***
G	Rha-Rha-C10-C14:1 (-C14:1-C10)		703 *480 [479, 391, 221, 169]*	703 *480, , 221*
H	Rha-Rha-C10-C8 (-C8-C10)	622 *103, 141, 169, 205, 451*	621 *141, 169, 311, 452 [479#, 451*, 311, 169, 141]*	616, 619 & 621 *102, 167, 451#, 452**
I	Rha-Rha-C10-C12# (-C12-C10)*	678 *103, 143, 145, 163, 169, 197, 205, 247, 479#, 507*, 553**	677 *163, 169, 197, 311, 479, 507 [507*, 479#, 367, 197, 169]*	679 *102, 143, 197, 507, 553**
J	Rha-Rha-C10-C12:1 (-C12:1-C10)	676 *103, 169, 205, 247, 479*	675 *103, 119, 195, 311, 479, 507# [505*, 479#, 365, 195,169]*	676 *102, 119, 195, 505*, 507#*
K	Rha-C8-C10# (-C10-C8)*	476 *103, 141, 169, 305#, 333**	475 *163, 168, 311 [333*, 311, 305#, 169, 141]*	475 [no other supportive fragment identified]

*[…] reported by the second author; # * values correspond to monomer bearing same sign; italics: ion fragments (sub-molecular)*

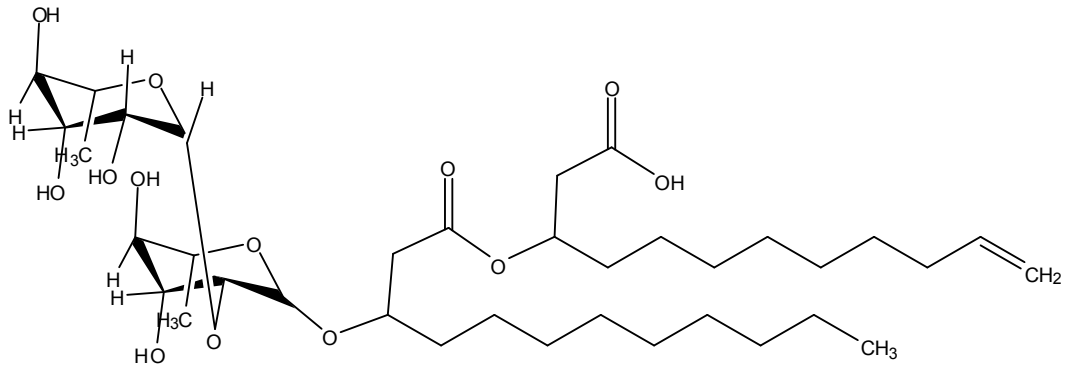
A: Rha-C10-C10



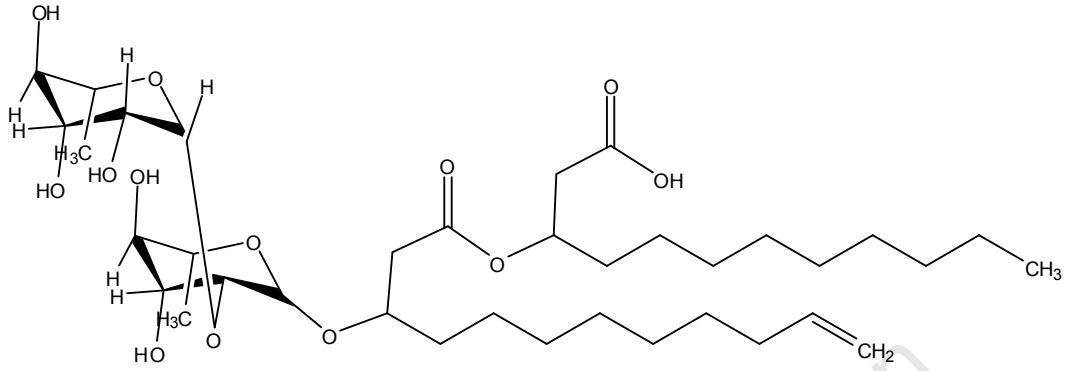
B: Rha-Rha-C10-C10



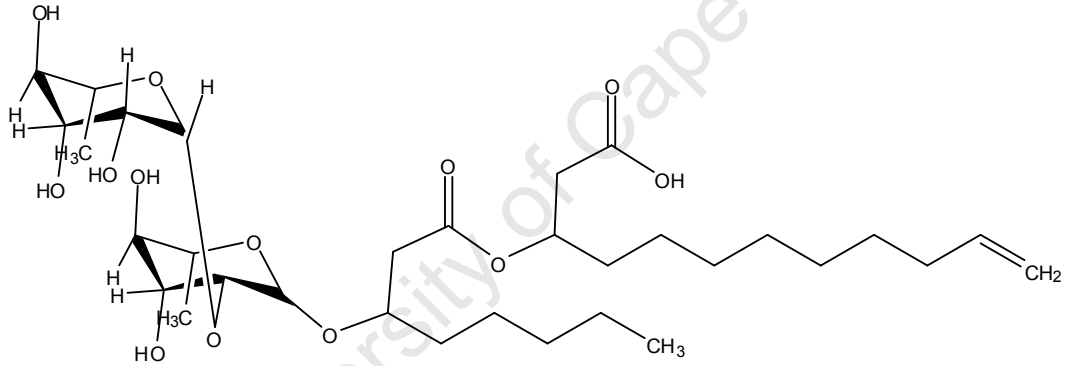
C.1: Rha-Rha-C12-C12:1



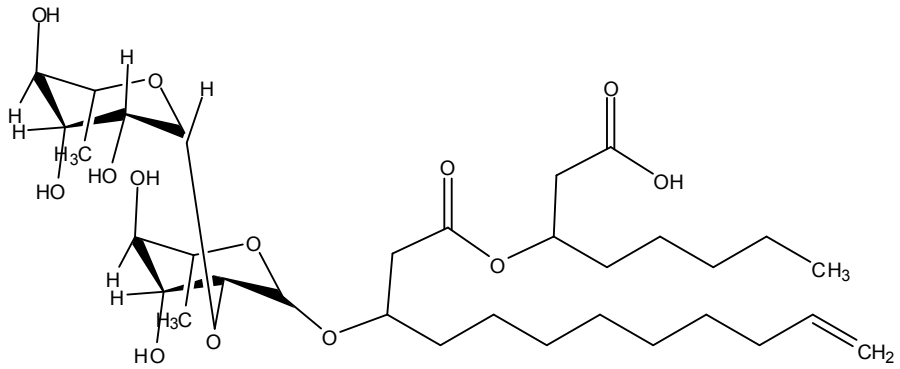
C.2: Rha-Rha-C12:1-C12



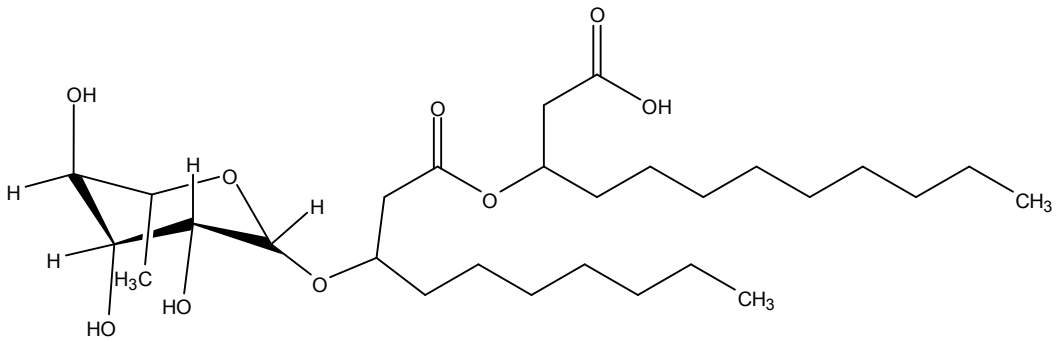
D.1: Rha-Rha-C8-C12:1



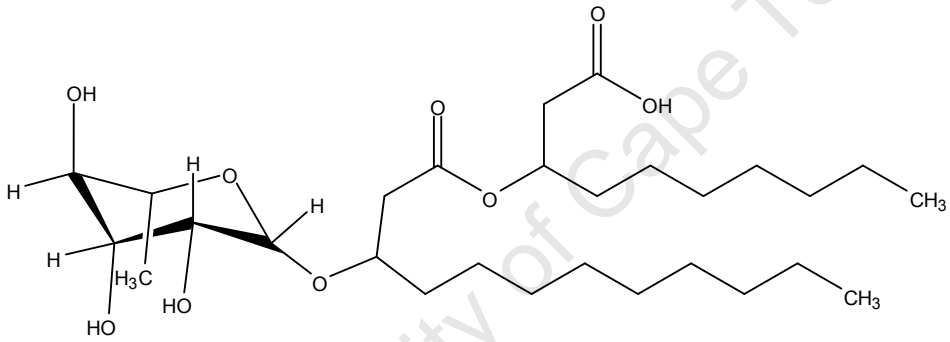
D.2: Rha-Rha-C12:1-C8



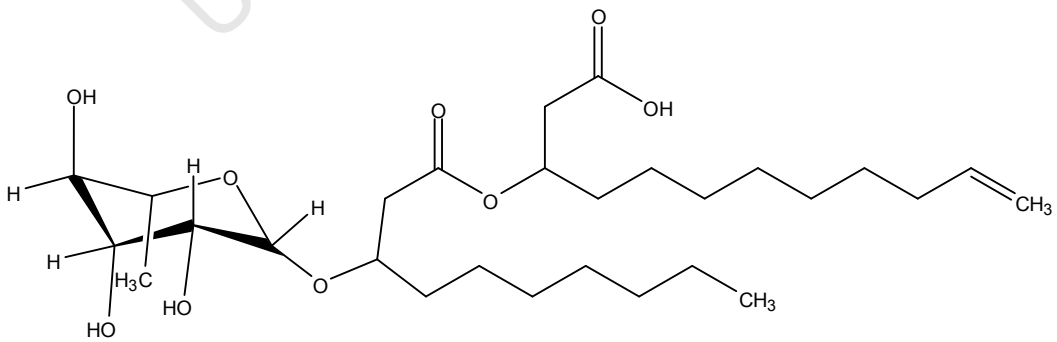
E.1: Rha-C10-C12#



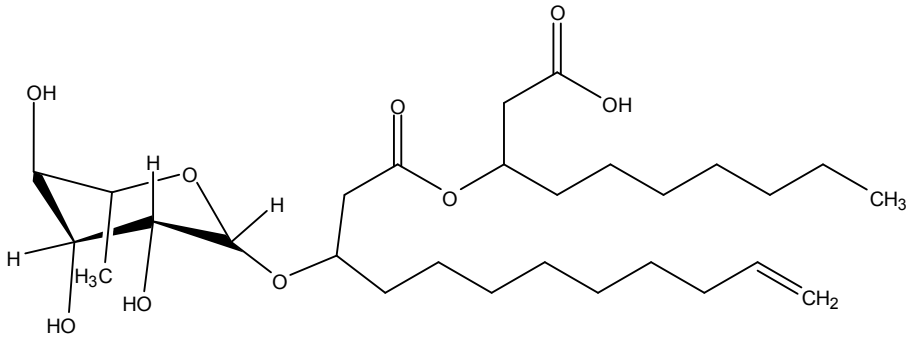
E.2: Rha -C12-C10*



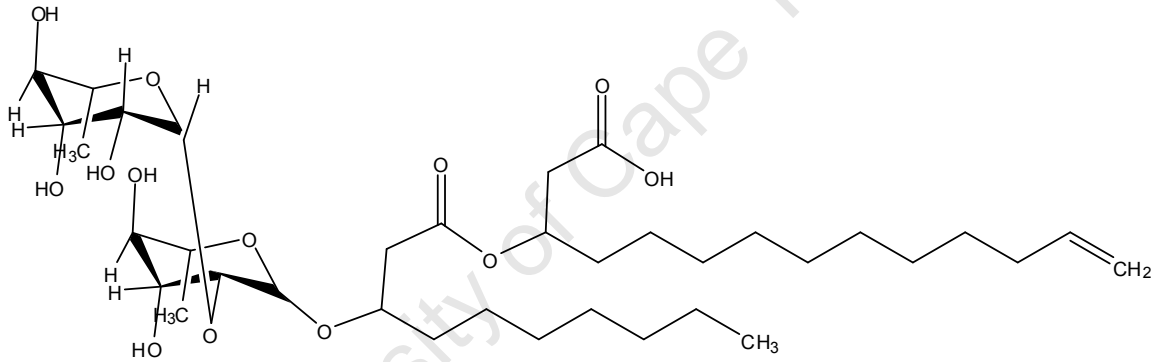
F.1: Rha-C10-C12:1



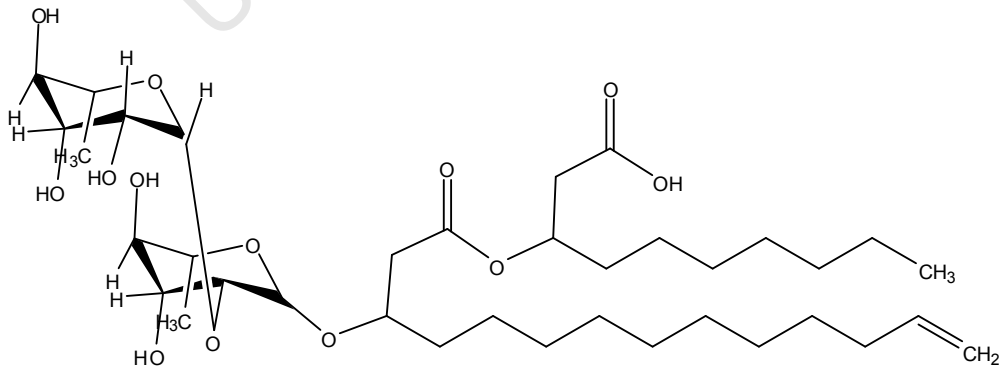
F.2: Rha-C12:1-C10



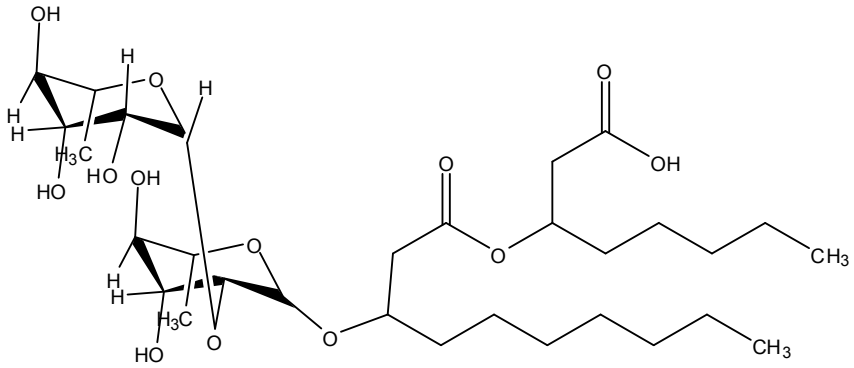
G.1: Rha-Rha-C10-C14:1



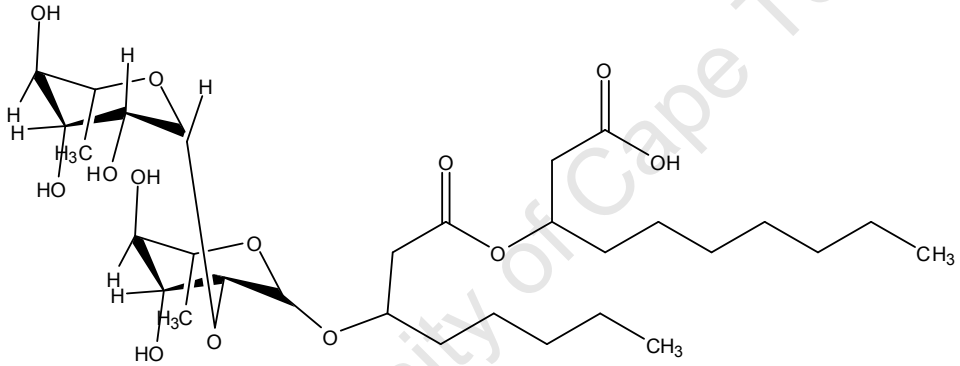
G.2: Rha-Rha -C14:1-C10



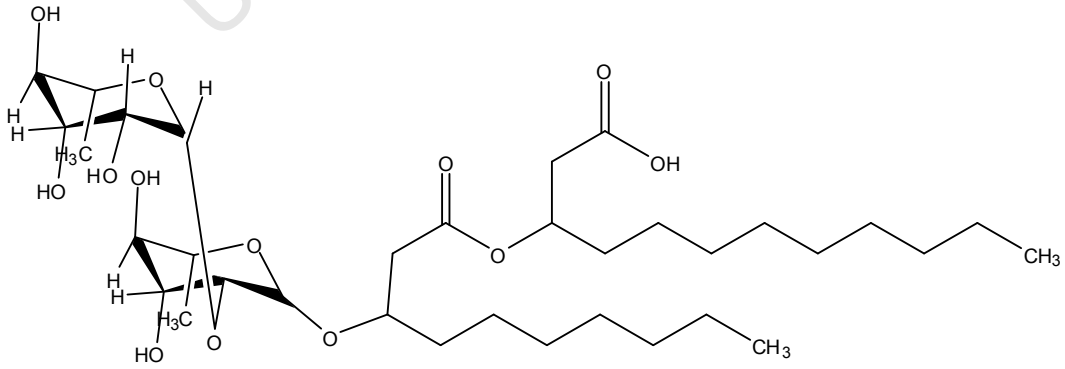
H.1: Rha-Rha-C10-C8



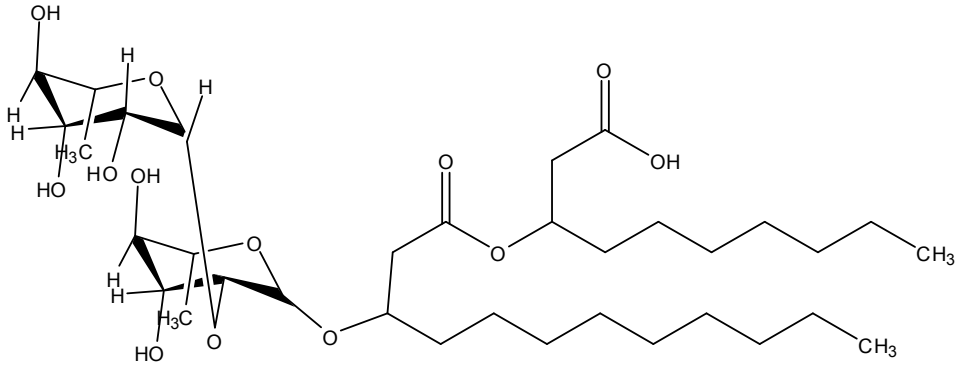
H.2: Rha-Rha -C8-C10



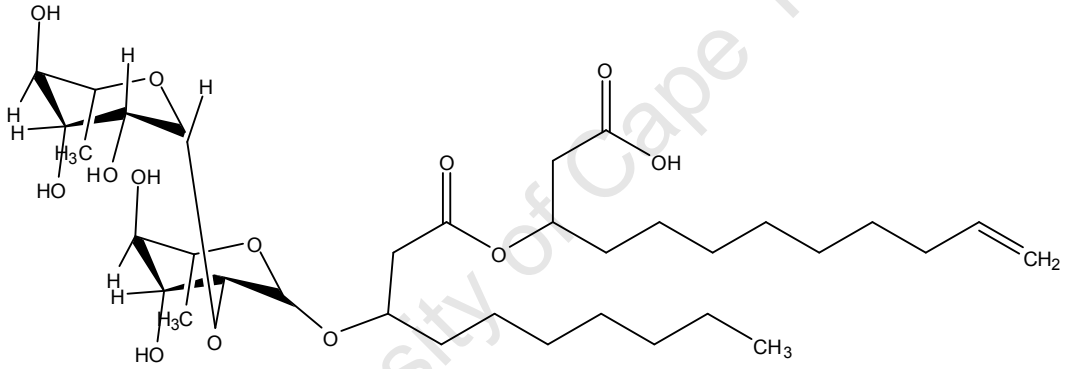
I.1: Rha-Rha-C10-C12#



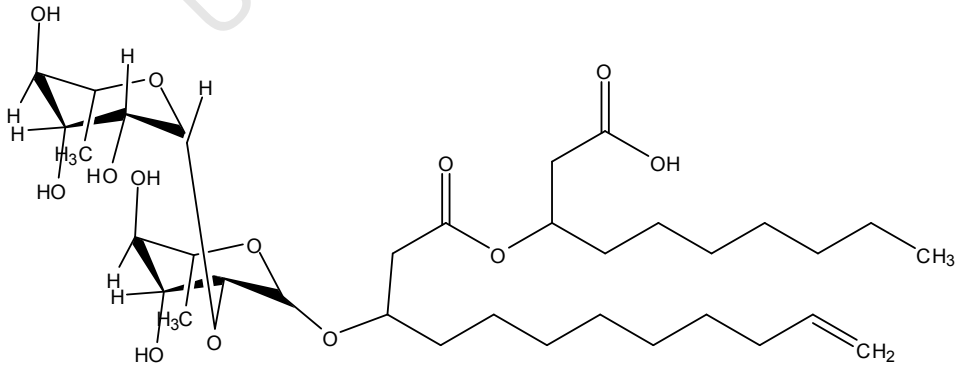
I.2: Rha-Rha -C12-C10*



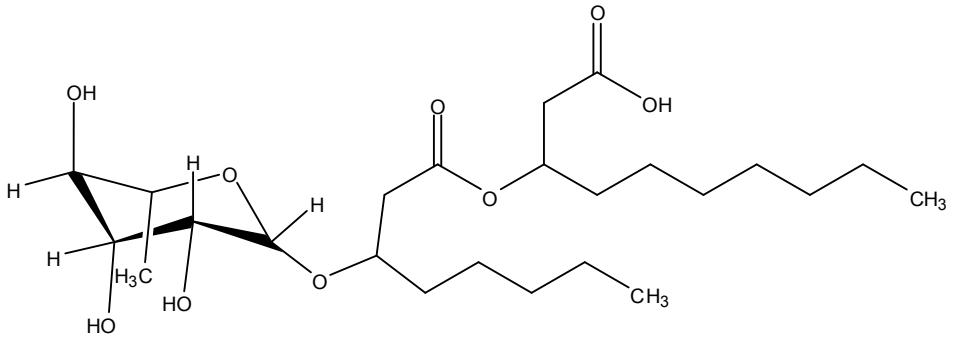
J.1: Rha-Rha-C10-C12:1



J.2: Rha-Rha -C12:1-C10



K.1: Rha-C8-C10#



K.2: Rha -C10-C8*

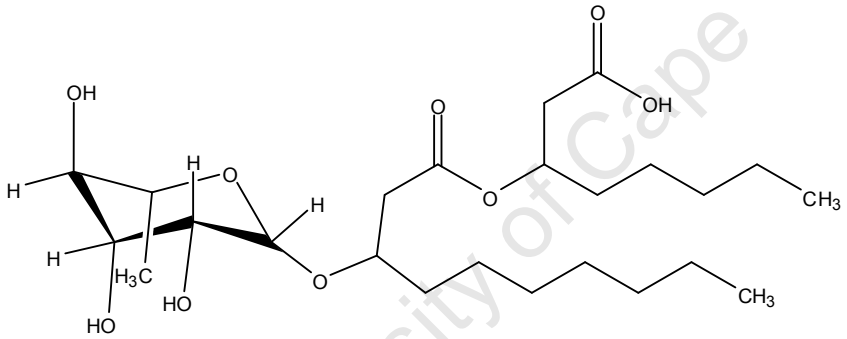


Figure 7.3: Rhamnolipid structures proposed to be present in the biosurfactant produced in this work. The structures labelled $A_{1,2}$, $B_{1,2}$, $C_{1,2}$, $D_{1,2}$, $E_{1,2}$, $F1_{,2}$, $G_{1,2}$, $H_{1,2}$, $I_{1,2}$, $J_{1,2}$ and $K_{1,2}$ refer to the rhamnolipids in Table 7.1.

Most of the pseudomolecular ion peaks found were reported by other authors (Monteiro *et.al.* 2007; Heyd *et al.* 2008; Haba *et al*. 2003). For ion fragments other than the main ions, most of the characteristic peaks were also recorded similar to those reported in literature in literature. The compound proposed to be Rha-C8-C10 and its dimer Rha-C10-C8, did not yield any sub-molecular ions, despite the fact that the peak at 475 m/z (Haba *et al.* 2002; Monteiro *et al.* 2007) is likely to be its main fragment. It was considered as resulting from secondary fragmentations of other identified molecular species, but not an independent rhamnolipid compound.

The compounds identified as Rha-C10-C10 at 503 m/z, Rha-Rha-C12-C12:1 (Rha-Rha-C12:1-C12) at 703 m/z, Rha-Rha-C10-C10 at 650 m/z and Rha-C10-C12 (Rha-C12-C10) at 531 m/z were best matched with those proposed by Haba *et al.* (2003) and Heyd *et al.* (2008). Rha-C10-C10 and Rha-Rha-10-C10, produced as single monomers, are reported to be among the four most abundant compounds during rhamnolipid biosynthesis by *Ps. aeruginosa* strains (Déziel *et al.* 2000; Kosaric 2001; Maier & Soberon-Chavez 2000; Chayabutra & Ju 2001; Tahzibi *et al.* 2004). In this case, Rha-C10-C10 corresponded to the main peak of m/z 503, while Rha-Rha-10-C10 corresponded to the main peaks 650 m/z, 651 m/z and 648 m/z.

This analysis has confirmed the presence of rhamnolipid in the product of *Ps. aeruginosa 2Bf*, as a mixture of similar compounds (Déziel *et al.* 1996). Both one and two rhamnose unit containing compounds were tentatively confirmed to be present in the biosurfactant produced. Chain lengths ranging from 8 to 14 carbon atoms were postulated to be present. A high noise level, expected from the limited purity for the crude product extract limited the quality of mass spectrometric results.

7.3.4 Infrared Spectrometry

The results obtained with Fourier-Transform Infrared suggested the presence of most functional groups that would be expected in glycolipid molecules (Christova *et al.* 2004). In some cases the characteristic bands were masked by other larger bands in their vicinity and were only visualised upon a suitable band separation.

In principle, IR spectroscopy is only sensitive to internal molecular vibration energies associated to different vibration modes specific to each type of functional group e.g. bond stretching, wagging, deformation, scissoring, etc. Thus, IR study is generally limited to the functional group's identification in the sample extracts analysed (Pinchas & Laulicht 1971). From FT-IR results presented in Table 7.2, the presence of certain functional groups characterising glycolipid molecules of the class of rhamnolipids was suggested. The data are compared to those reported in literature for similar molecular infrared analysis.

From the results in Table 7.2, the bands observed corresponding to different modes of vibration of -OH, C-O, C=O and –C=OO- groups were proposed to be due to the presence of both sugar rings and ester groups in the products. These esteric bonds in rhamnolipids make up the link between two carboxylic acid units. This observation

and the similarity of the results obtained tothose of Christova *et al.* (2004) and Tahzibi *et al.* (2004), presented in Table 7.2, support the presence of rhamnolipid compounds in the product. The hypothesised presence of unsaturated rhamnolipid molecules Rha-Rha-C10-C12:1 (-C12:1-C10), Rha-Rha-C14:1-C10 (-C10-C14:1), Rha-Rha-C12:1-C12 (-C12-C12:1), Rha-Rha-C12:1-C8 (-C8-C12:1) and Rha-C10-C12:1 (-C12:1-C10) was supprted by the revelation of C=C double bonds in the analysed biosurfactant. The aliphatic chain carbon-carbon and carbon hydrogen bonds were also detected in their different modes of vibration, as detailed in Table 7.2. The FT-IR analysis has permitted identification of key functional groups and bonds characteristic of glycolipids. The comparison with the findings of other authors has shown consistence of results with respect to the hypothesised compounds, thus supporting presence of rhamnolipid structures in the product. Figures 7.4 and 7.5 show the FT-IR results obtained in this study and by Guo *et al.* (2009), respectively.

Table 7.2: Comparison of FT-IR results of biosurfactant structural study reported in the literature with results from the present work

Christova *et al.* (2004);		**Tahzib *et al.* (2004)**		**Present Work**	
Band wave number (cm^{-1})	**Functional group and/or status (to which the band is assigned)**	**Band wave number (cm^{-1})**	**Functional group and/or status (to which the band is assigned)**	**Band wave number (cm^{-1})**	**Functional group and/or status (to which the band is assigned)**
1739	Ester carbonyl group (-C=O)	1736.5	Not reported reported	1741.38, 1746.73, 1743.45, 1730	Ester C=O stretching
		broad band between 3000 and 3700	Not reported reported	broad band in around 3386.35 ranging between 3050 and 3650	Presence of -OH groups (probably from the sugar moiety)
1718	Carboxylic group (-C=OO-)				
In the region 2700 – 3000 [2853.04 & 2924.61]	Several CH stretching bands of -CH2- and –CH3	2924.61 conjugated to 2855.04	Not reported reported	2924.36 conjugated to 2854.16 and 2958.50	CH3- & -CH2- stretching (evidence of aliphatic nature of lipid tale)
		1572.69	Not reported reported	1741.38	C=O stretch
		1454.91	Not reported reported	1632.73	possibility C=C presence (unsaturation)
		bands between 1300 and 780	Not reported reported	Between 1000 and 750	C-H conjugated to double bond (=C-H)
		722.62	Not reported reported	721.16	
		several bands between 720 and 450	Not reported reported	several bands between 720 and 450	Likely to be linked to the C-H rocking of saturated -CH2-
			Not reported reported	1466.82 & 1378.19	C-CH2 and C-CH3 deformation; 1466 from asymetric deformation and 1378 from symmetric deformation
				1097.95 & 1020.01	C-O stretching
				peak at 3600	free –OH groups

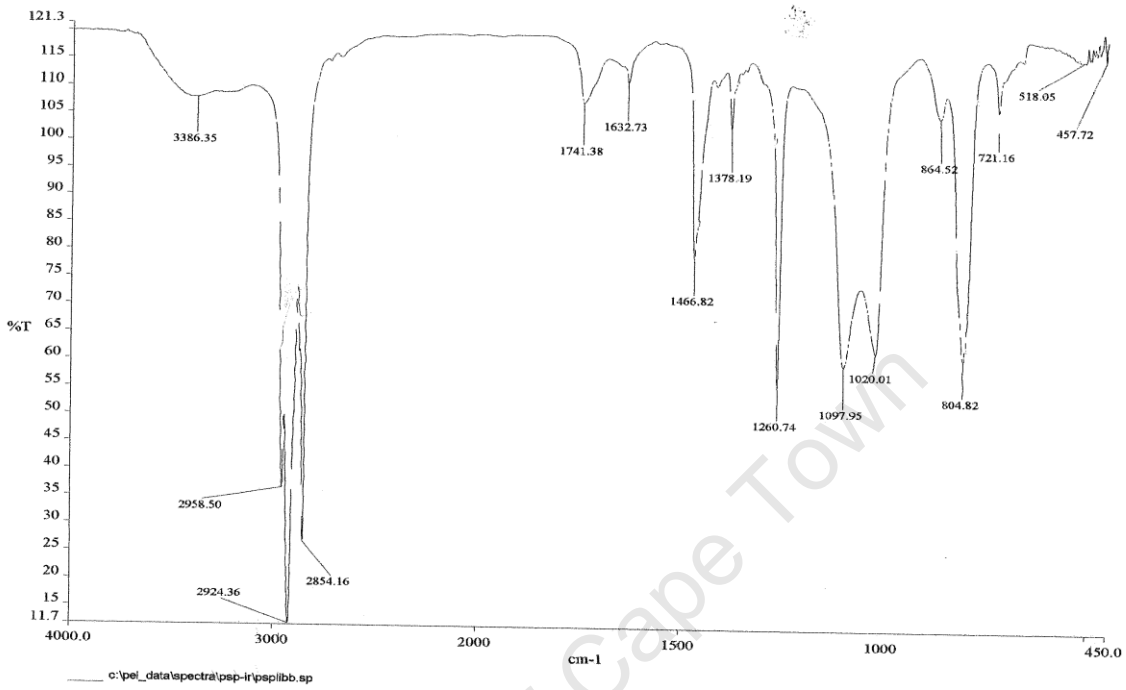


Figure 7.4: Sample FT-IR spectroscopy for the biosurfactant produced by *Ps. aeruginosa 2Bf* under the induction of the C_{14}-C_{17} alkane cut, in this study

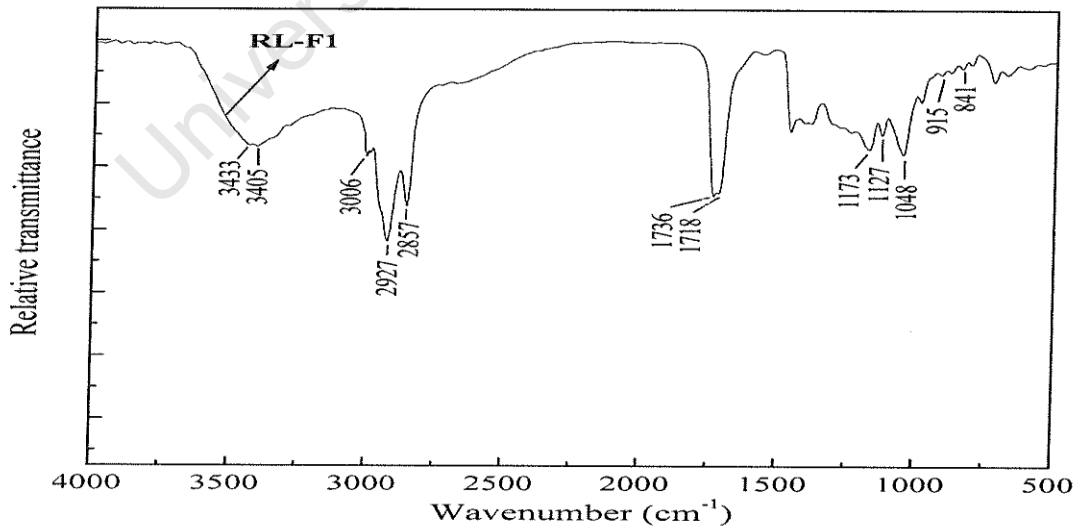


Figure 7.5: FT-IR spectroscopy for a typical rhamnolipid, labeled RL-F1, produced by *Ps. aeruginosa mutant MIG-N146* (Guo *et al.* 2009).

7.4 Antimicrobial activity of biosurfactants

The antibiotic activity demonstrated by *Ps. aeruginosa* (formerly *Bacillus pyocyaneus*) has been reported since 1899 by Emmerich and collaborators, as noted by Shigemi *et al.* (2002), whose work also reported an anti MRSA (Methicillin-resistant *Staphylococcus aureus*) and anti *Candida* biocidal activity.

Ps. aeruginosa 2Bf biosurfactant, identified in the present study as being in the form of a mixture of different rhamnolipid compounds, was initially hypothesized to possess an antimicrobial activity. In this section, the results obtained when testing *Ps. aeruginosa 2Bf* cultures and associated biosurfactant for biocidal activity against a *B. subtilis* are reported.

7.4.1 *Bacillus subtilis*

Bacillus subtilis is a Gram-positive, catalase-positive rod-shaped bacterium commonly found in soil. As a member of the genus *Bacillus*, *B. subtilis* has the ability to form a tough, protective endospore, allowing the organism to tolerate extreme environmental conditions. Unlike several other well-known species, historically *B. subtilis* has been classified as an obligate aerobe; however recent research has disputed this (Nakano & Zuber 1998).

B. subtilis is not considered a human pathogen. It may contaminate food but rarely causes food poisoning. *B. subtilis* produces the proteolytic enzyme subtilisin (Ryan & Ray 2004). Its spores can survive the extreme heating that is often used to cook food, and it is responsible for causing *ropiness,* a sticky, stringy consistency caused by bacterial production of long-chain polysaccharides, in spoiled bread dough.

B. subtilis can divide asymmetrically, producing an endospore that is resistant to environmental factors such as heat, acid, and salt, and which can persist in the environment for long periods of time. The endospore is formed at times of nutritional stress, allowing the organism to persist in the environment until conditions become favorable (Ryan & Ray 2004). Prior to producing the spore the bacterium might become motile, through the production of flagella, and take up DNA from the environment. This means that *B. subtilis* has the ability to take up exogenous DNA from the surrounding medium and integrate it in its genome (Nakano & Zuber 1998).

The test of antimicrobial activity against *B. subtilis* was used to test rhamnolipid biocidal activity proposed by Tuleva *et al.* (2002), as described in Section 4.5.9.

7.4.2 Test for antimicrobial activity

Blue agar plates were prepared with CTAB and methylene blue, and incubated for 48 hours before inoculation, to ensure no contamination. The plates were inoculated with *B. subtilis* from an overnight growth in liquid media. A small diameter filter paper on which culture supernatant from *Ps. aeruginosa 2Bf* was layered, was placed onto the agar medium at the centre of the plate. Zones of clearing in the bacterial lawn demonstrated antimicrobial activity. The plates were incubated at 37ºC for 24 hours. The results obtained are shown in Figures 7.6 and 7.7. This method provided a qualitative analysis.

In Fig. 7.6, a good growth of *B. subtilis* was observed over the solid nutrient agar medium supplemented with 3% glucose. At the center of the plate a small filter paper is visible, on which the separated un-concentrated biosurfactant (the separation was done according to the preparation described in Section 4.5.7) was applied prior to being laid onto the plate. An inhibition zone was clearly drawn around the paper. *B. subtilis* cells could not grow in this zone. Such an inhibition zone can also be used as a basis for quantitative study, knowing the exact amounts that are applied on to the filter paper. This work was limited to the qualitative investigation of the antibiotic potential of the biosurfactant produced by *Ps. aeruginosa 2Bf* in the present study.

The plates in Figure 7.7 correspond to the situation where biosurfactant was used in the crude form, after culture broth centrifugation at 10 000 g for 20 minutes (Fig. 7.7-B), and at 10 000 g for 5 minutes (Fig. 7.7-A). A clear zone of *B. subtilis* inhibition was evident on the two plates.

These results confirmed possession of an antimicrobial activity by the biosurfactant produced by *Ps. aeruginosa 2Bf* under alkane.

Figure 7.6: Inhibition of *B. subtilis* by *Ps. aeruginosa 2Bf*'s biosurfactant following the separation described in Section 4.5.7

Figure 7.7: *B*. *subtilis* inhibition with crude biosurfactant extract from *Ps. aeruginosa*

7.5 Conclusion

This chapter presented and discussed the results obtained during biosurfactant characterisation and identification. For the structural identification, thin layer chromatography, infrared, NMR and mass spectrophotmetric methods were exploited. To study the antimicrobial activity of the product, only one method, the *B. subtilis* inhibition method, was used.

Thin layer chromatography results confirmed the presence of a sugar component in the product structure. This sugar was proposed as rhamnose, since it presented the same $R_{f\,as}$ pure L-rhamnose. It was also found that three sugar structures other than rhamnose, and which were not identified, were also present in the product. Further TLC analyses revealed that glycolipid structures were present in the product. From the observation of TLC plates, approximately 6 different glycolipids were distinguished, but the product was proposed to contain more due to apparently overlapping spots. This multiplicity of rhamnolipid structures was reported earlier in similar biosurfactant studies. Guo *et al.* (2009) reported 14 different rhamnolipid structures produced by *Ps. aeruginosa* mutant MIG-N146 grown on corn oil, most of which were different from those usually found in literature, proposing that this difference could be caused by the microorganism, the culture mode, the medium composition, as well as the analytical methodology used (Guo *et al*. 2009).

The NMR results were not clear enough to allow conclusive identification. The lack of good accuracy in the case of NMR was attributed to the combined quality of instrument and sample used. A low resolution NMR was used, which requires high product concentration and/or sufficiently long sample analysis, not met in the present case. The limited data obtained suggested carbon backbones of C_8 to C_{14} in length.

Using mass spectroscopy, up to six different rhamnolipid structures were tentatively established, most of which were pairs of dimers. The results agreed with those formerly reported by three other research groups (Monteiro *et al.* 2007, Haba *et al.* 2003 and Heyd *et al.* 2008) reporting their spectrometric studies of different rhamnolipid structures. Some of the rahamnolipids were found to contain unsaturated chains at the C1. These results were consistent with Haba *et al.* (2003) who reported 15 different rhamnolipid structures produced by *Ps. aeruginosa 47T2 NCBIM 40044* grown on waste frying oil, some presenting unsaturation on carbon.

Infrared spectroscopy was used to confirm rhamnolipid presence in the product, and characteristic functional groups were identified. The presence of sugar was confirmed by identification of different –OH, -C-O and –C-H bonds, etc. (Heyd *et al.* 2008). The ester groups, carboxylic groups and double bond presence were confirmed in the biosurfactant structures by identification of characteristic carbonyl groups (Wu & Ju 1997). These results were compared to those suggested by Christova *et al.* (2004) and Tahzibi *et al.* (2003), who worked with different *Ps. aeruginosa* strains. Almost all the IR bands identified by these two groups were recognised in the IR spectrogram obtained in this work, in addition to other IR bands characteristic of both lipid and sugar structures. The chain length of identified rhamnolipds ranges from eight to fourteen, and both saturated and unsaturated rhamnolipid structures were found.

The antibiotic character of the biosurfactant produced was studied. In this study, the growth of *B. subtilis* on solid media was inhibited by the presence of the supernatant of *Ps. aeruginosa 2Bf* biosurfactant. These observations agree with the findings of Tuleva *et al.* (2002), who concluded upon similar tests. Tuleva *et al.* (2002) did not use any control. In this study, the method proposed by Tuleva *et al.* (2003) was used unmodified. Consistent with Tuleva *et al.*'s findings in 2002 for antibiotic activity possessed by rhamnolipids, the biosurfactant produced by *Ps. aeruginosa 2Bf* has shown a strong antimicrobial activity against *B. subtilis* cells grown at 37 ºC.

CHAPTER VIII: GENERAL CONCLUSION AND RECOMMENDATIONS

8.1 General conclusion

The general conclusion is presented in three main parts: overview of the context of the work, support for the hypotheses presented and the assessment of objectives realised.

8.1.1 Context of the study

Surfactants are a class of chemical compounds which can alter the surface properties and the interfacial tension between aqueous and oil phases, or between air and liquid phases. They are complex molecules with both a hydrophilic and a hydrophobic character. They range from low molecular weight glycolipid structures to very large lipoprotein structures. Their surface activity is essentially due to their dual affinity towards water molecules. Glycolipids form one of the most important classes of surfactants. The term biosurfactant applies to biologically produced surfactants. These are typically more specific and more environmentally friendly than their chemical counterparts, and are produced by many bacterial, fungal and yeast species, some of which use immiscible materials like linear hydrocarbons. Biosurfactants exhibit a diversity of chemical structures, which confer to them a broad range of applications in a large number of industrial areas, such as: food and beverage industry, agro-pharmaceutical industry, pigment and paint industry, de-scaling of crude oil, bioremediation of oil contaminated water and oil sites, etc.

The present work investigated the potential of alkane compounds to induce biosurfactant production by bacteria, and sought to optimise the production process in a batch reactor system. To achieve this goal, a bacterial species, previously isolated from oil contaminated sites and named *Pseudomonas aeruginosa 2Bf* after proper identification and characterisation at the molecular level, was used to produce a particular class of biosurfactants, identified as rhamnolipid. This biosurfactant consists of rhamnose sugar and decanoic acid, and forms a sub-class of glycolipid biosurfactants.

8.1.2 Verification of hypotheses

The verification of hypotheses helps to confirm or reject initial assertions, conclude the achievements of this study and to set the direction for further research.

- **Hypothesis 1**

It is possible to induce biosurfactant production by Ps. aeruginosa 2Bf with liquid n-alkanes as sole carbon source.

In Section 5.4, alkanes were confirmed to induce biosurfactant production. Poor biosurfactant production by *Ps. aeruginosa 2Bf* resulted on using glucose as sole carbon source, whereas satisfactory results were obtained with the C_{14}-C_{17} alkane blend as sole carbon source. In this case, the aqueous phase-kerosene mixture emulsification index reached 65%. This potential to induce biosurfactant production by *Ps. aeruginosa 2Bf* was found to depend on the growth media composition.

Further in Chapter 6, production of biosurfactant was investigated quantitatively in a series of bioreactor experiments. The presence of biosurfactant in the culture supernatant was confirmed by the low values of surface tension observed, the emulsification index recorded as well as the structural analysis presented in Section 7.3.

- **Hypothesis 2**

The choice of nitrogen source is a determinant step for biosurfactant production by bacteria.

This hypothesis was confirmed by the results obtained during the media study presented in Section 5.3. As shown in Figure 5.3, with a same carbon source, different growth patterns were obtained depending on the substance used for nitrogen source.

❖ **Hypothesis 3**

The mechanical foam control is preferred over chemical foam control.

In Section 6.3.3.2, chemical antifoam efficiency to control foaming in the reactor was compared to that of two different mechanical foam breakers. FB-1 was a two blade paddle mechanical foam breaker, while FB-2 was the modified two blade paddle with three slits mechanical foam breaker. Both of these performed better than the anti-foam reagents. Further investigation established FB-2 as the most efficient tool for foam control in the system.

❖ **Hypothesis 4**

Agitation and aeration rates are among the most important factors affecting the growth of Ps. aeruginosa 2Bf.

Oxygen transfer is one of the major challenges encountered during the alkane bioprocesses. To enhance oxygen transfer in such system, special attention must be paid to the agitation and aeration rates applied. Section 6.3 has largely addressed the importance of agitation and aeration rates in the growth of *Ps. aeruginosa 2Bf* and how they affect its biosurfactant producing capacity.

Further significant interaction between aeration and agitation was found to affect the process only at low agitation rates, whereas the interaction between agitation rate and type of foam control becomes significant only at high agitation rates.

❖ **Hypothesis 5**

The biosurfactants synthesised by Ps. aeruginosa 2Bf are rhamnolipids and possess an antibiotic activity.

In Section 7.3, the structure of *Ps. aeruginosa 2Bf*'s biosurfactant was analysed and found be a glycolipid. Further investigations, supported by the literature, permitted the identification of the product as a mixture of rhamnolipids. Octanoic, decanoic and dodecanoic acids were proposed to dominate in their structure. The last two were the most dominant.

The biocidal activity of the supernatant of *Ps. aeruginosa 2Bf* was tested against a *Bacillus subtilis* culture on solid media, as reported in Section 7.4. On plates where *B. subtilis* was grown on nutrient enriched solid agar media, an inhibition zone developed around a six millimetre diameter filter paper loaded with the crude extract of *Ps. aeruginosa 2Bf* biosurfactant. This *B. subtilis* growth inhibition confirmed possession of an antibiotic activity by the crude biosurfactant preparation.

8.1.3 Assessment of the objectives realised

8.1.3.1 General objectives

The following general objectives were set as the main goals to be achieved in the project:

- *Use a mixture of liquid alkanes to induce biosurfactant production by Ps aeruginosa 2Bf, successfully test the emulsification effect and carry out the structural identification of the product;*

- *Optimise the biosurfactant production conditions in a batch reactor system;*

- *Investigate the performance of mechanical foam control versus chemical control qualitatively.*

The C_{14}-C_{17} alkane cut was used to induce biosurfactant production by *Ps. aeruginosa 2Bf*. As shown in Section 5.4, the product obtained was tested and shown to possess good emulsification power. To test the emulsification capacity, a 1:1 mixture of kerosene and the culture supernatant was used. Emulsification of this mixture of immiscible liquid phases was quantified in terms of the emulsification index, as presented in Section 5.4. The emulsification effect was compared for suspension, supernatant and filtered supernatant, as presented in Table 5.5. Upon these experiments, centrifugation and filtration were found to reduce, to a certain extent, the emulsification capacity of the culture broth, indicating a role of biomass-associated biosurfactant as well as extracellular biosurfactant. This emulsification capacity was found to be dependent on the type of nitrogen used in the process.

The structural identification of the product was presented in Section 7.3. The quality of product's separation and purification limited the outcomes of this section of the study, especially in the case of the nuclear magnetic resonance analysis. NMR analysis required a high purification and a high product concentration in the sample. Two additional spectroscopic analyses, infrared and mass spectrometry, were used. Through combination of these analyses, satisfactory conclusion on the possible nature or identity of the product was drawn. The product's molecular structure was tentatively proposed to be rhamnolipid in nature. Six different rhamnolipid structures were identified as indicated in Section 7.3: Rha-C10-C10, Rha-Rha-C12-C12:1 (-C12:1-C12), Rha-C10-C12 (-C12-C10), Rha-C10-C12:1 (-C12:1-C10), Rha-Rha-C8-C12:1 (-C12:1-C8) and Rha-Rha-C10-C10.

Reactor mixing and aeration conditions, as well as the substrate concentration were optimised as detailed by the results presented in the Section 6.3. Low aeration rates (0.3 vvm) and intermediate agitation rates (500 to 600 rpm) were found to favour growth of *Ps. aeruginosa 2Bf* and its capacity to produce biosurfactants in the bench top bioreactor. High agitation rates of 800 rpm inhibited both growth and biosurfactant production.

8.1.3.2 Specific objectives

As outlined in Section 3.3.2, the following specific objectives were proposed for the present research work:

- *Propose the best way of monitoring growth of Ps. aeruginosa bacterial cells*

- *Establish the growth patterns of Ps. aeruginosa 2Bf on a mixture of liquid alkanes*

- *Monitor variation of essential parameters for bacterial growth and biosurfactant*

- *Study the process in both shake flasks and bioreactor systems characterisation, namely: pH, viscosity, alkane depletion, emulsification effect, surface tension and dissolved oxygen variation*

- *Monitor the main mass transfer parameters at least at three different times of the growth process in a three phase batch reactor system*

- Study the alkane utilisation during the process at the individual chain length level

- Study the effect of alkane concentration on cell growth and on biosurfactant production

- Use at least three different methods for the structural identification of the product.

As shown in Section 5.2, method selection was conducted to find the most efficient method to monitor *Ps. aeruginosa* growth. Four methods were analysed comparatively. The direct cell count was confirmed to be the most reproducible.

The process was studied in both shake flasks (Chapter 5) and the bench top bioreactor (Chapter 6). The trends of *Ps. aeruginosa 2Bf* growth were established. In Section 6.2, the results showed that non-controlled pH conditions favoured biomass and biosurfactant production. The results also showed that biosurfactant was mainly produced in the late exponential and stationary phases. Gas liquid mass transfer was enhanced by higher aeration rate (Section 6.3.4) and the presence of alkane. The alkane concentration impact on the process was investigated. Concentrations of alkanes of 10% and 20% w/v were found to be inhibitory to both biosurfactant production and biomass growth. The best results were obtained with the alkane concentration of 3% (Section 6.4.1). The reactor mixing conditions were found to influence the preferential consumption of different alkanes depending on their chain length and the microbial growth stage, as discussed in Section 6.4.2.

Four different methods, TLC, IR, MS and NMR, were used for the structural identification of the product. At end of this study, the product was proposed to be a mixture of six different rhamnolipids, as detailed in Section 7.3.

8.2 Recommendations

At the end of this thesis, the following recommendations were formulated:

- Biosurfactant should be directly quantified in mass terms so as to avail calculations on the biosurfactant (mass) productivity basis.

- Purification of biosurfactant should be optimised so as to allow definitive and more conclusive analyses for NMR and other spectroscopic techniques.

- Hydrocarbon sterilisation and feeding was done in many steps with contamination risks even during the filter-sterilisation process, as only a small amount of alkane could be sterilised at a time. This question should be addressed in further research to make hydrocarbon feeding more efficient.

- Off-gas analysis and monitoring would allow more information to be collected on the metabolic systems involved in the alkane degradation at different stages.

- Further studies of the process in a fed-batch reactor system should be conducted to improve biosurfactant productivity.

- Further studies should be conducted to test the anti-microbial capacity of *Ps. aeruginosa 2Bf*'s biosurfactant on more bacterial and fungal species, to determine its potential as anti-fungicide or biopesticide in the agro-pharmaceutical arena.

- The study of *Ps. aeruginosa 2Bf*'s biosurfactant should be extended to investigate its possible applications. Rhamnolipids are known to be efficient in metal sequestration and concentration, enhancement of the formation of nano-materials, use as ingredient in cosmetics, detergents, shampoos, and soaps, use in environmental bioremediation, enhanced oil recovery, use of rhamnolipid foaming to remove heavy metal contaminants from soils, wastewater, and other liquids, etc.

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APPENDICES

Appendix I

A. Calibration curve for the Du Nouy tensiometer

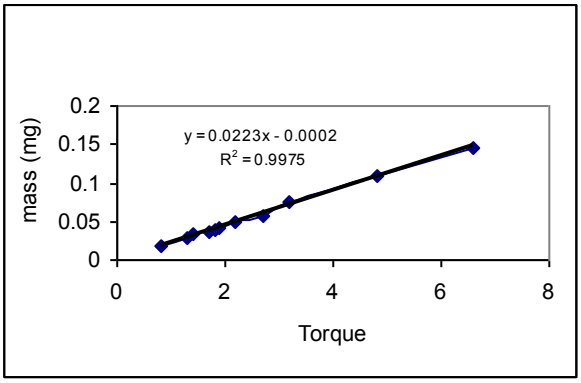

Figure A.1: Calibration curve for the Du Nouy tensiometer, which served as the basis for surface tension measurement

B. Determination of S.T. using the measured data (This is an example of how calculation were done)

Table A.1: Surface tension measurement

		Surface Tension measurement for the C14-C17 blend in shake flask (18 h)					
	Deion-Water	M1	M1'	M2	M2'	M3	M4
Calibration Constant	0.0223	0.0223	0.0223	0.0223	0.0223	0.0223	0.0223
Torque	43.00	26.00	28.10	29.75	26.10	26.10	26.60
Mass	0.9589	0.5798	0.6266	0.6634	0.5820	0.5820	0.5932
Density	0.9967	1.0018	0.9874	0.9873	0.9884	0.9965	0.9959
Volume	0.9620748	0.5787582	0.6346263	0.6719589	0.5888608	0.5840743	0.5956220
R	0.9542930	0.9542930	0.9542930	0.9542930	0.9542930	0.9542930	0.9542930
R^3 / v	0.9033091	1.5015786	1.3693902	1.2933098	1.4758174	1.4879118	1.4590645
R/r	53.6	53.6	53.6	53.6	53.6	53.6	53.6
F-value	0.943	0.908	0.913	0.917	0.91	0.908	0.910
ST	*75.4038*	*43.9008*	*47.7079*	*50.7305*	*44.1670*	*44.0696*	*45.0128*

For water, four different readings have been done giving exactly the same values. For other samples, the value given is an average of three readings. The calculations were made as follows:

Surface Tension = (Mass x 100 x f value)/ (4 x Π x Ring radius)

Mass = (Calibration constante) x (Torque value)

Calibration constant = 0.0222 for n-hexadecane and 0.223 for the C14-C17 alkane cut obtained from the calibration curves

Density of sample extract = (Mass of 20 ml sample extract) / (20ml)

F-value was obtained from Table A.2., through extrapolation where necessary

R = ring radius = 0.9543 cm

r = wire radius (this is the wire which the ring is made of)

R/r = 53.6 (this is the average ratio provided by the manufacturer)

C. Some values of the correction factor f for the ring method, following Harkins and Jordan (1930)

Table A.2: Correction factor data for the ring method of surface tension determination (Harkins and Jordan, 1930)

	R/r						
R^3/V	30	40	48	50	52	54	60
.80	.8937	.9230	.942	.9454	.949	.954	.9581
.82	.8817	.9211	.940	.9436	.947	.952	.9563
.84	.8894	.9190	.938	.9419	.946	.951	.9548
.86	.8874	.9171	.936	.9402	.944	.949	.9534
.88	.8853	.9152	.934	.9384	.942	.947	.9517
.90	.8831	.9131	.933	.9367	.940	.946	.9504
.92	.8809	.9114	.931	.9350	.939	.945	.9489
.94	.8791	.9097	.929	.9333	.937	.943	.9476
.96	.8770	.9074	.928	.9320	.936	.942	.9462
.98	.8754	.9064	.926	.9305	.934	.940	.9452
1.00	.8734	.9047	.925	9290	.933	.939	.9438
1.05	.8688	.9007	.921	.9253	.929	.936	.9408
1.10	.8644	.8970	.917	.9217	.925	.933	.9378
1.15	.8602	.8937	.914	.9183	.922	.930	.9352
1.20	.8561	.8904	.911	.9154	.920	.927	.9324
1.25	.8521	.8874	.908	.9125	.916	.924	.9300

Appendix II

GC set up

Column TRB-1
100% dimethylpolysiloxane ≡ DB-1, OV-1, HP-1, etc.

25m x 0.25mm x 0.50µm

Column temperature : 120ºC

Head Pressure : 9 psig

Split : 100:1

Carrier Gas : He at 40ml/min

Flame Gas : H2 at 44.624 ml/min
Air at 323.577 ml/min

Injector Temperature : 280ºC

Detector : FID → Temperature : 290ºC

Oven temperature : 100ºC held for 5min, with increments of 10ºC/min up to 260ºC and held for 5 min.

Appendix III

Data for method selection during biomass quantification

Table A.3: Biomass quantification method study data for CDW – normal Hexane

Time (h)	M1	M1'	Avrg	STDEV	M2	M2'	Avrg	STDEV	M3	M3'	Avrg	STDEV	M4	M4'	Avrg	STDEV
0	0.300	0.300	0.300	0.000	0.300	0.300	0.300	0.000	0.300	0.300	0.300	0.000	0.300	0.300	0.300	0.000
1.25	0.410	0.323	0.367	0.062	0.511	0.700	0.606	0.134	0.900	0.240	0.570	0.467	0.500	0.143	0.322	0.252
9.25	1.000	1.500	1.250	0.354	1.000	1.000	1.000	0.000	0.900	1.900	1.400	0.707	1.300	1.072	1.186	0.162
12.92	2.300	1.900	2.100	0.283	1.600	1.400	1.500	0.141	1.500	0.500	1.000	0.707	2.100	2.000	2.050	0.071
15.253	1.600	3.100	2.350	1.061	1.400	1.900	1.650	0.354	1.200	0.000	0.600	0.849	1.600	2.100	1.850	0.354
17.503	1.400	3.100	2.250	1.202	2.800	2.200	2.500	0.424	1.200	1.500	1.350	0.212	2.300	0.600	1.450	1.202
22.528	2.500	3.100	2.800	0.424	3.450	3.400	3.425	0.035	4.000	4.900	4.450	0.636	5.300	2.100	3.700	2.263
24.028	4.600	3.000	3.800	1.131	4.100	6.300	5.200	1.556	3.000	3.700	3.350	0.495	2.800	3.100	2.950	0.212
26.528	4.700	4.500	4.600	0.141	4.900	5.600	5.250	0.495	3.100	3.800	3.450	0.495	2.600	6.000	4.300	2.404
37.167	3.500	3.900	3.700	0.283	4.600	4.250	4.425	0.247	3.700	3.900	3.800	0.141	4.100	2.400	3.250	1.202

Table A.4: Biomass quantification method study data for CDW - Cyclohexane

Time (h)	M1	M1'	Avrg	STDEV	M2	M2'	Avrg	STDEV	M3	M3'	Avrg	STDEV	M4	M4'	Avrg	STDEV
0	0.290	0.290	0.290	0.000	0.290	0.290	0.290	0.000	0.290	0.290	0.290	0.000	0.290	0.290	0.290	0.000
1.25	0.294	0.230	0.262	0.045	0.310	0.720	0.515	0.290	0.730	0.850	0.790	0.085	0.390	0.320	0.355	0.049
9.25	3.000	1.500	2.250	1.061	1.300	1.000	1.150	0.212	3.400	1.230	2.315	1.534	0.540	0.350	0.445	0.134
12.92	1.200	3.800	2.500	1.838	2.600	2.100	2.350	0.354	1.500	3.500	2.500	1.414	1.810	2.670	2.240	0.608
15.253	4.100	3.900	4.000	0.141	2.800	2.400	2.600	0.283	1.200	4.050	2.625	2.015	3.000	3.130	3.065	0.092
17.503	1.400	4.100	2.750	1.909	2.800	2.820	2.810	0.014	1.200	4.600	2.900	2.404	3.300	4.100	3.700	0.566
22.528	4.900	4.600	4.750	0.212	3.150	3.400	3.275	0.177	4.000	5.000	4.500	0.707	3.860	4.400	4.130	0.382
24.028	4.800	4.900	4.850	0.071	3.500	6.300	4.900	1.980	3.000	3.700	3.350	0.495	3.800	4.200	4.000	0.283
26.528	4.900	4.500	4.700	0.283	4.900	5.100	5.000	0.141	3.100	3.800	3.450	0.495	3.600	4.400	4.000	0.566
37.167	4.850	4.900	4.875	0.035	4.600	4.500	4.550	0.071	3.700	3.900	3.800	0.141	3.850	4.400	4.125	0.389

Table A.5: Biomass quantification method study data for OD

Time (h)	M1	M1'	Avrg	STDEV	M2	M2'	Avrg	STDEV	M3	M3'	Avrg	STDEV	M4	M4'	Avrg	STDEV
0	0.870	0.870	0.870	0.000	0.870	0.870	0.870	0.000	0.870	0.870	0.870	0.000	0.870	0.870	0.870	0.000
1.25	1.700	1.275	1.488	0.301	1.530	1.530	1.530	0.000	0.425	0.850	0.638	0.301	0.850	1.521	1.186	0.475
9.25	2.550	2.975	2.763	0.301	1.804	3.109	2.456	0.922	1.000	2.975	1.988	1.397	0.425	2.200	1.313	1.255
12.92	2.975	3.750	3.363	0.548	2.509	3.825	3.167	0.931	1.275	1.403	1.339	0.090	1.275	2.975	2.125	1.202
15.253	3.170	3.925	3.548	0.534	3.028	4.128	3.578	0.778	2.030	1.275	1.653	0.534	1.700	2.950	2.325	0.884
17.503	2.500	3.425	2.963	0.654	3.400	4.121	3.760	0.510	2.700	1.275	1.988	1.008	2.975	3.225	3.100	0.177
22.528	5.525	4.250	4.888	0.902	4.250	4.250	4.250	0.000	4.710	4.250	4.480	0.325	8.500	3.825	6.163	3.306
24.028	4.675	6.375	5.525	1.202	4.500	6.375	5.438	1.326	6.800	5.525	6.163	0.902	4.250	2.975	3.613	0.902
26.528	7.500	6.375	6.938	0.795	5.950	4.675	5.313	0.902	5.525	5.950	5.738	0.301	4.675	5.100	4.888	0.301
37.167	7.075	5.100	6.088	1.397	5.950	4.675	5.313	0.902	5.525	5.950	5.738	0.301	4.675	7.225	5.950	1.803

Table A.6: Biomass quantification method study data for DCC

Time (h)	M1	M1'	Avrg	STDEV	M2	M2'	Avrg	STDEV	M3	M3'	Avrg	STDEV	M4	M4'	Avrg	STDEV
0	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
3	0.428	0.300	0.364	0.090	0.306	0.746	0.526	0.311	0.375	0.422	0.398	0.034	0.423	0.486	0.454	0.044
5	0.461	0.338	0.399	0.087	0.780	0.956	0.868	0.124	0.744	1.042	0.893	0.211	0.921	0.921	0.921	0.000
8	0.628	0.367	0.498	0.184	1.300	1.288	1.294	0.009	1.348	1.343	1.346	0.004	1.315	1.254	1.284	0.044
9	0.952	0.848	0.900	0.074	1.424	1.424	1.424	0.000	1.615	2.091	1.853	0.336	1.408	1.529	1.469	0.085
14	1.450	1.765	1.608	0.223	1.779	2.400	2.090	0.439	2.796	3.235	3.015	0.311	2.011	2.113	2.062	0.073
15	1.758	2.205	1.981	0.316	2.231	3.402	2.817	0.828	3.046	3.343	3.195	0.210	2.421	2.306	2.364	0.081
16	1.993	2.337	2.165	0.244	2.412	3.412	2.912	0.707	3.286	3.439	3.363	0.109	2.463	2.398	2.431	0.045
18	2.144	2.388	2.266	0.173	2.915	3.929	3.422	0.717	3.391	3.928	3.660	0.380	3.124	2.472	2.798	0.461
20	2.300	2.586	2.443	0.202	3.351	4.356	3.854	0.711	3.902	4.128	4.015	0.160	3.350	3.058	3.204	0.206
21	2.763	2.771	2.767	0.006	3.907	4.430	4.169	0.370	4.141	4.219	4.180	0.055	3.462	3.392	3.427	0.050
21.5	2.781	2.613	2.697	0.119	4.325	4.632	4.478	0.217	4.455	4.455	4.455	0.000	3.724	4.032	3.878	0.218
23	3.156	2.824	2.990	0.235	4.526	5.238	4.882	0.503	5.363	5.084	5.223	0.197	4.388	4.388	4.388	0.000
24	3.308	3.073	3.190	0.166	5.160	5.408	5.284	0.176	5.453	5.342	5.397	0.079	4.418	4.665	4.542	0.175
26	3.433	3.302	3.368	0.093	5.349	5.554	5.452	0.145	5.609	5.609	5.609	0.000	4.887	4.902	4.895	0.011
28	3.420	3.417	3.419	0.002	5.807	5.806	5.806	0.001	5.611	5.528	5.570	0.059	4.962	4.935	4.948	0.019
30	3.525	3.591	3.558	0.046	5.835	5.799	5.817	0.026	5.601	5.598	5.599	0.002	4.963	4.945	4.954	0.013
32	3.516	3.583	3.550	0.048	5.824	5.805	5.814	0.013	5.769	5.789	5.779	0.014	4.960	4.945	4.952	0.011
47	3.527	3.621	3.574	0.067	5.830	5.812	5.821	0.013	5.769	5.808	5.788	0.027	4.956	4.942	4.949	0.011

Appendix IV

A. Orcinol colorimetric method (Section 4.5.1)

A 333µl aliquot of the culture supernatant was extracted twice with 1ml of diethyl ether. The ether fractions were pooled and evaporated to dryness. A volume of 0.5 ml of H_2O was added. At this stage, 900µl of the orcinol solution (53% v/v H_2SO_4 + 0.19% w/v orcinol) were added to 100µl of each sample. After heating for 30 min at 80°C, the samples were cooled for 15 min at room temperature and the absorbance was measured at 421nm. The concentrations of rhamnolipids were compared to data with those of rhamnose standards between 0 and 50µg/ml (Koch *et al.* 1991; Tahzibi, Kamal & Assadi 2004), but calibrating the system with standard rhamnose solutions was unsuccessful.

Alternatively, the culture broth can be centrifuged for 10 min at 10'000 g. Thereafter, a 100 µl of the supernatant and 900µl of the orcinol reagent are mixed in a test tube. In this case, the mixture is heated at 100°C for 20 min then cooled at room temperature for 15 min. The absorbance is measured at 421 nm against standard rhamnose solutions (Koch et *al.*; Jeong et al. 2004).

B. Anthrone method and preparation of the anthrone reagent (Section 4.5.3)

The method uses the anthrone reagent, which is prepared as follows: 2g of anthrone are dissolved in a litre of concentrated sulphuric acid. Best results are obtained when the reagent is freshly prepared, although it may be used after standing for 7 to 8 days. In this case, a standard must be run with each determination, since the absorption decreases 5 to 10% on aging. Satisfactory preparations are available commercially, although the stability in H_2SO_4 varies widely.

Procedure: A 10 ml aliquot of anthrone reagent is added to a wide mouthed test tube and chilled in a water bath at 10 to 15°C. 5ml of the solution containing 100 to 200 λ of sugar is carefully layered over the H_2SO_4 and allowed to chill. The tubes are then shaken vigorously while still immersed in the bath. The samples are brought to room temperature and placed in a bath at 90°C for 16min. They are then cooled and read shortly afterwards at 625 mµ (Ashwell 1957). N.B.: λ is an old unit of mass equal to 1µg

Appendix V

HPLC method

The HPLC extract is generally prepared as follows (Chayabutra *et al.* 2001): 5 ml of the fermentation broth is adjusted to pH10 with NaOH 5N, extracted with 10 ml of hexane and centrifuged at 14'500 g for 15 min. The clear bottom aqueous phase is analysed for rhamnolipid concentration by HPLC.

In this case, pure L-rhamnose test showed maximum absorbtion at around 210 nm. The following Figure shows the results as displayed by the HPLC instrument.

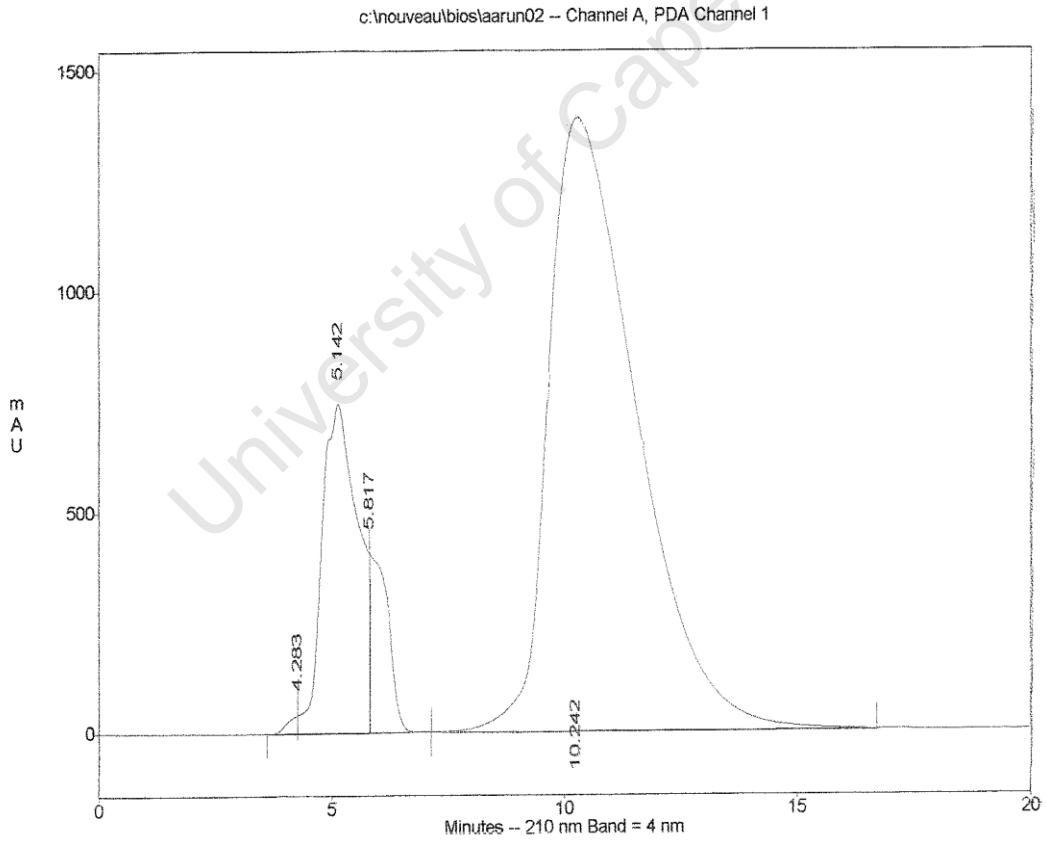

Figure A.2: HPLC analysis of standard L-rhamnose with a UV detector, at 210 nm

Appendix VI

A. Treatment applied to samples prior to NMR, FT-IR and MS spectrometric methods

This method was used by Tahzibi *et al.* (2004): 3ml culture sample were centrifuged 10 min at 10'000 g, the pH of the supernatant adjusted to 2.0 and the solution allowed to stand overnight at 4°C. An extraction was then done with a 2:1 mixture of chloroform and methanol. The solvent was evaporated and the residue dissolved in 3ml 0.1M $NaHCO_3$. This solution was used for spectroscopic analysis.

B. Sample spectrograms obtained with different spectroscopic methods

B1. Mass spectroscopy

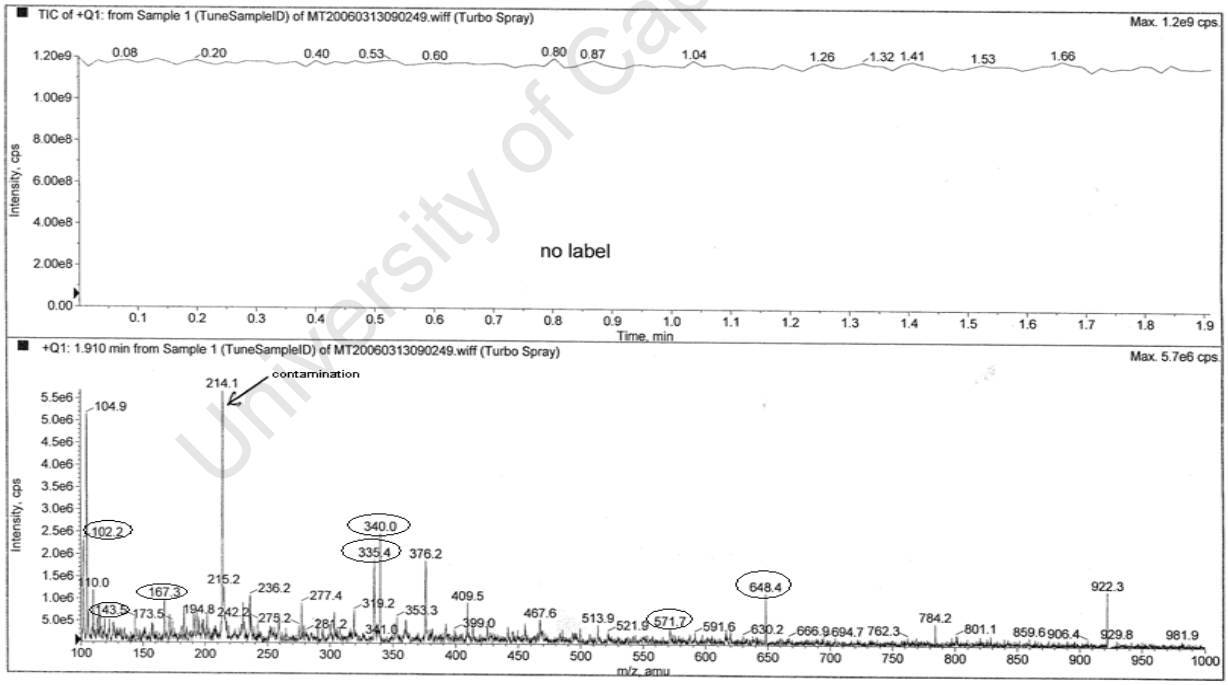

Figure A.3: Sample MS spectrum obtained with a sample scan between 100 m/z and 1000 m/z

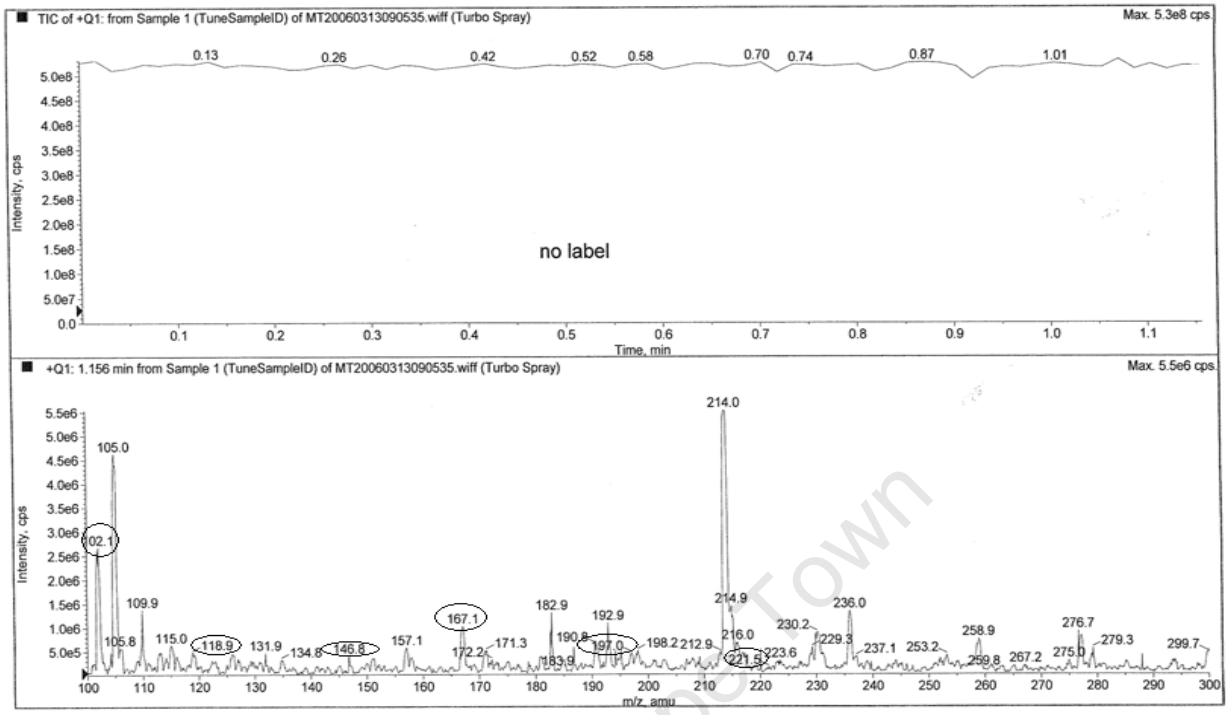

Figure A.4: Sample MS spectrum obtained with a sample scan between 100 m/z and 300 m/z

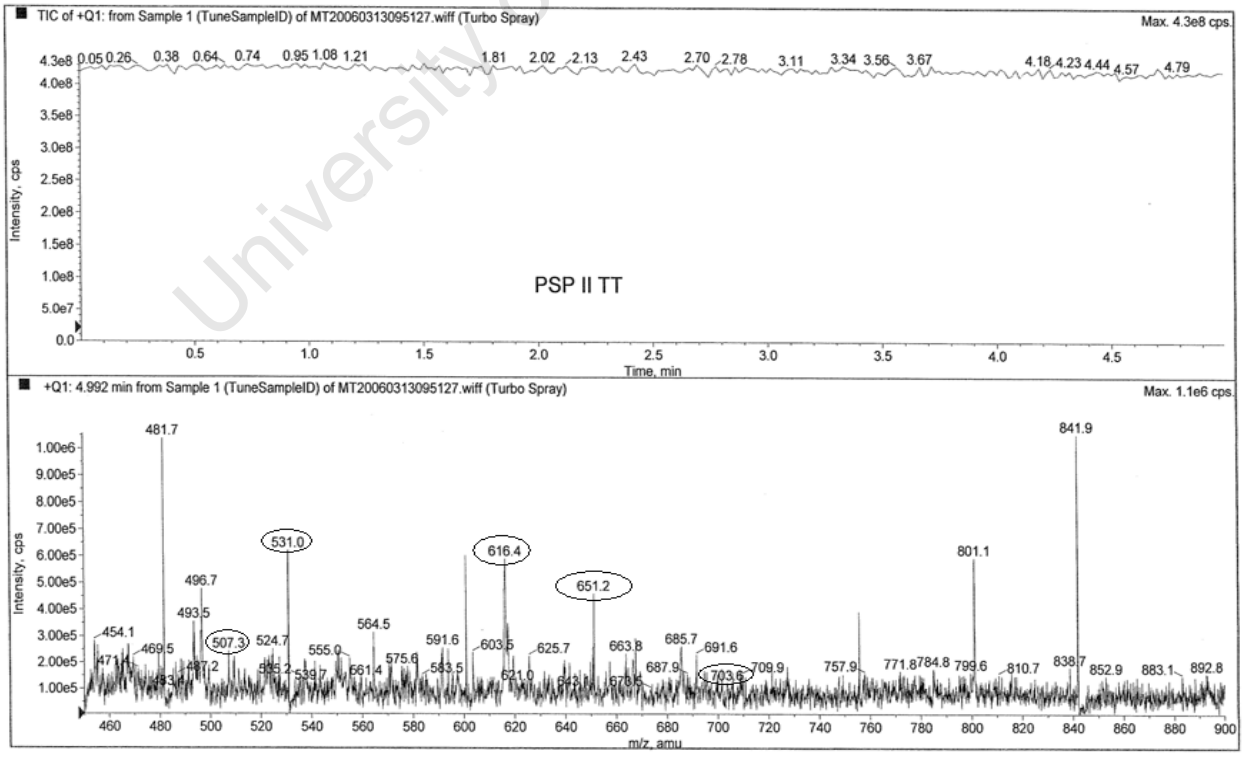

Figure A.5: Sample MS spectrum obtained with a sample scan between 450 m/z and 900 m/z

B2a. ^{1}H-NMR spectroscopy

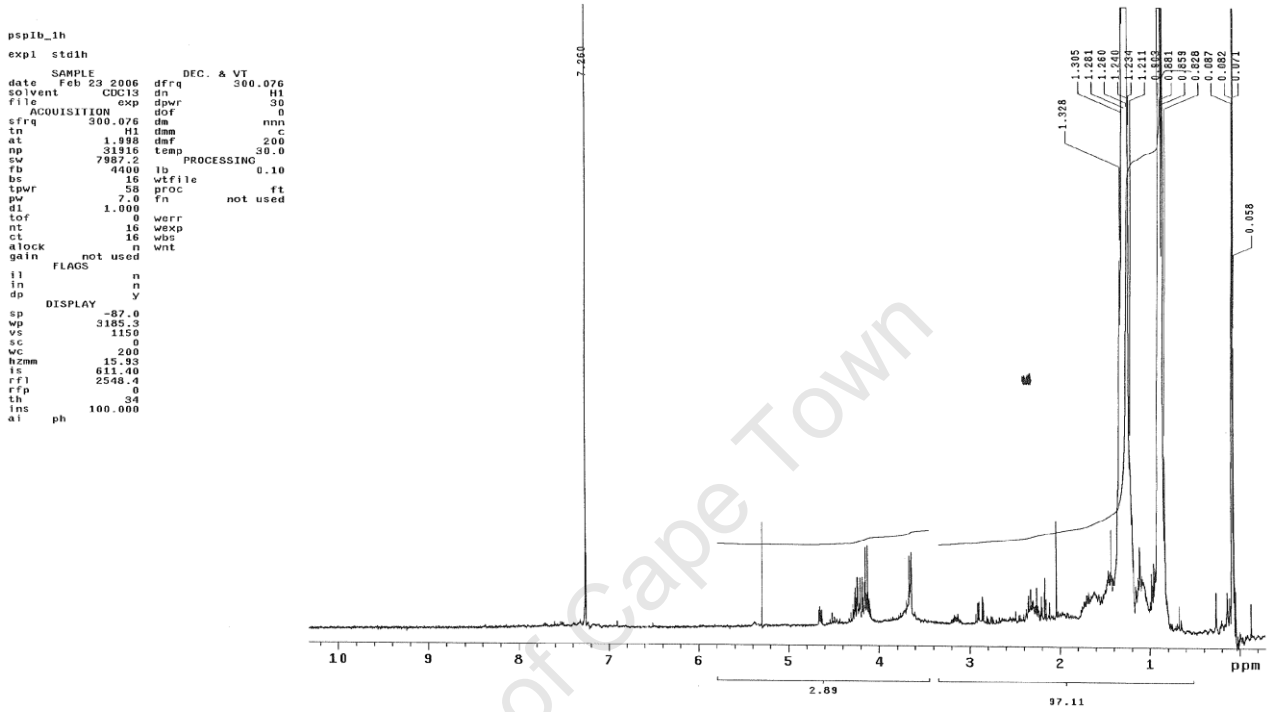

Figure A.6: First sample proton NMR spectrum obtained with proton NMR

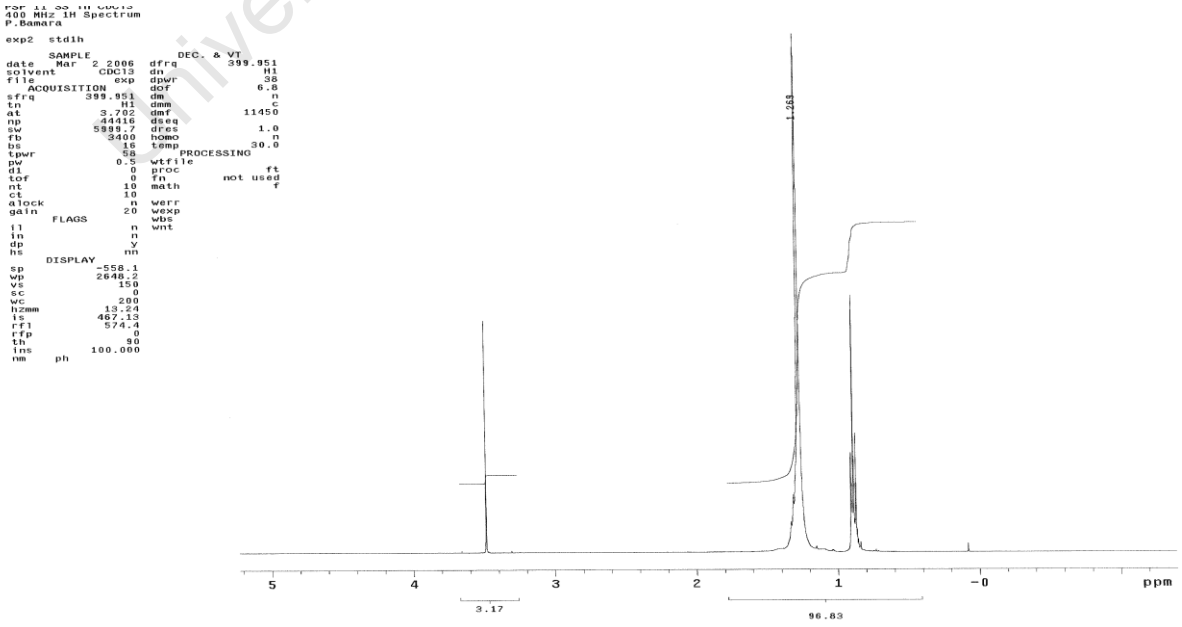

Figure A.7: Second Sample proton NMR spectrum obtained with proton NMR

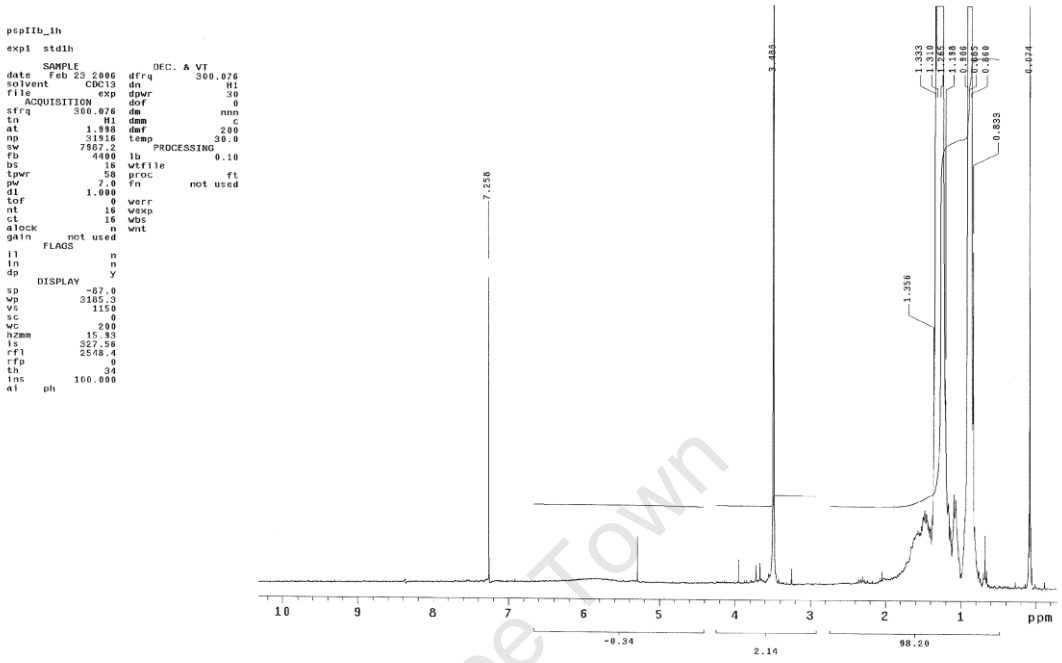

Figure A.8: Third sample proton NMR spectrum obtained with proton NMR

B2b. 13C-NMR spectroscopy

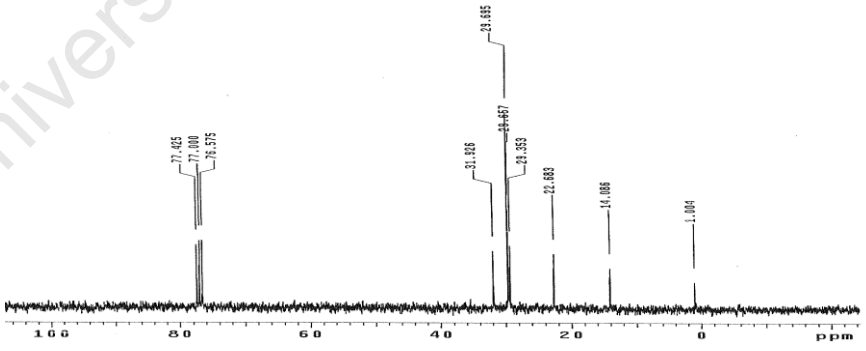

Figure A.9: First sample carbon-13 NMR spectrum

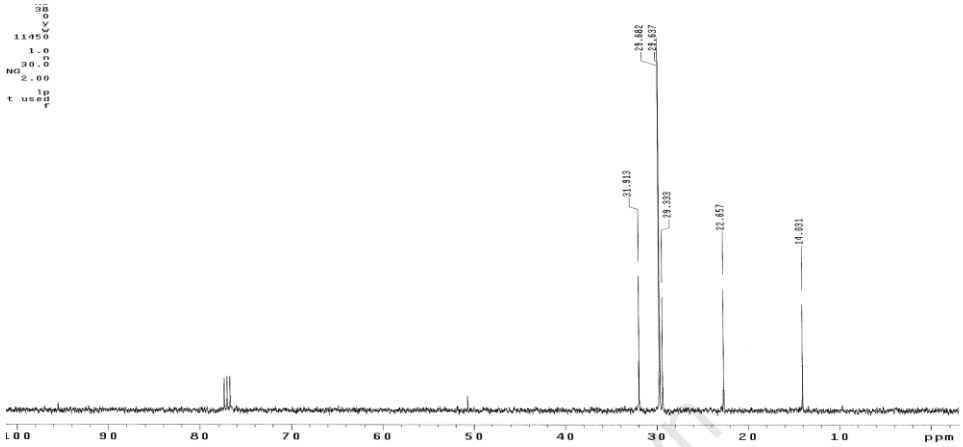

Figure A.10: Second sample carbon-13 NMR spectrum

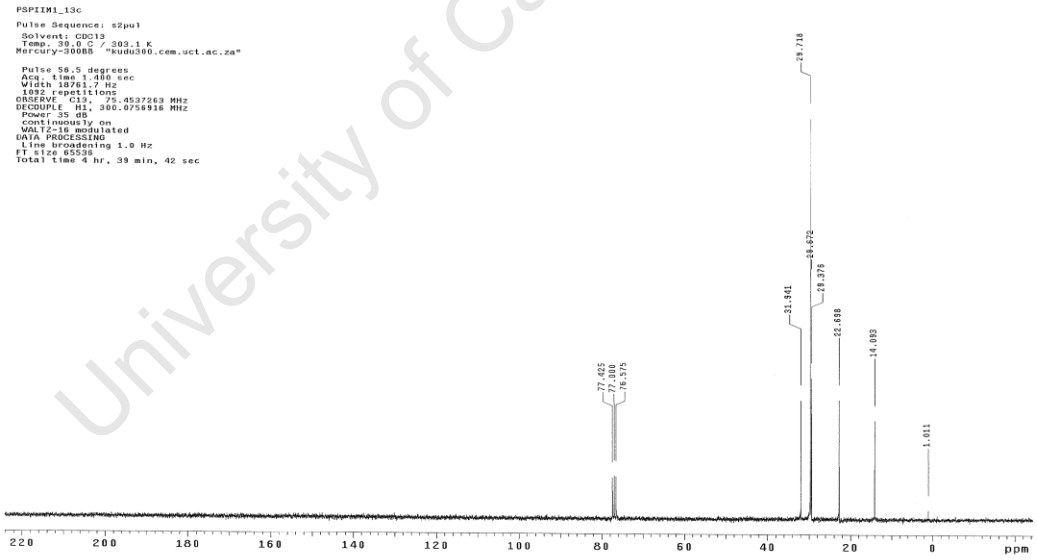

Figure A.11: Third sample carbon-13 NMR spectrum

Appendix VII

Alkane calibration curves

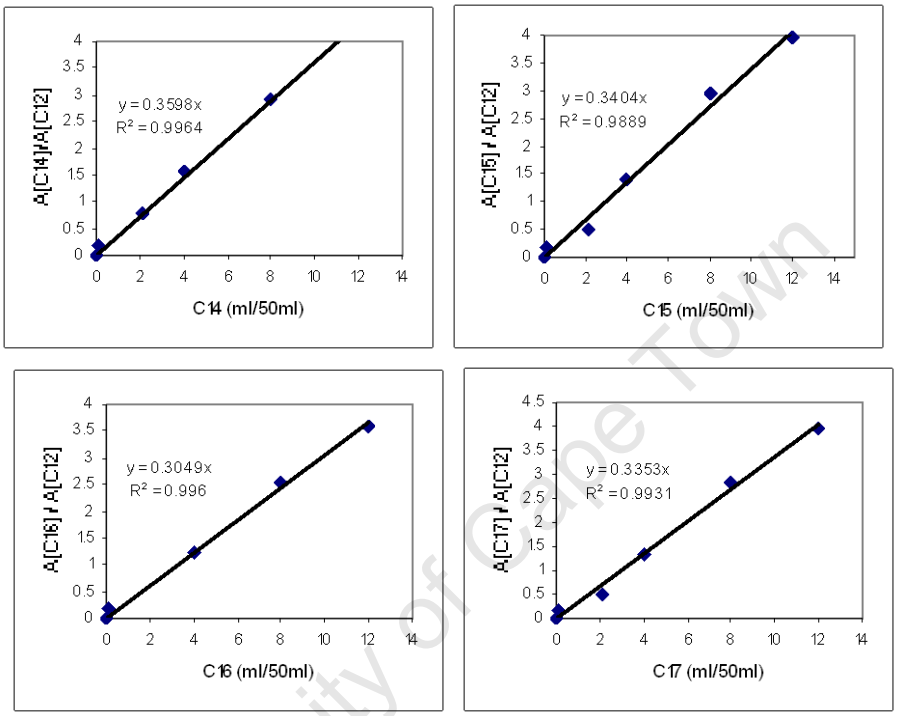

Figure A.12: Standard curves obtained during gas chromatography calibration for alkane, using n-dodecane as internal standard and MTBA as the solvent

Appendix VIII

Model Bioreactor data

Bioreactor data: 0.4 vvm, 500 rpm, pH monitored

Table A.7: Biomass data (CC) under the reactor operation at 0.4 vvm and 500 rpm

Time (h)	Cell nb	cell nb	cell nb	AVERAGE	STDEV	STDERR
0	3.14E+06	2.33E+06	3.78E+06	3.08E+06	726818.6	513938
7	2.34E+07	4.32E+06	1.60E+06	9.77E+06	11879147	8399825
15	1.27E+09	9.98E+08	3.12E+09	1.80E+09	1.16E+09	8.2E+08
18	6.51E+09	4.51E+09	7.89E+09	6.30E+09	1.7E+09	1.2E+09
20.5	1.22E+10	1.54E+10	1.14E+10	1.30E+10	2.12E+09	1.5E+09
22.2	2.55E+10	2.89E+10	2.31E+10	2.58E+10	2.89E+09	2E+09
24	2.60E+10	2.42E+10	2.81E+10	2.61E+10	1.97E+09	1.4E+09
24.5	2.53E+10	2.53E+10	2.64E+10	2.57E+10	6.35E+08	4.5E+08
24.75	2.53E+10	2.49E+10	2.58E+10	2.53E+10	4.51E+08	3.2E+08

Table A.8: Biomass data (DW) under the reactor operation at 0.4 vvm and 500 rpm

Time (h)	DW (g.l-1)	DW (g.l-1)	DW (g.l-1)	AVERAGE	STDEV	STDERR
0	0.022	0.028	0.017	0.022	0.006	0.003
2	0.029	0.023	0.034	0.029	0.006	0.003
14	0.321	0.271	0.276	0.289	0.028	0.016
16	0.697	0.590	0.490	0.592	0.103	0.060
18	0.990	0.560	1.220	0.923	0.335	0.193
20.5	1.340	1.470	1.710	1.507	0.188	0.108
21	1.540	1.490	1.620	1.550	0.066	0.038
22.2	1.620	1.604	1.715	1.646	0.060	0.035
23	1.640	1.740	1.660	1.680	0.053	0.031
24	1.670	1.780	1.650	1.700	0.070	0.040
24.5	1.690	1.710	1.730	1.710	0.020	0.012
24.75	1.730	1.740	1.760	1.743	0.015	0.009

Table A.9: pH, DO, S.T. and E_{index} data under the reactor operation at 0.4 vvm and 500 rpm

	pH	dO2	dO2 / 10	ST	ST/10	E_{index}	Eindex/10	C*
0	6.58	97.9	9.79	73.2	7.32	0	0	18.87
2	6.51	97.5	9.75	73.7	7.37	0.3	0.03	
14	6.29	88.3	8.83	73.2	7.32	3.6	0.36	8*
16	6.26	77.4	7.74	73	7.3	7	0.7	15**
18	5.3	77.25	7.725	72.1	7.21	13.2	1.32	
20.5	4.8	77.52	7.752	67.1	6.71	16.5	1.65	15.01
21	4.88	77.61	7.761	64.2	6.42	19.1	1.91	
22.2	4.92	77.59	7.759	60.2	6.02	22	2.2	13.19
23	4.95	77.8	7.78	56.1	5.61	23.8	2.38	
24	4.95	77.7	7.77	54.98	5.498	29	2.9	12.81
24.5	4.95	77.9	7.79	53	5.3	32	3.2	12.78
24.75	4.95	78.94	7.894	52.1	5.21	33.2	3.32	12.78

* measured at 8 h; ** measured at 15 h

Table A.10: Viscosity data under the reactor operation at 0.4 vvm and 500 rpm

Time (h)	Dynamic viscosity (mPa.s)
0	0.982
1.45	1.1
6	1.14
15	1.86
19	1.55
23	1.583
24	1.573

Table A.11: Gassing out data under the reactor operation at 0.4 vvm and 500 rpm

			0 h						18 h						24.75 h		
Time (s)	dO2 (%)	dt	dc	C	C*-C	Time (s)	dO2 (%)	dt	dc	C	C*-C	Time (s)	dO2 (%)	dt	dc	C	C*-C
0	98.700	10	-0.094	18.629	0.245	0	79.925	10	-0.021	13.120	3.295	0	88.900	10	11.358	11.358	1.418
10	98.200	10	-0.038	18.534	0.340	10	79.800	10	0.016	13.100	3.316	10	91.800	20	0.371	11.729	1.048
20	98.000	10	-0.019	18.496	0.377	20	79.900	10	0.049	13.116	3.300	30	91.100	10	-0.089	11.639	1.137
30	97.900	10	-0.019	18.478	0.396	30	80.200	10	0.005	13.165	3.250	40	90.400	15	-0.089	11.550	1.227
40	97.800	10	-0.038	18.459	0.415	40	80.230	10	0.154	13.170	3.245	55	89.700	14	-0.089	11.461	1.316
50	97.600	10	-0.019	18.421	0.453	50	81.170	10	-0.159	13.325	3.091	69	89.100	14	-0.077	11.384	1.393
60	97.500	10	-0.019	18.402	0.472	60	80.200	10	0.254	13.165	3.250	83	88.600	14	-0.064	11.320	1.457
70	97.400	10	-0.019	18.383	0.491	70	81.750	10	-0.090	13.420	2.996	97	88.000	14	-0.077	11.243	1.533
80	97.300	10	-0.038	18.364	0.510	80	81.200	10	-0.059	13.330	3.086	111	87.500	14	-0.064	11.179	1.597
90	97.100	10	-0.019	18.327	0.547	90	80.840	10	-0.039	13.270	3.145	125	87.000	14	-0.064	11.116	1.661
100	97.000	10	-0.019	18.308	0.566	100	80.600	10	-0.049	13.231	3.185	139	86.500	14	-0.064	11.052	1.725
110	96.900	10	-0.019	18.289	0.585	110	80.300	10	0.033	13.182	3.234	153	86.000	14	-0.064	10.988	1.789
120	96.800	10	-0.038	18.270	0.604	120	80.500	10	-0.033	13.215	3.201	167	85.500	14	-0.064	10.924	1.853
130	96.600	10	-0.019	18.232	0.642	130	80.300	10	-0.016	13.182	3.234	181	85.000	14	-0.064	10.860	1.916
140	96.500	10	-0.019	18.213	0.661	140	80.200	10	-0.016	13.165	3.250	195	84.400	14	-0.077	10.783	1.993
150	96.400	10	-0.019	18.194	0.679	150	80.100	10	-0.016	13.149	3.267	209	84.000	14	-0.051	10.732	2.044
160	96.300	10	-0.019	18.176	0.698	160	80.000	10	0.016	13.133	3.283	223	83.400	14	-0.077	10.656	2.121
170	96.200	10	-0.019	18.157	0.717	170	80.100	10	0.049	13.149	3.267	237	82.900	14	-0.064	10.592	2.185
180	96.100	10	-0.019	18.138	0.736	180	80.400	10	-0.115	13.198	3.217	251	82.400	14	-0.064	10.528	2.249
190	96.000	10	-0.038	18.119	0.755	190	79.700	10	-0.115	13.083	3.332	265	81.800	14	-0.077	10.451	2.325
200	95.800	10	-0.019	18.081	0.793	200	79.000	10	-0.131	12.968	3.447	279	80.600	14	-0.153	10.298	2.479
210	95.700	10	-0.019	18.062	0.812	210	78.200	10	-0.148	12.837	3.579	293	79.900	14	-0.089	10.208	2.568
220	95.600	10	-0.019	18.043	0.830	220	77.300	10	-0.131	12.689	3.726	307	79.400	14	-0.064	10.145	2.632
230	95.500	10	-0.019	18.025	0.849	230	76.500	10	-0.164	12.558	3.858	321	78.800	14	-0.077	10.068	2.709
240	95.400	10	-0.019	18.006	0.868	240	75.500	10	-0.181	12.394	4.022	335	78.100	14	-0.089	9.978	2.798
250	95.300	10	-0.019	17.987	0.887	250	74.400	10	-0.115	12.213	4.202	349	77.400	14	-0.089	9.889	2.887
260	95.200	10	-0.019	17.968	0.906	260	73.700	10	-0.148	12.098	4.317	363	77.000	14	-0.051	9.838	2.939
270	95.100	10	0.000	17.949	0.925	270	72.800	10	-0.148	11.951	4.465	377	76.100	14	-0.115	9.723	3.054
280	95.100	10	-0.019	17.949	0.925	280	71.900	10	-0.164	11.803	4.613	391	75.400	14	-0.089	9.633	3.143
290	95.000	10	0.000	17.930	0.944	290	70.900	10	-0.131	11.639	4.777	405	74.800	14	-0.077	9.557	3.220
300	95.000	10	-0.019	17.930	0.944	300	70.100	10	-0.197	11.507	4.908	419	74.000	14	-0.102	9.455	3.322
310	94.900	10	-0.019	17.911	0.963	310	68.900	10	-0.164	11.310	5.105	433	73.300	14	-0.089	9.365	3.411
320	94.800	10	-0.019	17.892	0.981	320	67.900	10	-0.181	11.146	5.269	447	72.600	14	-0.089	9.276	3.501
330	94.700	10	0.000	17.874	1.000	330	66.800	10	-0.164	10.966	5.450	461	71.800	14	-0.102	9.174	3.603
340	94.700	10	-0.019	17.874	1.000	340	65.800	10	-0.213	10.802	5.614	475	71.000	14	-0.102	9.071	3.705
350	94.600	10	0.000	17.855	1.019	350	64.500	10	-0.148	10.588	5.828	489	70.300	14	-0.089	8.982	3.795
360	94.600	10	-0.019	17.855	1.019	360	63.600	10	-0.181	10.440	5.975	503	69.500	14	-0.102	8.880	3.897

370	94.500	10	-0.019	17.836	1.038	370	62.500	10	-0.197	10.260	6.156	517	68.700	14	-0.102	8.777	3.999
380	94.400	10	-0.019	17.817	1.057	380	61.300	10	-0.213	10.063	6.353	531	67.800	14	-0.115	8.662	4.114
390	94.300	10	0.000	17.798	1.076	390	60.000	10	-0.164	9.849	6.566	545	67.000	14	-0.102	8.560	4.216
400	94.300	10	-0.019	17.798	1.076	400	59.000	10	-0.197	9.685	6.730	559	66.200	14	-0.102	8.458	4.318
410	94.200	10	-0.019	17.779	1.095	410	57.800	10	-0.197	9.488	6.927	573	65.300	14	-0.115	8.343	4.433
420	94.100	10	-0.019	17.760	1.114	420	56.600	10	-0.213	9.291	7.124	587	64.500	14	-0.102	8.241	4.536
430	94.000	10	0.000	17.741	1.132	430	55.300	10	-0.197	9.078	7.338	601	63.600	14	-0.115	8.126	4.651
440	94.000	10	-0.019	17.741	1.132	440	54.100	10	-0.197	8.881	7.535	615	62.700	14	-0.115	8.011	4.766
450	93.900	10	-0.019	17.723	1.151	450	52.900	10	-0.213	8.684	7.732	629	61.900	14	-0.102	7.909	4.868
460	93.800	10	-0.019	17.704	1.170	460	51.600	10	-0.213	8.470	7.945	643	60.900	14	-0.128	7.781	4.996
470	93.700	10	-0.019	17.685	1.189	470	50.300	10	-0.148	8.257	8.159	657	60.000	14	-0.115	7.666	5.111
480	93.600	10	-0.019	17.666	1.208	480	49.400	10	-0.213	8.109	8.306	671	59.100	14	-0.115	7.551	5.226
490	93.500	10	-0.019	17.647	1.227	490	48.100	10	-0.197	7.896	8.520	685	58.100	14	-0.128	7.423	5.353
500	93.400	10	-0.019	17.628	1.246	500	46.900	10	-0.230	7.699	8.717	699	57.200	14	-0.115	7.308	5.468
510	93.300	10	-0.019	17.609	1.265	510	45.500	10	-0.148	7.469	8.947	713	56.200	14	-0.128	7.180	5.596
520	93.200	10	-0.019	17.591	1.283	520	44.600	10	-0.181	7.321	9.094	727	55.300	14	-0.115	7.065	5.711
530	93.100	10	-0.019	17.572	1.302	530	43.500	10	-0.181	7.141	9.275	741	54.300	14	-0.128	6.938	5.839
540	93.000	10	-0.019	17.553	1.321	540	42.400	10	-0.181	6.960	9.455	755	53.400	14	-0.115	6.823	5.954
550	92.900	10	-0.019	17.534	1.340	550	41.300	10	-0.164	6.780	9.636	769	52.400	14	-0.128	6.695	6.082
560	92.800	10	-0.019	17.515	1.359	560	40.300	10	-0.197	6.616	9.800	783	51.500	14	-0.115	6.580	6.197
570	92.700	10	-0.019	17.496	1.378	570	39.100	10	-0.115	6.419	9.997	797	50.600	14	-0.115	6.465	6.312
580	92.600	10	-0.019	17.477	1.397	580	38.400	10	-0.164	6.304	10.112	811	49.700	14	-0.115	6.350	6.427
590	92.500	10	-0.019	17.458	1.416	590	37.400	10	-0.148	6.139	10.276	825	48.800	14	-0.115	6.235	6.542
600	92.400	10	-0.019	17.440	1.434	600	36.500	10	-0.148	5.992	10.424	839	47.900	14	-0.115	6.120	6.657
610	92.300	10	0.000	17.421	1.453	610	35.600	10	-0.148	5.844	10.572	853	47.000	14	-0.115	6.005	6.772
620	92.300	10	-0.019	17.421	1.453	620	34.700	10	-0.131	5.696	10.719	867	46.100	14	-0.115	5.890	6.887
630	92.200	10	-0.019	17.402	1.472	630	33.900	10	-0.148	5.565	10.851	881	45.200	14	-0.115	5.775	7.002
640	92.100	10	-0.038	17.383	1.491	640	33.000	10	-0.131	5.417	10.998	895	44.400	14	-0.102	5.673	7.104
650	91.900	10	-0.038	17.345	1.529	650	32.200	10	-0.181	5.286	11.130	909	43.500	14	-0.115	5.558	7.219
660	91.700	10	0.000	17.307	1.567	660	31.100	10	-0.082	5.105	11.310	923	42.700	14	-0.102	5.456	7.321
670	91.700	10	-0.019	17.307	1.567	670	30.600	10	-0.131	5.023	11.392	937	41.900	14	-0.102	5.353	7.423
680	91.600	10	-0.038	17.289	1.585	680	29.800	10	-0.131	4.892	11.524	951	41.100	14	-0.102	5.251	7.525
690	91.400	10	-0.019	17.251	1.623	690	29.000	10	-0.131	4.761	11.655	965	40.300	14	-0.102	5.149	7.628
700	91.300	10	-0.019	17.232	1.642	700	28.200	10	-0.115	4.629	11.786	979	39.500	14	-0.102	5.047	7.730
710	91.200	10	-0.038	17.213	1.661	710	27.500	10	-0.131	4.514	11.901	993	38.700	14	-0.102	4.945	7.832
720	91.000	10	0.000	17.175	1.699	720	26.700	10	-0.115	4.383	12.033	1007	37.900	14	-0.102	4.842	7.934
730	91.000	10	-0.019	17.175	1.699	730	26.000	10	-0.098	4.268	12.148	1021	37.200	14	-0.089	4.753	8.024
740	90.900	10	0.000	17.156	1.718	740	25.400	10	-0.131	4.170	12.246	1035	36.400	14	-0.102	4.651	8.126
750	90.900	10	-0.038	17.156	1.718	750	24.600	10	-0.098	4.038	12.377	1049	35.600	14	-0.102	4.548	8.228
760	90.700	10	0.000	17.119	1.755	760	24.000	10	-0.115	3.940	12.476	1063	34.900	14	-0.089	4.459	8.317
770	90.700	10	-0.019	17.119	1.755	770	23.300	10	-0.098	3.825	12.591	1077	34.200	14	-0.089	4.370	8.407
780	90.600	10	-0.019	17.100	1.774	780	22.700	10	-0.115	3.726	12.689	1091	33.500	14	-0.089	4.280	8.496
790	90.500	10	-0.019	17.081	1.793	790	22.000	10	-0.098	3.611	12.804	1105	32.700	14	-0.102	4.178	8.599
800	90.400	10	-0.019	17.062	1.812	800	21.400	10	-0.098	3.513	12.903	1119	32.000	14	-0.089	4.088	8.688
810	90.300	10	-0.038	17.043	1.831	810	20.800	10	-0.098	3.414	13.001	1133	31.300	14	-0.089	3.999	8.777
820	90.100	10	-0.019	17.005	1.869	820	20.200	10	-0.115	3.316	13.100	1147	30.600	14	-0.089	3.910	8.867
830	90.000	10	-0.019	16.987	1.887	830	19.500	10	-0.082	3.201	13.215	1161	30.000	14	-0.077	3.833	8.944

840	89.900	10	0.000	16.968	1.906	840	19.000	10	-0.098	3.119	13.297	1175	29.300	14	-0.089	3.744	9.033
850	89.900	10	-0.019	16.968	1.906	850	18.400	10	-0.098	3.020	13.395	1189	28.700	14	-0.077	3.667	9.110
860	89.800	10	-0.019	16.949	1.925	860	17.800	10	-0.098	2.922	13.494	1203	28.000	14	-0.089	3.577	9.199
870	89.700	10	-0.057	16.930	1.944	870	17.200	10	-0.098	2.823	13.592	1217	27.400	14	-0.077	3.501	9.276
880	89.400	10	0.000	16.873	2.001	880	16.600	10	-0.098	2.725	13.691	1231	26.800	14	-0.077	3.424	9.352
890	89.400	10	-0.057	16.873	2.001	890	16.000	10	-0.082	2.627	13.789	1245	26.100	14	-0.089	3.335	9.442
900	89.100	10	0.000	16.817	2.057	900	15.500	10	-0.098	2.544	13.871	1259	25.500	14	-0.077	3.258	9.518
910	89.100	10	-0.019	16.817	2.057	910	14.900	10	-0.115	2.446	13.970	1273	24.900	14	-0.077	3.181	9.595
920	89.000	10	0.000	16.798	2.076	920	14.200	10	-0.066	2.331	14.085	1287	24.300	14	-0.077	3.105	9.672
930	89.000	10	-0.019	16.798	2.076	930	13.800	10	-0.098	2.265	14.150	1301	23.700	14	-0.077	3.028	9.748
940	88.900	10	-0.019	16.779	2.095	940	13.200	10	-0.082	2.167	14.249	1315	23.100	14	-0.077	2.951	9.825
950	88.800	10	-0.019	16.760	2.114	950	12.700	10	-0.082	2.085	14.331	1329	22.700	14	-0.051	2.900	9.876
960	88.700	10	-0.019	16.741	2.133	960	12.200	10	-0.082	2.003	14.413	1343	21.900	14	-0.102	2.798	9.978
970	88.600	10	-0.038	16.722	2.152	970	11.700	10	-0.230	1.921	14.495	1357	21.300	14	-0.077	2.721	10.055
980	88.400	10	-0.019	16.685	2.189	980	10.300	10	0.000	1.691	14.725	1371	20.100	14	-0.153	2.568	10.208
990	88.300	10	-0.038	16.666	2.208	990	10.300	10	0.509	1.691	14.725	1385	19.400	14	-0.089	2.479	10.298
1000	88.100	10	-0.019	16.628	2.246	1000	13.400	10	1.871	2.200	14.216	1399	19.000	14	-0.051	2.428	10.349
1010	88.000	10	0.000	16.609	2.265	1010	24.800	10	2.676	4.071	12.345	1413	18.400	14	-0.077	2.351	10.426
1020	88.000	10	-0.019	16.609	2.265	1020	41.100	10	2.003	6.747	9.669	1427	17.800	14	-0.077	2.274	10.502
1030	87.900	10	-0.019	16.590	2.284	1030	53.300	10	1.477	8.750	7.666	1441	17.100	14	-0.089	2.185	10.592
1040	87.800	10	-0.019	16.571	2.303	1040	62.300	10	1.067	10.227	6.189	1455	16.600	14	-0.064	2.121	10.656
1050	87.700	10	-0.019	16.552	2.321	1050	68.800	10	0.886	11.294	5.122	1469	16.100	14	-0.064	2.057	10.719
1060	87.600	10	-0.019	16.534	2.340	1060	74.200	10	0.689	12.180	4.235	1483	15.600	14	-0.064	1.993	10.783
1070	87.500	10	-0.019	16.515	2.359	1070	78.400	10	0.410	12.870	3.546	1497	15.100	14	-0.064	1.929	10.847
1080	87.400	10	-0.019	16.496	2.378	1080	80.900	10	0.345	13.280	3.135	1511	14.600	14	-0.064	1.865	10.911
1090	87.300	10	-0.019	16.477	2.397	1090	83.000	10	0.246	13.625	2.791	1525	14.000	14	-0.077	1.789	10.988
1100	87.200	10	0.000	16.458	2.416	1100	84.500	10	0.213	13.871	2.544	1539	13.400	14	-0.077	1.712	11.064
1110	87.200	10	-0.019	16.458	2.416	1110	85.800	10	0.213	14.085	2.331	1553	12.900	14	-0.064	1.648	11.128
1120	87.100	10	-0.019	16.439	2.435	1120	87.100	10	0.164	14.298	2.118	1567	12.500	14	-0.051	1.597	11.179
1130	87.000	10	-0.019	16.420	2.454	1130	88.100	10	0.115	14.462	1.953	1581	12.100	14	-0.051	1.546	11.231
1140	86.900	10	-0.019	16.401	2.472	1140	88.800	10	0.082	14.577	1.839	1595	11.500	14	-0.077	1.469	11.307
1150	86.800	10	-0.019	16.383	2.491	1150	89.300	10	0.066	14.659	1.756	1609	11.000	14	-0.064	1.405	11.371
1160	86.700	10	0.000	16.364	2.510	1160	89.700	10	0.066	14.725	1.691	1623	10.600	14	-0.051	1.354	11.422
1170	86.700	10	-0.019	16.364	2.510	1170	90.100	10	0.049	14.791	1.625	1637	11.100	14	0.064	1.418	11.358
1180	86.600	10	-0.019	16.345	2.529	1180	90.400	10	0.033	14.840	1.576	1651	26.700	14	1.993	3.411	9.365
1190	86.500	10	0.000	16.326	2.548	1190	90.600	10	0.049	14.873	1.543	1665	43.600	14	2.159	5.571	7.206
1200	86.500	10	-0.019	16.326	2.548	1200	90.900	10	0.049	14.922	1.494	1679	55.500	14	1.520	7.091	5.686
1210	86.400	10	-0.019	16.307	2.567	1210	91.200	10	0.033	14.971	1.445	1693	64.300	14	1.124	8.215	4.561
1220	86.300	10	-0.019	16.288	2.586	1220	91.400	10	0.000	15.004	1.412	1707	66.200	14	0.243	8.458	4.318
1230	86.200	10	-0.019	16.269	2.605	1230	91.400	10	0.033	15.004	1.412	1721	77.200	14	1.405	9.863	2.913
1240	86.100	10	-0.019	16.250	2.623	1240	91.600	10	0.016	15.037	1.379	1735	80.000	14	0.358	10.221	2.555
1250	86.000	10	-0.019	16.232	2.642	1250	91.700	10	0.033	15.053	1.362	1749	82.700	14	0.345	10.566	2.210
1260	85.900	10	-0.019	16.213	2.661	1260	91.900	10	0.000	15.086	1.330	1763	84.600	14	0.243	10.809	1.968
1270	85.800	10	-0.019	16.194	2.680	1270	91.900	10	0.016	15.086	1.330	1777	86.400	14	0.230	11.039	1.738
1280	85.700	10	-0.019	16.175	2.699	1280	92.000	10	0.016	15.102	1.313	1791	87.400	14	0.128	11.167	1.610
1290	85.600	10	-0.019	16.156	2.718	1290	92.100	10	0.016	15.119	1.297	1805	88.400	14	0.128	11.294	1.482
1300	85.500	10	-0.019	16.137	2.737	1300	92.200	10	0.016	15.135	1.280	1819	89.100	14	0.089	11.384	1.393

1310	85.400	10	-0.019	16.118	2.756
1320	85.300	10	0.000	16.099	2.774
1330	85.300	10	-0.019	16.099	2.774
1340	85.200	10	-0.019	16.081	2.793
1350	85.100	10	-0.019	16.062	2.812
1360	85.000	10	-0.019	16.043	2.831
1370	84.900	10	-0.019	16.024	2.850
1380	84.800	10	0.000	16.005	2.869
1390	84.800	10	-0.038	16.005	2.869
1400	84.600	10	-0.019	15.967	2.907
1410	84.500	10	-0.019	15.948	2.925
1420	84.400	10	-0.019	15.930	2.944
1430	84.300	10	-0.019	15.911	2.963
1440	84.200	10	-0.019	15.892	2.982
1450	84.100	10	-0.019	15.873	3.001
1460	84.000	10	-0.019	15.854	3.020
1470	83.900	10	-0.019	15.835	3.039
1480	83.800	10	-0.019	15.816	3.058
1490	83.700	10	-0.019	15.797	3.076
1500	83.600	10	-0.019	15.779	3.095
1510	83.500	10	-0.019	15.760	3.114
1520	83.400	10	-0.019	15.741	3.133
1530	83.300	10	-0.019	15.722	3.152
1540	83.200	10	-0.019	15.703	3.171
1550	83.100	10	0.019	15.684	3.190
1560	83.200	10	-0.019	15.703	3.171

1310	92.300	10	0.000	15.152	1.264
1320	92.300	10	0.000	15.152	1.264
1330	92.300	10	0.016	15.152	1.264
1340	92.400	10	-0.016	15.168	1.248
1350	92.300	10	0.016	15.152	1.264
1360	92.400	10	0.033	15.168	1.248
1370	92.600	10	-0.016	15.201	1.215
1380	92.500	10	0.033	15.184	1.231
1390	92.700	10	-0.016	15.217	1.198
1400	92.600	10	0.000	15.201	1.215
1410	92.600	10	0.000	15.201	1.215
1420	92.600	10	0.000	15.201	1.215
1430	92.600	10	0.000	15.201	1.215
1440	92.600	10	0.016	15.201	1.215
1450	92.700	10	0.016	15.217	1.198
1460	92.800	10	-0.016	15.234	1.182
1470	92.700	10	0.000	15.217	1.198
1480	92.700	10	0.016	15.217	1.198
1490	92.800	10	0.000	15.234	1.182
1500	92.800	10	0.016	15.234	1.182
1510	92.900	10	-0.016	15.250	1.166
1520	92.800	10	0.016	15.234	1.182
1530	92.900	10	0.000	15.250	1.166
1540	92.900	10	0.000	15.250	1.166
1550	92.900	10	0.016	15.250	1.166
1560	93.000	10	0.000	15.267	1.149

1833	89.900	14	0.102	11.486	1.290
1847	90.500	14	0.077	11.563	1.214
1861	90.900	14	0.051	11.614	1.163
1875	91.200	14	0.038	11.652	1.124
1889	91.500	14	0.038	11.690	1.086
1903	91.800	14	0.038	11.729	1.048
1917	91.900	14	0.013	11.742	1.035
1931	92.100	14	0.026	11.767	1.009
1945	92.300	14	0.026	11.793	0.984
1959	92.500	14	0.026	11.818	0.958
1973	92.500	14	0.000	11.818	0.958
1987	92.600	14	0.013	11.831	0.945
2001	92.600	14	0.000	11.831	0.945
2015	92.800	14	0.026	11.857	0.920
2029	92.800	14	0.000	11.857	0.920
2043	92.900	14	0.013	11.869	0.907
2057	92.900	14	0.000	11.869	0.907
2071	92.900	14	0.000	11.869	0.907
2085	93.100	14	0.026	11.895	0.882
2099	93.200	14	0.013	11.908	0.869
2113	93.300	14	0.013	11.920	0.856
2127	93.300	14	0.000	11.920	0.856
2141	93.300	14	0.000	11.920	0.856
2155	93.400	14	0.013	11.933	0.843
2169	93.600	14	0.026	11.959	0.818
2183	93.700	14	0.013	11.972	0.805

F. Alkane utilisation data

Table A. 12: Amount of alkane remaining (g/l) under the reactor operation at 0.4 vvm and 500 rpm

Time (h)	C_{14}	C_{15}	C_{16}	C_{17}	TOT
0	13.21487	12.01101	6.736735	0.670226	32.63284
8	12.08942	11.66346	8.249909	0.642734	32.64552
15	10.69871	9.906768	7.814407	0.392177	28.81206
20.5	7.825802	8.593245	4.331391	0.173757	20.92419
22.2	7.555799	6.885053	0.809478	0.044114	15.29444
24	6.647997	7.158073	0.328667	0.02132	14.15606
24.5	6.593529	7.139725	0.31696	0.020838	14.07105

Table A.13: Amount of alkane utilised (g/l) under the reactor operation at 0.4 vvm and 500 rpm

Time (h)	C_{14}	C_{15}	C_{16}	C_{17}	TOT
0	0	0	0	0	0
8	1.125454	0.347549	0	0.027492	1.500496
15	2.516168	2.104239	0	0.278049	4.898456
20.5	5.389072	3.417762	2.405344	0.496469	11.70865
22.2	5.659075	5.125954	5.927257	0.626112	17.3384
24	6.566877	4.852934	6.408068	0.648906	18.47679
24.5	6.621345	4.871282	6.419775	0.649388	18.56179

Appendix IX

Data used for mass transfer study

Table A.14: Mass transfer study data for k_La, OUR, OTR and specific OUR

	k_La		OUR		SP-OUR		OTR	
	BOX	BO2	BOX	BO2	BOX	BO2	BOX	BO2
Time (h)								
0.0000	0.0222	0.0285	0.0018	0.0018	0.1059	0.0802	0.0032	0.0052
18.0000	0.0249	0.0228	0.0182	0.0181	0.0166	0.0196	0.0562	0.0571
24.7500	0.0170	0.0171	0.0080	0.0080	0.0045	0.0046	0.0163	0.0163

Table A.15: Mass transfer study data for OTR-OUR, biomass and *C*-C*

	Biomas		C*-C		OTR-OUR	
Time (h)	BOX	BO2	BOX	BO2	BOX	BO2
0.0000	0.0170	0.0224	0.2268	0.2454	0.0014	0.0034
18.0000	1.0955	0.9233	2.9894	3.2954	0.0380	0.0390
24.7500	1.7600	1.7433	1.4296	1.4182	0.0083	0.0083

Appendix X

Biomass and emulsification data for the reactor operation under 600 rpm, 0.3 vvm and 800 rpm, 0.3 vvm

A. Operation at agitation rate of 600 rpm and 0.3 vvm

Table A.16: Biomass and E_{index} data for the reactor run at 600 rpm and 0.3 vvm

	600 rpm and 0.3 vvm		
Time (h)	biomass (cell.ml^{-1})*	$E_{index}1$ (%)/10	$E_{index}2$ (%)/10
0	3.14E+06	0.00	0.00
2	3.50E+06	0.00	0.00
7	4.20E+06	0.23	0.20
14	2.68E+07	0.75	0.73
16	5.98E+07	0.92	1.20
18	1.02E+09	1.45	1.65
20.5	1.22E+10	1.75	1.73
22.2	2.55E+10	1.80	1.82
24	2.60E+10	1.97	1.87
24.5	2.53E+10	2.01	2.01
25.7	2.53E+10	2.50	2.79

* duplicate data for biomass not available

B. Operation at agitation rate of 800 rpm and 0.3 vvm

Table A17: Biomass and E_{index} data for the reactor run at 80 rpm and 0.3 vvm

	800 rpm and 0.3 vvm			
Time (h)	biomass (cell.ml^{-1})	biomass (cell.ml^{-1})	Eindex1 (%)/10	Eindex2 (%)/10
0	1.37E+06	1.37E+06	0.03	0.00
4	1.38E+06	1.40E+06	0.00	0.00
6	2.80E+06	1.40E+06	0.00	0.21
9	6.54E+06	1.60E+06	0.23	0.50
14	9.46E+06	4.10E+06	0.84	0.83
18	5.30E+07	9.40E+06	0.88	0.88
21	3.22E+08	3.30E+07	1.40	1.40
24	6.60E+08	7.30E+07	1.50	1.52
26	2.53E+09	1.00E+08	1.43	1.60

Appendix XI

Erythrocyte haemolysis method (Section 4.5.7)

This method is used as follows: 10 µl of 100-fold concentrated culture supernatant containing glycolipids are spotted on filter paper discs on top of an agar plate containing 5% sheep blood. In the result illustrated by the following picture, for instance, it can be observed that the culture supernatant contains abundant amounts of haemolysin, as the diameter of the haemolytic zone is 11mm (Tuleva *et al.* 1991). In this method, culture supernatants are concentrated as follows: the pH of 10ml of the culture supernatant is adjusted to 6.5 and $ZnCl_2$ is added to a final concentration of 75mM. The precipitated material is dissolved in 10ml of 0.1 M sodium phosphate buffer pH6.5 and extracted twice with an equal volume of diethyl ether. The pooled organic phases are evaporated to dryness and the pellets are dissolved in 100 µl of methanol. Concentrated culture of supernatant are spotted onto paper filter discs (6.0 mm Whatman discs), which are then put onto agar plates containing 5% sheep blood (Koch et al. 1991). Typically, 10ml of culture supernatant is concentrated down to 100µl and 10µl aliquots are applied to the paper discs. The blood agar plates are incubated at room temperature for 2 days; the haemolysis zones can then be measured if the purpose of the study is quantitative.

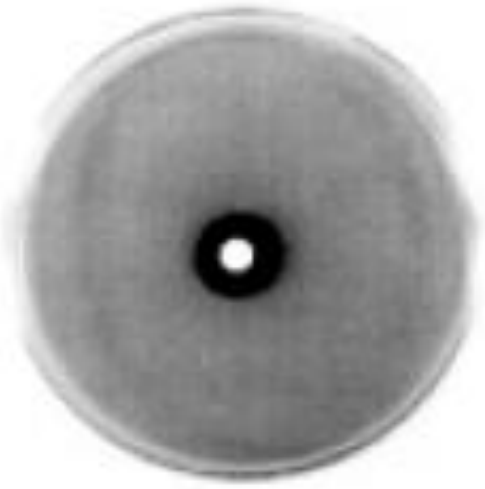

Figure A.13: Haemolytic activity of a 100-fold concentrated culture fluid of *Ps. putida* 21BN (Tuleva *et al*. 2002)

The visualisation can be made easier by the use of blue agar plates containing CTAB 0.2 $mg.ml^{-1}$ and methylene blue 5 $\mu g.ml^{-1}$. In this case, biosurfactants are recognised by the formation of blue halos around the colony.