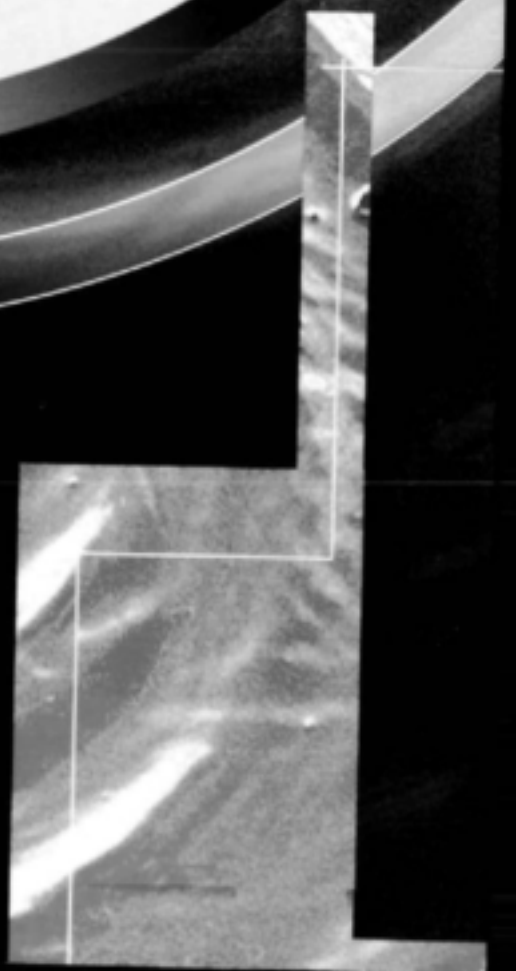
DEVELOPMENT OF A CONTINUOUS FLOW MEMBRANE BIOREACTOR CATALYSING THE SOLUBILISATION OF HYDROPHOBIC POLLUTANTS BY RHAMNOLIPID-PRODUCING BACTERIA

VS Brözel

WRC Report No. 846/1/03

Water Research Commission



DEVELOPMENT OF A CONTINUOUS FLOW MEMBRANE BIOREACTOR CATALYSING THE SOLUBILISATION OF HYDROPHOBIC POLLUTANTS BY RHAMNOLIPID-PRODUCING BACTERIA

Final Report

to the
Water Research Commission

by

Prof VS Brözel

Department of Microbiology
University of the Western Cape
Bellville

WRC Report No. 846/1/03
ISBN 1 86845 841 5

JANUARY 2003

Disclaimer

This report emanates from a project financed by the Water Research Commission (WRC) and is approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the WRC or the members of the project steering committee, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

Executive Summary

The contamination of water sources by chemical waste is inevitable in industrialised regions unless specific steps are put in place to contain, degrade or convert deleterious chemical wastes and side-products of a host of industries. Whereas the more biodegradable compounds are easily degraded in general waste water treatment processes, many compounds, especially aromatic-type structures, are recalcitrant, often have toxic properties and many display a low solubility in water. This low sollubility leads to poor degradation by micro-organisms. Certain bacterial members of degradative consortia produce surface-active agents, commonly termed biodispersants. Biosurfactants are thought to enhance cellular uptake and use of substances with low degrees of aqueous solubility. *Pseudomonas aeruginosa* is a small bacterial rod which produces a glycolipid biosurfactant called rhamnolipid. Rhamnolipid has a micelle-forming capability comparable to that of the generally used chemical detergent sodium dodecyl sulphate.

The aim of the project was to develop technology for the biodegradation of hydrophobic pollutants by emulsification using a membrane-supported biofilm producing the surfactant rhamnolopid produced by *Pseudomonas aeruginosa*.

- A number of rhamnolipid-producing *P. aeruginosa* were isolated from various locations in Cape Town.
- A protocol for the extraction and quantification of rhamnolipid (currently unavailable in the public domain) was developed and described in this report in detail.
- Rhamnolipid yields in batch suspended culture were best after 72h of growth.
- The genes encoding the enzymes responsible for rhamnolipid biosynthesis were obtained and cloned into an expression vector.
- The producer strain was shown to grow and form biofilms on the outside, but not the inside of the polysulphone membrane.

Future work should adress the optimisation of culturing of the producer strain under continuous flow conditions.

Acknowledgements

The research in this report emanated from a project funded by the Water Research Commission entitled:

"Development of a continuous flow membrane bioreactor catalysing the sollubilisation of hydrophobic pollutants by rhamnolipid-producing bacteria."

The steering committee responsible for this project, consisted of the following persons:

Dr G Offringa	Water Research Commission
Mr G Steenveld	Water Research Commission
Dr EP Jacobs	University of Stellenbosch
Prof P Swart	University of Stellenbosch
Dr SG Burton	Rhodes University
Prof DJG Rees	University of the Western Cape
Mr G Lok	ESKOM
Prof PD Rose	Rhodes University
Mr BA Hendry	Peninsula Technikon

The project was only possible with the co-operation of many individuals and institutions. The authors therefore wish to record their sincere thanks to the following:

- The project manager, Dr G. Offringa for his continued support of the project, and for his kind understanding during trying periods;
- The Department of Microbiology and Plant Pathology, University of Pretoria, for making available its research facilities for the completion of much of the research;
- Mssrs J.B. Weyers, F. du Plessis, R. MacDonald, C. Cooper, of the Department of Microbiology and Plant Pathology, University of Pretoria, for their selfless contributions to the project;
- Prof G. Wolfaardt, then of the Department of Applied Microbiology and Food Science, University of Saskatchewan, Canada, for his help with confocal scanning laser microscopy;
- Mr Andre Botha, Laboratory for Microscopy and Microscopic Analysis, University of Pretoria, for their help with environmental scanning microscopy.

Table of Contents

Table of Contents **4**

List of Tables **6**

List of Figures **7**

1 Introduction **10**

2 Literature survey: **11**

2.1 Introduction: *11*

2.2 The Structure of Biosurfactants *11*
2.2.1 The glycolipid surfactants *13*
2.2.2 The amino acid-containing lipid biosurfactants *15*
2.2.3 Biosurfactants containing polysaccharide-lipid complexes *15*

2.3 Applications of biosurfactants *16*

2.4 Production of biosurfactants *17*

2.5 Rhamnolipids *17*
2.5.1 Biosynthesis of rhamnolipids *18*
2.5.2 Production of rhamnolipids *20*

2.6 Microbial Biofilms *22*

3 Materials and Methods **23**

3.1 Isolation of rhamnolipid-producing P. aeruginosa *23*

3.2 Isolation and identification of putative biosurfactant-producing isolates *24*

3.3 Physiology of rhamnolipid-producing strains *25*
3.3.1 Effect of carbon source *25*
3.3.2 Effect of quorum sensing *25*
3.3.3 Effect of cell density *25*
3.3.5 Extraction of rhamnolipid *25*

3.4 Isolation of rhlAB operon by PCR *27*

3.5 Culturing biofilms on capillary membranes *30*

3.6 Culturing biofilms on glass wool *30*

3.7 Production of rhamnolopid in batch culture *30*

4 Results **32**

4.1 Isolation of rhamnolipid-producing Pseudomonas aeruginosa *32*

4.2 Isolation and identification of putative biosurfactant-producing isolates *33*
4.3 Physiology of rhamnolipid-producing strains *33*

4.4 Isolation and cloning of the rhlAB operon by PCR 34

4.5 Culturing biofilms on capillary membranes 45
4.5.1 Membrane properties 45
4.5.2 Staining of the membrane 45
4.5.3 Colonization of the inside of the membrane 46
4.5.4 Colonization of the outside of the membrane 46
4.5.5 Development of biofilms on the membrane 50

4.6 Culturing biofilms on glass wool 52

4.7 Production of rhamnolopid in batch culture 54

5 Discussion, Conclusion and Recommendations 55

5.1 Discussion 55

5.2 Conclusions 57

5.3 Recommendations 58

6 References 59

List of Tables

Table 1. Groups of biosurfactants, their microbial sources and relevant properties. (after Desai & Banat, 1997).....13

List of Figures

Figure 1 General structure of the three best characterized glycolipid biosurfactants..... 14
Figure 2 Structures of rhamnolipid produced by *Pseudomonas aeruginosa* (after Mata-Sandoval *et al.*, 1999). 18
Figure 3. Regulation of the *rhlAB* operon in *Pseudomonas aeruginosa*. 19
Figure 4. Sequences of the four primers used to amplify the *rhlAB* gene cluster..... 27
Figure 5. Sequences of the new primers used to amplify the *rhlAB* gene cluster..... 27
Figure 6. Partial *rhl* sequence showing the locations of two of the primers used for PCR. 29
Figure 7. Sequence of forward primer FFP1 used to amplify the *rhlAB* gene cluster together with reverse primer PMR (Fig. 5)..... 29
Figure 8. Production of rhamnolipid by isolate L3..... 34
Figure 9. Predicted secondary structure of the single stranded amplicon as obtained using RNAstructure 3.2 36
Figure 10. Electrophoretorgram of the PCR products obtained using primers FFP1 and PMR. Lane 8 shows the desired amplicon of 2.3 kb. 37
Figure 11. Electrophoretogram of PCR amplicons of recombinant clones. Lane 1: Lambda HingIII size marker, lanes 2 - 4: PCR amplicons of recombinant clones... 37
Figure 12. Electrophoretogram of plasmid extracts of recombinant clones. Lane 1: pGEM T-easy control vector, lanes 2, 8 and 9: blank, lanes 3 - 7 and 10 - 12: recombinant clones. 38
Figure 13 Figure 14 Electrophoretogram of restriction digests of recombinant vectors. Lane 1: Undigested pGEM T-easy, lanes 2 and 3: pGEM T-easy digested with EcoRI, lane 4: Recombinant vector digested with EcoR1, lane 5: Recombinant vector digested with EcoRV, lane 6: Recombinant undigested vector. 38
Figure 15. Electrophoretorgram of restriction digests of recombinant vectors. Lanes 1 - 3: Recombinant vector digested with EcoRV, lane 4: Recombinant vector digested with ScaI, lane 6: pGEM T-easy digested with ScaI. 39
Figure 16. Sequences of primers used to amplify the *rhlA* and *rhlB* genes separately..... 40
Figure 17. Electrophoretogram showing PCR amplicons obtained with the primers shown in Fig. 15. Lane 1: PCR with old primers amplifying the 2.3kb *rhlAB* gene product from *P. aeruginosa* PAO1 (faint upper band); Lane 2: the 2.3kb *rhlAB* gene product from *P. aeruginosa* PAO1 obtained using new primers ForBgl and RevHind (light upper band); Lane 3: the *rhlA* gene product obtained using primers ForBgl and RintKpn; and Lane 4: the *rhlB* gene product obtained using primers FintKpn and RevHind. 40
Figure 18. Electrophoretogram showing PCR amplicons be *rhlA* and *rhlB* in pGEM. Lane 1: Phage lambda cut with HindIII and EcoRI; Lane 2: pLAH31 uncut; Lane 3: pLAH31 cut for ligation with *rhlA* – BamHI and KpnI; Lane 4: pLAH31 cut for ligation with *rhlB* – HindIII and KpnI; Lane 5: pGEM/A uncut; Lane 6: pGEM/A cut with BglII and KpnI; Lane 7: pGEM/B uncut; Lane 8: pGEM/B cut with HindIII and KpnI; Lane 9: A insert from pGEM/A purified; Lane 10: B insert from pGEM/B purified. 41

Figure 19. Electrophoretogram showing Lane 1: Phage lambda cut with HindIII and EcoRI; Lane 2: *rhlA* PCR amplicon from pLAH31/A using primers for *rhlA*; Lane 3: *rhlA* PCR reaction mix from pLAH31 using primers for *rhlA;* Lane 4: H_2O with primers for *rhlA* PCR amplicon from pLAH31/A using primers for *rhlA*; Lane 5: *rhlA* PCR amplicon from genome of *P. aeruginosa;* Lane 6: *rhlB* PCR amplicon from pLAH31/B using primers for *rhlB*; Lane 7: *rhlB* PCR reaction mix from pLAH31 using primers for *rhlB;* Lane 8: H_2O with primers for *rhlB*; Lane 9: *rhlB* PCR amplicon from genome of *P. aeruginosa.* 42
Figure 20. SDS PAGE of whole-cel protein extracts of *Escherichia coli* harbouring pLAH31/B. Lane 1: recombinant TA1 – not induced; Lane 2: recombinant TA1 – induced; Lane 3: recombinant TA2 – not induced; Lane 4: recombinant TA2 – induced; Lane 5: recombinant TA4 – not induced; Lane 6: recombinant TA4 - induced; Lane 7: recombinant TA8 – not induced; Lane 8: recombinant TA8 – induced. 43
Figure 21. SDS PAGE of whole-cel protein extracts of *Escherichia coli* harbouring pLAH31/B. Lane 1: Molecular weight marker; Lane 2: recombinant TB1 – not induced; Lane 3: recombinant TB1 – induced; Lane 4: recombinant TB2 – not induced; Lane 5: recombinant TB2 – induced; Lane 6: recombinant TB4 – not induced; Lane 7: recombinant TB4 - induced Lane 8: DH5 *E. coli* containing pLAH31 44
Figure 22. View of the surface of the polysulphone capillary membrane after staining with acridine orange. The image was taken by scanning laser confocal microscope. 45
Figure 23. View of the surface of the polysulphone capillary membrane after staining with fluorescein. The image was taken by scanning confocal laser microscope..... 46
Figure 24. View of the voids in the polysulphone capillary membrane before innoculation with PAO1 and staining with acridine orange. The image was taken by scanning laser confocal microscope, focussing at various depths inside the voids. 47
Figure 25. View of the voids in the polysulphone capillary membrane after innoculation with PAO1 and staining with acridine orange. The image was taken by scanning laser confocal microscope, focussing at various depths inside the voids. 47
Figure 26. View of the voids in the polysulphone capillary membrane after innoculation with PAO1 and staining with acridine orange. The image was taken by scanning laser confocal microscope, focussing at various depths inside the voids. 48
Figure 27. View of the surface of the polysulphone capillary membrane after innoculation with PAO1 and staining with acridine orange. The image was taken by scanning laser confocal microscope, focussing at various depths inside the voids. 48
Figure 28. View of the surface of the polysulphone capillary membrane after innoculation with PAO1 and staining with acridine orange. The image was taken by scanning laser confocal microscope, focussing at various depths inside the voids. 49
Figure 29. View of the surface of the polysulphone capillary membrane after innoculation with PAO1 and staining with acridine orange. The image was taken by scanning laser confocal microscope, focussing at various depths inside the voids. 49
Figure 30. View of the surface of the polysulphone capillary membrane 5 d after innoculation with PAO1 and staining with acridine orange. The image was taken by scanning laser confocal microscope, focussing at various depths inside the voids.. 50

Figure 31. Sections from bottom up of the surface of the polysulphone capillary membrane 5d after innoculation with PAO1 and staining with acridine orange. The image was taken by scanning laser confocal microscope, focussing at various depths inside the voids. 51
Figure 32. View of the surface of the polysulphone capillary membrane showing a fracture in the skin at center top. 52
Figure 33. Low Vacume Scanning Electron Microscope image of a 26 h *P. aeruginosa* biofilm growing between two glass fibres. 52
Figure 34. Growth of *Pseudomonas aeruginosa* on glass wool as measured by absorbance (540 nm)(a), culturable count (b) and total cellular protein (c). 53
Figure 35. Rhamnolipid yield from a planktonic and biofilm culture of *Pseudomonas aeruginosa* growing on glass wool. 54

1 Introduction

The contamination of water sources by chemical waste is inevitable in industrialised regions unless specific steps are put in place to contain, degrade or convert deleterious chemical wastes and side-products of a host of industries. The more biodegradable compounds are easily degraded in general waste water treatment processes such as the activated sludge process, but many compounds, especially aromatic-type structures, are recalcitrant and often have toxic properties. These recalcitrant compounds require specialised treatment, and are generally termed xenobiotics. A number of developments have occurred in the isolation of microbial strains and consortia able to degrade such xenobiotics, often under very specific conditions.

The aromatic nature of many xenobiotics dictates that their solubility in water is extremely low. The central cause of the extremely low efficiency of biodegradation of such compounds by micro-organisms is precisely their low solubility in water, as microbes are essentially water-based and few tolerate high concentrations of non-polar solvents. It has become apparent that in cases where microbial consortia successfully degrade water-insoluble xenobiotics, at least one member of the consortium produces a surface-active agent, commonly termed a biodispersant. Although the exact mechanism is not clear, it is known that biosurfactants enhance cellular uptake and use of substances with low degrees of aqueous solubility. When associated with the cell, they promote transport across the membrane, and as extra-cellular compounds they help to solubilize the substrate. *Pseudomonas acruginosa* is a small bacterial rod which produces a glycolipid biosurfactant called rhamnolipid. Rhamnolipid has a similar micelle-forming capability to the generally used chemical detergent sodium dodecyl sulphate.

Therefore the aim of the project was to develop technology for the biodegradation of hydrophobic pollutants by emulsification using a membrane-supported biofilm producing the surfactant rhamnolopid produced by *Pseudomonas acruginosa*.

2 Literature survey:

2.1 Introduction:

Biosurfactants are amphipathic molecules produced by a wide variety of bacteria, yeast and filamentous fungi. These surface-active compounds include peptides, glycolipids, fatty acids and phospholipids (Desai & Banat, 1997). This group of compounds have the ability to interact with interfaces. An interface can be defined as a phase boundary between two phases in a heterogeneous system (Neu, 1996). The three general characteristics of surfactants are enrichment at interfaces, lowering of interfacial tension, and micelle formation.(Neu, 1996). They accumulate at interfaces and mediate between phases of different polarity such as oil/water, air/water, or water/solid. This process is based on the ability of the surfactant to reduce the surface tension by controlling the arrangement of liquid molecules, thus influencing the formation of H-bonds and hydrophobic and hydrophilic interactions.

The industrial demand for surfactants is high, since the market value for soaps and detergents reached US$12,8x10^9 in 1990, with a projected continual annual increase of between 5,9% and 35 % (Desai & Banat, 1997). Of this market, surfactants accounted for US$ 9.4x10^9 by 1994 and it is estimated that the demand for surfactants world-wide will increase by 35% by the end of the century (Fiechter,1992). The bulk of surfactants which are currently used are chemically synthesized from petroleum. Increase in microbial surfactants has increased due to their diversity, environmentally friendly nature, potential application in environmental protection and the possibility of production by fermentation using renewable resources. Biosurfactants are still more expensive to produce due to: (i) inefficient bio-processing methodology; (ii) poor strain productivity and (iii) expensive substrates. There is therefore a need to improve the understanding and also improve the ability to manipulate the metabolism of the producer strains so that cheaper substrates may be used and also to improve the process technology to facilitate product recovery.

2.2 The Structure of Biosurfactants

Biosurfactants are produced by certain bacteria and some yeasts and filamentous fungi during cultivation on various carbon sources, especially hydrophobic substances such as hydrocarbons.

They are low molecular mass surface-active molecules of a variety of chemical structure produced by living cells (Fiechter,1992). Therefore, biosurfactants can be classified in

terms of different properties: (i) The size of molecules spans a wide range from low-molecular weight surfactants through polymeric surfactants up to particulate surfactants;(ii) the biochemical nature of the molecules such as fatty acids, lipids, bacteriocins, peptides, and polysaccharides; (iii) the nature of the hydrophilic part of the surface- active compound such as the carboxylate of group of fatty acids, the glycerol of glycerolipids, the carbohydrates of glycolipids, and the amino acids of peptidyl lipids; (iv) the different locations of surface-active compounds in terms of intracellular, cell surface, and extra-cellular pool; (v) the species of the producer micro-organisms; and (vi) the type of carbon source used to produce the surface-active compound, such as hydrocarbons, water-soluble molecules, or both. (Neu, 1996).

Chemically synthesized surfactants are classified according to the nature of their polar grouping, but microbial surfactants have been grouped according to their chemical composition. In general their structure includes a hydrophilic moiety (amino acid, peptide, mono-, di- or polysaccharide), and a hydrophobic moiety, usually a fatty acid. Table 1 lists a range of currently known biosurfactants, their microbial sources and relevant properties.

Biosurfactants may be classified into three main groups based on the detail of their chemical structure within the basic framework: whereas the hydrophobic moiety consists of hydrocarbon chain of a fatty acid, the hydrophilic moiety may consist of the ester or alcohol function of neutral lipids, or the carboxylate group of fatty acids or amino acids, or the phosphate- containing portions of phospholipids, or the carbohydrate moiety of glycolipids.

The groups are: (i) glycolipid biosurfactants;

(ii) amino acid-containing lipid biosurfactants;

(iii) biosurfactants containing polysaccharide-lipid complexes.

Table 1. Groups of biosurfactants, their microbial sources and relevant properties. (after Desai & Banat, 1997).

Biosurfactant	Organisms	Surface tension (mN/m)	CMC	Interfacial tension (mN/m)
Glycolipids				
Rhamnolipids	*P. aeruginosa*	29		0.25
	Pseudomonas sp.	25–30	0.1–10	1
Trehalolipids	*R. erythropolis*	32–36	4	14–17
	N. erythropolis	30	20	3.5
	Mycobacterium sp.	38	0.3	15
Sophorolipids	*T. bombicola*	33		1.8
	T. apicola	30		0.9
	T. petrophilum			
Cellobiolipids	*U. zeae*, *U. maydis*			
Lipopeptides and lipoproteins				
Peptide-lipid	*B. licheniformis*	27	12–20	0.1–0.3
Serrawettin	*S. marcescens*	28–33		
Viscosin	*P. fluorescens*	26.5	150	
Surfactin	*B. subtilis*	27–32	23–160	1
Subtilisin	*B. subtilis*			
Gramicidins	*B. brevis*			
Polymyxins	*B. polymyxa*			
Fatty acids, neutral lipids, and phospholipids				
Fatty acids	*C. lepus*	30	150	2
Neutral lipids	*N. erythropolis*	32		3
Phospholipids	*T. thiooxidans*			
Polymeric surfactants				
Emulsan	*A. calcoaceticus*			
Biodispersan	*A. calcoaceticus*			
Mannan-lipid-protein	*C. tropicalis*			
Liposan	*C. lipolytica*			
Carbohydrate-protein-lipid	*P. fluorescens*	27	10	
	D. polymorphis			
Protein PA	*P. aeruginosa*			

CMC = critical micelle concentration.

2.2.1 The glycolipid surfactants

Most currently known biosurfactants are glycolipids. They are carbohydrates combined with long-chain aliphatic acids or hydro-aliphatic acids. The best known are rhamnolipids, trehalolipids and sophorolipids, the general structures of which are depicted in Figure 1.

Rhamnolipids, in which one or two rhamnose moieties are linked to one or two ∃-hydroxy-decanoic acid, are the best studied and show most promise to date. They are produced by strains of *Pseudomonas aeruginosa*, and were first described by Jarvis and Johnson (1949). It was held for a long time that four structure existed, with either one or two rhamnose and either one or two ∃-hydroxy-decanoate moieties. Recent studies have revealed that more structures exist in that the ∃-hydroxy-alkanoate may be C_8, C_{10} or C_{12}

(Mata-Sandoval *et al*., 1999). Generally rhamnolipids with two ∃-hydroxy-alkanoate units and two rhamnose units are referred to as rhamnolipid 1, and those with one sugar as rhamnolipid 2. Rhamnolipids with one ∃-hydroxy-alkanoate and one sugar are rhamnolipid 3, and with two sugars are rhamnolipid 4 (Desai & Banat, 1997). Rhamnolipids lower the interfacial tensions against n-hexadecane to 1 mN/m, and the surface tension to between 25 and 30 mN/m. They emmulsify alkanes and stimulate the growth of *P. aeruginosa* on hexadecane. The critical micelle concentration of rhamnolipid 1 is *ca*. 200 mg/l, and that of rhamnolipid 2 between 5 and 60 mg/l (Mata-Sandoval *et al*., 1999).

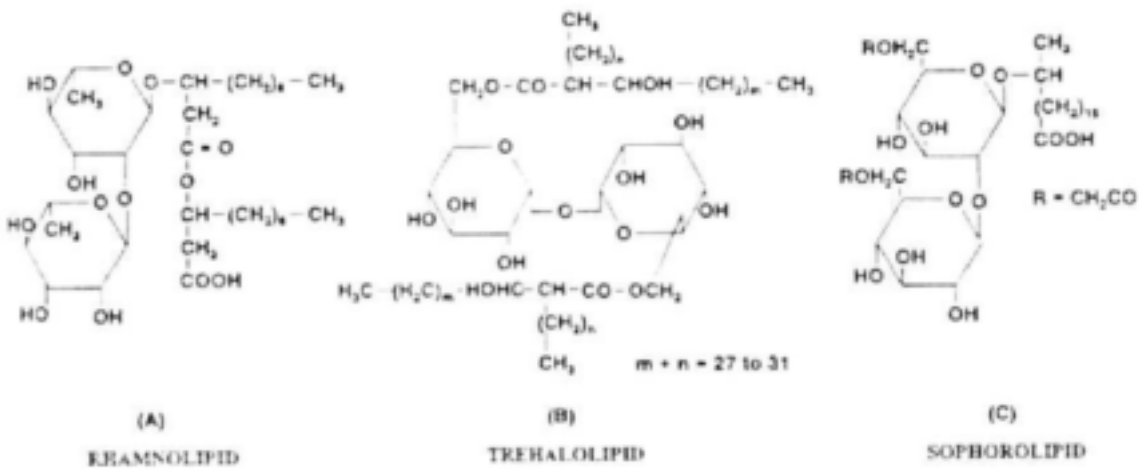


Figure 1 General structure of the three best characterized glycolipid biosurfactants.

Trehalolipids are a diverse group of disaccharide trehaloses linked to mycolic acids at the 6 and 6' ends. They are primarily produced by gram positive bacteria such as *Rhodococcus, Arthrobacter, Nocardia, Mycobacterium* and *Corynebacterium*. The mycolic acids are long chain ∀-branched ∃-hydroxy fatty acids. They lower the surface tension to between 1 and 40 mN/m, depending on the structures of the two fatty acids.

Sophorolipids consist of a dimeric sophorose unit linked to a long chain hydroxy fatty acid. They are primarily produced by yeasts of the genera *Torulopsis* and *Candida*, and are mostly mixtures of up to nine different fatty acid structures. Like the rhamnolipids, they are also produced in the absence of a hydrophobic carbon source, and are therefore not inducible by specific nutrient cues. They reduce the interfacial tension to between 5 and 40 mN/m.

2.2.2 The amino acid-containing lipid biosurfactants

The amino acid containing biosurfactants are lipo-peptides and lipoproteins, most of which are circular structures. Surfactin, produced by *Bacillus subtilis* is composed of a 7-amino acid ring structure: (Glu-Leu-Leu-Val-Asp-Leu-Leu) coupled to one molecule of 3 hydroxy-13-methyl-tetradecanoic acid. It is one of the most powerful biosurfactants, lowering surface tension to 28 mN/m at concentrations as low as 0.005 % (Arima *et al.*, 1968). Its CMC is 24 µM (Desai & Banat, 1997). Other biosurfactants in this group include the ornithine-containing lipid of *Pseudomonas rubescens*, a lysine-containing lipid of *Agrobacterium tumefaciens*, and an orthinine-taurine lipid of *Gluconobacter cerinus*. Recently Yakimov *et al* (1996) isolated lichenysinA from *B. licheniformis*, with a CMC of 12 µM. The lipopeptide structures are generally rather large, in the order of 1 kD, and production is energy intensive due primarily to the high amino acid content.

2.2.3 Biosurfactants containing polysaccharide-lipid complexes

These are polysaccharide backbones with fatty acid side-chains. In many cases the backbone is a hetero-polysaccharide. Generally, the hydrophilic component of the bio-surfactant consists of either a carbohydrate, a hydrophilic amino acid such as glutamate, aspartate, lysine, or arginine, or a hydrophilic peptide. The hydrophobic portion, generally consists of either a lipid structure, an isoprenoid structure such as cholesterol, a hydrophobic amino acid or peptides including amino acid such as phenylalanine, leucine, isoleucine, valine, or alanine (Thangamani, *et al.* 1994).

The best known polysaccharide bio-surfactant is emulsan produced by *Acinetobacter calcoaceticus*. Emulsan is a very effective for emulsifying hydrocarbons in water, even at concentrations as low as 0.001 % (Desai & Banat, 1997). Emulsan has a molecular mass of *ca.* 50 kD. An alanine-containing derivative, alasan, is also produced by *A. calcoaceticus*, and is 3 times as effective. Various yeast species such as *Candida tropicalis* produce a host of water soluble polymeric emmulsifiers containing amino acids (Fiechter, 1992).

2.3 Applications of biosurfactants

Biosurfactants have a broad range of functional properties including: emulsification, phase separation, wetting, foaming, solubilization, de-emulsification, corrosion-inhibition and viscosity reduction (Desai & Banat, 1997).

The areas of industrial application where chemical surfactants can be substituted by biosurfactants include agriculture, building and construction, food and beverage industries, industrial cleaning, leather, paper and metal industries, textiles, cosmetics, the pharmaceutical industry and petroleum and petrochemical industries. Surfactants are used in the food industry as emulsifiers in food additives in raw material processing. The texture and consistency of specific foods are contributed by emulsification . In agriculture, surfactants contribute to the wetability and fertilizer distribution in soil. In the cosmetic industry, surfactants are found in shampoos as well as many skin-care products. (Fiechter, 1992)

At present, biosurfactants are limited to application in the petroleum industry, mainly in microbial enhanced oil recovery (MEOR) (Lin *et al.* 1994). A major problem in oil recovery is the high surface- and interfacial tension between water and oil in the ground which needs to be decreased. Currently, petroleum sulfonates and lignosulfonates are being used. However, these products are relatively expensive and could be replaced by more inexpensive biosurfactants. In a study comparing the effectiveness of an anionic rhamnolipid bio-surfactant and a synthetic anionic surfactant in solubilizing hexadecane in multi-system, it was found that on a per mole basis, the rhamnolipid is capable of solubilizing about 20-times more hexadecane than the synthetic surfactant. The synthetic surfactant used in this study was the 377 Da, linear, anionic, alkyl benzene sulfonate (ABS). This was determined by measuring the critical micelle concentration (CMC), ie. the concentration at which the surfactant concentration is high enough to accommodate the aggregation of surfactant molecules into ordered micelle structures (Thangamani and Shreve, 1994).

A second area of growing application is in bioremediation of oil spills in soil. The low solubility of the predominant petroleum component, alkanes, hampers the interaction with bacteria in soil and water as alkane droplets and bacteria are mutually exclusive (Beal & Betts, 2000). Although the exact mechanism involved is still unclear, glycolipid biosurfactants such as rhamnolipid enhance the cellular uptake and use of substrates with low solubility in water (Mata-Sandoval *et al.*, 1999). Beal and Bates (2000) showed that

alkanes are taken up by an energy-dependant manner through the membrane directly from alkane droplets in contact with the cell. Various authors have reported that rhamnolipid-producing *P. aeruginosa*, while not being able to degrade pollutants such as polycyclic hydrocarbons themselves, enhanced biodegradation by other bacteria through the emmulsification action of the bio-surfactant (Arino *et al*., 1998).

2.4 Production of biosurfactants

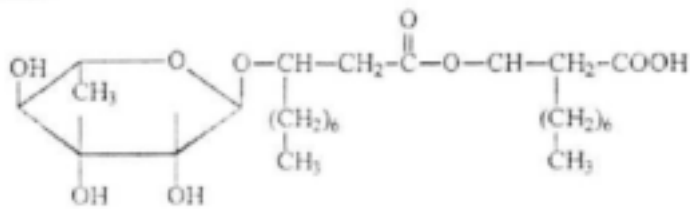
Biosurfactants are more expensive than synthetic surfactants due to: (i) inefficient bio-processing methodology; (ii) poor strain productivity and (iii) expensive substrates. However, the advantages of biosurfactants over chemically synthesized surfactants are greater. These advantages are as follows:(i) the biosurfactants possess novel characteristics and physical properties; (ii) they are produced on renewable substrates; (iii) they have the capacity to be modified by genetic engineering, biological and biochemical techniques; thereby being able to meet specific requirements; (iv) they are bio-degradable; (Fiechter, 1992) and (v) they are not toxic to the micro-organisms that produce them, therefore they can be used in bioremediation (Mercadé, *et al.* 1996). These advantages are important since chemical surfactants cause environmental problems due to their resistance to biodegradation and their toxicity when allowed to accumulate in the natural ecosystem.

2.5 Rhamnolipids

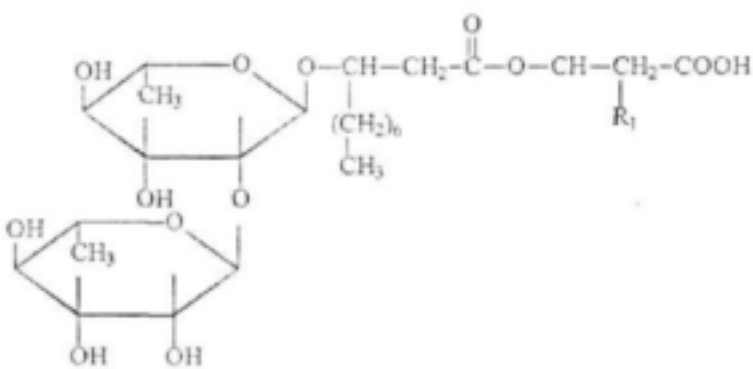
The rhamnose-containing glycolipid biosurfactants produced by *P. aeruginosa* were first described in 1949 by Jarvis and Johnson. Four rhamnolipids were subsequently distinguished. L-Rhamnosyl-L-rhamnosyl-∃-hydroxydecanoyl-∃-hydroxydecanoate and -L-rhamnosyl-∃-hydroxydecanoyl-∃-hydroxydecanoate are referred to as rhamnolipid 1 and rhamnolipid 2, respectively. These rhamnolipids are also the principal glycolipids produced in liquid cultures. Rhamnolipids 3 and 4, possess only one ∃-hydroxydecanoyl moiety and one / two rhamnose groups, and are synthesized by resting cells only (Ochsner *et al.*1994, 1996). Mata-Sandoval *et al.* (1999) later reported that the fatty acid side chain can contain between 8 and 12 carbons as shown below in Figure 2.

The production of rhamnolipids is regulated, and the formation of rhamnolipid by *P. aeruginosa* in a mineral salt medium with 2% alkanes as a carbon source is increased after NO_3^- limitation during the stationary growth phase (Koch *et al.*1991). It was also

found, when analysing *P. aeruginosa* cells grown in nitrate, that an increased glutamine synthetase activity resulted in a more enhanced biosurfactant production. However, an increase in ammonium and glutamine concentrations resulted in repression of both glutamine synthetase activity as well as biosurfactant production. Thus it was deduced that the control of rhamnolipid production was linked to the control of nitrogen metabolism.



Monorhamnolipid ($RhC_{10}C_{10}$)



Dirhamnolipids ($Rh_2C_{10}C_n$)
n = 8, 10, 12

Figure 2 Structures of rhamnolipid produced by *Pseudomonas aeruginosa* (after Mata-Sandoval *et al.*, 1999).

2.5.1 Biosynthesis of rhamnolipids

The biosynthesis of these rhamnolipids was studied *in vivo* by using various radioactive precursors such as [^{14}C] acetate and [^{14}C] glycerol. In this pathway, the synthesis of rhamnolipids proceeds by sequential glycosyl transfer reactions, each catalysed by a specific rhamnosyl transferase with thymidine-diphospho-rhamnose (TDP-rhamnose) acting as a rhamnose donor and 3-hydroxdecanoyl-3-hydroxdecanoate or L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate acting as the rhamnose acceptor (Ochsner, *et*

*al.*1994, 1996). These reactions are as follows: TDP-rhamnose + ∃-hydroxydecanoyl-∃-hydroxydecanoate $\xrightarrow{\text{transferase 1}}$ TDP + L-rhamnosyl-∃-hydroxydecanoyl-∃-hydroxydecanoate and TDP-L-rhamnose + L-rhamnosyl-∃-hydroxydecanoate $\xrightarrow{\text{transferase 2}}$ 2TDP + L-rhamnosyl-L-rhamnosyl-∃-hydroxydecanoyl-∃-hydroxydecanoate. These glycolipids were shown to be secreted into the medium during the stationary phase of growth. (Koch, *et al.*1991).

Rhamnosyl transferase activity is conferred by two inducible gene products, RhlA and RhlB which are located at the cytoplasmic membrane (Ochsner & Reiser, 1995). The two genes (*rhlA* and *rhlB*) form an operon, the expression of which is controlled by an activator protein RhlR which is activated by a homoserine lactone. The homoserine lactone (synthesized constitutively by the RhlI gene product) is cell density dependant so that expression of the *rhlAB* operon only takes place at high cell densities (Pesci *et al*,. 1997). The regulatory mechanism of the *rhlAB* operon is depicted in Figure 3.

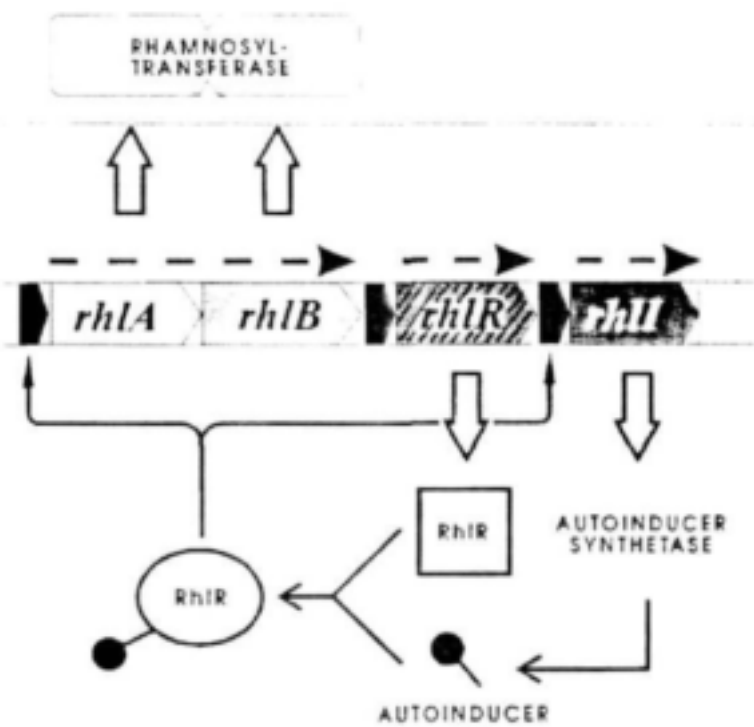


Figure 3. Regulation of the *rhlAB* operon in *Pseudomonas aeruginosa*.

2.5.2 Production of rhamnolipids

Hydrocarbons and lately plant-derived oils are commonly used as the substrate for the production of biosurfactants (Haba *et al.*, 2000). It was thought that the function of the biosurfactants was related to the uptake of hydrocarbons (Guerra-Santos, *et al.* 1984). In *P. aeruginosa* rhamnolipid is, however, produced regardless of whether or not the cell is under pressure to assimilate a hydrophobic carbon source (Desai & Banat, 1997).

The carbon source is an important process parameter since changing the substrate often changes the structure of the product, thereby altering the properties of the surfactant. Thus, the carbon source should be determined by the intended application. (Fiechter, 1992). However, the use of carbohydrates as substrates does have it's advantages over hydrocarbons, since the latter requires more sophisticated equipment and more power input for adequate dispersion of the insoluble hydrocarbons. Additionally, more heat is produced during cultivation on hydrocarbons, therefore, requiring greater cooling surfaces within the bioreactor. (Guerra-Santos, *et al.* 1986)

In the pursuit to develop a continuous biosurfactant production process, the following was determined. Of the trace elements, iron concentrations directly influenced the biosurfactant production of *P. aeruginosa.* Thus it has been established that high Fe^{2+} concentrations ie. 2mg/g glucose and above, resulted in the cessation of biosurfactant production.

The advantages of a continuous culture over batch culture production, include the following : (i) long term incubations were avoided , therefore resulting in a high productivity level per unit of reactor volume; (ii) constant flow; and (iii) the exact control of culture conditions which id extremely important for high bio-surfactant production in a continuous culture.

The production of extra-cellular rhamnolipids by *P. aeruginosa* depends on various nutritional as well as environmental factors (Guerra-Santos *et al.* 1986*)*. It was found that high rhamnolipid biosurfactant productivity results under conditions of nitrogen limitations during the stationary phase of growth, and the utilization of glucose, glycerol or *n*-paraffins as a carbon source. (Ochsner *et al.* 1995) Other factors such as salt limitation viz. magnesium, calcium, potassium, and sodium, pH, and the temperature are also important in rhamnolipid biosynthesis (Guerra-Santos *et al.* 1986). Haba *et al.*

(2000) found that a C:N ratio of 8:1 led to the highest rhamnolipid yield on waste frying oil as carbon source, ie 2.7 g/l, or 0.34 g rhamnolipid per gram biomass.

The aeration rate and pH of cultivation directly influences foam formation. At pH 6.5 and above the foam formation was higher than at pH below 6.5. Thus, an optimum pH for biosurfactant production was obtained at pH 6.25 (Guerra-Santos, *et al.* 1986), since the slightest change above or below this value resulted in a tremendous drop in biosurfactant production. Gas exchange filters and / mechanical foam breakers are required to control foam formation since antifoam agents were shown to be inadequate (Fiechter, 1992).

The previously mentioned factors are justified as follows, in the quest for improved methods of biosurfactant production the analysis of *P. aeruginosa* yielded the following result. An increased biosurfactant recovery level was characterized under certain qualitative as well as quantitative environmental conditions. Important qualitative factors include the growth under non-carbon limited conditions, the use of nitrate as a nitrogen source and the omission of complex medium components such as yeast extract. (Guerra-Santos *et al.* 1984). In the case of the quantitative factors, a unique pattern of the affect of particular components on biosurfactant production was not established. In addition to the latter responses for the medium components, it was also determined that a minimal concentration of the respective salts viz. magnesium, calcium, potassium, sodium and trace elements yielded a high biosurfactant production. A complete characterization of the quantitative factors was achieved by establishing the optimal temperature of 32EC to 34EC and the pH 6.2 to 6.4 to yield a high rhamnolipid biosynthesis (Guerra-Santos *et al.* 1986).

A "protein-like activator for *n*-alkane oxidation" was isolated from *P. aeruginosa* (Hisatsuka, *et al.* 1972). This activator has a molecular weight of about 14,000 and constitutes 147 amino acid residues. It was found to emulsify hexadecane and water and stimulated the growth of *P. aeruginosa* on hexadecane. However, it was observed that the *P. aeruginosa* rhamnolipids and the protein-like activator interacted with each other during emulsification of hexadecane, thus it remains unclear as to whether this activator is an integral factor in the uptake of water insoluble substances (Koch, *et al.*1991).

2.6 Microbial Biofilms

Biofilms are defined as matrix-enclosed bacterial populations adherent to each other and / surfaces or interfaces. The cells of ubiquitous bacterial species such as *P. aeruginosa* respond to favourable nutrient conditions by adhering to available surfaces and by binary fission and exopolymer production to develop mature biofilms. *P. aeruginosa* are present as rod-shaped vegetative cells that grow predominantly as a matrix-enclosed sessile mode of growth, thus being protected from adverse environmental conditions and from biological and chemical antibacterial agents (Costerton, *et al.*1995).

Conditions within a biofilm differ from both the bulk phase and cells in biofilms behave differently to those in suspension (Fletcher, 1991). Biofilm cells are covered in extra-cellular polymeric substances (EPS), the composition of which is still not quite clear. The older literature states that the EPS is purely polysaccharide material, but is has emerged that proteins also form part of the EPS matrix.

P. aeruginosa embeds itself in exopolymer composed of alginate. Alginate is a linear copolymer of ∃-1,4-linked *D*-mannuronic acid and its C-5 epimer *L*-guluronic acid. Alginate in the form of a biofilm matrix polymer plays an important role in diseases such as cystic fibrosis, and urinary tract infections, also in the fouling of man-made materials and at surfaces in the natural environment (Davies, *et al.*1993). The activation of a critical alginate promoter, *algD*, by *P. aeruginosa* has been shown to take place during nitrogen limitation, during membrane perturbation induced by ethanol, and when cells are exposed to media of high osmolarity. This activation is dependent on the presence of a response regulator protein, AlgR.

High osmolarity activates transcription of *algD* and *algC*, thus leading to enhanced alginate production. Activation of the *algC* promoter in cells grown on a surface, is assumed to be as a result of decreasing water or nutrient levels at the point of contact with the substratum (Davies & Geesey,1995). Work in our group has shown that *P. aeruginosa* responds to growth as a biofilm be expressing a wide range of genes (Weyers, 1998). Subsequently a number of reports have confirmed these findings (O'Toole & Kolter, 1998). We have recently found that the whole-cell proteome (2D electrophoresis) of biofilm-associated cells differs from that of planktonic cells (Steyn *et al.*, unpublished). More importantly, planktonic cells in the vicinity of a biofilm differ substantially from planktonic cells where no surface is present. This implies that there is active exchange between cells in the biofilm and the surrounding liquid, and that the

biofilm is not a static entity. This has been confirmed by other workers using real-time microscopic analysis (Kolter, personal communication).

3 Materials and Methods

3.1 *Isolation of rhamnolipid-producing* P. aeruginosa

A variety of samples that could possibly contain biosurfactant-producing micro-organisms were collected both in Bellville (Western Cape) and Pretoria (Gauteng). Samples were taken from oil spills, from under tarmac, at petrol stations, from sewage works and garden compost. Serial dilutions in sterile tap water were plated onto both SW agar (Ochsner *et al.*, 1994).

SW agar of Ochsner *et al.*, (1994):

$NaNO_3$	140 mg
$FeSO_4.7H_2O$	30 µg
H_3PO_4 (conc.)	55 µl
$MgSO_4.7H_2O$	22 mg
KCl	28 mg
NaCl	28 mg
$CaCl_2.2H_2O$	1.4 mg
$ZnSO_4.7H_2O$	82.5 µg
$MnSO_4.H_2O$	82.5 µg
H_3BO_3	16.5 µg
$CoCl_2.6H_2O$	8.3 µg
$CuSO_4.5H_2O$	8.3 µg
$NaMoO_4.2H_2O$	5.5 µg
Cetyltrimethylammonium bromide	200 mg
Methylene blue	5 mg
Hexadecane	10 g
Agar	15 g
dH_2O	1000 ml
pH	6.3

The second approach was to inoculate samples into selective enrichment broth containing either hexadecane or engine oil as carbon source to enrich for biosurfactant-producing organisms. *Pseudomonas* was selected for by adding a selective supplement for *Pseudomonas* (Oxoid SR 103, Oxoid, Basingstoke, England), containing cetrimide, fucidin and cephaloridine. Flasks (250 ml) containing 100 ml broth were incubated at 28 °C for 7 d while shaking at 180 rpm.

Composition of selective enrichment broth:

$NaNO_3$	140 mg
$FeSO_4.7H_2O$	30 µg
H_3PO_4 (conc.)	55 µl
$MgSO_4.7H_2O$	22 mg
KCl	28 mg
NaCl	28 mg
$CaCl_2.2H_2O$	1.4 mg
$ZnSO_4.7H_2O$	82.5 µg
$MnSO_4.H_2O$	82.5 µg
H_3BO_3	16.5 µg
$CoCl_2.6H_2O$	8.3 µg
$CuSO_4.5H_2O$	8.3 µg
$NaMoO_4.2H_2O$	5.5 µg
Hexadecane or Motor oil	10 ml
Cetrimide	5 mg
Fucidin	5 mg
Cephaloridine	25 mg
dH_2O	1000 ml
pH	6.3

The third approach was to suspend samples (2 g) in 10 ml sterile tap water and leave to soak to allow hydration and release of cells into the aqueous phase. Aliquots (1 ml) were inoculated into sterile SW broth (as SW agar but omitting agar, cetyltrimethylammonium bromide and methylene blue), and supplementing with either motor oil or sunflower oil in place of the hexadecane.

3.2 Isolation and identification of putative biosurfactant-producing isolates

Samples demonstrating visible emulsification of oil were streaked onto Pseudomonas Isolation Agar (Difco, USA) to obtain *P. aeruginosa*. Pure cultures were re-inoculated into SW broth with motor oil (see 3.1 above) and cultures demonstrating emulsification of oil within three days were retained. They were tested for gram reaction, cell morphology, motility, cytochrome oxidase, catalase and ability to ferment glucose by standard methods (Gerhardt *et al.*, 1994).

3.3 *Physiology of rhamnolipid-producing strains*

3.3.1 Effect of carbon source

Glycerol and glucose were compared to motor oil as some recent reports indicate that higher yields and faster production rates may be obtained. Five ml of minimal medium with a 1% glycerol together with 0.05ml oil as a carbon source.

3.3.2 Effect of quorum sensing

Supernatant from stationary phase broth cultures was added to minimal medium plus oil. The supernatant would contain homoserinelactones that influence the expression of the stationary phase genes, of which the *rhl* operon is one. Five ml minimal medium containing 0.5ml oil was supplemented with 5ml filter-sterilized supernatant from isolates grown in LB broth until stationary phase.

3.3.3 Effect of cell density

The effect of initial inoculum density on initiation of rhamnolipid synthesis was tested by inoculating with concentrated stationary phase cells. Five ml of minimal medium with 0.5ml oil was inoculated with cells harvested by centrifugation from 5ml of LB broth gown to stationary phase.

3.3.4 Investigation of the factors affecting rhamnolipid production

During attempts to study rhamnolipid production quantitatively it was found that the assay as uniformly described in the bulk of the relevant literature was not reliable. It does not effectively exclude sugars so that artifactual data is obtained. Rhamnolipid was also not precipitated quantitatively, yielding erratic results. Various precipitation and extraction approaches were tested, and finally a reliable albeit lengthy protocol was developed as described below:

3.3.5 Extraction of rhamnolipid

1) Spin down 500µl of culture for 5 minutes and keep supernatant.
2) Add 50µl of concentrated HCL to the supernatant. The pH needs to be lowered to below 2 so that the rhamnolipid could solidify and precipitate. (the pH is beneath the rhamnolipid molecule's pKa value)
3) Incubate at 4°C for 5 days. The incubation at the lower temperature seems to enhance the precipitation of the rhamnolipid.

4) Centrifuge for 10 minutes at 10 000 x g to precipitate the rhamnolipid and other molecules. Remove supernatant.
5) Wash pellets with a pH 2 solution (dilute HCl) so as to remove any traces of glucose. (The anthrone test is extremely sensitive)
6) Add 1.5ml of a 10:1 Chloroform:Methanol mix to the pellet and vortex. The chloroform dissolves the lipid part of the rhamnolipid while the methanol dissolves the rhamnose sugars. A too high an amount of methanol causes ineffective division between the two phases. It also dissolves other unwanted sugars which could influence the Anthrone reaction. Water can be added to ensure the effective separation of the two phases. A variety of solvents were tested but the chloroform:methanol mixture gave the best results.
7) Let the mixture stand for 30min to facilitate the separation.
8) Remove the water phase (on top) plus some of the solvent (to avoid losing product located at the interface) and repeat step 6.
9) Let the mixture stand for 30min to facilitate the separation.
10) Remove the water phase and discard.
11) Pool the solvent aliquots
12) Evaporate the chloroform in a water bath at 80 to 90 °C.
13) The residue has a honey-like consistency. Dissolve in 500µl of a 0.05M sodium bicarbonate solution (pH 8.6) before subjection to the anthrone assay.

The dissolved extract can now be subjected to the Anthrone biochemical quantification of the sugar moiety of rhamnolipid.

Anthrone Reaction:

Prepare fresh Anthrone reagent by adding 0.2g anthrone to100ml of H_2SO_4 (98%)

1) Add 1ml of reagent (very cautiously) to 0.5ml of sample and place tube in ice water
2) After 1min vortex tubes and place in a water bath (95°C) for 15 minutes
3) Cool down to room temperature
4) Load 200µl in a micro-well on a microtiter-plate and determine the absorbance at 620nm.
5) The reading needs to be taken within one hour of completion of the reaction.

In this technique the isolation of the rhamnolipid from the media is extremely important, as even the tiniest amounts of *any* residual sugar will contribute to artificially high results.

3.4 *Isolation of* rhlAB *operon by PCR*

Primers were designed to anneal to the ends of the *rhlAB* gene cluster as shown below. Primers RHL3 and 4 were designed to obtain the *rhlAB* gene from genomic DNA of *P. acruginosa* (Fig. 4), and primers RHL1 and 2 were extended to include restriction endonuclease sites. The expected gene product was 2.26 kb.

```
RHL1: 5' CCTCTAGAGGGAGGTGTGAAATGCGGCGC 3'
RHL2: 5' AAGAATTCGCGCAGCCTTGCCGATACGGC 3'
RHL3: 5' GGGAGGTGTGAAATGCGGC 3'
RHL4: 5' CGCGCAGCCTTGCCGATAC 3'
```

Figure 4. Sequences of the four primers used to amplify the *rhlAB* gene cluster.

Genomic DNA was prepared by standard methods. The PCR was performed using Expand High Fidelity (Boehringer Mannheim) to decrease the level of mismatch incorporation. The 2.2 kb amplicon was excised from an agarose gel, purified by the freeze-squeeze method and re-amplified by PCR. The PCR product was cloned it into the pCR Script vector (Stratagene). This was transformed into XL1blue cells and sequenced by automated sequencing.

Three new primers were designed (two forward and one reverse)(Fig. 5) for the amplification of the *rhlAB* genes. The complete expected region of amplification is 2300 base pairs. The two forward primers differed in their Tm values so as to get the best possible amplification of the specific target region.

```
PMR: 5' CGGCAAAATCATGGCAACCCTA 3'   (Reverse)
PJF: 5' GCGATAGCTGTTTGCCTGTTC 3'    (Forward)
PBF: 5' GGAGGTGTCAAATGCGGCGCGAAAG 3' (Forward)
```

Figure 5. Sequences of the new primers used to amplify the *rhlAB* gene cluster.

Usually the closer the Tm values of the forward and reverse primers to each other the better the reaction should work. Primability was lowered to obtain a higher Tm value for the second forward primer. The two forward primers were used independently from each other. Different reaction conditions were changed so as to get the desired band size.

Magnesium, dNTP, primer and template concentrations were changed. Furthermore, the reaction conditions were also modified, such as adding a hot start to the PCR process, lowering the annealing temperature and changing elongation as well as annealing time. Reactions with different Taq polymerase enzymes were also set up.

AGATCTACGCCAACGAAGGCGTGGCGCAGATGCTCTTCCTGCAATCCGACGAGGCCTGCGAAGTGTCCTAT
AAGGACCGTGGCGGCAAATACCAGGGCCAGCGCGGCGTGACCCTGCCAAAAGCCTGACGCCAGAGCGTTTC
GACACCGGAAACCGGCCTGGCGCCCGGTTTTTTCATGCCTTTTCCGCCAACCCCTCGCTGTCCCCCGCCGG
CCGCTCTGGCCCGCCTTATCGCGGGCGGGCAGGGGCTTATGCGCAGGCGGCCGCCCGTCCTGTGAAATCTG
GCAGTTACCGTTAGCTTTCGAATTGGCTAAAAAGTGTTCATCGGCTACGCGTGAACACGGACGCCAATCGT
TTGCGCAGGCCGATCTGCAAGACCCACACAAGCCCCTCGCCTGAAGGGGTACGCATCCGCCGTGGCTGGTC
CGCGCGGATGGCCGCTGAGTTACTTGTCTGCCGTTCGAACAACAAGAACGAACTCTACGTAATGCCGGGAT
ACCCGTGGCAGCGATAGCTGTTTGCCTGTTCGAAAATTTTT***GGGAGG***TGTGAA***ATG***CGGCGCGAAAGTCTG
TTGGTATCGGTTTGCAAGGGCCTGCGGGTACATGTCGAGCGCGTTGGGCAGGATCCCGGGCGCAGCACGGT
GATGCTGGTCAACGGCGCGATGGCGACCACCGCCTCGTTCGCCCGGACCTGCAAGTGCCTGGCCGAACATT
TCAACGTGGTGCTGTTCGACCTGCCCTTCGCCGGGCAGTCGCGTCAGCACAACCCGCAGCGGGGGTTGATC
ACCAAGGACGACGAGGTGGAAATCCTCCTGGCGCTGATCGAGCGCTTCGAGGTCAATCACCTGGTCTCCGC
GTCCTGGGGCGGTATCTCCACGCTGCTGGCGCTGTCGCGCAATCCGCGCGGCATCCGCAGCTCGGTGGTGA
TGGCATTCGCCCCTGGACTGAACCAGGCGATGCTCGACTACGTCGGGCGGGCGCAGGCGCTGATCGAGCTG
GACGACAAGTCGGCGATCGGCCATCTGCTCAACGAGACCGTCGGCAAATACCTGCCGCCGCGCCTGAAAGC
CAGCAACCATCAGCACATGGCTTCGCTGGCCACCGGCGAATACGAGCAGGCGCGCTTTCACATCGACCAGG
TGCTGGCGCTCAACGATCGGGGCTACCTGGCTTGCCTGGAGCGGATCCAGAGCCACGTGCATTTCATCAAC
GGCAGCTGGGACGAATACACCACCGCCGAGGACGCCCGCCAGTTCCGCGACTACCTGCCGCACTGCAGTTT
CTCGCGGGTGGAGGGCACCGGGCATTTCCTCGACCTGGAGTCCAAGCTGGCCGCGGTACGCGTGCACCGCG
CCCTGCTCGAGCACCTGCTGAAGCAACCGGAGCCGCAGCGGGCGGAACGCGCGGCGGGATTCCACGAGATG
GCCATCGGCTACGCCTGAACCCTTGACCTGCGAAGACCCGGCCTGGCCGGGCTTTGCGGTTGCATAACGCA
CGGAGTAGC

1501 ***rhlB*** -> ***STOP***

ACCATGCACGCCATCCTCATCGCCATCGGCTCGGCCGGCGACGTATTTCCCTTCATCGGCCTGGCCCGGAC
CCTGAAATTGCGCGGGCACCGCGTGAGCCTCTGCACCATCCCGGTGTTTCGCGACGCGGTGGAGCAGCACG
GCATCGCGTTCGTCCCGCTGAGCGACGAACTGACCTACCGCCGGACCATGGGCGATCCGCGCCTGTGGGAC
CCCAAGACGTCCTTCGGCGTGCTCTGGCAAACCATCGCCGGGATGATCGAGCCGGTCTACGAGTACGTCTC
GGCGCAGCGCCATGACGACATCGTGGTGGTCGGCTCGCTCTGGGCGCTGGGCGCACGCATCGCTCACGAGA
[illegible]
CACCCCAAGTTCAACGTGCCCGAGCAGATGCCGCTGGCGATGCGCAAGCTGCTCTGGCGCTGCATCGAGCG
CTTCAAGCTGGATCGCACCTGCGCGCCGGATATCAACGCGGTGCGGCGCAAGGTCGGCCTGGAGACGCCGG
TGAAGCGCATCTTCACCCAATGGATGCATTCGCCGCAGGGCGTGGTCTGCCTGTTCCCGGCCTGGTTCGCG

CCGCCCCAGCAGGATTGGCCGCAACCCCTGCACATGACCGGCTTCCCGCTGTTCGACGGCAGTATCCCGGG
GACCCCGCTCGACGACGAACTGCAACGCTTTCTCGATCAGGGCAGCCGGCCGCTGGTGTTCACCCAGGGCT
CGACCGAACACCTGCAGGGCGACTTCTACGCCATGGCCCTGCGCGCGCTGGAACGCCTCGGCGCGCGTGGG
ATCTTCCTCACCGGCGCCGGCCAGGAACCGCTGCGCGGCTTGCCGAACCACGTGCTGCAGCGCGCCTACGC
GCCACTGGGAGCCTTGCTGCCATCGTGCGCCGGGCTGGTCCATCCGGGCGGTATCGGCGCCATGAGCCTGG
CCTTGGCGGCGGGGGTGCCGCAGGTGCTGCTGCCCTGCGCCCACGACCAGTTCGACAATGCCGAACGGCTG
GTCCGGCTCGGCTGCGGGATGCGCCTGGGCGTGCCATTGCGCGAGCAGGAGTTGCGCGGGGCGCTGTGGCG
CTTGCTCGAGGACCCGGCCATGGCGGCGGCCTGTCGGCGTTTCATGGAATTGTCACAACCGCACAGTATCG
CTTGCGGTAAAGCGGCCCAGGTGGTCGAACGTTGTCATAGGGAGGGGGATGCGCGATGGCTGAAGGCTGCG
TCCTGAACGGTGCTGGCATAACAGATAGGGTTGCCATGATTTTGCCGTATCGGCAAGGCTGCGCGCTTGAC

Figure 6. Partial *rhl* sequence showing the locations of two of the primers used for PCR.

We then performed a computer analysis (Amplify 1.2 on Apple Macintosh) of the binding of the primers to the target region. A new forward primer, FFP1 (Fig. 7) was designed with the help of the computer program, as well as blasting the new primer at then an almost complete genomic sequence for *Pseudomonas aeruginosa*. The new forward primer's Tm was closer to the reverse primer and had a higher stability. Different enzymes as well as different annealing temperature were tried. New genomic DNA was extracted to circumvent the possibility of degraded DNA giving these non-specific bands.

FFP1 5' TGAAATGCGGCGCGAAAGTCT 3'

Figure 7. Sequence of forward primer FFP1 used to amplify the *rhlAB* gene cluster together with reverse primer PMR (Fig. 5)

PCR amplicons were also cloned into the pGEM Teasy vector system (Promega, Wisconsin, USA). The vector has 5' T overhangs, facilitating better ligation with PCR products which tend to have 3' A overhangs. Ligations and transformations were as per manufacturers instructions. Ligation mixtures were transformed into competent *Escherichia coli* JM109 cells. The presence of the PCR amplicon in white colonies was confirmed by colony PCR using the relevant PCR primers. White colonies were isolated and plasmid DNA extracted. Plasmid DNA was digested with restriction endonucleases

EcoRI (multiple cloning site), EcoRV (inside *rhlAB* operon) or ScaI (inside *bla* gene on vector).

3.5 Culturing biofilms on capillary membranes

The membrane was placed in a type A flow cell, exposed to water, EtOH and MGL and viewed under autofluorescence on confocal scanning laser microscope. Three stains / probes were evaluated; acridine orange (AO), fluorescein and RH795 (Molecular Probes Inc, USA). The inoculum was prepared by inoculating *P. aeruginosa* PAO 1 into minimal glucose lysine broth and incubating while shaking at 30 °C to an OD_{540} of 0.8 of which 1 ml was inoculated into the flow cell (inside and outside of the membrane, for the inside inoculum the membrane was cut open). Flow and nutrients were supplied after 5 min. Inoculum prepared as above was exposed to the membrane by reversing the flow for a short period. Medium was then flowed through the membrane from the lumen outward.

3.6 Culturing biofilms on glass wool

Cultures of *P. aeruginosa* were cultured in Luria Bertani broth (Sambrook *et al*., 1989) in the presence of glass wool having a diameter of 15 µm (Merck, Darmstad, Germany). The ratio of glass wool to liquid was 0.05g/ml. The surface area per gram was 1.4×10^{11} μm^2, so that each ml of culture was exposed to 7×10^{9} μm^2. Growth was monitored by measuring absorbance (540 nm), culturable count, total cellular protein (Bradford assay) and cell size (light microscopy) over time. Planktonic and biofilm biomass were always separated, and controls without glass wool were included. Biofilms were also inspected by light microscopy and by environmental scanning microscopy. The rhamnolipid produced was also assayed in biofilms and planktonic cultures by the method described above in 3.3.

3.7 Production of rhamnolopid in batch culture

Rhamnolipid was produced in batch culture by pre-culturing *P . aeruginosa* on PTYG agar (see below) for 24h at 37°C. Kay's Pre-culture Media was inoculated from the PTYG plate and incubated at 37°C for 24h with constant shaking at 200rpm. MSM media (200 ml)(see below) was autoclaved in a 1 liter Erlenmeyer flask. Ten ml sterile 30% glucose solution and 1 ml 24h pre-culture grown in Kay's medium were added per 100 ml cooled sterile MSM. Flasks were Incubate at 37°C and 22°C with constant shaking at 200rpm.

PTYG Agar: add the following to 1 liter dH_2O

Tryptone	5g
Yeast Extract	10g
$CaCl_2$ $2H_2O$	0.07g
Agar	15g

Kay's Mineral Media:

1) Prepare Solution A, Solution B, and Solution C separately in dH_2O.
2) Add 1 ml Solution B to 100 ml of Solution A before autoclaving.
-Adjust pH to 7.0

1) Add 1 ml Solution C to 100 ml of Solution A after autoclaving and before inoculation.

Solution A	100 ml
$(NH_4)H_2PO_4$	0.3 g
K_2HPO_4	0.2 g
Glucose - $C_6H_{12}O_6$	0.2 g

Solution B 100x
0.045 g $FeSO_4$ is dissolved in 100 ml dH2O and autoclave.

Solution C 100x
10 g $MgSO_4 \cdot 7H_2O$ is dissolved in 100 ml dH_2O and autoclave.

Mineral Salts Production Media (MSM)

1. Prepare Solution A and Solution B separately in dH_2O.
2. Add 1ml of Solution B to 1000ml of completed Solution A

Solution A	1000ml
$NaNO_3$	2.5 g
$MgSO_4 \cdot 7H_2O$	0.4 g
KCl	1.0 g
NaCl	1.0 g
$CaCl_2 \cdot 2H_2O$	0.05 g
H_3PO_4 (85%)	10 ml

Adjust the pH to 7.0 with KOH Pellets

Solution B 1000X	100ml
$FeSO_4 \cdot 7H_2O$	0.05g
$ZnSO_4 \cdot 7H_2O$	0.15g
$MnSO_4 \cdot H_2O$	0.15g

H_3BO_3	0.03g
$CoCl_2 \cdot 6H_2O$	0.015g
$CuSO_4 \cdot 5H_2O$	0.015g
$Na_2MoO_4 \cdot 2H_2O$	0.010g
Glucose	1000ml
$C_6H_{12}O_6$	300g

Rhamnolipid harvesting and extraction

Precipitation:

a) Remove the culture from the shaker. The appearance should be greenish yellow with a thick head of white foam. Presence of pigment is an indicator of rhamnolipid production.
b) Centrifuge the culture at 15 000 x g for 10 minutes to pellet the cells.
c) Collect and combine the supernatant (liquid) in one flask.
d) Acidify the supernatant with concentrated HCl until the pH is less than 2 and rhamnolipid begins to precipitate out, indicated by a color change from bright yellow to cloudy greyish/yellow
e) Confirm that the pH is less than 2 with litmus paper.
f) Store the acidified supernatant at 4°C for 5 days to obtain maximum precipitation of rhamnolipid before extracting.

a) Pellet the precipitated rhamnolipid as described for pelleting of cells.
b) Dissolve rhamnolipid pellets in a generous amount of 10:1 chloroform to methanol mixture. Add to separatory funnel, rinsing centrifuge bottles with solvent mixture to ensure complete transfer of rhamnolipid. If no aqueous phase develops, a small amount of dH_2O can be added to the separatory funnel.
c) Remove the organic phase from the separatory funnel and recover the rhamnolipid by removing the solvent by rotary evaporation. Extract the remaining aqueous phase twice more, using the same chloroform/methanol mixture.
d) Continue to evaporate the solvent mixture with the rotary evaporator until the rhamnolipid has a thick, honey-like consistency.

4 Results

4.1 *Isolation of rhamnolipid-producing* Pseudomonas aeruginosa

The initial isolation procedure as described in the proposal did not yield many isolates. Several samples obtained by the second and third methods did demonstrate emulsification of engine oil after 5 days of incubation. It is well established that *P. aeruginosa* produces rhamnolipids primarily during the stationary phase. This means that

where the carbon and energy source is not miscible in water, the initial growth rate is highly limited.

The emulsion appeared to break after about 7 days as a white viscous ball developed in the liquid. Microscopical examination revealed that the micelles decreased in size with culture age, and that the apparent broken emulsion consisted of tiny micelles surrounded by bacterial cells.

4.2 Isolation and identification of putative biosurfactant-producing isolates

Five biosurfactant-producing isolates identified as *P. aeruginosa* were obtained. All were gram negative rods, catalase and cytochrome oxidase positive, motile and oxidised but did not ferment glucose.

4.3 Physiology of rhamnolipid-producing strains

Effect of carbon source: The isolates took longer to begin with emulsification than the standard growth media of minimal medium plus oil. This would indicate that they used the glycerol first before attempting to use the oil as carbon source. There was no noticeable difference in the amount of emulsification between the addition of glycerol and the standard culture media.

Effect of quorum sensing: The addition of the supernatant had a complex effect and did influence the start of emmulsification (emulsification was slow to start). The oil formed finer droplets directly following inoculation, indicating the presence of rhamnolipid in the LB broth. The degree of emmulsification varied between the different the isolates, implying different reactions to the addition of the supernatant and therefore to the homoserinelactone of the control strain.

Effect of cell density: Although a lot of cells died off in the first two days, emulsification started very early (two days) and showed the highest degree of emulsification,. indicating that quorum sensing does play a role in rhamnolipid production, but that it is rather dependant on fresh homoserine lactone produced *in situ*. The implication is that successful production using wild type strains will depend to a large degree on maintaining a high density of cells.

Of the five isolates three were chosen to be used for further studies due to the superior degree and rate of emulsification observed. Rhamnolipid production was shown to begin with onset of the stationary phase (Fig. 8). The production of rhamnolipid was also limited by the availability of glucose as shown be the halt in increase upon depletion of available glucose (Fig. 8).

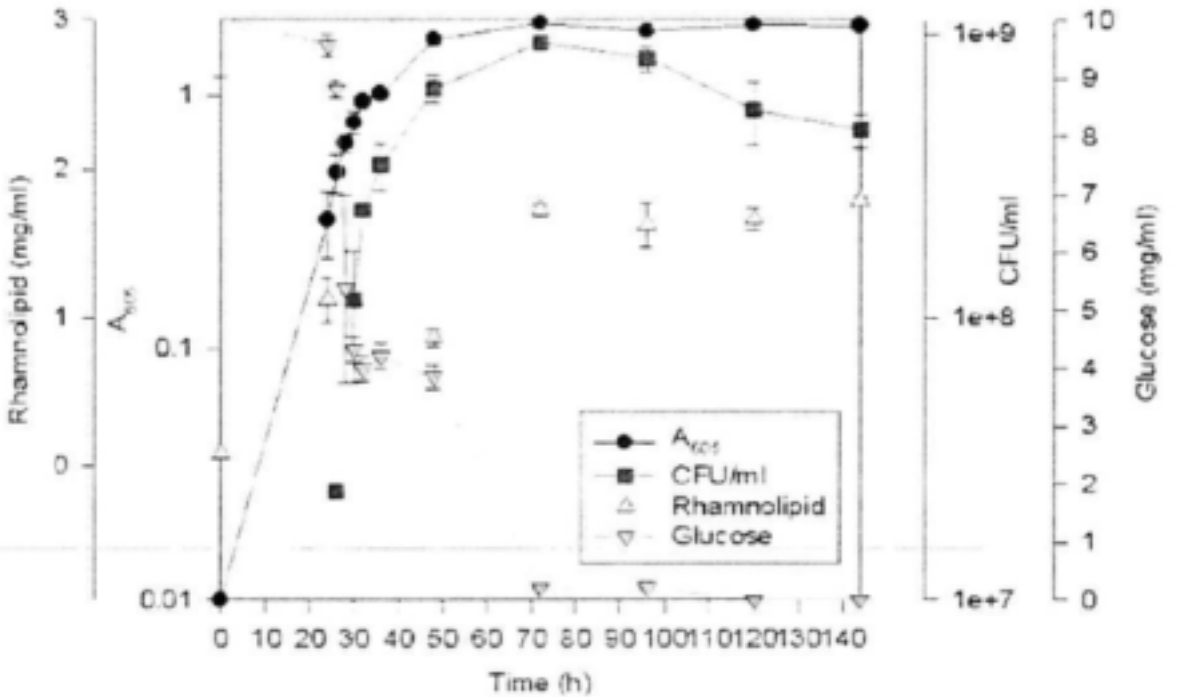


Figure 8. Production of rhamnolipid by isolate L3

4.4 Isolation and cloning of the rhlAB *operon by PCR*

Rhamnolipid is only produced late during the stationary phase in *P. aeruginosa*. For this reason we aimed to place the expression of *rhlAB*, the genes coding for rhamnosyl transferase, under control of a biofilm-specific promoter. This would ensure expression of rhamnosyl transferase, and therefore production of rhamnolipid during growth on the membrane.

A range of PCR products were obtained following the PCR, and despite various modifications, we never succeeded in obtaining only the 2.26 kb product. We therefore excised the 2.2 kb band from an agarose gel, purified it by the freeze-squeeze method and re-amplified it by PCR. The PCR product was cloned it into the pCR Script vector (Stratagene). This was transformed into XL1blue cells and sequenced by automated

sequencing. Unfortunately the cloned gene carrying an insert of 2.3 kb did not match anything on the databases at the time. It must be taken into consideration that at the time only *ca*.10% of the *P. aeruginosa* genome sequence was available within the public domain. It appeared that primers as RHL 3 and 4 have a high degree of homology with other areas on the genome, possibly a repeating sequence.

PCR products showed multiple bands and none of them indicated the correct size of 2.3 kB. There were also signs of primer dimers being formed. Different reaction conditions were changed so as to get the desired band size. Magnesium, dNTP, primer and template concentrations were changed. Furthermore, the reaction conditions were also modified, such as adding a hot start to the PCR process, lowering the annealing temperature and changing elongation as well as annealing time. Reactions with different Taq polymerase enzymes were also set up. This indicated the preference of the reaction mix and conditions for certain polymerase enzymes. Only the taq DNA polymerase from Southern Cross Biotechnologies yielded amplicons, but again a series of smaller products was obtained.

A computer analysis was performed (Amplify 1.2 on Apple Macintosh) of the binding of the primers to the target region. The program indicated that for both forward primers there were multiple annealing sites explaining the possible multiple bands in the PCR reactions. A new forward primer, FFP1 (Fig. 7) was designed with the help of the computer program, as well as blasting the new primer at then an almost complete genomic sequence for *Pseudomonas aeruginosa*. The new forward primer's Tm was closer to the reverse primer and had a higher stability. Different enzymes as well as different annealing temperature were tried. The reaction had a preference for Southern Cross enzyme only. A higher annealing temperature indicated better results although there were still some multiple bands forming in the PCR. New genomic DNA was extracted to circumvent the possibility of degraded DNA giving these non-specific bands. PCR products formed cleaner bands with the new genomic DNA, but the products were still made up of multiple bands and large amounts of primer dimers.

During July 1999 the complete genome sequence of *P. aeruginosa* was made available, albeit not assembled yet. It became apparent that the primers designed were specific to the *rhlAB* operon, and did not anneal anywhere else on the genome. Initial computer analysis of the possible secondary structures of the expected amplicon (RNA structure

3.2) indicated a high degree of folding as shown in Fig. 9, indicating that this could indeed be the reason for multiple band formation.

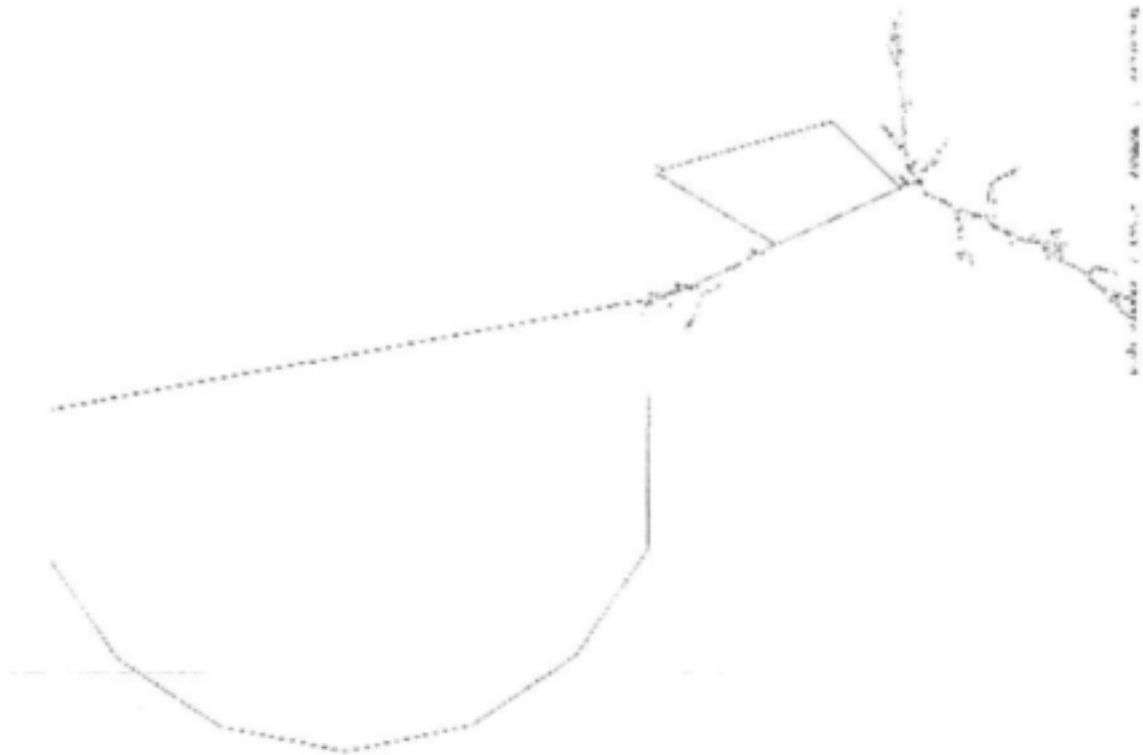


Figure 9. Predicted secondary structure of the single stranded amplicon as obtained using RNA structure 3.2

Again reaction conditions and concentrations of the reaction components were changed. The biggest band we now got was close to 2kb. The size of some of these bands indicated to us the possibility of the interference of secondary structure of the amplified region with the PCR.

Addition of DMSO (dimethylsulphoxide) at 5% yielded an amplicon of 2.3 kb, the desired size (Fig. 10). Most of the secondary bands also disappeared. Different positions on the PCR block gave us different concentrations of the desired product indicating high sensitivity to minor temperature changes. The stronger the desired product band, the less secondary bands were as well as less primer dimers were obtained. One set of reaction conditions yielded a perfect singular band with no secondary bands and virtually no primer dimers (Fig. 10 lane 8).

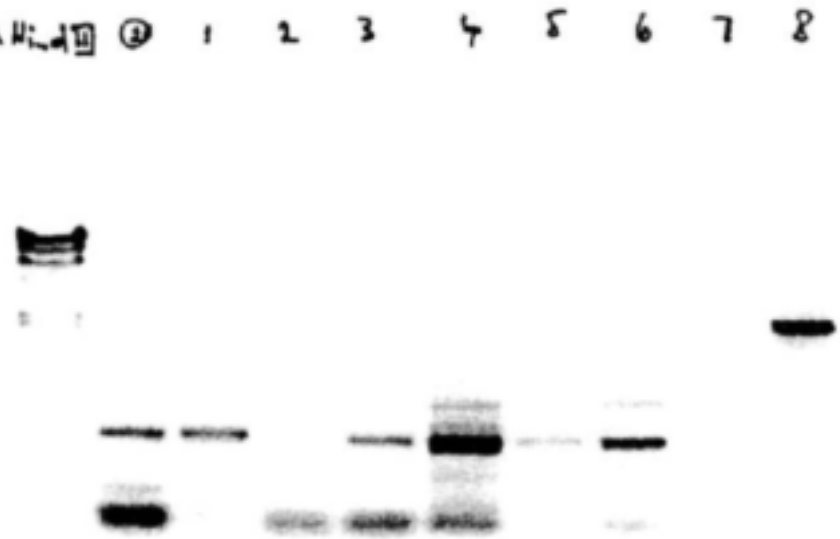


Figure 10. Electrophoretogram of the PCR products obtained using primers FFP1 and PMR. Lane 8 shows the desired amplicon of 2.3 kb.

The PCR product was excised and extracted, and prepared for cloning into the pCRscript cloning vector. Colony PCR of white (recombinant colonies) did not yield the desired amplicon (Fig. 11).

Figure 11. Electrophoretogram of PCR amplicons of recombinant clones. Lane 1: Lambda HindIII size marker, lanes 2 - 4: PCR amplicons of recombinant clones.

Restriction digestion of plasmid extracts from recombinant clones did not yield the expected 2.3 kB fragment (Fig. 12). On the contrary, digestion with EcoR1 which cuts in the multiple cloning site yielded only one fragment which was smaller than the pGEM T-easy vector (Fig. 13).

Figure 12. Electrophoretogram of plasmid extracts of recombinant clones. Lane 1: pGEM T-easy control vector, lanes 2, 8 and 9: blank, lanes 3 - 7 and 10 - 12: recombinant clones.

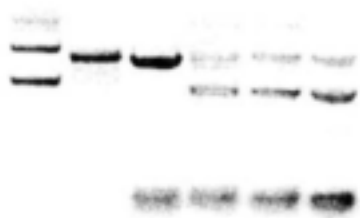


Figure 13 Figure 14 Electrophoretogram of restriction digests of recombinant vectors. Lane 1: Undigested pGEM T-easy, lanes 2 and 3: pGEM T-easy digested with EcoRI, lane 4: Recombinant vector digested with EcoR1, lane 5: Recombinant vector digested with EcoRV, lane 6: Recombinant undigested vector.

Similarly EcoRV, which digests once in the *rhlAB* operon and not once in the vector did not yield any linearized fragments, indicating that the specific sites were not present (Fig. 14). Digestion of pGEM T-easy with ScaI, which cuts once within the *bla* gene but not in the *rhlAB* operon, yielded the expected 3 kB fragment. Digestion of recombinant clones yielded a significantly smaller fragment (Fig. 14), indicating that the recombinant clones were in fact smaller than the native plasmid. This indicated that the insert was being excised with a section of the vector by a recombinational event, although the *E. coli* JM109 host was RecA negative. We are currently in discussion with the group of Prof H.

Huismans where a similar phenomenon has been discovered for a large, G-C rich PCR product.

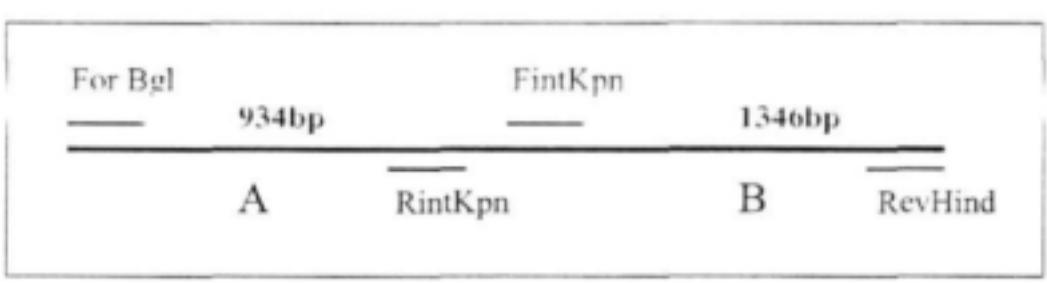
Figure 15. Electrophoretogram of restriction digests of recombinant vectors. Lanes 1 - 3: Recombinant vector digested with EcoRV, lane 4: Recombinant vector digested with ScaI, lane 6: pGEM T-easy digested with ScaI.

The strategy was altered and an expression vector specifically designed for large DNA inserts (pLAH31) was chosen. The advantages of the plasmid include:

1. Origin of replication for both *E. coli* and *P. aeruginosa* making it possible to clone into *E. coli* first.
2. The plasmid can be transferred from *E. coli* to *P. aeruginosa* by conjugation using a helper plasmid in tri-parental mating.
3. His tag expression makes it easier to isolate and detect proteins.
4. Expression in *E. coli* is inducible with the *lacI* promoter and in *Pseudomonas* the protein is expressed constitutively.

New primers were designed to clone the 2.3kb insert in two stages into pLAH31. The RhlA and RhlB proteins needed to be cloned separately since in the case of the whole 2.3kb insert the RhlB protein is expressed under the control of the *rhlA* promoter. We therefore need to determine if RhlB is expressed by Western blotting. (The RhlB protein is the rhamnosyltransferase responsible for rhamnolipid containing one rhamnosyl sugar).

The primers were also designed with the CTX vector system in mind whereby the *rhlAB* genes could be cloned into CTX and integrated into a specific site on the genome of *P. aeruginosa*, thereby eliminating the need for antibiotic selection. This could prove useful in an industrial system.



ForBgl 5'-GGGAAGATCTTGAAATGCGGCGCGAAAGTCT-3'- a forward primer with BglII restriction site built in.
RintKpn 5'-CGGGGTACCGGTCTTCGCAGGTCA-3' - an internal reverse primer with KpnI restriction site.
FintKpn 5'-CGGGGTACCCACCATGCACGCCAT-3' - an internal forward primer with KpnI built in.
RevHind 5'-CCCAAGCTTCGGCAAAATCATGGCAACCC-3' - a reverse primer with HindIII restriction site built in.

Figure 16. Sequences of primers used to amplify the *rhlA* and *rhlB* genes separately.

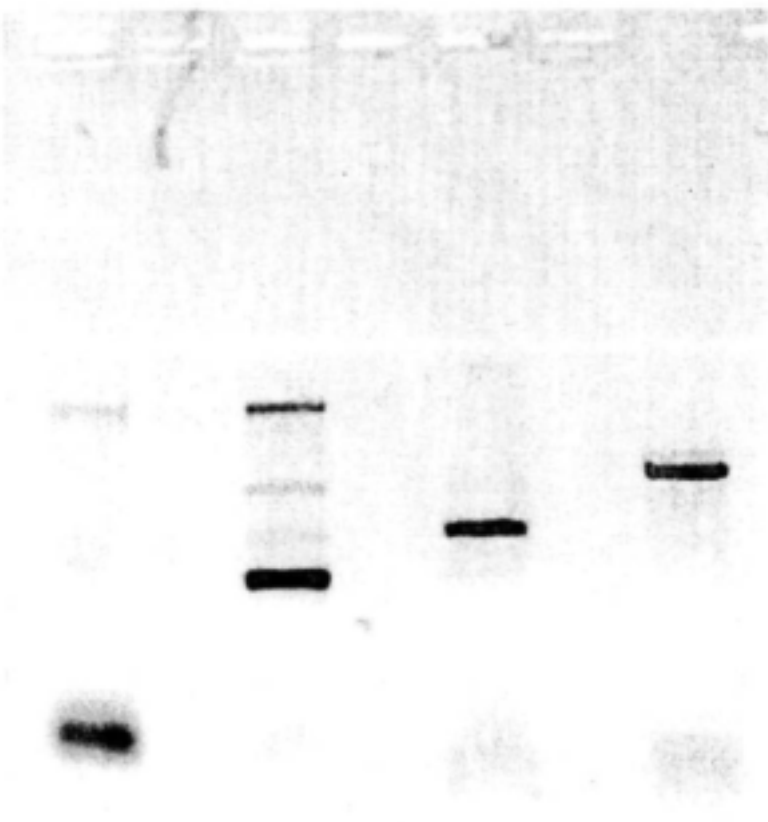


Figure 17. Electrophoretogram showing PCR amplicons obtained with the primers shown in Fig. 15. Lane 1: PCR with old primers amplifying the 2.3kb *rhlAB* gene product from *P. aeruginosa* PAO1 (faint upper band); Lane 2: the 2.3kb *rhlAB* gene product from *P. aeruginosa* PAO1 obtained using new primers ForBgl and RevHind (light upper band); Lane 3: the *rhlA* gene product obtained using primers ForBgl and RintKpn; and Lane 4: the *rhlB* gene product obtained using primers FintKpn and RevHind.

The PCR products of the *rhlA* and *rhlB* genes were cloned into pGEM T easy to make working with the insert and further cloning steps easier. Restriction digestion of the inserts with their own restriction sites from the pGEM plasmid was performed to ensure

presence of the two amplicons (Fig. 17). Sequencing of the insert in pGEM with the M13 primer set confirmed the amplicons to be *rhlA* and *rhlB*.

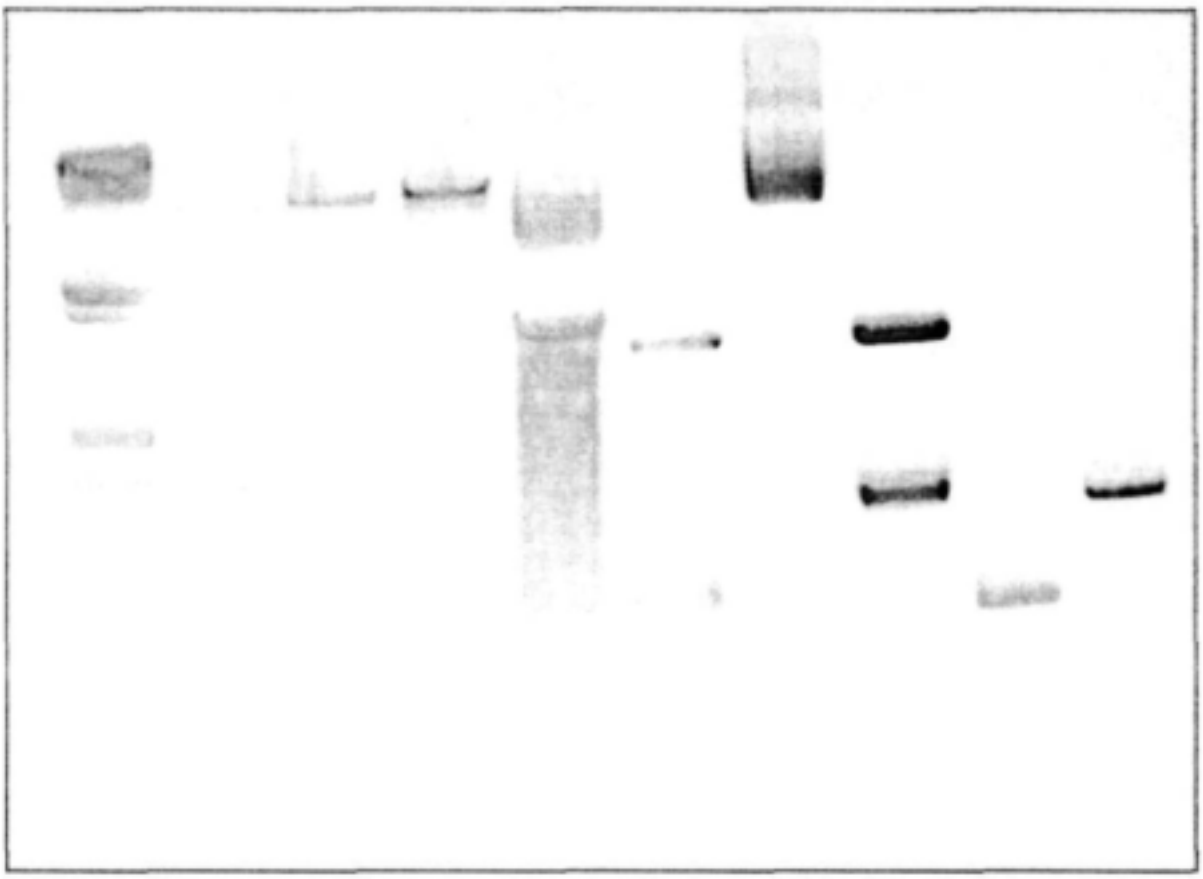


Figure 18. Electrophoretogram showing PCR amplicons be *rhlA* and *rhlB* in pGEM. Lane 1: Phage lambda cut with HindIII and EcoRI; Lane 2: pLAH31 uncut; Lane 3: pLAH31 cut for ligation with *rhlA* – BamHI and KpnI; Lane 4: pLAH31 cut for ligation with *rhlB* – HindIII and KpnI; Lane 5: pGEM/A uncut; Lane 6: pGEM/A cut with BglII and KpnI; Lane 7: pGEM/B uncut; Lane 8: pGEM/B cut with HindIII and KpnI; Lane 9: A insert from pGEM/A purified; Lane 10: B insert from pGEM/B purified.

Sequencing of the A and B inserts with both forward and reverse M13 primers proved that the inserts were definitely the *rhlA* and *rhlB* genes from *P. aeruginosa*.

Ligations were set up with pLAH 31 and the be *rhlA* and *rhlB* inserts separately. PLAH31 is a low copy number plasmid and insert to vector ratio as well as vector to cell ratio is very important. (pLAH31 is a 22kb plasmid with approximately 5 copies per cell). Once the be *rhlA* gene BglII site is ligated to the BamHI site for pLAH31, both sites are destroyed. Restriction digests to confirm the presence of the insert is therefore no longer possible. PCR and sequencing were therefore performed to confirm the presence of the correct inserts (Fig. 18).

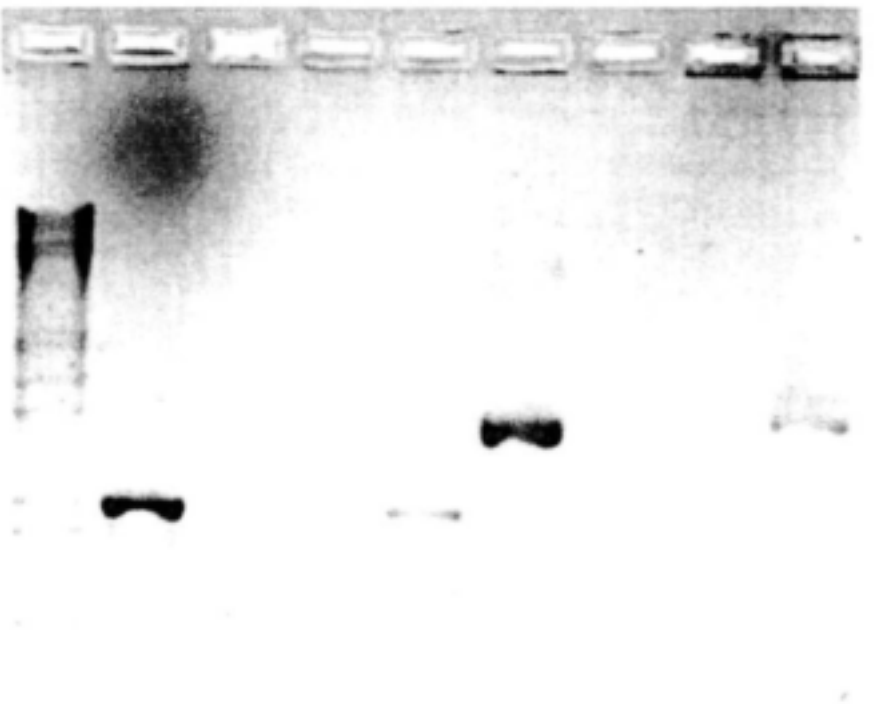


Figure 19. Electrophoretogram showing Lane 1: Phage lambda cut with HindIII and EcoRI: Lane 2: *rhlA* PCR amplicon from pLAH31/A using primers for *rhlA*; Lane 3: *rhlA* PCR reaction mix from pLAH31 using primers for *rhlA;* Lane 4: H_2O with primers for *rhlA* PCR amplicon from pLAH31/A using primers for *rhlA*; Lane 5: *rhlA* PCR amplicon from genome of *P. aeruginosa;* Lane 6: *rhlB* PCR amplicon from pLAH31/B using primers for *rhlB*; Lane 7: *rhlB* PCR reaction mix from pLAH31 using primers for *rhlB;* Lane 8: H_2O with primers for *rhlB*; Lane 9: *rhlB* PCR amplicon from genome of *P. aeruginosa.*

Expression:

The *rhlA* and *rhlB* genes in the pLAH31/A and /B plasmids were induced to be expressed via the Olac promoter. Induction was with IPTG for 5 hours after which proteins were extracted and separated on a SDS-PAGE gel (Figs. 19 & 20).

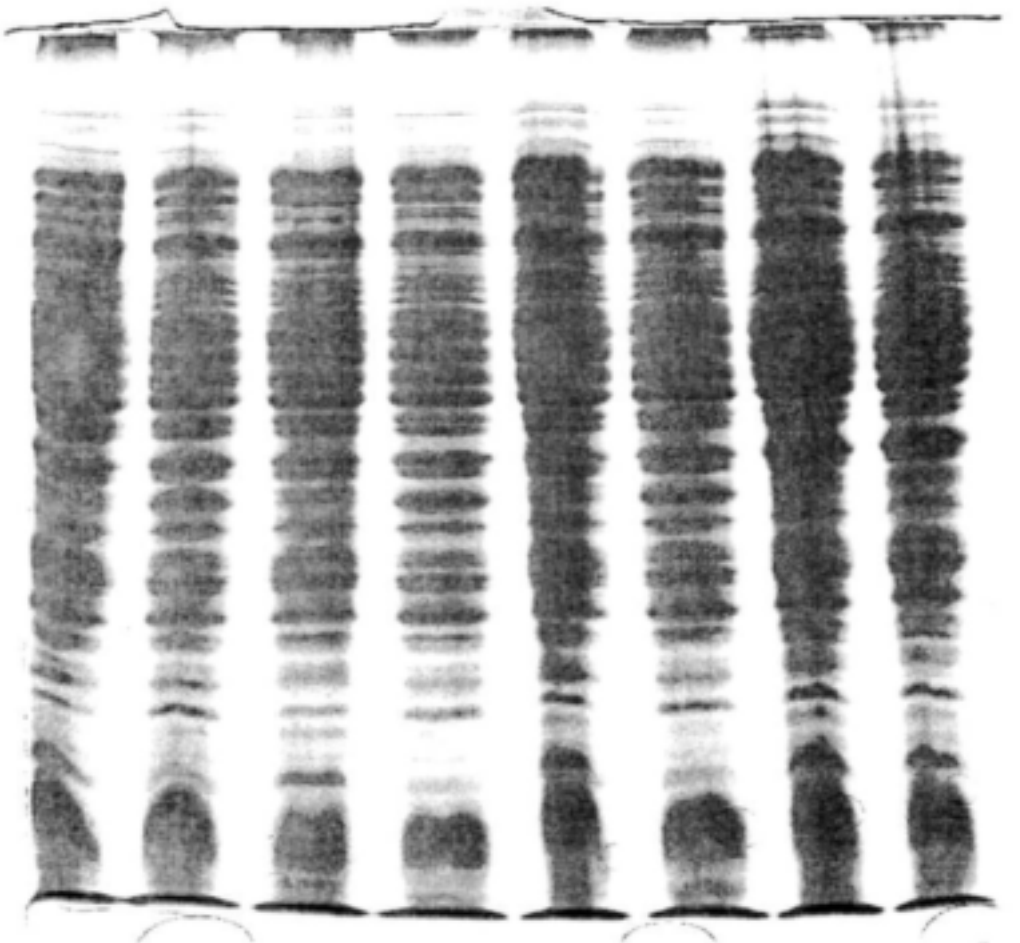


Figure 20. SDS PAGE of whole-cell protein extracts of *Escherichia coli* harbouring pLAH31/B. Lane 1: recombinant TA1 – not induced; Lane 2: recombinant TA1 – induced; Lane 3: recombinant TA2 – not induced; Lane 4: recombinant TA2 – induced; Lane 5: recombinant TA4 – not induced; Lane 6: recombinant TA4 - induced; Lane 7: recombinant TA8 – not induced; Lane 8: recombinant TA8 – induced.

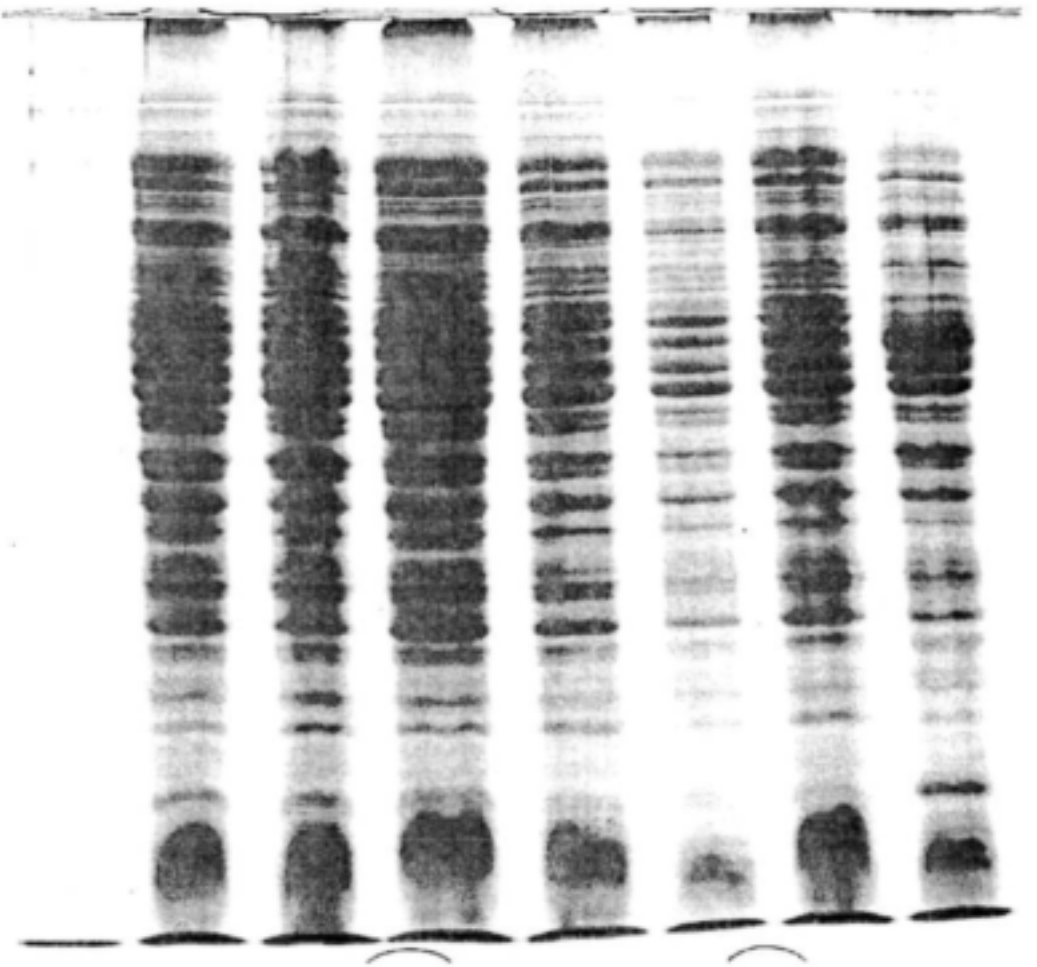


Figure 21. SDS PAGE of whole-cel protein extracts of *Escherichia coli* harbouring pLAH31/B. Lane 1: Molecular weight marker; Lane 2: recombinant TB1 – not induced; Lane 3: recombinant TB1 – induced; Lane 4: recombinant TB2 – not induced; Lane 5: recombinant TB2 – induced; Lane 6: recombinant TB4 – not induced; Lane 7: recombinant TB4 - induced Lane 8: DH5 *E. coli* containing pLAH31

The TA recombinants definitely showed expression of the RhlA protein under induction (around 32 kDa). The TB recombinants is possibly recombinant but unfortunately the main proteins of *E. coli* fall in to the 40 to 60 kDa group. The RhlB protein is 47 kDa. So its presence may be masked by other *E. coli* proteins. Western Blot with anti-Histidine antibodies will be used to show whether the RhlA and RhlB proteins are truly expressed.

Cloning of the *rhlAB* 2.3kb insert into pLAH31 continues.

4.5 Culturing biofilms on capillary membranes

4.5.1 Membrane properties

There appeared to be no change in the surface properties of the membrane when exposed to the three conditions . The different conditions did not have any effect on the membrane pore size, with average pore size of 12- 20 μM.

4.5.2 Staining of the membrane

It was possible to visualize the pore structure of the membrane with acridine orange (Fig. 21) whereas fluorescein gave inferior results and needed to be washed out before the pore structure could be visualized (Fig. 22). The visible intensity was also reduced when stain RH795 was used. No pictures of the inside of the membrane could be taken due to reflection from the skin on the inside of the membrane.

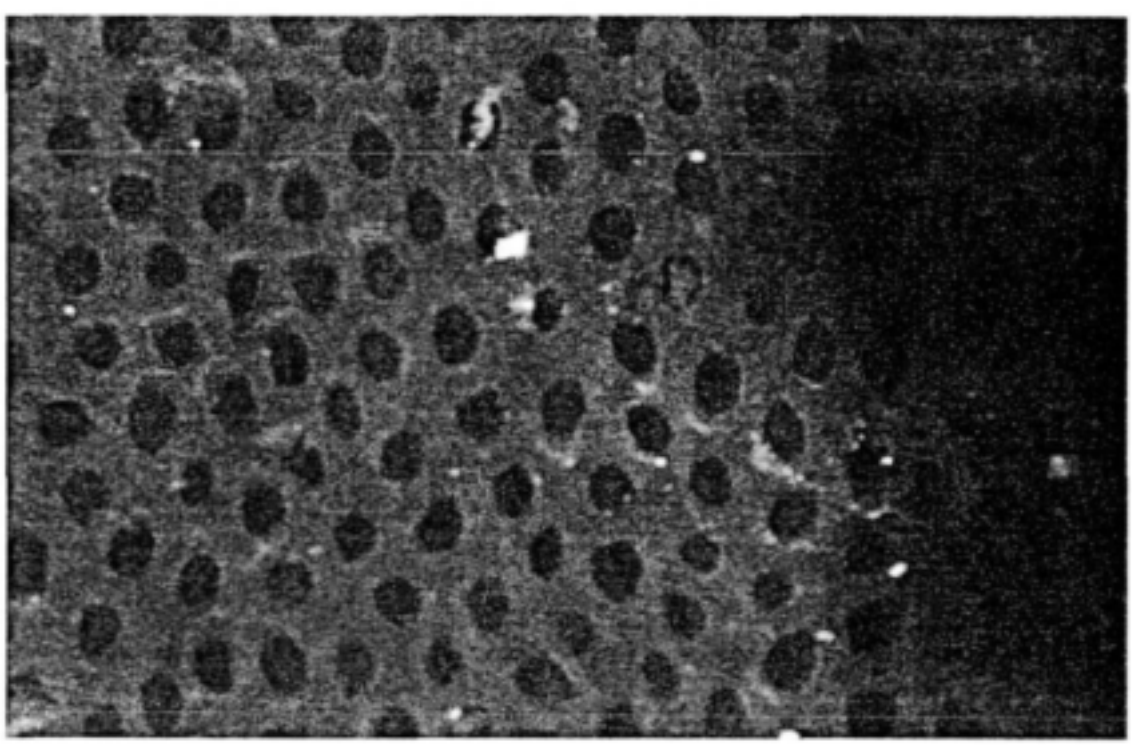


Figure 22. View of the surface of the polysulphone capillary membrane after staining with acridine orange. The image was taken by scanning laser confocal microscope.

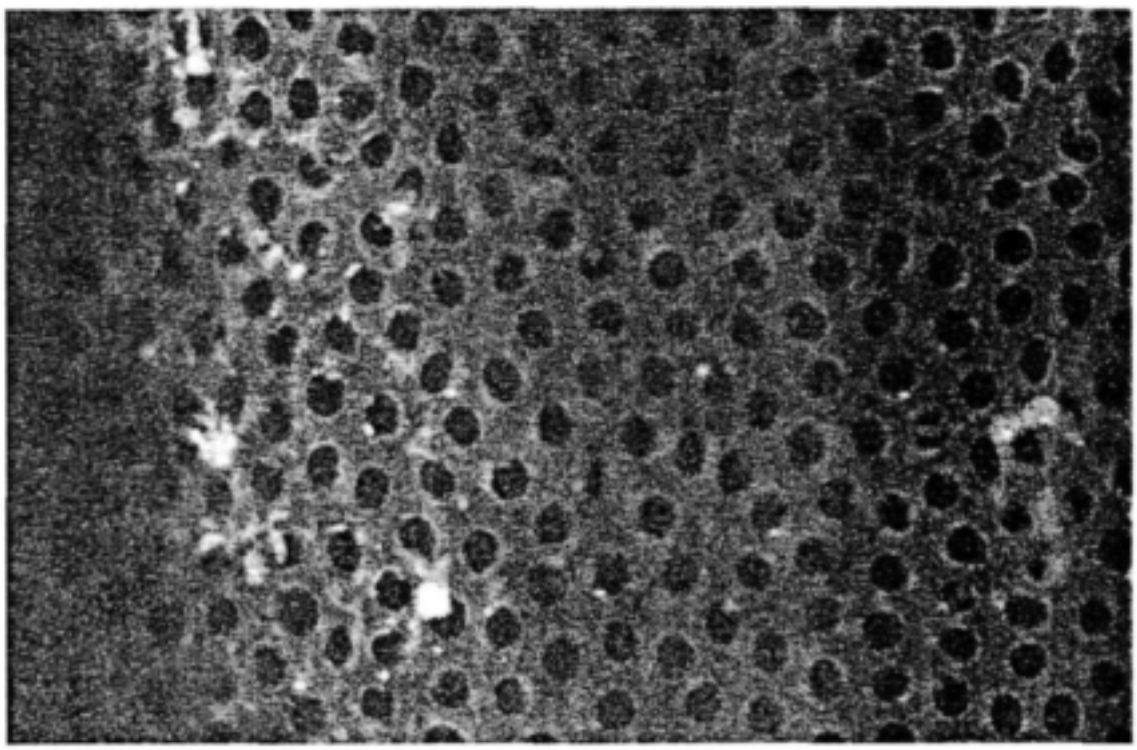


Figure 23. View of the surface of the polysulphone capillary membrane after staining with fluorescein. The image was taken by scanning confocal laser microscope.

4.5.3 Colonization of the inside of the membrane

No significant colonization was observed on the inside of the membrane, indicating that the inner surface was not conducive to adhesion by *P. aeruginosa*.

4.5.4 Colonization of the outside of the membrane

It was difficult to visualize bacteria inside the voids of the membrane. This could be due to the distance of the membrane from the cover slip. Bacteria did colonize the voids in the membrane, although it was difficult to obtain good images (Figs. 24 - 25). Bacteria did, however, colonize the surface, attaching at first at the edges around the microvoids (Figs. 26 - 27), then forming biofilms on the intervoidal surface (Figs. 28 - 30).

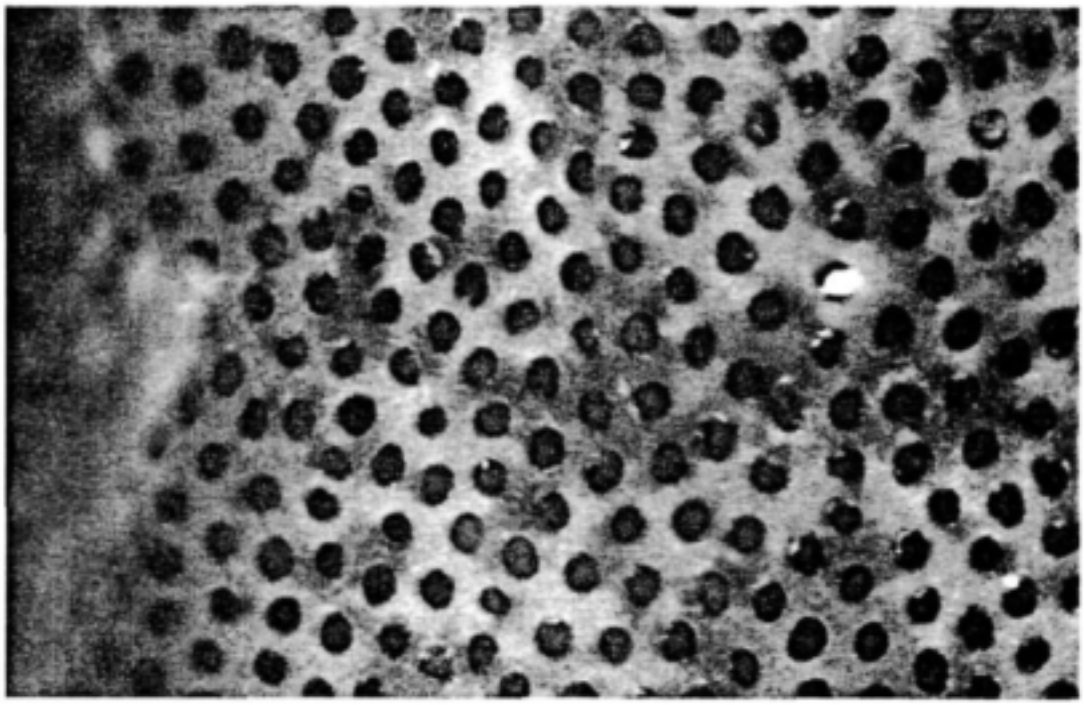


Figure 24. View of the voids in the polysulphone capillary membrane before inoculation with PAO1 and staining with acridine orange. The image was taken by scanning laser confocal microscope, focusing at various depths inside the voids.

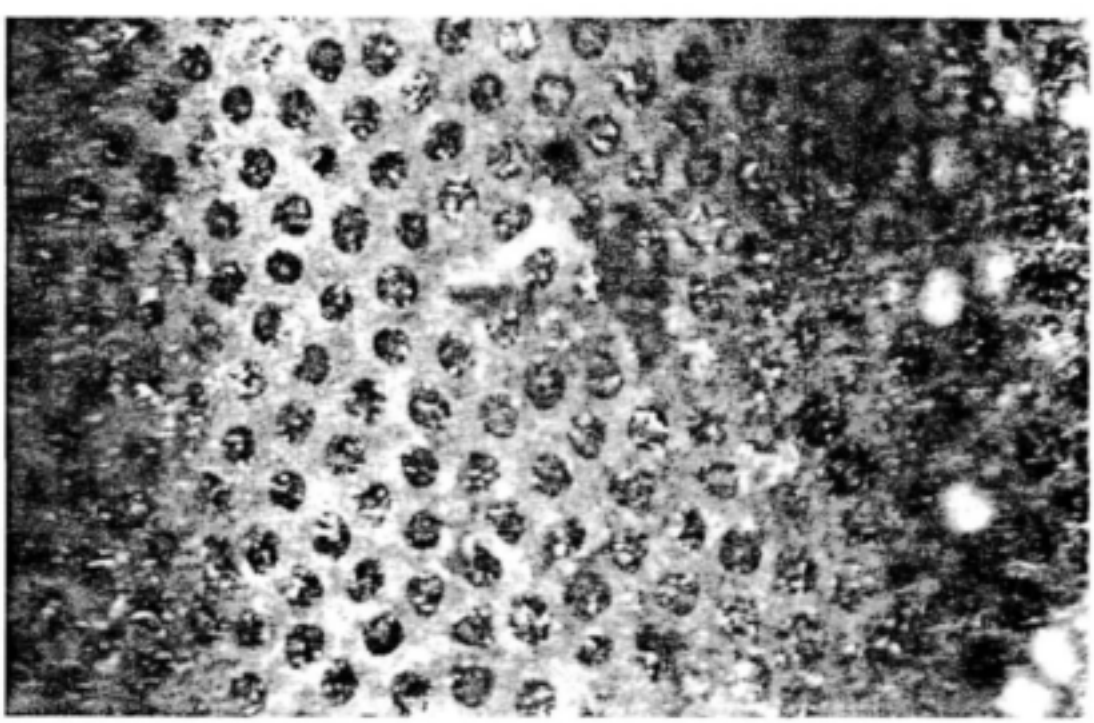


Figure 25. View of the voids in the polysulphone capillary membrane after inoculation with PAO1 and staining with acridine orange. The image was taken by scanning laser confocal microscope, focusing at various depths inside the voids.

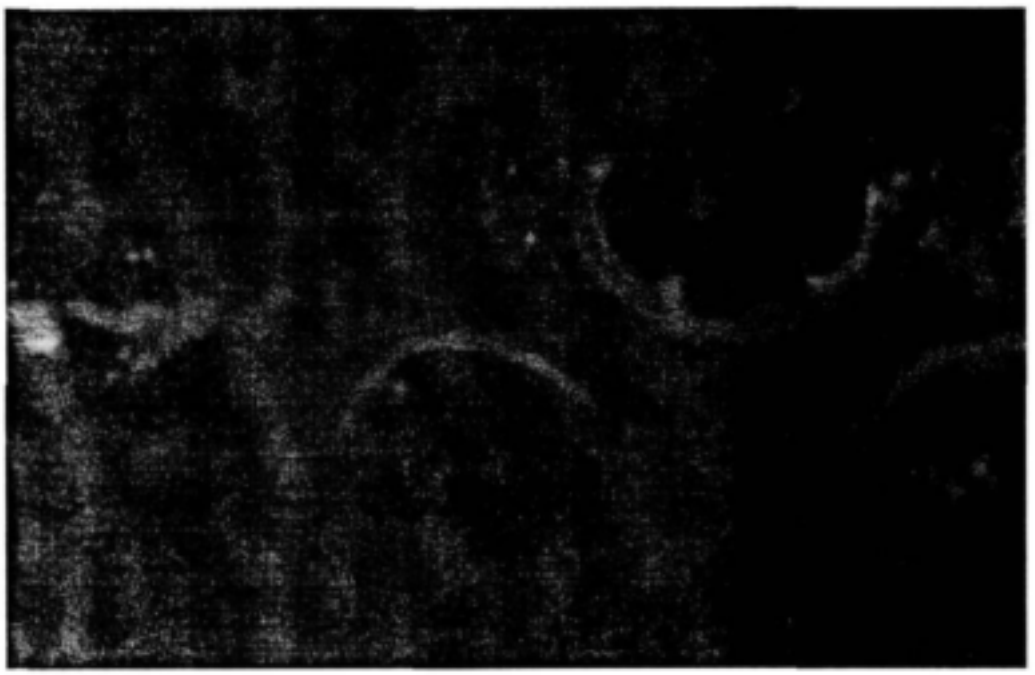


Figure 26. View of the voids in the polysulphone capillary membrane after inoculation with PAO1 and staining with acridine orange. The image was taken by scanning laser confocal microscope, focusing at various depths inside the voids.

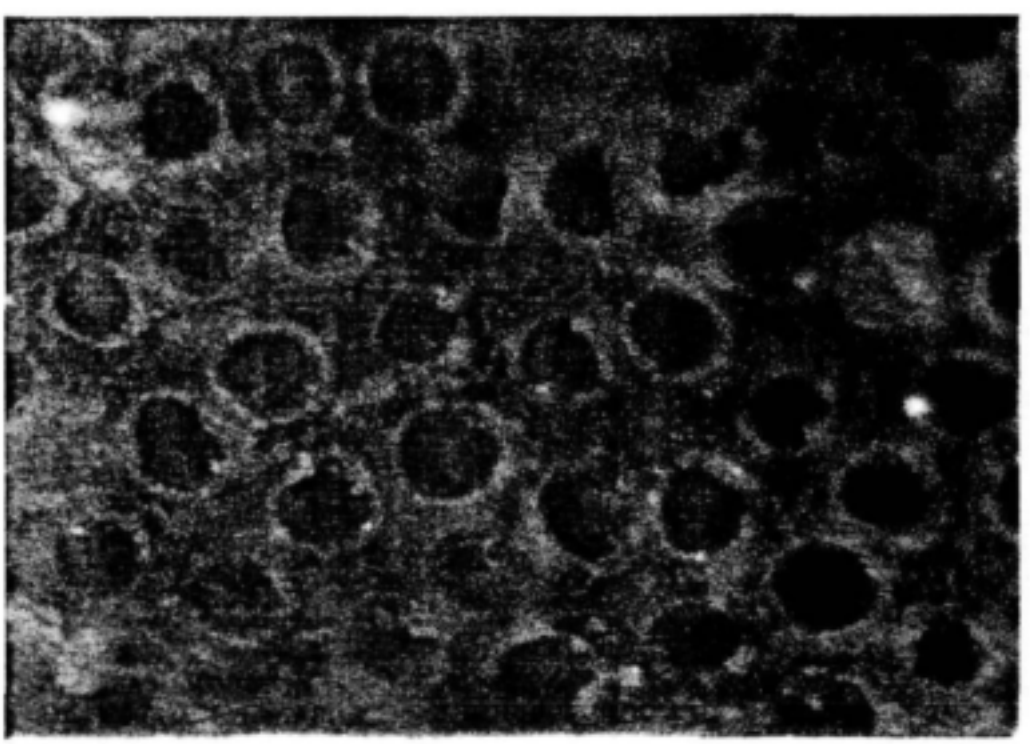


Figure 27. View of the surface of the polysulphone capillary membrane after inoculation with PAO1 and staining with acridine orange. The image was taken by scanning laser confocal microscope, focusing at various depths inside the voids.

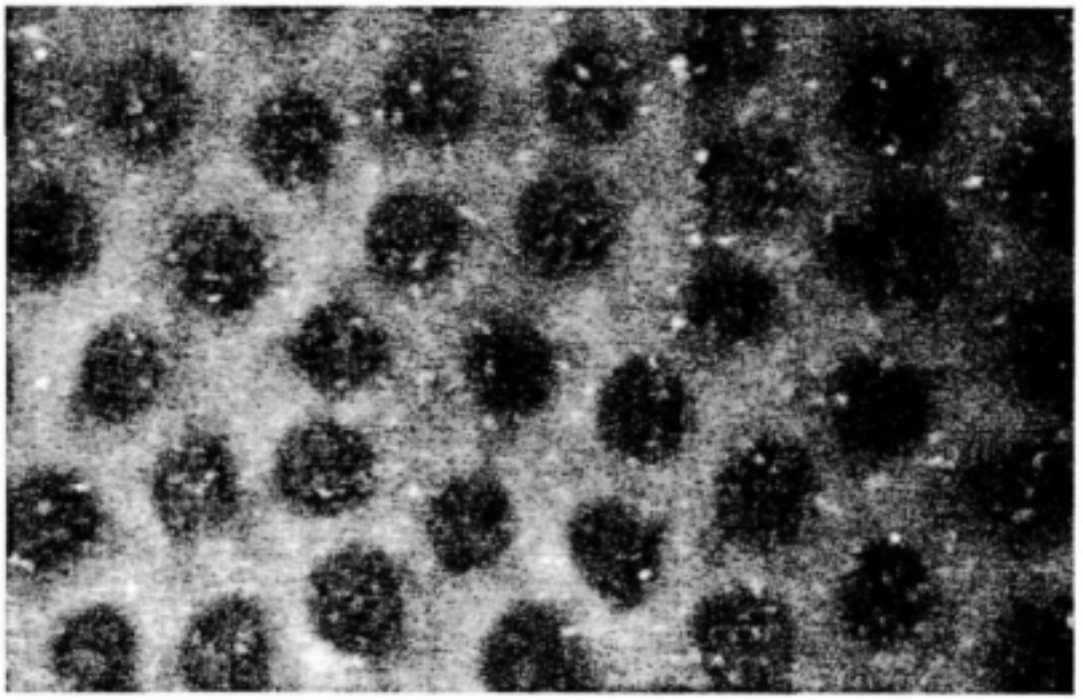


Figure 28. View of the surface of the polysulphone capillary membrane after inoculation with PAO1 and staining with acridine orange. The image was taken by scanning laser confocal microscope, focusing at various depths inside the voids.

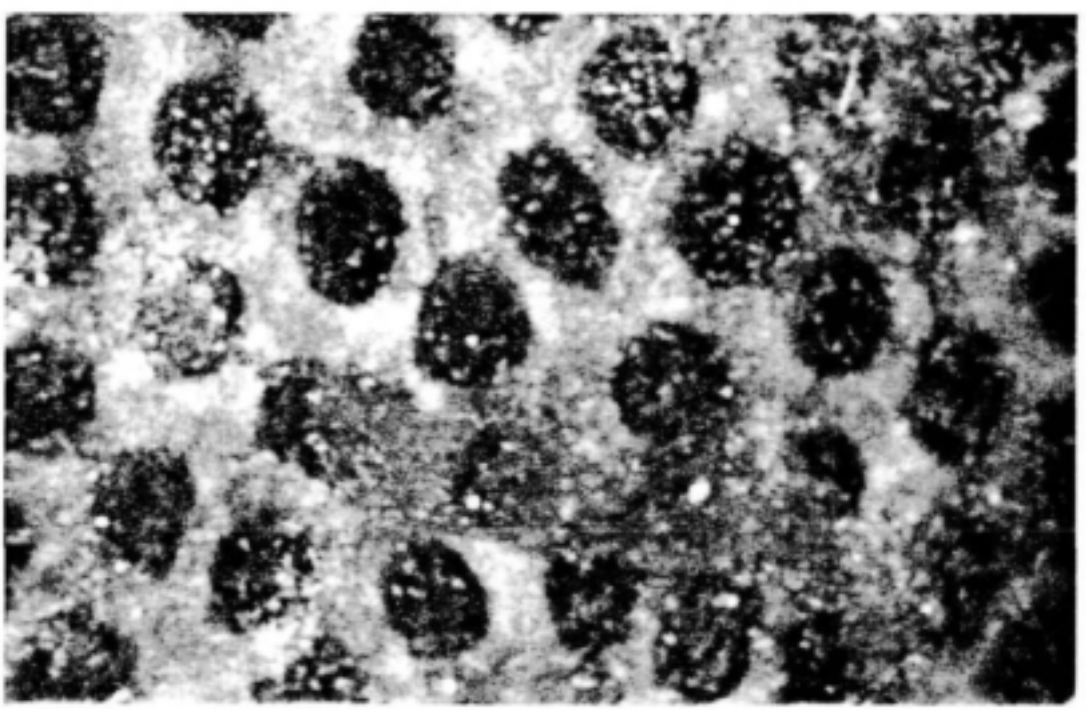


Figure 29. View of the surface of the polysulphone capillary membrane after inoculation with PAO1 and staining with acridine orange. The image was taken by scanning laser confocal microscope, focusing at various depths inside the voids.

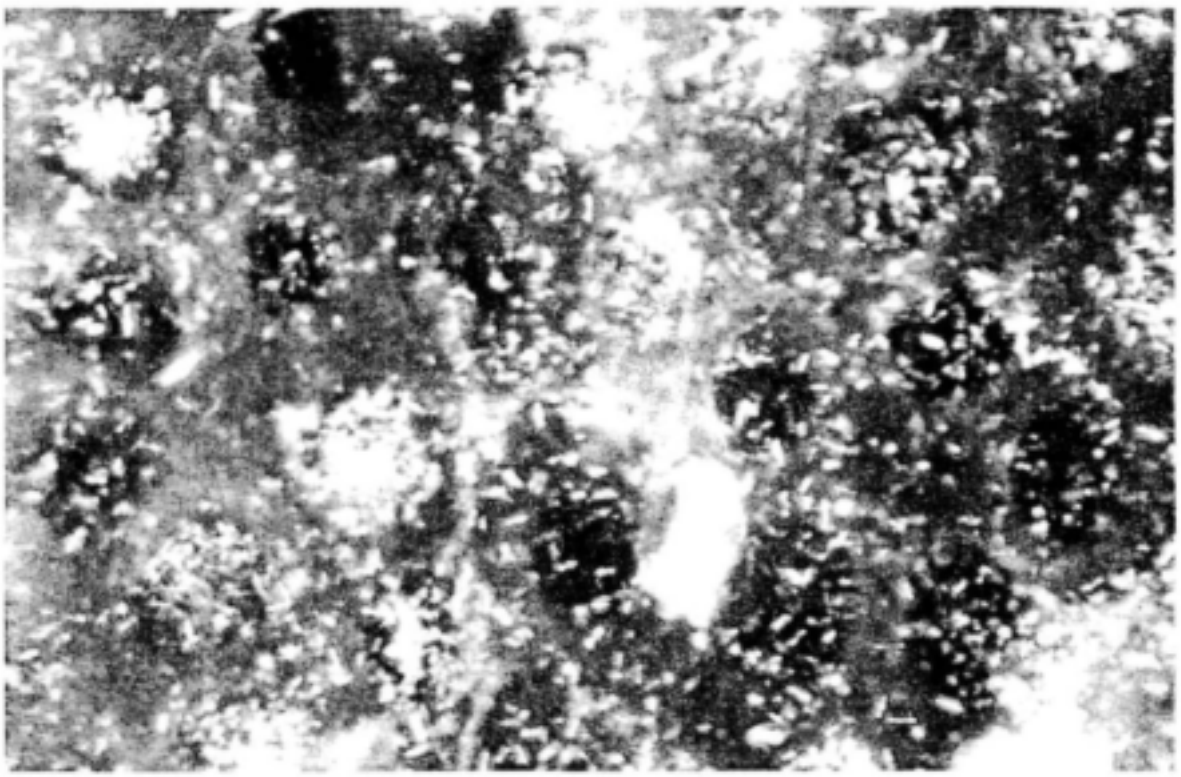


Figure 30. View of the surface of the polysulphone capillary membrane 5 d after inoculation with PAO1 and staining with acridine orange. The image was taken by scanning laser confocal microscope, focusing at various depths inside the voids.

4.5.5 Development of biofilms on the membrane

Following colonization at the surface, the attached cells multiplied to form patches of biofilm. These patches developed primarily on the intervoidal surfaces, but also extended sparsely across the void spaces (Fig. 22). This lead to development of micro-colonies on the intervoidal areas and growth into the void spaces (Fig. 23). The cells in biofilm on the inter-void spaces were quite densely populated, whereas those in biofilm covering the voidal spaces were more sparsely positioned (Fig. 24). The biofilm was never observed to block flow though the membrane.

It is important to note that, whereas prolific colonization was observed at the void wall surface, there was no colonization on surfaces of fractures which formed in the skin of the membrane (Fig. 31).

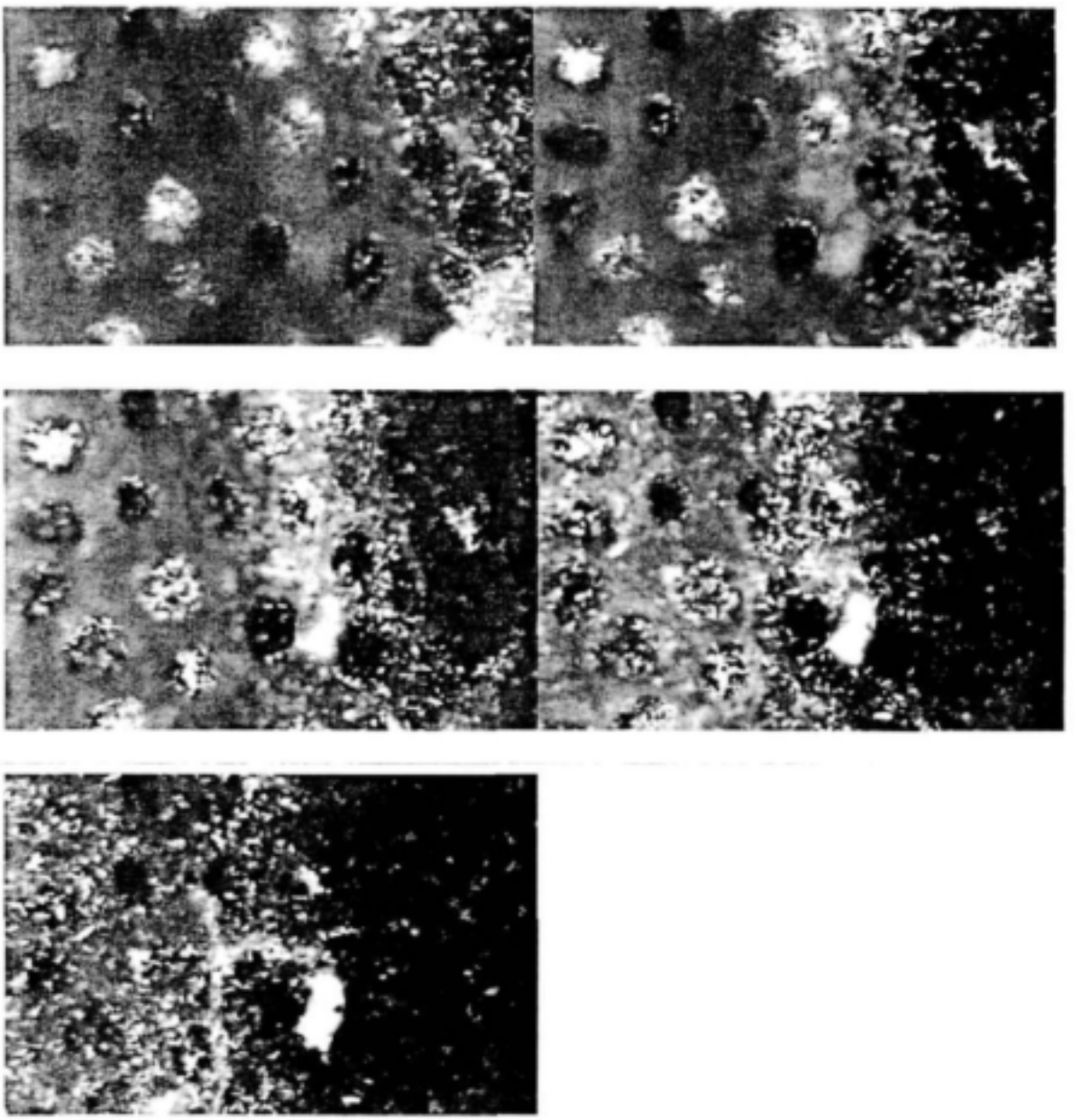


Figure 31. Sections from bottom up of the surface of the polysulphone capillary membrane 5d after inoculation with PAO1 and staining with acridine orange. The image was taken by scanning laser confocal microscope, focusing at various depths inside the voids.

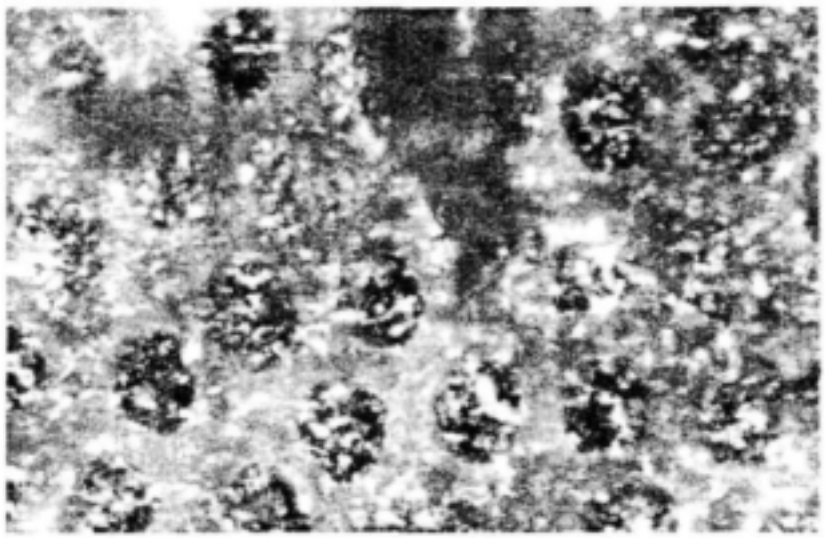


Figure 32. View of the surface of the polysulphone capillary membrane showing a fracture in the skin at center top.

4.6 *Culturing biofilms on glass wool*

Copious biofilms formed on the glass wool with several hours (Figs 32 - 33). The structure of the biofilms remained open, with few zones of dense population, allowing good exchange of components (Fig. 32).

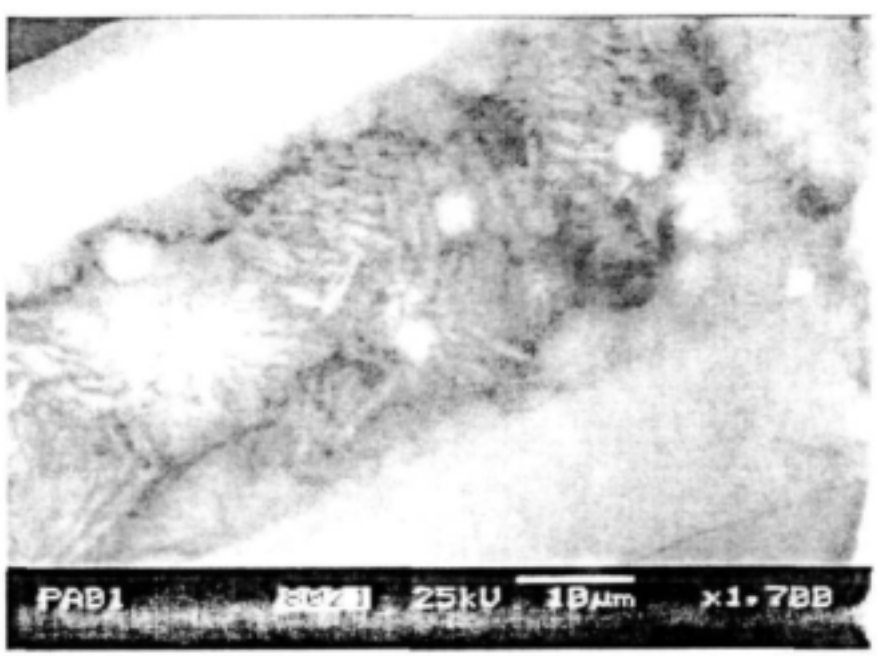


Figure 33. Low Vacume Scanning Electron Microscope image of a 26 h *P. aeruginosa* biofilm growing between two glass fibres.

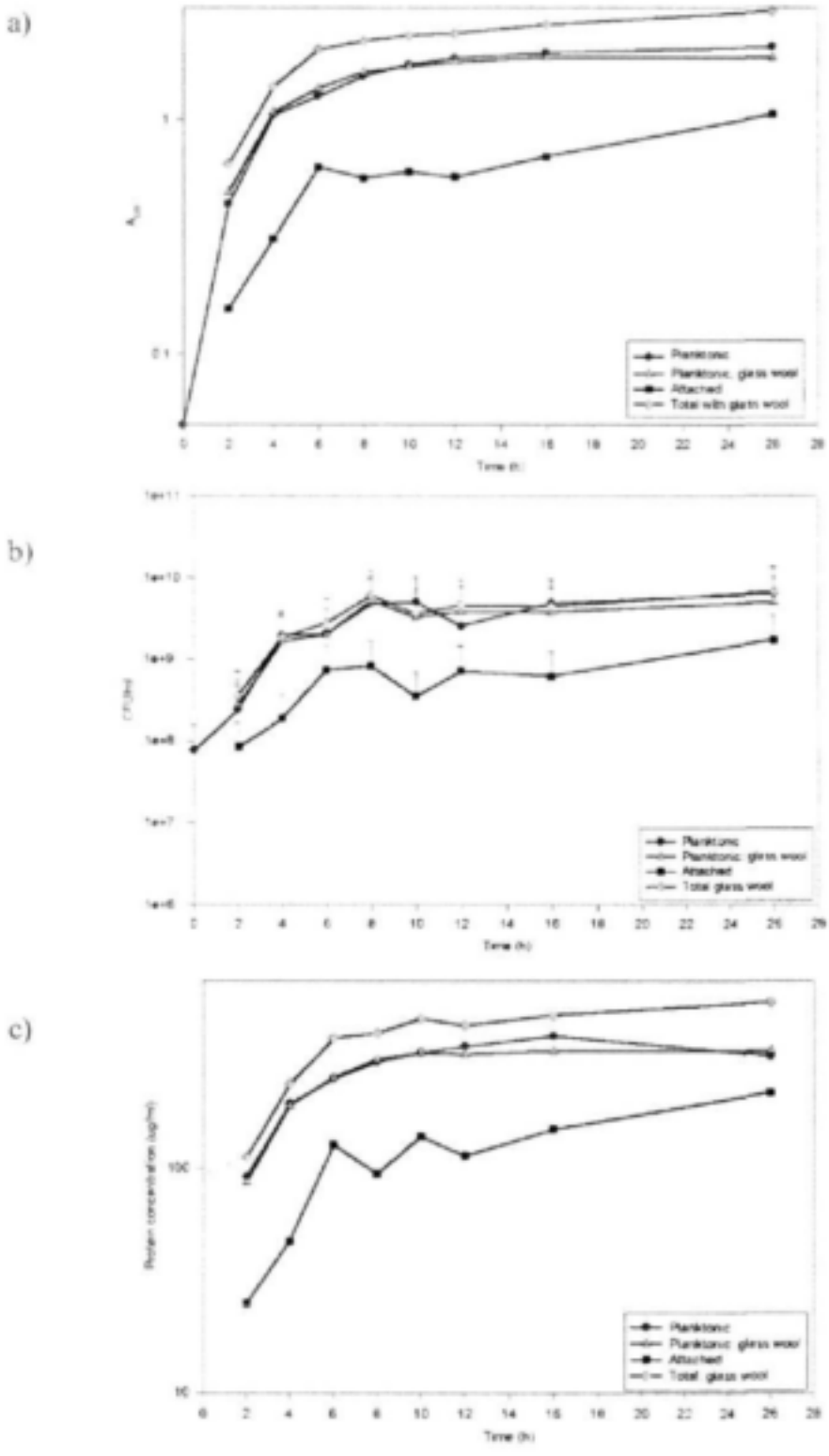


Figure 34. Growth of *Pseudomonas aeruginosa* on glass wool as measured by absorbance (540 nm)(a), culturable count (b) and total cellular protein (c).

The rhamnolipid yield was higher in the batch biofilm culture grown on glass wool than in planktonic batch culture (Fig. 34). This may be because the quorum sensing regulator *rhlI* is up-regulated in about 5% of biofilm cells (DeKievit et *al.*, 2001). We speculate that rhamnolipid plays a role in facilitating detachment of cells of *P. aeruginosa* from areas of the biofilm that are becoming too crowded, thereby contributing to the control of biofilm thickness.

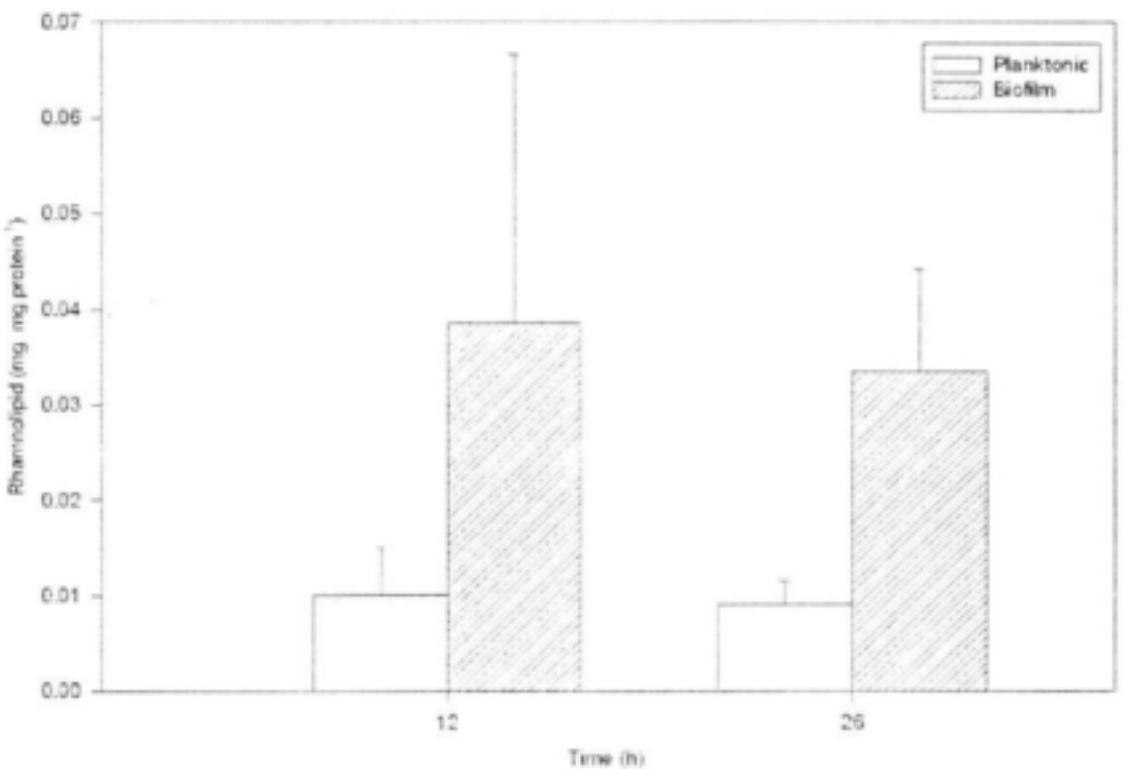


Figure 35. Rhamnolipid yield from a planktonic and biofilm culture of *Pseudomonas aeruginosa* growing on glass wool.

4.7 Production of rhamnolopid in batch culture

The rhamnolipid yield in batch culture was best at 37 °C and in complex medium. Culturing in the minimal medium plus glucose yielded poor results, but a fed-batch approach did improve the yield. Cultivation in a crude airlift reactor, using a simple air pump, yielded good results. Batch culturing in PPGAS medium at high aeration and 37 °C, and incubation for 5 d gives the highest yield.

5 Discussion, Conclusion and Recommendations

5.1 Discussion

The oil-emmulsifying *P. aeruginosa* isolates all produced rhamnolipid as confirmed by the anthrone reaction of acid-precipitated, chloroform-soluble substance. The assay for rhamnolipid was refined by introducing extended precipitation at pH < 2 at 4°C. Whereas rhamnolipid in high concentration precipitates rapidly, so that 30 min suffice under production conditions, this is insufficient for complete precipitation as required in quantitative studies. An important factor for the success of quantitative assays is complete removal of all reducing sugars by discarding the entire aqueous phase together with the meniscus following chloroform extraction as free sugars react strongly with the anthrone reagent, giving false positive results.

Rhamnolipid was produced from onset of the stationary phase, irrespective of the residual carbon source concentration. This indicates a relationship between expression of rhamnosyl transferase and regulation of gene expression in stationary phase, possibly requirement for RpoS, the stationary phase sigma factor. We have recently found that a RpoS deletion mutant produces less rhamnolipid that the wild type, but that some rhamnolipid is still produced (unpublished results). This may indicate either that expression is enhanced by RpoS levels, or that RpoS has a positive effect on the intracellular level of at least one precursor molecule.

The cloning of the *rhlAB* operon by PCR posed great difficulties. The operon proved difficult to amplify by PCR and single strand analysis revealed complex secondary structure formation, suggesting that the partially replicated single stranded amplicons could fold during the reaction. This is supported by the high GC content, and by the positive effect of DMSO on amplification.

The greatest challenge was cloning the amplicon. Initial experiments yielded confounding results as plasmid DNA from white colonies failed to yield digestion products when digested with restriction endonucleases specific to the multiple cloning site of both pCR script and pGEM T-easy. Digestion inside the native vector with ScaI revealed that vectors from white colonies were smaller than the original plasmid from blue colonies. This implied that the entire amplicon plus the entire multiple cloning site were removed by some form of recombination event, despite the deletion of the *recA* gene in the *E. coli* JM109 host.

The capillary membrane did support some surface growth, especially at low flow rates. It became apparent that the bacteria did not colonize the voids within the membrane, but grew to form small microcolonies on the membrane matrix and over the voids. An interesting finding was that no bacteria appeared to colonize areas where the membrane had been torn open. This finding has sparked interaction between Drs Wolfaardt (who assisted greatly with setting up the flow cells and the confocal scanning laser microscopy), and Dr Jacobs as it poses a possible strategy for biofouling control.

P. aeruginosa formed copious biofilms on glass wool. The biofilm was well structured with the typical frond or mushroom structure reported for many biofilms, and allowing for exchange of substances between cells in the biofilm and the surrounding liquid. Stationary phase was reached within 8 h, and as this is a prerequisite for rhamnolipid production, it was thought that glass wool would be a suitable biofilm support for continuous production. It was found that the rhamnolipid yield was improved in biofilm cultures on a per biomass basis. AlTahhan *et al.* (2000) reported that a proportion of the rhamnolipid adheres to the cell surface of *P. aeruginosa*. It may be that a further proportion of rhamnolipid is entrapped in the extra-cellular polymeric matrix surrounding biofilm-associated cells.

These results suggest that production in suspended batch culture be the most appropriate approach as the biomass yield is higher in suspended culture, and stationary phase is only truly achieved under batch conditions.

5.2 Conclusions

- A number of *P. aeruginosa* producing rhamnolipid were isolated and characterized. Whereas *P. aeruginosa* does grow to some extent on the capillary membranes, growth was not good.

- The amount of rhamnolipid produced by biofilm-growing cells was higher than that produced by cells growing in suspension.

- Attempts at constructing a genetically modified strain overproducing rhamnolipid have not succeeded to date, but work is in progress.

- Production of rhamnolipid by suspended batch culture was successful, and does not require intricate technology or expensive substrates.

5.3 Recommendations

- The production of rhamnolipid for bioremediation applications can be performed in suspended batch culture, possibly using an airlift reactor.

- The large-scale local production of rhamnolipid should be encouraged as it does not require intricate technology or expensive substrates.

- Production of rhamnolipid using immobilised / biofilm culture should only be considered once a stable, genetically modified overproducing strain is constructed.

- In order to construct such a modified strain, the factors contributing to the instability of the *rhlAB* operon in small plasmids should be investigated, and ways devised to circumvent the problem..

6 References

Al-Tahhan, R.A., Sandrin, T.R., Bodour, A.A. and Maier, R.A. 2000 Rhamnolipid-induced removal of lipopolysaccharide from *Pseudomonas aeruginosa*: effect on cell surface properties and interaction with hydrophobic substances. *Appl. Environ. Microbiol.* 66, 3262 - 3268.

Arima, K., Kakinuma, A. and Tamura, G. 1968. Surfactin, a crystalline peptide lipid surfactant produced by *Bacillus subtilis*: isolation, characterization and its inhibition of fibrin clot formation. *Biochem. Biophys. Res. Commun.* 31: 488 - 494.

Arino, S., Marchal, R. and Vandecasteele, J.P. 1996. Identification and production of a rhamnolipidic biosurfactant by a *Pseudomonas* species. Appl. Microbiol . Biotechnol. 45:162-168.

Arino, S., Marchal, R. and Vandecasteele, J.P. 1998. Involvement of a rhamnolipid-producing strain of *Pseudomonas aeruginosa* in the degradation of polycyclic aromatic hydrocarbons by a bacterial community. *J. Appl. Microbiol.* 84: 769 - 776.

Beal, R. and Betts, W.B. 2000 Role of rhamnolipid biosurfactants in the uptake and mineraiization of hexadecane in *Pseudomonas aeruginosa*. *J. Appl. Microbiol.* 89: 158 - 168.

Carrillo, P.G., Mardaraz, C., Pitta-Alvarez, S.I. and Giulietti, A.M. 1996. Isolation and selection of biosurfactant-producing bacteria. W J. *Microbiol. Biotechnol.* 12:82-84.

Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R. and Lappin-Scott, H.M. 1995. Microbiol biofilms. *Annu. Rev. Microbiol.* 45:711-745.

Davies, D.G., Chakrabarty, A.M. and Geesey, G.G. 1993. Exopolysaccharide production in biofilms:sustratum activation of alginate gene expression by *Pseudomonas aeruginosa*. *Appl. Enviro. Microbiol.* 59: 1181-1186.

Davies, D.G. and Geesey, G.G. 1995. Regulation of the alginate biosynthesis gene *algC* in *Pseudomonas aeruginosa* during biofilm development in continuous culture. *Appl.Environ. Microbiol.* 61: 860 – 867.

De Kievit, T. R., R. Gillis, S. Marx, C. Brown, and B. H. Iglewski. 2001. Quorum-sensing genes in *Pseudomonas aeruginosa* biofilms: Their role and expression patterns. *Appl. Environ. Microbiol.* **67:**1865-1873.

Desai, J.D. and Banat, I.M. 1997 Microbial production of surfactants and their commercial potential. *Microbiol. Mol. Biol. Rev.* 61: 47-68.

Fiechter, A. 1992. Biosurfactants: moving towards industrial application. *Trends Biotechnol.*

10:208-217.

Fletcher, M. 1991. The physiological activity of bacteria attached to solid surfaces. *Adv. Micro. Physiol.* 32:53-85.

Gerhardt, P., Muray, R.G.E., Wood, W.A. and Krieg, N.R. 1994. Methods for general and molecular bacteriology. ASM Press, Washington DC, USA.

Guerra-Santos, L., Käppeli, O. and Fiechter, A. 1984. *Pseudomonas aeruginosa* biosurfactant production in continuous culture with glucose as carbon source. *Appl. Environ. Microbiol.* 48: 301-305.

Guerra-Sontos, L.H., Käppeli, O. and Fiechter, A.1986. Dependence of *Pseudomonas aeruginosa* continuous culture biosurfactant production on nutritional and environmental factors. *Appl Microbiol. Biotechnol.* 24:443-448.

Haba, E., Espuny, M.J., Busquets, M. and Manresa, A. 2000 Screening and production of rhamnolipids by *Pseudomonas aeruginosa* 47T2 NCIB40044 from waste frying oils. *J. Appl. Microbiol.* 88: 379 - 387.

Iqbal, S., Khalid, Z.M. and Malik, K.A. 1995. En emulsification of crude oil and hyperproduction of biosurfactants by a gamma ray-induced mutant of *Pseudomonas aeruginosa*. *Letts. Appl. Microbiol.* 21:176-179.

Jarvis, F.G. and Johnson, M.J. 1949. A glycolipid produced by *Pseudomonas aeruginosa*. *J. Am. Chem. Soc.* 71: 4124 - 4126.

Koch, K.A., Käppeli, O., Fiechter, A. and Reiser, J. 1991. Hydrocarbon assimilation and biosurfactant production in *Pseudomonas aeruginosa* mutants. *J. Bacteriol.*173:4212-4219.

Kolter, R. 2000. Personal communication. Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts, USA.

Lin, S.C., Carswell, K.S., Sharma, M.M. and Georgiou, G. 1994. Continuous production of the lipopeptide biosurfactant of *Bacillus licheniformis* JF-2. *Appl. Microbiol. Biotechnol.* 41: 281 - 285.

Mercadé, M.E., Monleón, L., de Andrés, C., Rodón, I., Martinez, E., Espuny, M.J. and Manresa, A. 1996. Screening and selection of surfactant-producing bacteria from waste lubricating oil. *J. Appl. Bacteriol.* 81:161-166.

Marin, M., Pedregosa, A. And Laborda, F. 1996. Emulsifier production and microscopic study of emulsions and biofilms formed by the hydrocarbon-utilizing bacteria *Acinetobacter cacoaceticus* MM5. *Appl. Microbiol. Biotechnol.* 44: 660-667.

Mata-Sandoval, J.C., Karns, J. and Torrents, A. 1999. High performance liquid chromatography method for the characterization of rhamnolipid mixtures produced by *Pseudomonas aeruginosa* UG2 on corn oil. *J. Chrom. A* 864: 211 - 220.

Neu, T.R. 1996. Significance of bacterial surface-active compounds in interaction of bacteria with interfaces. *Microbiol. Rev.* 61 (1):151-166.

Ochsner, U.A., Fiechter, A. and Reiser, J. 1996. Isolation, characterization, and expression in *Escherichia coli* of the *Pseudomonas aeruginosa rhlAB* genes encoding a rhamnosyltransferase involved in rhamnolipid biosurfactant synthesis. *J. Biol. Chem.* 269:19787-19795.

Ochsner, U.A., Koch, A.K., Fiechter, A. and Reiser, J.1994. Isolation and characterization of a regulatory gene affecting rhamnolipid biosurfactant synthesis in *Pseudomonas aeruginosa*. *J Bacteriol.* 176: 2044-2054.

Ochsner, U.A., Reiser, J., Fiechter, A. and Witholt, B. 1995. Production of *Pseudomonas aeruginosa* rhamnolipid biosurfactants in heterologous hosts. *Appl. Environ. Microbiol.* 61: 3503-3506.

O'Toole, G.A. and Kolter, R 1998. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Mol. Microbiol.* 30: 295 – 304.

Pakula, R. And Freeman, A. 1996. A new and continuous biofilm reactor for immobilized oil-degrading filamentous fungi. *Biotechnol. Bioeng.* 49: 20-25.

Pesci, E.C., Pearson, J.P., Seed, P.C. and Iglewski, B.H. 1997. Regulation of *las* and *rhl* quorum-sensing systems in *Pseudomonas aeruginosa*. *J. Bacteriol.* 179: 3127 - 3132.

Steyn, B., MacDonald, R. Oosthuizen, M.C., Theron, J. and Brözel, V.S. 2000 Proteomic analysis of *Pseudomonas aeruginosa* biofilms: The use of glass wool as a substrate for attachment and growth. Paper in preparation.

Thangamani, S. and Shreve, G.S. 1994. Effect of anionic biosurfactant on hexadecane partitioning in multiphase systems. *Environ. Sci. Technol.* 28:1993-2000.

Weyers, J.B. 1998 Construction of a promoter gene library for the detection of attachment-specific promoters in *Pseudomonas aeruginosa*. MSc dissertation, University of the Western Cape.

Yakimov, M.M., Frederickson, H.L. and Timmis, K.N. 1996. Effect of heterogeneity of hydrophobic moieties on surface activity of lichnysinA, a lipopeptide biosurfactant from *Bacillus licheniformis* BAS50. *Biotechnol. Appl. Biochem.* 23: 13 - 18.

Zhang, Y. and Miller, R.M. 1992. Enhanced octadecane dispersion and biodegradation by a *Pseudomonas* rhamnolipd surfactant (biosurfactant). *Appl. Environ. Microbiol.* 58: 3276 - 3282

Zhang, Y. and Miller, R.M. 1995. Effect of rhamnolipid (biosurfactant) structure on solubilization and biodegradation of *n*-alkanes. *Appl. Environ. Microbiol.* 61: 2247-2251.

Other related WRC reports available:

Development of transverse-flow capillary-membrane modules of the modular and block types for liquid separation and bioreactors

SE Domröse, DA Finch and RD Sanderson

The transverse-flow membrane modules developed in this project entail an arrangement of capillary membranes in a cross-flow mode with regard to the flow. Transverse-flow membrane modules may be used for many commercial and industrial applications, because of the higher mass-transfer coefficient modular design, individual stacking freedom and possible uses as bioreactors.

A working transverse-flow, capillary membrane module of 24 m^2 membrane area has been developed which has potential for a variety of uses. In addition to the normal separation role, the module may be used to add gases (O_2, CO_2, H_2S) to or strip them from the liquid, thereby providing compelling reasons for its use as a bioreactor. Unfortunately the module is still somewhat sophisticated and might be expensive to manufacture at this stage.

However, the project has paved the way for the production of very large modules, with 200 capillary membranes per layer, 333 layers high (1 m high) which would have surface areas of 250 m^2 for externally-skinned membranes, which can be constructed using the same methods as those used for the present 24 m^2 model. This product is seen to have good potential for a wide range of water-related applications.

Report Number: 847/1/98 **ISBN: 1 86845 412 6**

TO ORDER: Contact **Rina** or **Judas** - Telephone No. 012 330 0340
Fax Number: 012 331 2565
E-mail: publications@wrc.org.za

Water Research Commission
Private Bag X03, Gezina, 0031, South Africa
Tel: +27 12 330 0340, Fax: +27 12 331 2565
Web: http://www.wrc.org.za