Isolation and characterization of n-alkane utilizing bacteria, which produce bioemulsifiers

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Abstract

Bacterial strains were isolated by enrichment cultures from oil-contaminated soil samples. In the present study, several strains, capable of growing on crude oil, were isolated. Isolates were screened for their inherent abilities to produce bioemulsifiers when they were grown on hydrocarbon substrates. Three strains, designated 2Bf, 2Bf*, and 2Bg were observed to produce a stable emulsion with emulsion indices (E1) of 69%, 70% and 66% respectively. The three strains were identified to belong to the genus *Pseudomonas* using standard biochemical tests; and one of the strains, 2Bf, was further characterized using 16S rDNA PCR and was observed to show a 99% identity with the previously identified strain *P. aeruginosa* PAO1. Hence the strain 2Bf was designated as *P. aeruginosa*. In addition, the strain 2Bf was shown to accumulate commercially important inclusion bodies known as polyhydroxyalkanoates (PHAs). This was shown both phenotypically by Nile Blue A staining and genetically by PCR amplification of the *phaC* gene.

The growth of the strain *P. aeruginosa* 2Bf on hydrophobic substrates was studied along with the biosurfactant production. The maximum cell growth and biosurfactant production, as measured by a specific colorimetric method (i.e. Orcinol assay), was observed to occur at about 40 hours of incubation in the shake flask cultures. Little effect was observed on the viable cell counts and biosurfactant production, when the carbon source applied was 1, 5, 10 and 15% (v/v) of the C_{14}-C_{17} n-alkane fraction. The growth of *P. aeruginosa* 2Bf in laboratory-scale bioreactor cultivation was observed to be different

to that in shake flasks. A marked reduction in log phase was witnessed when the strain 2Bf was grown in a batch fermentor. The culture from a fermentor was also observed to change its pigmentation and a milky color was observed at 65 hours of fermentation.

The bioemulsifiers produced by the strain *P. aeruginosa* 2Bf were extracted and partially characterized physicochemically. The analysis of a crude extract by thin layer chromatography (TLC) revealed the presence of carbohydrate, lipid and protein components. Therefore, the bioemulsifier by the strain 2Bf was temporarily identified as a peptidoglycolipid. The protein component of the bioemulsifier was shown to play a major role in stabilizing emulsions. The molecular weight of the protein moiety was estimated to be 14 400 Da by SDS-PAGE protein electrophoresis. The peptidoglycolipid bioemulsifier was observed to be most active at alkaline pH (pH 7-14), showing reduced activity at lower pH regimes. The crude extract of the bioemulsifier was also shown to be heat stable, retaining about 97% of its activity at 100°C after 1 hour.

Chapter 1

Literature review

Contents

1.1 Research in context

It is clear that all human activities depend upon the input and consumption of external energy. Industrial activities, modern agriculture and all other activities needed to feed most of the world's population, consume huge amount of energy, most of which is generated by the combustion of fossil fuels. Petroleum is one of the principal fossil fuels currently used for such purposes.

Since the late 1970s the increased demand for petroleum as a source of energy has resulted in an increase in world production from 29 to over 2400 million metric tons per year (Gutnick & Rosenberg, 1977). Such a dramatic increase in the production, refining, and distribution of crude oil has also brought about an ever increasing problem of environmental pollution and the generation of large amounts of the petrochemical by-products. The main problem is the massive transportation of petroleum from areas of high production to those of high consumption (Gutnick & Rosenberg, 1977). In 1991, it was estimated that two million tonnes of oil enter the sea each year, about 20% of which originates from oil pumping operations, transport and refining activities, and 25% results from non-tanker shipping and natural leakages (Timmis *et al.*, 1998). A petrochemical corporation in South Africa, SASOL, produces linear alkanes (C_5-C_{40}) in large volumes as a by-product of their petrochemical industry. These alkanes are cheap and of limited market value, thus it would be strategic to develop in South Africa processes utilizing these alkanes as raw materials for the conversion to value added chemicals. The production of value-added products from alkanes requires as a first step the introduction of a functional group, such as a hydroxyl group, into the inactivated hydrocarbon backbone. Microorganisms, which grow on these hydrocarbons, can achieve this much

more efficiently than synthetic chemical catalysts. This project thus centers on exploiting the availability of a low cost abundant substrate, and the isolation of bacteria, which can utilize alkanes as their carbon source, to develop processes for the bacterial conversion of linear alkanes in the range C_{12}-C_{17} to biosurfactants, or as secondary interest to polyhydroxyalkanoates (PHAs). The bacterial conversion of C_{12}-C_{17} to PHAs was considered in a preliminary investigation as it confers the additional advantage of producing two products from a single fermentation.

Recently, the degradation of petroleum hydrocarbons by microorganisms has received much attention due to their potential to control the impact of hydrocarbon contamination in sensitive marine and terrestrial habitats (Chang *et al.*, 2000; Van Hamme & Ward, 2001; Sonderkamp *et al.*, 2001, Engelhardt *et al.*, 2001; Francy *et al.*, 1991). Moreover, many of the oil degrading microorganisms are capable of enhancing oil emulsification by producing extracellular metabolites known as biosurfactants or bioemulsifiers. These biosurfactants are of great interest because of their physicochemical and biological properties, which can be used in food, pharmaceuticals and food industries.

Nowadays, the surfactant industry is estimated to exceed US $ 9 billion per year (Maier & Sober• n-Chávez, 2000). However, surfactants currently marketed are almost exclusively synthetic. This is because the cost of production is higher for biosurfactants than synthetic ones. For example, Lang and Wullbrandt (1999) estimated that rhamnolipid biosurfactant cost US $ 5-20/kg when produced in fermentation quantities of 20 to 100 m^3. In contrast ethoxylate or alkyl polyglycoside synthetic surfactants cost US $ 1-3/kg. Although biosurfactants are more costly, they have gained considerable attention as alternatives to the commercially available chemical surfactants due to their

low toxicity, biodegradability and their structural diversity which can be used in a wide range of applications such as agents for emulsification, lubrication, wetting, foaming, dispersion etc. Research aimed at selecting efficient and novel bioemulsifier producers, decreasing the cost of production by the use of cheaper substrates and optimizing production strategy, will no doubt see the application of bioemulsifiers become more wide spread and attractive.

1.2. Microbial degradation of hydrocarbons

Earlier in the 1960s, petroleum was considered as a cheap carbon source and it was utilized as a carbon source for microbial growth (single cell protein), which was deemed as a possible solution to the alleged world food shortage for the predicted global population growth (Atlas & Bartha, 1992). Applied studies concentrated on fermentor designs so as to optimize microbial growth on low to middle molecular weight hydrocarbons (Atlas & Bartha, 1992). Such fermentor designs were aimed at large-scale single cell production with agitation and aeration systems that allowed high rates of microbial growth on soluble and highly emulsified hydrocarbon substrates. The focus on hydrocarbon utilization resulted in basic research elucidated the metabolic pathways involved in the degradation of alkanes, cycloalkanes, and aromatic hydrocarbons (Bernheimer & Avigad, 1970; Fukui & Tanaka, 1971; Atlas & Bartha, 1992). However, by the late 1960s, it became evident that there would be foreseeable shortages and an eventual energy crisis, because of the limited and non-renewable petroleum resources (Atlas & Bartha, 1992). Hence petroleum could no longer serve as the cheap substrate for microbial biomass production to feed the world.

Hydrocarbons are organic compounds consisting of only carbon and hydrogen and are sparingly soluble in water. Some hydrocarbons are aliphatic, in which the hydrocarbon atoms are joined in an open chain, while others contain aromatic groups and are regarded as derivatives of benzene. Certain microorganisms (for example *Nocardia*, *Pseudomonas*, *Mycobacterium*, *Bacillus*, *Acinetobacter*, *Corynebacterium*, *Rhodococcus* and certain yeasts and molds) can use hydrocarbons for growth (Noordman & Janssen, 2002; Navon-Venezia *et al.*, 1995; Toren *et al.*, 2002; Bernheimer & Avigad, 1970; Barkay *et al.*, 1999).

Microbial degradation of oil pollutants has been shown to occur by attack on the saturated and low-molecular weight aromatic fraction of the oil (Atlas & Bartha, 1992). Very high molecular weight aromatics, resins, and asphaltenes are considered to be intractable or show only very low rates of biodegradation, though some studies have reported the biodegradation of some of these hydrocarbons (Leahy & Colwell, 1990). Fought *et al.* (1990) reported that microorganisms that degrade aromatic hydrocarbons are distinct from those that attack aliphatic hydrocarbons. Thus, completely different microorganisms may carry out the degradation of different groups of hydrocarbons.

Mostly, the degradation of saturated aliphatic hydrocarbons is an aerobic process (Fukui & Tanaka, 1971), while the unsaturated ones containing a terminal double bond are not refractory to anoxic degradation and can be oxidized by certain sulphate-reducing bacteria and other anaerobic bacteria. Biodegradation of alkanes normally proceeds with the monoterminal attack carried out by a monooxygenase to form a primary alcohol followed by conversion to an aldehyde and a mono carboxylic acid (Atlas & Bartha, 1992). Further degradation of a mono carboxylic acid proceeds by

β-oxidation with the subsequent formation of shorter fatty acids and acetyl coenzyme A (Fig. 1-1).

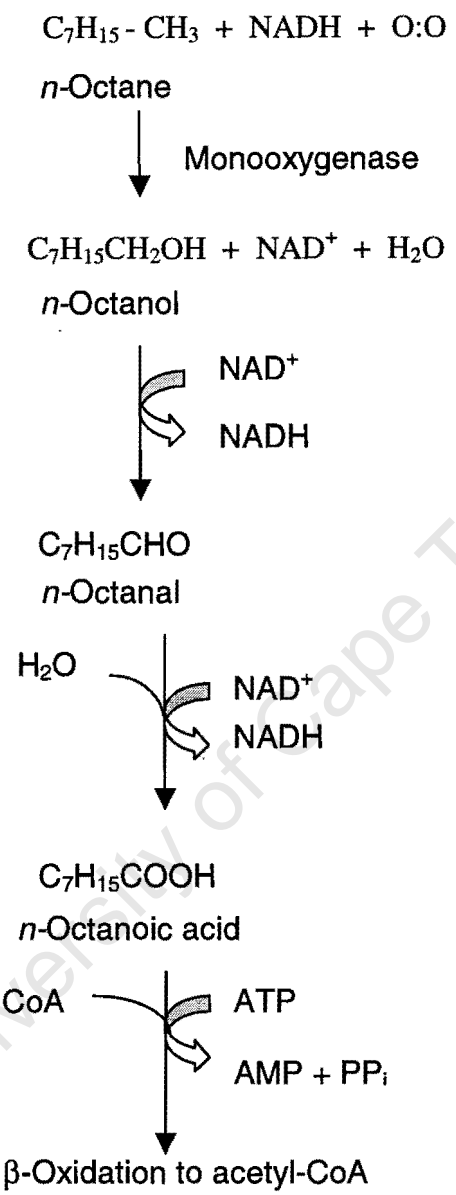


Fig. 1-1. The metabolic pathway of hydrocarbon degradation by oil degrading microorganisms.

The degradation of aromatic hydrocarbons, some of which are large molecules, invariably involve the formation of either protocatechuate or catechol, or other structurally related compound, as an initial stage (Atlas & Bartha, 1992). These single-ring compounds are regarded as starting substrates because oxidative catabolism

proceeds only after large molecules have been cleaved to these more simple forms (Atlas & Bartha, 1992).

The low water insolubility of hydrocarbon substrates, which increases their adsorption to surfaces and limits their bioavailability, often lowers the biodegradation process by microorganisms.

1.3. Problems associated and mechanisms evolved by microorganisms with growth on hydrophobic substrates

The bioavailability of sparingly soluble hydrophobic compounds for microbial degradation is often very low and invariably limits the rate of degradation in aqueous and soil systems (Angelova & Schmauder, 1999; Volkering *et al.*, 1992). Communities exposed to hydrocarbons become adapted, exhibiting selective enrichment and genetic changes resulting in increased proportions of hydrocarbon degrading bacterial plasmids encoding hydrocarbon catabolic genes (Leahy & Colwell, 1990). Certain microorganisms produce biosurfactants to increase the bioavailability and consequently the biodegradation of hydrophobic compounds. This can be achieved in different ways and depends on the organisms and type of biosurfactant produced. These biosurfactants can either be cell-associated, which can cause an interaction between bacteria and oil droplets, or extracellular, which leads to emulsification of hydrocarbons in water (Noordman & Janssen, 2002). Surfactants converge or cluster at interfaces because of their amphipathic nature, i.e. they contain both hydrophobic and polar groups (Ron & Rosenberg, 2001). Surfactants are substances that adsorb to and change the conditions that are present at interfaces, and emulsifiers are a subclass of surfactants that stabilize dispersions of one liquid in another, such as the oil-in-water emulsions (Ron &

Rosenberg, 2001). Surfactants are surface active agents with wide ranging properties including the lowering of surface and interfacial tensions of liquids. Surface tension is defined as the free surface enthalpy per unit area (OECD, 1995) and is the force acting on the surface of a liquid leading to minimization of the area of that surface. Both synthetic and natural surfactants are capable of reducing the surface tension of water from 72 mN/m to around 27 mN/m. Because surfactants have both hydrophilic (polar) and hydrophobic/lipophilic (nonpolar) portions in the molecule, they function by residing at the oil-water interface. The hydrophilic (polar) part of a surfactant is usually referred to as the 'head', whereas the nonpolar hydrophobic portion is known as the 'tail'. These amphipathic molecules enable the formation of specialized structures vital to their action, and the presence of surfactants can lead to an increase in the concentration of hydrophobic compounds in the water phase. This is achieved through the formation of oil/water emulsions and solubilization, where biosurfactant molecules aggregate to form micelles above the Critical Micelle Concentration (CMC). CMC is that concentration of surfactant favouring micelle formation. Between 50 and 100 surfactant molecules aggregate to form micelles. Micelles arise when the lipophilic part of the surfactant molecule that is unable to form hydrogen bonding in an aqueous phase causes an increase in the free energy of the system. One way to alleviate this free energy increase is for the hydrocarbon tail to be isolated from water by adsorption onto surfaces, absorption into an organic matrix or by the formation of micelles vesicles where the hydrocarbon moiety of the surfactant becomes situated towards the center with the hydrophilic part in contact with water (Haigh, 1996). An emulsion is a dispersed system containing at least two immiscible liquid phases. In an emulsion, one immiscible phase forms droplets that are internally dispersed in the second immiscible phase, which constitutes the continuous phase of emulsion. It is generally believed that due to excess

free energy associated with the surface of the droplets, emulsions are inherently thermodynamically unstable (Lubetkin, 1999). To minimize the excess free energy, the dispersed droplets, therefore, intrinsically strive to come together and reduce their surface area, ultimately fusing to form one large drop and destroying the emulsion. In order to reduce the inherent tendency to fuse, an emulsifying agent is added to the immiscible phases to improve emulsion stability. It is generally believed that an emulsifying agent forms a film around each dispersed droplet in an emulsion. It is also believed that an emulsifying agent is adsorbed at least as a monolayer at an interface between two phases. Thus, the emulsifying agent serves to maintain the surface area of the dispersed phase and eliminates the inherent tendency of the dispersed droplets to coalesce, and thereby increase the emulsion stability (Lubetkin, 1999).

Microorganisms synthesise a wide range of bioemulsifier compounds, which have been divided broadly into two classes: low and high–molecular mass bioemulsifiers. Biosurfactants consist of a hydrophobic moiety containing saturated, unsaturated and hydroxylated fatty acids or fatty alcohols; and a hydrophilic moiety consisting of mono, oligo or polysaccharides, peptides or proteins (Lang, 2002). Even though bioemulsifiers are invariably produced to emulsify hydrocarbon substrates, there are some exceptions to these observations. *Bacillus subtilis*, for example, was reported to produce a high yield of very active surfactant when the growth medium contained a carbohydrate as the carbon source (Cooper *et al.*, 1981). *Corynebacterium fuscians* CF15 was able to stabilize emulsions of water and hydrocarbon when grown on a medium, which did not contain hydrocarbon (Cooper *et al.*, 1982). The ability of these organisms to synthesize a biosurfactant or an emulsifier with renewable substrates such as carbohydrates helps to minimize the cost of using hydrocarbons as growth substrates, which prohibits the large-

scale production of biosurface-active agents. Biosurfactants are of great interest because of their physicochemical and biological properties in bioremediation, pharmaceutical, food, cosmetics and other industries (Banat *et al.*, 2000).

1.4. Natural Roles of Biosurfactants

Surfactants produced by many different organisms, have different chemical and physical properties. Hence, they have different natural roles in the growth of the producing microorganism. Likewise, each emulsifier probably provides advantages in a particular ecological niche (Ron & Rosenberg, 2001).

The growth rate of microorganisms growing on sparingly soluble hydrocarbons can be limited by the interfacial surface area between water and oil (Shreve *et al.*, 1995). Hence, surface active agents produced by certain organisms increase the bioavailability of the insoluble hydrocarbons by lowering the interfacial tension or by the production of oil-water emulsions. One of the main reasons for the prolonged persistence of hydrocarbon substrates is their low water solubility, which increases their adsorption to surfaces and limits their availability to microorganisms (Cerniglia, 1993; Miller & Zhang, 1997). Surfactants that lower interfacial tension are very effective in desorbing the high-molecular-weight hydrophobic compounds and making them available for biodegradation. Arino *et al.* (1998) reported the involvement of a rhamnolipid biosurfactant in the degradation of polyaromatic hydrocarbons (PAHs) by *Pseudomonas aeruginosa*. The apparent aqueous solubilities of phenanthrene, flourathene, and pyrene were observed to increase 6.6, 25.7, and 19.9 fold respectively in the presence of 500 µg alasan per ml (Barkay *et al.* 1999).

Certain biosurfactants bind and reduce the toxicity of heavy metals. Herman *et al.* (1997) have shown the capability of a rhamnolipid biosurfactant in removing cadmium, lead and zinc from soil. A combination of rhamnolipid complexing of cadmium and the rhamnolipid interaction with the cell surface to alter cadmium uptake may signify the mechanism by which rhamnolipid reduces the toxic effects of heavy metal (Ron & Rosenberg, 2001). The high molecular weight polysaccharide bioemulsifiers interact with metals by binding to them, as has been shown for the binding of uranium by emulsan of *Acinetobacter calcoaceticus* (Zosim *et al.*, 1983).

Several lipopeptide surfactants are potent antibiotics (Marahiel *et al.*, 1997; Yakimov *et al.*, 1995). These include cyclic lipopeptide of *B. subtilis*, (Peypoux *et al.*, 1999), the extracellular hydrophobic peptide, streptofactin produced by *Streptomyces tendae* (Richter *et al.*, 1998) and the polymyxins produced by *B. polymyxa* and related bacilli (Suzuki *et al.*, 1969). These peptide antibiotics were reported to play a role in sporulation (Grossman, 1988).

Other natural roles of surfactants that have been described are: pathogenesis, regulating the attachment-detachment of microorganisms to and from surfaces, emulsifier production and quorum sensing, and the role of bioemulsifiers in the formation of biofilms (Ron & Rosenberg, 2001).

1.5. Chemical and surface properties of biosurfactants

The molecular structure of biosurfactants includes a hydrophobic and a hydrophilic moiety. The hydrophobic moiety contains saturated, unsaturated, and/ or hydroxylated

fatty acids or fatty alcohols; and the hydrophilic moiety consists of mono, oligo and polysaccharides, peptides or proteins (Lang, 2002). The high molecular weight class of biosurfactants is generally less efficient in lowering surface and interfacial tension, but are effective hydrocarbon emulsifiers (Rosenberg & Ron, 1999).

1.5.1. The low-molecular-weight biosurfactants

The low molecular mass bioemulsifiers are generally glycolipids or lipopeptides (Rosenberg & Ron, 1999).

1.5.1.1. Glycolipids

The best studied glycolipid bioemulsifiers, rhamnolipids, trehalolipids and sophorolipids, are disaccharides that are acylated with long-chain fatty acids or hydroxy fatty acids (Fig. 1-2). Essentially, all the trehalolipids of *Rhodococcus erythropolis* are cell-bound and their function is to enhance attachment of cells to the substrate (Noordman & Janssen, 2002). The surfactant properties of the fractionated cell-bound lipids of *R. erythropolis* were measured by Kretschmer *et al.* (1982). The minimal interfacial tensions obtained with corynomycolic acids, trehalose monocorynomycolates, and trehalose dicorynomycolates were 6, 16 and 17 mN/m respectively. However, the critical micelle concentration (CMC) for the trehalose lipids (approx. 2 mg/l) was more than ten times lower than for the free corynomyolic acids.

Different species of the yeast *Torulopsis* produce extracellular sophorolipids. The sophorolipids lower surface and interfacial tensions, although they are not effective emulsifying agents (Cooper and Paddock, 1984). Davila *et al.* (1997) observed that the

pure lactonic sophorose lipid (10 mg/l) decreased the interfacial tension between *n*-hexadecane and water from 40 mN/m to about 5 mN/m, relatively independently of the pH (6-9), salt concentration and temperature (20-90°C).

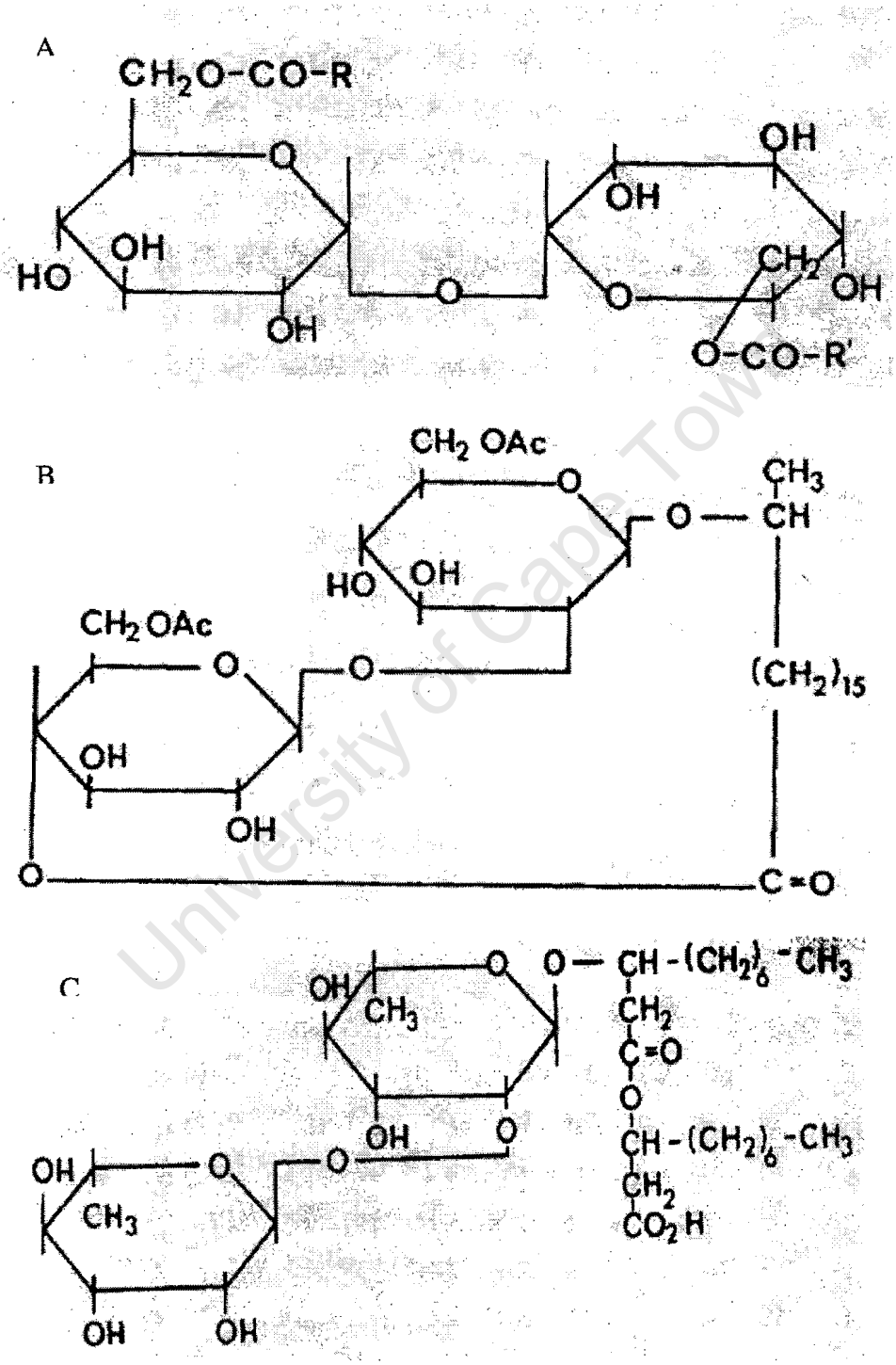


Fig. 1-2. Glycolipid biosurfactants (Rosenberg & Ron, 1999). A, Trehalose-6,6'-dicarboxylic ester; B, sophorose lipid; C, rhamnolipid.

Certain species of *Pseudomonas* are known to produce rhamnolipids consisting of rhamnose and β-hydroxydecanoic acid. The pure rhamnolipid lowered the interfacial tension against n-hexadecane to about 1 mN/m and had a CMC of 10-30 mg/l depending on pH and salt conditions (Lang & Wullbrandt, 1999).

1.5.1.2. Lipopeptides

B. subtilis produces a cyclic lipopeptide antibiotic called surfactin or subtilisin (Bernheimer & Avigad, 1970). The structure of surfactin is shown in Fig 1-3. Surfactin lowers the surface tension to 27mN/m in water, and has a CMC of 25 mg/l (Rosenberg & Ron, 1999).

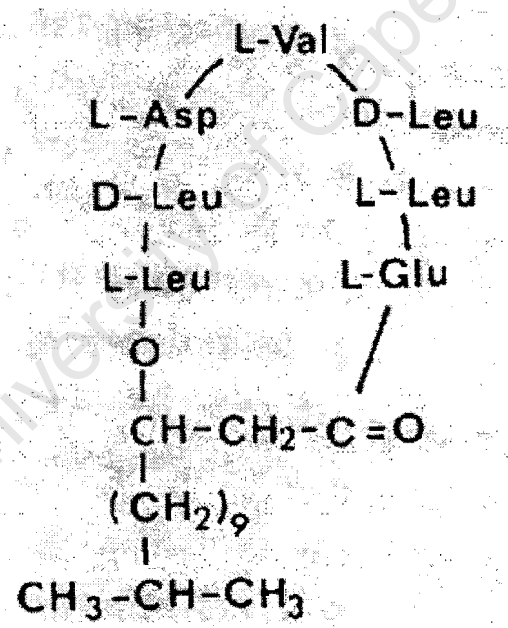


Fig. 1-3. A lipopeptide biosurfactant, surfactin (Rosenberg & Ron, 1999).

Bacillus polymyxa and related bacilli, produce a lipopeptide antibiotic called polymyxin (Rosenberg & Ron, 1999). Certain *Pseudomonas* strains produce viscosin, a peptidolipid biosurfactant that lowers surface tension down to 27 mN/m (Neu & Poralla, 1990).

Surfactant properties of cellular phospholipids of *R. erythropolis* grown on *n*-alkanes were studied by Kretschmer *et al.* (1982). The phosphatidylethanolamine fraction was the most potent, lowering the interfacial tension between water and hexadecane down to 1mN/m with a CMC of 30mg/l.

1.5.2. Bioemulsans: Polysaccharide-protein surfactants

Many bacterial species produce amphipathic polysaccharides, proteins, lipopolysaccharides, lipoproteins or complex mixtures of these biopolymers that stabilize the oil-in-water emulsions (Rosenberg & Ron, 1999). RAG-1, which is produced by *Acinetobacter,* is an emulsan complex of an anionic hetropolysaccharide and protein (Rosenberg *et al.*, 1979). Its surface property is largely due to the presence of fatty acids; comprising 15% of the emulsan dry weight, which are attached to the polysaccharide backbone via O-ester and N-acyl linkages (Belsky *et al.*, 1979). Under these conditions, approximately 80% of the emulsan produced is released in the stationary phase (Goldman *et al.*, 1982). RAG-1 is an effective emulsifier at low concentrations (0.01%-0.001%), representing a ratio of 1:100-1:1000 emulsan to hydrocarbon ratio, and exhibits considerable substrate specificity (Rosenberg *et al.*, 1979). RAG-1 emulsan does not emulsify pure aliphatic, aromatic, or cyclic hydrocarbons; however, all mixtures having the appropriate mixture of aliphatic and aromatic (or cyclic alkane) are efficiently emulsified (Rosenberg & Ron, 1999).

Acinetobacter radioresistens produces the bioemulsifier alasan, which is a complex of an anionic polysaccharide and protein with a molecular weight of approximately 1000 KDa (Navon-Venezia *et al.*, 1995). The protein component of alasan appears to

play an important role in both the structure and activity of the complex (Navon-Venezia *et al.*, 1998). Recently, one of the alasan proteins, with an apparent molecular mass of 45 kDa, was purified and shown to constitute most of the emulsifying activity (Toren *et al.*, 2002b). This protein is homologous to that of *Escherichia coli* OmpA and is highly effective in stabilizing oil-in-water emulsions and in solublizing hydrocarbons, including polycyclic aromatic hydrocarbons (Toren *et al.*, 2002a). Alasan lowers interfacial tension from 69 mN/m to 41 mN/M at 20°C and has a CMC of 200 µg/ml (Barkay *et al.*, 1999).

Table 1-1. Table showing the different biosurfactants isolated from different microorganisms along with their main properties

Surfactant	Producing microorganisms	Main property	Details of structure	Location	References
Low mol. weight					
Rhamnolipid	*P. aeruginosa*	Good bioemulsfier and effcient in reducing surface and interfacial tensions	A glycolipid consisting of rhamnose and hydroxy-decanoic acid.	Extracellular	Lang & Wullbrandt, 1999
Sophorolipid	*Torulopsis. bombicola*	Lower surface and interfacial tensions, although they are not effective bioemulsifiers	Consists of sophrose unit linked glycosidically to a hydroxyl fatty acid residue	Extracellular	Cooper & Paddock, 1983
Trehalolipid	*R. erythropolis*	lower interfacial and surface tensions, and make good emulsions when cells are grown in hydrocarbon substrates	consists of trehalose linked to dicarboxylic ester	Cell -bound	Kretschmer *et al*., 1982
Surfactin	*B. subtilis*	A lipopeptide antibiotic with potent suface active properties capable of reducing surface and interfacial tensions	cyclic lipopeptide	Extracellular	Bernheimer & Avigad, 1970
Polymyxin	*B. polymyxa*	A lipopeptide antibiotic with surface-active properties of a cationic detergent	Consists of a cyclic decapeptide with a branched chain fatty acid connected to the terminal 2,4-diaminobutyric acid (Dab).		Suzuki *et al*., 1965
Viscosin	*P. aeruginosa*	Lowers the surface tension to 27 mN/m	Consists of peptide and lipid components		Neu & Poralla, 1990
Corynomycolic Acids	*Nocardia erythropolis* *Candida lepus*	Efficient in lowering surface and interfacial tensions	Consists of complex fatty acids containing hydroxyl groups and alkyl branches		MacDonald *et al*., 1981 Cooper *et al*., 1981
Phospholipids	*Acinetobacter spp.* *Thiobacillus thiooxidans*	Good emulsifying agent and able to generate optically clear micro-emulsions of alkanes in water	Are phospholipid-rich, mainly phosphatidylethanolamine		Kaeppeli & Finnerty, 1980 Beebe & Umbreit, 1971

High mol. Weight					
RAG-1 emulsan	*Acinetobacter calcoaceticus*	An effective emulsifier at low concentrations (0.01%-0.001%)	RAG-1 is composed of an anionic heteropolysaccharide and protein	Extracellular	Belsky *et al.*, 1979
Alasan	*Acintobacter radioresistens*	Good emulsifying agent, and lowers interfacial tension from 69 mN/m to 41 mN/m	Alasan consists of an unusual polysaccharide component in that it contains covalently bound alanine	Extracellular	Navon-Venezia *et al.*, 1995
Peptidoglycolipid	*P. aeruginosa*	Good emulsifying agents, but not efficient in lowering both surface and interfacial tension	consists of protein, carbohydrate and lipid components	Extracellular	Ilori & Amund, 2001
Biodispersant	*A. calcoaceticus*	Effectively disperses limestone and titanium dioxide	Composed of anionic poly-saccharides	Extracellular	Rosenberg *et al.*, 1988
Mannoprotein	*Saccharomyces cerevisiae*	Efficient bioemulsifying agent		Extracellular	Cameron, 1988
Liposan	*C. lipolytica*	Efficient bioemulsifier	Composed of 83% carbohydrate and 17% protein	Extracellular	Cirigliano & Carman, 1984
Biosurf. TypeI	*Nocardia sp.*	Strong properties as emulsifying agent and as an emulsion-stabilizing agent		Extracellular	Kim *et al.*, 2000
Biosurf TypeII	*Nocardia sp*	Strong ability to reduce surface tension		Extracellular	Kim *et al.*, 2000

1.6. Commercial applications of microbial surfactants

Currently, biosurfactants are mainly used in studies on enhanced oil recovery and hydrocarbon bioremediation. Biosurfactants also have potential applications in agriculture, cosmetics, pharmaceuticals, detergents, personal care products, food processing, paper processing and paint industries (Banat *et al.*, 2000).

1.6.1. Biosurfactants and bioremediation

The problem of oil spills is not only destroying the habitats of aquatic animals and fish, but also creates health problems for local residents and causes long term devastation and deterioration of the environment (Harvey *et al.*, 1990). Bartha (1986) reported that approximately 0.08-0.4% of the total worldwide petroleum production reaches the oceans. One of the problems associated with the biodegradation of hydrophobic compounds, which include petroleum hydrocarbons, is that they bind to soil particles and have limited solubility in water, resulting in limited availability to soil microorganisms, which in turn can retard and/or stop the degradation process. It is generally assumed that, if you can increase the availability of the hydrophobic compounds to the microorganisms, you can increase the rate of bioremediation. By definition bioremediation is the use of living organisms, mainly predominantly microorganisms, to degrade the environmental pollutants into less toxic forms. Biodegradation is the degradation of matter in the natural environment in the absence of any human intervention. Bioremediation, in contrast, involves human intervention and requires the technology of pollution treatment, using biological systems to transform and convert various pollutant species in the environment to less toxic or non-toxic forms.

Bioremediation often involves the acceleration of the bacterial degradative process by improving the conditions (e.g. pH, temperature, and moisture content), and genetically manipulating the prevailing microorganisms to be efficient hydrocarbon utilizers (Ron & Rosenberg, 2001). Different bioremediation strategies are adopted in facilitating the biodegradation processes of hydrophobic compounds, some of which are:

Addition of nutrients. Bioremediation involves the addition of nutrients to help indigenous microorganisms. Fertilizers are applied to oil-polluted environments to provide nitrogen, phosphorus and other nutrients to the microbial degradation process in situ. Atlas & Bartha (1973) tested the effectiveness of oleophilic fertilizers for stimulating oil biodegradation in nearshore areas off the coast of New Jersey in situ as well as in vitro experiments. They reported that the degradation of oil pollutants was 30 to 40% higher in oleophilic-fertilized oil slicks compared to unfertilised slicks. Bioremediation usually involves the addition of surfactant and oleophilic fertilizers, which provide nitrogen and phosphorus nutrients to the microbial degradation process in situ.

Seeding or bioaugmentation. Seeding involves the introduction of genetically manipulated or best selected strains into the natural environment to increase the rate of biodegradation of pollutants. Such an approach is adopted when indigenous microbial populations may not be capable of degrading the wide range of hydrocarbon contaminants.

Oxygenation or aeration. The initial steps in the aerobic biodegradation of hydrocarbons by microorganisms involve the oxidation of the substrate by

oxygenases for which molecular oxygen is required. Oxygen is important in aerobic bioremediation as oxygen is the electron acceptor and is required for the oxidation-reduction reactions that transform the organic contaminants (petroleum hydrocarbons) to carbon dioxide and water. In situ bioremediation is typically limited by the amount of available oxygen. Therefore, aeration or sparging (mass transfer of oxygen into the ground water) is crucial in facilitating the degradation rates of hydrophobic contaminants (Atlas & Bartha, 1992).

Addition of bioemulsifiers. In order for bacteria to utilize an organic contaminant, the contaminant must first diffuse through the outer cell wall and inner cytoplasmic membrane of the bacterium where it can be broken down by the bacteria's multi-enzyme complexes and thereby made available to the bacteria for uptake. The process of diffusion through two barriers is extremely slow, particularly with more complex molecules such as diesel, crude oil and PAHs. By extracting the multi-enzyme complexes and biosurfactants from bacteria and applying them directly to the contaminant, the diffusion process is bypassed, and the rate of molecular degradation is significantly increased (ETEC, 1991). Thus application of bioemulsifiers would result in increased surface area, while application of enzymes would result in increased surface area or bioavailability of organic contaminants.

Application of monoxygenases. Monooxygenases and dioxygenase are complex proteins that catalyse oxidation-reduction reactions in the hydrocarbon degradation process. They have been used to improve the bioremediation process by transforming complex, recalcitrant aromatic and aliphatic hydrocarbons that are not easily biodegradable, thereby accelerating biological clean up (Atlas & Bartha,

1992). Thus, they are used to enhance the remediation of long–chain aliphatic hydrocarbons (oil) and aromatic benzene rings (gasoline compounds).

Optimization of the environmental conditions. Although microorganisms are isolated from extreme conditions, most of them grow optimally over a narrow range of environmental conditions such as temperature, pH and moisture content. Therefore, it is important to achieve optimal conditions for bacterial growth and thereby facilitate the degradation processes. If the soil is acidic, it is possible to increase pH by adding lime. Plastic covering can be used to enhance solar warming in late spring, summer, and autumn. Available water is also essential for all living organisms, and irrigation is required to achieve optimal moisture contents.

Generally, the goals of bioremediation are providing cheap, contaminant specific treatments to decrease the deleterious effects of environmental pollutants (Head, 1998). Chemical surfactants, which have been used in offshore ocean spills, cause major pollution problems in shallow coastal waters because of their toxicity and persistence in nature (McIntosh, 1989). Microbial degradation of oil allows the slow removal of oil from the environment, particularly from the beaches or the shallow waters (Harvey *et al.*, 1990). Furthermore, microorganisms present the possibility that organic pollutants can be completely mineralized to inorganic materials (Head, 1998). However, despite its benefits over other technologies, bioremediation has not been widely adopted to treat environmental pollution. One reason is that physical treatments, such as excavation and removal of contaminated soil, high temperature incineration of oil contaminants etc, are rapid and their outcome is generally predictable in the short term (Head, 1998). The unpredictability of bioremediation arises from a lack of understanding of the behavior of

microbial populations in natural systems, and how they interact with the physical, chemical, and biological factors to control their activities against pollutants (Head, 1998). Biosurfactants facilitate growth on bound substrates by desorbing them from surfaces or increasing their apparent water solubility (Marcoux *et al.*, 2000). Low molecular weight biosurfactants, which have low critical micelle concentrations (CMCs), enhance the apparent water solubility of hydrocarbons by incorporating them into the hydrophobic cavities of micelles (Miller & Zhang, 1997). Harvey *et al.* (1990) tested a biosurfactant from *P. aeruginosa* for its ability to remove oil from contaminated Alaskan gravel samples under various conditions. They reported increased oil removal (about 2-3 fold) in comparison to water alone, demonstrating the capacity of biosurfactants to remove oil from a naturally occurring substrate. Much less is known on how the high molecular weight biosurfactants enhance the biodegradation of hydrophobic compounds (Ron & Rosenberg, 2001). Recently, Barkay *et al.* (1999) have demonstrated that alasan enhanced the apparent solubilities of polyaromatic hydrocarbons (PAHs) 5-20 fold and significantly increases their rate of biodegradation. Burd & Ward (1996) have shown that a bioemulsifier consisting of protein and lipopolysaccharides produced by *Pseudomonas marginalis* PD-14B enhanced the dispersion of PAH crystals and increased growth on these substrates.

At present, bioremediation enhanced processes, using bio-surfactants, have relied on the direct introduction of biosurfactants into the contaminated site. The advantage of in situ production (growth at the site of contamination) is that it would be more cost effective, visibly and ecologically more acceptable with less transport/labour. Bacteria that are efficient in biosurfactant production can be used to function in a bacterial consortium, supplying the emulsifier for other bacteria that carry out the degradation of

hydrocarbons. Some biosurfactants enhance the degradation of poorly soluble organic compounds, such as polycyclic aromatics, by increasing their apparent bioavailability. Their biodegradation is limited because of their hydrophobicity, low aqueous solubility and strong adsorptive capacity in soil (Volkering *et al.*, 1992). For example, alasan, a high molecular weight bioemulsifier produce by *A. radioresistens* KAS 3, was observed to increase the aqueous solubility and biodegradation rates of PAHs (Barkay *et al.*, 1999). In the presence of 5000 µg of alasan/ml, the apparent aqueous solubilities phenanthrene, flouranthrene, and pyrene were increased 6.6-, 25.7-, and 19.8-fold, respectively. Van Dyke *et al.* (1993) conducted a study surveying a variety of biosurfactants for their ability to remove hexachlorobiphenyl from soil. Out of thirteen biosurfactants tested, seven removed the hexachlorobiphenyl more efficiently compared to the control. In addition they found two strains of *P. aeruginosa* and one strain of *Acinetobacter calcoaceticus* RAG-1, which produced the most efficient biosurfactants. The rhamnolipid biosurfactant produced by *P. aeruginosa* 19SJ was observed to increase the apparent aqueous solubility of naphthalene from 31 mg/l to solubilities greater than 1000 mg/l. This supports a potential role for biosurfactants in increasing the solubility of such compounds, and thus enhancing bioremediation. Similarly, Zhang *et al.* (1997) studied the role of two biosurfactants in increasing the solubility of phenanthrene, and reported increases in both solubility and degradation rate of phenanthrene in the presence of biosurfactant.

It is well known that microbial cells chelate metals from solution. However, although there are reports of exopolysaccharide used for this purpose (Kaplan *et al.*, 1987; Scott *et al.*, 1988; Marques *et al.*, 1990), little is known about the use of biosurfactants to chelate metals. Exopolysaccharides however, differ from biosurfactants in their size and

minimal surface activity (Banat *et al.*, 2000). The advantages of using a biosurfactant as chelating agent over exoplymers includes their smaller size and their biodegradability (Banat *et al.*, 2000). Two mechanisms were suggested for the desorption of heavy metals from soil by the addition of biosurfactants (Miller, 1995). The first is through complexion of the free form of the metal present in solution which decreases the solution phase activity of the metal and therefore promotes desorption. The second occurs under conditions of reduced interfacial tension; where the biosurfactant concentrates at the solid interface, which may allow direct contact between the biosurfactant and the sorbed metal. The removal of metals from soil by rhamnolipid biosurfactants produced by *P. aeruginosa* ATCC 9027 was investigated by Tan *et al.* (1994). They reported 92% complexation of Cd^{2+} in a 0.5 mM solution of $Cd(NO_3)_2$ using a 5 mM solution of rhamnolipid (22 μg/mg rhamnolipid).

1.6.2. Biosurfactants in the crude oil industry

Most crude oils are highly viscous with viscosities ranging from 10 000 to about 500 000 centipoises at ambient temperatures. Their highly viscous nature brings difficulties in their extraction, production, recovery, treatment and/ or transportation. For example the residual crude oil adhered to wall surfaces of large transport vessels can amount to 5% or as much 12 tons from the original load. Often large volumes of water are used for cleaning and recovering the residual oil, but this results in large quantities of oil contaminated cleaning water, thus making this method both environmentally costly and economically inefficient (Banat *et al.*, 1991). Moreover, it is difficult to extract viscous crude oil from underground sources, and because of the high viscosity, transportation from production fields to refineries via pipelines becomes extremely difficult. Therefore, it is necessary to reduce the viscosity of the fluid to less than 500 cp

in order to ensure environmentally sound and economically efficient ways of extraction, production, recovery, transportation and treatment of highly viscous hydrocarbons. Furthermore, it is also necessary that the viscous hydrocarbons have a reduced pressure drop, preferably of less than or equal to about 20 psi/mile when transported in a well conduit or a pipeline. Conventional techniques such as heating, dilution with solvents, annular transportation, watery lubrication etc, which have been used to achieve these pressure drop and viscosity levels, are costly and less efficient. Surfactants have been used for lowering the viscosity of heavy oils, which will in turn facilitate oil recovery, transportation and pipelining (Bertrand *et al.*, 1994). Hayes *et al.* (1986) have shown the ability of emulsan to lower the viscosity of Boscan (Venezuelan heavy oil) from 200 000 to 100 cP, making it feasible to pump heavy oil in 26 000 miles of commercial pipeline. Earlier on, Zajic *et al.* (1974) reported the emulsification of heavy grade VI fuel oil by an emulsifying agent produced by a *Pseudomonas* strain. Another application of biosurfactant is oil storage tank cleaning. In a small-scale field investigation, Banat *et al.* (1991) tested a biosurfactant produced by a bacterial strain (Pet 1 006) for its ability to clean oil storage tanks and to recover hydrocarbons from the emulsified sludge. Two tonnes of biosurfactant-containing whole-cell culture were used to mobilise and clean 850 m^3 oil sludge. They reported, approximately 91% (774 m^3) of this sludge was recovered as re-sellable crude oil and 76 m^3 non-hydrocarbon materials remained as impurities to be manually cleaned. Such a clean-up process is therefore economically feasible and environmentally sound leading to less disposal of oily sludge in the natural environment.

1.6.3. Biosurfactant as therapeutic agents

Biosurfactants have some therapeutic applications. Rhamnolipid produced by *Pseudomonas* (Itoh *et al.*, 1971), lipopeptides produced by *Bacillus subtilis* (Sandrin *et al.*, 1990; Leenhouts *et al.*, 1995; Vollenbroich *et al.*, 1997a), and *Bacillus licheniformis* (Jenny *et al.*, 1991; Fiechter, 1992; Yakimov *et al.*, 1995) and mannosyleryhtritol lipids from *Candida antractica* (Kitamoto *et al.*, 1993) have all been shown to have antimicrobial activities. Surfactin, for example, has various pharmacological uses such as inhibiting fibrin clot formation and haemolysis (Bernheimer and Avigad, 1970), inhibiting cyclic adenosine 3',5'-monophosphate phospho-diesterase (Hosono & Suzuki, 1983), and having anti-fungal properties (Vater, 1986). Another anti-fungal biosurfactant, Iturin, a lipopeptide produced by *Bacillus subtilis*, was found to affect and disrupt the morphology and membrane structure of yeast cells (Thimon *et al.*, 1995). Itokawa *et al.* (1994) reported the potential of surfactin against human immunodeficiency virus 1 (HIV-1). Vollenbroich *et al.* (1997b) suggested that the anti-viral action of surfactin is due to a physicochemical interaction between the membrane-active surfactant and the virus lipid membrane. Such reports of antibiotic activity (Neu *et al.*, 1990) and inhibition of HIV virus growth in white blood cells have opened new applications for biosurfactants. Yet, to date, commercial production of biosurfactants for use as antimicrobial agents has not taken place (Banat *et al.*, 2000).

1.6.4. Biosurfactants for agricultural use

The use of pesticides has long been a threat to environmental pollution, such as eutrofication in fresh water bodies. These concerns have prompted global efforts to seek for alternative biological control systems. Stanghellini *et al.* (1996), while investigating the effects of synthetic surfactants on controlling the root rot fungal infections of

cucumbers and peppers caused by *Pythium aphanidermatum* and *Phytophora capsici*, observed lysis of fungal zoospores due to certain bacterial metabolites in the nutrient solution. They suspected that the metabolites were biosurfactants, as their mode of action was similar to synthetic surfactants. Later on, the bacterium was identified as *Pseudomonas* and the biosurfactant as a rhamnolipid (Stanghellini & Miller, 1997). The biosurfactant was found to have a zoosporicidal activity against species of *Pythium*, *Phytophora*, and *Plasmopara* at concentrations ranging over 5-30 μg/ml. Biosurfactants have also been utilised in mobilizing sparingly soluble organophosphorus fertilizers (Banat *et al.*, 2000). Patel & Gopinathan (1986) reported that a glycolipopeptide bioemusifier, produced by two *Bacillus* strains, was able to form a stable emulsion in the presence of the pesticide fenthion. The bioemulsifier had shown some activity against other liquid immiscible organophosphorus pesticides, but not against solid organophosphorus or organochlorine pesticides.

1.6.5. Biosurfactants and personal care

Biosurfactants have drawn attention in the personal care market because of their improved moisturizing properties and skin compatibility (Brown, 1991). For example, Yamane (1987) has reported the specific skin-compatability and the commercial utility of a product, containing one mole sophorolipids and 12 moles propylene glycol, as a skin moisturizer. At present, Kao Chemical Corporation (Japan) uses sophorolipids industrially as humectants for cosmetic makeup brands such as Sofia (Banat *et al.*, 2000). Kosaric (1992) has predicted on the expanding role of biosurfactants in many different products used in the cosmetic industry. Recently much higher yields of sophorolipids, up to 300 g/l (Davila *et al.*, 1997) and 422 g/l (Daniel *et al.*, 1998), have

been reported from *Candida bombicola* in two different two-stage fermentation techniques using rapeseed oil as the main carbon source.

1.6.6. Other applications of biosurfactants

Some other potential commercial uses of biosurfactants are in mining (Rosenberg *et al.*, 1988; Polman *et al.*, 1994), food industry (Shepherd *et al.*, 1995; Busscher *et al.*, 1996), pulp and paper industry (Rosenberg *et al.*, 1989), textiles, ceramics (Horowitz & Currie, 1990) and uranium ore processing (McInerney *et al.*, 1990).

1.7. Substrate Versus product yield relationship

Despite the relative advantages conferred by biosurfactants over the chemical surfactants, biosurfactants are more costly to produce than synthetic ones. This provoked several studies in maximizing biosurfactant production and thereby reducing their cost of production. Such studies include the use of cheap substrates and the optimisation of culture conditions to obtain maximum possible biosurfactant production at a low cost (Table 1-2). The substrates used in these studies include n-alkanes, alcohols, oils and glucose. Some of the highest yields of biosurfactants have been obtained from processes using rape seed, canola or soybean oil as carbon substrates. Davila et al. (1997) reported yields of 150 g/l, making potential commercial exploitation of sophorolipid biosurfactants from *T. bombicola* using 100 g/l of rapeseed oil. More than 100 g/l of rhamnolipid production were also obtained from 160 g/l soybean oil (Lang & Wullbrandt, 1999). In a number of cases n-alkanes have been used as substrates for the production of biosurfactants (Rapp *et al.*, 1979; Philip *et al.*, 2002; Wagner *et al.*, 1983, Hisatsuka *et al.*, 1972). Reports indicate that, yields of trehalose lipids were optimised to 4 g/l when the bacteria, *R. erythropolis*, was grown on 10 % (w/v) alkanes. Wagner *et*

al., reported yields about 12.8 g/l of rhamnolipid biosurfactant from *P. aeruginosa* using 80 g/l of n-C_{14}-C_{15}. The use of cheap substrates can drastically decrease the production cost of biosurfactants. Haba *et al.* (2000) have used cheap industrial wastes, such as used frying oils as substrates for the production of rhamnolipids by *P. aeruginosa*. They were able to obtained yields of 2.7 g/l of rhamnolipid biosurfactant from such substrates.

Table 1-2. Substrate versus yield relationship of the different biosurfactants produced by different microorganisms

Biosurfactant	Producing microorganisms	Substrate used	Production	References
Trehalose lipids	*R. erythropolis*	10% (w/V) n-alkanes	4 g/l	Rapp *et al.*, 1979
	R. ruber IEGM 231	3% (v/v) hexadecane	9.9 g/l	Philip *et al.*, 2002
		3% (v/v) pentadecane	4.01 g/l	Philip *et al.*, 2002
		3% (v/v) tetradecane	1.79 g/l	Philip *et al.*, 2002
Sophorose lipids	*C. bombicola* ATCC 222214	100 g/l glucose/rapeseed oil	150 g/l	Davila *et al.*, 1997
Rhamnolipids	*P. aeruginosa* DSM 7107	160 g/l soybean oil	112 g/l	Lang & Wulbrandt, 1999
	P. aeruginosa DSM 2874	80 g/l n-C14/C15	12.8 g/l	Wagner *et al.*, 1983
	P. aeruginosa MUB	20 g/l n-C14/C15	2.9 g/l	Wagner *et al.*, 1983
	P. aeruginosa 47T2NCIB	Waste frying oil	2.7 g/l	Haba *et al.*, 2000
	P. aeruginosa BS2	20 g/l sucrose	1.9 g/l	Babu *et al.*, 1996
	P. aeruginosa UW-1	6% (v/v) canola oil	24.3 g/l	Sim *et al.*, 1997
Surfactin	*B. subtilis*	4% (w/v) glucose	3.5 g/l	Wei & Chu, 1998
Emulsan RAG-1	*A. calcoaceticus* ATCC 31012	8.0 g/l ethanol	3.1 g/l	Choi *et al.*, 1996
Biodispersan	*A. calcoaceticus* A2	1% ethanol	0.5 mg/l	Elkeles *et al.*, 1994
Alasan	*A. radioresistens* KA53	0.5% (v/v) ethanol	4.6 mg/ml	Navon-Venezia *et al.*, 1995
Surfactin	*B. subtilis* ATCC 21332	4% (w/v) glucose	0.8 g/ml	Cooper *et al.*, 1981
Protein like activator (PA)	*P. aeruginosa* S_7B_7	1% (v/v) n-hexadecane	100 mg/l	Hisatsuka *et al.*, 1972
		1% (v/v) n-tetradecane	70 mg/l	Hisatsuka *et al.*, 1972
		1% (v/v) n-pentadecane	100 mg/l	Hisatsuka *et al.*, 1972
Peptidoglycolipid	*P. aeruginosa* P-20	1% (v/v) hexadecane	7 mg/l	Koronelli *et al.*, 1983
Mannoprotein	*S. cerevisiae*	1% (w/v) glucose	17.8 g/g (wet wt. of cells)	Cameron *et al.*, 1998

1.8. Aims of this study

The main objective of the project was to isolate and characterize novel South African bacterial strains, that are capable of efficient utilization of the n-alkane fraction (C_{12}-C_{17}), which is a byproduct of the SASOL petrochemical industry. These waste alkanes are produced in large volumes but have limited market value, so that their bioconversion into specialty compounds would be of interest to the industry. Thus, the study involved the screening of bacterial isolates capable of efficient growth on the n-alkane fraction, for the production of extracellular biosurfactants. The most efficient biosurfactant producer would be identified and growth studies for optimal biosurfactant production would be carried out. Preliminary characterization of biosurfactant would be performed to determine the chemical nature and the emulsifying properties of the compound so that possible industrial applications can be identified.

Chapter Two

Isolation, identification and preliminary characterization of oil degrading bacteria

Contents

2.1. Summary

From crude oil polluted soil samples, several phenotypically different bacterial strains, capable of growing on crude oil, were isolated based on enrichment principal. Three of these isolates were found to produce strong emulsions using kerosene as a substrate. The three strains, designated 2Bf, 2Bf*, and 2Bg, were observed to produce extracellular bioemulsifying agents with an emulsion index (E1) of 69%, 70%, and 66% respectively. The three strains were identified to belong to the genus *Pseudomonas* using standard biochemical tests. One isolate, 2Bf, was further identified using 16S rDNA PCR, and showed 99% identity with the previously identified *Pseudomonas aeruginosa* PAO1 AE004844). Hence the strain was identified as *Pseudomonas aeruginosa* and designated as *P. aeruginosa* 2Bf. The bioemulsifying agent excreted by the strain 2Bf was found to be in effective in lowering the surface tension. In addition *P. aeruginosa* 2Bf appeared to synthesize commercially important compounds known as polyhydroxyalkanoates (PHAs). This was suggested phenotypically, by Nile Blue staining, and genetically, by PCR detection of the *pha*C gene. These results suggest that the isolated strain, *P. aeruginosa* 2Bf is important in utilizing hydrocarbons, and is capable of producing commercially important compounds such biosurfactants and PHA.

2.2. Introduction

The role of microorganisms in the degradation of hydrocarbons and the production of biosurfactants is of great interest to the fields of environmental and industrial biotechnology. As a result, the isolation and characterization of novel bioemulsifier producing strains has increased significantly in recent years (Ilori & Amund, 2001). Typically the isolation of oil degrading microorganisms is achieved using the enrichment culture technique. This technique is based on controlling environmental and nutritional conditions so as to favour the growth of a specific organism or group of organisms of interest. For the isolation of oil degrading organisms from contaminated soil, crude oil can be used as the sole carbon source for growth in a minimal medium (MM), so that those bacteria that are better adapted to using the compounds present in crude oil will eventually outcompete others. The organism with the strongest growth rate under a defined set of conditions will have a competitive advantage over others present in an inoculum, so that after subsequent inoculations it will outcompete the others and prevail as the dominant species. In a number of cases, oil-degrading microorganisms have been isolated from crude oil polluted soil samples (Ilori & Amund, 2001; Harvey *et al.*, 1990; Navon-Venezia *et al.*, 1995; Francy *et al.*, 1991; Toren *et al.*, 2002b).

Organisms that have been exposed to hydrocarbons develop adaptations to live in such an environment. Often, they do so by undergoing genetic modifications, which will enable them to utilize hydrocarbons as an energy source (Leahy & Colwell, 1990). Moreover, certain oil degrading microbes produce bioemulsifiers to facilitate the uptake of hydrocarbons (Ilori & Amund, 2001; Francy *et al.*, 1991; Zhang & Miller, 1995). These bioemulsifiers can be accumulated as cell bound components or can be released as

extracellular compounds (Ilori & Amund, 2001). For biotechnological applications, microbial strains that produce extracellular bioemulsifiers are commercially more attractive than those that produce the cell-associated emulsifiers, since the extraction of cell-bound bioemulsifiers is a more complex and costly procedure compared to the purification of extracellularly secreted bioemulsifiers.

Many oil-degrading organisms have been observed to accumulate intracellular, energy storage materials known as polyhydroxyalkanoates (PHAs) (Poirier *et al.*, 1995; Steinbüchel & Füchtenbusch, 1998; Lee, 1996). The synthesis of PHAs by organisms invariably takes place when a nutritional component such as nitrogen, phosphorus, sulfur, oxygen, or magnesium is limiting in the presence of excess carbon source (Poirier *et al.*, 1995; Steinbüchel & Füchtenbusch, 1998; Lee, 1996). Most prokaryotic organisms synthesize poly(3-hydroxybutyric acid), poly(3HB) and other PHAs as storage compounds, and accumulate these polyesters as insoluble inclusions in the cytoplasm (Rehm & Steinbüchel, 1999). Bacterial PHAs have attracted much attention because they can be used for the synthesis of truly biodegradable thermoplastics or elastomers (Hrabak, 1992). Because of their biodegradability and non-toxic nature, PHAs are considered for several applications in the packaging industry, medicine, pharmacy, agriculture and food industry or as raw materials for the synthesis of enantiometrically pure chemicals and the production of paint (Anderson & Dawes, 1990).

So far, more than 20 PHA synthesis operons have been cloned and analysed from a variety of bacteria revealing that the proteins required for PHA biosynthesis pathways have diverged considerably (Madison & Huisman, 1999). Nevertheless, PHA synthase is

a crucial enzyme in all PHA synthesis pathways (Anderson & Dawes, 1990; Madison & Huisman, 1999; Rehm & Steinbüchel, 1999). There are many phenotypic detection methods for intracellular PHA granules, which are applied for the screening of PHA producers. These includes Sudan Black staining (Schlegel *et al.*, 1970) and Nile blue A staining (Ostle & Holt, 1982) which result in dark blue or fluorescent granules respectively. In this study, Nile blue A was used to stain the PHA synthesized by presently isolated strain, because, Nile blue A was shown to be more specific stain for poly-beta-hydroxybutyrate than Sudan black B (Ostle & Holt, 1982). However, these methods cannot distinguish between bacteria that specifically accumulate PHA granules and those that accumulate lipid compounds (Sheu *et al.*, 2000). Hence, a genotypic detection method was devised which is based on the PCR detection of the gene that encodes the PHA synthase enzyme (*phaC*) (Sheu *et al.*, 2000) thus circumventing the major drawbacks inherent in phenotypic detection methods. The synthesis of PHAs requires the enzyme PHA synthase (PhaC), which uses ß-hydroxyacyl-coenzyme A substrates for polymerisation (Sheu *et al.*, 2000).

The aim of this study was twofold. Firstly, to isolate bacterial strains that were capable of efficient utilization of hydrocarbons. Secondly, to determine their potential to produce commercially important compounds such as bioemulsifiers and PHAs with the view to commercial applications. Microorganisms were isolated from crude oil polluted soil samples using an enrichment culture procedure. Crude oil was used as the sole carbon source to isolate those organisms that can efficiently utilize hydrocarbon substrates. The isolated bacterial strains were then screened for their ability to produce extracellular bioemulsifiers. The isolates that gave the highest emulsifying activity were identified

using biochemical and molecular methods. One of the strains producing the highest levels of bioemulsifier was also characterized for its ability to synthesize PHAs both phenotypically and genetically.

2.3. Materials and Methods

2.3.1 Isolation of oil degrading bacteria and culture media

Oil polluted soil samples were collected aseptically from the CALTEX Oil Refinery in Cape Town, South in Africa 250 ml autoclaved bottles with screw tops (Duran, Germany). Samples were taken in duplicate from three different sites as described below.

1. Caltex Oil Refinery Company, around the vicinity of the crude oil tank 202 (Site 1). The two samples from this site were designated 1A and 1B.
2. Caltex Oil Refinery, near the receiving station where an oil leakage occurred on the 7th of May 2002 (Site 2). Duplicate samples from this site were designated 2A and 2B.
3. Caltex Oil Refinery Company, near the Land Farm, which used to be a dumping site for hydrocarbon waste ten years ago (Site 3). Duplicate samples from this site were designated 3A and 3B.

The minimum salt medium (MSM) used for culture selection and biodegradation of 1% (v/v) of crude oil (Caltex, South Africa) contained, per liter, 1g of $(NH_4)_2SO_4$, 1g of K_2HPO_4, 0.5g of KH_2PO_4 and 10 ml of the salt stock solution. The salt stock solution contained, per liter: 25g of $MgSO_4.7H_2O$, 2.8g of $FeSO_4.7H_2O$, 1.7g of $MnSO_4$, 0.6g of NaCl, 0.1g of $Na_2MoO_4.2H_2O$, 0.1g of $ZnSO_4.7H_2O$ and 8.54 ml of 32% HCl solution (Demain & Solomon, 1986). The final pH of the medium was adjusted to 7.0 using 10N NaOH. For the initial cultures, 5g of the oil-polluted soil samples were added into 50 ml sterile MSM supplemented with 1% (v/v) filter sterilized (i.e. through 0.22 • m acetate filter, OSMONICS, USA) crude oil as carbon source. The culture was then incubated at 30°C on a reciprocal shaker (60 rpm). Each culture was subcultured four times, after a

period of four days by transferring 10% (v/v) of the culture solution under strictly sterile conditions. Yeast extract was added at concentration of 0.05 g/l to the starter enrichment culture to help stimulate initial growth of fastidious organisms. Amphotericin was added to inhibit the growth of fungi, at concentrations of 0.01 g/l only at the initial stage. Amphotericin was prepared by dissolving 0.01g of amphotericin powder in 1 ml of 0.1M NaOH and was sterilized by filtering through 0.22 µm acetate filter (Osomonics, USA). Serial dilutions of 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} were made from each culture by vigorously vortexing each step of the dilution; and spread on nutrient agar plates, and incubated at 30°C overnight. Bacterial colonies were counted and identified according to their morphology, shape and colour.

After the isolation and screening processes, the C_{14}-C_{17} n-alkane fraction (where each of the alkanes were present in equal proportion, i.e. 1:1:1:1), obtained from SASOL, South Africa, was used as carbon source in place of crude oil for further studies.

2.3.2. Screening for extracellular production of bioemulsifiers

Strains isolated from a single colony were incubated at 30°C with shaking for four days in 50 ml of MSM media supplemented with 1% (v/v) crude oil. After incubation, 10ml of the culture was centrifuged for ten minutes at 3000 x g at room temperature. After centrifugation, 5ml of the supernatant, carefully retrieved from below the surface of the oil layer, was used for emulsification assays. Emulsification power was measured by vortexing an equal volume of the culture supernatant with kerosene (Fluka) for one minute in a 10 ml Sterilin tube, and determining the % of volume occupied by the emulsion (Haba *et al.*, 2000; Van Hamme & Ward, 2001; Ilori & Amund, 2001). The

mixture was allowed to settle for one hour and the volume, as a function of height of emulsion, was then measured to get the emulsion index. The emulsion index (E1) is the height of the emulsion layer divided by the total height and multiplied by 100.

2.3.3 Biochemical identification

Biochemical tests were performed on the three best bioemulsifier producers (i.e. 2Bf, 2Bf* and 2Bg). Standard methods were used for motility, gram staining, oxidation-fermentation test, and oxidase and catalase activity tests, to identify isolates up to genus level (Reid *et al.*, 2001). The strains used as controls in some of the biochemical tests are presented on Table 1-1.

Table 1-1. Table Showing the strains used as controls in biochemical tests.

Biochemical tests	Strains used	Purpose	Strain genotypes
Gram staining	*Staphylococcus albus*	As gram positives cocci	MCB prac. strain
	Bacillus subtilis	As gram positive rod	MCB prac. strain
	E. coli k12	As gram negative rod	MCB prac. strain
Oxidase test	*P. aeruginosa*	As a positive control	MCB prac. strain
	E. coli K12	As a negative control	Wild type
Catalase test	*Staphylococcus aureus*	As a positive control	Ap^R, Em^R
	Streptococcus faecalis	As a negative control	Wild type

MCB = Department of Molecular and Cell Biology, University of Cape Town.
Prac = practical

2.3.4. Determination of the 16S rDNA gene sequences

DNA extracts were prepared from the isolated strain (2Bf) grown in LB (Luria-Broth). An overnight (1ml) culture was centrifuged at 10 000 x g for 5 minutes and the pellet was washed in 1ml Tris-EDTA buffer (pH 7), and finally resuspended in 0.5 ml TE buffer (pH 8). Cells were lysed by boiling for 10 minutes at 100°C in water bath. Approximately

450 µl of supernatant was recovered after centrifugation to remove the cell debris. The crude DNA extract was then stored at -20°C.

PCR amplification of the 16S rDNA gene was performed for the strain 2Bf, using the universal forward primer (F1) (5' CGC CAG GGT TTT CCC AGT CAC GAC AGA GTT TGA TCC TGG CTC AG 3') and reverse primer (R5) (5' CAG GAA ACA GCT ATG AC ACG GIT ACC TTG TTA AGA CTT 3') (Weisberg *et al.*, 1991). The PCR reaction mixture contained the following: 0.3 µl of 25 mM of each dNTP, 2.5µl of 10 µM of each primer, 1.5 µl of 25 mM $MgCl_2$, 5 µl of buffer (10x), 0.5 µl of 1U Supertherm DNA polymerase, 100ng of chromosomal DNA template and distilled water to make up a 50 µl PCR reaction. All the PCR reaction mixtures were supplied by Roche. The amplification was carried out using a GeneAmp 9700 PCR machine (Applied Biosystems) as follows: the reaction mixture was heated for 2 minutes at 96°C, followed by 25 cycles of 30 s at 95°C, 30 s at 56°C, 90 s at 72°C and finally 3 minutes extension step at 72°C. The PCR product was purified using the Wizard SV Gel and PCR Clean-Up System, Promega Corporation, according to the manufacturer's instructions. The purified PCR product was sequenced using MegaBace sequencer machine (Department of Molecular and Cell Biology, UCT). The primers used for sequencing were: F3 (5' CGC CAG GGT TTT CCC AGT CAC GCC AGC CC GGT AAT AC GAC 3') and R3 (5' CAG GAA ACA GCT ATG AC CAC GAG CTG ACG ACA ICC ATG 3') (Weisburg *et al.*, 1991). Database searches with the determined sequences were conducted using the BLAST program (Altschul *et al.*, 1990) against the databases at genebank, NCBI, USA.

2.3.5. Surface tension measurements

Surface tension measurements were conducted on *Pseudomonas aeruginosa* 2Bf cultures grown in MSM containing either of the to carbon substrates; 2% (w/v) glucose or 1% C_{14}-C_{17} alkane fraction. *Escherichia coli* XL-Blue grown on 2% (w/v) glucose was used as a negative control. Growth was carried out in 500 ml flasks containing 200 ml of culture. Cultures were incubated for 4 days at 30°C with shaking. The cultures were centrifuged at 1 200 x g for 15 minutes at room temperature and filtered through Whatman filter paper, and 100 ml of the supernatant fluid was used for surface tension measurements along with methanol standards between 0 to 29.9% (w/w). The surface measurements were carried out using a bubble pressure tensiometer (Department of Chemical Engineering, UCT) against methanol standards at 30°C.

2.3.6. Detection of PHA granules by Nile blue staining

P. aeruginosa 2Bf and the negative control, *E. coli* XL1-Blue, were grown in 5 ml LB overnight at 30°C. The positive control, *Pseudomonas oleovorans* ATCC29347 was grown overnight in MSM media supplemented with 1% octane as carbon source. One ml of each culture was washed with an equal volume of 5 ml MSM medium. The pellet from each of the cultures was resuspended in MSM media after which 0.2 ml was used as a starter inoculum into 50 ml of nitrogen limited (0.4 g/l $(NH_4)_2SO_4$) MSM media. The strain *P. aeruginosa* 2Bf and the positive control *P. oleovorans* ATCC29374 were grown on media containing 2% C_{14}-C_{17} alkane fraction as carbon source, while *E. coli* XL-Blue was grown on 2% (w/v) glucose as the carbon source in the presence of 20 • g/ml of tetracycline. The cultures were incubated at 30°C for 4 days.

After four days, bacterial smears were prepared aseptically from each of the cultures (Reid *et al.*, 2001). The smears were stained with 1% (w/v) Nile Blue A (Sigma) according to the protocol by Ostle & Holt (1982). Heat fixed smears of bacterial cells were stained with the 1% (w/v) Nile Blue A solution at 55°C for 10 minutes in staining jar. After being stained, the slides were washed with tap water to remove excess stain and with 8% aqueous acetic acid for 1 min. The stained smear was washed and blotted dry, remoistened with tap water, and covered with a glass cover slip. The cover slip is necessary, as standard immersion oil will extract some of the fluorescence. The cover slip thus protects the stained cells from immersion oil. The stained slides were then observed under an epifluorescent microscope using an exciter filter that provides an excitation wavelength of approximately 460nm (EM unit, UCT). Photographs were obtained using the Axiocam camera and the Axiovision program. A positive result shows a bright orange fluorescence of the PHA granules, with individual granules often visible within a cell.

2.3.7 PCR detection of PHA synthase gene (*phaC*)

DNA extraction

DNA extracts were prepared from *P. aeruginosa* 2Bf, and the negative control *E. coli* XL1-Blue were grown in 5 ml LB media overnight. The positive control *P. oleovorans* ATCC29374 was grown overnight in 5 ml MSM supplemented with 2% (v/v) octane as carbon source. Each of the cultures was centrifuged and the cell pellet was used to extract DNA using the DNA extraction kit (Roche Applied Science) according to the manufacturers instructions.

PCR of *phaC* gene

Two slightly modified degenerate primers (all 26-mers) based on the primers designed by Sheu *et al.* (2000), were synthesized to PCR amplify *phaC* genes from different bacteria for PCR of *phaC* genes from different bacteria (Sheu *et al.,* 2000). The modified primers were 5'-ATC AAC AA(GA) T(TA)C TAC (AG)TC (CT)T(CG) GAC CT-3' (designated phaCF1, corresponding to nt 741-766 of *Rhodococcus eutropha phaC*) and 5'-AGG TAG TTG T(TC)G AC(CG) (AC)(AC)(GA) TAG (TG)TC CA-3' (designated phaCR4, corresponding to nucleotide 1237-1212 of *R. eutropha phaC*), and were synthesized using the Oligo 1000M DNA Synthesizer (Beckman Instruments Inc) by the Department of Molecular and Cell Biology, University of Cape Town.

The PCR reaction mixture contained 4.0 µl of 25 mM of each dNTP, 2.0 µl of 10 µM of each primer, 1.5, or 2.0 or 3.0 µl of 25 mM $MgCl_2$, 5 µl of Supertherm polymerase buffer (10x), 0.4 µl 1U Supertherm DNA polymerase, 100 ng of chromosomal DNA template, 4.0 µl of 3% dimethyl sulfoxide (DMSO) in 50 µl PCR reaction. All the PCR mixes were supplied by Roche. The thermal cycle programme, run on a GeneAmp PCR System 9700 (Applied BioSystems) consisted of one cycle of 94°C for ten minutes, 51°C for 2 minutes, 72°C for 2 minutes, and 35 cycles of 94°C for 20 s, 57°C for 45 s (decreased by 1 s per cycle), 72°C for 1 minute, and then incubation at 72°C for 5 minutes, and a final incubation at 4°C. The PCR-amplified DNA fragments were observed by agarose gel electrophoresis in 0.8% agarose gel. In this experiment, *P. oleovorans* ATCC29347 and *E. coli* XL1-Blue were used as positive and negative controls, respectively.

2.4. Results

2.4.1. Enrichment cultures

After four sequential enrichment cultures, supplemented with crude oil as the sole carbon source, a number of phenotypically distinct strains were isolated based on the shape, size, texture, color etc of the colonies plated on nutrient agar plates (Table 2-2). The numbers obtained on the different dilutions are included to illustrate the difficulty in getting accurate dilution series of bacteria grown in the presence of hydrophobic substrates.

Table 2-2. Table showing the strains isolated from the three sites at the Caltex Oil Refinery, Milnerton. The different morphologies are indicated by letters.

Sample Site	Dilutions	Number of different colony types isolated									
		a	b	c	d	e	f	f*	g	h	i
1A	10^{-3}	12	3	12	M*	0	0	0	0	0	0
	10^{-4}	5	1	0	0	0	0	0	0	0	0
	10^{-5}	3	0	0	0	0	0	0	0	0	0
	10^{-6}	1	0	0	0	0	0	0	0	0	0
1B	10^{-3}	195	140	0	0	0	0	0	0	0	0
	10^{-4}	37	23	0	0	0	0	0	0	0	0
	10^{-5}	7	1	0	0	0	0	0	0	0	0
2A	10^{-3}	0	0	0	0	258	0	0	0	0	0
	10^{-4}	0	0	0	0	24	0	0	0	0	0
	10^{-5}	0	0	0	0	2	0	0	0	0	0
2B	10^{-3}	86	1	0	M*	0	0	0	0	0	0
	10^{-4}	9	0	0	0	0	155	18	600	0	0
	10^{-5}	0	0	0	0	0	1	0	35	0	0
3A	10^{-3}	0	0	0	0	0	0	0	M*	1	6
	10^{-4}	0	0	0	0	0	0	0	475	1	0
	10^{-5}	0	0	0	0	0	0	0	87	0	0
	10^{-6}	0	0	0	0	0	0	0	6	0	0
	10^{-7}	0	0	0	0	0	0	0	2	0	0
3B	10^{-3}	0	0	0	0	0	0	0	120	9	0
	10^{-4}	0	0	0	0	0	0	0	32	3	0
	10^{-5}	0	0	0	0	0	0	0	3	0	0

M* = many in number

Colonies that appeared phenotypically identical to other strains isolated from the different sites were given the same representations (i.e. they were represented with the same symbol). However, because they are isolated from different soil samples, they were regarded as different isolates and were dealt separately. The duplicate soil samples from sites 1 and 3 appeared to have same colony types, while the type of colonies observed between the duplicate samples in site 2 were very different. In fact, replicate 2B had colonies that appeared phenotypically similar to site 1. Surprisingly, in replicate 2A, a single colony was observed which appeared to be phenotypically distinct from all the other soil samples. These differences may be due to a particular strain getting the competitive advantage early in that enrichment culture, and then outgrowing the other strains.

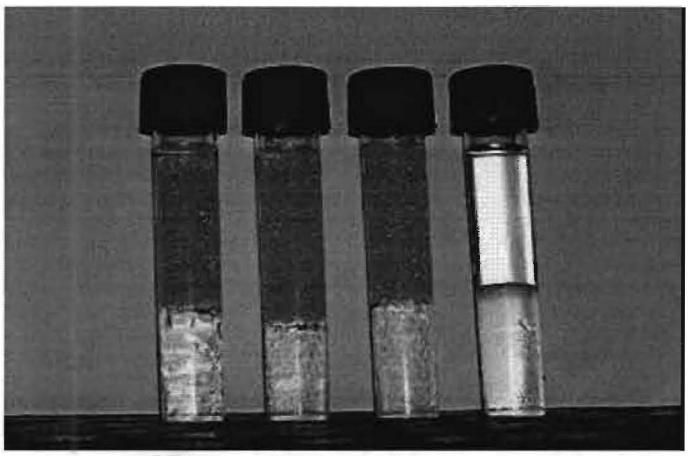
2.4.2. Screening for strains with bioemulsifying potential

The newly isolated strains were tested for the production of extracellular bioemulsifying agents according to the method outlined in section 2.3.2, using kerosene as a substrate. Kerosene is routinely used as a standard to assess the emulsifying activities of bioemulsifiers in many instances (Haba *et al.*, 2000; Van Hamme & Ward, 2001; Ilori & Amund, 2001). Three colonies designated 2Bf, 2Bf*, and 2Bg (all from Site 2) were observed to be good bioemulsifiers with an emulsion index (E_I) of 69%, 70% and 66% respectively (Table 2-3 & Fig. 2-1). It is important to note that all the three best strains were obtained from the same soil sample. Isolates 1Ab and 1Ba were observed to have an emulsion index of 25% and 35% respectively, while others didn't show any emulsifying activities (Table 2-3). The emulsions obtained from 2Bf, 2Bf*, and 2Bg were observed to be stable for about two months. The strains, 1Aa and 1Ba, which appeared to be

phenotypically similar, were observed to exhibit rather different bioemulsifying properties (0% and 35% respectively). Therefore, the morphological characterization of the colonies, which was used as a preliminary characterization of isolates, clearly has its own limitations as a method of differentiation though it is fast and easy.

Table 2-3. Strains isolated from the three sites at Caltex, and their emulsifying power after 1h (E1) after growth in 1% (v/v) crude oil for four days.

Isolate	Site	Emulsion index (E_1)
1Aa	1	0
1Ab	1	25%
1Ac	1	0
1Ad	1	0
1Ba	1	35%
1Bb	1	0
2Ae	2	0
2Ba	2	0
2Bb	2	0
2Bd	2	0
2Bf	2	69%
2Bf*	2	70%
2Bg	2	66%
3Ah	3	0
3Ai	3	0
3Aj	3	0
3Bh	3	0
3Bi	3	0



2Bf 2Bf* 2Bg -ve control

Fig. 2-1. Graphical presentation of emulsifying abilities of oil degrading bacteria isolated from soil samples obtained from Site 2, Caltex. The percentage of emulsion were: 69%, 70%, and 66% for 2Bf, 2Bf* and 2Bg respectively.

2.4.3. Identification of the three best bioemulsifier producing strains

2.4.3.1 Primary characterization of strains 2Bf, 2Bf* and 2Bg

The standard biochemical methods for preliminary bacterial classification indicated that under the conditions used, the three best bioemulsifying strains (2Bf, 2Bf* and 2Bg) were from the genus *Pseudomonas* (Table 2-4). Since they were all from the same genus, it was decided to concentrate on one of the three strains. Hence, strain 2Bf was chosen for further study, because of its ability to produce slightly higher levels of bioemulsifying agents.

Table 2-4. Primary characterization and identification of the three best bioemulsifier producing strains.

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

2.4.3.2. Partial 16S rDNA PCR

The 16S rRNA sequences are highly conserved in different species, hence it used as a means of comparison for the classification of microorganisms. Using the universal primers (Weisburg *et al.*, 1991) a 1.5 kb rDNA fragment was amplified from the strain 2Bf by PCR (Fig 2-2). The PCR product was sequenced using F3 and R3 universal primers and analysed using the BLAST search. Sequence analysis revealed 97% sequence identity to four strains of *P. aeruginosa* and 99% to the strain *Pseudomonas aeruginosa* PAO1 16S rRNA gene (Table 2-5) (Stover *et al.*, 2000). Hence the newly isolated strain was designated *P. aeruginosa* for future work.

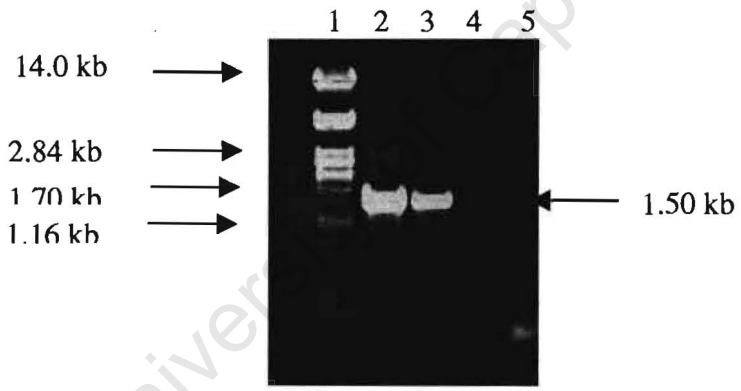


Fig. 2-2. Agarose gel electrophoresis of PCR products following PCR amplification of the strain 2Bf using 16S rRNA universal primers. Lane 1 contained λ DNA digested with restriction enzyme *Pst*I. Lanes 2 and 3 contained PCR products synthesized with $MgCl_2$ concentrations of 2 and 2.5 mM respectively. Lane 5: No DNA, represents negative control.

Table 2-5. Sequence similarities of the 16S rDNA sequence of the strain 2Bf to sequences in database.

Organisms to which significant sequence similarity was found	% Identity
P. aeruginosa PAO1 (Accession no. AE004844)	99% (1302/1315)
P. aeruginosa MO2 (Accession no. AY162138)	97% (1277/1315)
P. aeruginosa BHP7-6 (Accession no. AY162139)	97% (1277/1315)
P. aeruginosa OLB-1 (Accession no. PSP387904)	97% (1277/1315)
P. aeruginosa AL98 (Accession no. PAC249451)	97% (1277/1315)
Gamma proteobacterium clone (AF529330)	97% (1277/1315)

2.4.4. Surface tension measurements

Not all bioemulsfying agents produced by microorganisms are capable of reducing the surface tension of the medium. Therefore, the bioemulsifying agent produced by the strain *P. aeruginosa* 2Bf, which was observed to be good bioemulsifier, was tested for its ability to lower the surface tension of the medium. For such measurements, the strain *P. aeruginosa* 2Bf was grown for four days in MSM supplemented with either 2% (w/v) glucose or 1% (v/v) C_{14}-C_{17}. As it is mentioned in Chapter 1, certain microorganisms are capable of producing bioemulsifying agents to some degree both in hydrophobic and hydrophilic substrates. Hence, for this reason, the strain was grown on the hydrophobic substrate (1% (v/v) C_{14}-C_{17}) and a hydrophilic substrate (2% (w/v) glucose). The emulsification assays, which were performed prior to surface tension measurements, revealed positive results with an emulsion index (E1) of about 70% for both cultures. This shows the bioemulsifying agent produced by the strain 2Bf was secreted into the culture medium regardless of the hydrophobicity of the substrate. However, the surface tension measurements conducted under the same growth conditions revealed that the

strain *P. aeruginosa* 2Bf was ineffective in lowering the surface tension of the media (Table 2-6).

Table 2-6. Surface tension measurements of the strain *P. aeruginosa* 2Bf grown on 1% (v/v) C_{14}-C_{17} or 2% (w/v) glucose.

Bacterial strain	Substrate used	Surface tension (mN/m)
P. aeruginosa 2Bf	1% (v/v) C_{14}-C_{17}	70.22
P. aeruginosa 2Bf	2% (w/v) glucose	70.28
E. coli	2% (w/v) glucose	71.12

The surface tension of supernatants obtained from cultures of *P. aeruginosa* 2Bf grown in MSM supplemented with either 1% (v/v) C_{14}-C_{17} or 2% (w/v) glucose as energy sources were very similar, giving values of 70.22 mN/m and 70.28 mN/m, respectively. These values in turn were only slightly lower than that of distilled water (which is 71.8 mN/m) and the negative control, *E. coli* (71.12 mN/m) suggesting that, while *P. aeruginosa* 2Bf produces an effective bioemulsifier, it does not lower the surface tension of the medium under these conditions.

2.4.5. PHA synthesis by *Pseudomonas aeruginosa* 2Bf

2.4.5.1. Nile Blue staining

Some oil degrading bacteria were reported to accumulate PHA granules when supplied with excess carbon source under nitrogen-limited conditions (Ramsay *et al.*, 1990). We were also interested to investigate whether the strain *P. aeruginosa* 2Bf was also capable of accumulating PHA granules when grown on alkanes so as to produce two specialty compounds simultaneously. Thus in the present study, the strain, *P. aeruginosa* 2Bf was

grown in nitrogen limited (0.4 g/l $(NH_4)_2SO_4$) MSM media containing 2% (v/v) C_{14}-C_{17} alkane fraction as carbon source.

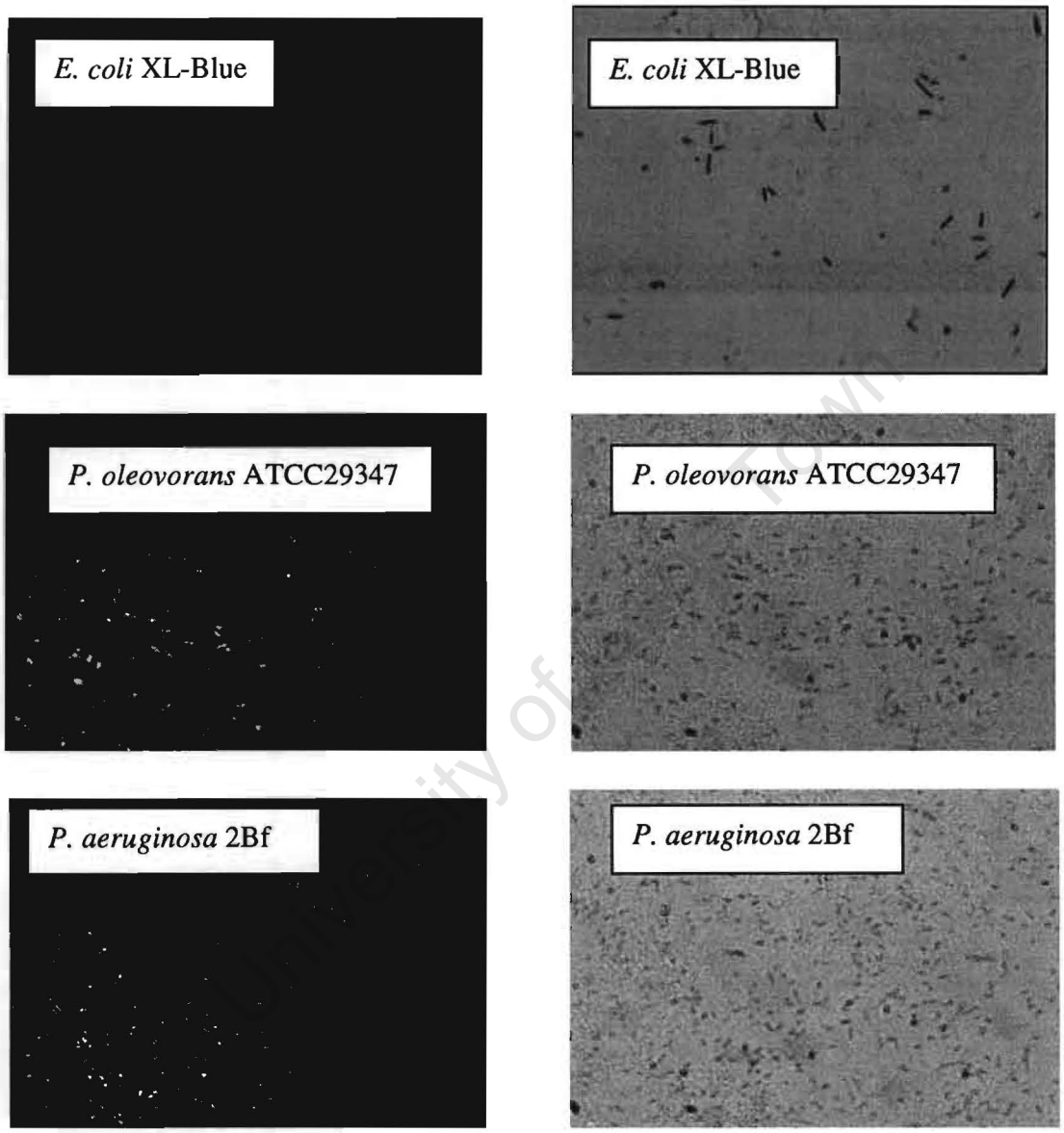


Fig. 2-3. Nile Blue staining of *Pseudomonas aeruginosa* 2Bf compared to the positive control strain *P. oleovorans* ATCC29347 and the negative control E. coli XL1_Blue. Pictures on the left hand side were captured from the fluorescent microscope, while those on the right hand side were captured from normal light microscope (magnification, 1000 X). The bright orange fluorescence is indicative of the presence of PHA granules.

As shown in Fig. 2-3, *P. aeruginosa* 2Bf was observed to be Nile Blue positive. A bright orange fluorescence, both from fluorescent pictures captured for *P. aeruginosa* 2Bf and

the positive control *P. oleovorans* ATCC29347, indicate the accumulation of PHA granules, while no fluorescence was observed in the negative control, *E. coli* XL-Blue.

2.4.5.2. Molecular approach in detecting *phaC* gene

The synthesis of PHAs requires the enzyme PHA synthase (PhaC) which is encoded by the gene *phaC*.

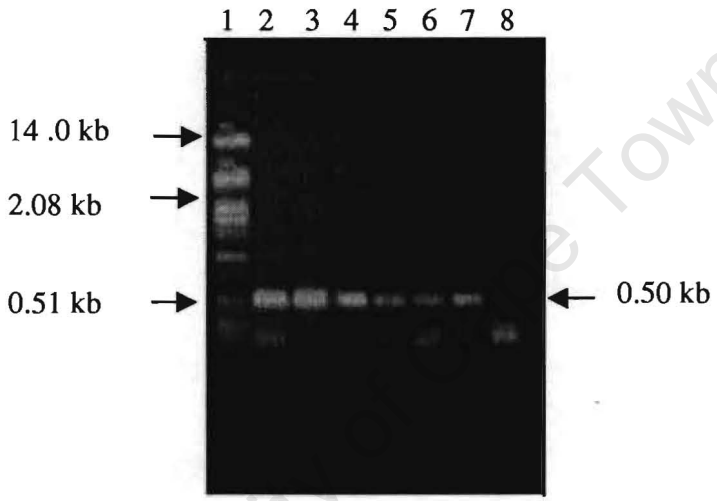


Fig. 2-4. PCR amplification of the *phaC* gene. Lane 1 contained λ DNA digested with restriction enzyme *Pst*I. Lanes, 2, 3 and 4 contained PCR products from *P. aeruginosa* 2Bf obtained using $MgCl_2$ concentrations of 2, 2.5 and 3.0 mM respectively. Lanes, 5, 6 and 7 contained PCR products from *P. oleovorans* ATCC29347 using $MgCl_2$ concentrations of 2, 2.5 and 3.0 mM respectively. Lane 8 was the negative control.

Hence, the genotypic detection of the gene *phaC* based on PCR amplification can be used to detect the inherent ability of microorganisms to accumulate PHAs granules. Thus, the presence of the *pha*C gene, encoding the PHA synthase enzyme, in the strain *P. aeruginosa* 2Bf was confirmed by PCR amplification, using primers designed to conserved regions in the *phaC* genes from a number of bacterial genera (Sheu *et al.*, 2000) (Fig. 2-4). The size of the PCR product, as shown in Fig. 2-4, was approximately 0.5 kb which agrees with the predicted size of the *phaC* gene from different bacteria

(Sheu *et al.*, 2000). Furthermore, it is the same size as the PCR product obtained from the positive control, *P. oleovorans* ATCC29347 (lanes 5-7). The lower bands indicated in Fig. 2-4 are the primer-dimers. The PCR amplification of the *phaC* gene was conducted using three different concentrations of $MgCl_2$ (i.e. 2, 2.5, and 3 mM) to improve the yield of PCR products. The results confirm the presence of the *phaC* gene in *P. aeruginosa* 2Bf, and support the previous results that the fluorescence detected by Nile Blue staining was due to PHA accumulation by the strain.

2.5. Discussion

Several phenotypically distinct bacterial strains were isolated from oil-polluted soil samples using crude oil as carbon source. Although the aim of this project was to produce value-added products from n-alkanes, crude oil was used as the carbon source for the isolation of bacterial colonies using enrichment cultures, because the soil samples used were collected from crude oil polluted sites, and the microorganisms from the soil samples would be more adapted to using crude oil. After four subsequent subcultures, dilutions of the enrichment cultures were made accordingly and spread into nutrient agar plates for selecting the different isolates. MSM solid medium, supplied with 1% (v/v) crude oil as carbon source, would have been more appropriate for selection than nutrient agar. However it was difficult to prepare MSM solid medium because the less dense crude oil on the surface of the media and incurs difficulties in separating colonies. On the other hand, it is worth mentioning the problems of using the nutrient-rich nutrient agar medium for selection because certain microorganisms, which might have been out competed in the enrichment cultures, may appear as dominant strains in the nutrient agar plates and vice versa. Therefore, all the strains isolated from the different soil samples were screened for their inherent abilities to produce bioemulsifying agents. Three strains, which were all identified to be from genus *Pseudomonas*, were shown to produce good bioemulsifying agents. The most effective producer of bioemulsifying agents, strain 2Bf, was identified as *P. aeruginosa* and the 16S rDNA sequence was shown to have 99% identity to the previously identified strain *P. aeruginosa* PAO1 (Stover *et al.*, 2000). Hence we concluded that the strain 2Bf is one of *P. aeruginosa* species and have chosen to designate it as *P. aeruginosa* 2Bf.

In several reports, *P. aeruginosa* was shown to produce a rhamnolipid biosurfactant when grown on *n*-Hexadecane (Hisatsuka *et al.*, 1971; Haba *et al.*, 2000; Lang & Wullbrandt, 1999). Rhamnolipids stimulate the growth of *P. aeruginosa* on hydrocarbon substrates by lowering the surface tension and emulsifying hydrocarbons and thereby increase the bioavailability of hydrophobic substrates (Haba *et al.*, 2000). However, although in the present study, *P. aeruginosa* 2Bf showed good emulsifying activity, it was inefficient in lowering the surface tension, unlike the above reports. Hence it is possible to speculate that the bioemulsifier produced by this particular strain is probably not a rhamnolipid biosurfactant, or it may have different properties. However, some studies have revealed that certain strains of *P. aeruginosa* produce a peptidoglycolipid biosurfactant in place of rhamnolipids (Ilori & Amund, 2001; Hisatsuka *et al.*, 1972; Koronelli *et al.*, 1983). Although only preliminary studies have been performed on the peptidoglycolipid bioemulsifier of *P. aeruginosa*, several studies have been conducted on the polymeric bioemulsifiers produced by *Acinetobacter calcoaticus*, *A. radioresistens*, and on the mannoproteins by certain yeast species (Cameron *et al.*, 1998; Desai & Bannat, 1997; Rosenberg & Ron, 1999; Goldman *et al.*, 1982; Navon-Venezia *et al.*, 1995). High molecular weight (polymeric) bioemulsifiers were reported to be less effective in reducing surface and interfacial tension, but they are highly efficient bioemulsifiers (Ron & Rosenberg, 2001). Hence, it is possible that the strain isolated in this study produces a bioemulsifier such as a peptidoglycolipid biosurfactant. The stain *P. aeruginosa* was shown to produce emulsifying agents both in hydrophobic (C_{14}-C_{17}) and hydrophilic (glucose) substrates. Certain microorganisms such as *B. subtilis* (Wei & Chu, 1998; Cooper *et al.*, 1981), *P. aeruginosa* (Babu *et al.*, 1996), *S. cerevisiae* (Cameron *et al.*, 1998), *C. fuscians* (Cooper *et al.*, 1982) were shown to produce bioemulsifying agents in

non-hydrophobic substrates. The ability of the strain *P. aeruginosa* 2Bf to produce an emulsifying agent with a renewable substrate (i.e. glucose) could be considered as an added advantage in minimizing the cost of using hydrocarbons as growth substrates.

In addition to the bioemulsifier, the strain *P. aeruginosa* 2Bf was suggested to accumulate PHA granules when grown on n-C_{14}-C_{17} alkane fractions under nitrogen-limited conditions. A bright orange fluorescence from pictures captured for *P. aeruginosa* 2Bf after Nile Blue A staining of the cells, indicates the accumulation of PHA granules. The presence of the PHA synthase gene was confirmed genetically by the amplification of the *phaC* gene using primers designed to conserved regions of *phaC* genes from various bacterial strains (Sheu *et al.*, 2000). The presence of the PHA synthase gene does not always correlate with the ability of the organism to accumulate PHA granules detected by Nile Blue A staining. Sheu *et al.* (2000), for example, have noted that five strains out of thirty-eight strains isolated from the environment, which were PHA positive based on PCR, were not observed to accumulate PHA granules as detected by Nile Blue A staining. They suggested that this might be due to inappropriate growth conditions used in the culture medium or to a low yield of PHA granule accumulation resulting in negative detection by Nile Blue A staining. This outcome could also reflect the possibility that there may be some bacterial isolates that harbour non-functional PHA synthase genes. Therefore, the use of both methods (i.e. phenotypic and genetic) would be important in determining the potential of a microorganism to accumulate PHA granules.

Enrichment techniques using crude oil as a carbon substrate were therefore useful for the isolation of novel microorganisms with the ability to utilise alkane fractions efficiently.

These bacteria were screened for the production of bioemulsifiers, and the strain capable of producing the most effective bioemulsifier was identified as *P. aeruginosa*. This strain was also found to be capable of accumulating PHAs after growth on the alkane fractions for four days.

Chapter 3

Analysis of growth and bioemulsifier production by *P. aeruginosa*

Contents

3.1. Summary

The growth of the strain *Pseudomonas aeruginosa* 2Bf on different hydrocarbon substrates was studied along with the biosurfactant production. Five different growth measurements: OD, viable cell counts, microscopic cell counts, dry weight biomass and total protein content, were used to monitor the growth of *P. aeruginosa* 2Bf. Viable cell count was found to be more appropriate and reliable in monitoring the growth of *P. aeruginosa* 2Bf on hydrocarbon substrates compared to others.

The maximum cell growth and biosurfactant production, as measured by a specific colorimetric method (i.e. Orcinol assay), was observed to occur at about 40 hours of incubation. Little effect was observed on the cell numbers and biosurfactant production, when the C_{14}-C_{17} n-alkane fraction carbon source supplied was either 1, 5, 10, or 15% (v/v), although slightly greater cell numbers and yields of biosurfactant were observed when 10% (v/v) of C_{14}-C_{17} was used as carbon source.

The growth of *P. aeruginosa* 2Bf was observed to be different in the laboratory-scale bioreactor cultivation to that in shake flasks. A marked reduction in log phase was observed when the strain *P. aeruginosa* 2Bf was grown in batch fermentor. The culture from a fermentor was observed to change its pigmentation and a milky appearance was observed at 65 hours of fermentation. Such an abrupt change in color was possibly attributed to the production of extracellular wax esters by *P. aeruginosa* (Wu & Ju, 1998).

3.2. Introduction

In order to optimize the production of bioemulsifiers by microorganisms, it is important to understand the production of biosurfactants in relation to microbial growth. Hence, accurate and suitable growth measurements need to be applied in delineating microbial growth on hydrocarbon substrates. There are several techniques used to express microbial growth. Turbidimetric measurements are most commonly used since they generally are rapid and easy for indirect estimation of biomass, however they can be problematic when applied to growth on hydrocarbon substrates due to the interferences of water insoluble oil, and because of the tendency of cells to attach to hydrophobic substrates (Marino *et al.*, 1998). Dry weight biomass measurements (Green *et al.*, 2000), enumeration of colony forming units (CFUs) on agar (Ilori & Amund, 2001), and total protein content as an indirect biomass measurements (Haba *et al.*, 2000) are methods, which have been successfully used to delineate the growth of different microorganisms on *n*-alkanes.

Various methods have been published on assaying bioemulsifier production, which in turn depend on both the properties of the bioemulsifier as well as the substrate. As described by Ilori & Amund (2001) and in Chapter 2 (Section 2.3.2), the emulsion index (E1) can be used as a crude measurement of emulsion potential. A variation of this basic method is described by Patil & Chopade (2001) in which they used a turbidimetric assay to express emulsion potential. In contrast to the first method, this method is suited to assaying bioemulsifier that can generate very fine emulsions of the substrates used. Quantitative methods, including the quantitation of carbohydrate or protein depending on the nature of the bioemulsifier, have also been used to assess the

production of bioemulsifiers by microorganisms. The Orcinol assay, for example, which is based on the quantitation of the rhamnose sugar, has been used to assess the production of rhamnolipids (Koch *et al.*, 1991).

In the present study, all the growth measurements were tested for their efficacy to monitor the growth of *P. aeruginosa* on *n*-alkanes. The production of the bioemulsifier by this strain was monitored by both the Orcinol and emulsifying assays in relation to the growth of *P. aeruginosa* 2Bf. In addition, since it has been reported in strain *P. aeruginosa* 47T2NCIB that the carbon to nitrogen ratio (C/N) affects biomass and biosurfactant production (Haba *et al.*, 2000), cultures were also supplemented with different concentrations of the carbon source (i.e. C_{14}-C_{17} *n*-alkane fraction) to determine the specific effects of C/N ratio on biomass and biosurfactant production by *P. aeruginosa* 2Bf. In order to extract and analyze the emulsifier (Chapter 4), produced by *P. aeruginosa* 2Bf, it was also grown in a 6 litre fermentor. Growth assays, including enumeration of colony forming units, dry weight biomass and turbidimetric readings were performed during the fermentation process for comparison purposes.

3.3. Materials and methods

3.3.1. Growth conditions

Growth of *P. aeruginosa* 2Bf was analysed with respect to OD_{600}, viable cell counts, dry weight biomass and total protein content during growth in MSM supplemented with C_{14}-C_{17} *n*-alkane fraction (SASOL, South Africa) as carbon source. An overnight culture of *P. aeruginosa* 2Bf was prepared by inoculating 5 ml of LB media with a single colony off MSM agar plates supplemented with 1% (v/v) C_{14}-C_{17} *n*-alkane fraction. One ml of the overnight culture was washed with MSM by centrifugation at 10 000 x g for 5 minutes The pellet was recovered and resuspended in 1 ml MSM, and 100 µl was used to inoculate in 100 ml MSM culture media supplemented with the C_{14}-C_{17} *n*-alkane fraction as energy source with concentrations ranging between 1% and 15% (v/v) as described in the text. Cultures were grown at 30°C with shaking. In addition, the pure alkanes, n-tetradecane and n-hexadecane (Fluka) were also used as carbon source as described in the text.

Protein content as an indirect measurement of biomass

The protein content (mg/l) of culture samples was measured using the Bio-Rad method according to the manufacturers instructions against a standard of bovine serum albumin. Samples were prepared in duplicate by centrifuging 5 ml of the culture at 5 000 x g for 8 minutes. The pellets were then resuspended in 2 ml MSM medium each. One sample was used to determine the protein concentration on whole-cell suspensions, while the other sample was used to determine protein concentration of the cell-free

extract (CFE) following sonication. Sonication was done using a Virsonic Sonifier at 10 amplitude microns for 3 minutes (Department of Molecular and Cell Biology, UCT) for five minutes to disrupt and burst open cells to release intracellular components.

Turbidimetric methods (OD measurements)

The optical density at 600nm (OD) of the culture samples was measured in a spectrophotometer (Beckman DU 530, Life Science UV/Vis spectrophotometer). Dilutions were made in sterile distilled water so that the OD_{600} reading did not exceed 0.6 units. The samples, prepared in duplicate prior to taking the readings, were allowed to settle for 5 minutes so as to minimize interference from the presence of C_{14}-C_{17} *n*-alkane fraction.

Viable cell counts

Colony forming units per 1 ml (CFU/ml) were determined by plating different dilutions of the culture samples, prepared in sterile distilled water, on agar plates. Prior to dilution and plating, samples were vortexed vigorously to ensure a homogeneous preparation. Experiments were done in triplicate, and results show the average of the three counts taken.

Microscopic counts (Total cell counts)

Cell concentrations were also determined by direct microscope counts, using a counting chamber (Thoma counting chamber). With a depth of 0.1 mm, and an area of 0.00065 mm^2 per mini square each. Dilutions of cell suspensions were made in order to achieve a cell density between 5 and 50 for accurate counting, which was done under

100x magnification. Prior to counting, the diluted cell suspensions of *P. aeruginosa* 2Bf were put in ice for ten minutes in order to minimize problems incurred in counting due to motility of cells. The average cell counts from 10 different mini squares was calculated, the dilution factor was accounted for accordingly, and the total cell counts were expressed as cells/ml.

Dry weight biomass determination

The dry weight biomass was determined in duplicate by filtering 5 ml of the sample culture through a pre-weighed filter paper. The filter paper was dried at 60°C for three days, after which it was weighed consecutively until a constant reading was achieved. The cell dry weight biomass was then estimated by subtracting the weight of the pre-weighed filters from the final weight. Weight measurements were taken using a four decimal point sensitive weighing balance (Mettler, Switzerland).

3.3.2. Analysis of the bioemulsifier

Emulsification assay

The emulsification assay, as described in chapter 2, was used to assess the extracellular production of the bioemulsifier by *P. aeruginosa* 2Bf. The emulsion index (E1) was used as an indicator of the amount of bioemulsifier produced at different stages of growth.

Orcinol assay

The Orcinol assay, described by Koch *et al.* (1991), with some modifications, was used to directly detect the amount of carbohydrate present in the sample as an indication of

the bioemulsifier concentration. To 333 µl of the culture supernatant prepared by centrifugation at 10 000 x g for ten minutes, 1 ml of chilled acetone was added and allowed to stand at 4°C over night. It was centrifuged for 10 minutes at 10 000 x g, after which the pellets were dried in vacuum evaporator for 1 hour to remove residual acetone. The pellets were then resuspended in 100 µl of distilled water. To 100 µl of each sample, 900 µl of a solution containing 0.19% (w/v) Orcinol (in 53% (v/v) H_2SO_4) was added. After heating for 30 minutes at 80°C, the samples were cooled for 15 minutes at room temperature, and the A_{421} was measured. The carbohydrate concentrations were calculated by comparing the absorbance with those of rhamnose standards between 0 and 50 µg/ml.

3.3.3. Batch fermentation of *P. aeruginosa* 2Bf

The fermentation was carried out in a bioreactor with a working volume of 6L, under the following conditions: temperature, 30°C; agitation, 500 revolutions per minute (rpm), and initial aeration, 0.8 vvm. The pH was controlled at 6.5 (±0.5) by automatic addition of 2N NaOH. The media composition was the same as for the shake flasks, but this time the MSM salt solution was autoclaved separately and added into the MSM medium afterwards, to minimize precipitation of culture constituents.

The inoculum was prepared in two stages prior to inoculation of the fermentor;

1. A single colony was taken off the MSM agar plates, and inoculated into 60 ml MSM broth, and incubation for 30 hours at 30°C with shaking.

2. Thereafter, the 60 ml culture was transferred into 600 ml MSM broth. The 600 ml culture was incubated for 15 hours at 30°C with shaking, before being used as a starter culture for the fermentation process.

During the fermentation process, 10 ml aliquots were withdrawn at specific time intervals and the following measurements performed on each: viable cell counts, OD_{600}, dry weight biomass, and Orcinol assays were done from each of the samples.

3.4. Results

3.4.1. Growth measurements

The presence of hydrophobic substrates in the growth medium causes problems with certain growth measurements. Hence suitable and accurate methods need to be established for measuring the growth of microorganisms on hydrophobic substrates such as n-alkanes. In this study, five different growth measurements were performed as preliminary trials to follow the growth of *P. aeruginosa* 2Bf, in order to assess the feasibility of the different methods, and to select a suitable and appropriate strategy to express growth of *P. aeruginosa* 2Bf on n-alkane substrates.

As an indication of biomass, the total protein content was measured on two different fractions on crude CFE (prepared by sonication) and whole cell suspensions. As can be seen in Fig. 3-1, there were significant differences in the total protein content measured on the two different fractions over the growth period.

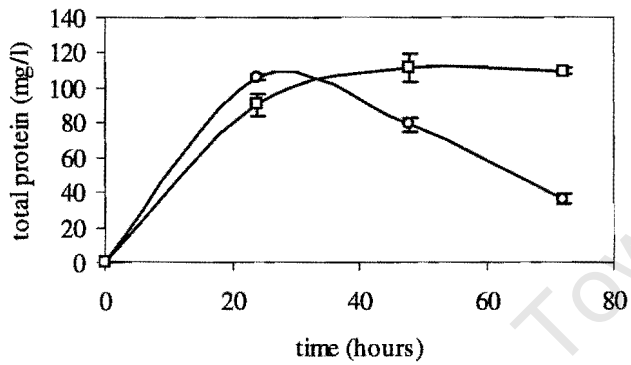


Fig. 3-1. Growth curve of *Pseudomonas aeruginosa* 2Bf, showing changes in the protein content from crude CFE (○), and whole cell suspensions (□) sample cultures. These results are the average of two readings.

The protein content was relatively higher for CFE compared to the whole cell suspensions, until 40 hours of incubation when, the protein content from whole cell suspensions was observed to exceed that of the CFE, reaching a maximum protein content 101 mg/l in the late stationary phase. In contrast, the protein content was observed to decline to 35 mg/l for CFE readings during the stationary phase. We suspect that it is because older cells may be relatively harder to lyse by sonication as compared to younger ones, which ultimately results in underestimating the total protein for older cells. This may also reflect a larger proportion of proteins accumulating at the cell surface at later growth stages, leading to higher protein readings for whole cell suspensions. These results suggest that this method is not consistent, and is subject to the stage at which the culture is being assayed.

A relatively better growth curve was obtained from OD_{600} measurements (Fig. 3-2). OD_{600} measurements were taken after letting the samples stand for five minutes so that the hydrophobic C_{14}-C_{17} *n*-alkane fraction is separated into a surface layer, and does not interfere with the turbidity measurement. However, the tendency of cells to adhere to hydrophobic substrates may be expected to result in underestimation of the OD_{600} reading, which may be more pronounced during the initial incubation hours where the ratio of n-alkanes to cells is much higher as compared to the late growth phase.

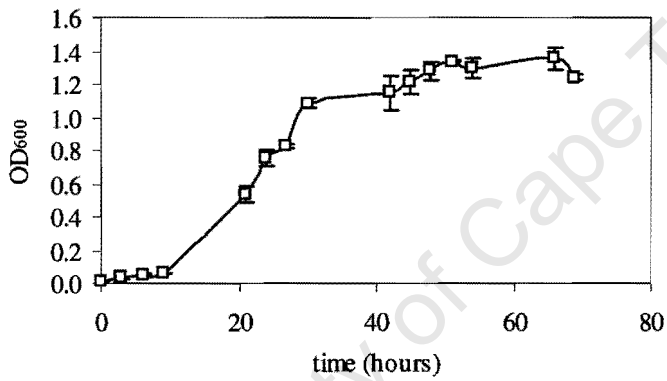


Fig. 3-2. Growth curve of *Pseudomonas aeruginosa* 2Bf as function of OD_{600} measurements at different stages of growth. These results represent the average of two readings performed in duplicate.

More reasonable results were observed from viable cell counts (Fig. 3-3). Although it is a relatively tedious method, viable cell counts appeared to provide more reliable and repeatable measurements to depict the growth cycle of the strain, *P. aeruginosa* 2Bf. However, one may underestimate the true number of viable cells due to their clumping in oil media as evidenced by the sporadic viable colony sizes. A maximum of 5.07 x 10^9 cfu/ml was obtained at the onset of the stationary phase at about 44 hours of incubation.

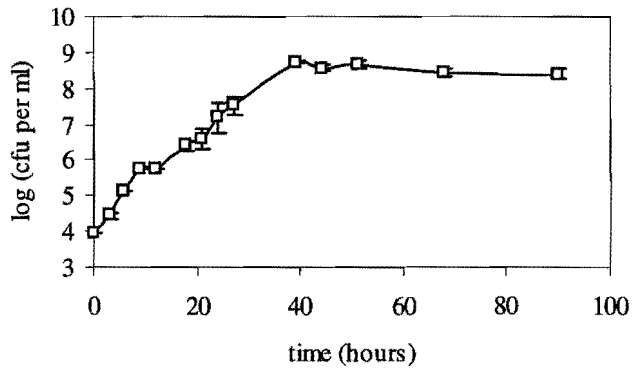


Fig. 3-3. Growth curve of *P. aeruginosa* 2Bf showing changes in viable cell number at different stages of growth. These results were the averages of three counts.

The growth pattern observed from total cell count (microscopic counts) was slightly different than the viable colony counts (Fig. 3-4). The total number of cells obtained from microscopic counts (1.68 x 10^{10} cells/ml) was higher than from viable colony counts (5.07 x 10^{9} cells/ml) possibly because, in addition to the viable count method potentially underestimating the true number, microscopic counts also include non viable cells.

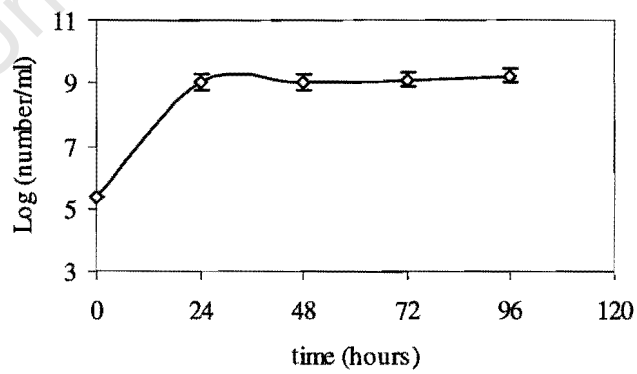


Fig. 3-4. Growth curve of *P. aeruginosa* 2Bf showing changes in total cell number (microscopic counts) at different stages of the growth cycle. These results were the average 10 counts made on the different mini squares.

The use of dry weight biomass as a measure of cell growth yielded a very different profile. First a sharp increase in biomass was observed between 30 to 40 hours of incubation, followed by a noticeable decrease from 1.3 g/l at 66 hours to 0.8 g/l at 90 hours of incubation, during the stationary phase (Fig. 3-5).

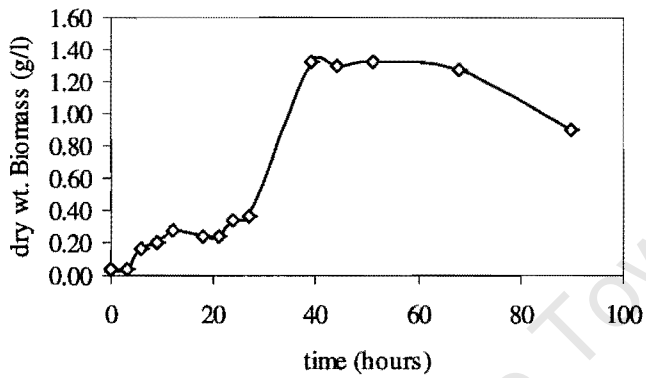


Fig. 3-5. Growth curve of *P. aeruginosa* 2Bf showing changes in dry weight biomass measurements. Values are the average of two independent readings.

Since this significant increase and decrease was not reflected in either the viable cell counts (Fig. 3-3) nor in the total cell counts (Fig. 3-4), this may be attributed to the accumulation of polyhydroxyalkanoate (PHA) inclusion bodies by the strain *P. aeruginosa* 2Bf, as indicated in Chapter 2. The maximum dry weight biomass estimated was 1.4 g/ml at about 40 hours of incubation.

From all the above methods, the enumeration of colony forming units (viable cell counts) was found to be a more reliable and appropriate method as compared to others. Hence the viable cell count was endorsed in this study to monitor the cell growth along with the production of the bioemulsifier by *P. aeruginosa* 2Bf.

3.4.2. pH

The pH of the culture medium was monitored along with the growth of *P. aeruginosa* 2Bf on 1% (v/v) C_{14}-C_{17} *n*-alkane fraction. The pH of the culture was measured using pH paper. The pH of the culture was observed to drop during the exponential phase (Fig. 3-6), dropping rapidly from 7 to 4, within 24 hours, after which it remained relatively constant at about 4 throughout the stationary phase.

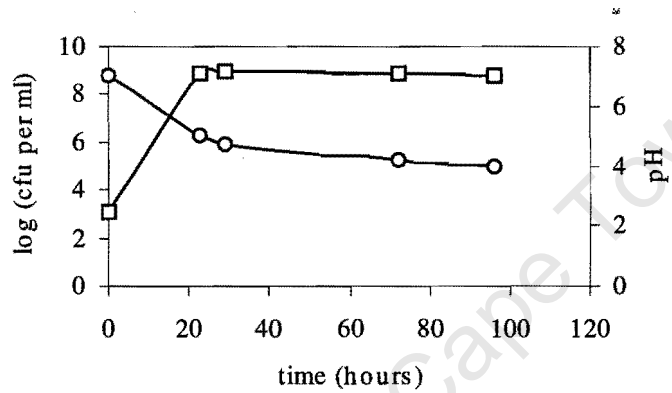


Fig. 3-6. Growth profile of *P. aeruginosa* 2Bf, expressed as cfu/ml (•) in relation to culture pH (O).

3.4.3. Effect of carbon source concentration

It has been reported in *P. aeruginosa* 47T2NCIB that the carbon to nitrogen ratio (C/N) affects biomass and biosurfactant production (Haba *et al.*, 2000). In this study, cultures were supplemented with different concentrations of the carbon source (i.e. C_{14}-C_{17} *n*-alkane fraction, ranging from 1% (v/v) to 15% (v/v)) to determine the specific effects of C/N ratio on biomass and biosurfactant production by *P. aeruginosa* 2Bf. As shown in Fig. 3-7, little effect was observed, on the viable cell numbers and biosurfactant production when the C_{14}-C_{17} *n*-alkane fraction was supplied as either 1, 5, 10 or 15% (v/v), although marginally higher number of viable cells and yields of bioemulsifier

production were recorded when 10% C_{14}-C_{17} *n*-alkane fraction was used. The highest cell number and bioemulsifier production (according to the Orcinol assay) recorded were 1.94 x 10^{10} cells/ml and 12.5 mg/l respectively. In all cases, the biosurfactant production was observed to run in parallel with cell counts, indicating that production of the bioemulsifier by *P. aeruginosa* 2Bf is growth rate dependent (Fig. 3-7).

Specific growth rate was calculated for the different supplementations during exponential growth as follows: The concentration of biomass, x (cell number/ml), increases as function of time due to conversion of food substrates to biomass;

$\mathbf{dx / dt = \mu x_0}$ (equation 1) where dx = change in cell growth,

dt = change in time, μ = specific growth rate,

and x_0 = initial growth

Equation 1, can be rearranged as follows to bring the same variables together

$\mathbf{dx / x_0 = \mu dt}$ (equation 2), by integrating equation 2, the specific growth rate, μ can be calculated as follows:

$$\mathbf{\int dx / x_0 = \int \mu dt} \quad \text{(equation 3)}$$

$$\mathbf{\ln x - \ln x_0 = \mu t} \quad \text{(equation 4)}$$

$$\mathbf{\ln x = \mu t + \ln x_0} \quad \text{(equation 5)}$$

The results calculated from equation 5, were plotted and the value μ was estimated by linear regression analysis.

The specific growth rates calculated in the presence of 5%, 10% and 15% (v/v) of the alkane fraction during the log phase were more or less comparable, and were 0.27/hour, 0.26/hour and 0.22/hour respectively, while the specific growth rate (0.17/hour)

calculated for the 1% (v/v) supplementation of the alkane fraction appeared to be lower than the other formulations. These results signify that the rate of increase in cell numbers as function of time was lower for 1% as compared to other formulations more probably because of substrate limitations in case of 1% (v/v) alkane fraction as compared to the others. It would be helpful to establish the substrate utilization rate by the analysis of alkane fractions in order to have a concrete understanding on what is happening in these cases. It is also important in biological treatment systems to establish basic relationships between the growth rate of the microorganisms involved in bioconversion process and the substrate utilization.

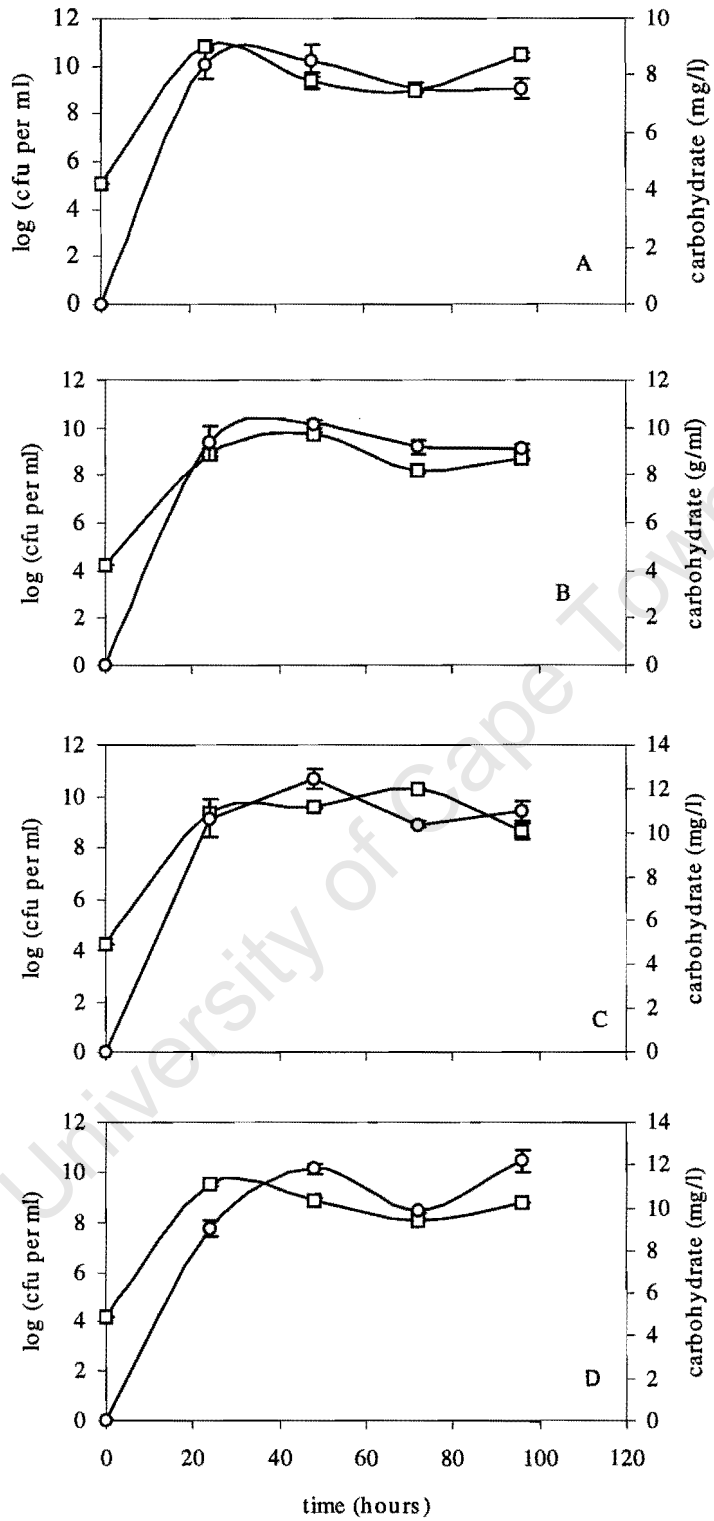


Fig. 3-7. The effect of different concentrations C_{14}-C_{17} on the viable cell growth (± S.E) (□) and biosurfactant production, measured as carbohydrate (± S.E) (○). A, 1% (v/v) of C_{14}-C_{17}; B, 5% (v/v) of C_{14}-C_{17}; C, 10% (v/v) of C_{14}-C_{17}; and D, 15% (v/v) of C_{14}-C_{17}.

3.4.4. Effect of filtration on emulsification activity

The presence of hydrophobic substrate in the culture medium makes it difficult to completely remove all traces of cells by centrifugation, because a proportion of cells remain associated with the buoyant hydrophobic substrate. The presence of cells in turn would be expected to affect/influence the bioemulsification results if the bioemulsifier is also found cell-associated. Thus, in order to more accurately determine the extracellular bioemulsifying activity, the centrifuged culture supernatant was filtered through a 0.22 µm biologically inert filter (Osmonics, USA) in order to remove any traces of cells completely. Biosurfactant production was analysed on both filtered and non-filtered culture supernatants, by both the Orcinol assay, and by assaying for emulsifying activity (Fig. 3-8).

The amount of bioemulsifier, measured by the Orcinol assay, between the filtered and non-filtered samples were very similar (Fig. 3-8A), but significant differences were found for the emulsifying activity index. Non-filtered samples showed significantly more emulsifying activity (almost 3-fold more) than filtered samples, which had cells completely removed by filtration (Fig. 3-8B). The fact that the Orcinol results for filtered and non-filtered samples were similar nullifies the significant contribution of residual cells to bioemulsifier activity according to the Orcinol method; unless it was unable to detect bioemulsifier bound to cells which is unlikely given the Orcinol assay protocol. Hence, from these observations, one can assume either there are very few cells present in the non-filtered sample or there is no cell-bound bioemulsifier. However, these results are very different to those obtained for the emulsion index (Fig. 3-8B). Non-filtered samples showed significantly more activity than the filtered ones

(in the order of 3 fold). Furthermore, maximum emulsifying activity was recorded at approximately 25-30 hours of incubation for non-filtered sample, while the much lower activity from filtered samples was observed to peak approximately 30 hours later at 50 hours. Interestingly, the maximum emulsifying activity of filtered samples coincided with the Orcinol assay peak, suggesting that these may represent the same fraction. The maximum activity represented by the non-filtered sample on the other hand appeared to be lost by filtration. This suggests that the bioemulsifier produced by *P. aeruginosa* 2Bf may be composed of two separate activities on which one is lost on filtering, while the other is retained to some degree; or may be it is made up of two components.

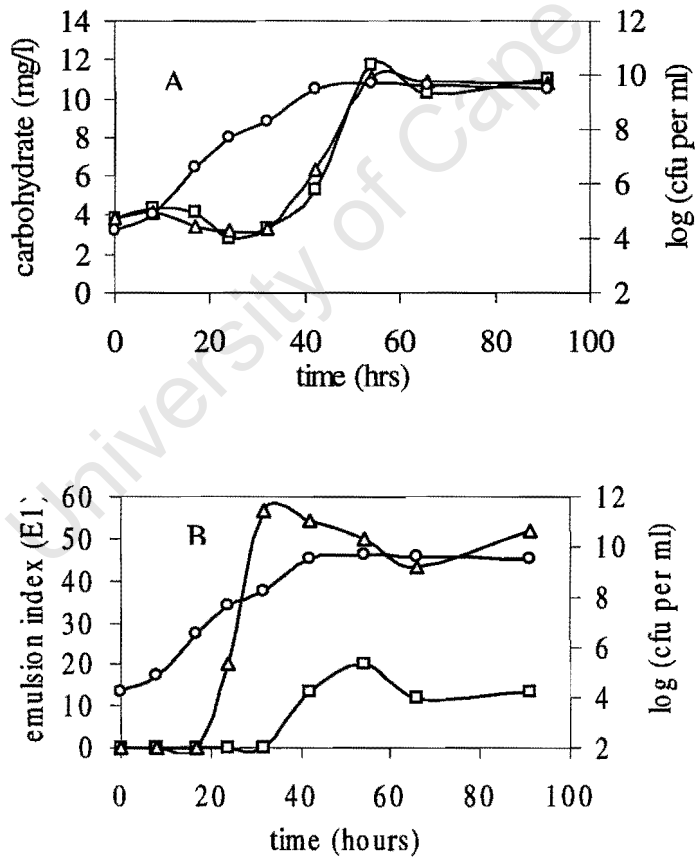


Fig. 3-8. Comparisons of the bioemulsifier activity between non-filtered (△) and filtered (□) culture samples along with the growth determined by viable cell counts (○): A, Orcinol assay and B, emulsifying activity.

3.4.5. *n*-Tetradecane and *n*-hexadecane as substrates

Pure *n*-alkanes, *n*-tetradecane and *n*-hexadecane, were used as carbon sources at 1% (v/v) for the purpose of comparison to the mixed alkane fractions. *n*-Tetradecane and *n*-hexadecane were compared as substrates with respect to cell growth and the production of the bioemulsifier as measured by the Orcinol assay (Fig. 3-9). There were no significant differences observed in the maximum cell number ultimately achieved and in bioemulsifier production between the two substrates. The maximum number of cells recorded, which were reached at 36 hours of incubation, were 2.33×10^9 cfu/ml and 1.63×10^9 cfu/ml for n-tetradecane and n-hexadecane, respectively. This is comparable with the highest number of cells obtained from 1% (v/v) n-alkane fraction, which was approximately 5.07×10^9 cfu/ml. The specific growth rates, 0.40 per hour for n-tetradecane and 0.41 per hour for n-hexadecane, were similar, but the specific growth rates calculated from pure alkanes appeared to be greater than the C_{14}-C_{17} alkane fraction, which was 0.17 per hour when 1% (v/v) of the alkane fraction was supplied as carbon source. The maximum production of the bioemulsifier was delayed for about 8 hours in the case of *n*-tetradecane as compared to *n*-hexadecane, but reached the same value after 48 hours of incubation. The maximum amounts of bioemulsifier production recorded were 9.56 mg/l and 9.48 mg/l from *n*-tetradecane and *n*-hexadecane respectively at 47 hours of incubation, which was lower than the yield from the C_{14}-C_{17} alkane fractions (12.5 mg/l).

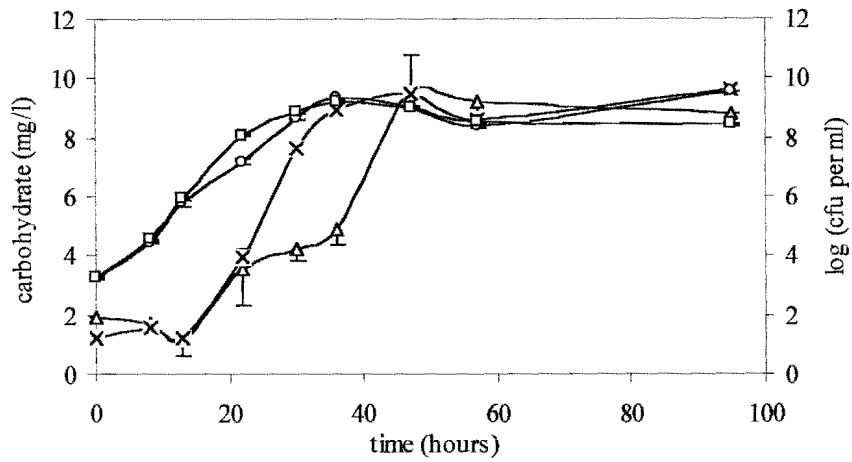


Fig. 3-9. Growth of *P. aeruginosa* 2Bf on *n*-tetradecane and *n*-hexadecane. Symbols: ○, cell number (*n*-tetradecane); □, viable cell number (*n*-hexadecane); △, bioemulsifier concentration determined as carbohydrate (*n*-tetradecane); and ×, bioemulsifier concentration determined as carbohydrate (*n*-hexadecane). Values are the average of two independent readings.

3.4.6. Laboratory-scale bioreactor fermentation of the strain *P. aeruginosa* 2Bf

The growth of *P. aeruginosa* 2Bf gave a different profile in a 6 litre bioreactor process to 100 ml shake flasks. A marked reduction of the log phase was observed from about 35-45 hours of incubation in the case of shake flasks to less than 20 hours in the bioreactor. The amount of the bioemulsifier produced by cultures grown in a 6 litre fermentor, measured by Orcinol assay, was observed to be three-fold higher than the shake flasks (Fig. 3-10), reaching a maximum of 31.5 mg/l of bioemulsifier. However, the maximum number of cells obtained from the fermentor (1.46×10^{10} cfu/ml) was fairly similar to those obtained in the shake flask (1.94×10^{10} cfu/ml). A sharp increase in the amount of the bioemulsifier was witnessed late after 51 hours of fermentation, when the culture suddenly turned a milky color.

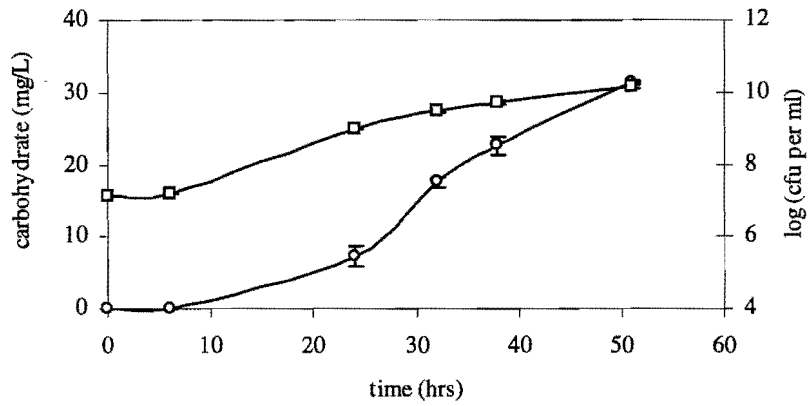


Fig. 3-10. Growth of *P. aeruginosa* 2Bf in a batch fermentor. Symbols: □, viable cell number; and ○, amount of the bioemulsifier produced, measured as carbohydrate. These results represent the average of two independent readings.

The OD_{600} measurements were also observed to increase rapidly after 40 hours of incubation (Fig. 3-11).

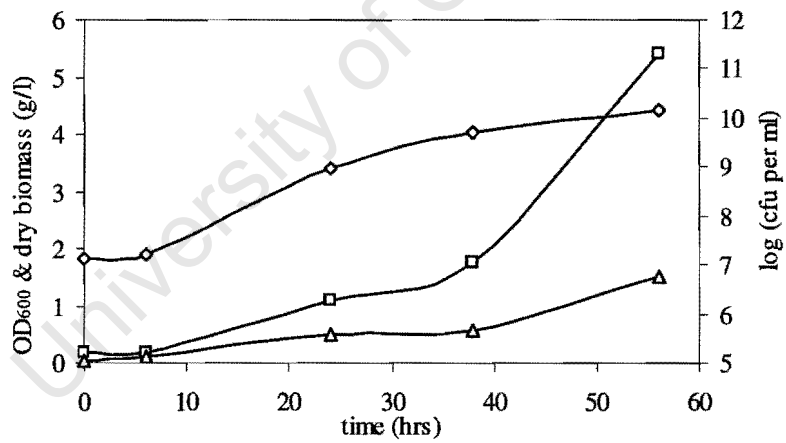


Fig. 3-11. Comparisons between OD_{600} (□), dry weight biomass (△), and viable cell number (◇) when the strain *P. aeruginosa* 2Bf was grown on a batch fermentor.

Biomass as measured by dry weight also increased between 40 and 60 hours, which may be due to the intracellular accumulation of PHAs at high cell density, leading to errors in biomass estimation.

Comparisons and correlations were established between viable cell counts, OD, and dry weight biomass measurements (Fig. 3-12). Strong correlations were obtained between the three different growth measurements. The correlation coefficient (R^2 value) between OD and viable cell counts (0.9843) was slightly higher than between viable and dry weight (0.9382) or dry weight biomass and OD (0.9810). From these relationships, OD measurement, which is rapid and rather easy, can be used to delineate the growth of the strain *P. aeruginosa* 2Bf in hydrocarbon substrates.

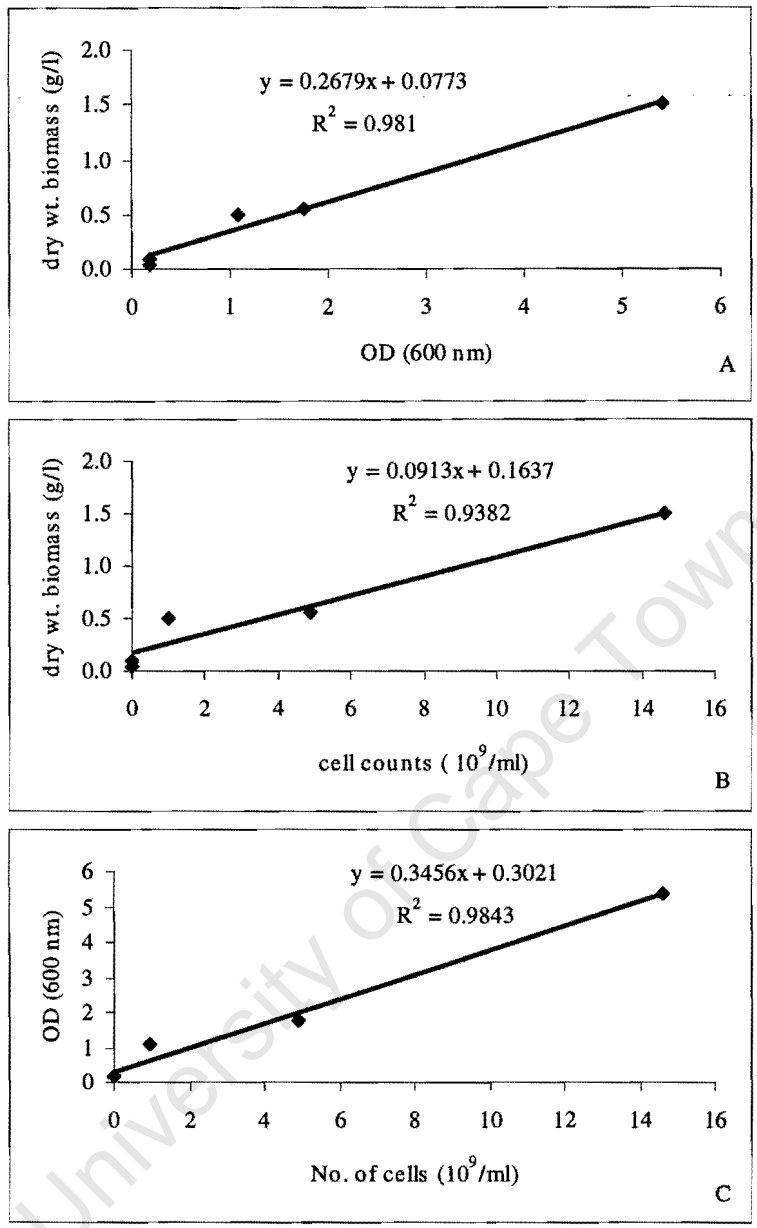


Fig. 3-12. Correlation between (A) turbidimetric measurements and dry weight biomass, (B) viable cell counts and dry weight biomass and (C) viable cell counts and turbidimetric measurements after a 56 hour fermentation of *P. aeruginosa* 2Bf grown on MSM medium supplemented with 10% (v/v) C_{14}-C_{17}.

3.5. Discussion

The purpose of this study was to obtain an appropriate and reliable method to use to measure the growth of *P. aeruginosa* 2Bf grown on *n*-alkanes, and relate it to biosurfactant production. In this study, the viable cell count method was found to be more reproducible and realistic compared to the other methods, even though one may underestimate the true number of viable cells due to their clumping in oil media as evidenced by the sporadic viable colony size. The protein measured for the CFE was observed to decline during the stationary phase, while the protein measured for the whole cell suspension correlated well with viable counts and OD_{600} measurements (Fig. 3-1). These results show the difficulties encountered in lysing older cells by sonication as compared to younger ones, which ultimately results in underestimating the total protein for older. Therefore, these results suggest that measurements using protein content is not consistent, and is subject to the stage at which the culture is being assayed. OD_{600} measurements, though rapid and easy, may result in underestimation of the biomass due to the tendency of cells to adhere to hydrophobic substrates, which may be more pronounced during the initial incubation hours where the ratio of C_{14}-$C1_7$ n-alkanes to cells is much higher as compared to the late growth phase. A rather different pattern was observed from dry weight biomass. The dry weight biomass was observed to increase abruptly at about 40 hours, and to decline sharply after 60 hours of incubation (Fig. 3-5). This phenomenon was not observed in either the viable cell numbers and total cell counts. This could be attributed to the accumulation of PHA inclusion bodies by the strain *P. aeruginosa* 2Bf as shown in Chapter 2. Hence the accumulation of these compounds by the organism, which has been reported to constitute up to 80% of the dry weight of the cell (Kim & Lenz, 2001), in the presence

of excess carbon source and their subsequent utilization by cells when the carbon source is depleted at later stages of the growth (after 60 hours) may incur biases when using dry weight biomass to measure cell growth. Therefore, the potential accumulation of PHA storage compounds by the strain should also be taken into consideration when using dry weight biomass estimation.

From all growth measurements, except for the total protein content, the growth of *P. aeruginosa* 2Bf was observed to reach its maximum point in the range of 35-45 hours of incubation. Such an exception as seen in total protein content could be attributed to the inefficiency of sonication to burst open older cells.

In the shake flask experiments, under the conditions used, the maximum amount of the biosurfactant produced, as measured by a specific colorimetric method (i.e. Orcinol assay), was observed to be 12.46 mg/l (when 10% (v/v) of C_{14}-C_{17} alkane fraction was supplied as carbon source, see Fig. 3-6). Lang & Wullbrandt (1999) reported the production of 112 g/l rhamnolipid, by *P. aeruginosa* 7107, from 163 g/l soybean oil. Haba *et al.* (2000), in their investigation on the effect of C/N ratio on cell growth and biosurfactant production of the strain *P. aeruginosa* 47T2, obtained the highest rhamnolipid production yield of 3.4 g/l at a C/N ratio of 8. The relatively lower yield in this study by the strain *P. aeruginosa* 2Bf may be because the biosurfactant produced is a peptidoglycolipid (as shown later in Chapter 4) other than rhamnolipid, and the optimum conditions for production of the polymeric biosurfactant by *P. aeruginosa* 2Bf would therefore be expected to be different from rhamnolipids. Goswami *et al.* (1994) reported maximum production of 70 mg/l of total carbohydrate concentration from the

cell free culture broth, during the stationary phase, which they accounted for carbohydrate content of a glycoprotein bioemulsifier C_{16}PSF produced by *P. cepacia* N1. Using different concentrations of the carbon source was observed to have little effect. Slightly greater cell number and biosurfactant yields were observed when 10% (v/v) of C_{14}-C_{17} was added as carbon source compared to the other formulations (Fig. 3-7). These results suggest that, under these conditions, the concentration of the carbon source was not the growth-limiting factor, and that other nutrients or any other growth conditions could be affecting the growth. These results also suggest that, alkane concentrations added up to 15% (v/v) did not limit the growth of *P. aeruginosa* 2Bf despite the hydrophobic nature and the possible presence of impurities in alkane fraction. Under these growth conditions, concentrations greater than 1% (v/v) of the alkane fraction are basically a waste of substrate. In all cases, the biosurfactant production by *P. aeruginosa* 2Bf was observed to run in parallel with viable cell numbers, indicating that production of the bioemulsifier by *P. aeruginosa* 2Bf is growth associated. Higher emulsifying activity was observed during the log phase just before the stationary phase (after which it started declining, see Fig. 3-7). This may be possibly attributed to the effect of pH. It was shown that, at approximately 30 hours, the pH of the culture drops to about 4 at the onset of the stationary phase and, as shown in Chapter 4, the activity of the extracted crude bioemulsifier was observed to be weaker at lower pH values. Hence, the drop in the emulsifying activity detected in these growth experiments could be due to the lower pH of the culture medium during the stationary phase. *n*-Tetradecane and *n*-hexadecane were compared as substrates with respect to cell growth and the production of the bioemulsifier as measured by the Orcinol assay (Fig. 3-9). There were no significant differences observed in maximum cell number

ultimately achieved and bioemulsifier production between the two substrates. The highest number cells obtained from these pure n-alkane substrates was comparable with the highest number of cells obtained from 1% (v/v) n-alkane fraction, though the specific growth rate measured from the pure n-alkanes was much higher from n-alkane fraction.

The growth of *P. aeruginosa* 2Bf in a bioreactor showed a rather different pattern to that in shake flasks. A marked reduction of the log phase was observed from about 35-45 hours of incubation in shake flasks to less than 20 hours in the bioreactor. This phenomenon could mainly be due to the differences in culture pH and dissolved oxygen encountered between batch fermentation and shake flasks. When growing in the batch fermentor, there was a continuous supply of oxygen with agitation throughout the growth. The pH of the culture in the bioreactor was also adjusted to 7.0 by the addition of NaOH throughout the fermentation process. Hence, the monitoring of pH and oxygen in the case of laboratory-scale fermentation is expected to favour bacterial growth, and to result in higher biomass yields. Moreover, 3-fold higher yield of bioemulsifier, as measured by the Orcinol assay, was obtained after 50 hours of growth in the bioreactor. Unlike the shake flasks, the fermentation culture was observed to undertake an abrupt change in color at 56 hours of fermentation. It turned milky and had a waxy particulate appearance. Wu & Ju (1998) have reported the secretion of extracellular wax esters by the strain *P. aeruginosa* ATCC 10145 grown on *n*-hexadecane. They observed the secretion of waxy particles, consisting of proteins and carbohydrates, by *P. aeruginosa* during the fermentation process. Such a phenomenon was witnessed when they tried to reduce the excessive foaming caused by rhamnolipids at higher pH (ca. >6.8) by undertaking several runs at a lower pH, i.e. 6.5±0.1. This

resulted in low yields of rhamnolipid production and the formation of large quantity of waxy particles with sizes up to 3–5 mm. The waxy polymeric material was found to contain less than 2.5% (w/w) proteins and less than 9% carbohydrates, of which only one sugar component was observed and tentatively identified as glucose by the TLC analysis (Wu & Ju, 1998). The material was found predominantly to be hydrocarbon-based, with oxidized functional groups such as esters, alcohols, and carboxylic acids being identified by FTIR (Wu & Ju, 1998). Hence this phenomenon may result in overestimating the amount bioemulsifier measured by Orcinol assay during the fermentation process due to the excess carbohydrate from the waxy material.

Chapter 4

Extraction and preliminary chemical characterization of the bioemulsifier(s)

Contents

4.1. Summary

The bioemulsifier produced by the strain *P. aeruginosa* 2Bf was extracted and characterized physicochemically. The analysis of a crude extract of bioemulsifier by thin layer chromatography showed the presence of carbohydrate, lipid and protein components. Hence the bioemulsifier produced by the strain *P. aeruginosa* 2Bf was temporarily identified as a peptidoglycolipid. Certain strains of *P. aeruginosa* produce a peptidoglycolipid in place of rhamnolipid biosurfactant. The peptidoglycolipid was observed, as shown by protease digestion, to have a protein component greatly involved in the emulsification activities. SDS Polyacrylamide gel electrophoresis showed that the molecular weight of the protein component of the bioemulsifier was estimated to be 14 400 Da. The peptidoglycolipid obtained in this study was observed to be most active at alkaline pH (pH 7-14), showing reduced activity at lower pH regimes. The crude extract of bioemulsifier was shown to be heat stable, retaining about 97% of its activity at 100°C after 1 hour. The broad pH and temperature stability observed for this bioemulsifier indicate its potential use in areas such as bioremediation, cosmetics or the food industry.

4.2. Introduction

Microorganisms produce different biosurfactants when they are grown on hydrophobic substrates. Differences may even exist between strains of *P. aeruginosa* (Goswami & Singh, 1991), although it has been shown in several reports that *P. aeruginosa* mainly produces rhamnolipid biosurfactants (Haba *et al*, 2000; Hisatsuka *et al.*, 1971). However, certain strains were reported to produce peptidoglycolipid in place of rhamnolipid (Ilori & Amund, 2001; Koronelli *et al.*, 1983; Hisatsuka *et al.*, 1972), which would require different isolation and extraction methods to be employed for the different biosurfactants (Noordman & Janssen, 2002). In the present study, the extracellular bioemulsifier produced by *P. aeruginosa* 2Bf was concentrated using two different extraction techniques, namely methanol:chloroform (2:1) extraction and acetone precipitation. The former was used previously by Juan *et al.* (1999) to extract rhamnolipid mixtures from their culture, while the acetone precipitation was used to extract the peptidoglycolipid bioemulsifiers from *P. aeruginosa* (Ilori & Amund, 2001; Hisatsuka, 1972).

In this investigation, the chemical composition of the bioemulsifier produced by *P. aeruginosa* 2Bf, was analyzed by thin layer chromatography. The emulsifying activity of the extracted bioemulsifier was characterized physicochemically with respect to pH and temperature. The ability of the bioemulsifier to emulsify a range of selected hydrocarbon substrates was also investigated. Such a study of the characteristics will help to delineate the potential uses of the bioemulsifier in areas such as bioremediation, cosmetics, medicine, and food industries.

4.3. Materials and methods

4.3.1. Extraction of the bioemulsifier

4.3.1.1. Chloroform:methanol extraction

Bioemulsifier was extracted from 1litre of the *P. aeruginosa* 2Bf culture, which had been incubated for 50 hours. After 50 hours of incubation, the pH of the culture was brought up from 4 to 8 using 10N NaOH, and the cell biomass was removed by centrifugation for 20 minutes at 10000 rev/min. The pH of the supernatant was then adjusted to pH 2 (using 3M H_2SO_4), after which equal volume of chloroform:methanol (2:1) was added (Juan *et al.*, 1999). The mixture was shaken for 10 minutes, and then it was left to stand for overnight in a separating funnel. Three distinct phases or layers were observed after the extraction stage. Each layer was carefully collected and dealt with accordingly. Special attention was given to the middle phase as one may expect to get more of the bioemulsifier in between the hydrophobic (organic phase) and hydrophilic (aqueous layer) because of its detergent-like properties. Hence the middle phase was carefully collected and centrifuged for 10 minutes at 10 000 x g. Once again, after centrifugation, the three-layer phenomenon was prevalent, but this time, the middle phase was more like a pellet and was collected carefully using a spatula and placed in a clean plastic vial. It was then left to dry in the fume hood, after which it was resuspended in distilled water. In addition, the organic phase was evaporated until chloroform was evaporated completely. After evaporation, a faint yellow solution was recovered and stored at room temperature for further analysis.

4.3.1.2. Acetone precipitation

The isolated strain, *P. aeruginosa* 2Bf was grown in 1L MSM supplemented with 10% C_{14}-C_{17} alkane fraction. The culture was incubated at 30°C for five days in a 5L flask with shaking. After incubation, the pH of the culture was adjusted up from 4 to 8 using 10N NaOH, and the cell biomass was removed by centrifugation for 20 minutes at 10 000 x g. The pH of the supernatant was then adjusted to pH 2 (using 3M H_2SO_4). The bioemulsifier was recovered from the supernatant by acetone precipitation (Ilori & Amund, 2001). Three times volume of acetone was added into the supernatant, and then it was allowed to stand for overnight at 4°C. The precipitate was then collected by centrifugation at 10 000 x g, after which it was dried in the fume hood and resuspended in sterile distilled water. Some of the crude emulsifier recovered by acetone precipitation was partially purified following the method described by Ilori & Amund (2001). The protein complex of the bioemulsifier was precipitated with $(NH_4)_2SO_4$ (analytical grade) at 80% saturation. The precipitate was collected by centrifugation at 10 000 x g for 15 minutes, redissolved in sodium phosphate buffer (0.05M, pH 7) and dialyzed against the buffer at 4°C for 12 hours. After dialysis the fraction was stored at 4°C.

4.3.2. Identification of the bioemulsifier

4.3.2.1. Thin layer chromatography (TLC)

Thin-layer chromatography (TLC) was used to analyse the extracted bioemulsifiers. Samples were applied to a silica-gel plate (20 cm x 20 cm) (Merck, Germany). Chromatograms were developed with two different solvent systems (Komagata &

Suzuki, 1987); methanol-chloroform-acetic acid (65:25:4, v/v) for the lipid component, and methanol-water-6N HCl-pyridine (80:26:4:10, v/v) for the carbohydrate and protein components. The TLC plate was then placed in a shallow pool of solvent in a developing chamber so that only the very bottom of the plate was in the liquid. It was run for about half an hour until the solvent reached the edge of the TLC plate. The spots were visualized by spraying with 0.02 Rhodamine 6G ethanol solution, ninhydrin (1% in acetone) and α-naphthol for detecting lipids, proteins and sugars respectively (Komagata & Suzuki, 1987). The Rf values of the developed spots were then calculated accordingly;

$$Rf = \frac{\text{Distance travelled by the compound}}{\text{Distance travelled by the solvent front}}$$

4.3.2.2. Estimation of the molecular weight of the protein component

The apparent molecular weight of the protein component of the bioemulsifier was estimated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE, 8%) (Laemmli, 1970). The gel was stained with Commassie blue reagent. The following marker proteins (Amersham) were used as standards: phosphorylase b (97 000 Da), albumin (66 000 Da), ovalbumin (45 000Da), carbonic anhydrase (30 000 Da), trypsin inhibitor (20 100 Da) and α-lactalbumin (14 400 Da).

4.3.3. Physico-chemical characterization of the bioemulsifier

4.3.3.1. Effects of pH and temperature

The pH of the extracted bioemulsifier was adjusted to a range between 2.0 and 14.0 with 2N HCl or 1N NaOH, after which its emulsifying activity was determined as in Chapter 2. Surface tension measurements of the crude emulsifier were taken at pH 7 and pH 14. For surface tension measurements, the crude emulsifier extracted from a 1litre MSM culture supplemented with 10% (v/v) C_{14}-C_{17} alkane fraction by acetone precipitation. The precipitate was resuspended in 250 ml distilled water and the emulsifying activity was determined to confirm the activity of the crude emulsifier. Then, it was poured into two 100 ml separate flasks and one of the flasks was adjusted to pH 7 while the other was adjusted to pH 14, after which surface tension measurements were taken accordingly as in Chapter 2.

Samples of the bioemulsifier were also exposed to heat for 1 hour in a water bath at different temperatures ranging from 20 to 100°C, and its emulsifying activity was determined thereafter.

4.3.3.2. Substrate specificity of the bioemulsifier

The emulsifying activity of the bioemulsifier prepared by acetone precipitation was investigated with a range of selected hydrocarbons to serve as substrates for emulsification. Hexadecane, dodecane, tetradecane, olive oil, castor oil, palm oil, and

almond oil were used as substrates for emulsification, and the test was performed as described in Chapter 2.

4.3.3.3. The role of the protein component

The bioemulsifier concentrated by acetone precipitation, was denatured by a specific enzyme, proteinase K (Roche Applied Science), to ascertain whether or not the protein moiety was essential for emulsifying activity. Twenty µl of proteinase K (200 mg/ml) was added to 200 µl of the crude extract. It was incubated at 37°C for 1 hour, after which the emulsification activity was determined.

4.4. Results

4.4.1. Extraction methods

Two different extraction methods were used to recover the bioemulsifier produced by *P. aeruginosa* 2Bf grown in MSM supplemented with 10% C_{14}-C_{17} for 4 days at 30°C. These were the methanol:chloroform extraction (2:1) and the acetone precipitation. The middle (pellet) and the organic (yellow solution) phases extracted by methanol:chloroform (2:1) were analysed with respect to their emulsifying activity. Emulsifying activities were observed from the two phases. However, higher emulsifying activity was obtained from the middle phase fraction (emulsion index of 70% at pH 7) than the organic fraction (emulsion index of 64% at pH 7). Greater emulsifying activity was observed from crude emulsifier recovered by acetone precipitation, with an emulsion index of 78% at pH 7.

4.4.2. Chemical composition of the bioemulsifier

The analysis of the crude bioemulsifier (recovered by acetone precipitation) by thin layer chromatography (TLC) showed the presence of carbohydrate, protein and lipid components (Fig 4-1) with R_f values of 0.86, 0.83, and 0.73 respectively. The carbohydrate and protein components would appear to be co-migrating, and therefore are probably components of the same chemical compound, while the lipid component cannot be compared to the others because a different solvent system had to be used for its migration.

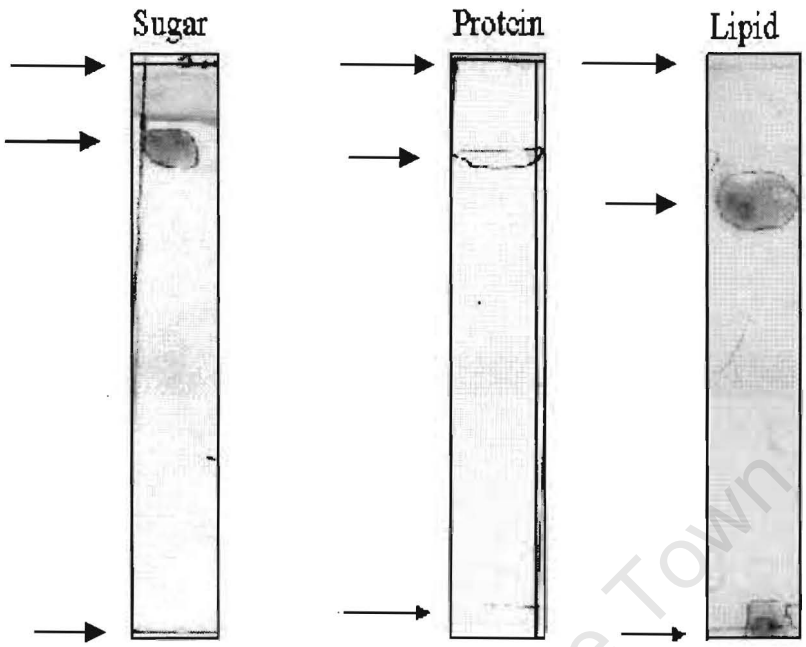
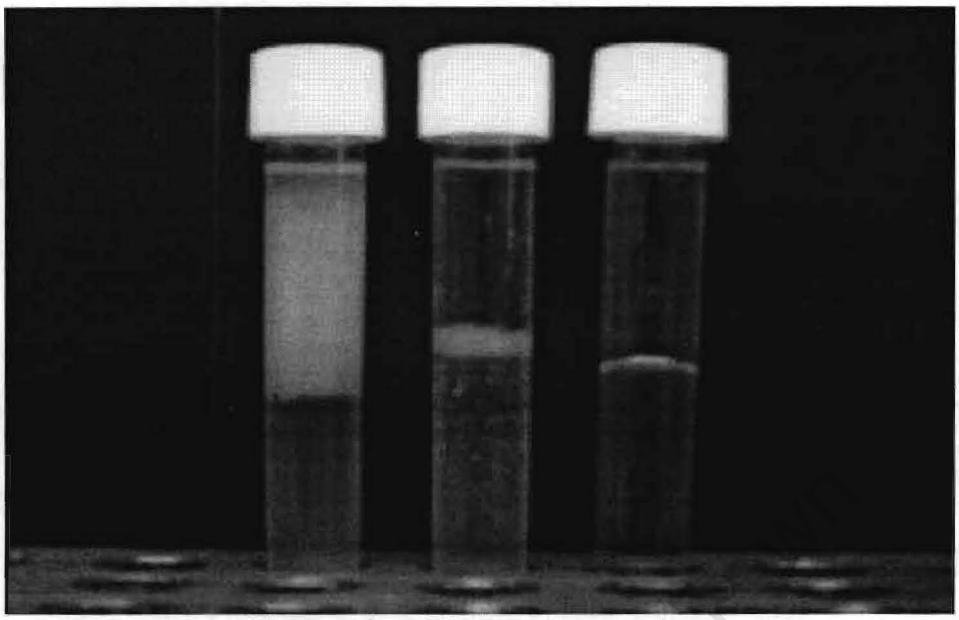


Fig. 4-1. Thin layer chromatogram of the extracted bioemulsifier. Solvent systems: methanol-chloroform-acetic acid, 65:25:4 (v/v) for lipid analysis; and methanol-water-6N HCl-pyridine, 80:26:4:10 (v/v) for carbohydrate and protein analysis. Red arrows show the solvent front, blue arrows show the new position of the compound, and black arrows show the origin.

4.4.3. The role of the protein component

The crude extract was hydrolyzed by a proteinase K enzyme, to find out whether or not the protein component of the bioemulsifier plays a major role in maintaining the emulsification properties of the bioemulsifier. After denaturation by proteinase K enzyme for 1 hour, no emulsifying activity was observed in the aqueous deproteinized bioemulsifier extract (Fig. 4-2).



Untreated Denatured negative control

Fig. 4-2. The role of the protein component of the bioemulsifier in maintaining the emulsifying activity. Untreated control contained 5 ml crude extract; denatured tube contained 5 ml crude extract treated with proteinase K for 1 hour, and the negative control contained 5 ml of distilled water.

4.4.4. Molecular weight of the protein component

A single SDS-PAGE band was obtained from the bioemulsifier extracted by either of the two extraction methods, which co-migrated with the α-lactalbumin protein standard (Fig. 4-3). The apparent molecular weight of the protein was estimated to be 14 400 Da. The crude extract of bioemulsifier was further partially purified by ammonium sulphate precipitation (Ilori & Amund, 2001), and this resulted in the same 14 400 Da protein band. However, although some of the background was eliminated by this step, the protein yield was considerably reduced.

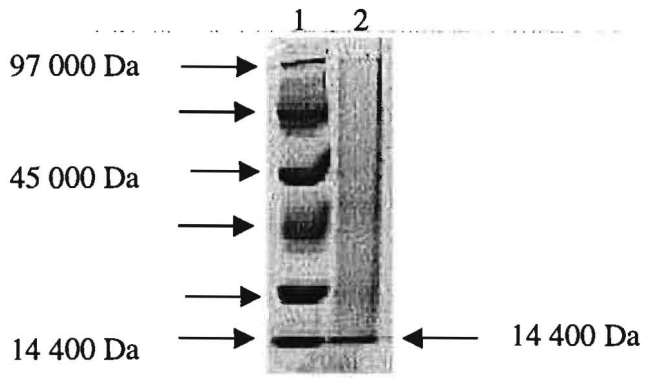


Fig. 4-3A. Polyacrylamide SDS-gel electrophoresis of the crude extract of the bioemulsifier. Lane 1, 10 µl of protein electrophoresis standards: phosphorylase b (M_r = 97,000), albumin (M_r = 66,000), ovalbumin (M_r = 45,000), carbonic anhydrase (M_r = 30,000), trypsin inhibitor (M_r = 20,100) and α-lactalbumin (M_r = 14,400).
Lane 2, 10µl of the bioemulsifier produced by *P. aeruginosa* 2Bf, extracted with chloroform:methanol (2:1).

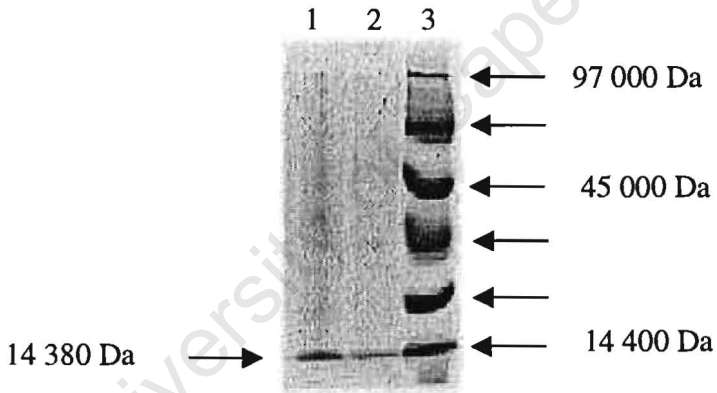


Fig. 4-3B. Polyacrylamide gel electrophoresis of the crude extract of the bioemulsifer: Lane 1, 10µl of the partially purified bioemulsifier produced by *P. aeruginosa* 2Bf, extracted with chilled acetone.
Lane 2, 10µl of the bioemulsifier produced by *P. aeruginosa* 2Bf, extracted with chilled acetone and partially purified by ammonium sulphate precipitation.
Lane 3, 10 µl of electrophoresis standards.

4.4.5. Effect of pH and temperature

Emulsifying activity of the bioemulsifier was observed over the entire pH range studied (Fig. 4-4). The bioemulsifier was most active at alkaline pH (7-14). Low emulsifying

activity was however observed at acidic pH (2-6). The effect of pH on the emulsifying activity was different for the bioemulsifier extracted by the different methods. The bioemulsifier, extracted by chloroform:methanol extraction method, was observed to lose its activity at lower pH values (less than 7), while there was reduced activity observed at lower pH when the bioemulsifier was extracted by acetone precipitation. The pattern was more or less the same at higher pH values (i.e. 7-14), though the emulsion index (E1) was slightly higher for the bioemulsifier recovered from acetone precipitation. This would indicate that the acetone precipitation method is less stringent, resulting in a higher yield of bioemulsifier. It also suggests that the acetone extract is a mixture of bioemulsifiers, one of which is destroyed by the chloroform:methanol extraction method.

Surface tension measurements of the crude emulsifier extracted by acetone precipitation were also accomplished at pH 7 and pH 14. Prior to surface tension measurements the activity of the resuspended crude emulsifier was confirmed and was shown to be still active with an emulsion index (E1) of about 72% at both pH 7 & 14. The crude bioemulsifier obtained from the strain *P. aeruginosa* 2Bf was observed to be less efficient in reducing the surface tension for the medium. Surface tensions of 69.6 mN/m and 69.2 mN/m were measured for the crude emulsifier at pH 7 and pH 14 respectively.

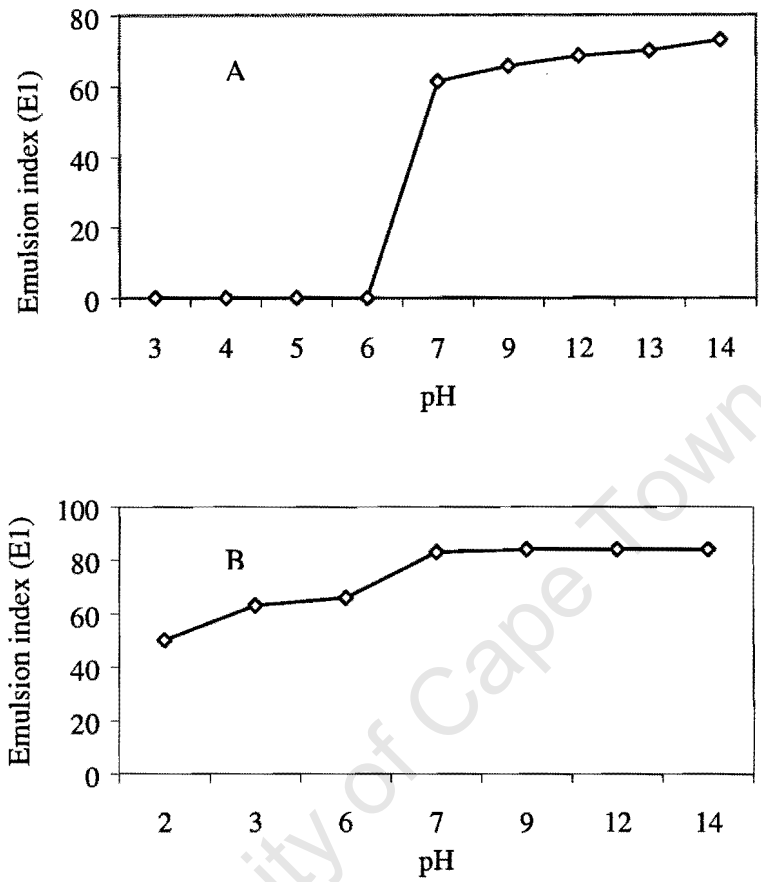


Fig. 4-4. Effect of pH on emulsifying activity of the bioemulsifier. A, bioemulsifier extracted by chloroform:methanol (2:1); and B, by acetone precipitation.

The heat stability of the crude extract prepared by acetone method was also tested at alkaline pH. The emulsifying activity of the bioemulsifier was stable after heating for 1 hour at temperatures up to 100°C (Fig. 4-5). At 100°C, about 97% of the activity was retained when compared to untreated control samples. Slightly higher activity than the controls was observed at a temperature range between 60 and 80^0C.

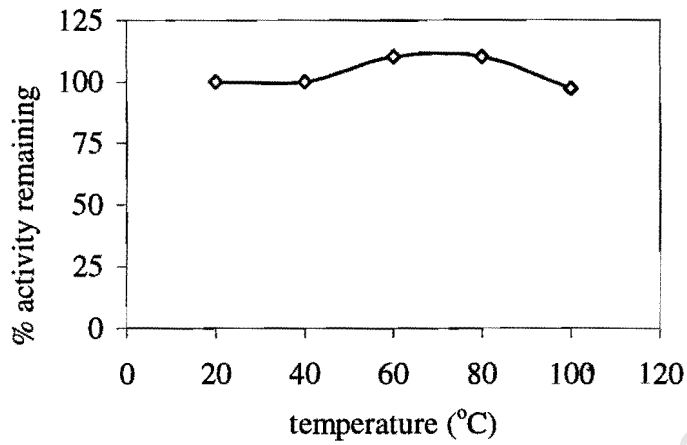


Fig. 4-5. Effect of temperature on the activity of the bioemulsifier (100% value = emulsion index (E1) of 77 shown by the unheated control extract).

4.4.6. Substrate specificity

The substrate specificity of the bioemulsifier prepared by acetone extraction was studied over a range of liquid hydrocarbon substrates at room temperature at pH 7. The results presented in Fig. 4-6 revealed that *n*-tetradecane was the substrate showing the greatest emulsion, with an E1 of 78%. Hexadecane and dodecane were also observed to be good substrates for emulsification, with E1 of 68% and 74% respectively. No emulsion was observed from castor oil at pH 7, while at pH 14, about 67% of emulsion was obtained from castor oil. Palm oil, olive oil, and almond oil all were effectively emulsified, with E1's of approximately 50%.

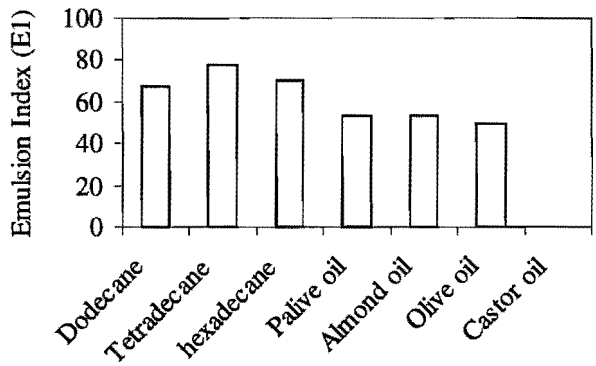


Fig. 4-6. Substrate specificity of the bioemulsifier produced by *P. aeruginosa* 2Bf.

4.5. Discussion

The bioemulsifier produced by the strain *P. aeruginosa* 2Bf was found to contain carbohydrate, lipid, and protein, hence it was classified as a peptidoglycolipid. Several strains of *P. aeruginosa* have been reported to produce peptidoglycolipids instead of rhamnolipids (Ilori & Amund, 2001; Hisatsuka *et al.*, 1972; Koronelli *et al.*, 1983). The peptidoglycolipid was observed to have a protein component greatly involved in emulsification activities. Ilori & Amund (2001) described a peptidoglycolipid bioemulsifier from the *P. aeruginosa* strain isolated in Nigeria which had a protein moiety with a molecular weight of 14 332 Da. The protein component of the bioemulsifier produced by the *P. aeruginosa* strain from Watata was found to contain 147 amino acids with a molecular weight of 14 300 Da (Hisatsuka *et al.*, 1972). Furthermore, the protein fraction of the bioemulsifier produced by *P. aeruginosa* P-20 was reported to have 52 amino acids (Koronelli *et al.*, 1983). Goswami *et al.* (1994) isolated an emulsifying protein from *Pseudomonas cepacia* N1 with an apparent molecular weight of 14 000 Da. In terms of molecular weight, the protein moiety produced by *P. aeruginosa* 2Bf reported in this study, appeared closely similar to those described by Ilori & Amund (2001) and Hisatsuka *et al.* (1972). The bioemulsifier had lipid and carbohydrate components that were not yet identified. Like most high molecular weight bioemulsifiers, although it was an efficient emulsifier, the bioemulsifier obtained in the present study was observed to be less efficient in lowering the surface tension of the medium.

The peptidoglycolipid was most active at alkaline pH and fairly active at acidic pH. There was collapse in emulsifying activity of the bioemulsifier extracted by chloroform:methanol at pH lower than 7. This could indicate that the bioemulsifier is a complex molecule, and that the chloroform has denatured or extracted one of the components of this complex. The acetone extraction procedure is a one-step technique, frequently used for concentrating proteins in the active form and this may have preserved the original complex. Different bioemulsifiers are known to have different pH optima. The optimum pH of the previously isolated peptidoglycolipid bioemulsifier was reported to be 5 (Navon-Venezia *et al*., 1995) and that of *Bacillus cereus* was only active at pH below 7 (Cooper & Goldberg, 1987). The peptidoglycolipid obtained in this study was observed to be heat stable, and retained about 97% of its activity after 1 hour at 100°C. In terms of heat stability, the peptidoglycolipid isolated in this study appeared more stable than the previously isolated peptidoglycolipid (Ilori & Amond, 2001), which was reported to lose 50% of its activity at 100°C. The bioemulsifier produced by *P. aeruginosa* 2Bf was also comparatively more stable than liposan produced by *Candida lipolytica* which has been described to be stable at temperatures up to 70°C and has about 60% loss in activity after heating at 100°C (Cirigliano & Carman, 1984). The broad pH and temperature stability observed for the bioemulsifier produced by the strain 2Bf indicate its potential use in areas such as bioremediation, and in the cosmetic and food industries.

Chapter 5

General conclusion

Contents

5.1. Characterization of the strain *P. aeruginosa* 2Bf

The strain *P. aeruginosa* 2Bf was isolated from oil-contaminated soil by enrichment culture and was shown to grow well on C_{14}-C_{17} alkane fractions. *Pseudomonas* and *Rhodococcus* often are isolated from hydrocarbon-contaminated sites and hydrocarbon degrading cultures (Van Hamme & Ward, 2001). These two genera have broad affinities for hydrocarbons and can degrade selected alkanes, alicyclics, thiphenes, and aromatics (Van Hamme & Ward, 2001). The strain isolated in the present study was observed by thin layer chromatography (TLC), to produce a bioemulsifier containing both glycolipid and protein moieties, which could be a peptidoglycolipid. The protein component had a molecular weight of 14 400 Da. Microorganisms are known to produce many extracellular proteins (Hisatsuka *et al.*, 1972). The molecular weight of the protein component of the presently isolated strain was appeared to be similar with that described by Ilori & Amund (2001), however it was observed to have a different pH optimum for the emulsifying activity. Good emulsifying activity at alkaline pH may be a novel characteristic of the peptidoglycolipid bioemulsifier isolated in this study as compared to other biosurfactants. These studies have been performed on a crude extract of the bioemulsifying agents produced by *P. aeruginosa* 2Bf, and there are indications that this crude extract could either be a mixture of two bioemulsifiers, or is a complex high-molecular weight molecule (See Chapter 3 and Chapter 4). Therefore, detailed chemical analysis of this bioemulsifier should be undertaken, using techniques such as TLC, HPLC, FT-IR, NMR, in order to fully characterize its chemical structure and composition. Moreover, analysis of the amino acid composition of the purified protein component of the bioemulsifier should be undertaken in order to study the differences in

amino acid sequence in relation to previously characterized peptidoglycolipid bioemulsifiers. The peptidoglycolipid bioemulsifier had lipid and carbohydrate components that were not identified. Lipids such as monoglycerides, 1,2 – diglyceride and corynomycolic acids have been reported to take part in bioemulsification (Cooper & Goldberg, 1987). The peptidoglycolipid bioemulsifier reported by Koronelli *et al.* (1983) was observed to have a peptide portion consisting of seven amino acids, a carbohydrate component represented by rhamnose and a lipid portion represented by a mixture of fatty acids with a number of C-atoms from 11 to 18.

The peptidoglycolipid produced by *P. aeruginosa* 2Bf was shown to be stable over a broad range of pH and temperature regimes, and to be capable of emulsifying a range of oils, including olive, palm and almond oil, indicating its potential applications in areas such as bioremediation, food, and cosmetics and other industries.

5.2. Growth of *P. aeruginosa* 2Bf and optimization of biosurfactant production

The growth of *P. aeruginosa* 2Bf on n-alkanes was monitored using viable colony counts, which was proven to be a reliable and appropriate method compared to other growth measurements (Chapter 3). Although OD measurement is a rapid and simple method of biomass estimation, it is still not highly accurate because the sparingly soluble hydrophobic substrates interfere with turbidimetric measurements resulting in biases and inaccuracies. Moreover, microbial cell surfaces are often hydrophobic and hence they cling or adhere to hydrophobic substrates making it even more difficult to undertake OD

measurements. Attempts were made in the present study, to establish a correlation between OD, dry weight and viable cell counts. This was carried out by growing cells in fed-batch fermentation. Strong correlations were observed between the three growth measurements. The strain *P. aeruginosa* 2Bf was observed to exhibit a rather different growth pattern in batch fermentation when compared to shake flasks. A marked reduction in log phase, possibly due to the efficient aeration in the bioreactor, was witnessed in the case of batch fermentation (Chapter 3). Moreover, unlike the shake flasks, the fermentation culture was observed to undertake an abrupt change in color at 56 hours of fermentation. It turned milky had a waxy particulate appearance possibly due to the production of wax esters as reported by Wu & Ju (1998)

The biosurfactant production, measured as carbohydrate using the Orcinol assay, was observed to reach its maximum value in line with the maximum cell number reached, at about 40 hours of incubation (Chapter 3). This suggests that the production of the peptidoglycolipid biosurfactant is growth associated. The maximum amount of the peptidoglycolipid biosurfactant produced by *P. aeruginosa* 2Bf was 12 mg/l in shake flasks, but it was increased to approximately 32 mg/l in a 6 litre bioreactor. This is still approximately 3-fold lower than the amount of glycoproteins produced by *P. cepacia* N1 (Goswami *et al.*, 1994). Attempts were made, in shake flasks, to increase the biosurfactant production by altering the amount of the substrate (C_{14}-C_{17}) added. Little effect was observed in cell growth and biosurfactant production when the amount of the substrate used were 1%, 5%, 10% and 15% (Chapter 3). There was a slight increase in the number of viable cells and the amount of biosurfactant produced when 10% of the carbon substrate was used. Therefore, further bioreactor studies need to be performed, which would include studies of other parameters such dissolved oxygen, amount and type

of nitrate used, phosphorus, pH, and temperature in order to optimize the biosurfactant production by the strain *P. aeruginosa* 2Bf. Factors reported to control the production of biosurfactants include the quality and quantity of carbon and nitrogen constituents in culture (Desai and Banat, 1997; Lang and Philp, 1998). The type of nitrogen present (whether NH_4^+, NO_3^-, urea or amino acid) influences the biosurfactant produced (Robert *et al.*, 1989; Haba *et al.*, 2000). Use of inexpensive or cheap substrates can drastically decrease the production cost of biosurfactants and meet one of the requirements for a competitive process for biosurfactant production. Makkar & Cameotra (1999) described the possible use of unconventional carbon sources for biosurfactant production, including urban as well as agro-industrial wastes. They suggested that these wastes could be used as substrates for large-scale production of biosurfactants with suitable engineering and microbiological modifications. Industrial wastes, such as used frying oils, have also been used as substrates for the production of rhamnolipids by *P. aeruginosa* NCIB 40044 (Haba *et al.*, 2000). The strain, P. aeruginosa 2Bf, has been shown to be an efficient utilizer of hydrocarbon substrates such as aliphatic alkanes, which are produced as by-products of the petrochemical industry. This strain could therefore be used for the industrial production of bioemulsifiers.

5.3. Discussion of protein enhancer

The presence of a protein component involved in emulsifying activity (Chapter 4) provokes the need for undertaking studies on molecular genetics to optimize the production of the biosurfactant. An understanding of the structural and regulatory genes for biosurfactant production would facilitate the adoption of cost-effective innovations

for large-scale production. The structures of biosurfactants can also be genetically engineered for specific applications. The essential role played by a 14 000 Da protein component of the *P. aeruginosa* 2Bf bioemulsifier in the emulsifying activity is interesting. The protein may represent an activator similar to that reported by Hardegger *et al.* (1994), who have isolated and characterized the *pra* gene from *P. aeruginosa* PG201 encoding the 16 kDa extracellular protein activator (PA), which is structurally and functionally very similar to that of the *P. aeruginosa* S_7B_1 strain characterized previously by Hisatsuka et al. (1977). They have also cloned and expressed the *pra* gene in *E. coli*, and have obtained substantial amounts of the recombinant protein. The gene sequence of the lipopeptide surfactin, produced by *Bacillus subtilis*, has been determined (Sullivan, 1998). Preliminary genetic information on the high molecular weight polymer, emulsan, has also been established despite the complexity of the molecule (Sullivan, 1998). Recently, a cluster (27 kb) of genes responsible for the biosynthesis of the amphipathic, polysaccharide bioemulsifier emulsan from the oil-degrading *Acinetobacter lwoffii* RAG-1 was isolated and characterized (Nakar & Gutnick, 2001). In their report they presented results of analysis of a 27 kb gene cluster, termed *wee*, encoding the genes required for biosynthesis of the emulsan biopolymer. Once the structural genes involved in biosurfactant production in *P. aeruginosa* 2Bf are identified and characterized, they may be able to be deployed under the regulation of strong promoters in non-pathogenic heterologous hosts to optimize production (Sullivan, 1998).

5.4. Applications in Bioremediation

Like all other high molecular weight bioemulsifiers, the peptidoglycolipid produced by the strain *P. aeruginosa* 2Bf, was observed to form very stable emulsions with a variety of substrates, including almond, olive and palm oils, but was inefficient in lowering the surface tension (Chapter 4). The formation of stable oil-in-water emulsions by microorganisms can enhance oil degradation, as this increases nutrient availability (Bredholt *et al.*, 1998). It is interesting to note that the production of stable emulsions enables bacteria to adhere to hydrophobic surfaces very strongly. For example, the addition of the high molecular weight bioemulsifier, emulsan, either initially or at various times after inoculation enabled the non-adherent *Acinetobacter calcoaceticus* MR-481 cells to grow on hexadecane (Rosenberg & Rosenberg, 1981). They have proven that growth was not the result of reversion of MR-481 from nonadherent to adherent cells, and that adherence is a crucial factor in the growth of *A. calcoaceticus* RAG-1 on hexadecane in the absence of extracellular emulsification of the substrate.

To optimize the use of biosurfactants for soil remediation, more insight is required in the effect of biosurfactants on contaminant bioavailability. It is necessary to determine which processes are stimulated by biosurfactants and why biodegradation by certain bacteria is stimulated whereas biodegradation by others is negatively affected. Arino *et al.* (1998) reported the inhibitory effect of rhamnolipid produced on the growth of bacteria such as *Rhodococcus erythropolis, Bacillus cereus* and *Pseudomonas fluorescence*. They have also indicated the role of the strain *Pseudomonas aeruginosa* GL1 in stabilizing the polcyclic aromatic hydrocarbons (PAH) degrading community by utilizing the PAH

metabolites. Noordman & Janssen (2002) determined the biodegradation of hexadecane by five biosurfactant-producing bacterial strains (*Pseudomonas aeruginosa* UG2, *Acinetobacter calcoaceticus* RAG1, *Rhodococcus erythropolis* DSM 43066, *R. erythropolis* ATCC 19558, and strain BCG112) in the presence and absence of exogenously added biosurfactants. They found that the degradation of hexadecane by *P. aeruginosa* was stimulated only by the rhamnolipid biosurfactant produced by the same organism. While the biodegradation of hexadecane by the four other strains was not stimulated by the addition of their own biosurfactants, nor was the degradation enhanced by rhamnolipid. This suggests that *P. aeruginosa* has a mode of hexadecane uptake different from those of the other organisms.

Interesting observations relate to the effect of nitrogen limitation that appears to stimulate biosurfactant production and overproduction by some microorganisms (Suzuki *et al.*, 1974; Guerra-Santos *et al.*, 1984). This has implications for bioremediation as systems are often supplemented with nitrates and phosphates to reduce the effect of N and P limitation and enhance microbial activity. This may affect *in-situ* biosurfactant production and requires further investigation. Holden & Firestone (1997) suggest a need to determine the factors controlling the desired metabolic capability; the distribution of degrading communities relative to contaminant source and the overall activity of relevant communities. Hence, the basic understanding of the nature and solubilization of contaminants, the composition of bacterial communities, and the role of biosurfactants, as well as cost-effective production of these substances are required to optimize and increase the effectiveness of remediation systems.

In summary, the strain *P. aeruginosa* 2Bf, which was isolated from oil-contaminated South African soil, was observed to degrade crude oil and produce a bioemulsifier with strong stable emulsifying activities. The broad range of pH and temperature stability of the bioemulsifier and its ability to emulsify a wide range of hydrocarbon substrates, also indicate its potential use in bioremediation and other applications. The presence of a protein component of the 2Bf bioemulsifier which is involved in emulsifying activity entices the need for molecular studies to optimize the production of the bioemulsifier. These outcomes indicate the potential uses of the presently isolated strain in ameliorating the effects of oil pollution as well as in the production of value-added products from waste alkanes. At present, despite the comparative advantages conferred by bioemulsifiers over the synthetic ones, the use of bioemulsifiers is limited by the cost of production and insufficient experience in applications (Rosenberg & Ron, 1999). However, it seems inevitable that bioemulsifiers will replace the currently used chemical emulsifiers because of the increased awareness in environmental conservation, as well as the increasing demand for natural products.

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