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CHARACTERISATION OF THE ACTIVITY OF BIOSURFACTANTS PRODUCED BY *PSEUDOMONAS* SPECIES ISOLATED FROM FOODS

Nathalie Madalena Melro Fernandes

A dissertation submitted to the Faculty of Science, University of the Witwatersrand, in fulfilment of the requirements for the degree of Master of Science.

Johannesburg, 2009

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before any degree or faculty in any other University.

_____

Nathalie Madalena Melro Fernandes

_____ day of _____ 2009

ABSTRACT

Spoiled food products were screened for biosurfactant-producing *Pseudomonas* strains, which were then evaluated for antimicrobial activity and the ability to grow on two model hydrocarbons. Strains isolated from 14 different spoiled food products were screened for biosurfactant production using the drop collapse assay, of which 5.6% tested positive. None exhibited emulsifying activity. The strains were isolated predominantly from leafy vegetable products, and bottled mineral water and low-fat milk; the latter two of which had the lowest APC and PC, respectively. The sources of biosurfactant-producers suggest an attachment role for biosurfactants, rather than one of directly increasing the availability of hydrophobic nutrients to spoilage bacteria. Biosurfactant-producing strains were evaluated for antibacterial activity against potentially pathogenic and food spoilage bacteria using the spot-on-lawn assay, of which 56% exhibited activity against only Gram-positive bacteria, predominantly *B. cereus* ATCC 10702. Strains found to be active against *B. cereus* ATCC 10702 were subjected to treatments with protease, heat and organic solvents in order to determine their stability, and 6 of those isolates were further characterised by TLC. The combined data suggested that the majority of compounds produced by the strains isolated in this study produce cyclic lipopeptides, some of which may be novel. Identification by 16S rRNA sequencing identified the antibacterial strains as *Pseudomonas* (8), *B. pumilis* (9) and *Proteus vulgaris* (1). Phylogenetic analysis of the sequences also showed that the strains are closely related to other bacteria with biosurfactant-producing, antimicrobial and biodegradative activities. The ability of 8 biosurfactant-producing strains (3 *Pseudomonas*, 1 *Proteus*, 4 *B. pumilis*) and 2 consortia (Gram-negative and -positive) to grow in minimal media supplemented with *n*-hexadecane (MMH) or mineral motor oil (MMMO) was evaluated. All of the strains and both consortia reached high cell numbers (>7 – 9 log CFU/ml); however, no significant differences ($P<0.05$) were observed between individual strains inoculated into either medium. The Gram-negative strains and consortium grew at lower rates in MMMO than in MMH, and when compared with the Gram-positive strains and consortium. *P. fulva* 381C grew at a significantly lower ($P<0.05$) rate when grown in MMMO. Furthermore, the consortia did not achieve significantly higher ($P<0.05$) cell numbers or grow better than individually inoculated strains in either medium. This study is the first to document antibacterial activity by *P. fulva*, and hydrocarbon and *n*-alkane utilisation by strains of *P. fulva* and *Proteus vulgaris*, respectively.

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ABSTRACT

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PREFACE

Some aspects of work conducted for this dissertation have been presented as posters elsewhere:

CHAPTER 2

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CHAPTER 3

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CHAPTER 1

Introduction

1.1. BIOSURFACTANTS PRODUCED BY *Pseudomonas* SPECIES

A. Introduction to biosurfactants

Boundaries are of major importance to the behaviour of any heterogeneous system as an entity (Rosenberg *et al*., 1988; Rosenberg and Ron, 1999; Ron and Rosenberg, 2001). Given their large surface-to-volume ratio, it follows that microorganisms produce an assortment of surface-active compounds (surfactants), which adsorb to and alter the prevailing conditions at interfaces. Surfactants are able to accumulate at interfaces because they are amphipathic, meaning that they contain both hydrophobic and hydrophilic groups (Karanth *et al*., 1999; Ron and Rosenberg, 2001).

B. Classes, chemical structure and properties of biosurfactants

Biosurfactants can be divided into low-molecular-mass molecules, which lower surface and interfacial tensions effectively, and high-molecular-mass molecules, which adsorb to hydrophobic surfaces (Rosenberg and Ron, 1999; Ron and Rosenberg, 2001, 2002). Emulsifiers are a subclass of surfactants that stabilise dispersions of one immiscible liquid in another, e.g. oil-in-water emulsions, and may not necessarily reduce surface tension (Ron and Rosenberg, 2001). Table 1.1 presents a list of select microbially produced surfactants that have been studied. Pseudomonads are known producers of biosurfactants and it is these molecules that will be discussed in more detail.

i. Low-molecular-mass biosurfactants

The low-molecular-mass biosurfactants are glycolipids, lipopeptides, fatty acids (including corynomycolic and spiculisporic acids), phospholipids and neutral lipids (Mulligan, 2005). *Pseudomonas* spp. are known to produce predominantly glycolipids and lipopeptide biosurfactants (Table 1.1).

- **Glycolipids**

Most of the known biosurfactants are glycolipids. Among these, the best studied are the rhamnolipids, trehalolipids and sophorolipids. They are typically disaccharides that are acetylated with long-chain fatty acids or hydroxyl fatty acids (Ron and Rosenberg, 2001).

The best studied of these molecules are the rhamnolipids (Hauser and Karnovsky, 1954), which were first described in *Pseudomonas aeruginosa* (Jarvis and Johnson, 1949). It has since been shown that some isolates of the non-pathogenic pseudomonads *P. putida* and *P. chlororaphis* also produce a variety of rhamnolipids (Tuleva *et al*., 2001; Gunther *et al*., 2005). Rhamnolipids are typically dimers comprising 3-hydroxy fatty acids and a mono- or di-rhamnose moiety, linked by a β-glycosidic bond (Soberón-Chávez *et al*., 2005) (Fig 1.1). Rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate (Rha-C_{10}-C_{10}), a mono-rhamnolipid, and rhamnosyl-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate (Rha-Rha-C_{10}), di-rhamnolipid, are the two most commonly produced rhamnolipids in liquid culture, under normal growth conditions (Desai and Banat, 1997; Rosenberg and Ron, 1999; Soberón-Chávez *et al*., 2005) and up to 28 homologues have been identified (Benincasa *et al*., 2004). Rhamnolipids have been shown to lower surface tensions to 29 mN/m, emulsify hydrocarbons and stimulate the growth of *P. aeruginosa* on *n*-hexadecane (Hisatsuka *et al*., 1971; Guerra-Santos *et al*., 1986; Parra *et al*., 1989).

- **Lipopeptides**

Lipopeptides are a remarkable group of biosurfactants due to their many valuable properties, including their activity as potent antibiotics (Marahiel *et al*., 1993; Yakimov *et al*., 1995; Tsuge *et al*., 1996; Nielsen *et al*., 2002). For example, many cyclic lipopeptides (CLPs), including surfactin, gramicidin S and the polymyxins, are produced by members of the genus *Bacillus*. Interestingly, the synthesis of such, and other, antibiotic surfactants during the early stages of sporulation is a characteristic shared among most, if not all, *Bacillus* spp. (Katz and Demain, 1977; Grossman, 1995; Driks, 1999).

Several cyclic lipodepsipeptides (CLDPs) have also been isolated from *Pseudomonas* spp. All of these compounds typically comprise a 3-hydroxy fatty acyl residue linked to an *N*-terminal group

in a ring structure of 8 – 9 amino acids. *Pseudomonas viscosa* (Kochi *et al*., 1951) and strains of *P. fluorescens* biovar II (Hildebrand, 1989) produce the CLDP biosurfactant viscosin, which has been reported to lower surface tensions to 26.5 mN/m (Neu *et al*., 1990). A novel biosurfactant produced by a different strain of *P. fluorescens* has since been identified and named viscosinamide (Fig 1.2A). The AA2 amino acid in the cyclic peptide is D-glutamic acid and D-glutamine in viscosin and viscosinamide, respectively (Nielsen *et al*., 1999). Viscosinamide has been shown to lower the surface tension of sterile distilled water (Nielsen *et al*., 2002). A series of 8 depsinonapeptide biosurfactants, massetolides A – H (Fig 1.2B), were first isolated from unidentified marine strains of *Pseudomonas* sp. (Gerard *et al*., 1997) and differ from viscosin either in one of the amino acid units or in the fatty acid moiety, or in both. More recently, the CLP tensin was isolated from yet another strain of *P. fluorescens* (Nielsen *et al*., 2000) (Fig 1.2C). A comparison of CLP structures of biosurfactants produced by Gram-positive and Gram-negative bacteria demonstrates similarity with other CLPs, such as surfactin from *B. subtilis* (Arima *et al*., 1968) and the white line inducing principle (WLIP) (Fig 1.2D) from *P. reactans* (Mortishire-Smith *et al*., 1991). Viscosin and WLIP are structurally identical with the exception of the chirality of Leu5, which has a D and L configuration in WLIP and viscosin, respectively (Fig 1.3).

ii. High-molecular-mass biosurfactants

The high-molecular-mass biosurfactants are commonly polyanionic heteropolysaccharides containing both polysaccharides and proteins. They include polymeric microbial surfactants and particulate surfactants and generally exhibit better emulsifying activity than the low-molecular-mass biosurfactants (Ron and Rosenberg, 2001; Mulligan, 2005).

- **Polymeric substances**

The best studied polymeric biosurfactants are the emulsans, liposan, mannoprotein and other polysaccharide-containing complexes. The majority of these molecules are produced by members of the genera *Acinetobacter*, *Candida* and *Pseudomonas* (Desai and Banat, 1997). Hisatsuka *et al*. (1972, 1977) reported the isolation of a protein-like activator that was capable of emulsifying hydrocarbons from *P. aeruginosa*. This compound, referred to as protein A, has a molecular mass of 14 300 and comprises 147 amino acids, of which 51 are serine and threonine (Hisatsuka *et al*.,

1977). A bioemulsifier produced by *P. fluorescens*, emulsan 378, was first isolated during growth on gasoline. The compound comprises 50% carbohydrate, 19.6% protein and 10% lipid, where trehalose and lipid-*o*-dialkyl monoglycerides are the main constituents of the carbohydrate and lipid, respectively (Desai *et al*., 1988). Emulsan 378 has been shown to reduce the surface tension of an aqueous solution to 27 mN/m and has a critical micellar concentration (CMC) of less than 10mg/ml in 0.9% (w/v) NaCl (Persson *et al*., 1988).

- **Particulate compounds**

Phalle *et al*. (1995) isolated a particulate biosurfactant, Biosur-PM, from *P. maltophilia* CSV89, which was found to comprise 50% protein and 12 – 15% sugar. Biosur-PM has been found to lower the surface tension of water to 53 mN/m and has a CMC of 80 mg/l. The study also showed that the biosurfactant emulsified aromatic hydrocarbons preferentially to aliphatic hydrocarbons. Kinetic data of Biosur-PM production indicate that its synthesis is associated with growth and is pH-dependent, suggesting that it may be a cell-wall component. Another particulate surfactant, PM-factor, was isolated from a culture of *P. marginalis* PD-14B (Burd and Ward, 1996). PM-factor was found to emulsify aromatic and aliphatic hydrocarbons, crude oil and creosote. Furthermore, the emulsifying activity was found to be stimulated by polymyxin B, Ca^{2+} and Mg^{2+}. The biosurfactant reportedly contains a low amount of aromatic amino acids in the protein moiety, and 3-deoxy-D-mannooctulosonic acid, heptose, hexosamine, phosphorus, and 3-hydroxy fatty acid, suggesting that PM-factor contained lipopolysaccharide.

C. Natural roles of low-molecular-mass biosurfactants

As discussed in the preceding sections, biosurfactants are produced by a range of diverse microorganisms and have a variety of different chemical structures and surface properties. This diversity makes it impossible to generalise about the natural roles of biosurfactants, despite their clear significance in the physiology of their producer microorganism. There have only been a few studies in which non-biosurfactant-producing mutants have been created and compared with the parent strains (Itoh and Suzuki, 1972; Koch *et al*., 1991), making it impossible to identify common functions of biosurfactants in producing microorganisms. As a result, most of the concepts have been deduced from a consideration of the surface active properties of

biosurfactants and experiments where biosurfactants have been added to microorganisms growing on hydrophobic substrates (Ron and Rosenberg, 2001). A select few of the hypothetical natural roles for biosurfactants are described below.

i. Increasing the surface area of hydrophobic water-insoluble substrates

Studies have demonstrated that for bacteria growing on hydrocarbons, when the surface area between water and oil becomes limiting, growth proceeds arithmetically rather than exponentially (Shreve, 1995). There exists indirect evidence that emulsification is a natural process effected by extracellular agents. There is; however, a difficulty in understanding how emulsification can provide an evolutionary advantage for the producer microorganism if the process is cell-density-dependent and occurs in an open system. A feasible theory is that bioemulsifiers play a role in oil degradation by creating a microenvironment for each cell, instead of producing macroscopic emulsions in the bulk liquid (Rosenberg and Ron, 1999; Ron and Rosenberg, 2001).

ii. Increasing the bioavailability of hydrophobic water-insoluble substrates

A major reason for the recalcitrance of hydrophobic compounds is their low water solubility, which increases their adsorption to surfaces and consequently limits their availability for biodegradation (van Loosdrecht *et al*., 1990; van Delden *et al*., 1998). By increasing the water solubility and thus availability of bound substrates, biosurfactants can effectively improve the growth of degrading microorganisms on hydrophobic substrates (Déziel *et al*., 1996). Potent low-molecular-mass biosurfactants are particularly effective in solubilising bound hydrophobic molecules as they possess a low CMC and incorporate hydrocarbons into the hydrophobic pockets of micelles, thereby increasing their solubility (Miller and Zhang, 1997).

iii. Regulating the attachment-detachment of microorganisms to and from surfaces

The ability of microorganisms to establish themselves in an ecological niche where they can multiply successfully is a basic survival strategy. Although attention on biosurfactant-producing bacteria has been predominantly focused on those able to degrade hydrocarbons, biosurfactant production also occurs in natural environments, such as the phyllosphere, which constitutes a very large microbial habitat. Bunster *et al*. (1989) showed that some bacteria produce biosurfactants in order to increase the wettability, and consequent colonisation, of the

hydrophobic phylloplane. The production of biosurfactants may also facilitate the movement of bacteria across the phylloplane (Hutchinson and Johnstone, 1993; Lindow and Brandl, 2003). Neu (1996) reviewed that secreted biosurfactants can form a conditioning film on an interface which can stimulate the attachment of certain microbes to that interface and inhibit the attachment of others. For example, the presence of cell-bound biosurfactant increased the cell surface hydrophobicity of *P. aeruginosa* significantly (Zhang and Miller, 1994) but reduced that of *Acinetobacter* strains (Rosenberg and Rosenberg, 1983). More recently, it was shown that rhamnolipids increased the cell surface hydrophobicity of *Pseudomonas* spp. by stimulating the release of lipopolysaccharide (LPS), an integral component of the cell surface (Al-Tahhan *et al*., 2000). The rhamnolipid precursors in *P. aeruginosa*, 3-(3-hydroxyalkanoyloxy)alkanoic acids (HAAs), have also been suggested to affect cell surface hydrophobicity of *P. aeruginosa* (Caiazza *et al*., 2005). Such results suggest that biosurfactants are used by their producer microorganism to regulate their cell surface properties and thus their ability to attach or detach as required. However, little else is known about the interaction of biosurfactants with bacterial cells and it is important to understand this interaction if biosurfactants are to be used more efficiently in bioremediation processes.

iv. Role of biosurfactants in biofilms and motility

The *lasR-lasI* quorum sensing system in *P. aeruginosa* (Fig 1.4) has previously been shown to play a role in the later stages of biofilm differentiation, when the cell density is sufficient to form a quorum (Davies *et al*., 1998). Studies have demonstrated that rhamnolipids produced by *P. aeruginosa* play several roles in the structural development of a biofilm, including promoting the formation of microcolonies in the initial phase, maintaining the channels between macrocolonies, facilitating migration-dependent structural development in the later phase and regulating the attachment-detachment of cells to biofilms (Davey *et al*., 2003; Pamp and Tolker-Nielsen, 2007). Boles *et al*. (2005) demonstrated that rhamnolipids were required for the detachment of hyper-detaching variants and suggested that this detachment mechanism could be used to disrupt existing biofilms. They also found that rhamnolipid could induce detachment in *P. aeruginosa* biofilms. Similarly, Kuiper *et al*. (2004) found that the surface-active CLDPs putisolvin I and putisolvin II, produced by *P. putida* PCL1445, could influence biofilm development on polyvinyl

chloride, inhibit biofilm formation by several *Pseudomonas* strains and disrupt established *Pseudomonas* biofilms.

Rhamnolipids, and biosurfactants produced by other bacteria, also contribute to swarming motility. Studies by Köhler *et al*. (2000) and Déziel *et al*. (2003) suggest that quorum sensing and rhamnolipid production contribute to swarming in *P. aeruginosa*. Caiazza *et al*., 2005 showed that rhamnolipid production regulates swarming motility by adjusting the movement of tendrils; the defined groups in which bacteria migrate. This control is suggested to be mediated by RhlR, a transcriptional regulator, which activates the genes responsible for producing rhamnolipids (Ochsner *et al*., 1994a, b). Evidence for the additional role of swarming in biofilm formation was provided by Shrout *et al*. (2006), who showed that swarming motility plays a role in the early stages of biofilm formation. It was also demonstrated that under certain nutritional conditions, the degree of biofilm formation is regulated by swarming motility.

v. Antimicrobial activity

Many low-molecular-mass biosurfactants are known to exhibit antimicrobial activity; however, this useful property has not yet been extensively reviewed, despite a range of applications in the biomedical sciences and in the biological control of root pathogenic microorganisms (Singh and Cameotra, 2004). The antimicrobial activity of the biosurfactants discussed thus far results from the ability of the molecules to alter the permeability of the phospholipid bilayer membrane in a detergent-like manner. In the case of both the rhamnolipids and lipopeptides, the biosurfactants intercalate into the membrane, forming transmembrane pores or disintegrating the membrane. This results in the collapse of the transmembrane electrochemical gradients and subsequent cell lysis (Stanghellini and Miller, 1997; Shai, 2002; Makotvitzki *et al*., 2006). The antimicrobial activities of selected surfactants produced by *Pseudomonas* spp. are discussed below.

- **Glycolipids**

The rhamnolipids produced by *P. aeruginosa* (Itoh *et al*. 1971) are known to possess a range of antimicrobial activities. Antimicrobial activity is commonly estimated on the basis of minimal inhibitory concentration (MIC) values and is defined as the lowest concentration of antimicrobial agent that is able to inhibit visible microbial growth. Rhamnolipids have been shown to exhibit

antimicrobial activity against predominantly Gram-positive, a few Gram-negative bacteria, and fungi. Good antimicrobial activities (MIC ≤ 50µg/ml, except where comparative values for microorganisms are not available) against select bacteria and fungi are listed in Table 1.2. These glycolipids are also known to exhibit antiviral (Lang and Wagner, 1993), antimycoplasmal (Abalos *et al*. 2001) and antiamoebal (Cosson *et al*. 2002) activities. Wang *et al*. (2005) found that rhamnolipids could inhibit the growth of the harmful algal bloom spp. *Hetersigma akashiwo* (0.4mg/L) and *Proracentrum dentatum* (> 1mg/L). It was found that the rhamnolipids disrupted the plasma membrane, which also facilitated further damage to the organelles, including the chloroplasts and mitochondria. Stanghellini and Miller (1997) demonstrated that rhamnolipids could induce the lysis of zoospores of the plant pathogens *Pythium aphanidermatum*, *Pythophthora capsici* and *Plasmopara lactucae-radicis* from 5µg/ml.

- **Lipopeptides**

CLPs, produced by predominantly fluorescent pseudomonads, are also known to possess antibacterial, antifungal and antiviral activities. Viscosin, which is produced by *P. fluorescens*, exhibits antibacterial and antiviral activity (Neu and Poralla, 1990). Gerard *et al*. (1997) found that viscosin exhibited good antimicrobial activity against the human pathogen *Mycobacterium tuberculosis* (10 – 20µg/ml) and *M. avium-intracellulare* (10 – 20µg/ml). In the same study, massetolide A was found to be particularly effective against *M. tuberculosis* (5 – 10µg/ml) and *M. avium-intracellulare* (2.5 – 5µg/ml); however no activity was shown against a selection of other human pathogenic bacteria, which included *Escherichia* (*E*.) *coli* and *Staphylococcus* (*S*.) *aureus*. CLPs produced by fluorescent pseudomonads, including viscosinamide and tensin, have been shown to play a role in the biocontrol of root pathogenic microfungi (Nielsen *et al*., 1999; Thrane *et al*., 1999, 2000; Nielsen *et al*., 2002). Using vital fluorescent stains, Thrane *et al*. (1999) found that various pathogenic microfungi, including *Pythium ultimum* and *Rhizoctonia solani*, responded to viscosinamide treatment, which manifested as increased branching, occasional hyphal swelling and increased septation. Thrane *et al*. (2000) later demonstrated the reduction in *P. ultimatum* mycelial density, oospore formation and intracellular activity by viscosinamide-producing *P. fluorescens* DR4. In a study by Nielsen *et al*. (2000), hyphae of *R. solani* became hyaline and swollen upon challenge with tensin. Although tensin-challenged

mycelium showed reduced outgrowth on the medium surface, biomass production was considerably higher in the tensin-containing medium than in the control media.

1.2. *Pseudomonas*-PRODUCED BIOSURFACTANTS AND BIOREMEDIATION

Biosurfactants have several advantages over their synthetic counterparts. Some of these include their biodegradability, specificity, low toxicity, and efficiency at high temperatures, pH and salinity (Kosaric, 2001). These properties make biosurfactants suitable for use in several industrial and environmental contexts, some of which are outlined in Table 1.3.

Given the well-documented ability of biosurfactants to increase the surface area and bioavailability of water-insoluble substrates (discussed in 1.1.C.i. and 1.1.C.ii.), an obvious application of biosurfactants is that in pollution bioremediation. Bioremediation refers to the acceleration of natural biodegradative processes in contaminated environments by improving the availability of materials (e.g. nutrients and oxygen), conditions (pH and moisture content) and prevailing organisms (Ron and Rosenberg, 2001). Biosurfactants can efficiently be used in handling industrial emulsions, the control of oil spills, the biodegradation and detoxification of industrial effluents and in the bioremediation of contaminated sites (Kosaric, 2001; Ron and Rosenberg, 2001).

A. Soil bioremediation methods

Water treatment is relatively easy to perform; however, the bioremediation of soil poses considerably more challenging and complex problems. One of the major problems is the large area over which the pollution may be distributed, as *ex situ* treatment of the contaminated soil becomes too costly. Treatment; however, can be achieved in two ways. The first is the biostimulation or bioaugmentation of the native microflora, which is achieved by adding nutrients to the soil. This increases the number of microorganisms which are able to metabolise or co-metabolise the pollutant of interest. The second is the production of a microbial community *ex situ*, which is adapted to and capable of metabolising the pollutant (Kosaric, 2001). The addition or *in situ* production of surfactants can aid in the bioremediation of polyaromatic

hydrocarbon (PAH)-contaminated sites through the solubilisation or emulsification of PAHs thereby increasing the bioavailability and subsequent biodegradation of PAHs (Tiehm, 1994). Contradictory results regarding the efficiency of these compounds on PAH degradation are found throughout the literature (Rouse *et al*., 1994). What follows is a review of the application of biosurfactants, specifically rhamnolipids, to the bioremediation (*in* and *ex situ*) of petroleum hydrocarbon- and oil-contaminated sites.

i. Petroleum hydrocarbons

Petroleum hydrocarbons typically comprise alkanes, cycloalkanes, aromatic hydrocarbons, polycyclic aromatic hydrocarbons (PAHs), asphaltenes and resins. Although alkanes can have many isomers as the number of carbons increase, relatively few exist in petroleum. Alkanes are occasionally referred to as aliphatic hydrocarbons, and the low-molecular-mass alkanes are the most easily degraded by microorganisms (Mulligan, 2005). PAHs are components of creosote and occur primarily as a result of incomplete combustion processes such as petroleum refining and coke production, and as by-products of industrial activities including chlorine bleaching of cellulose pulp, pesticide and herbicide production, and chemical, plastics, iron and steel industries (Kosaric, 2001). As many of these compounds are toxic and proven carcinogens, their release into water and soil in prohibited (Kosaric, 2001, Samanta *et al*., 2002; Mulligan, 2005). PAHs pose a threat to human health and the environment, thus it is imperative that should PAHs appear in industrial waste effluents, they be treated and detoxified. However, the compounds become increasingly difficult to degrade as the number of rings increases as this decreases volatility and aqueous solubility, and increases adsorptive capacity (Mihelcic *et al*., 1993; Volkering *et al*., 1995).

Studies that have investigated the effect of rhamnolipids on the biodegradation of hydrocarbon contaminants have produced mixed results. There has been particular focus on hydrocarbons with low solubility. It is suggested that enhanced biodegradation by rhamnolipids is achieved through one of two mechanisms. The first of these is to increase the solubility of the compound in question thereby increasing their bioavailability to degrading microbial cells. The second mechanism involves the interaction of the compound with microbial cells, which results in an increase in the hydrophobicity of the cell surface, allowing hydrophobic substrates to associate

more easily (Shreve *et al*., 1995; Zhang and Miller, 1995). Studies demonstrating both mechanisms follow.

The addition of rhamnolipids to soil and liquid systems contaminated with hydrocarbons mixtures has been shown to degrade the majority of hydrocarbons. Jain *et al*. (1992) demonstrated that rhamnolipids produced by *P. aeruginosa* UG2 added to soil contaminated with a hydrocarbon mixture enhanced the degradation of all hydrocarbons, except 2-methyl-naphthalene, after 2 months. Van Dyke *et al*. (1993) also showed enhanced recovery of hydrocarbons from sandy-loam (25 – 70%) and (40 – 80%) silt-loam soil upon the addition of rhamnolipids. *In situ* studies on oil-contaminated desert sands in Kuwait found that up to 82.5% and 90.5% reduction of total petroleum hydrocarbons (TPH) and *n*-alkanes, respectively, could be achieved within 12 months (Al-Awadhi *et al*., 1994). Maier and Soberón-Chávez (2000) found that added rhamnolipids enhanced the biodegradation of hexadecane, octadecane, *n*-paraffin and phenanthrene in liquid systems, and hexadecane, tetradecane, pristine, creosote and a hydrocarbon mixture in soil systems. Benincasa (2007) showed that TPH were degraded by indigenous microflora to 85% within the first 20 days when rhamnolipids produced by *P. aeruginosa* LBI were added.

Studies have demonstrated that adding biosurfactants increase the solubility (and subsequent degradation) of many PAHs, including naphthalene (Déziel *et al*., 1996), phenanthrene (Burd and Ward, 1996; Providenti *et al*., 1996; Zhang *et al*., 1997; Noordman *et al*., 1998; Garcia-Junco *et al*., 2001) and pyrene (Bordas *et al*., 2005). By simply adding rhamnolipids, recoveries of up to 90% have been achieved. Such studies indicate that added rhamnolipids play a role in the solubilisation of the PAH, enabling degradation by different, degrading bacteria. Some also show that rates of degradation increase with an increase in rhamnolipid concentration (Bordas *et al*., 2005; Lai *et al*., 2009).

There have also been studies providing evidence for the second mechanism proposed for enhanced biodegradation by rhamnolipids. Beal and Betts (2000) demonstrated that a rhamnolipid-producing strain increased the cell surface hydrophobicity of *P. aeruginosa* more than did a non-rhamnolipid-producing during growth on hexadecane. Al-Tahhan *et al*. (2000)

found that the interaction of rhamnolipid with the *Pseudomonas* cell surface resulted in a loss of LPS, an integral component of the cell surface, and a corresponding increase in the relative hydrophobicity of the cell. Dean *et al*. (2001) showed the enhanced degradation of soil-bound phenanthrene by only one (*Pseudomonas* strain R) of two (*Pseudomonas* strain R and isolate P5-2) degrading bacteria when either rhamnolipid or rhamnolipid-producing *P. aeruginosa* ATCC 9027 or both were added.

Other studies have shown the effect of adding rhamnolipids in combination with nutrients. Churchill *et al*. (1995) showed that the addition of rhamnolipid and an oleophilic fertiliser (Inipol EAp-22) enhanced the biodegradation rate of aromatic and aliphatic hydrocarbons in aqueous phase and soil reactors and by pure bacterial cultures. Rahman *et al*. (2002) demonstrated that the *ex situ* bioremediation of a gasoline-contaminated soil was enhanced by the addition of rhamnolipids produced by *Pseudomonas* sp. DS10-129 together poultry litter and coir pith. Similarly, Straube *et al*. (2003) investigated the addition of biosurfactant-producing *P. aeruginosa* 64, nutrients (slow-release nitrogen) and a bulking agent (ground rice husks), to enhance the bioremediation of PAH- and pentachlorophenol-contaminated soil during landfarming. Large scale pan experiments showed that decreases in the total PAH (86%) and benzo[a]pyrene (BaP) (87%) toxicity were achieved after 16 months by this biostimulation/ bioaugmentation approach, as compared to a 12% decrease in PAHs for the control. Rahman *et al*. (2003) examined the bioremediation of *n*-alkanes in petroleum sludge, and found that $C_8 - C_{11}$, $C_{12} - C_{21}$ and $C_{22} - C_{31}$, $C_{32} - C_{40}$ alkanes in 10% petroleum sludge were degraded 100%, 83 – 98%, 80 – 85% and 57 – 75%, respectively, following 56 days with the addition of a bacterial consortium, nutrients and rhamnolipids. Although rates of biodegradation decreased as the chain length increases, rates were still significant even for $C_{32} - C_{40}$ alkanes, indicating the use of rhamnolipid addition on the enhancement of biodegradation. Ueno *et al*. (2006) compared the effect of biostimulation and bioaugmentation with *P. aeruginosa* WatG on TPH degradation in sterilised and unsterilised diesel-contaminated soil. TPH degradation in the bioaugmentation samples was found to be 51 and 46% in unsterilised and sterilised soil microcosms (1 week), respectively, and that di-rhamnolipids were produced only in the presence of diesel oil. Whang *et al*. (2009) found that the addition of either a crude rhamnolipid preparation or nutrient mixture enhanced the degradation of crude oil sludge (1% v/v) to 95%, while the addition of both

enhanced degradation of the sludge to 98% in 4 weeks Degradation was achieved by a bacterial consortium comprising two species of *P. aeruginosa* and one of *R. erythropolis*.

Comparisons of the solubilisation and/or degradation of PAHs by biosurfactants or chemical surfactants have generally showed greater solubilisation by biosurfactants. However, degradation rates can vary, further demonstrating that added biosurfactants increase PAH solubility but do not usually play a role in degradation. Vipulanandan and Ren (2000) compared the solubilisation of naphthalene by a rhamnolipid, the anionic surfactant sodium dodecyl sulphate (SDS) and the non-ionic surfactant Triton X-100. The solubility of naphthalene was increased 30-fold in the presence of the biosurfactant, but its biodegradation took 40 days as compared with 100h in the presence of Triton X-100. It seemed that the rhamnolipid was utilised as the carbon source instead of the PAH. Liang *et al*. (2006) compared the desorption and biodegradation of phenanthrene in fresh-contaminated and aged-contaminated sediment slurry by a rhamnolipid mixture (Rha-mix) and SDS. It was shown that the Rha-mix enhanced desorption and biodegradation of phenanthrene more efficiently than SDS and that the rates were lower for aged-contaminated as compared with fresh-contaminated sediment, suggesting that the aging process could significantly decrease the bioavailability of phenanthrene. Lai *et al*. (2009) compared the efficiencies with which 3 different surfactants were able to remove TPH from soil heavily contaminated with oil. The study found that TPH removal efficiency for soil contaminated with *ca*. 3g TPH/kg dry soil by rhamnolipids, Tween 80 and Triton X-100 was 14%, 6%, and 4%, respectively. TPH removal efficiency for soil contaminated with *ca*. 9g TPH/kg dry soil was 62%, 40%, and 35%, respectively. Removal efficiency increased with an increase in biosurfactant concentration but did not vary for the contact time of 1 and 7 days.

ii. Oil

Oil spill accidents are becoming increasingly common and have resulted in ecological and economical catastrophes. The Exxon Valdez spill near Prince William Sound in 1989 is an example of major coastline contamination. The ability of biosurfactants to emulsify hydrocarbon-water mixtures has been shown to enhance the biodegradation of hydrocarbons *in situ*, thus making them potentially useful tools for oil spill pollution control (Banat, 1995; Desai and Banat, 1997).

Chakrabarty (1985) described the production of an emulsifier by *P. aeruginosa* SB30 that could disperse oil into fine droplets, which could enhance biodegradation. Harvey *et al*. (1990) tested a biosurfactant from the same *Pseudomonas* strain for its ability to remove oil from Alaskan gravel samples contaminated from the Exxon Valdez spill. The authors reported oil displacement (2- to 3-fold) in comparison to the water control and a reduction in contact time from 1.5 – 2min to 1min for water. The efficacy of *in situ* bioremediation on the Exxon Valdez oil spill was later demonstrated by Bragg *et al*. (1994). Shafeeq *et al*. (1989) reported the production of biosurfactants by *P. aeruginosa* S8, which was isolated from oil-contaminated sea water, and the degradation of hexadecane, heptadecane, octadecane and nonadecane *in situ* by up to 47, 58, 73 and 60%, respectively. Chhatre *et al*. (1996) described the degradation of 70% of Gulf and Bombay High crude oil by 4 isolates from crude oil. Additionally, one of the isolates produced an emulsifying rhamnolipid that consequently enhanced biodegradation of the crude oil.

A recent development is the possibility of biosurfactants to disperse oil slicks (Holakoo and Mulligan, 2002). At 25ºC and a salinity of 35 ‰, a solution of 2% rhamnolipids, applied at a dispersant to oil ratio (DOR) of 1:2, immediately dispersed 65% of a crude oil. The co-addition of 60% ethanol and 32% octanol with 8% rhamnolipids applied at a DOR of 1:8 improved the dispersion to 82%. The dispersion efficiency was found to decrease in fresh water and at lower temperatures; however, this could be improved by altering the formulation. Comparisons of the dispersion behaviour to the control indicated that the rhamnolipids have excellent potential as oil dispersing agents.

In a study that compared the efficiency with which different surfactants (SDS, rhamnolipid and saponin) were able to remove crude oil from contaminated soil using a soil washing process, Urum *et al*. (2006) found that rhamnolipid was able to remove 44% of the crude oil from contaminated soil. Rhamnolipid and SDS were shown to remove aliphatic preferentially to aromatic hydrocarbons, corresponding to their higher removal of crude oil, as compared with saponin, which removed 27% of the crude oil. The efficiency of rhamnolipids, together with organic lipophilic nutrients and molasses, to enhance the removal of crude oil from artificially contaminated sea water was investigated by Nikolopoulou and Kalogerakis (2008). They found

that the addition of rhamnolipids increased the removal of $C_{19} - C_{34}$ alkanes to 96% within 18 days and reduced the lag phase.

Although the studies report mixed results, they do advocate for the potential commercial uses of biosurfactants. Application is currently limited by production and inefficient experience. However, considering the increasing awareness of water control and environmental conservation and the expanding demand for natural products, it appears inevitable that high-quality biosurfactants will play an increasingly important role in many of the applications that have been outlined above.

1.3. *Pseudomonas* SPECIES IN FOOD

In order to expand our current knowledge, more information regarding the application of biodegradative *Pseudomonas* spp. is required. As demonstrated by the preceding subchapter (1.2), scientists have commonly turned to contaminated sites as sources for the isolation of biosurfactant-producing, hydrocarbon-degrading microorganisms. Such studies have produced varied results.

An alternative and largely untapped source of biosurfactant-producing *Pseudomonas* spp. is food. *Pseudomonas* spp. comprise the largest genus of bacteria that exists in fresh and spoiled foods, especially red meats, poultry and seafood products and vegetables (Jay, 2000, 2005). Many of these foods contain oils, potentially necessitating the presence of biosurfactants which can increase the bioavailability of those oils to initial and spoilage microflora. Members of the genus *Pseudomonas* are ubiquitous saprophytic opportunistic plant, animal and humans pathogens typical of soil and water bacteria. Primary sources and routes of these microorganisms to fresh foods include handling by handlers, handling tools such as knives, the use of non-sterile containers and the handling and storage environment. In the case of fresh meats, animal hides, the gastrointestinal tract and lymph nodes are also noteworthy sources of contaminating microorganisms (Jay, 2000).

A. Fresh meats, poultry and fish

i. Red meats and poultry

It has consistently been observed that although the initial microflora of fresh meat and poultry products varies greatly, this variety yields to only a few genera by the time spoilage becomes obvious. Spoilage results when microbial loads reach *ca.* 7 – 8 log CFU/cm^2 and organoleptic deterioration occurs. The spoilage rate depends on several factors, such as the numbers and types of contaminating psychrotrophs, storage temperature, intrinsic parameters (e.g. pH), and type of packaging. However, under aerobic conditions *Pseudomonas* spp., especially *P. fluorescens*, *P. fragi*, *P. lundensis*, *P. putida* and related spp., dominate the spoilage flora of fresh meat and poultry (Lambert *et al*., 1991; Arnaut-Rollier *et al*., 1999a, b; Ellis and Goodacre, 2001; Jay *et al*., 2003; Ercolini *et al*., 2006). Microorganisms quantitatively represented at the time of spoilage are already present on the fresh red meats and poultry in substantial numbers, and the larger the percentage of pseudomonads in the initial flora, the sooner spoilage becomes obvious (Jay, 2000).

Such foods have pH values within the growth range of most of the microorganisms found on these foods (Jay, 2000). They also contain adequately high concentrations of low-molecular-mass nutrients, particularly simple sugars, to support the early growth of bacteria which are able to utilise these simple constituents, such as pseudomonads (Gill and Newton, 1977). As growth progresses and these low-molecular-mass nutrients are depleted, proteolysis – mediated by certain spoilage microorganisms – is speculated to be a key metabolic process for liberating further nutrients to support bacterial growth (Nychas *et al*., 1988; Greer, 1989). This process of proteolysis has significant effects on the degradation of different types of muscle proteins (Greer, 1989) and is thought to be necessary for the penetration of meats by bacteria (Gill and Penney, 1977). As the dominant microorganisms in aerobic refrigerated foods, many strains of psychrotrophic *Pseudomonas* spp. are the major producers of extracellular proteolytic enzymes (Fox *et al*., 1989; Suhren, 1989).

ii. Fish

As with fresh meat and poultry, the initial microflora of fish varies and reflects its ecological environments. Fish originating from temperate and tropical waters yield microbial loads of *ca*. 2 – 4 and 3 – 6 log CFU/cm^2 of skin and gill surface, respectively (ICMSF, 2000). The bacterial flora of temperate fish are dominated by the psychrotropic Gram-negative genera, including *Pseudomonas, Acinetobacter*, *Flavobacterium*, *Moraxella* and *Shewanella*. *P. fluorescens*, *P. putrefaciens*, other fluorescent pseudomonads and other pseudomonads exhibit the highest degree of spoilage activity, and late spoilage is dominated by group II pseudomonads, represented by *P. fragi* (Hui, 1992). Members of the *Aeromonadaceae* and *Vibrionaceae* also occur commonly (Shewan, 1977; Fraser and Sumar, 1998; Chytiri *et al*., 2004) and dominate the spoilage flora at higher spoilage temperatures (ICMSF, 2000).

In contrast to red meats and poultry, fish contain comparatively high levels of proteins and other nitrogenous constituents, including free amino acids and volatile nitrogen compounds such as urea, trimethylamine oxide and histamine. The carbohydrate content of fish; however, is negligible (Jay, 2000). Autolysis dominates the earlier stages of spoilage and is succeeded by microbial degradation in the later stages of spoilage. As the growth of spoilage bacteria increases, the microorganisms initially utilise the simpler products and, in the process, release various volatile off-odour components including aldehydes, esters, ketones and non-dihydrogen sulphide sulphides (Fraser and Sumar, 1998).

B. Dairy and eggs

i. Milk and dairy products

Since the implementation of refrigerated storage of raw and processed milk, psychrotrophic bacteria have become an increasing problem for the dairy industry (Sørhaug and Stepaniak, 1997). Although *Pseudomonas* spp. represent a low proportion (*ca.* 10%) of the microflora of freshly drawn milk, they are the predominant microorganisms (70 – 90%) in raw or pasteurised milk at the time of spoilage during refrigerated storage (Sørhaug and Stepaniak, 1997; Wiedmann *et al*., 2000) and are typically present as a result of post-process contamination (Cousin, 1982; Meer *et al*., 1991). They comprise mainly *P. fluorescens, P. fragi, P. putida* and *P. putrefaciens*

(Gilmour and Rowe, 1990). Spoilage may also be caused by other post-process contaminants, spore-formers and thermoduric species such as *Arthrobacter*, *Bacillus*, *Clostridium*, *Corynebacterium*, *Microbacterium* and *Streptococcus* (Cousin, 1982; Meer *et al*., 1991; Sørhaug and Stepaniak, 1997).

In addition to rapid growth at low temperatures, many *Pseudomonas* spp. also produce heat stable lipases and proteases which survive pasteurisation and ultra high temperature (UHT) treatment (sterilised at 135 – 148ºC for 2 – 5s). Most proteases produced by psychrotrophs are readily able to degrade κ-, α_{81}- and β-caseins and are associated with organoleptic changes in processed milk and cheese, while lipases typically produced by *Pseudomonas* spp. hydrolyse triglycerides, thereby resulting in flavour defects associated with fat hydrolysis in dairy and UHT products (MacPhee and Griffiths, 2002).

ii. Eggs

Freshly laid eggs are generally sterile; however, many microorganisms may be found on the external surface within a short period of time and, under the appropriate conditions, may enter the eggs, grow and cause spoilage. As mentioned with meat, poultry and fish, although the microflora on the egg shell varies depending on several factors, spoilage tends to be caused by the same microorganisms. Fluorescent pseudomonads are most commonly associated with the spoilage of eggs during and after the removal of eggs from storage (Lorenz and Starr, 1952; Ayres, 1960). This is mainly due to the production of fluorescent pigments which facilitate penetration of the egg shell and subsequent resistance to the internal antimicrobial properties of the egg white. Other bacteria able to act as primary invaders include *Alcaligenes*, *Citrobacter*, *Flavobacterium* and *Proteus* spp., while secondary invaders include members from the genera *Enterobacter*, *Escherichia* and *Moraxella* (Elliott, 1958; Ayres, 1960; Board, 1965; Braun and Fehlhaber, 1995).

Once in the yolk, bacteria grow in the nutrient-rich medium, producing by-products of protein and amino acid metabolism such as dihydrogen sulphide and other foul-smelling compounds. Significant bacterial growth typically results in the yolk becoming less viscous and discoloured (Jay, 2000). The most common form of bacterial spoilage is process referred to as “rotting”.

Green rots are caused by *Pseudomonas* spp. (*fluorescens*); colourless rots by *Pseudomonas*, *Acinetobacter* and *Moraxella*; black rots by *Proteus*, *Pseudomonas*, *Aeromonas* and *Alcaligenes* spp.; pink rots by *Pseudomonas* spp.; red rots by *Pseudomonas* and *Serratia* spp. and so-called "custard rots" by *Proteus vulgaris* and *P. intermedium*. Bacteria can also result in a condition known as mustiness, which is suggested to be caused by *P. graveolens* and *Proteus* spp., with the former producing the most characteristic spoilage pattern (ICMSF, 2000; Jay, 2000). The type of bacterial rot depends on the spoilage bacteria. For example, green rot results from the fluorescence of the egg white produced by non-proteolytic *P. fluorescens* whereas pink rot results from lecithinase-producing *P. fluorescens*. Black rot occurs due to the potent proteolytic ability of bacteria such as *Proteus, Pseudomonas, Aeromonas* and *Alcaligenes* spp. (Stadelman, 1994).

C. Fruits and vegetables

Vegetables have a high water content and a relatively low carbohydrate and fat content. The pH range of most vegetables is within the growth range of a large number of bacteria, but that of fruits is low. Thus, it is not surprising that bacteria are common agents of spoilage (Jay, 2000).

The demand for minimally processed refrigerated fruits and vegetables has increased considerably over the last few years, predominantly because consumers perceive such products as being fresh, healthy, tasty and convenient (Wiley, 1994; Garret *et al*., 2003). Understandably, such ready-to-use vegetables retain much of their native microflora after minimal processing, where bacterial counts have been reported to range from *ca*. 3 – 7.5 log CFU/g (ICMFS, 2000; Allende *et al*., 2004; Tournas, 2005). Minimally processed fresh (MPF) vegetables may also be subsequently contaminated from a number of sources, including post-harvest handling and processing (Beuchat, 1996; Beuchat and Ryu, 1997). MPF vegetables are typically stored in modified atmosphere packaging (MAP), which inhibits the growth of aerobic spoilage microorganisms of fresh processed vegetables but may allow or stimulate the growth of pathogens. The bacterial flora of MPF vegetables stored in MAP has previously been shown to be predominantly psychrotrophs such as *Pseudomonas* spp., but also *Aeromonas* (*hydrophila*), *Campylobacter*, *Clostridium*, *Enterobacter*, *Escherichia*, *Salmonella* and *Yersinia* spp., and *Listeria monocytogenes* (Szabo *et al*., 2000; Viswanathan and Kaur, 2001). Numerous reports have shown that bacterial counts in unprocessed and MPF vegetables usually range from *ca*. log

5 – 7 CFU/g (Allende *et al*., 2004; Johnston, 2005). The spoilage of MPF vegetables during refrigerated storage results from soft rot, which is largely due to psychrotrophic *Pseudomonas* spp. For example, *P. cichorii* causes bacterial spot in cabbage, *P. marginalis* in endives, lettuce and potatoes, and *P. maulicola* in broccoli and cauliflower (Tournas, 2005). At the time of spoilage, bacterial numbers can reach over 8 log CFU/g (Brocklehurst *et al*., 1987; Nguyen-The and Prunier, 1989).

Spoilage microorganisms produce hydrolytic enzymes which hydrolyse plant components, such as pectin. Pectinolytic enzymes such as polygalacturonase, pectin esterase and pectate transeliminases break down pectin, resulting in the deterioration of the plant tissue. This decay sequentially manifests as softening and liquefaction of the tissue; the hallmark of bacterial spoilage. Pectin degradation occurs in the early stage of spoilage and may be followed by the degradation of cellulose, which is mediated by cellulases produced by some microorganisms (Codner, 1971; Tournas, 2005).

Considering the unambiguous preponderance of *Pseudomonas* spp. in foods and their impressive capacity for biosurfactant production, as illustrated in preceding subchapters, foods appear to be a potential source of biosurfactant-producing bacteria worth exploring.

1.4. MOTIVATION

Biosurfactants are surface-active compounds that are produced by a variety of diverse organisms and play many roles, which cannot be generalised. Biosurfactants are perhaps best known for being capable of efficiently increasing the surface area and bioavailability of hydrophobic substrates, and they are well known for their ability to enhance the biodegradation of various organic pollutants (petroleum hydrocarbons, including PAHs, and oil). Biosurfactants also exhibit antimicrobial activity, but this property has not been reviewed extensively. Biosurfactants have several advantages over their synthetic counterparts, making them potentially useful in environmental control and in the biomedical sciences. In developing the application of biosurfactants for these markets, scientists have commonly isolated biosurfactant-producing microbes from contaminated sites. Results from such studies; however, have been inconsistent, necessitating the identification and exploration of novel sources of biosurfactant-producing microorganisms. Considering the unambiguous preponderance of *Pseudomonas* spp. in foods and their impressive capacity for biosurfactant production, in this study, our approach is to isolate biosurfactant-producing members of the genus *Pseudomonas* from supermarket food products. By sampling this alternative source for biosurfactant-producing *Pseudomonas* spp., it may be possible to isolate biosurfactant-producing bacteria, whether novel or existing, that offer specific activities applicable in pollution remediation and in the biomedical sciences.

1.5. OBJECTIVE

Characterise the antimicrobial and biodegradative activities of biosurfactants produced by *Pseudomonas* spp. isolated from food products.

1.6. AIMS OF STUDY

1. Evaluate selected food products available for purchase from a retail supermarket for the prevalence of *Pseudomonas* and growth media using total plate (aerobic) and *Pseudomonas* selective plate counts.

2. Characterise isolates from 14 food products morphologically and biochemically.

3. Screen isolates for biosurfactant production using the drop collapsing test, and for emulsification activity using an emulsification assay.

4. Screen biosurfactant-producing isolates for antimicrobial activity by spotting biosurfactant-producing isolates and supernatants of spent biosurfactant-producing cultures onto indicator plates prepared with target microorganisms; and evaluate the stability of the antimicrobial compounds in terms of whether they are protein-based, heat stable and stable in organic solvents. Additionally, identify positive isolates by 16S rDNA sequencing.

5. Screen four Gram-positive and -negative biosurfactant-producing isolates for biodegradative activity against *n*-hexadecane and motor oil. Activity will correspond to growth, which will be inspected visually and measured by plate counts.

Table 1.1: Selected microbially produced surfactants.

Surfactant type	Producing microorganisms	Reference
Low molecular mass		
Glycolipids		
Rhamnolipids	*Pseudomonas aeruginosa*	Desai and Banat, 1997; Banat *et al*., 2000; Mulligan *et al*., 2005; Monteiro *et al*., 2007 Wu *et al*., 2007; Prieto *et al*., 2008
	Pseudomonas sp.	Desai and Banat, 1997; Nayak *et al*., 2008; Oliveria *et al*., 2008
	Serratia rubidea	Matsuyama *et al*., 1990
Trehalolipids	*Rhodococcus* sp.	Rapp *et al*., 1979; Cooper *et al*., 1989; Shulga *et al*, 1990; Singer and Finnerty, 1990; Abu-Ruwaida *et al.*, 1991a
	Nocardia sp.	Margaritis *et al*., 1979, 1980
	Mycobacterium sp.	Cooper *et al*., 1989
Sophorolipids	*Torulopsis bombicola*	Inoue and Ito, 1982; Cooper *et al*., 1989
	Torulopsis apicola	Tulloch *et al*., 1967; Hommel *et al*., 1987
	Candida petrophilum	Cooper *et al*., 1983
	Candida lipolytica	Lesik *et al*., 1989
Arthrofactin	*Arthrobacter* sp.	Morikawa *et al*., 1993
Streptofactin	*Streptomyces tendae*	Richter *et al*., 1998
Lipopeptides, lipoproteins		
Lipopeptide	*Bacillus licheniformis*	McInerney *et al*., 1990; Lin *et al*., 1994; Yakimov *et al*., 1995; Horowitz *et al*., 1990
	Bacillus pumilis	Naruse *et al*., 1990
	Pseudomonas putida	Kuiper *et al*., 2004
Viscosin	*Pseudomonas fluorescens*	Neu *et al*., 1990
Viscosinamide	*Pseudomonas fluorescens*	Nielsen *et al*., 1999
White line inducing principle (WLIP)	*Pseudomonas reactans*	Mortishire-Smith *et al*., 1991
Tensin	*Pseudomonas fluorescens*	Nielsen *et al*., 2000
Massetolides	*Pseudomonas* sp.	Gerard *et al*., 1997
	Pseudomonas fluorescens	de Bruin *et al*., 2008
Surfactin	*Bacillus subtilis*	Arima *et al*., 1968
	Bacillus pumilis	Morikawa *et al*., 1992

	Lactobacillus	Velraeds-Martine *et al*. 1996
Gramicidin S	*Bacillus brevis*	Marahiel *et al*., 1977
Polymyxins	*Bacillus polymyxa*	Suzuki *et al*., 1965
Serrawettin	*Serratia marcescens*	Matsuyama *et al*., 1991
Fatty acids, neutral lipids, phospholipids		
Fatty acids	*Corynebacterium lepus*	Cooper *et al*., 1978, 1989
	Penicillium spiculisporum	Ishigami *et al*., 1982
	Arthrobacter paraffineus	Duvnjak *et al*., 1982
Neutral lipids	*Nocardia erythropolis*	MacDonald *et al.*, 1981
	Clostridium pasteurianum	Cooper *et al*., 1980
Phospholipids	*Thiobacillus thiooxidans*,	Beeba and Umbreit, 1971
	Corynebacterium insidiosum	Akit *et al*., 1981
High molecular mass		
Polymeric substances		
Emulsan	*Acinetobacter calcoaceticus*	Rosenberg *et al*., 1979; Zosim *et al*., 1982
	Pseudomonas fluorescens	Desai *et al*., 1988
Biodispersan	*Acinetobacter calcoaceticus*	Rosenberg *et al*., 1988
Alasan	*Acinetobacter radioresistens*	Navon-Venezia *et al*. 1995
Liposan	*Candida lipolytica*	Cirigliano and Carman, 1984, 1985
Mannosylerythritol lipids	*Candida antarctica*	Kitamoto *et al*., 1990
Mannan-lipoprotein	*Candida tropicalis*	Kaepelli *et al*., 1984
Protein PA	*Pseudomonas aeruginosa*	Hitsatsuka *et al*., 1972; 1977
Particulate compounds		
Particulate surfactant (PM)	*Pseudomonas marginalis*	Burd and Ward, 1996
Biosur PM	*Pseudomonas maltophila*	Phalle *et al*., 1995
Whole cells	Variety of bacteria	Fattom and Shilo, 1985; Rosenberg, 1986

Table 1.2: Antimicrobial activities of rhamnolipids isolated in various studies.

Target microorganism	MIC (µg/ml)	Reference
Gram negatives		
Alcaligenes faecalis	32	Abalos *et al*., 2001
Enterobacter aerogenes	4	Benincasa *et al*., 2004
Escherichia coli	16	Abalos *et al*., 2001
	250	Benincasa *et al*., 2004
	ND	Onbasli and Aslim, 2008
Proteus mirabilis	8	Benincasa *et al*., 2004
Pseudomonas aeruginosa	32	Benincasa *et al*., 2004
Salmonella typhimurium	16	Benincasa *et al*., 2004
Serratia marcescens	32	Abalos *et al*., 2001
Gram positives		
Arthrobacter oxidans	16	Abalos *et al*., 2001
Bacillus cereus v. mycoide	4	Benincasa *et al*., 2004
	64	Abalos *et al*., 2001
Bacillus subtilis	8	Benincasa *et al*., 2004
	64	Abalos *et al*., 2001
	ND	Onbasli and Aslim, 2008
Clostridium perfringens	256	Abalos *et al*., 2001
Micrococcusluteus	32	Abalos *et al*., 2001
	128	Benincasa *et al*., 2004
Mycobacterium phlei	16	Abalos *et al*., 2001
Staphylococcus aureus	8	Benincasa *et al*., 2004
	128	Abalos *et al*., 2001
Staphylococcus epidermidis	8	Abalos *et al*., 2001
	250	Benincasa *et al*., 2004
Streptococcus faecalis	4	Benincasa *et al*., 2004
	64	Abalos *et al*., 2001
Fungi		
Alternaria alternata	4	Benincasa *et al*., 2004
Aspergillus niger	16	Abalos *et al*., 2001
Botrytis cinerea	18	Abalos *et al*., 2001
Candida albicans	32	Benincasa *et al*., 2004
Cercospora kikuchii	50	Kim *et al*., 2000
Chaetomium globosum	32	Abalos *et al*., 2001; Benincasa *et al*., 2004
Cladosporium cucumerinum	25	Kim *et al*., 2000

Colletotrichum orbiculare	25	Kim *et al*., 2000
Cylindrocarpon destructans	50	Kim *et al*., 2000
Gliocladium virens	16	Abalos *et al*., 2001
	32	Benincasa *et al*., 2004
Magnaporthe grisea	50	Kim *et al*., 2000
Penicillium crysogenum	32	Abalos *et al*., 2001; Benincasa *et al*., 2004
Phytophthora capsici	10	Kim *et al*., 2000
Rhizoctonia solani	18	Abalos *et al*., 2001

ND, not determined

Table 1.3: Potential applications of biosurfactants. †

Function	Application field
Emulsifiers and dispersants	Cosmetics, paints, petroleum production/products, elastomers and plastics, additives for rolling oil, leather industry, food industry, waxes and polishes
Solubilisers and microemulsions	Pharmaceuticals, toiletries, food and beverage industry
Wetting and penetrating agents	Paints, pharmaceuticals, textile industry, industrial cleaning
Detergents	Household, agriculture products (herbicides, pesticides), high tech production
Foaming agents	Cosmetics, ore floatation, casting
Thickening agents	Paints
Metal sequestering agents	Mining
Vesicle forming materials	Cosmetics, drug delivery systems
Microbial growth enhancers	Sewerage sludge treatments for oily wastes, fermentation
Demulsifers	Waste treatment
Viscosity reducing agents	Pipeline transportation
Dispersants	Coal-oil mixture, coal-water slurry
Resource recovery agents	Tertiary recovery of oil

† Adapted from Rosenberg and Ron, 1999; Banat *et al*., 2000; Kosaric, 2001

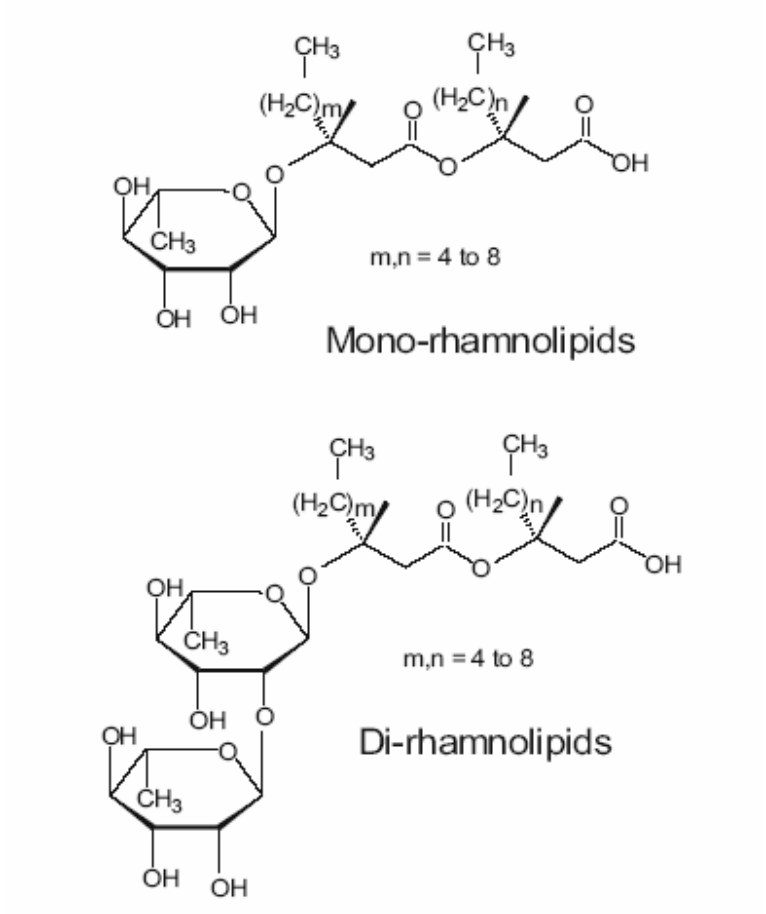
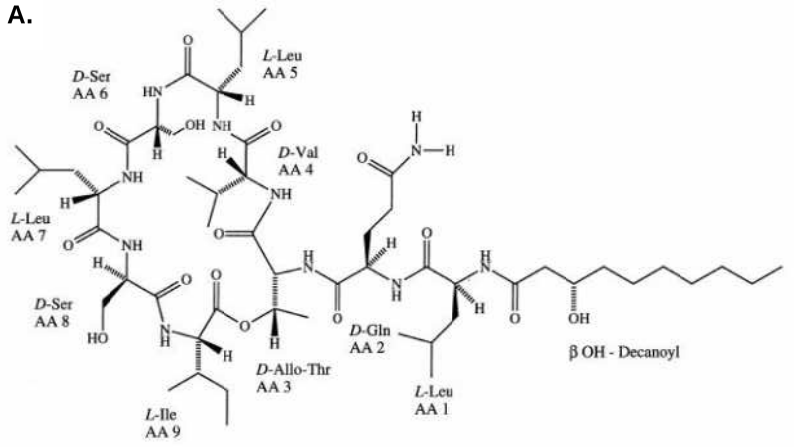
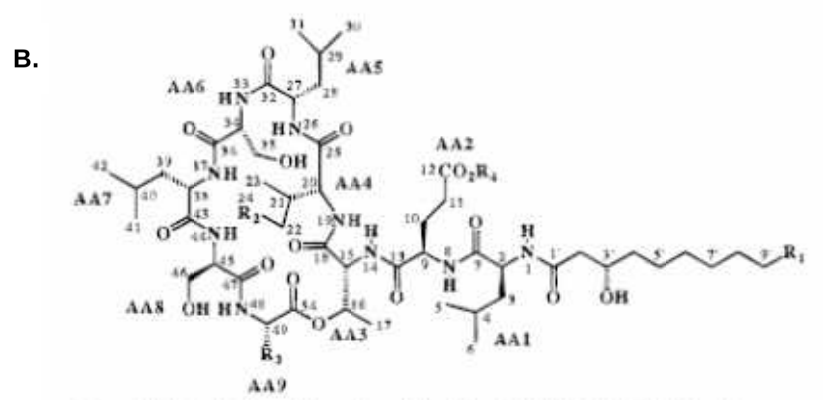


Figure 1.1: Chemical structures of rhamnolipids produced by *P. aeruginosa (*Soberón-Chávez *et al*., 2005).





Massetolide A (1) $R_1 = CH_3$-, $R_2 = CH_3$, $R_3 = CH(CH_3)CH_2CH_3$, $R_4 = H$

Massetolide B (2) $R_1 = CH_3CH_2$-, $R_2 = CH_3$, $R_3 = CH(CH_3)CH_2CH_3$, $R_4 = H$

Massetolide C (3) $R_1 = CH_3CH_2CH_2$-, $R_2 = CH_3$, $R_3 = CH(CH_3)CH_2CH_3$, $R_4 = H$

Massetolide D (4) $R_1 = CH_3$-, $R_2 = CH_3$, $R_3 = CH_2CH(CH_3)_2$, $R_4 = H$

Massetolide E (5) $R_1 = CH_3$-, $R_2 = H$, $R_3 = CH(CH_3)_2$, $R_4 = H$

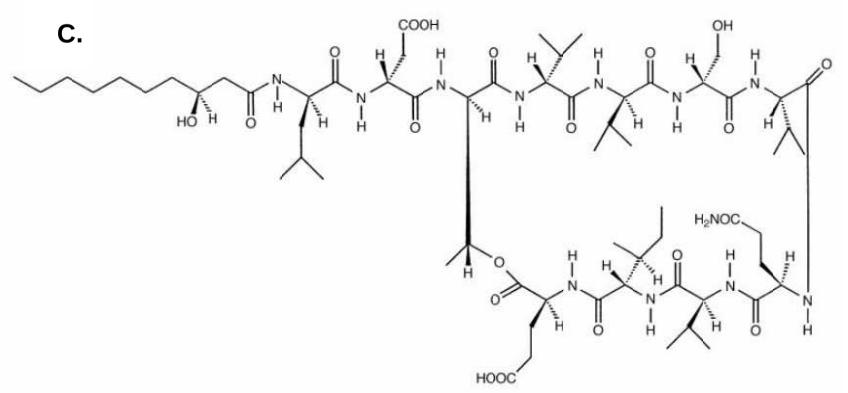
Massetolide F (6) $R_1 = CH_3$-, $R_2 = H$, $R_3 = CH_2CH(CH_3)_2$, $R_4 = H$

Massetolide G (7) $R_1 = CH_3CH_2$-, $R_2 = H$, $R_3 = CH(CH_3)CH_2CH_3$, $R_4 = H$

Massetolide H (8) $R_1 = CH_3CH_2CH_2$-, $R_2 = H$, $R_3 = CH(CH_3)CH_2CH_3$, $R_4 = H$

Viscosin (9) $R_1 = CH_3$-, $R_2 = H$, $R_3 = CH(CH_3)CH_2CH_3$, $R_4 = H$

Ester (10) $R_1 = CH_3$-, $R_2 = CH_3$, $R_3 = CH(CH_3)CH_2CH_3$, $R_4 = CH_3$



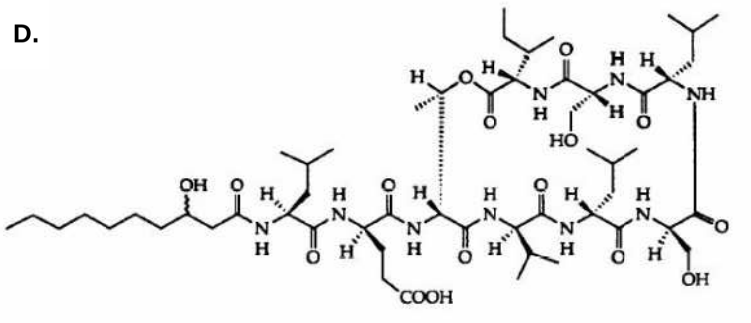


Figure 1.2: Chemical structure of (**A**) viscosinamide (Nielsen *et al*., 1999), (**B**) the massetolides (Gerard *et al*., 1997), (**C**) tensin (Nielsen *et al*., 2000) and (**D**) WLIP (Mortishire-Smith *et al*., 1991).

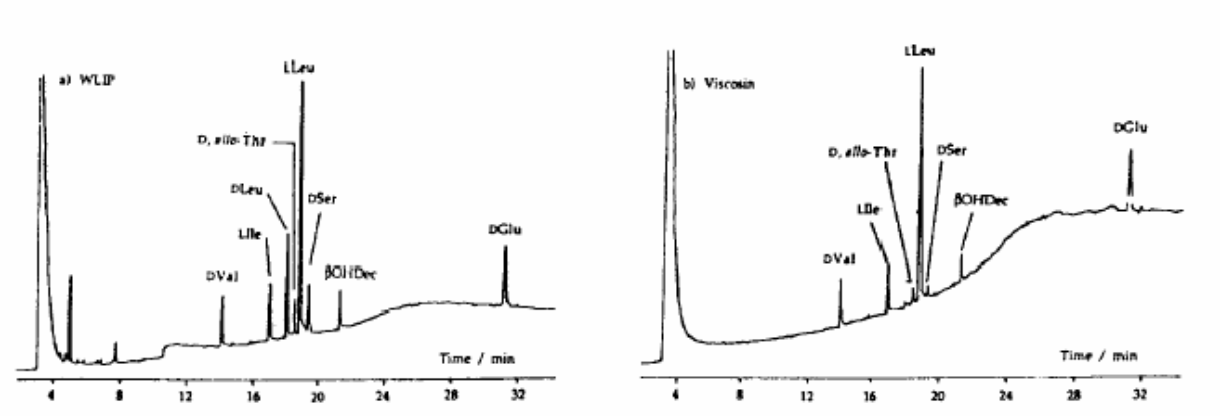


Figure 1.3: Chiral gas chromatography analyses of the N, O-trifluoroacetyl isopropyl ester derivatives of the total hydrolysates of a) WLIP and b) viscosin. The extended hydrolysis period resulted in the low intensities of the D-*allo*-threonine and D-serine peaks observed in the viscosin trace (Mortishire-Smith *et al*., 1991).

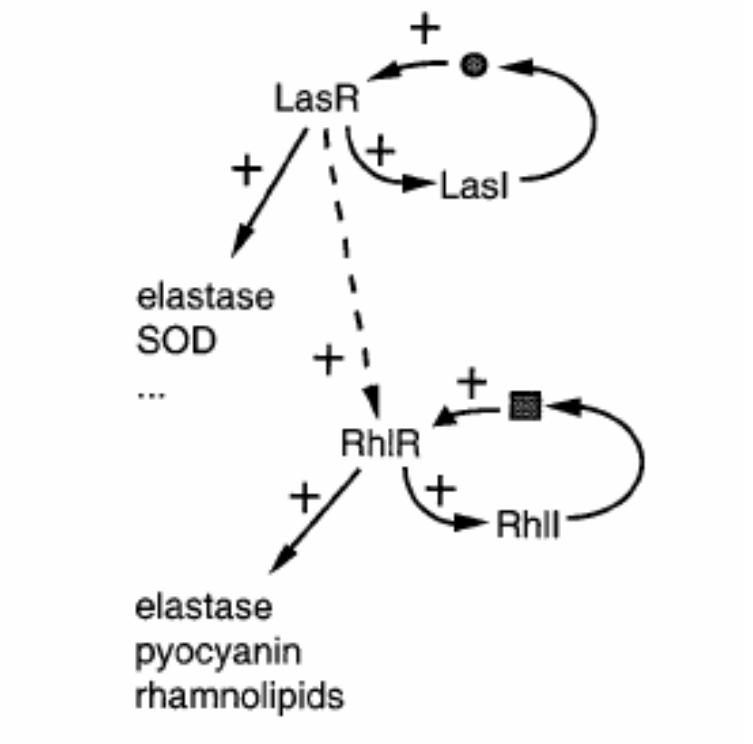


Figure 1.4: Quorum sensing in *P. aeruginosa*. In *P. aeruginosa*, two quorum-sensing systems, *las* and *rhl*, control the transcription of secreted compounds, including proteases (LasB elastase), pyocyanin and rhamnolipids. The systems include a transcriptional regulator (LasR and RhlR, respectively) and an autoinducer synthase (LasI and RhlI, respectively). When the bacterial density is sufficient to form a quorum, the accumulation of signaling autoinducer molecules (● and ■) in the medium induces the *las* and *rhl* pathways, resulting in the transcription of virulence genes. The *las* quorum-sensing system can, to a degree, induce the *rhl* system. SOD, superoxide dismutase (Cosson *et al*., 2002).

CHAPTER 2

Food as a novel source for biosurfactant-producing bacteria, with emphasis on *Pseudomonas* strains

ABSTRACT

The aim of this study was to evaluate whether food could be a novel source of biosurfactant-producing bacteria. A total of 14 food products in the categories of water, dairy, meat, fish and vegetables were exposed to an elevated temperature of 30˚C for three days in order to induce spoilage. Aerobic plate counts (APC) and *Pseudomonas* counts (PC) were performed by standard plating methods using tryptone soy agar and *Pseudomonas* selective agar, respectively. Vegetable, fish and meat product samples were found to have the highest APC, ranging from *ca*. 8.5 – 9.5 log CFU/g, in descending order. By contrast, the water samples had the lowest APC of 4.45 log CFU/ml. Similarly, PCs from vegetable, fish and meat products ranged from *ca*.8 – 9 log CFU/g. The lowest PC was recorded for dairy product samples (<2 log CFU/ml). Preliminary identification of the isolates was achieved by Gram staining and standard biochemical tests. The spoilage microflora of bottled water samples were predominated by *Bacillus* (27%), RT V (36%) and RT VI (27%) Gram-negative bacilli (GNB). Milk samples were predominated by *Lactobacillus* (47%), RT V (24%) and RT VI (24%) GNB, while meat and fish products were predominated by RT II (78 and 96%, respectively) GNB, which includes *Aeromonas*/*Vibrio* strains. RT IV (28%), V (21%) and VI (33%) GNB were the predominant populations identified from vegetable products. *Pseudomonas* was identified at high proportions from the *Pseudomonas* selective medium in only the water and vegetable products, highlighting the need to confirm the identities of isolates from selective media. A total of 568 strains were screened for biosurfactant activity. The isolates were tested using the drop collapse assay, which is based on the collapsibility of a drop of bacterial cell suspension placed on an oil droplet. Of these, 32 (6%) tested positive for biosurfactant activity. In addition, none of the 32 isolates tested positive for emulsifying activity. This study demonstrated the possibility of isolating biosurfactant-producing bacteria from foods, thus providing a further alternative source of these bacteria other than soils.

INTRODUCTION

Biosurfactants are an interesting group of secondary metabolites produced by a wide variety of microorganisms. They are amphipathic molecules and thus exhibit surface activities. Their structures vary greatly, and are dependent upon their carbon substrate and hydrophobic and polar domains (Ron and Rosenberg, 2001). Many of the known biosurfactants are glycolipids. The best studied of these molecules are the rhamnolipids, which were first described in *Pseudomonas aeruginosa* (Jarvis and Johnston, 1949). It has since been found that several isolates of *Pseudomonas* produce a range of biosurfactants, including lipopeptides and lipoproteins (low-molecular-mass), and polymeric and particulate compounds (high-molecular-mass) (Ron and Rosenberg, 2001, 2002; Mulligan, 2005).

The diversity of origin, chemical structure and surface active properties of biosurfactants makes it difficult to generalise about the roles of biosurfactants, despite their clear significance in the physiology of their producer organism. Most notably, biosurfactants have been found to both increase the surface area and bioavailability (solubility) of hydrophobic water-insoluble substrates. As a result of this property, most research has been conducted on developing biosurfactants for application in bioremediative efforts.

In developing biosurfactants for commercial application, scientists have traditionally isolated biosurfactant-producing microorganisms from contaminated soil sites (Banat, 1993; Whyte *et al*., 1997; Stelmack *et al*., 1999; Kanaly and Harayama, 2000; Christofi and Ivshina, 2002; Prabhu and Phale, 2003; Mulligan, 2005; Vasileva-Tonkova *et al*., 2006; Singh *et al*., 2007; Whang *et al*., 2008). However, results from such studies, have been inconsistent, thus necessitating the identification and investigation of novel sources of biosurfactant-producing bacteria (Rouse *et al*., 1994; Desai and Banat, 1997; Christofi and Ivshina, 2002). One such source may be food. Each food product harbours its own unique microflora at any give point in time during production and storage, and this microflora is an effect of a number of factors including the initial flora, processing and storage conditions. Of the initial microflora, some of the constituent isolates will be able to produce spoilage metabolites and consequently contribute to spoilage of the food product. Despite the variety in microfloras and the factors affecting them in different products,

this variety typically yields to spoilage patterns which may emerge in different products under similar conditions (Gram *et al*., 2002). This is exemplified by *Pseudomonas* spp., which have consistently been found to comprise the largest genus of bacteria that exists in fresh and spoiled aerobic, refrigerated food; including red meats, poultry, seafood products, and vegetables (Jay, 2000; Szabo *et al*., 2000; Chytiri *et al*., 2004; Ercolini *et al*., 2006; Wiedmann *et al*., 2006). Many of these foods contain oils, potentially necessitating the presence of biosurfactants which can increase the bioavailability of those oils to spoilage bacteria. Considering the unambiguous preponderance of *Pseudomonas* spp. in foods and their impressive capacity for biosurfactant production, foods appear to be a logical potential source of biosurfactant-producing bacteria worth exploring.

Thus, the aim of this study was to isolate potential biosurfactant-producing bacteria, including *Pseudomonas*, from foods.

MATERIALS AND METHODS

Sample collection and processing.

A total of 14 food products, including water, milk and cream, red meats, fish and vegetables, in their original packaging, were collected from a local supermarket on 3 separate occasions. All of the samples were stored at 4ºC until incubation at an elevated temperature of 30ºC for three days to induce spoilage. The food products selected for this study are listed in Table 2.1.

Sample processing and bacteriological examination.

For each sample, 20g was aseptically removed from the original sample and homogenised for 2min in 180ml diluent (0.1% bacteriological peptone, 0.85% NaCl) in a Colworth 400 Stomacher (Seward Medical, London, UK). A tenfold serial dilution (10^0 to 10^{-8}) was prepared in the same diluent and plated in duplicate using the spread-plate technique on three different media within 20min of the dilution series being prepared. The media used include tryptone soy agar (TSA; Biolab, Midrand, South Africa); *Pseudomonas* selective agar, supplemented with cefalotin sodium (PSA; Merck, Modderfontein, South Africa) and cetrimide agar (CA; Scharlau,

Barcelona, Spain), which were used to determine aerobic (APC), *Pseudomonas* (PC) and *P. aeruginosa* (PAC) plate counts, respectively. Plates were then inverted and incubated aerobically at 25ºC for 24 – 48h. Plates exhibiting between 30 and 300 colony forming units (CFU) or the highest number, if below 30, were counted and the log CFU per gram or ml (log CFU/g or ml) determined.

Selection of isolates.

Predominant populations were obtained from the TSA media as previously described (von Holy and Holzapfel, 1988), while presumptive *Pseudomonas* isolates were selected using Pake's disk (Harrigan and McCance, 1976). A total of 963 isolates were collected in this way, including 154 from the TSA plates, and 809 from the PSA and CA plates. All isolates were streak-plated onto TSA to ensure purity.

Preliminary identification of isolates.

Morphology of the isolates was examined by using light microscopy and the Gram-stain technique (Shewan *et al*., 1960). Biochemical tests included an oxidase test (Kovacs, 1956) as well as a catalase test (3% H_2O_2), which was performed on stock TSA plates. Oxidative or fermentative acid production from glucose on OF medium (Biolab) according to Hugh and Leifson (1953) was also performed. The medium was incubated at 30ºC for 24 – 48h. Identification was determined using the dichotomous key of Fisher *et al*. (1986), and Gram-negative bacilli were further classified into reaction type (RT) groups based on oxidative-fermentative reactions, shown in Table 2.2.

Screening of isolates for biosurfactant and emulsifying activities.

Due to similar populations arising on PSA and CA, as determined by preliminary identification as described previously, predominant populations on TSA and PSA only were selected for further screening for potential biosurfactant-producing strains. The test for biosurfactant production among the isolates obtained from the TSA and PSA was a test for the ability of a drop of cell suspension from each isolate to collapse on the surface of an oil drop, as described by Jain *et al*. (1991). For this assay, 40µl of cooking oil was added to the surface of a sheet of laboratory parafilm and allowed to equilibrate to room temperature (*ca*. 25°C) for 1h. A loopful of cells of

each isolate cultured on TSA (30ºC, overnight) was suspended in 150µl sterile distilled water and 50µl of the cell suspension was then added to the surface of an oil drop. The shape of the drop on the surface of oil was inspected visually after 1 – 2min. Biosurfactant-producing isolates produced collapsed drops while those that did not remained stable. Sterile distilled water, and sterile distilled water plus 1% sodium dodecyl sulphonate (SDS, 1:1), functioned as negative and positive controls, respectively.

To assay for emulsifying activity, supernatants of the biosurfactant-producing isolates were obtained by subjecting each isolate (grown at 30°C, 150rpm, overnight in tryptone soy broth) to centrifugation (5min, 5000rpm) (Risøen *et al*., 2004), and 500µl was added to an equal volume of cooking oil in an Eppendorf microfuge tube and vortexed for 30s. Tryptone soy broth (TSB, Biolab), and TSB + 1.0% Tween (1:1) functioned as the negative and positive controls, respectively. The rationale behind using cooking oil for the emulsification assay was based on two considerations. Firstly, emulsification was investigated in a food system. Secondly, several studies have demonstrated that the addition of vegetable oil can accelerate and enhance the removal of PAHs and chlorinated aliphatic hydrocarbons (Borden, 2007; Gong *et al*., 2007; Pannu *et al*., 2004). The presence/absence of an emulsion layer of each of the test isolates was observed after the amount of time taken for the emulsion layer in the TSB control to separate (*ca*. 20min).

RESULTS

Bacterial counts.

In order to determine the microbial ecology of spoiled food products, APCs, PCs and PACs were determined. Spoiled vegetable, fish and meat product samples were found to have the highest APC, ranging from *ca*. 8.5 – 9.5 log CFU/g, in descending order (Fig. 2.1). By contrast, the water samples had the lowest APC of 4.45 log CFU/ml (Fig. 2.1). Similarly, PCs from vegetable, fish and meat products ranged from *ca*. 8 to 9 log CFU/g (Fig. 2.1). The lowest PC was recorded for dairy product samples (<2 log CFU/ml) (Fig. 2.1).

Identification of isolates.

Predominant spoilage populations.

The percentage distribution of aerobic predominant populations isolated from the 14 different food products is shown in Figure 2.2A. Predominant populations of *Bacillus*, reaction type (RT) V and VI Gram-negative bacilli (GNB) each were isolated from the water samples in similarly-sized proportions (27%, 36% and 27%), while RT IV GNB comprised the remaining 9% of the predominant isolates. Predominant isolates from dairy product samples included *Lactobacillus* spp. (47%), RT V (24%) and VI (24%) GNB (Fig. 2.2A). The meat products selected for this study were a combination of fresh beef and lamb meats, and ground beef and lamb meat products. The isolates selected from meat product samples were predominately RT II GNB (78.4%), with 6 of the 9 other bacterial groups each constituting between 1 and 4% (Fig. 2.2A). RT II GNB were predominantly isolated from fish product samples (96%) (Fig. 2.2A). *Enterococcaceae*/*Staphylococcus* comprised the remaining 4% of strains. Isolates from vegetable product samples were predominantly RT VI, V and IV GNB, each group of which represented 21 to 33% of the spoilage microflora (Fig. 2.2A). The remaining 19% comprised *Bacillus* spp. and RT II GNB. RT VI and IV GNB include *Pseudomonas* spp., particularly *P. cichorii* and *P. fluorescens*.

Putative *Pseudomonas* and *P. aeruginosa* populations.

Pseudomonas spp. occur in RTs III, IV and VI, while *P. aeruginosa* occurs within RT IV GNB. For water and vegetable products, the majority of strains selected by the Pake's disk on PSA were identified as predominantly RT IV GNB (100 and 79%, respectively), and RT II (>96%) GNB from meat products and fish (Fig. 2.2B). Alternatively, the strains isolated from meat and fish products from CA, also using Pake's disk, were identified as predominantly RT II GNB (88 – 91%), while that of vegetables were identified predominantly as RT IV (50%) and RT VI GNB (25%) (Fig. 2.2C).

Screening for biosurfactant and emulsifying activity.

Of the 568 isolates, only 32 (6%) exhibited biosurfactant activity (Fig. 2.3). The majority of the biosurfactant-producing strains (72%) isolated in this study were obtained from spoiled vegetable products, as well as bottled mineral water (19%) and low fat milk (9%) (Table 2.3). The

biosurfactant-producing isolates obtained were found to be predominantly Gram-negative bacteria (84%). Of these, RT IV GNB predominated (67%), followed by RT VI (22%), RT V (7%) and RT III (4%) GNB (Table 2.3). Gram-negative isolates were *Pseudomonas* spp., while Gram-positive isolates included *Bacillus* and *Lactobacillus* spp. None of the biosurfactant-producing isolates showed emulsifying activity with cooking oil (Fig. 2.4) with respect to the positive control, TSB plus Tween 80 (1:1).

DISCUSSION

The high APCs (*ca*. 8.5 – 9.5 log CFU/g) obtained for spoiled vegetable, fish and meat product samples were expected as all three categories of food products have pH values within the growth range of a number of Gram-negative psychrotrophs commonly found in them. Additionally, these foods have adequate nutrient and water contents to support the growth of their spoilage microflora, which may reach numbers of over 7 log CFU/g (ICMFS, 2000; Jay, 2005). These food product samples also produced the highest PCs (*ca*. 8 to 9 log CFU/g). This was also expected as *Pseudomonas* spp. comprise the largest genus of bacteria that exists in fresh and spoiled aerobic, refrigerated foods (ICMFS, 2000; Jay, 2005). The low APC reported for bottled water samples correlates with other studies, which have reported that, after bottling, the autochthonous bacterial population present in the water obtained from the source increases from 1 – 2 log CFU/ml to 4 – 5 log CFU/ml over a period of 1 – 3 weeks post bottling (Hunter, 1993; Tamagnini and Gonzalez, 1997). This type of growth is typical of bacteria in low-nutrient sources. Spoiled dairy product samples had the lowest PC (<2 log CFU/ml). Studies have shown that pasteurised milk is spoiled predominantly by thermoduric Gram-positive bacteria, as pasteurisation destroys most Gram-negative bacteria, especially psychrotrophs, including *Pseudomonas* spp. (Cousin, 1982; Meer, 1991).

The relatively high incidence of *Bacillus* (27%) in spoiled bottled water samples is surprising as studies have reported that the autochthonous flora of water is composed predominantly of the Gram-negative bacteria *Pseudomonas*, *Moraxella*, *Acinetobacter* and *Flavobacterium* spp. *Pseudomonas* spp. most frequently dominates the microflora (Guillot and Leclerc, 1993; Ferreira *et al*., 1996). One factor that could contribute to the occurrence of *Bacillus* in drinking water is

the extraction procedure (Hunter, 1993). *Bacillus* species are common soil inhabitants (Winogradsky, 1924; Henrici, 1934; Garbeva *et al*., 2003) and it is possible that the drilling process during sampling contaminated the aquifer supplying the bottled drinking water. Considering that natural mineral water may not be subjected to any treatments that alter or eliminate its biological components (EC-directive, 1980; Leclerc and Moreau, 2002), it is expected that bacteria present in the underground water supplies, whether autochthonous or introduced by drilling, would be isolated in subsequently bottled mineral water. If the aquifer is the source of the isolated *Bacillus*, it is unlikely that the isolates originated from endospores, as these have not been reported widely from aquifer systems (Leclerc and Moreau, 2002). Other factors potentially affecting the bacterial flora of drinking water include the type and size of the container, the method of bottling and storage, and the age of the water (Hunter, 1993).

The predominance of *Lactobacillus* in spoiled dairy products is accepted as the natural microflora of dairy products has commonly been reported to be predominated by *Lactobacillus*, as some strains are thermoduric and survive the pasteurisation process (Christiansen *et al*., 2006). The incidence of Alcaligenes could be attributed to contamination as *Alcaligenes* (RT V and VI GNB) is a psychrotrophic post-pasteurisation contaminant which typically originates from equipment surfaces and water supplies (Meer *et al*., 1991).

Aeromonas spp. (RT II GNB) has been reported as one of the most important spoilage bacteria of ground beef (Neyts *et al*., 2000). However, its predominance (78.4%) in the spoiled red meat samples is worth noting as it is currently classified as a potential emerging foodborne pathogen (Daskalov, 2006). *Aeromonas* is also widely distributed in both raw and processed water, and it is possible that the high incidence of *Aeromonas* observed in this study is due to contamination originating from water supplies used in the processing of the meat (Holmes *et al*., 1996). Fresh red meats are also commonly spoiled by *Pseudomonas*, *Moraxella* and *Acinetobacter* (Jay, 2005; Ercolini *et al*., 2006).

The almost exclusive predominance of RT II GNB in spoiled fish samples correlates with studies which have shown that Gram-negative bacteria, including *Aeromonas* and *Vibrio* spp., dominate fish spoilage at elevated temperatures between 10 and 37°C (Chytiri *et al*., 2004; Jay, 2005).

RT IV and VI, which predominate the spoiled vegetable products, include *Pseudomonas* spp., particularly *P. cichorii* and *P. fluorescens*, which are known to are known to dominate the spoilage microflora of MPF vegetables (Szabo *et al*., 2000; Viswanathan and Kaur, 2001). The relatively high incidence of *Alcaligenes* could also be attributed to contamination of the products during handling (Meer *et al*., 1991).

Pseudomonas and *P. aeruginosa* were putatively selected for by PSA and CA, respectively, using Pake's disk. *Pseudomonas* spp. occur in RTs III, IV and VI, while *P. aeruginosa* occurs within RT IV GNB. Considering the high incidences of non-*Pseudomonas* strains isolated particularly from spoiled meat and fish samples (>88%) using both PSA and CA, which are developed to select for *Pseudomonas* spp. and *P. aeruginosa*, respectively, the results obtained from these two media highlight the need to confirm the identities of bacterial colonies resulting on such selective media.

Studies investigating the prevalence of biosurfactant-producing bacteria have reported variable values. Jennings and Tanner (2000) investigated 4 different uncontaminated soils and found that biosurfactant-producing bacteria constituted 0.7 – 35% of the aerobic population. The frequency of surfactant-producing fluorescent *Pseudomonas* spp. isolates from the sugar beet rhizosphere in two Danish soils was 6 and 60% (Nielsen *et al*., 2002). In a study that evaluated the frequency of culturable biosurfactant-producing bacteria in undisturbed and contaminated Southwestern soils, it was found that 3.4% tested positive for biosurfactant-production (Bodour *et al*., 2003). Tran *et al*. (2007) reported that only 1.3% of the culturable *Pseudomonas* population in the rhizosphere of black pepper in Vietnam produced biosurfactants. The frequency of biosurfactant-producing bacteria isolated in this study (6%) is comparable to other, such related studies.

It is not surprising that biosurfactant-producing bacteria were isolated from the leafy vegetables evaluated in this study. The phylloplane is considered an unfavourable environment for colonising bacteria due to rapidly fluctuating temperature, relative humidity and moisture, and low nutrient availability. The production of biosurfactants by colonising bacteria not only increases the wettability of leaves, but also increases the nutrient availability on the phylloplane

(Bunster *et al*., 1989; Lindow and Brandl, 2003), thus assisting in the colonisation of the leaf surface. Bunster *et al*. (1989) found that biosurfactant production occurred in 50% of the *Pseudomonas* strains tested from leaf surfaces. This correlated with our results which found that all of the biosurfactant-producing strains isolated in this study from vegetables were putatively identified as *Pseudomonas* species. Only 13% of putative *Pseudomonas* strains from leafy vegetable products were positive for biosurfactant production, which could be a result of washing during minimal processing of the product. The occurrence of such significant proportions of biosurfactant-producing strains on the phylloplane suggests an attachment role for biosurfactants. This speculation is supported when the other sources of biosurfactant-producing bacteria are considered. The three biosurfactant-producing strains isolated from low fat milk were putatively identified as *Lactobacillus*. This finding corresponds with other studies, which have reported the isolation of biosurfactant-producing strains of probiotic lactic acid bacteria from dairy products (Rodrigues *et al*., 2006). Lactobacilli inhabit the gastrointestinal tract of healthy mammals and comprise the dominant indigenous microflora of the urogenital tract (Velraeds *et al*., 1996a). It has been hypothesised that biosurfactants produced by such bacteria may facilitate detachment of uropathogenic bacteria, such as *Enterococcus faecalis* (Velraeds *et al*., 1996b). We cannot yet infer the significance of biosurfactant-producing isolates also having been isolated from bottled water. Haemolytic activity has been recorded for bacterial strains, including *Bacillus* and *Pseudomonas*, isolated from mineral water (Pavlov *et al*., 2004; Korzeniewska *et al*., 2005). Haemolytic activity has been used as a selection criterion for the detection of potential biosurfactant-producing bacteria (Carrillo *et al*., 1996; Lin, 1996); however, this activity is not definitively indicative of biosurfactant-production, but could reflect other pathogenicity factors (Pavlov *et al*., 2004).

To our knowledge, there has only been one study that recorded the isolation of a biosurfactant-producing *Bacillus subtilis* isolate from a food product. In the study by Joshi *et al*. (2008), a biosurfactant-producing strain of *B. subtilis* was isolated from a fermented rice product, idly batter. Furthermore, the isolate was also found to produce an antifungal compound. In efforts to improve the economy of the production of biosurfactants, biosurfactants have been produced from economical, renewable substrates including vegetable oils, used vegetable oils, native oils and even potato substrates from the potato processing industry (Makkar and Cameotra, 2002;

Nitschke and Pastore, 2003; Nitschke *et al*., 2005; Nitschke and Pastore, 2006). To further investigate the potential of food as a source of functional biosurfactants, it might be worthwhile to investigate the prevalence of biosurfactant-producing bacteria in foods both when fresh and when spoiled. Such data could also clarify the role that biosurfactants play in food products.

The biosurfactant-producing isolates obtained in our study were found to be predominantly Gram-negative bacteria (84%). Of these, RT IV GNB predominated (67%), followed by RT VI (22%), RT V (7%) and RT III (4%) GNB. Gram-negative isolates were *Pseudomonas* spp., while Gram-positive isolates included *Bacillus* and *Lactobacillus* spp. *Bacillus*, *Lactobacillus* and *Pseudomonas* spp. are all recognised producers of biosurfactants (Persson *et al*., 1988; Desai and Banat, 1997; Singh and Cameotra, 2004; Mulligan, 2005). Our results suggest that although the percentage isolation of biosurfactant-producing isolates is only 6%, food products may be a novel source of biosurfactant-producing isolates and much work is required in order to discover the full potential of food as such a source.

None of the biosurfactant-producing isolates showed emulsifying activity with respect to the positive control; however, biosurfactant activity is not mutually inclusive of emulsifying activity. Bioemulsifiers are a subclass of biosurfactants that only stabilise suspensions of one liquid in another and may not necessarily reduce surface tension (Ron and Rosenberg, 2001, 2002). Many studies have shown the emulsifying activity of biosurfactant-producing bacteria isolated from hydrocarbon-contaminated sites (Bodour *et al*., 2003; Rahman *et al*., 2003; Bento *et al*., 2005b; Batista *et al*., 2006). The use of cooking oil for the emulsification assay served to give some preliminary indication of emulsification ability as vegetable oil has previously been shown to enhance the bioremediation of PAHs and other hydrophobic contaminants (Borden, 2007; Gong *et al*., 2007; Pannu *et al*., 2004). Vegetable oil can be used as a biodegradable, low-cost soil amendment in biological and non-biological treatment processes.

However, the variation in emulsifying activity of bacterial strains isolated from different sources could be due to various factors. As with biosurfactants, bioemulsifiers can be substrate-specific, emulsifying different hydrophobic compounds at different rates (Ilori *et al*., 2005; Bodour *et al*., 2003). Thus, the screening media used could have affected the outcome of the assay or emulsion

stability could have lasted only a few minutes and was not observed after 20 minutes. Additionally, emulsifying activity could be affected by the substrate on which the bioemulsifier was produced (Ilori *et al*., 2005). It is also possible that emulsification activity is not required for growth on spoiled foods. If the latter is true, this could further support the theory that the production of biosurfactants by strains in the present study plays an attachment role.

CONCLUSION

High proportions of *Bacillus*, *Lactobacillus* and *Pseudomonas* spp. were isolated from the spoiled food products evaluated, confirming the hypothesis that food is a potential source of biosurfactant-producing bacteria. Of all the isolates screened for biosurfactant activity, only a percentage of those obtained from predominantly leafy vegetable, but also water and low fat milk products tested positive. These findings are novel and strongly suggest that certain food products may be a source of biosurfactant-producing isolates worth investigating.

Table 2.1: Food products selected for evaluation from a local supermarket.

Category	Product
Water	Valpre spring water
Milk and milk products	Fresh cream
	Low fat (2%) milk
Red meats	Beef stir fry
	Lamb stir fry
	Beef burger patty
	Lamb burger patty
	Lamb knuckle
Seafood	Fresh Canadian salmon
	Fresh rainbow trout
Vegetables	Pre-packed butter lettuce
	Pre-packed crisp lettuce
	Pre-packed coleslaw mix
	Pre-packed spinach

Table 2.2. The typical constituent genera and/or species of the oxidative-fermentative reaction types (RT).

Reaction Type	Glucose metabolism	Oxidase test	Constituent genera/species
I	Fermentative	–	*Enterobacteriaceae* *Chromobacterium violaceum*
II		+	*Aeromonas* (*hydrophila, caviae, sobria, salmonicida*) *Plesiomonas shigelloides* *Vibrio* (*cholerae,* NAG) *Actinobacillus* (*lignieresil, suis*) *Chromobacterium violaceum* *Pasteurella* (*aerogenes, haemolytica, pneumotropica*) *Kingella kingae*
III	Oxidative	–	*Acinetobacter calcoaceticus* (biotype *anitratus* and *haemolyticus*) *Pseudomonas maltophilia*
IV		+	*Pseudomonas* (*aeruginosa, fluorescens, putida*) *Pseudomonas* (*pseudomallei*, *cepacia*) *Pseudomonas* (*stutzeri*, *mendocina*) *Pseudomonas putrefaciens* *Pseudomonas xylosoxidans* *Pseudomonas* (*meningospeticum, breve*)
V	Inactive	–	*Acinetobacter calcoaceticus* (biotype *lwoffi* and *alcaligenes*)
VI		+	*Alcaligenes* (*faecalis, odorans, denitrificans*) *Pseudomonas* (*alcaligenes, pseudoalcaligenes*) *Pseudomonas* (*acidovorans, testosteroni*) *Bordetella bronchiseptica* *Flavobacterium odoratum* *Moraxella osloensis* *Moraxella* (*phenylpyruvia*, *atlantae*, *urethralis*)

Table 2.3: Sources, putative identities and degree of oil droplet collapse of biosurfactant-producing strains.

Isolate no.	Origin	Putative identity	Degree of droplet collapse
001T	Bottled water	RT V	+
002T	Bottled water	RT V	+
007T	Low fat (2.0%) milk	*Lactobacillus*	+
009T	Bottled water	RT VI	+
010T	Bottled water	*Bacillus*	+
012T	Bottled water	*Bacillus*	+ +
013T	Low fat (2.0%) milk	*Lactobacillus*	+
016T	Low fat (2.0%) milk	*Lactobacillus*	+ +
020T	Bottled water	RT VI	+ +
100T	Butter lettuce	RT VI	+ + +
119T	Crisp lettuce	RT IV	+ + +
133T	Crisp lettuce	RT VI	+ + +
145T	Coleslaw	RT IV	±
226C	Butter lettuce	RT VI	+ +
227C	Butter lettuce	RT IV	+ + +
228C	Butter lettuce	RT IV	+ + +
229C	Butter lettuce	RT IV	+ + +
231C	Butter lettuce	RT IV	+ +
283C	Crisp lettuce	RT IV	+ + +
326C	Butter lettuce	RT IV	+
331C	Crisp lettuce	RT IV	+
340C	Crisp lettuce	RT VI	+ +
345C	Coleslaw	RT IV	+ + +
347C	Coleslaw	RT IV	+ + +
348C	Coleslaw	RT IV	+ + +
364C	Spinach	RT IV	+ + +
381C	Spinach	RT III	+ + +
384C	Spinach	RT IV	+ + +
390C	Coleslaw	RT IV	+ + +
398C	Coleslaw	RT IV	+ +
408C	Spinach	RT IV	+ +
412C	Spinach	RT IV	+ +

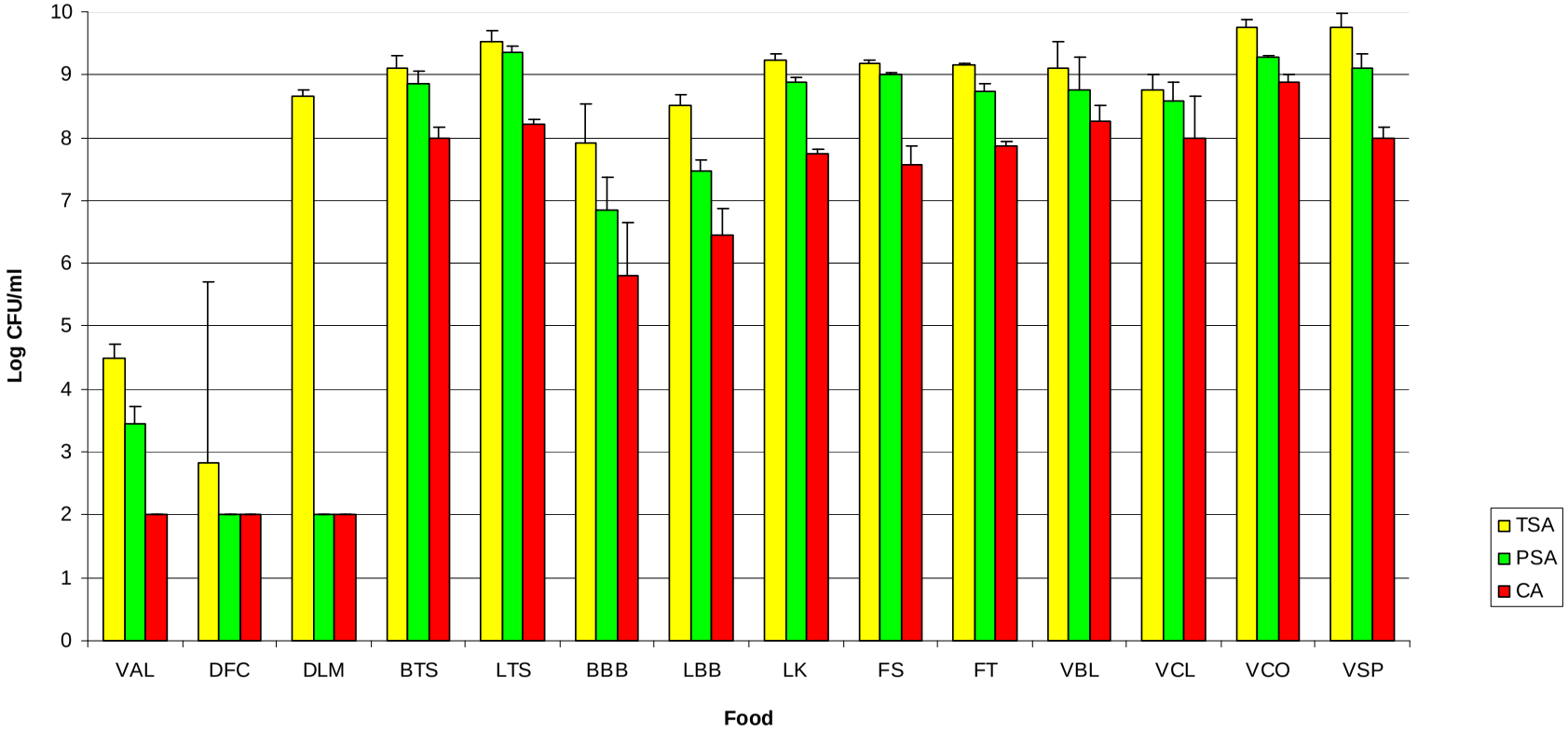
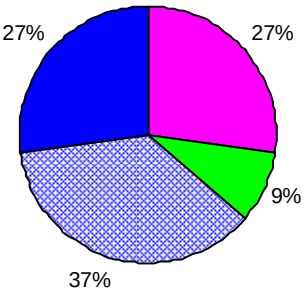
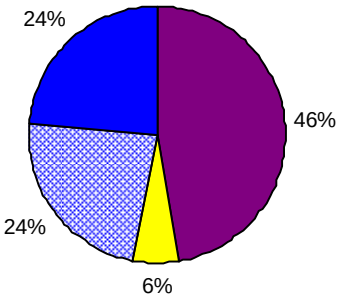


Figure 2.1: Average bacterial counts of all of the food products obtained from each of the three media. VAL, Valpre spring water; DFC, fresh cream; DLM, low fat (2%) milk; BTS, beef stir fry; LTS; lamb stir fry; BBB, beef burger patty; LBB, lamb burger patty; LK, lamb knuckle; FS, fresh salmon; FT, fresh trout; VBL, pre-packed butter lettuce; VCL, pre-packed crisp lettuce; VCO, coleslaw mix; VSP, spinach.

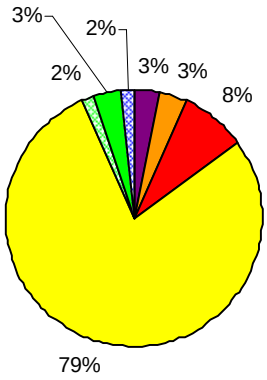
A.



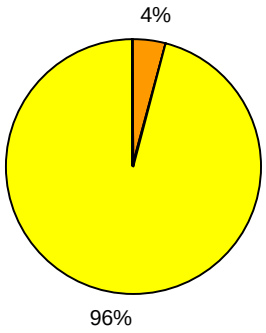
Water



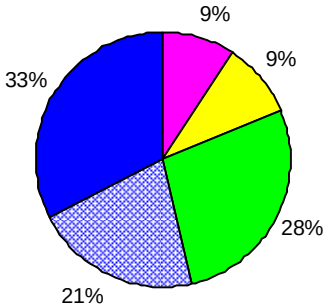
Dairy



Meat

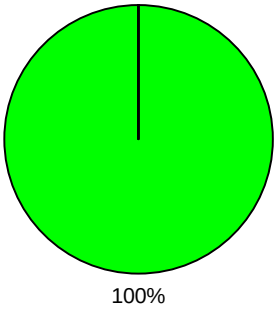


Fish

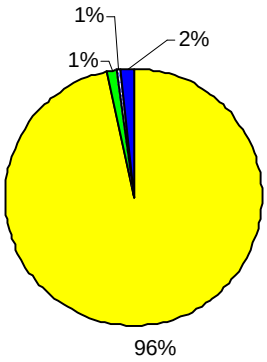


Vegetable

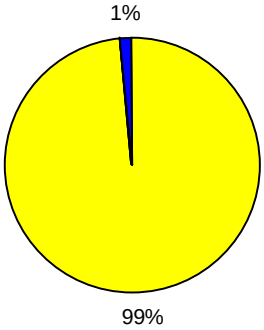
B.



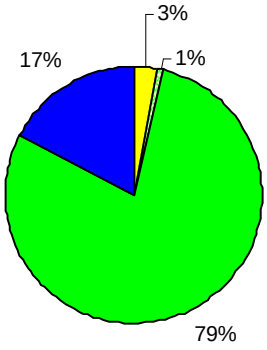
Water



Meat



Fish



Vegetable

C.

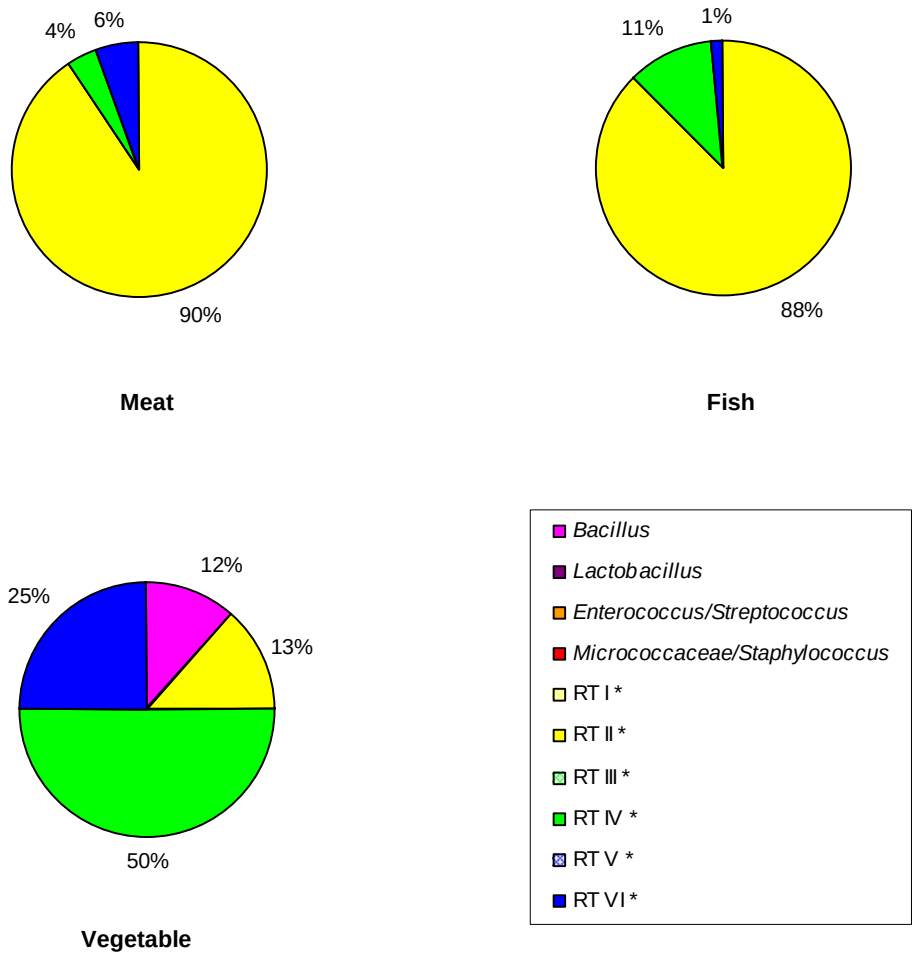


Figure 2.2: Putative identities of isolates isolated from APCs on TSA (**A.**), PCs on PSA (**B.**), PACs on CA (**C.**).

A.



B.



Figure 2.3: Assay screening for biosurfactant-producing bacteria, indicating negative (●) and positive isolates (**A.**) and a representative assay showing oil droplet collapse upon addition of a biosurfactant-producing isolate and a non-biosurfactant-producing isolate (**B.**) compared to negative (dsH_2O) and positive (1% SDS) controls.

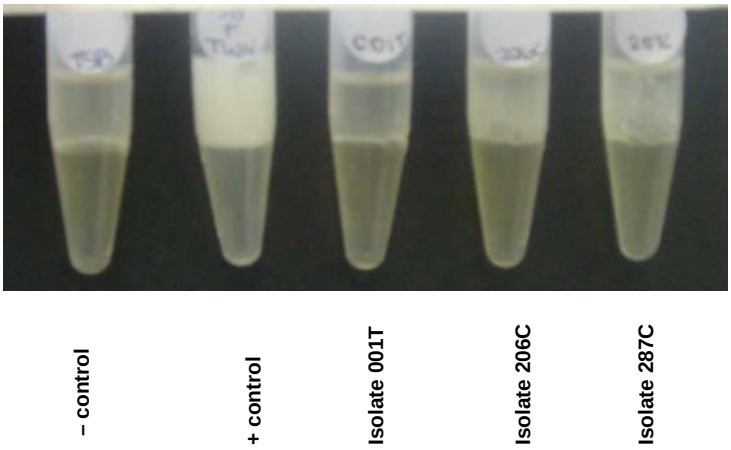


Figure 2.4: Emulsification assay showing activity of selected biosurfactant-producing isolates compared to a positive control (TSB + Tween 80, 1:1).

CHAPTER 3

Partial characterisation and antimicrobial activity of compounds from biosurfactant-producing bacteria isolated from food

ABSTRACT

The aim of this study was to screen biosurfactant-producing bacterial strains isolated from foods for antimicrobial activity against a range of Gram-positive and Gram-negative bacteria, and to partially characterise some of those compounds. The 32 biosurfactant-producing strains isolated (see Chapter 2) were evaluated for antibacterial activity against Gram-negative and Gram-positive bacteria using the spot-on-lawn assay. Of the 32 isolates, 18 (56%) exhibited activity against only Gram-positive bacteria, predominantly *B. cereus* ATCC 10702, and to some extent, *S. aureus* ATCC 25923. To confirm the identities of antimicrobial and biosurfactant-producing strains, 15 isolates were selected for 16S rRNA sequencing based on differing colony morphologies and/or origins. It was found that 6 (40%) isolates were identified as *B. pumilis*, 7 (47%) as *Pseudomonas* sp. and 2 (13%) as *Proteus vulgaris*. This study is the first to document antimicrobial activity by a strain of *P. fulva*. The 16 strains exhibiting activity against *B. cereus* ATCC 10702 were subjected to treatments with protease, heat and organic solvents in order to determine their stability. It was found that compounds produced by the majority of the *B. pumilis* strains (78%) retained their antibacterial activity after all 3 treatments, while 56% of the *Pseudomonas* strains lost their activity after the 3 treatments. Of the 16 isolates selected for the stability assays, 6 were selected for further characterisation by TLC and it was found that the majority of the isolates tested (67%) possibly contained peptides, but that all of the isolates stained ninhydrin-negative. The data suggests that the majority of compounds produced by the strains isolated in this study produce cyclic lipopeptides, some of which may be novel. Phylogenetic analysis of 16S rDNA sequences also showed that the 15 biosurfactant-producing strains are closely related to sequences of other bacteria with biosurfactant-producing and antimicrobial activities. This study demonstrated that a significant proportion of biosurfactants produced by bacteria isolated from foods exhibited activity against Gram-positive bacteria of medical and food safety relevance, and advocated strongly for further characterisation of the identified molecules, given their potential applications.

Several low-molecular-mass microbially-produced surfactants are also known to exhibit some biological properties of therapeutic and biomedical relevance. However, these useful properties have not yet been exhaustively explored. Some of the better known biosurfactants with antimicrobial activities are produced by *Pseudomonas* and *Bacillus* spp., and are discussed below.

Lipopeptides form the most widely reported class of biosurfactants exhibiting antimicrobial activity. Surfactin, produced by *Bacillus subtilis*, presents the first and best-documented antimicrobial lipopeptide. It has well known antibacterial and antifungal activities (Thimon *et al*., 1992; Amihou *et al*., 2001) and also exhibits broader biological activities, such as the inhibition of fibrin clot formation (Arima *et al*., 1968), the formation of ion channels in lipid bilayer membranes (Sheppard *et al*., 1991), blocking the activity of cyclic adenosine monophosphate (Hosono and Suzuki, 1983), the inhibition of platelet and spleen cytosolic phospholipase A2 (PLA2) (Kim *et al*., 1998), antiviral (Itokawa *et al*., 1994; Kracht *et al*., 1999) and antitumour (Kameda *et al*., 1974) activities. Iturin and pumilacidin are other antimicrobial lipopeptides produced by some strains of *B. subtilis* and *B. pumilis*, respectively. Iturin is a potent antifungal lipopeptide (Besson *et al*., 1976; Amihou *et al*., 2001), while pumilacidins A - G are a group of antiviral biosurfactants (Naruse *et al*., 1990). Other cyclic lipopeptides (CLPs) produced by *Pseudomonas* spp., viz. massetolides A – H (Gerard *et al*., 1997), viscosin (Neu and Poralla, 1990) and viscosinamide (Nielsen *et al*., 1999), have all been shown to exhibit antibacterial (Gram-positive and -negative), antifungal and antiviral activities.

Rhamnolipids, produced by *Pseudomonas aeruginosa* (Itoh *et al*., 1971), are known to possess a range of biological activities, including antibacterial (predominantly against Gram-positive bacteria), antifungal, antiviral (Lang and Wagner, 1993), antimycoplasmal (Abalos *et al*., 2001) and antiamoebal (Cosson *et al*., 2002) activities. Additionally, rhamnolipids have been shown to inhibit the growth of the harmful algal bloom spp. *Hetersigma akashiwo* and *Proracentrum dentatum* (Wang *et al*., 2005) and induce the lysis of zoospores of the plant pathogens *Pythium*

aphanidermatum, *Pythophthora capsici* and *Plasmopara lactucae-radicis* from 5µg/ml (Stanghellini and Miller, 1997).

The antimicrobial activity of biosurfactants results from the ability of the molecules to alter the permeability of phospholipid bilayer membrane in a detergent-like manner. Both rhamnolipids and lipopeptides intercalate into the membrane, forming transmembrane pores or disintegrating the membrane. This results in the collapse of the transmembrane electrochemical gradients and subsequent cell lysis (Stanghellini and Miller, 1997; Shai, 2002; Makotvitzki *et al*., 2006).

Given the increasing prevalence of drug-resistant pathogenic bacteria, alternative lines of therapy are required. Certain biosurfactants present a promising alternative to synthetic medicines and antimicrobial agents, and may be used as safe and effective therapeutic agents. Thus, the aim of this study was to screen biosurfactant-producing bacterial isolates for antimicrobial activity against a range of Gram-positive and Gram-negative bacteria.

MATERIALS AND METHODS

Biosurfactant-producing strains and growth conditions.

As discussed in the previous chapter (Chapter 2), 32 strains of bacteria capable of producing biosurfactants were isolated. These are listed in Table 2.3. For subsequent experiments, all isolates were grown up in TSB and incubated overnight at 30°C with shaking at 150rpm.

Screen for antimicrobial activity.

Antimicrobial activity of biosurfactant-producing isolates was assessed against a variety of target bacteria by the spot-on lawn assay (Hoover and Harlander, 1993) and conducted in duplicate. These included potential pathogens: *Bacillus cereus* ATCC 10702 (Lindsay *et al*., 2002), *B. cereus* DL5 (Lindsay *et al*., 2002), *Escherichia coli* ATCC 25922, *Salmonella enteritidis* S61 (Geornaras, 2000), *S. typhimurium* S48 (Geornaras, 2000) and *Staphylococcus aureus* ATCC 25923, and food spoilage strains: *B. subtilis* EL39 (Kirschner, 1993), *B. subtilis* 168 (Lindsay *et al*., 2006), *Lactobacillus brevis* (Deva, 2007), *Pseudomonas aeruginosa* ATCC 27853

(Panayides, 2009), *P. fluorescens* DSM 50090 (Geonaras, 2000) and *P. putida* dss2 (Lindsay *et al*., 2008). For this assay, indicator plates of each target bacterium were prepared by adding a 10% volume exponential phase (OD_{600}, 0.6) culture of the respective target bacterium to molten TSA cooled to 55°C. The indicator agar mixture was poured into Petri dishes (90mm diameter), allowed to set, stored at 4ºC and used within 24h. The indicator plates were spotted with 20 – 50µl of an overnight culture of each biosurfactant-producing isolate and allowed to incubate at 30°C for 24 – 48h. Zones of inhibition were then examined and recorded. Distilled water was the negative control while 1% SDS and 50% sodium-hypochlorite solution served as the positive controls.

Effects of chemicals and heat on antimicrobial activity.

To determine the stability and composition of the antimicrobial substances, the cell free supernatants (CFS) containing the antimicrobial compounds were subjected to treatments with an enzyme, heat and organic solvents. For this assay, 16 isolates (001T, 002T, 007T, 009T, 010T, 012T, 013T, 016T, 119T, 133T, 228C, 229C, 231C, 240C, 283C, 340C) that were shown to possess antimicrobial activity against *B. cereus* 10702 were selected. CFS were obtained by subjecting each isolate to centrifugation (5000rpm, 5min) followed by sterile filtration through a 0.22µm membrane filter (Risøen *et al*., 2003). For the enzymatic reactions, the CFS were incubated with an equal volume of proteinase K (20mg/ml) for 4h at 30°C. The enzyme was then heat-inactivated by incubating the experimental samples at 50°C for 10min. Thermal stability of the antimicrobial compounds was evaluated by incubating 100µl of CFS at 50°C and 100°C for 10min. To test for the stability of the compounds in organic solvents, 1/10 of three different organic solvents (chloroform, methanol and propanol) was added to CFS and vigourously mixed when plated (Risøen *et al*., 2003). Indicator plates of *B. cereus* ATCC 10702 were spotted with 20 – 50µl of each sample and allowed to incubate at 30°C for 24 – 48h. Zones of inhibition were then examined and recorded. The negative and positive controls for the enzyme treatment were untreated CFS and TSB incubated with enzyme (activated), respectively. For the heat treatment, the controls were CFS untreated (negative) and heat-treated (positive) at 100°C for 30min, respectively. For the treatment with organic solvents, the controls were CFS only (negative) and solvent only (positive), respectively.

Thin layer chromatography (TLC) analysis of the biosurfactants.

A modified method of Matsuyama *et al*. (1987) for TLC was employed to evaluate the presence of peptides in the antimicrobial biosurfactants of selected isolates. For this assay, 6 isolates (001T, 002T, 010T, 113T, 228C and 119T) were selected based on differing profiles from the stability assay. A 10µl volume of CFS of each of the isolates was spotted onto silica gel aluminium TLC plates (20×20cm, 250µm, F_{254}; Merck) and developed in chloroform:methanol:water (65:15:2, vol/vol/vol) (Sim *et al*., 1999). For detection of peptides the plates were air dried, sprayed with ninhydrin (0.2% in 95% ethanol; Merck), air dried once more and heated at 110°C for 10min. Plates were also viewed under UV light before staining with ninhydrin. Migration distances of sample spots, before and after staining, relative to the mobile phase (R_f values) were calculated. *B. subtilis* EL39 functioned as the positive biosurfactant-producing control, as determined by the oil droplet collapse assay (Chapter 2).

16S rDNA sequencing and construction of phylogenetic trees.

In order to confirm the identities of biosurfactant-producing isolates, molecular identification by 16S rRNA sequencing was done. For identification by the polymerase chain reaction (PCR), 13 of the 18 isolates showing antimicrobial activity, as well as another two isolates, were selected based on differing colony morphologies and/or origins. The isolates selected for 16S rRNA sequencing were: 001T, 007T, 010T, 012T, 013T, 016T, 100T, 119T, 133T, 145T, 228C, 283C, 345C, 364C and 381C. Isolates were prepared for DNA extraction by using a modified boiling method as described by Scarpellini *et al*. (2004). A 2.5µl volume of supernatant of each isolate was used for PCR. The primer set used for the amplification of the 16S rDNA was U1392R (5'-ACG GGCGGT GTG TAC-3'; Ferris *et al*., 1996; McGarvey *et al*., 2004) and Bac27F (5'-AGA GTT TGA TCC TGG CTC AG-3'; Inagaki *et al*., 2003; McGarvey, *et al*. 2004) in combination with 2× PCR Master Mix (Fermentas Life Sciences, http://www.fermentas.com), according to the manufacturers instructions and yielded a 1300bp product (Sambrook *et al*., 1989). PCR amplifications were performed using the following conditions: initial denaturation of template DNA at 94°C for 3min; then 35 cycles consisting of denaturation (94°C, 30s), annealing (60°C, 45s) and extension (1min 30s, 72°C), and a final extension at 72°C for 7min. The resulting sequences were analysed by BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) against 16S rDNA sequences from GenBank (GenBank database of the National Center for Biotechnology

Information, http://www.ncbi.nlm.nih.gov/GenBank/). The 16S rDNA sequences were deposited in GenBank with accession numbers FJ477095 – FJ477109. The sequences were aligned using DNAMAN (Lynnon Biosoft, version 4.0) and phylogenetic trees were constructed with a bootstrap value of 500.

RESULTS

16S rDNA sequencing and phylogenetic analysis.

Molecular identification of the 15 biosurfactant-producing isolates selected for 16S rRNA sequencing identified the strains as *B. pumilis* (001T, 007T, 010T, 012T, 013T, 016T, 020T), *P. fluorescens* (100T, 229C), *P. cichorii* (133T, 283C), *P. fulva* (381C), *P. tolaasii* (119T) and *Proteus vulgaris* (145T, 345C) (Table 3.1). The 6 *B. pumilis* strains selected for 16S rDNA sequencing were all isolated from spoiled bottled mineral water and low fat (2%) milk, the *Pseudomonas* strains from butter and crisp lettuce, and the *P. vulgaris* strains from pre-packaged coleslaw. Sequence analysis showed high sequence similarities (<97%) with bacterial strains with activities relevant to this study (Fig. 3.1).

All of the *B. pumilis* strains isolated in this study shared 99 – 100% sequence similarity with strains that have been previously shown to produce biosurfactants [*B. pumilis* f-2-3 (FJ561295), BSFD2-1 (FJ594457)], exhibit antiphytopathogenic activity [*B. pumilis* TAD166 (FJ225309)] and degrade lambda-cyhalothrin, an insecticide [*Bacillus* sp. DF-1 (EU600242)]. With the exception of strain 001T, all of the isolates also shared 98% sequence identity with *B. pumilis* BM5-2 (EU880542), which was shown to produce biosurfactants, while isolates 001T and 012T shared 98 – 100% sequence similarity with *B. pumilis* 1Re36 (EF178454) and SSR07 (EU373329); strains reported to exhibit activity against phytopathogens. Strains 001T (FJ477095), 007T (FJ477096), 013T (FJ477099) and 016T (FJ477100) were 98 – 100% similar to *B. pumilis* gf-4 (EU239356), a previously reported cyhalothrin-degrader, and *B. pumilis* VII (EU779998), which degraded dairy wastewaters (Fig. 3.3A). The strains showed 91 – 95% sequence similarity to reference strains *B. subtilis* ATCC 6633 (AY616162) and *B. cereus* ATCC 10987 (AJ577290).

The *Pseudomonas* strains included in the *P. fluorescens* group comprised 100T (FJ477101), 119T (FJ477102) and 229C (FJ477104) (Fig. 3.3C). 16S rDNA sequence analysis revealed that both *P. fluorescens* 229C and *P. tolaasii* 119T share 99% sequence similarity with antifungal strain *Pseudomonas* sp. IMER-A2-24 (FJ434132) and H460 (EF693759), isolated from medicinal herbs and plant bulbs, respectively, *Pseudomonas* sp. PSB-UG2-4 (EU849100), which solubilised phosphate, and *P. fluorescens* 2132 (EU360313) which was isolated from milk. *P. fluorescens* 100T also shared 99% sequence similarity with *Pseudomonas* sp. BE08 (AY456701), which has recorded antiphytopathogenic activity, and *Pseudomonas* sp. PSB-UG2-4, which also solubilised phosphate. Similarly, *P. fluorescens* 229C shared 99% sequence similarity with *Pseudomonas* sp. TAD025 (FJ225177), which was shown to be antiphytopathogenic. *P. tolaasii* 119T (FJ477109) was found to share 99% sequence similarity with *Pseudomonas* sp. PCL1171 (AY236959), TAD054 (FJ225205), CH33-4 (EU057891) and Pi 3-58 (AB365069); strains all previously shown to have antifungal activity. Interestingly, *Pseudomonas* sp. CH33-4 (EU057891) was isolated from a salamander. *P. tolaasii* 119T also shared 99% sequence similarity with *P. trivialis* BIHB 750 (FJ179366), LW3 (EU257208) and BFXJ-8 (EU013945), which have been shown to solubilise phosphate, and degrade chlorimuron-ethyl and phenol, respectively. Strains 100T, 119T and 229C shared 98 – 99% sequence similarity with reference strains *P. fluorescens* ATCC 13525 (AF094725) and *P. tolaasii* NCPPB 2129 (AF320988) (Fig. 3.3C).

P. fulva 381C (FJ477107) and *P. putida* 364C (FJ477106) are included in the *P. putida* group. Analysis of 16S rDNA sequences showed that strain 381C shared 99% sequence similarity with *Pseudomonas* sp. BHP7-11 (AY162141), EC-S102 (AB200256), SP28 (FM202488) and *P. putida* F29 (EF204245). The first 3 of these strains have been shown to produce a biosurfactant, exhibit antifungal activity and solubilise phosphate, while *P. putida* F29 was isolated from raw milk (Fig. 3.3D). *P. putida* 364C shared 99% sequence similarity with *P. putida* 214-D (EF615008), which was isolated from black pepper and produced a biosurfactant, and *P. putida* D19 (EF204247), which was isolated from raw milk. Strain 364C also shared 98% sequence identity with *P. putida* WH3 (FJ262368), 23975 (FJ227304), JM9 (FJ472861), PNP-1, *P. oryzihabitans* HAMBI2374 (AF501336), *P. metavorans* (AB302395), *Pseudomonas* sp. Pds-4 (EU312075) and WSCIII (AY344806); all of which were shown to degrade xenobiotics (Fig. 3.3D).

Proteus vulgaris 145T (FJ477108) and 345C (FJ477109) were found to share 98 – 99% sequence similarity with *P. vulgaris* YRR06 (EU373433) and *P. mirabilis* Ps4 (AM231709), which have been shown to be antimicrobial, and 98% sequence similarity with the reference strain *P. vulgaris* ATCC 29905 (DQ885527) (Fig. 3.3E).

Inhibitory spectrum of antimicrobial compounds.

Of the 32 isolates that demonstrated biosurfactant activity, 18 (56%) also exhibited antimicrobial activity (Table 3.1). The isolates showing antibacterial activity were active only against several Gram-positive bacteria. The majority of the strains (89%) were active against *Bacillus cereus* ATCC 10702, while only 5 of those were active against Gram-positive *B. subtilis* EL39, 4 against *B. subtilis* 168 and 3 against *Staphylococcus aureus* ATCC 25923 (Fig. 3.1, Table 3.1). As shown in Table 3.1, all of the *B. pumilis* strains were active only against *B. cereus* ATCC 10702, while the *P. cichorii*, *P. fluorescens* and *P. tolaasii* strains exhibited slightly broader spectrum activity against *B. cereus* ATCC 10702, *B. subtilis* EL39, *B. subtilis* 168 and *S. aureus* ATCC 25923. The *P. fulva* and *Proteus vulgaris* isolates were active only against *S. aureus* 25928.

Effects of chemicals and heat on antimicrobial activity.

CFS containing antimicrobial activity were subjected to several conditions in order to gain some insight into the stability of the compounds. As shown in Table 3.1, 7 of the 9 (78%) *B. pumilis* strains were stable after treatment with proteinase K (20mg/ml), heat (up to 100°C) and all three organic solvents. One (11%, *B. pumilis* 002T) was stable only after heat treatment and treatment with organic solvents, and the other (*B. pumilis* 010T) was unstable after all three treatments (Table 3.1). In the case of the *Pseudomonas* strains, 4 of 7 (57%) were unstable after all three treatments, 2 (29%; *P. cichorii* 283C and *P. fluorescens* 228C) were stable after all three treatments and one (*P. tolaasii* 119T) was stable only after heat treatment and treatment with organic solvents.

TLC analysis of the biosurfactants.

TLC was employed to determine whether selected CFS containing putative antimicrobial biosurfactants contained peptides. The TLC plates were viewed under UV light before ninhydrin staining and spots were observed for 4 of the 6 isolates, with *P. fluorescens* 228C and *P. tolaasii*

119T producing no spots (Fig 3.2). The strains demonstrating spots under UV light, including the positive control *B. subtilis* EL39, had R_f values of 0.5 – 0.6. Staining with ninhydrin was negative for all isolates, including the controls (Fig 3.2).

DISCUSSION

Based on the results in this study, a few trends relating to antimicrobial activity were observed. Firstly, all of the strains evaluated were active only against Gram-positive bacteria. This was expected as Gram-negative bacteria tend to be resistant to many antimicrobial molecules due to the lipopolysaccharide layer of the outer membrane, as it acts as an effective permeability barrier against hydrophobic molecules and macromolecules (Nikaido and Vaara, 1985). Secondly, the activity of the *B. pumilis* strains had a narrow spectrum of antibacterial activity which was limited to closely related bacteria as antibacterial activity was observed only against *B. cereus* ATCC 10702. Similarly, the Gram-negative *P. fulva* and *Proteus vulgaris* strains were active only against *S. aureus* ATCC 25923, which indicated a narrow spectrum of activity towards Gram-positive *S. aureus*. Gram-negative *P. cichorii*, *P. fluorescens* and *P. tolaasii* exhibited a broader spectrum of activity against only Gram-positive bacteria that included *B. cereus* ATCC 10702, *B. subtilis* EL39, *B. subtilis* 168 and *S. aureus* ATCC 25923.

Biologically active peptides – or CLPs – of biomedical and biotechnological important are produced non-ribosomally (Schwarzer *et al*., 2003) and are characterised by the presence of D-amino acids (Luo *et al*., 2003). The incorporation of D-amino acids results in unusual stereochemistry, which confers peptides resistance to proteolysis (Luo *et al*., 2003; Mitchell and Smith, 2003). The ninhydrin stain used in the TLC experiments typically stains amines, but not unusual amino acids, such as D-amino acids. Thus, the TLC results in this study showing negative ninhydrin staining support the hypothesis that the majority of the compounds produced by our strains were CLPs.

Bacillus pumilis has been reported to produce a range of antimicrobial compounds, including micrococcin (Fuller, 1955; Rosendahl and Douthwaite, 1994), pumilacidin (Naruse *et al*., 1990), pumilin (Bhate, 1955) and tetaine (Borowski *et al*., 1952). The antimicrobial compound (or

compounds) produced by the majority of the *B. pumilis* strains were stable after treatment with proteinase K and organic solvents, and were heat stable, suggesting that the compound could be a lipoprotein, such as pumilacidin. Pumilacidin is a group of CLPs with a structure very similar to that of surfactin (Naruse *et al*., 1990; Kalinovskaya *et al.*, 2002) with antifungal and antiviral activities (Naruse *et al*., 1990). The antibacterial activity of pumilacidin has not yet been evaluated. The remaining two isolates (002T and 010T) lost their antibacterial activity after treatment with proteinase K, suggesting that their active compounds are more proteinaceous, or are more sensitive to protein-degrading enzymes. These compounds could be related to micrococcin or tetaine. However, their antimicrobial activity profiles did not fit that of the compounds identified in this study. Tetaine is a dipeptide that has broad antimicrobial activity against both Gram-negative and Gram-positive bacteria (Krynski and Becla, 1963; Kenig *et al*., 1976), and *Candida albicans* (Milewski *et al*., 1983), while micrococcin is a thiopeptide antibiotic produced by *B. pumilis* (Fuller, 1955) and *Staphylococcus* sp. (Cundliffe and Thompson, 1981; Thompson and Cundliffe, 1991; Carnio *et al*., 2001). It has broad antimicrobial activity against Gram-positive bacteria (Carnio *et al*., 2001) and has been found to inhibit the growth of the malaria parasite *Plasmodium falciparum* (McConkey *et al*., 1997; Rogers *et al*., 1998).

One isolate of *P. fluorescens* (228C) exhibited broad spectrum activity against Gram-positive bacteria and retained its antibacterial activity after treatment with proteinase K, high temperatures and organic solvents, suggesting that the compound in question could be rhamnolipid-like (Fujita *et al*., 1988; Pearson *et al*., 1997). Strains of *P. fluorescens* are known to produce glycolipids (Healy *et al*., 1996) and the results from the TLC assay support this speculation. The other two isolates (229C and 231C) were found to be active against only one Gram-positive target bacterium, namely *B. cereus* ATCC 10702. Additionally, their antibacterial activity was lost upon treatment with proteinase K, heat (100°C) and organic solvents. These data suggest that the compound (or compounds) is not protein-based or is lipophilic. The compound produced by one or more of the isolates could be similar to mupirocin, an antimicrobial compound produced by *P. fluorescens* (Sutherland *et al*., 1985). Mupirocin is reported to be highly active against Gram-positive bacteria, particularly *S. aureus*, but less active against Gram-negative bacteria (Sutherland *et al*., 1985; Nicholas *et al*., 1999). However, mupirocin has not been evaluated for

surface activity. Some strains of *P. fluorescens* are known to produce two very closely related surface-active CLPs, namely viscosin (Neu *et al*., 1990) and viscosinamide (Nielsen *et al*., 1999). The antibacterial activity of viscosin is limited to mycobacteria (Neu *et al*., 1990) while the activity of viscosinamide is limited to phytopathogenic fungi (Nielsen *et al*., 1999; Thrane *et al*., 2000). This discussion, together with 16S rDNA sequence analysis, demonstrates that *P. fluorescens* produce a range of biologically active biosurfactants with a range of applications, including as antimicrobial agents (De Souza *et al*., 2003; Singh and Cameotra, 2004), in the control of phytopathogenic fungi (Nielsen *et al*., 1999; Thrane *et al*., 2000) and in the degradation of xenobiotic compounds (Wikström *et al*., 1996; Whiteley *et al*., 2001; Dua *et al*., 2002).

Both *P. tolaasii* 119T and 340C isolated in this study exhibited broad spectrum activity against Gram-positive bacteria. The antibacterial activity of one isolate, 119T, was lost only after treatment with proteinase K, while the other, 340C, was lost after all three treatments. Although the stability and TLC results suggest that the molecules identified from the two *P. tolaasii* strains do not contain peptides, only one antimicrobial toxin – tolaasin – has been identified from *P. tolaasii* (Nair and Fahy, 1973; Hu *et al*., 1998) to date. Tolaasin is an extracellular, heat-stable polypeptide that exhibits a broad spectrum of activity against predominantly Gram-positive bacteria, fungi and plants (Rainey *et al*., 1991). The varying results obtained for the molecules identified from isolates 113T and 340C could possibly have been a result of phenotypic variation, a strategy commonly employed by bacteria to withstand various environmental conditions, which includes the alteration of several biochemical properties, including the loss of the ability to produce tolaasin (Cutri *et al*., 1984; Grewal *et al*., 1995).

This study reports the first recorded incidence of antimicrobial activity of an isolate of *Pseudomonas fulva*. It was found that strain *P. fulva* 381C, isolated from pre-packaged spinach, was active against only *S. aureus*. However, *P. fulva* 381C is closely related (99% sequence identity) to *Pseudomonas* sp. EC-S102, a strain which was active against a phytopathogenic water mould, *Peronosporomycetes*. *P. fulva* is an environmental *Pseudomonas* and is placed in the *P. putida* group (Yamamoto *et al*., 2000). Strains of *P. putida* are known to produce rhamnolipids (Tuleva *et al*., 2002) and the CLPs putisolvin I and putisolvin II (Kuiper *et al*.,

2004). The novel CLPs putisolvins I and II are produced by *P. putida* 1445 which was isolated with naphthalene-degrading *P. putida* PCL1444 and, in addition to reducing surface tension, are known to inhibit the formation and degrade existing *Pseudomonas* biofilms (Kuiper *et al*., 2004). The identification of a putative antibacterial biosurfactant produced by a strain of *P. fulva* is interesting as 16S rDNA sequence analysis showed that *P. fulva* 381C is closely related to strains able to produce biosurfactants and with antimicrobial activities.

Biologically active substances have been reported from many phytopathogenic fluorescent *Pseudomonas* spp. However, the activity of most of these toxic compounds is predominantly antifungal. Antibacterial activity has previously been reported for *P. cichorii* (Shirata *et al*., 1981) but, to date, no agent has been clearly identified. Hu *et al*. (1998) found that the unknown, active compound produced by several strains of *P. cichorii* were stable after heat but inactivated by protease treatments. Both of the *P. cichorii* strains isolated in this study exhibited broad spectrum activity against Gram-positive bacteria. Although isolate 133T lost its antibacterial activity after all three treatments, the TLC results suggest that the antibacterial compound contains a peptide fraction. Alternatively, isolate 283C retained its antibacterial activity after all three treatments. Given the differing stability profiles, it cannot be determined which one of the 2 compounds identified in this study are those identified in the study by Hu *et al*. (1998), if indeed there are 2 different compounds. If there are, it is possible that the active agent identified from *P. cichorii* 133T is a non-protein, water-soluble toxin, and that from *P. cichorii* 283C is a lipoprotein, as the compound is resistant to proteinase K and soluble in organic solvents; somewhat like syringomycin-like toxins (Hu *et al*., 1998).

Proteus spp. are opportunistic pathogens generally known for causing urinary tract infections (UTIs) and are perhaps best known for swarming motility, which is characterised by cellular differentiation and multicellular migration (Allison *et al*., 1992; Givskov *et al*., 1997). Swarming motility has also been recorded for spp. of other genera, including *Aeromonas, Bacillus, Clostridium, Escherichia, Pseudomonas, Salmonella, Serratia, Vibrio* and *Yersinia* (Allison and Hughes, 1991; Rather, 2005). Many bacterial species that are capable of swarming require the production of biosurfactants, which act as wetting agents (Matsuyama and Nakagawa, 1996; Sharma and Anand, 2002). In many cases, such as *B. subtilis*, *P. aeruginosa* and *Serratia* spp., the production of biosurfactants is regulated by their respective quorum sensing systems

(Solomon *et al*., 1996; Lazazzera *et al*., 1997; Lindum *et al*., 1998; Köhler *et al*., 2000; Daniels *et al*., 2004), which play important roles in biofilm formation and maintenance (Bassler, 1999; Kjelleberg, and Molin, 2002; Parsek and Greenberg, 2005). However, biosurfactant production by *Proteus* spp. has only been identified from oil-utilising strains isolated from diesel oil-contaminated soil (Balogun and Fagade, 2008). Alternatively, peptides or amino acids, especially glutamine, are proposed to have a signalling function in *Proteus* spp. (Fraser and Hughes, 1999). In terms of the biological activities of *Proteus* spp., the only antimicrobial activity reported is the isolation of two antibacterial compounds, phenylacetic acid and phenylacetaldehyde, produced by *P. mirabilis* isolated from screwworm larvae (Erdmann and Khalil, 1986). Of other interest, is the production of haemolysin by *Proteus* spp., a virulence factor whose expression is correlated with swarming migration (Allison *et al*., 1992). Haemolysins are pore-forming toxins which are produced by a range of Gram-positive and Gram-negative bacteria (Rozalski *et al*., 1997); the cytotoxic activity of which is limited to erythrocytes of human and animal origin (Philips, 1955; Rozalski and Kotelko, 1987; Rozalski *et al*., 1997). 16S rDNA sequence analysis showed that *P. vulgaris* 145T and 345C shared sequence similarity to two *Proteus* strains which reportedly exhibited antimicrobial activity. However, it is not known if this particular activity was attributable to haemolysins. Although the majority of *Proteus* spp. are known particularly for causing UTIs, *P. vulgaris* has also been reported to cause gastroenteritis resulting from the ingestion of contamination meat or other food (Cooper *et al*., 1971; Schoub *et al*., 1977). This is significant when considering that the two strains of *P. vulgaris* isolated in this study were isolated from pre-packaged coleslaw. Given that the identity of the antibacterial compound identified in this study cannot be speculated upon, further investigation of this compound would prove valuable and informative.

CONCLUSION

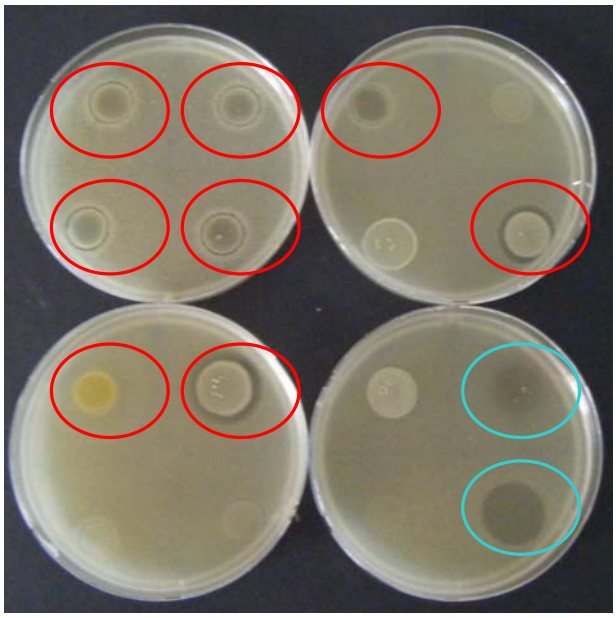
A high percentage of the biosurfactants evaluated for antimicrobial activity tested positive, particularly against potentially pathogenic Gram-positive bacteria, confirming the hypothesis that at least a percentage of biosurfactants produced by bacteria isolated from foods exhibit antimicrobial activity of medical importance. The stability and TLC data suggest that the majority of the putative antibacterial biosurfactants are cyclic lipopeptides of pharmaceutical interest. Phylogenetic analysis of the strains selected for 16S rDNA sequencing also support the proposal to further investigate the identified molecules, given their potential biomedical applications.

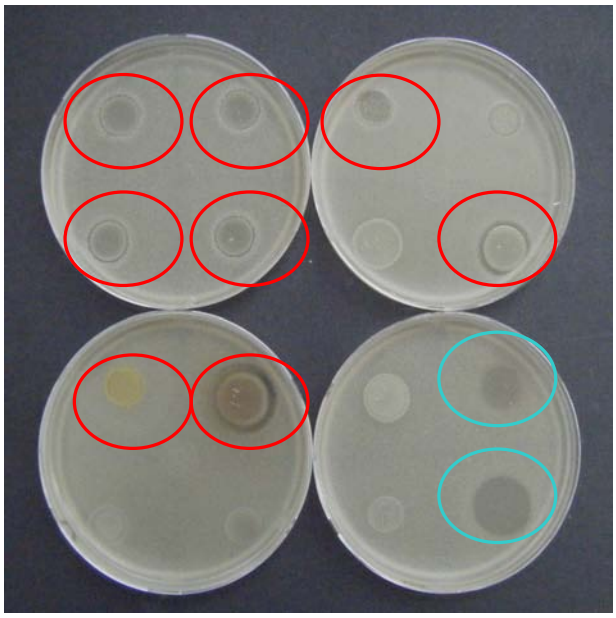
Table 3.1: Table summarising results of antimicrobial and antimicrobial stability assays.

Isolate	Identification by 16S rRNA sequencing	Target bacteria	Stability		
			Proteinase K (20µg/ml)	Temperature (100°C)	Organic solvents (3)
001T	*Bacillus pumilis*	1	+	+	+
002T	*Bacillus pumilis*	1	–	+	+
007T	*Bacillus pumilis*	1	+	+	+
009T	*Bacillus pumilis*	1	+	+	+
010T	*Bacillus pumilis*	1	–	–	–
012T	*Bacillus pumilis*	1	+	+	+
013T	*Bacillus pumilis*	1	+	+	+
016T	*Bacillus pumilis*	1	+	+	+
020T	*Bacillus pumilis*	1	+	+	+
119T	*Pseudomonas tolaasii*	1, 3, 4	–	+	+
133T	*Pseudomonas cichorii*	1, 3, 4	–	–	–
145T	*Proteus vulgaris*	13	ND	ND	ND
228C	*Pseudomonas fluorescens*	1, 4, 13	+	+	+
229C	*Pseudomonas fluorescens*	1	–	–	–
231C	*Pseudomonas fluorescens*	1	–	–	–
283C	*Pseudomonas cichorii*	1, 3, 4	+	+	+
340C	*Pseudomonas tolaasii*	1, 3, 4	–	–	–
381C	*Pseudomonas fulva*	13	ND	ND	ND

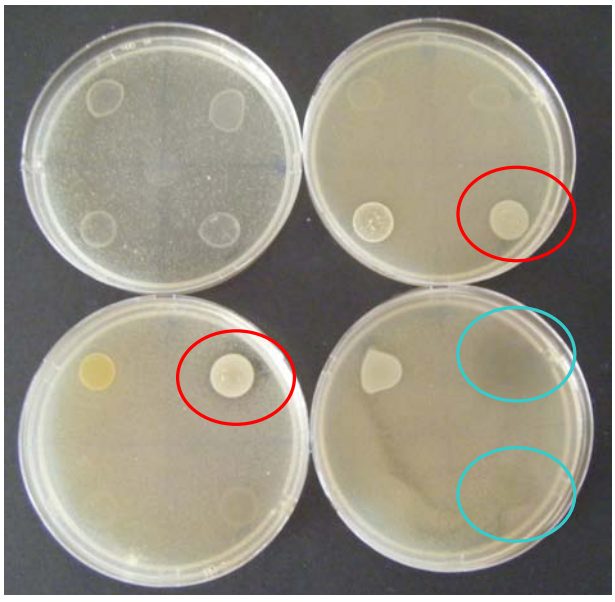
1 = *B. cereus* ATCC 10702, **3** = *B. subtilis* 168, **4** = *B. subtilis* EL39, **13** = *S. aureus* 25928, **ND** = not determined

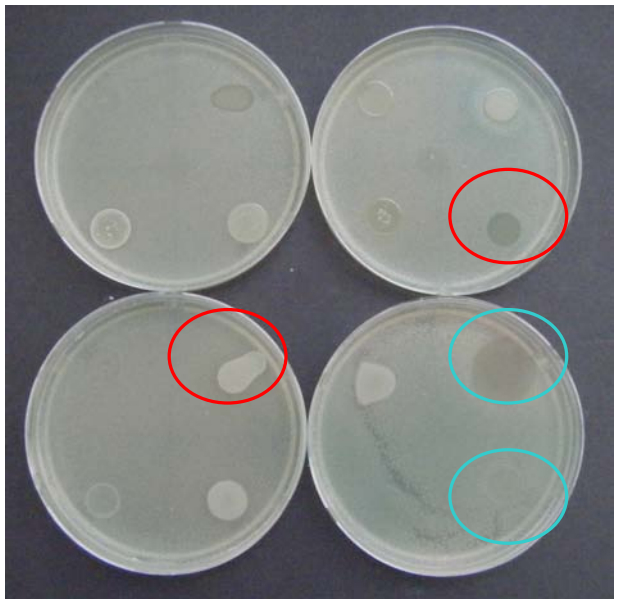
B. cereus ATCC 10702





B. subtilis 168





24h post incubation

48h post incubation

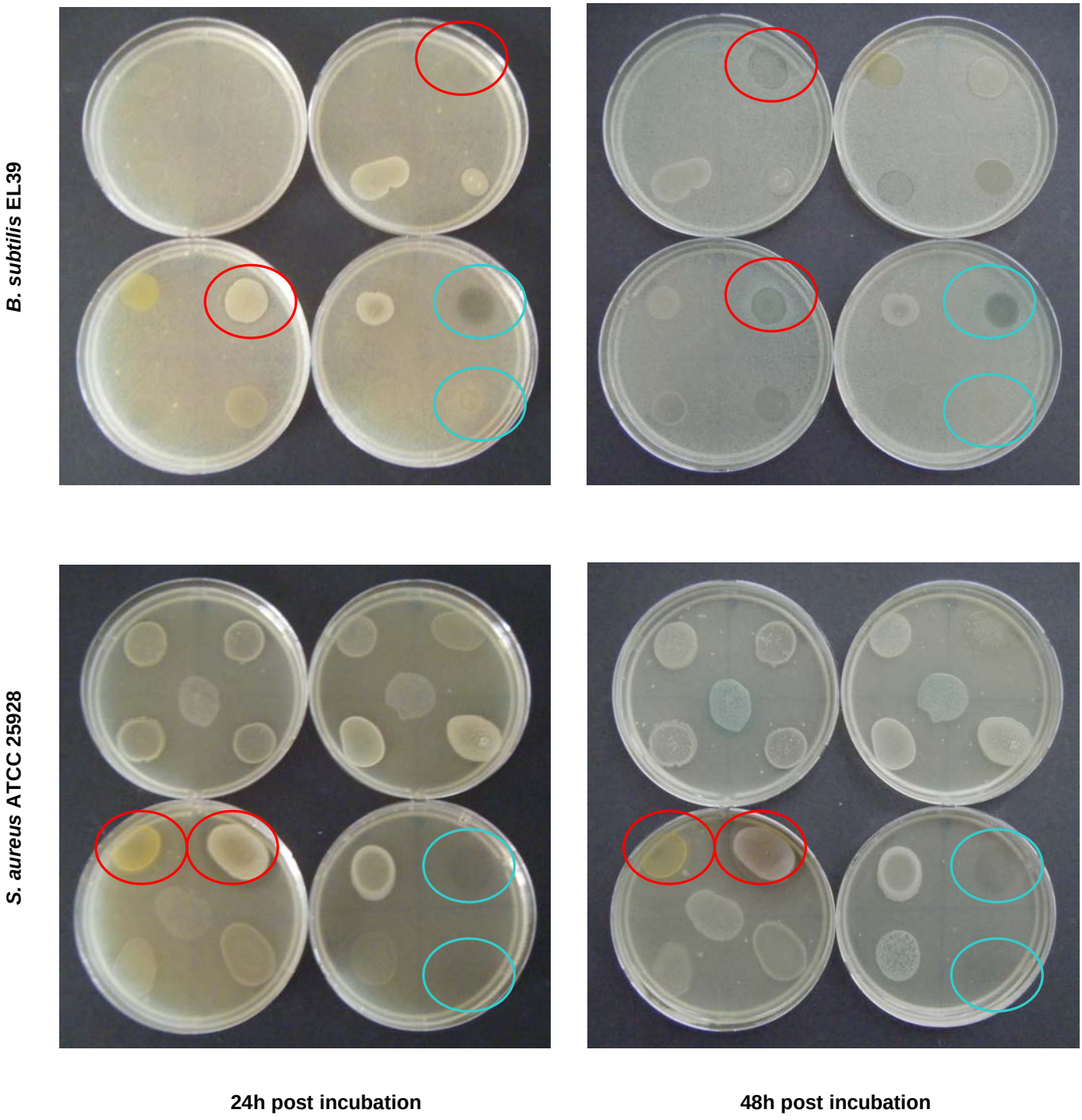


Figure 3.1: Indicator plates of *B. cereus* ATCC 10702, *B. subtilis* 168, *B. subtilis* EL39 and *S. aureus* 25928 exhibiting zones of inhibition resulting from biosurfactant-producing isolates (○) and positive controls (1% SDS and 50% bleach, ○).

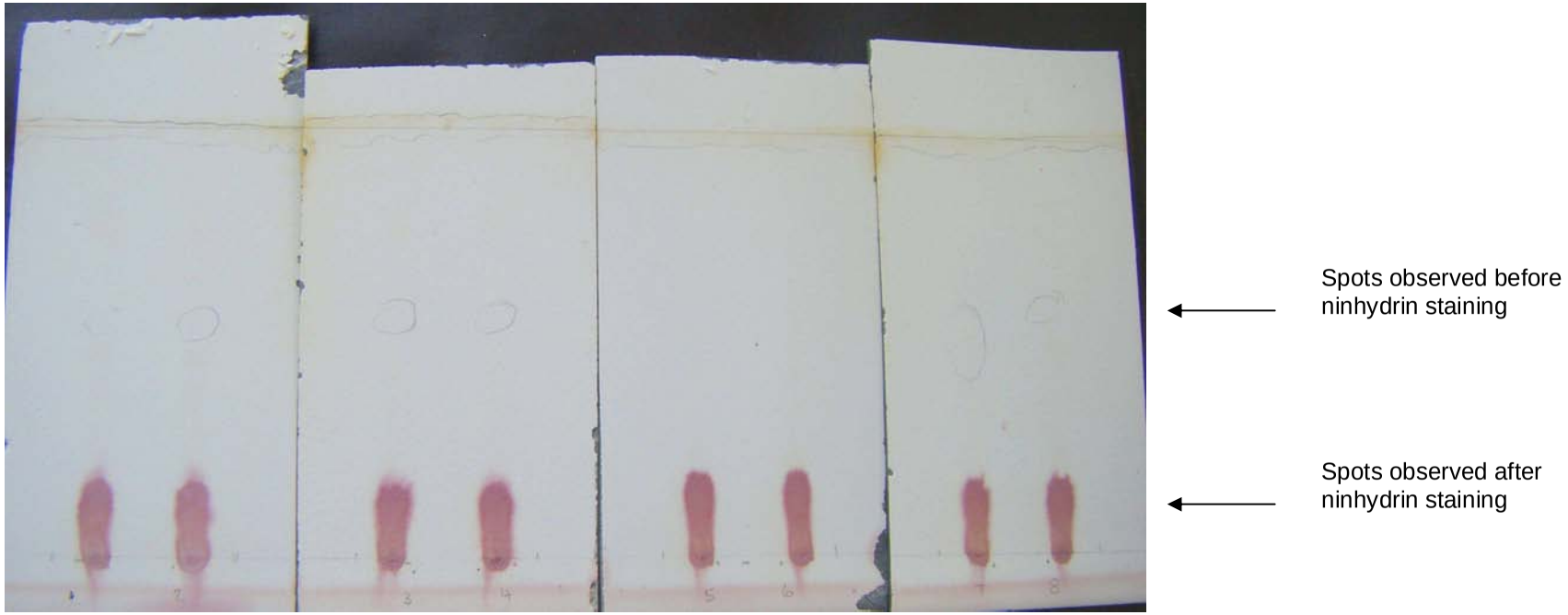
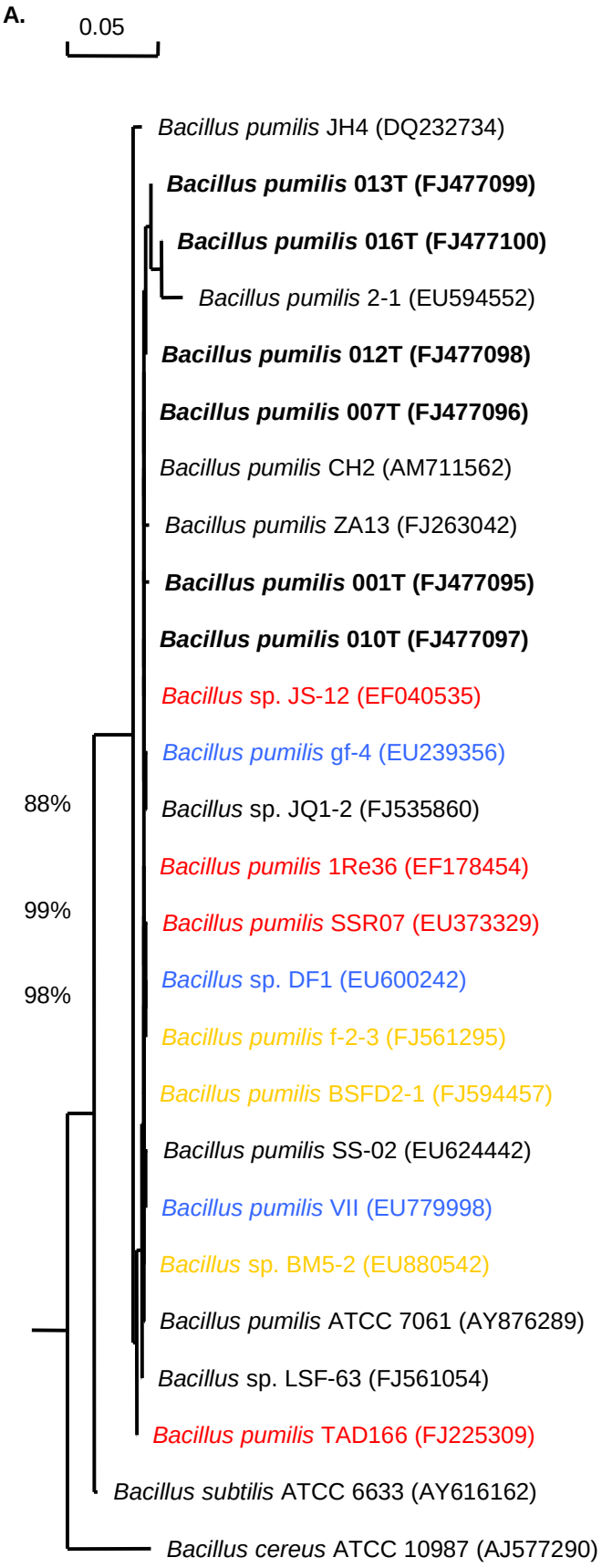
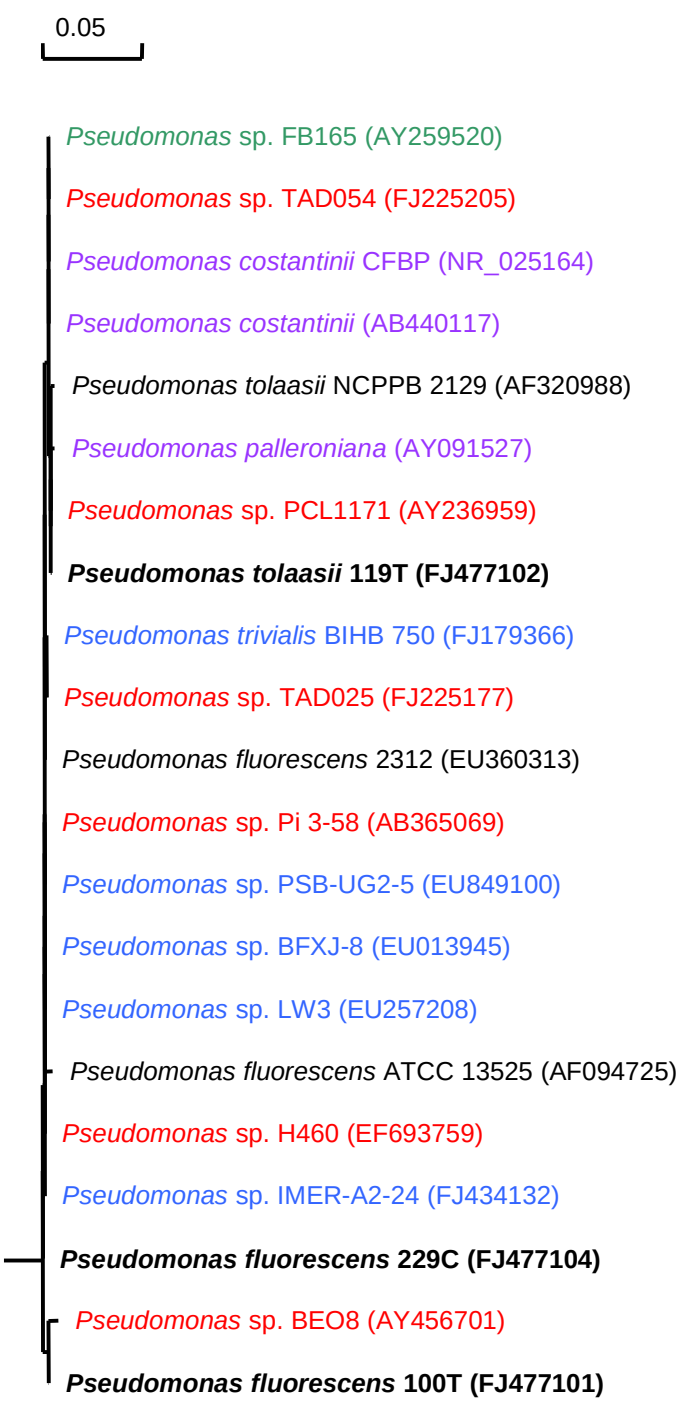


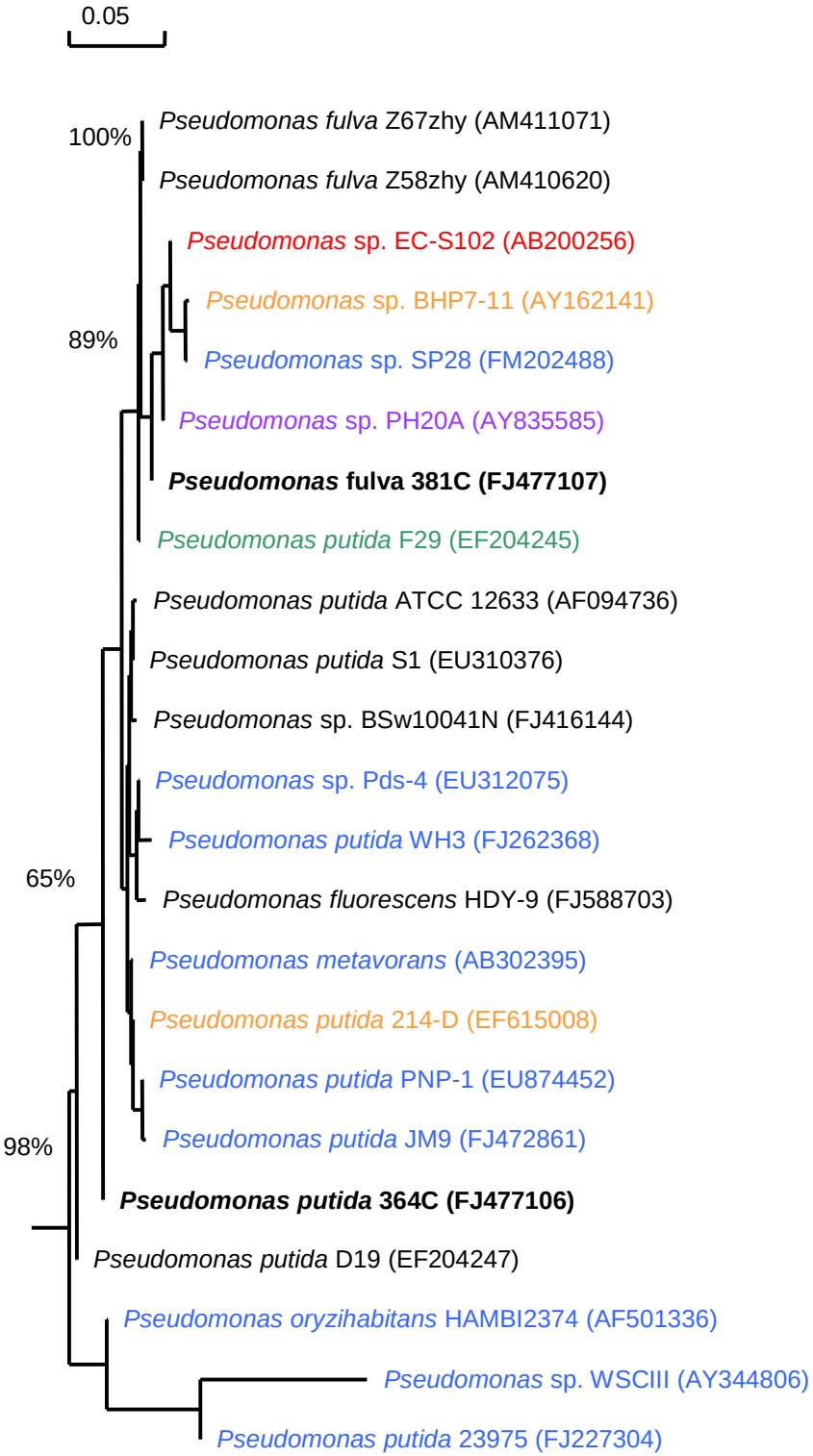
Figure 3.2: Thin-layer chromatograms obtained in a chloroform:methanol:water (65:15:2, vol/vol/vol) solvent system. Lane 1, *B. pumilis* 001T, lane 2, *B. pumilis* 002T, lane 3, *B. pumilis* 010T, lane 4, *P. cichorii* 113T, lane 5, *P. fluorescens* 228C, lane 6, *P. tolaasii* 119T, lane 7, negative control (TSB), lane 8, positive control (*B. subtilis* EL39).


A.
0.05
Bacillus pumilis JH4 (DQ232734)
Bacillus pumilis 013T (FJ477099)
Bacillus pumilis 016T (FJ477100)
Bacillus pumilis 2-1 (EU594552)
Bacillus pumilis 012T (FJ477098)
Bacillus pumilis 007T (FJ477096)
Bacillus pumilis CH2 (AM711562)
Bacillus pumilis ZA13 (FJ263042)
Bacillus pumilis 001T (FJ477095)
Bacillus pumilis 010T (FJ477097)
Bacillus sp. JS-12 (EF040535)
Bacillus pumilis gf-4 (EU239356)
88%
Bacillus sp. JQ1-2 (FJ535860)
Bacillus pumilis 1Re36 (EF178454)
99%
Bacillus pumilis SSR07 (EU373329)
Bacillus sp. DF1 (EU600242)
98%
Bacillus pumilis f-2-3 (FJ561295)
Bacillus pumilis BSFD2-1 (FJ594457)
Bacillus pumilis SS-02 (EU624442)
Bacillus pumilis VII (EU779998)
Bacillus sp. BM5-2 (EU880542)
Bacillus pumilis ATCC 7061 (AY876289)
Bacillus sp. LSF-63 (FJ561054)
Bacillus pumilis TAD166 (FJ225309)
Bacillus subtilis ATCC 6633 (AY616162)
Bacillus cereus ATCC 10987 (AJ577290)

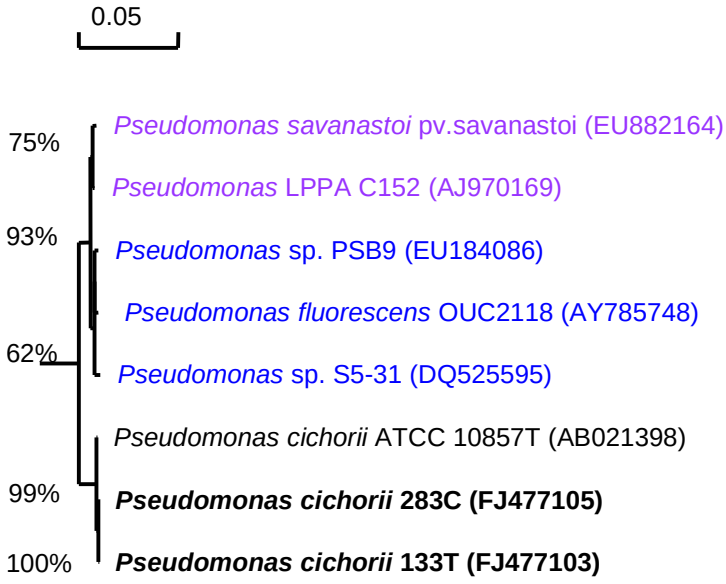
B.


0.05
Pseudomonas sp. FB165 (AY259520)
Pseudomonas sp. TAD054 (FJ225205)
Pseudomonas costantinii CFBP (NR_025164)
Pseudomonas costantinii (AB440117)
Pseudomonas tolaasii NCPPB 2129 (AF320988)
Pseudomonas palleroniana (AY091527)
Pseudomonas sp. PCL1171 (AY236959)
***Pseudomonas tolaasii* 119T (FJ477102)**
Pseudomonas trivialis BIHB 750 (FJ179366)
Pseudomonas sp. TAD025 (FJ225177)
Pseudomonas fluorescens 2312 (EU360313)
Pseudomonas sp. Pi 3-58 (AB365069)
Pseudomonas sp. PSB-UG2-5 (EU849100)
Pseudomonas sp. BFXJ-8 (EU013945)
Pseudomonas sp. LW3 (EU257208)
Pseudomonas fluorescens ATCC 13525 (AF094725)
Pseudomonas sp. H460 (EF693759)
Pseudomonas sp. IMER-A2-24 (FJ434132)
***Pseudomonas fluorescens* 229C (FJ477104)**
Pseudomonas sp. BEO8 (AY456701)
***Pseudomonas fluorescens* 100T (FJ477101)**

C.


0.05
100%
89%
65%
98%
Pseudomonas fulva Z67zhy (AM411071)
Pseudomonas fulva Z58zhy (AM410620)
Pseudomonas sp. EC-S102 (AB200256)
Pseudomonas sp. BHP7-11 (AY162141)
Pseudomonas sp. SP28 (FM202488)
Pseudomonas sp. PH20A (AY835585)
***Pseudomonas* fulva 381C (FJ477107)**
Pseudomonas putida F29 (EF204245)
Pseudomonas putida ATCC 12633 (AF094736)
Pseudomonas putida S1 (EU310376)
Pseudomonas sp. BSw10041N (FJ416144)
Pseudomonas sp. Pds-4 (EU312075)
Pseudomonas putida WH3 (FJ262368)
Pseudomonas fluorescens HDY-9 (FJ588703)
Pseudomonas metavorans (AB302395)
Pseudomonas putida 214-D (EF615008)
Pseudomonas putida PNP-1 (EU874452)
Pseudomonas putida JM9 (FJ472861)
***Pseudomonas putida* 364C (FJ477106)**
Pseudomonas putida D19 (EF204247)
Pseudomonas oryzihabitans HAMBI2374 (AF501336)
Pseudomonas sp. WSCIII (AY344806)
Pseudomonas putida 23975 (FJ227304)

D.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75%
93%
62%
99%
100%
Pseudomonas savanastoi pv.savanastoi (EU882164)
Pseudomonas LPPA C152 (AJ970169)
Pseudomonas sp. PSB9 (EU184086)
Pseudomonas fluorescens OUC2118 (AY785748)
Pseudomonas sp. S5-31 (DQ525595)
Pseudomonas cichorii ATCC 10857T (AB021398)
***Pseudomonas cichorii* 283C (FJ477105)**
***Pseudomonas cichorii* 133T (FJ477103)**

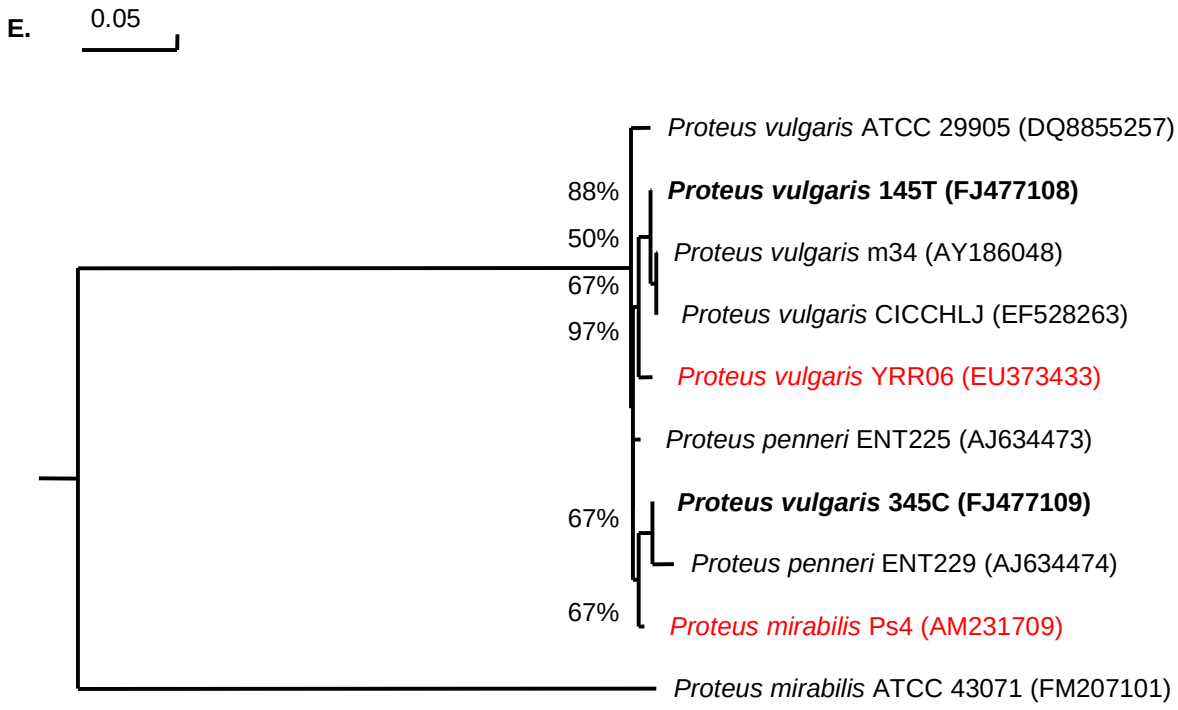


Figure 3.3: Phylogenetic relationships between 16S rDNA sequences of 15 selected surfactants, arranged into five different groups, namely *Bacillus pumilis* (**A.**), *Pseudomonas fluorescens* (**B.**), *Pseudomonas putida* (**C.**), *Pseudomonas syringae* (**D.**) and *Proteus* (**E.**). Included are reference strains and highly similar sequences of relevance, such as those that produce biosurfactants (yellow), exhibit antimicrobial activities (red), degrade xenobiotics (blue), were isolated from food (green) and are pathogenic (purple). The branch length indicates the percentage of sequence dissimilarity. Numbers at the nodes indicate bootstrap values and only values of above 50% are shown.

CHAPTER 4

Utilisation of *n*-hexadecane and motor oil by biosurfactant-producing *Pseudomonas*, and other bacterial strains, isolated from food

ABSTRACT

The aim of this study was to evaluate the utilisation of model hydrocarbon compounds, such as *n*-hexadecane and mineral motor oil, by Gram-negative and -positive biosurfactant-producers isolated from foods. For this study, 8 different biosurfactant-producing strains (3 *Pseudomonas*, 1 *Proteus*, and 4 *Bacillus*) were selected and evaluated for individual growth in minimal media supplemented with either *n*-hexadecane (MMH) or mineral motor oil (MMMO). Additionally, the growth of two consortia, comprising either the 4 Gram-negative strains or 4 Gram-positive strains, in combination, was evaluated. It was found that all 8 of the individual biosurfactant-producing strains and the two consortia grew well in minimal media supplemented with either hydrocarbon, reaching maximum cell numbers of *ca.* 7 – 9 log CFU/ml. However, no significant differences ($P<0.05$) were observed between individual Gram-negative or Gram-positive strains inoculated into either MMH or MMMO. The negative control, a non biosurfactant-producer, reached significantly lower ($P<0.05$) counts of *ca.* 4 and 0 log CFU/ml in MMH and MMMO, respectively. Growth rates for the majority of cultures were between 0.20 – 0.26 log CFU/ml/h; however, the Gram-negative strains and consortium grew at significantly lower ($P<0.05$) rates in MMMO than in MMH, and when compared with the Gram-positive strains and consortium. *Pseudomonas fulva* 381C grew at a significantly lower ($P<0.05$) rate (0.10 log CFU/ml/h) than the other Gram-negative strains and consortium when grown in MMMO. The consortia did not achieve significantly higher ($P<0.05$) cell numbers or grow better than individually inoculated strains in either medium. The negative control grew at a significantly lower ($P<0.05$) rate than the strains and consortia in MMH and the population declined to 0 in MMMO. This study is the first to document hydrocarbon and *n*-alkane utilisation by strains of *P. fulva* and *Proteus vulgaris*, respectively. The results demonstrated that hydrocarbon substrates can efficiently be utilised by bacteria isolated from foods and advocates for the further investigation of bacteria from such sources for bioremediative activities.

INTRODUCTION

Given the well-documented ability of biosurfactants to increase the surface area and bioavailability of water-insoluble substrates, an obvious application of biosurfactants is that of bioremediation. Bioremediation refers to the enhancement of natural biodegradative processes and is achieved by improving the availability of materials (e.g. nutrients and oxygen), conditions (pH and moisture content) and prevailing microorganisms (Ron and Rosenberg, 2002).

Polycyclic aromatic hydrocarbons (PAHs) are common environmental contaminants that result primarily from the incomplete combustion of organic matter associated with coal and crude oil processing. Many PAHs and other petroleum hydrocarbons have toxic, mutagenic and/or carcinogenic properties (Samanta *et al*., 2002), thus posing a threat to human health and the environment. One of the main approaches for eliminating polluting hydrocarbons from contaminated sites is their biodegradation by microorganisms (Wong *et al*., 2004). However, the biodegradation of PAHs is restricted by their limited bioavailability, which is largely due to their hydrophobicity and strong adsorptive capacity in soil (Wong *et al*., 2004). Microorganisms are able to degrade the natural hydrocarbon compounds, particularly the main saturated and unsaturated alkanes, monoaromatic and low-molecular-mass PAHs (Gibson and Subramanian, 1984; Ward *et al*., 2003). PAHs become increasingly difficult to degrade as the number of rings increase due to a decrease in volatility and increase in adsorptive capacity (Milhelcic *et al*., 1993; Volkering *et al*., 1995).

A well-documented microbial strategy for increasing the bioavailability of insoluble PAHs is the production of biosurfactants. Many microorganisms that are able to degrade hydrocarbons produce biosurfactants, either as cell surface components or as extracellular molecules (Ward *et al*., 2003). Several studies have shown that the addition of surfactants can improve pollutant desorption and bioavailability (Maier and Soberón-Chávez, 2000; Christofi and Ivshina, 2002; Doong and Lei, 2003; Shen *et al*., 2004; Wong *et al*., 2004; Mulligan *et al*., 2005), while others reported no effect or inhibition by surfactants (Christofi and Ivshina, 2002; Shin *et al*., 2004; Wong *et al*., 2004; Avramova *et al*., 2008). Contradictory results may be attributable to several factors, including the diverse interactions among hydrocarbon-degrading microbial communities,

varied hydrocarbon structures, varied degrading microorganisms, unpredictable production of surfactants depending on growth conditions and phases, and possible toxicity to degrading microorganisms (Rouse *et al*., 1994; Christofi and Ivshina, 2002; Wong *et al*., 2004).

Biosurfactants have several advantages over their synthetic counterparts that make them suitable for use in several industrial and environmental contexts. Some of these advantages include their biodegradability, low toxicity, specificity and efficiency at high temperatures, pH and salinity (Kosaric, 2001; Makkar and Rockne, 2003). The aim of this study was to evaluate the utilisation of *n*-hexadecane and mineral motor oil by Gram-negative and Gram-positive biosurfactant-producers isolated from foods.

MATERIALS AND METHODS

Biosurfactant-producing strains and growth conditions.

For this assay, 4 Gram-negative and 4 Gram-positive biosurfactant-producing strains isolated from foods were selected based on their positive oil droplet collapse test results (+++) (Table 2.3). The Gram-negative strains were *Pseudomonas fluorescens* 100T, *P. putida* 364C, *P. fulva* 381C and *Proteus vulgaris* 345C, while the Gram-positive strains were *Bacillus pumilis* 007T, 012T, 013T and 016T. All of the isolates were cultured on TSA and incubated at 30°C overnight until subsequent use in the growth assays.

Growth assays to evaluate utilisation of hydrocarbons.

The growth of Gram-negative (3 *Pseudomonas* sp. strains and 1 *Proteus vulgaris* strain) and Gram-positive (4 *B. pumilis* strains) bacteria, singly and in mixed consortia, was monitored. For this assay, 10^2 cells/ml of each strain were inoculated into separate 100ml minimal media (2.50 g/l NH_4Cl, 5.46 g/l KH_2PO_4, 4.76 g/l Na_2HPO_4, 0.20 g/l $MgSO_4$, 0.10 g/l NaCl and 0.20 g/l yeast extract at pH 7), supplemented with 0.4% *n*-hexadecane (Merck) or 0.4% mineral motor oil (Helix Super 20W-50, Shell) as the carbon substrate (Barathi and Vasudevan, 2001). *n*-Hexadecane and mineral motor oil were used as they are model hydrocarbons that have been used previously (Volkering *et al*., 1997; Balba *et al*., 1998; Banat *et al*., 2000; Noordman and Janssen,

2002; Tuleva *et al.*, 2002; Straube *et al.*, 2003; Vasileva-Tonkova *et al.*, 2006; Adoki and Orugbani, 2007). Mixed consortia were similarly prepared in minimal medium by inoculating overnight cultures of the 4 Gram-negative or 4 Gram-positive strains together in separate 100ml flasks. Growth was monitored every 24h by cell counts. To obtain a viable cell count, a tenfold serial dilution (10^{-1} to 10^{-8}) was prepared in the minimal media and plated in duplicate using the pour-plate technique on nutrient agar (2%; Biolab). Plates were then inverted and incubated aerobically at 30ºC for 24h. A flask inoculated with B. *subtilis* 168, a non-biosurfactant-producer (Lee *et al*. 2005), and an uninoculated flask, were also included as negative controls.

Statistical analysis of the data from the duplicate assays was performed using the SAS Enterprise Guide System (version 3.0., SAS Institute, Inc.). An analysis of variance was done by the ANOVA; GPLOT procedure.

RESULTS

Utilisation of the two different hydrocarbon substrates by the 8 different isolates and two consortia is shown by an increase in the number of viable cells (Figs. 4.1, 4.2) as well as by visual inspection (Figs. 4.3 – 4.5).

All of the isolates and both consortia were found to grow well in minimal media supplemented with 0.4% *n*-hexadecane (MMH) or 0.4% mineral motor oil (MMMO), reaching maximum cell counts of *ca.* 7 – 9 log CFU/ml (Figs. 4.1 **–** 4.5). However, no significant differences were observed between individual Gram-negative or Gram-positive strains inoculated into either MMH or MMMO. The Gram-positive strains, including the Gram-positive consortium, achieved significantly higher ($P<0.05$) maximum cell numbers when grown in MMH (average, 7.84 log CFU/ml) than when grown in MMMO (average, 7.74 log CFU/ml). It was also found that the consortia did not reach significantly higher ($P<0.05$) maximum cell counts than individually inoculated strains in either MMH or MMMO. Additionally, the individually inoculated isolates and consortia achieved significantly higher ($P<0.05$) maximum counts than the negative control. The negative control, a non biosurfactant-producer, achieved maximum counts of *ca.* 4 log

CFU/ml in MMH subsequent to a short death phase (24 – 48h), but when grown in MMMO, the control population declined from 2.5 – 0 log CFU/ml (Fig. 4.1, 4.2). The uninoculated control did not show any growth or spontaneous degradation of the hydrophobic substrates, as determined by visual inspection (Figs. 4.3 – 4.5).

Determination of growth rates demonstrated that there were no significant differences ($P<0.05$) between the Gram-positive strains grown in MMH and MMMO, and the Gram-negative strains grown in MMH (0.20 – 0.26 log CFU/ml/h) (Table 4.1). It was found that the Gram-negative strains, including the consortium, grew at significantly higher ($P<0.05$) rates in MMH (average, 0.24 log CFU/ml/h) than in MMMO (average, 0.18 log CFU/ml/h) (Figs. 4.1, 4.2, Table 4.1). Additionally, *P. fulva* 381C grew at a significantly lower rate ($P<0.05$) (0.10 log CFU/ml/h) than the other Gram-negative strains and consortium in MMMO (Fig. 4.1, Table 4.1). Results also found that the Gram-negative strains, including the consortium, grew at significantly lower ($P<0.05$) rates (0.10 – 0.22 log CFU/ml/h) than the Gram-positive strains (0.21 – 0.26 log CFU/ml/h) in MMMO (Table 4.1). The consortia did not grow significantly better ($P<0.05$) than individually inoculated strains in either MMH or MMMO (Figs. 4.1, 4.2, Table 4.1). The individually inoculated isolates and consortia also grew significantly better ($P<0.05$) than the negative control, which was shown to undergo a brief death phase (0 – 24h) when grown in MMH, before reaching a growth rate of 0.06 log CFU/ml/h until the assay was discontinued (Table 4.1). The negative control did not grow in MMMO and the population declined at a rate of 0.05 log CFU/ml/h (Table 4.1).

DISCUSSION

A previous study (Chapter 3) showed that all the strains used in this study produced biosurfactants. Studies have shown that the production of biosurfactants is often associated with the growth of microorganisms, especially those utilising *n*-alkanes, on hydrocarbons (Itoh and Suzuki, 1972; Rosenberg and Ron, 1981; Oberbremer and Müller-Hurtig, 1989; Hommel, 1990). The most common bacterial genera known to be responsible for petroleum hydrocarbon and oil degradation comprise mainly *Achromobacter*, *Acinetobacter*, *Alcaligenes*, *Arthrobacter*, *Bacillus*,

Flavobacterium, *Micrococcus*, *Mycobacterium*, *Nocardia*, *Pseudomonas*, *Rhodococcus* and coryneforms (Leahy and Colwell, 1990; Watkinson and Morgan, 1990; Balba *et al*., 1998; Ajayi *et al*., 2008). This study showed varying degrees of utilisation by all 8 strains evaluated, including *Pseudomonas fulva* and *Proteus vulgaris*. The negative control, a non-biosurfactant-producer, showed significantly lower ($P<0.05$) utilisation of *n*-hexadecane and growth inhibition by mineral motor oil. Similarly, a previous study also showed that non-surfactin-producing *B. subtilis* 168 did not degrade *n*-alkanes (Kim *et al*., 2000). The secondary exponential phase is likely to be due to the germination of spores, which is activated by adverse environmental conditions, such as starvation (Katz and Demain, 1977; Grossman, 1995; Driks, 1999).

The ability of some *Pseudomonas* spp. to degrade hydrocarbon pollutants is well-documented. Wilson and Bradley (1996) evaluated the degradation of *n*-alkanes in petrol (Slovene diesel) by free and immobilised *Pseudomonas fluorescens*, and found that that the immobilised systems degraded significantly more *n*-alkanes than the free systems. The immobilised systems degraded 14.8 – 24.5% of C_{12} alkanes and 52.4 – 91.2% of the C_{13} – C_{18} alkanes, whereas the free system was found to degrade 52.3 and 11.6% of C_{12} and C_{13} alkanes, respectively, but not C_{13} – C_{18} alkanes. Barathi and Vasudevan (2001) reported the production of biosurfactant/s by an indigenous strain of *P. fluorescens* growing, in decreasing order, on hexadecane, hexane, decane, kerosene and crude oil. Maximum viable cell numbers of 1.2 – 2.2 log CFU/ml on the different hydrophobic carbon substrates were observed. These counts are significantly lower than those obtained by *P. fluorescens* 100T grown on *n*-hexadecane in this study, suggesting that strain 100T is a better *n*-hexadecane utiliser. The production of biosurfactants by another strain of *P. fluorescens* (HW-6), isolated from industrial wastewater, was also observed when the strain was grown on hexane (Vasileva-Tonkova *et al*., 2006). The glycolipid biosurfactants were also found to efficiently emulsify aromatic hydrocarbons, kerosene and *n*-paraffins. Several studies have shown high rates of degradation of petroleum hydrocarbons by strains of *P. putida*. Robinson *et al*. (1990) found that *P. putida* (biotype B) was able to degrade the extractable fraction of sorbed toluene, which consisted of >90% of the total sorbed toluene, while Guerin and Boyd (1992) found that *P. putida* 17484, could degrade both sorbed and aqueous phase naphthalene. In a study that evaluated the effects of bioremediation programs of sterile agricultural soils contaminated with crude petroleum, Nwachukwu (2001) found that the inoculation of a *P. putida* strain,

together with the addition of appropriate inorganic nutrients, grew on (up to 10 log CFU/ml) and completely degraded the oil within 9 weeks. Tuleva *et al*. (2002) reported the production of rhamnolipids by *P. putida* 21BN when grown on *n*-hexadecane, while Kumar *et al*. (2006b) showed that *P. putida* IR1 also produced biosurfactants upon its growth on 2-, 3- and 4-ring PAHs, but not hexadecane and octadecane, as a sole carbon and energy source. Doong and Lei (2003) evaluated the effects of 5 surfactants (4 non-ionic, 1 anionic) on *P. putida* NCIMB 9816 inoculated into a soil system containing [^{14}C]pyrene, naphthalene and phenanthrene. They found that the addition of surfactants decreased the mineralisation rate of [^{14}C]pyrene and enhanced the bioavailability of naphthalene, phenanthrene and pyrene with efficiencies ranging from 21.1 – 60.6%, 33.3 – 62.8% and 26.8 – 70.9%, respectively. Additionally, only the biodegradable surfactants (Triton X-100 and Brij 30), were utilised as sole carbon sources. This study suggests that biodegradable biosurfactants, such as those produced by *P. putida* 364C (and the other strains evaluated in this study), play a significant role in increasing the bioavailability of hydrophobic substrates. Our study is the first to report the utilisation of hydrocarbons by a strain of *P. fulva*. It is worth noting; however, that *P. fulva* is closely related to hydrocarbon-degrading *P. putida*. Additionally, the *P. fulva* strain isolated in this study was 97% similar to strains of xenobiotic-degrading *Pseudomonas* spp., according to 16S rRNA sequencing (Chapter 2).

Some species of *Proteus* have been known to degrade and grow on oil, but not *n*-alkanes, in such mixed consortia. The first recorded incidence of oil degradation by a *Proteus* sp. is by Kadri *et al*. (1986), who identified *P. mirabilis* in the Kuwait Bay. However, most of the studies on oil degradation by *Proteus* spp. have been conducted in Nigeria due to its oil production industry, and such studies have focused on degradation by bacterial consortia also including *Aeromonas*, *Bacillus*, *Micrococcus*, *Pseudomonas* and *Serratia*. Benka-Coker and Ekundayo (1996) reported the degradation of blend crude oil by such a bacterial consortium, while Adoki and Orugbani (2007) found that consortia grown on different refined petroleum products (including engine oil, diesel, kerosene and petrol) reached maximum cell counts of 4.5 – 6.7 log CFU/ml. The Gram-negative consortium from this study was able to reach *ca.* 1 log CFU/ml higher than that of Adoki and Orugbani (2007), suggesting that our consortium is able to utilise engine (motor) oil slightly better. To our knowledge, the production of biosurfactant by oil-utilising *Proteus* spp. has previously been reported only by Balogun and Fagade (2008). In their study it was found that

7% of oil-utilising *Proteus* spp. isolated from diesel oil-contaminated soil produced biosurfactants. Our study; however, is the first to document the utilisation of an *n*-alkane (*n*-hexadecane) by *Proteus*. It is possible that the production of biosurfactant/s by *Proteus* spp. has facilitated their growth and utilisation of hydrocarbons in this study.

Many *Bacillus* spp. are known to degrade hydrocarbons, including *B. subtilis* (Kim *et al*., 2000; Morán *et al*., 2000; Haghihat *et al*., 2008), *B. cereus* (Jennings and Tanner, 2000), *B. thuriengensis* (Jennings and Tanner, 2000), *B. sphaericus* (Jennings and Tanner, 2000), *B. licheniformis* (Kumar *et al*., 2006a; Haghighat *et al*., 2008) and *B. circulans* (Das *et al*., 2008). Calvo *et al*. (2004) showed that *B. pumilis* 28-11, isolated from solid waste oil, grew well in the presence of 0.1% (w/v) crude oil and naphthalene under aerobic conditions and used these hydrocarbons as carbon and energy sources. Viable cell counts of *ca*. 10 log CFU/ml were recorded in stationary phase. The production of a cell surface-associated biosurfactant was suggested to have enabled the utilisation of crude oil, while naphthalene utilisation appeared to be under control of inducible enzyme systems. The utilisation of naphthalene, as well as phenanthrene, fluoranthene or pyrene, as sole carbon sources was also reported by Toledo *et al*. (2006), with the 5 different *B. pumilis* strains, including 28-11, reaching cell counts of 5.5 – 8.8 log CFU/ml. With regards to cell counts, these counts correspond with or are approximately 2 log CFU/ml higher than those obtained in this study for *B. pumilis*. That said, it is not possible to directly compare counts as the other studies cited did not evaluate the growth of their strains of *B. pumilis* on *n*-hexadecane or mineral motor oil. Such counts merely highlight the observation that different species and different strains within a particular species have differing abilities to utilise certain hydrophobic substrates.

This study further showed that neither of the two consortia was able to achieve significantly higher ($P<0.05$) cell numbers or grow better than individually inoculated strains in either medium. Some studies have motivated for the degradation of hydrocarbons by bacterial consortia. Bento *et al*. (2005a) found that a bacterial consortium consisting of *Bacillus* (*B. pumilis*, *B. cereus*, *B. sphaericus*, *B. fusiformis*), *Acinetobacter* and *Pseudomonas* sp. isolated from diesel-contaminated soil in Long Beach, California (USA) (Bento *et al*., 2005b), were found to degrade light ($C_{12} – C_{23}$) and heavy ($C_{23} – C_{40}$) fractions of the total petroleum hydrocarbons

(TPH) 72.7 and 75.2%, respectively. Co-cultures of *P. fluorescens* and *P. putida*, immobilised in fibrous-bed bioreactors, were shown to degrade benzene, toluene, ethylbenzene and xylenes (BTEX) as sole carbon and energy sources (Shim and Yang, 1999; Shim *et al*., 2002; Shim *et al*., 2005). However, BTEX degradation showed substrate inhibition kinetics.

Such results further motivate for the determination of hydrocarbon-utilisation by HPLC and/or gas chromatography as cell numbers and substrate utilisation do not always necessarily correlate. An interesting experiment for the future would be to assess the degradation of hydrocarbons by bacterial consortia containing both Gram-negative and Gram-positive bacteria.

CONCLUSION

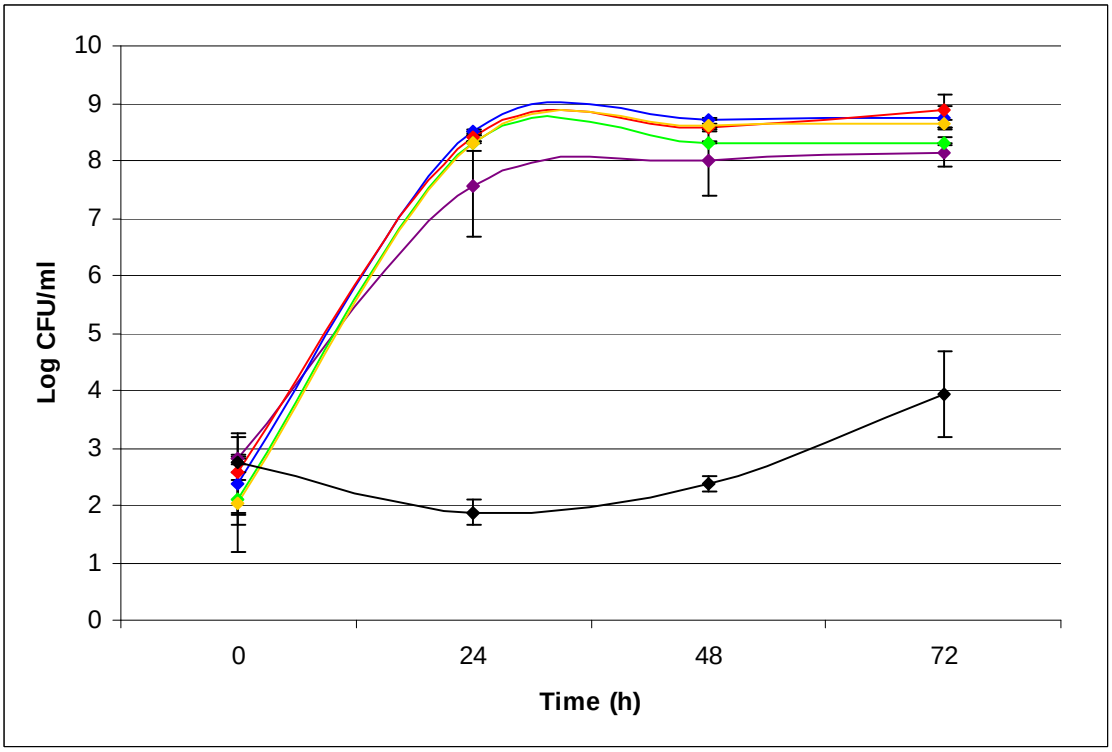
Results from this study showed that *Pseudomonas* strains isolated from foods, and producing biosurfactants, were able to grow well in minimal media supplemented with either *n*-hexadecane or mineral motor oil. Furthermore, this study is the first to show hydrocarbon and *n*-alkane utilisation by strains of *P. fulva* and *Proteus vulgaris*, respectively. Other biosurfactant-producing bacteria isolated from foods, including *Proteus vulgaris* and 4 strains of *Bacillus pumilis*, also grew in hydrocarbon-supplemented medium and maximum cell numbers of up to 8.56 log CFU/ml were recorded. Overall, the Gram-positive strains reached significantly higher cell numbers and growth rates than the Gram-negative strains when grown in minimal media supplemented with 0.4% mineral motor oil. Additionally, the Gram-negative strains grew better in the minimal media supplemented with 0.4% *n*-hexadecane than with 0.4% mineral motor oil. These results motivate for the further investigation of hydrocarbon-utilisation by biosurfactant-producing bacteria isolated from spoiled food.

Table 4.1: Table summarising growth rates of selected *Bacillus*, *Pseudomonas* and *Proteus* isolates grown in minimal media supplemented with 0.4% *n*-hexadecane or mineral motor oil.

Culture (+0.4% *n*-hexadecane)	**Growth rate (log CFU/ml/h)**	**Culture (+0.4% mineral motor oil)**	**Growth rate (log CFU/ml/h)**
B. pumilis 007T	0.23	*B. pumilis* 007T	0.24
B. pumilis 012T	0.22	*B. pumilis* 012T	0.21
B. pumilis 013T	0.25	*B. pumilis* 013T	0.26
B. pumilis 016T	0.23	*B. pumilis* 016T	0.24
G +	0.27	G +	0.24
Ps. fluorescens 100T	0.20	*Ps. fluorescens* 100T	0.17
Ps. putida 345C	0.26	*Ps. putida* 345C	0.18
Ps. fulva 364C	0.24	*Ps. fulva* 364C	0.10
Proteus vulgaris 381C	0.26	*Proteus vulgaris* 381C	0.22
G -	0.26	G -	0.25
Negative control	-0.04 (0 – 24h) 0.06 (48 – 72h)	*Negative control*	-0.05

G+ (Gram-positive), consortium comprising 007T + 012T + 013T + 016T; G- (Gram-negative), consortium comprising 100T + 345C + 364C + 381C

A.



B.

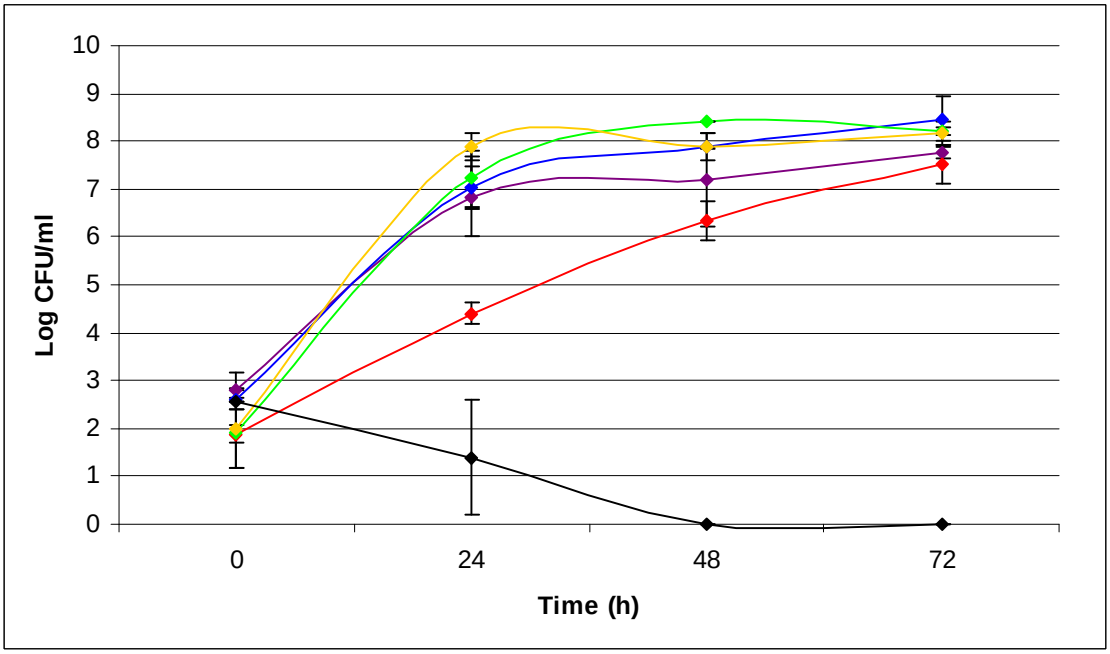
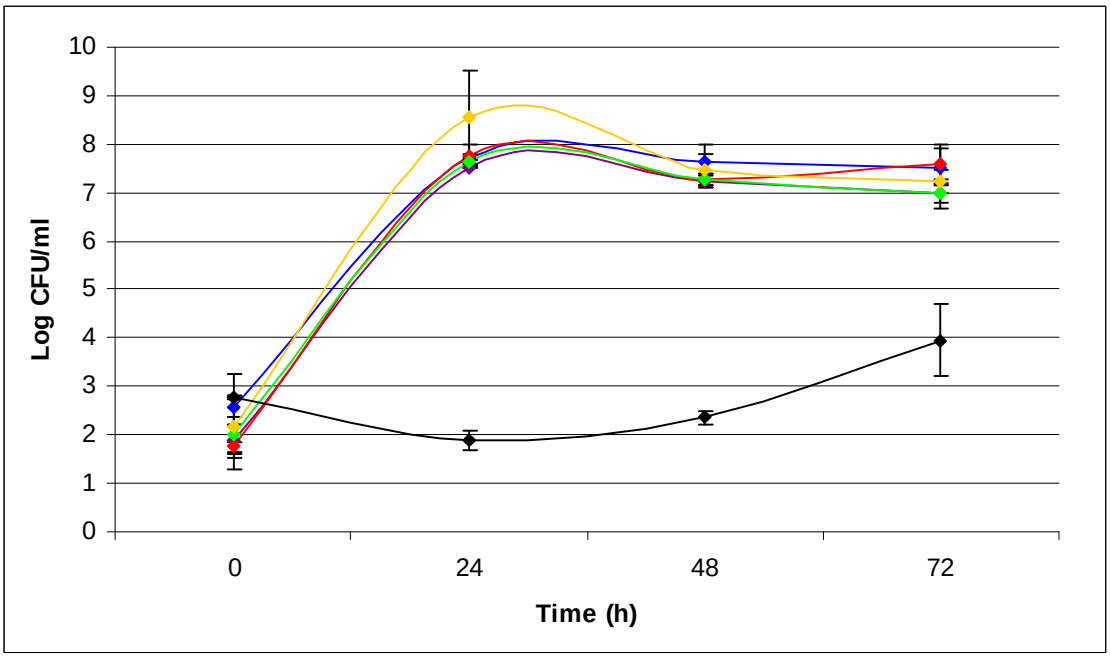


Figure 4.1: Growth of selected *Pseudomonas* and *Proteus* isolates, singly [isolate 100T (♦); isolate345C (♦); isolate 364C (♦); isolate 381C (♦)] or in combination [100T + 345C + 364C + 381C (♦)], in 0.4% *n*-hexadecane (**A.**) and 0.4% motor oil (**B.**). The negative control is *B. subtilis* 168 (♦), a non-biosurfactant-producer.

A.



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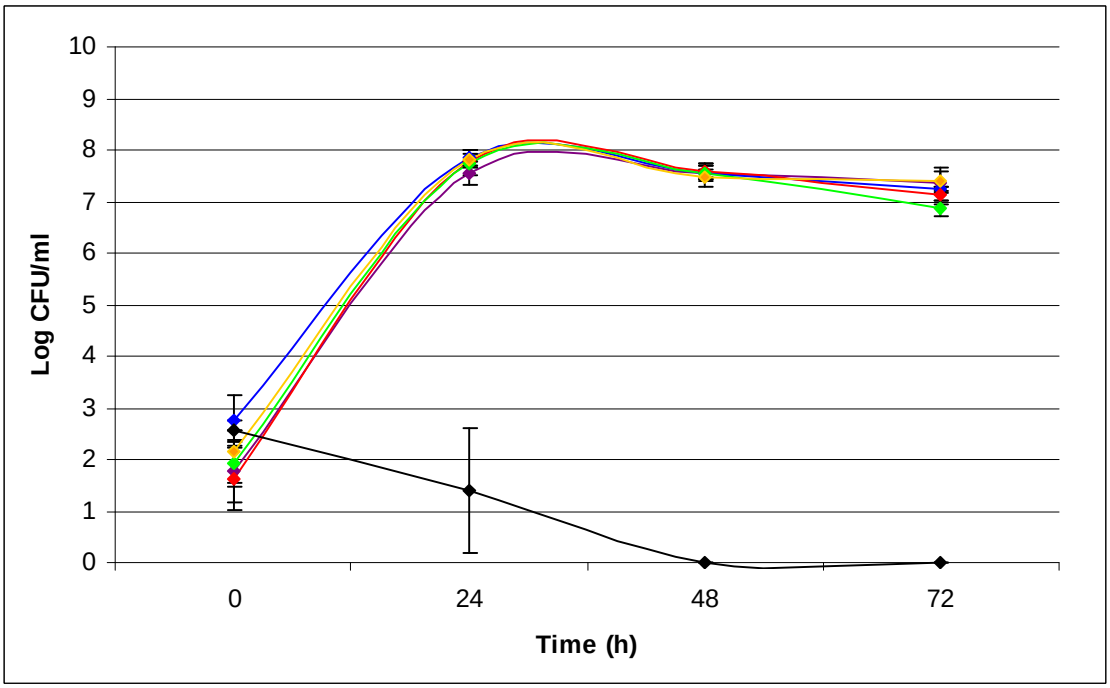
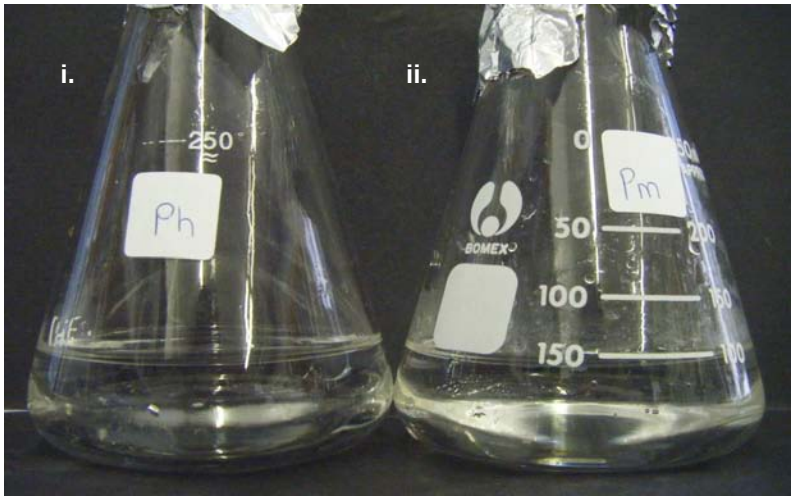
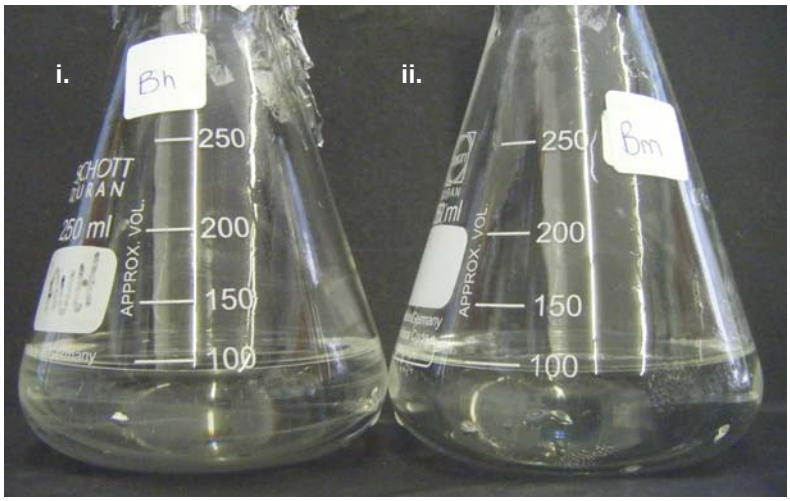


Figure 4.2: Growth of selected *Bacillus* isolates, singly [isolate 007T (♦); isolate 012T (♦); isolate 016T (♦)] or in combination [007T + 012T + 013T + 016T (♦)], in 0.4% *n*-hexadecane (**A.**) and 0.4% motor oil (**B.**). The negative control is *B. subtilis* 168 (♦), a non-biosurfactant-producer.

A.



B.



C.

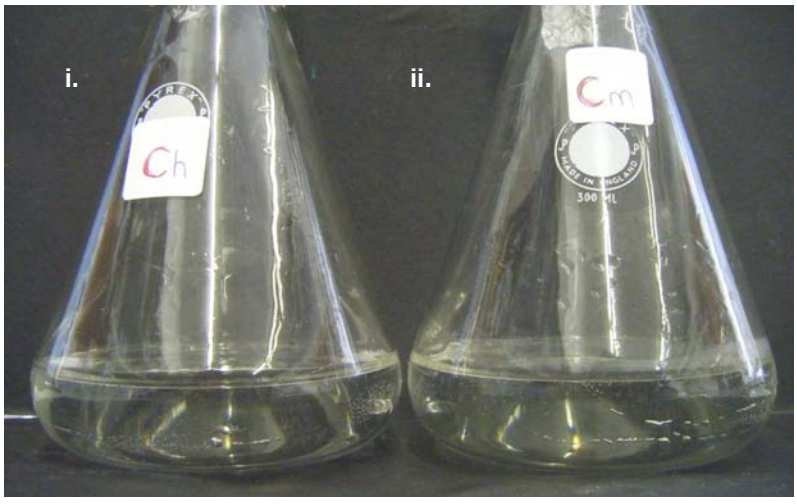
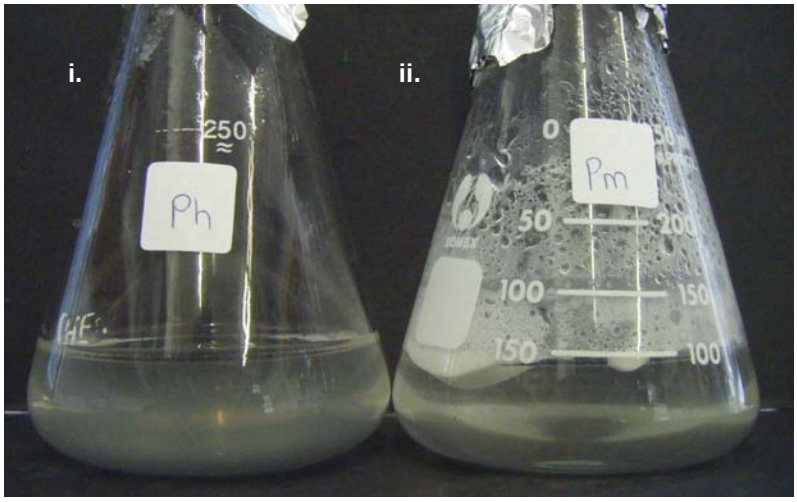
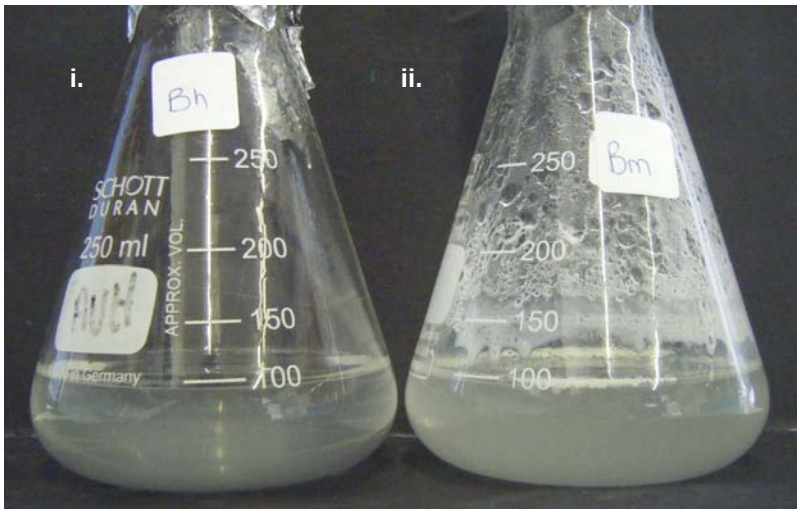


Figure 4.3: Photographs showing growth of representative Gram-negative (**A.**), Gram-positive (**B.**) and negative control (**C.**) in minimal media supplemented with 0.4% *n*-hexadecane (**i.**) or mineral motor oil (**ii.**) at 0h post-inoculation.

A.



B.



C.

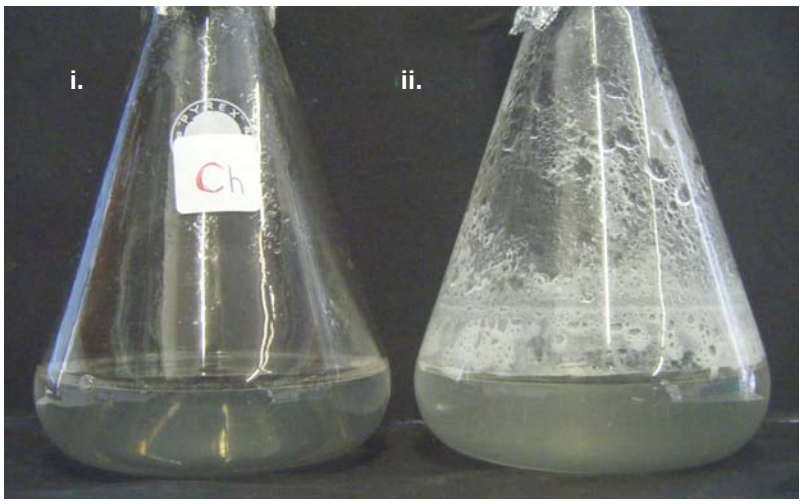


Figure 4.4: Photographs showing growth of representative Gram-negative (**A.**), Gram-positive (**B.**) and negative control (**C.**) in minimal media supplemented with 0.4% *n*-hexadecane (**i.**) or mineral motor oil (**ii.**) at 24h post-inoculation.

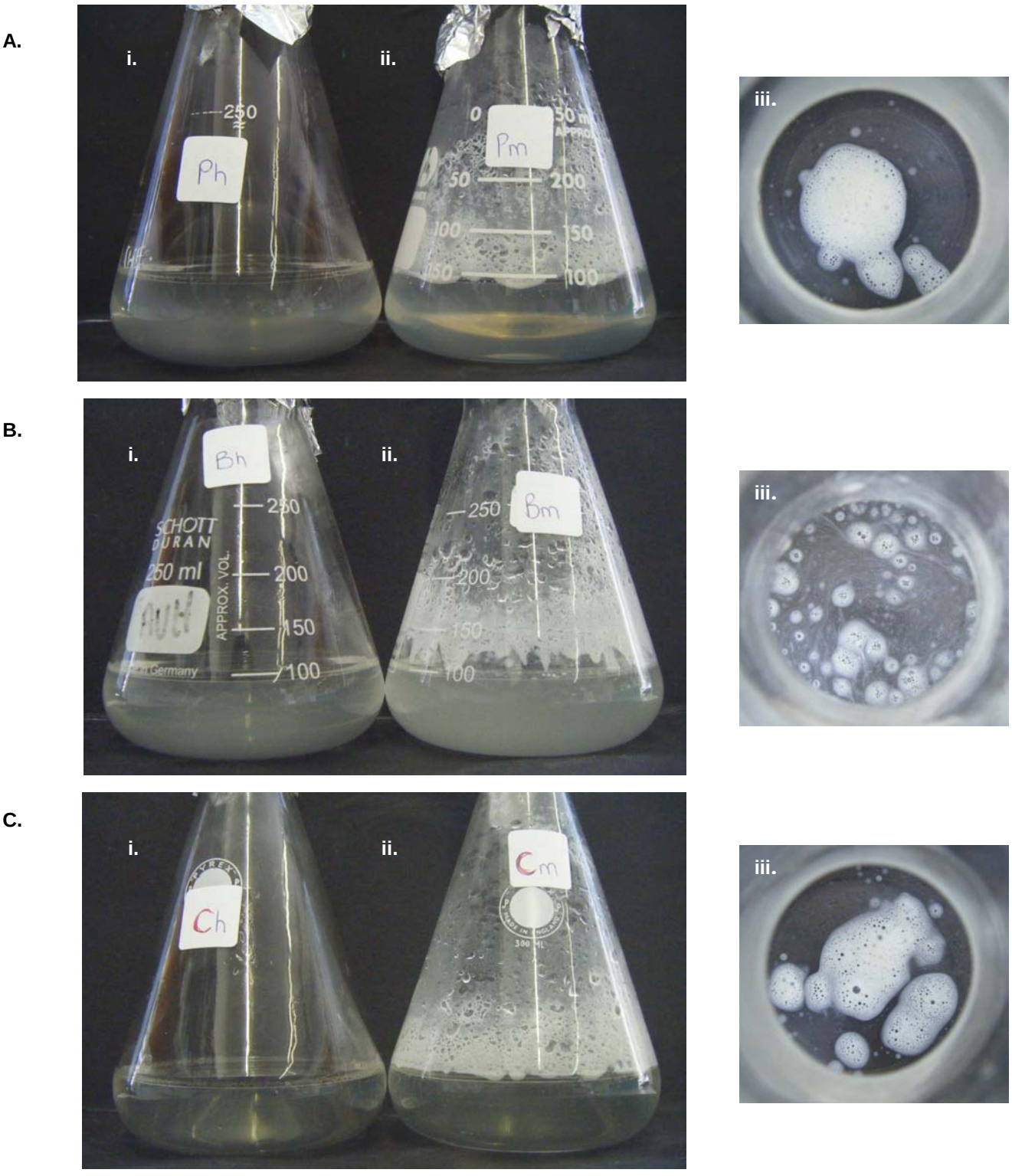


Figure 4.5: Photographs showing growth of representative Gram-negative (**A.**), Gram-positive (**B.**) and negative control (**C.**) in minimal media supplemented with 0.4% *n*-hexadecane (**i.**) or mineral motor oil (**ii.**) at 72h post-inoculation. Magnified images (**iii.**) show emulsification of mineral motor oil, as seen through the top of the flasks.

CHAPTER 5

Summarising discussion and conclusion

Surfactants are amphipathic compounds which have a diverse range of application in various industries worldwide. However, studies have indicated that chemical surfactants pose several problems, including poor specificity, low biodegradability and toxicity to organisms in the environment (Cooper *et al*., 1986). Due to the advantages over chemical surfactants, biologically (microbially) produced surfactants, biosurfactants, represent an attractive alternative to chemical surfactants. Due to their surface-active properties, one of the primary industries in which biosurfactants are used is that of the bioremediation of hydrophobic pollutants (Mulligan, 2005; Singh *et al*., 2007). In developing biosurfactants for this industry, studies have typically sourced biosurfactant-producing bacteria from contaminated soil environments (Tuleva *et al*., 2002, Mulligan, 2005; Vasileva-Tonkova *et al*., 2006, Singh *et al*., 2007; Whang *et al*., 2008). In this study, food products were evaluated as potential sources of bacteria, with emphasis on *Pseudomonas* spp., able to produce biosurfactants

Bacterial counts and spoilage populations

Prior to evaluating 14 different food products for the production of biosurfactants by food spoilage bacteria, bacterial numbers and the microbial ecologies of the food products were determined (Chapter 2). Results of bacterial counts and identification of selected isolates generally showed consistency with the literature, with a few exceptions.

Spoiled vegetable, fish and meat products were found to have the highest aerobic and *Pseudomonas* plate counts (APC and PC, respectively), which was expected as the pH values, nutrient and water content of such foods support the growth of Gram-negative psychrotrophs, predominantly *Pseudomonas*, to high numbers (Jay, 2000, 2005). In contrast, the lowest APC and PC were recorded for bottled mineral water (4.5 log CFU/ml) and dairy products (<2 log CFU/ml), respectively (Fig. 2.1). Hunter (1993), and Tamagnini and Gonzalez (1997) showed that the APC of bottled water increased to 4 – 5 log CFU/ml over several weeks post bottling. Although *Pseudomonas* spp. typically dominate the spoilage flora of raw and pasteurised milk at the time of spoilage (Wiedmann *et al*., 2000), the low bacterial counts obtained in this study could reflect a low level of post-process contamination by heat susceptible *Pseudomonas* spp.

Spoilage populations were putatively identified by biochemical methods and that of dairy products was found to be predominantly *Lactobacillus* and that of fish reaction type (RT) II Gram-negative bacteria (GNB). Spoiled vegetable products were predominated by RT IV, V and VI GNB, each of which constituted *ca.* 21 – 33% of the total population (Fig. 2.2). These results are in accordance with those of related studies (King *et al*., 1991; Christiansen *et al*., 2006; Jay, 2005). It was also found in the study that *Bacillus* and RT VI GNB, which includes *Alcaligenes* spp., constituted an unusually high proportion of the spoilage populations of bottled mineral water (27%) and dairy products (24%), respectively. The atypical presence of *Bacillus* in bottled mineral water is possibly attributable to contamination during the sampling and/or extraction process, as previously shown by Hunter (1993). Similarly, the relatively high proportion of RT VI GNB identified from spoiled dairy products was most likely due to contamination from equipment surfaces or water supplies, as *Alcaligenes* has been reported as a common post-pasteurisation contaminant (Meer, 1991).

Food as a novel source for biosurfactant-producing bacteria, with emphasis on *Pseudomonas* strains

The present study is the first to report the production of biosurfactants from bacteria isolated from spoiled food products. Other studies, which have investigated the prevalence of biosurfactant-producing bacteria isolated from various soils, have reported frequencies ranging from 0.7 – 60% (Jennings and Tanner, 2000; Nielsen *et al*., 2002; Bodour *et al*. 2003; Tran, 2007). Results from the oil droplet collapse assay in this study showed that 6% of isolates obtained from the 14 different spoiled food products were able to produce biosurfactants, demonstrating that the frequency isolation of biosurfactant-producing strains is comparable to related studies and that spoiled food products are a novel source of biosurfactant-producing isolates worth investigating (Chapter 2).

The 32 biosurfactant-producing strains were isolated only from leafy vegetables, bottled mineral water and low fat milk, with the majority of strains isolated from leafy vegetables (72%) (Table 2.3). Biosurfactant-producing strains isolated from leafy vegetables were identified as predominantly RT IV GNB, which includes *Pseudomonas* (*P*.) *aeruginosa*, *P. fluorescens* and *P. putida*. Strains isolated from bottled mineral water were predominantly *Bacillus*, RT IV and VI

GNB, the latter group of which include other *Pseudomonas* spp., while all of the strains isolated from low fat milk were putatively identified as *Lactobacillus* spp. *Pseudomonas*, *Bacillus* and *Lactobacillus* spp. are all well-known producers of biosurfactants (Velraeds *et al*., 1996a; Singh and Cameotra, 2004; Rodrigues *et al*., 2006; Nitschke and Costa, 2007). Bacteria have evolved to produce surface-active products which fulfil a variety of roles that cannot be generalised. Some of these roles include increasing the surface area and bioavailability hydrophobic substrates, regulating the attachment-detachment of bacteria to surfaces and antimicrobial activity. The first of these roles is probably the best known as it allows bacteria to feed on water-insoluble substrates. The results from this study; that only vegetable, bottled mineral water and dairy yielded biosurfactant-producing strains, suggest that the production of biosurfactants by the biosurfactant-producing strains may have occurred in order to facilitate the attachment of bacteria to food product surfaces, and not to directly facilitate the utilisation of fats and oils present in foods by initial and spoilage microflora as initially hypothesised (Chapter 1).

Biosurfactant-producing isolates were evaluated for their ability to emulsify cooking oil as this activity was investigated in the context of food. Cooking oil was used also to serve as a preliminary indication of emulsification activity, for which none of the isolates tested positive (Fig. 2.4). However, this is not altogether surprising as biosurfactant activity is not mutually inclusive of emulsifying activity (Ron and Rosenberg, 2002). That said, emulsifying activity has frequently been exhibited by biosurfactant-producing bacteria isolated from hydrocarbon-contaminated sites (Bodour *et al*., 2003; Rahman *et al*., 2003; Bento *et al*., 2005b; Batista *et al*., 2006). Emulsification activity can be substrate-specific and may occur at different rates on different hydrophobic substrates. The observed variation in activity of bacterial strains isolated from different sources could suggest that such activity is not required for growth on spoiled foods, further supporting the speculation that the production of biosurfactants by strains in the present study plays an attachment role. Considering that several biosurfactant-producing bacteria were shown to utilise two model hydrocarbons (Chapter 4), further investigation into the emulsification activity of the biosurfactant-producing strains is suggested. Such an investigation would include the evaluation of a variety of hydrocarbons, including those employed in the hydrocarbon utilisation assay.

Partial characterisation and antimicrobial activity of compounds from biosurfactant-producing bacteria isolated from food

Many biosurfactants have also been shown to exhibit antimicrobial activity and, considering the increasing prevalence of drug-resistant pathogenic bacteria, present a potential alternative to synthetic medicines and antimicrobial agents. To test the antimicrobial potential of biosurfactant-producing strains isolated in the study, the 32 strains were evaluated against potential bacterial pathogens and food spoilage strains (Chapter 3). The spot-on-lawn assay found that 18 of the 32 (56%) isolates were active against only the Gram-positive target bacteria (Table 3.1), which was expected as Gram-negative bacteria tend to be resistant to many antimicrobial molecules due to the lipopolysaccharide layer of the outer membrane (Nikaido and Vaara, 1985).

Furthermore, the majority of the antibacterial strains were active against the potential pathogen *Bacillus* (*B.*) *cereus* ATCC 10702. The *B. pumilis* strains exhibited very narrow spectrum activity against *B. cereus* ATCC 10702, while the *Pseudomonas* strains exhibited slightly broader spectrum activity against *B. cereus* ATCC 10702, *B. subtilis* and *Staphylococcus* (*S.*) *aureus*. The *P. fulva* and *Proteus vulgaris* isolates were active against only the *S. aureus* strain (Fig. 3.1, Table 3.1). This study is also the first to document antimicrobial activity by *P. fulva*, an environmental bacterium of the *P. putida* group; however, this requires further investigation.

The identities of the 18 antibacterial isolates was confirmed by 16S rRNA sequencing and it was found that 40% of the isolates were correctly identified by biochemical methods, highlighting the need to confirm identities of bacterial strains by additional methods. Molecular identification by 16S rRNA sequencing identified 8 strains as *Pseudomonas* spp. (one by inference), 9 as *B. pumilis* (two by inference) and one as *Proteus vulgaris*. Phylogenetic analysis of 16S rRNA sequences also showed that the strains identified by 16S rRNA sequencing share high sequence similarity with other bacteria exhibiting biosurfactant-producing and antimicrobial activities (Chapter 3).

Although the original focus of this study is on *Pseudomonas*, results have highlighted the importance of other bacterial species as biosurfactant-producing food spoilage bacteria, primarily *B. pumilis*. *B. pumilis* is a well-documented food spoilage bacterium (Jay, 2005) and has been

reported to produce several antimicrobial agents (Borowski *et al*., 1952; Bhate, 1955; Fuller, 1955; Naruse *et al*., 1990; Rosendahl and Douthwaite, 1994), some of which are also known to exhibit surface activity. As a result, this part of the study (Chapter 3) was extended to include the *B. pumilis* strains isolated in the previous part of the study, Chapter 2. *Proteus vulgaris* is also known to be involved in food spoilage, particularly that of eggs (Ayres and Taylor, 1956) and fish (Shalaby, 1997), and given that it has not been reported to produce biosurfactants, unless when isolated from oil-contaminated soil (Balogun and Fagade, 2008), or to exhibit antimicrobial activity, further investigation of one of the strains previously isolated (Chapter 2) was of interest.

The 16 strains that were shown to be active against *B. cereus* ATCC 10702 were selected for stability assays, in which the strains were subjected to treatments with protease, heat and organic solvents. Of those 16 isolates, 6 were selected for further characterisation by thin layer chromatography (TLC) in order to gain some structural information of the active compounds. The combination of antimicrobial, stability and TLC data (Fig. 3.1) suggest that the majority of compounds produced by the strains isolated in this study produce cyclic lipopeptides (CLPs), with few exceptions. Additionally, some of the putative CLPs may be novel.

One of the *P. fluorescens* strains isolated from butter lettuce (228C) is suspected to produce a rhamnolipid-like molecule (Fujita *et al*., 1988; Pearson *et al*., 1997). As mentioned, antimicrobial activity for *P. fulva* had not been documented before the present study; however, given that no stability or TLC data was obtained for this strain, it is not possible to speculate whether the molecule produced by the strain is rhamnolipid-like or a CLP, like the putisolvins produced by *P. putida* PCL 1445 (Kuiper *et al*., 2004). The incongruent stability and TLC results obtained for the *P. cichorii* and *P. tolaasii* strains could be a result of phenotypic variation of compounds previously reported (Nair and Fahy, 1973; Shirata *et al*., 1981) or could reflect the production of at least two different compounds for each of the strains. The majority of the *B. pumilis* strains are suspected to produce surfactin-like CLPs (Naruse *et al*., 1990). Structural characterisation of the compounds would be required in order to definitively identify the compounds.

Utilisation of *n*-hexadecane and motor oil by biosurfactant-producing *Pseudomonas*, and other bacterial strains, isolated from food

A previous chapter of this exploratory study, Chapter 2, demonstrated that bacterial strains isolated from spoiled food products were able to produce biosurfactants. In Chapter 4, the ability of 8 biosurfactant-producing strains (3 *Pseudomonas*, 1 *Proteus* and 4 *B. pumilis*) to grow in minimal media supplemented with either *n*-hexadecane (MMH) or mineral motor oil (MMMO) was evaluated.

It was found that all 8 of the individual biosurfactant-producing strains and the two consortia grew well in minimal media supplemented with either hydrocarbon, reaching maximum cell numbers of *ca.* 7 – 9 log CFU/ml (Figs. 4.1, 4.2). However, no significant differences ($P<0.05$) were observed between individual Gram-negative or Gram-positive strains inoculated into either medium. Similarly, in terms of growth rates, the Gram-negative strains and consortium grew at significantly lower ($P<0.05$) rates in MMMO than in MMH, and when compared with the Gram-positive strains and consortium. *Pseudomonas fulva* 381C grew at a particularly low rate when grown in MMMO. Generally, the growth of individual *Pseudomonas* and *Bacillus* strains in hydrocarbons corresponded with or was higher than that of other strains investigated in similar studies. Our *P. fluorescens* 100T grew to higher cell numbers than an indigenous strain of *P. fluorescens* grown in *n*-hexadecane, as reported by Barathi and Vasudevan (2001). Similarly, the maximum cell numbers achieved by the *B. pumilis* strains isolated in this study were equal to or *ca.* 2 log CFU/ml higher than those observed in other studies investigating the growth of *B. pumilis* in other hydrocarbons (Calvo *et al*., 2004; Toledo *et al*., 2006). The consortia did not achieve significantly higher ($P<0.05$) cell numbers or grow better than individually inoculated strains in either medium (Figs 4.1, 4.2). The negative control achieved lower counts and growth rates, and population numbers were found to decline when grown in MMMO.

These results highlight the variation in the ability of different species and strains within a particular species to utilise certain hydrophobic substrates. Additionally, they demonstrate that strains isolated from food matrices are able to grow using hydrocarbon substrates at a rate that is comparable to strains isolated from contaminated sites. An interesting observation is that biosurfactants produced by strains growing on hydrocarbon substrates typically tend to be

glycolipid biosurfactants; however, the significance of this remains to be determined. In addition to the first recorded incidence of antibacterial activity for *P. fulva*, this study is also the first to document hydrocarbon and *n*-alkane utilisation by strains of *P. fulva* and *Proteus vulgaris*, respectively.

Our results, together with phylogenetic analyses of antimicrobial biosurfactants isolated in this study (Chapter 3), demonstrated that many of the antimicrobial biosurfactants shared high (>97%) sequence similarity with other strains shown to produce biosurfactants and exhibit antimicrobial and biodegradative activities. Although groups of biosurfactants have different structures and are produced by various microorganisms, data demonstrates that biosurfactants are convergent in function – even when those compounds are isolated from varying and unrelated matrices such as food.

Conclusion

In this study, it was shown that biosurfactant-producing *Pseudomonas* (and other) strains were isolated from spoiled foods at a frequency that motivates the further investigation of this novel source of biosurfactant-producing bacteria. Future work based on this study could include determining the prevalence of biosurfactant-producing bacteria in fresh and subsequently spoiled food products. This would allow for the determination of whether fresh or spoiled food products yield higher numbers of biosurfactant-producing bacteria. It could also provide evidence, supporting or opposing, for the attachment role of biosurfactants in food products.

It was demonstrated that the biosurfactant-producing bacteria isolated in this study exhibited other activities of note. Over 50% of those biosurfactant-producing isolates exhibited a degree of antimicrobial activity against Gram-positive bacteria of medical and food safety relevance. Future studies investigating the antimicrobial activity of bacterial isolates should incorporate the screening of more target microorganisms as it would expand the known antimicrobial spectrum of these compounds. Additionally, high performance liquid chromatography (HPLC) or nuclear magnetic resonance (NMR) imaging would facilitate the determination of the structure and subsequent accurate identification of the compounds of interest. In the case of putatively novel

compounds such as those speculated to have been produced in this study, such techniques would be invaluable.

Results showed that the strains pre-selected for the growth assay on 2 different hydrocarbon substrates were able to reach cell numbers in excess of 7 log CFU/ml. Given that no other parameters were measured, the fate of the hydrocarbons is unknown. In future, parameters such as biosurfactant production and hydrocarbon degradation should be determined when evaluating the utilisation of hydrocarbon substrates by biosurfactant-producing bacteria. Such data obtained, by, for example, HPLC or gas chromatography would allow the establishment of any correlations between biosurfactant production and hydrocarbon degradation, and to determine the fate of the hydrocarbon substrates. The data could also facilitate better comparisons of the growth of strains isolated in this study between those isolated from conventional, i.e. pollutant-contaminated, sources.

This study showed that food was a novel source of biosurfactant-producing bacterial strains, including *Pseudomonas* and *Bacillus* species, and that these isolates may also produce novel antimicrobial compounds, and be useful for the bioremediation of organic pollutants, advocating for the further characterisation of these molecules identified in this study.

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