Università degli Studi di Milano – Bicocca
Dottorato di Ricerca in Scienze Ambientali – XX ciclo

Surface active compounds by *Gordonia* and their applications in environmental remediation

Tesi finale di:

Andrea Franzetti – Matricola R00627

Coordinatore: Prof. Demetrio Pitea
Tutor: Prof. Giuseppina Bestetti

SETTORE SCIENTIFICO-DISCIPLINARE:
BIO/19 MICROBIOLOGIA GENERALE

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To survive on the earth, human beings require the stable, continuing existence of suitable environment. Yet the evidence is overwhelming that the way in which we now live on the earth is driving its thin, life-supporting skin, and ourselves with it, to destruction. To understand this calamity we need to begin with a close look at the nature of the environment itself. Most of us find this a difficult thing to do, for there is a kind of ambiguity in our relation to the environment. Biologically, human beings participate in the environmental system as subsidiary parts of the whole. Yet the human society is designed to exploit the environment as a whole, to produce wealth. The paradoxical role we play in the natural environment – at once participant and exploiter- distorts our perception of it.

Berry Commoner - Ecologist, The Closing Circle (1972)

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CHAPTER 3. FEASIBILITY STUDIES FOR APPLICATION OF GORDONIA SP. BS29 IN ENVIRONMENTAL REMEDIATION

"In the year of 1657 I discovered very small living creatures in rain water."

Antonie van Leeuwenhoek (1632 – 1723) - First microbiologist

CHAPTER 1. INTRODUCTION

The word "surfactant" is a contraction of the descriptive phrase surface-active agents to indicate compounds which concentrate and alter the conditions prevailing at interfacial surfaces (air-water, oil-water, solid-liquid). Surfactants find application in almost every industrial sector as emulsifying and wetting agents (Desai and Banat, 1997). Most of the surfactants available on the market are chemically synthesised from petroleum (Banat et al., 2000).

Many prokaryotic and eukaryotic microorganisms synthesise a wide variety of structurally different amphiphilic molecules and produce both extracellular and cell-bound compounds (Ron and Rosenberg, 1999). Neu (1996) divided microbial surface-active compounds (SACs) into low molecular weight SACs, also termed biosurfactants, and high molecular weight SACs including amphiphilic and polyphilic polymers. The former lower surface and interfacial tensions, whereas the later are usually more effective in stabilizing oil-in-water emulsions, and thus they are known as bioemulsans (Rosenberg and Ron, 1997).

Many SACs exhibit activities equivalent to synthetic surfactants but they have the advantage of lower toxicity, higher biodegradability and the property of being produced from renewable and inexpensive substrates (Makkar and Cameotra, 2002). These characteristics result in greater acceptability, especially in applications that cause the dispersion of SACs in the environment. In recent years, both low and high molecular weight SACs have been evaluated as substitutes for chemically synthesised surfactants to improve the rate of oil and heavy metals removal in soil and water remediation technologies. They find potential application within chemico-physical technologies, such as "in situ soil flushing" and "ex situ

soil washing" for remediation of the unsaturated zone, "pump and treat" technology for aquifer remediation (Barkay et al., 1999), and also in bioremediation to improve the biodegradation rate of organic compounds (Rosenberg and Ron, 1997; Mulligan, 2005). A wide range of other different potential commercial exploitations have been described in the oil industry (such as microbial enhanced oil recovery, oil transportation and tank cleaning), as well as in medicine, cosmetic and food industries (Rosenberg and Ron, 1997; Banat et al., 2000).

Thus, the selection and characterisation of new SAC-producing microorganisms is actively underway.

However, economy is often the bottleneck of biotechnological process, and this is also the case of the biosurfactant production. The production economy of every microbial metabolite is governed by three basic factors: (1) initial raw material costs, (2) availability of suitable and economic production and recovery procedures, (3) product yields of the producer microorganisms. Different strategies have been adopted to make the process cost-competitive: (1) use of cheap and waste substrates (2) development of efficient bioprocesses, including optimization of fermentative condition and recovery process, (3) development of overproducing strains (Mukherjee et al 2006). However, a systematic and effective combination of these strategies has not been adopted up to now. Thus, the prices of microbial surfactants are not competitive with those of the chemical ones and these products have not been commercialized extensively.

For these reasons the aims of this research project were (1) to isolate new SAC producing microorganisms (2) to characterize the properties of the biosynthesized molecules and their conditions of production, (3) to evaluate the potentialities of the isolate and the produced SACs in environmental restoration technologies and (4) to optimize, at laboratory scale, the cultural conditions for the SACs production.

This thesis reports the results of an experimentation carried out in collaboration between the Microbiology research group of the Environmental Sciences Department of the University of Milano – Bicocca and the Biomedical Science and Technology Department of the University of Cagliari. Moreover, a further collaboration between Environmental

Sciences Department of the University of Milano – Bicocca and the group of Prof. I.M. Banat and Prof. R. Marchant of the School of Biomedical Sciences of the Ulster University (Coleraine – Northern Ireland, UK) concerned the study of the ecology and the survival of thermophilic bacteria in cold soil environments. The results came out from this collaboration are here reported as well.

1.1. SUMMARY OF THE THESIS

This chapter reports the outline of the results presented in the next chapters.

CHAPTER 2 deals with the isolation and the characterization of new SAC-producing strains. From a site, chronically contaminated by diesel fuel, three bacterial strains (M22, BS25 and BS29) belonging to the *Gordonia* genus were isolated. Results showed that those *Gordonia* strains were able to grow using a wide range of straight and branched aliphatic hydrocarbons as carbon and energy source and to produce at least two classes of surface-active compounds. Emulsifying agent(s) was released in the culture medium when bacteria grew both on hydrocarbons and water-soluble substrates. Cell-bound biosurfactant(s), that reduce the surface tension, was produced on hydrocarbons; however, its production was significantly lower on water soluble substrates. Solvent extraction and Thin Layer Chromatography analyses allowed us to classify the cell-bound biosurfactants as glycolipids and to confirm that the chemical nature of the extracellular bioemulsans is different. The relation among growth phase, surface-active compound production and cell-surface properties was analysed in kinetic experiments on hydrocarbons. *Gordonia* sp. BS29 synthesised, and released extracellularly, bioemulsan(s) during the exponential phase with *n*-hexadecane as carbon and energy source. The production of biosurfactant(s) started in the exponential phase and its concentration increased during the following linear growth. Furthermore, the adhesion of bacterial cells to hydrocarbons decreased during growth. Our results let to hypothesize a change in the mode by which *Gordonia* cells access the substrate during growth on hydrocarbons.

The results reported in **CHAPTER 3** deals with the evaluation of the application potentialities of the isolates and the biosynthesised SACs in

environmental restoration technologies. Microbial amphiphiles and their producing strains can be use both as enhancers of biodegradation rate in bioremediation technologies of hydrocarbon contaminated soil and chemico-physical technologies of soil washing. The applicability of our strains and SACs were evaluated for the following applications: bioremediation of (i) aliphatic and (ii) aromatic hydrocarbon contaminated soil and (iii) washing of soil contaminated by crude oil. Microcosm bioremediation experiments were carried out and the effect of BS29 and M22 bioaugmentation and the bioemulsans on biodegradation rate and extent were evaluated also in comparison to the effect of the rhamnolipids, biosurfactants known for their capacities of enhancing bioremediation. Over time, the humidity was kept constant (25%) and the analyses of residual hydrocarbons, total microorganisms and hydrocarbon-degrading microorganisms were carried out. Hydrocarbon biodegradation data were analysed by Analysis of Variance (ANOVA) to compare the tested conditions with the control without any addition. The dependent variables for the comparison were the biodegradation rate and the residual concentration of each contaminant at the end of the experiments. The bioremediation results showed that the bioemulsan is able to effectively reduce final concentration of recalcitrant branched hydrocarbons even not at the same extent of rhamnolipids.

Batch soil washing experiments were carried and we compared the effect of the BS29 bioemulsans with the ones of the sole water and a mixture of rhamnolipids (biosurfactant already experimented and used in soil washing techniques) on the removal of the pollutants from soil. Experimental Design Techniques were utilised for an efficient experiment planning and to evaluate the effect of the operational parameters on pollutants removal. The investigated factors were (i) the time of washing and (ii) the ratio between the volume of washing solution and the weight of soil. The use of a crude solution of the BS29 bioemulsans in different conditions resulted in a mean of crude oil removal of 43.1%, while the mean removal in rhamnolipid experiments was 50.8%. ANOVA test showed that both of them are significantly different (α=0.05) from the mean of sole water removal (19.5%). Furthermore, in bioemulsan experiments, a significant effect of time of washing was observed with a positive correlation with the extent of the oil removal. On the contrary, in the tested experimental conditions,

neither the solution:soil ratio nor the time of washing significantly affects the removal in rhamnolipids experiments.

The results obtained from the optimisation procedure for the maximisation of biosurfactant concentration in cultural broth are reported in **CHAPTER 4**. The optimisation process involved three consecutive steps. In the first step a two level $2^{(8-2)}$ Fractional Factorial Design (FFD) was used to identify cultural factors that have a significant influence on biosurfactant(s) biosynthesis. Concentrations of inorganic phosphorous and sodium chloride were found as most important factors affecting yield of biosurfactant biosynthesis Then, on the selected factors, a steepest ascent procedure and a Central Composite Design (CCD) were applied to obtain a second order polynomial function which fitted the experimental data in the vicinity of the optimum. The factors taken into account were inorganic nutrients, such as phosphorous, ammonium and micronutrients, the carbon sources and the time of growth. With the optimised broth composition we obtained a more than 16-fold increase in the biosurfactant concentration compared to the normal BH broth, reaching a Critical Micelle Diluton (CMD) value (129.43 (95% confidence interval: 119.47 – 139.40)) among the highest in literature. In **CHAPTER 5** the whole conclusions of the project are drawn and the perspectives for the future are put forward. **CHAPTER 6** presents the results of the studies dealing with the ecology and the survival of thermophilic bacteria in cold soil environments. Population analysis of thermophiles in rainwater and air samples has shown distinct differences supporting the view that long distance global transport and deposition in rainwater are a possible source of replenishment of the soil thermophile populations.

Discovery consists of seeing what everybody has seen and thinking what nobody has thought.

Albert von Szent-Gyorgyi (1893 - 1986) –Nobel prize in Medicine

CHAPTER 2. ISOLATION AND CHARACTERIZATION OF GORDONIA SP. STRAINS ABLE TO SYNTHESISE SURFACE-ACTIVE COMPOUNDS

2.1. INTRODUCTION

2.1.1. Microbial amphiphiles

2.1.1.1. Low- and high-molecular-weight biosurfactants

Microbial amphiphiles can be classified as low- and high-molecular-weight biosurfactants (Ron and Rosemberg, 2001). The former, significantly, reduce interfacial and surface tensions, the latter, since they are very efficient in stabilizing oil/water emulsion, are also called bioemulsans (Neu, 1996).

The low-molecular-weight biosurfactants are, generally, glycolipids or lipopeptides. Glycolipids are synthesized by a wide variety of microorganisms. *Rhodococcus erythropolis* produces cell-bond trehalose dimycolates (Lang and Philp, 1998), several species of *Pseudomonas* produce rhamnolipids (Lang, 2002) while different species of the yeast *Candida* produce extracellular sophorolipids (Rosenberg and Ron, 1999). The best-studied glycolipids, rhamnolipids, trehalolipids and sophorolipids, are disaccharides that are acylated with long-chain fatty acids or hydroxy fatty acids.

The surfactin is a cyclic lipopeptide produced by *Bacillus subtilis* which has been reported to be the most active biosurfactant (Arima et al. 1968). The surfactin has a CMD in water of 25 mg/l and can reduce the surface tension to 27 mN/m. It shows, also, potent antibacterial activities. The structure consists in a peptide cycle of seven amino acids linked to a fatty acid chain (Ron and Rosenberg, 1999).

Different bacterial genera produce exocellular polymeric surfactants composed of polysaccharides, proteins, lipopolysaccharides, lipoproteins or complex mixtures of these biopolymers. Rosenberg and Ron (1998), extensively, studied the production of the emulsan RAG-1 by different species of *Acinetobacter*. RAG-1 emulsan is a complex of an anionic heteropolysaccharide and protein (Rosenberg and Kaplan, 1987). Its surface activity is due to the presence of fatty acids, comprising 15% of the emulsan dry weight, which are attached to the polysaccharide backbone via O-ester and N-acyl linkages (Belsky et al. 1979). Another bioemulsan, Alasan, produced by a strain of *Acinetobacter radioresistens*, is a complex of an anionic polysaccharide and protein with a molecular mass of approximately 1 MDa (Navon-Venezia et al. 1995).

2.1.1.2. Natural role

The debate on the natural roles of microbial amphiphiles is currently underway. Since they have different structures, are produced by a different microorganisms and exhibit different surface properties, thus several different roles have been proposed. At this stage it is impossible to make any generalization or identify one or more common roles to all microbial surfactants (Ron and Rosenberg, 2001). Microbial surfactants can mediate and facilitate the growth of bacteria on hydrocarbons both increasing the surface area between oil and water by emulsification and increasing pseudosolubility of hydrocarbons by partition into micelles (Volkering et al., 1997, Miller and Zhang, 1997). However, there are some conceptual difficulties in understanding the evolutionary advantages of producing extracellular emulsifying agents, since it is impossible to obtain an oil/water emulsion available only for emulsifier-producing strain in open system.

Rhamnolipids have been shown to reduce cadmium toxicity probably by complexation of cadmium and rhamnolipids interaction with cells (Sandrin et al., 2000) while Alanas can efficiently bind uranium (Zosim et al., 1983). Moreover, rhamnolipids are considered to be regulated and correlated with the virulence factors of *Pseudomonas aeruginosa* (Olvera et al., 1999).

As already known for surfactin, several lipopeptide surfactants are potent antibiotics. These include also the extracellular hydrophobic peptide, streptofacin by *Streptomyces tandae* (Richter et al., 1999). The involvement of

the surfactin in sporulation of *B. subtilis* has been also proposed (Grossman, 1995).

Moreover, roles in regulating bacterial attachment to surface have been supposed. Due to the amphiphilic nature of the microbial surfactants, they can increase or decrease the hyrophobicity of the bacterial cell exposing the hydrophobic moieties outside or inside of the cells. When excreted, they can alter interface properties allowing or inhibiting the attachment to surfaces.

2.1.2. The genus *Gordonia*

2.1.2.1. Taxonomy

In 1971, Tsukamura proposed *Gordona* as a new genus for coryneform bacteria isolated from sputa of patients with pulmonary disease or soil (Tsukamura, 1971). Members of this genus are distinguished from fast-growing mycobacteria by their slight acid fastness and the absence of arylsulfatase, while they are distinguished from the genus *Nocardia* by their ability to reduce nitrate and the absence of a mycelium. Six years later, Goodfellow and Alderson discarded this taxon and transferred *Mycobacterium rhodochrous* and the "rhodochrous complex," including representatives of the genus *Gordona*, to the genus *Rhodococcus* (Goodfellow et al., 1977). However, studies of the mycolic acid (high-molecular-weight α-branched 3-hydroxy fatty acid) and menaquinone composition revealed heterogeneous variations within the genus *Rhodococcus* (Goodfellow, 1986; Tomiyasu et al., 1986). Additional analysis of 16S rRNA similarities led to a revival of the genus *Gordona* by Stackebrandt et al. (1988) with the following species: *Gordona bronchialis*, *Gordona rubripertincta* [formerly *Gordona rubra*], *Gordona terrae* and, additionally, *Rhodococcus sputi* (Tsukamura, 1978) as *Gordona sputi*. In 1997, the etymologically correct name *Gordonia* instead of *Gordona* was proposed by Stackebrandt et al. (1997). According to their newly proposed hiercharchic classification system for the actinomycete line, *Gordonia* is the type genus of *Gordoniaceae* (the *Gordonia* family) within the suborder *Corynebacterineae*. At present, the genus *Gordonia* comprises 27 validly published species. Meanwhile, gordoniae have been shown to be ubiquitously distributed in nature.

2.1.2.2. Biodegradation and bioremediation

Members of *Gordonia* are important in bioremediation processes due to their capacity to degrade hydrocarbons, toxic widespread environmental pollutants, xenobiotics, and natural compounds which are not readily biodegradable. *Gordonia* are known to be deeply involved in wastewater treatment and in biofilters (Berdinger et al. 1993). Several *Gordonia* strains, able to transform and/or degrade aliphatic and aromatic hydrocarbons with different substituent and different degree of branching, have been isolated. The incorporation of the long aliphatic chains of the mycolic acids into the cell wall is associated with hydrophobicity and surface adhesion (Pagilla et al., 2002) and it may play a role in the degradation of hydrophobic pollutants. Several species of the genus *Gordonia* strains are described as able to growth on the surface of rubber materials (Arenskötter et al., 2001) and utilise hydrophobic hydrocarbons. Chatterjee and Dutta (2003) described *Gordonia* sp. strain MTCC 4818, isolated from creosote-contaminated soil, which was able to metabolize several phthalic acid esters as sole sources of carbon and energy. Phthalic acid esters exhibit hormonal action due to their binding activity toward the estrogen receptor (Nakai et al., 1999) and can cause proliferation of MCF-7 cells (Soto et al.,1995). Obviously, this strain was able to hydrolyze both ester bonds of butyl benzyl phthalate and utilized the released benzyl alcohol and butanol for growth. The esterase activity was shown to be induced by butyl benzyl phthalate and/or monobenzyl phthalate, indicating a specificity of the enzyme for this xenobiotic. Surprisingly, *Gordonia* sp. strain MTCC 4818 was unable to metabolize the phthalic acid, which, therefore, accumulated as a dead-end product in the culture broth.

2.1.2.3. Anabolism

Gordonia species also synthesised a variety of compounds which are useful for various applications. The production of surface active compounds has been, often, reported connected to the capability of biodegrading unsoluble compounds (Banat et al., 2000). Biosurfactant production by *G. amarae* is the best studied up to now since this phenomenon is associated with foaming events in activated sludge in wastewater treatment plants (De los Reyes, 1998)

Many *Gordonia* isolates present red pigmented colonies as index of capability of synthesising carotenoids. This ability is shared with a large group of gram-positive bacteria, including species of the genera *Corynebacterium, Flavobacterium, Micrococcus,* and *Mycobacterium* (Jagannadham et al., 1996). A cell component responsible for the degree of hydrophobicity of the cell surface was characterised (Moormann et al., 1997). This molecule, called Gordonin, belongs to the class of glycosylated peptidolipids. Gordonin is able to confer a hydrophilic cell surface by exposing the hydrophilic head of the molecule to the exterior of the cell. The authors also reported that gordonin could act as a surfactant, but the extracellular release of the product has not been demonstrated.

2.2. RESULTS

In this chapter, the isolation of three new hydrocarbon-degrading *Gordonia* strains is described. The aim of the present study is to gain more insights into the physiology of SACs in microorganisms belonging to *Gordonia* genus; therefore, the catabolic abilities of selected strains and the relationship among SAC production, phase of growth and cell-surface properties were investigated and a change in the hydrocarbon accession mode was proposed.

2.2.1. Bacteria isolation and 16S rRNA gene analysis

Two strains (BS25 and BS29) were isolated from diesel-contaminated capillary fringe soil samples, whereas another strain (M22) was isolated from a NAPL sample recovered from a groundwater. All the strains were aerobic, Gram-positive, catalase-positive, and nonmotile. On TSA medium, M22 grew forming red-pigmented, rough colonies. BS25 and BS29 formed red-pigmented, smooth colonies with shiny surface; however, also rough colonies with irregular margins were occasionally observed.

The almost complete sequence of 16S rRNA gene of each strain was determined and compared with the Ribosomal Database Project II (Cole et al., 2005). This analysis assigned all isolates to the *Gordonia* genus belonging to the *Corynebacterineae* suborder, the mycolic acid-containing group within the *Actinomycetales* order (Stackebrandt et al., 1997).

The 16S rRNA gene sequences of the three isolates and that of the type strains of all *Gordonia* species, validly described, were used to construct a phylogenetic tree (Figure 2.2-1). BS25 and BS29 strains were phylogenetically closely related to *G. terrae*^{T} (99.8% similarity), whereas M22 strain was closely related (100.0% similarity) to *G. amicalis*^{T}.

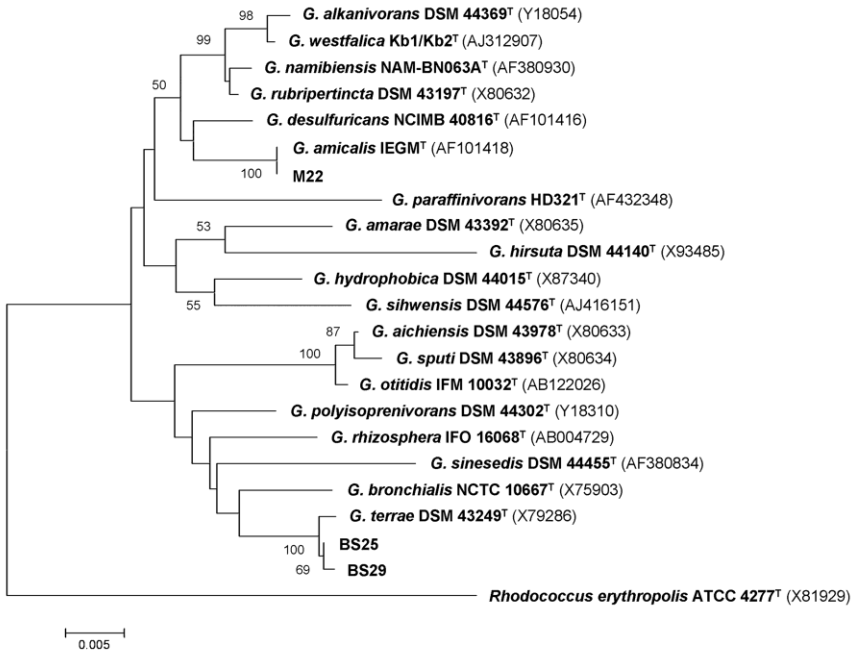

***Figure* 2.2-1** *Unrooted phylogenetic tree based on 16S rRNA gene comparison. The positions of M22 (EF064794), BS25 (EF064795), BS29 (EF064796) strains and the type strains of Gordonia species are shown. Bootstrap probability values that were less than 50% were omitted from the figure. The scale bar indicates substitutions per nucleotide position. The GenBank accession numbers are reported in parenthesis.*

2.2.2. Catabolic abilities

The ability of each *Gordonia* strain to grow using different hydrocarbons as carbon and energy sources was tested. The isolates were able to grow using a wide range of straight hydrocarbons (n-C10-28), branched aliphatic hydrocarbons (pristane) and diesel. However, the isolates did not show a significant biomass increase on n-hexane, cyclohexane, naphthalene, 1-methyl- and 2-methyl-naphthalenes, and phenanthrene after seven days.

Biodegradation kinetics was also carried out to better evaluate the hydrocarbon degradation potential of the selected strains. n-heptadecane, pristane and squalene were chosen as middle-range hydrocarbons. These compounds cover a wide range of molecular weight and different degrees of branching. Figure 2.2-2 shows, as examples, data from kinetic experiments with *Gordonia* sp. BS29 on n-heptadecane. In liquid cultures, all selected strains were able to completely degrade n-heptadecane and pristane within one week. They also degraded squalene, but about 30% of the initial hydrocarbon, still remained after seven days.

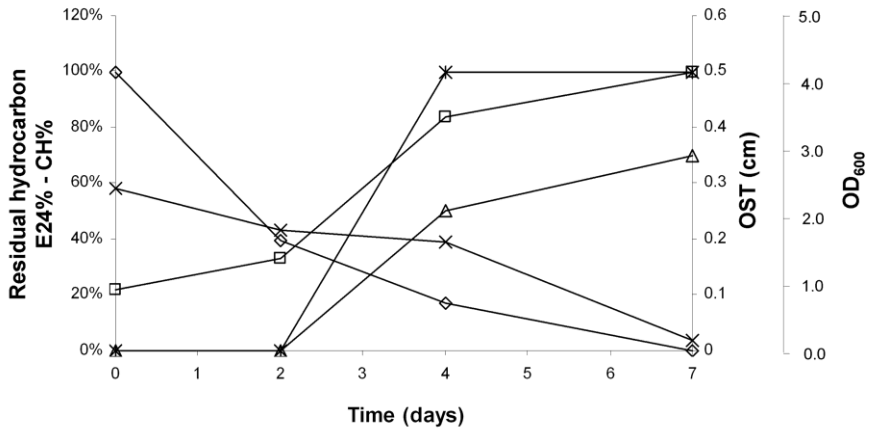

Figure 2.2-2 *Biodegradation kinetics by Gordonia sp. BS29 on n-heptadecane. The hydrocarbon was supplied at initial concentration of 1.0 g/l. Residual hydrocarbon (◊), emulsification activity (E24%) (Δ), cell surface hydrophobicity (CH%) (×), diameter (cm) of clear zone as determined by oil spreading technique (OST) (*) and optical density at 600 nm (OD600) (□) in whole culture broths. Average values are presented.*

2.2.3. Production of surface-active compounds

In order to evaluate the spectrum of carbon and energy sources that the selected bacteria utilise as substrate to produce SACs, each *Gordonia* strain was grown on different hydrocarbons (n-C10-17, branched alkanes and diesel) or water-soluble substrates (alcohol, organic acids, sugars). The ability of whole culture broths (with cells) and culture filtrates (without cells) to produce a stable kerosene-water bioemulsion was tested. As shown in Table 2.2—1, high emulsification activities were detected in whole culture broths and in cell-free filtrates when the isolates were grown on

both hydrocarbons (up to 70.0%) and water-soluble substrates (up to 73.3%). The bioemulsions remained stable for more than four months.

Table 2.2—1 *Emulsification activity of whole culture broths and cell-free culture filtrates of Gordonia M22, BS25 and BS29 grown on different carbon and energy sources for 7 days at 30°C. Values are means based on two separate experiments with two independent measurements each.*

Carbon sources (20 g/l)	**E24%**					
	***Gordonia* sp. M22**		***Gordonia* sp. BS25**		***Gordonia* sp. BS29**	
	C	**F**	**C**	**F**	**C**	**F**
n-C10	9.8	35.5	48.3	70.0	43.1	64.9
n-C12	3.2	45.8	53.8	65.6	50.4	59.8
n-C16	56.3	52.5	49.5	41.5	39.6	44.0
n-C17	37.3	57.5	39.6	38.2	37.2	28.3
Pristane	44.1	68.3	57.9	61.8	57.5	63.5
Diesel	2.2	42.4	46.7	44.0	40.4	63.4
Fructose	28.3	26.5	43.3	38.4	46.3	50.4
Glucose	0.0	0.0	ND[a]	ND[a]	14.6	1.7
Sucrose	25.9	20.9	25.0	47.6	30.2	10.6
Ethanol	16.7	22.5	23.3	48.4	12.0	39.0
Sodium acetate	0.0	0.0	ND[a]	ND[a]	40.9	45.0
Potassium citrate	ND[a]	ND[a]	53.8	31.1	73.3	35.0
Sodium citrate	ND[a]	ND[a]	56.7	60.5	62.6	51.1
Palmitic acid	8.3	43.2	53.4	50.8	45.0	38.7

[a] Biomass increase was not detected.

E24%: Emulsification activity; C: Culture broth; F: Cell-free culture filtrate; ND: not determined.

Table 2.2—2 shows surface tension in the culture broths of each *Gordonia* strain (M22, BS29 and BS25) grown on different hydrocarbons or on water-soluble carbon sources. The CMD was also determined as the reciprocal of the dilution factor to achieve the critical micelle concentration. This parameter is proportional to the amount of biosurfactant produced. High biosurfactant production was observed in culture broths of isolates grown on hydrocarbons, which resulted in low surface tension of water; high CMD values were also found. When the *Gordonia* sp. BS29 was grown on n-hexadecane, the surface tension achieved in culture broth was 30.0 mN/m

and the CMD values was 26, whereas the interfacial tension against diesel was 1.1 mN/m. Cell-free culture filtrates did not show significant reduction in surface tension.

The reduction of surface tension and CMD values were considerably lower in culture broths on water-soluble compounds than that obtained on hydrocarbons also when comparable or higher biomass increases were observed (Table 2.2—2).

Table 2.2—2 *Optical density at 600 nm, surface tension and critical micelle dilution of whole culture broths of Gordonia M22, BS25 and BS29 strains grown on different carbon and energy sources for 7 days at 30°C.*

Carbon sources	***Gordonia* sp. M22**			***Gordonia* sp. BS25**			***Gordonia* sp. BS29**		
	OD_{600}	**ST (mN/m)**	**CMD**	**OD_{600}**	**ST (mN/m)**	**CMD**	**OD_{600}**	**ST (mN/m)**	**CMD**
***n*-C16**	2.9	43.8	6	2.6	30.1	13	1.7	30.0	26
Diesel	1.5	35.8	7	2.0	32.0	7	1.4	33.5	14
Sucrose	1.1	62.3	<1	1.9	48.6	<1	1.3	53.5	<1
Fructose	0.84	49.8	<1	0.57	51.3	<1	1.7	47.1	<1
Ethanol	0.34	57.1	<1	0.26	56.0	<1	0.26	61.3	<1
Potassium citrate	0.029	ND[a]	ND[a]	3.7	51.4	<1	4.0	43.7	<1

[a] Biomass increase was not detected.

OD_{600}: Optical Density at 600 nm; ST: Surface Tension; CMD: Critical Micelle Dilution; ND: not determined.

2.2.4. Kinetics of surface-active compound production

In biodegradation kinetic experiments, parameters concerning SAC production were also measured over time in culture broths of *Gordonia* strains. Emulsification activity and the diameter of clear zone, as measured with OST, are parameters semiquantitatively related to the total amount of SACs. The relative cell surface hydrophobicity, measured as BATH, was determined as well. Figure 2 shows, as examples, the kinetics of SAC production by *Gordonia* sp. BS29 on n-heptadecane.

Gordonia sp. BS29 presented detectable concentrations of SACs in whole culture broths four days after the inoculation (E24% = 50%; OST= 0.5 cm). Considering the low initial concentration of hydrocarbons (1.0 g/l), these

data indicate a good production. Youssef et al. (2004) classified as positive results values greater than 0.5 cm when the initial concentration of carbon source was 10 g/l. In *Gordonia* sp. BS29, cell hydrophobicity decreased from 60% to 39% when SACs were found in the culture broth, and it further decreased to 5% seven days after inoculation. Similar results were obtained for the three *Gordonia* strains on each carbon source tested (n-heptadecane, pristane and squalene).

These data seem to indicate a relationship between hydrophobicity of microbial cells and SAC production. Therefore, the relationships among SAC production, phase of growth and cell surface properties were specifically addressed by a kinetic experiment carried out growing *Gordonia* sp. BS29 strain on n-hexadecane as a model compound for microbial degradation of middle molecular weight hydrocarbons. Since absorbance of cells grown on hydrocarbons was difficult to determine, due to their low buoyant density, and since the cells could not be recovered as a pellet after centrifugation, bacterial growth was monitored by total DNA quantification (Figure 2.2—1). SAC production was quantified by measuring the following parameters: i) emulsification activity in whole culture broths and cell-free filtrates, ii) surface tension and biosurfactant concentration by dilution test (CMD) of whole culture broths. The surface properties of bacterial cells during the growth on hydrocarbons were also monitored by determining the relative cell surface hydrophobicity and the Ziehl-Neelsen stain reaction. Furthermore, the distribution of microorganisms in culture broths was evaluated by CLSM.

The *Gordonia* sp. BS29 grew exponentially during the first 43 hours after inoculation in minimal medium with n-hexadecane, with a generation time of about 8 hours. This phase was followed by a linear growth. Growth phase-dependent production and release of emulsifying agents were observed and the highest emulsifying activity was detected at the end of the exponential phase both in whole culture broths and cell-free filtrates. The emulsification activity was constant during the linear growth in whole culture broths, whereas it slowly decreased in cell-free filtrates (Figure 2.2—1 A). Twenty-four hours after the inoculation the surface tension achieved in culture broths was 31.1 mN/m and remained nearly constant during the following six days, reaching the minimum value four days after inoculation (29.7 mN/m). The biosurfactant concentration, as determined

by dilution test, was lower than the critical micelle concentration during the first two days of growth. The surfactant accumulated during the linear phase and the maximum concentration was achieved after seven days (CMD = 8) (Figure 2.2—1 B).

The *Gordonia* cells showed a cell surface hydrophobicity of 58% after 24 hours-growth in LD rich medium. Furthermore, the cells released the carbol fuchsin dye upon washing with acid-alcohol (data not shown). The cell surface hydrophobicity rapidly increased after inoculation in mineral salts medium with n-hexadecane, reaching the highest value (100%) after 3 hours. Then, the cell surface hydrophobicity rapidly decreased during the exponential and linear phases and reached the lowest value in seven days (2%), when the cells showed no significant adhesion to the hydrocarbons. During the first 16 hours of exponential growth on n-hexadecane, the cells were coccoid, in small clusters and not acid-fast. In an 18 hr old culture, most of the cells were rod-shaped and few acid-fast cells were observed. The proportion of acid-fast cells increased during the next days and most of the cells were acid-fast in a 43 hr old culture, when the exponential phase ended and in a 168 hr old culture during the linear growth.

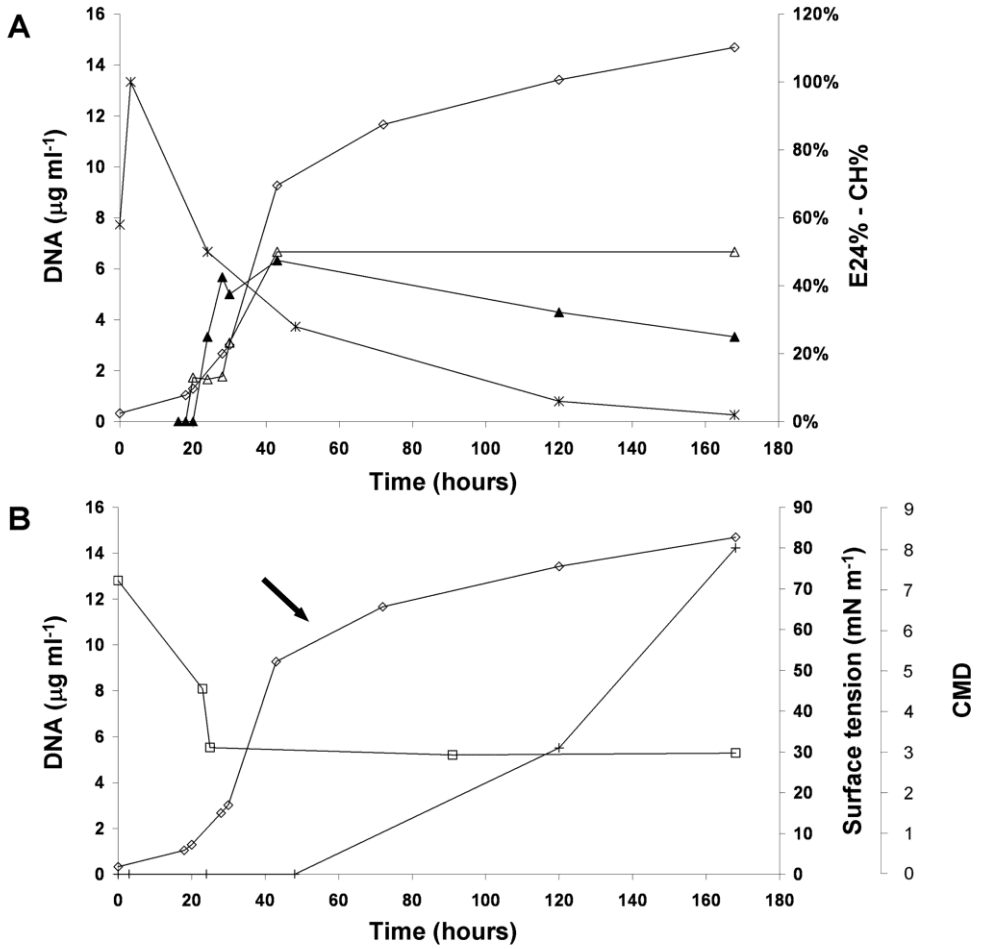

Figure 2.2—1 *Kinetics of SAC production by Gordonia sp. BS29 strain on n-hexadecane. The hydrocarbon was supplied at initial concentration of 20.0 g/l. (A) Total DNA content (◊), cell surface hydrophobicity (CH%) (*), emulsification activity (E24%) in the whole culture broth (Δ) or in the cell-free culture filtrate (▲); (B) total DNA content (◊), surface tension (mN/m) (□), critical micelle dilution (CMD) (+). Average values are presented. The arrows indicate the shift-time from exponential phase to linear growth.*

CLSM images were acquired 3 hours (Figure 2.2—2 A) and 7 days (Figure 2.2—2 B) after inoculation. Most of cells adhered to the hydrocarbon drops and only few bacteria were in the water phase three hours after the inoculation, while most of the bacteria were dispersed in the culture broth after 7 days of growth.

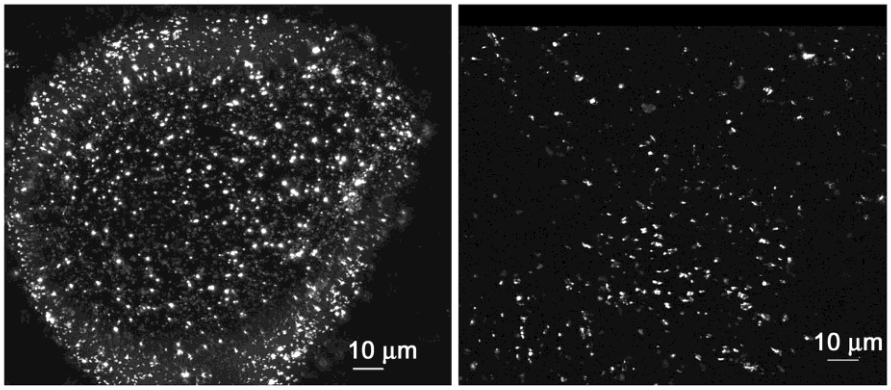

Figure 2.2 – 2 Confocal Laser Scanning Microscope images of Gordonia sp. BS29 cells grown on n-hexadecane. The hydrocarbon was supplied at initial concentration of 20.0 g/l. Images were acquired after 3 hours (A) and 7 days (B) growth at 30°C.

2.2.5. Preliminary chemical characterisation

The biomass and the cell-free filtrates of *Gordonia* sp. BS29 grown on n-hexadecane were extracted with different solvents and analysed by TLC.

The presence of biosurfactant(s) in the chloroform-methanol extracts from bacterial biomass was demonstrated by a positive OST test (> 15 cm). The TLC analysis with chloroform/methanol/water as running solvent showed the best separation of the components. Two different spots (Rf1= 0.27; Rf2 = 0.51) were detected by glycolipid specific α-naphthol staining; both of them did not show UV fluorescence. Furthermore, when the *Gordonia* sp. BS29 was grown on citrate (a substrate that did not allow biosurfactant production) no spots were detected in the chromatographs of the extracts from bacterial biomass.

The bioemulsan(s) was present in the n-butanol extracts (E24% = 83%) while no emulsification activity was detected in chloroform-methanol ones. The TLC analyses with chloroform/glacial acetic acid/methanol/water as running solvent of n-butanol extracts showed eight clear spots (Rfs from 0.07 to 0.93), both UV-fluorescent and positive to α-naphthol staining.

2.3. DISCUSSION

In this work, three new bacterial strains (M22, BS25 and BS29) belonging to the genus *Gordonia* were isolated from diesel-contaminated soil. *Gordonia*

was recently recognised as an emerging genus in industrial and environmental biotechnology (Arenskötter et al., 2004).

The isolates are able to grow using a wide range of straight and branched aliphatic hydrocarbons as carbon source. They are able to completely degrade n-heptadecane in batch cultures. Furthermore, they also show very appreciable capability of degrading pristane and squalene that, for their high degree of branching, are considered extremely recalcitrant to biodegradation and often remain in the environment as residual contaminants after bioremediation (Nocentini et al., 2000). The SACs produced by the *Gordonia* sp. strains (M22, BS25 and BS29) were studied by growing the cells on water-soluble and insoluble carbon and energy sources. The kinetics of SAC production was analysed and a change in the hydrocarbon accession mode was proposed.

2.3.1. Production of surface-active compounds

In *Corynebacterineae*, the SAC production has been investigated thoroughly in the *Rhodococcus* genus. Several members produce biosurfactants, mainly trehalose lipids, and bioemulsans when grown on water-insoluble substrates (Lang and Philp, 1998; Bredholt et al., 1998).

Very little is known about the SACs produced by the *Gordonia* members and most of the studies have been carried out on *G. amarae*. This species is commonly found in foaming-activated sludge wastewater treatment plants, where both cells and their extracellular biosurfactant participate in the formation of stable foams (Iwahori et al., 2001; Pagilla et al., 2002). A SAC-producer *Gordonia* strain has been recently isolated from an oil field (Nazina et al., 2003).

The three *Gordonia* strains, described in this work, produce at least two different types of SACs. Extracellular bioemulsan(s) (able to produce stable emulsions, but not to reduce surface tension) is synthesised on both hydrocarbons and water-soluble carbon sources, whereas cell-bound biosurfactant(s) (able to reduce surface tension) is produced only on hydrocarbons. Furthermore, the two products show different production kinetics. The release of emulsifying agents in mycolic acid-containing actinomycetes, grown on hydrocarbons, has been previously attributed to the extraction of amphiphilic compounds from the cell surface by

hydrocarbons acting as hydrophobic solvents (Duvnjak and Kosaric, 1985; Bredholt et al., 1998). The *Gordonia* strains release extracellular emulsifying agent(s) in the culture medium when grown both on insoluble and water-soluble substrates; thus, a different releasing mechanism should be supposed.

The preliminary chemical characterisation allows us to suppose that *Gordonia* sp. BS29 incorporates biosurfactants belonging to glycolipid class in its cellular envelops, as extensively demonstrated in *Rhodococcus* members (Lang and Philp, 1998). The bioemulsans are more polar compounds than the biosurfactants since the former are extracted by n-butanol while the latter are extracted with chloroform-methanol. Furthermore, the α-naphthol staining and the UV fluorescence suggest the presence of sugar moieties and chromophores (such as aromatic moieties) in the bioemulsan molecules. The preliminary chemical characterisation confirms that the extracellular bioemulsans and the cell-bound biosurfactants are structurally different compounds.

To the best of our knowledge, the biosurfactants and the emulsifying agents synthesised by the *Gordonia* sp. M22, BS25 and BS29 strains are the most effective compounds currently identified in *Gordonia* members.

2.3.2. Cell surface properties and cell-hydrocarbon interaction

In order to gain insight into the mode by which microorganisms belonging to the *Gordonia* genus access hydrocarbons, the cell surface properties and SAC production were investigated during growth on hydrocarbons as carbon and energy sources.

On n-hexadecane, *Gordonia* sp. BS29 exhibits a complex growth curve characterised by an exponential growth phase followed by a linear one. Similar curves have been previously described also in other bacteria grown on aliphatic and polycyclic hydrocarbons (Ron and Rosenberg, 2001; Bouchez-Naitali et al., 1999, 2001; Wick et al., 2001). In both cases, the biomass increases arithmetically when access to hydrocarbons is limited by a mass transfer.

In mycolic acid-containing actinomycetes, mycolic acids are thought to be located in the outer layer of the cell wall, where they form the basis of an outer lipid permeability barrier. *Gordonia* species are generally described as

slightly acid-fast. On n-hexadecane, the *Gordonia* sp. BS29 cells are not acid-fast during the early exponential phase, whereas most of the cells retain the basic fuchsin, after alcohol-acid destaining, at the end of exponential growth and during linear phase. Thus, changes in cell permeability properties occur during growth on hydrocarbons. On the contrary, *R. erythropolis* E1 cells result acid-fast at any time during cultivation on pristane (Sokolovska et al., 2003).

Gordonia sp. BS29 cells, grown in rich medium, show a moderate cell surface hydrophobicity. After switch to mineral medium with hydrocarbons, the BS29 cell surface hydrophobicity rapidly increases resulting in cell adhesion to hydrocarbon drops, as demonstrated both by CLSM analysis and by visual inspection of the cultures. This "adhesion phase" has already been described in *Rhodococcus* sp. strain 094 (Bredholt et al., 1998). Interestingly, the BS29 cells develop a hydrophilic surface during growth on hydrocarbons and, at the same time, they disperse rendering the culture turbid. The hydrophilic surface may be due to surface amphiphiles which expose their hydrophilic portion toward the medium masking the highly hydrophobic character of the mycolic acid layer, as previously described both in *Corynebacterineae* (Moormann et al., 1997 ; Sutcliff, 1998). Thus, *Gordonia* sp. BS29 shows a complex change in cell surface properties during growth on hydrocarbons. Our results are thus consistent with the hypothesis that microorganisms can use SACs to regulate their cell surface properties in order to attach and detach from surfaces, such as hydrocarbons (Ron and Rosenberg, 2001).

Several strategies are used by bacteria to overcome the low solubility of n-alkanes and enhance their transport (Van Hamme at al., 2003; Bouchez-Naitali et al., 1999). The major mechanism in rhodococci is likely to be direct contact of hydrophobic cells with large oil drops (Lang and Philp, 1998; Whyte et al., 1998). On the contrary, *Pseudomonas* strains release their surfactants in culture broth enhancing hydrocarbons apparent solubility. Their hydrophilic surface allows cells to interact with hydrophilic outer layer of hydrocarbon-containing micelles (Van Hamme and Ward, 2001).

Overall, our results allow us to hypothesize a change in the mode of substrate accession during growth on hydrocarbons of *Gordonia* cells. To the best of our knowledge, this behaviour has never been described in

literature so far. During early exponential phase, *Gordonia* strains present high values of cellular surface hydrophobicity and remain attached to large hydrocarbon drops showing a direct access to hydrocarbons; during the late exponential phase of growth, the cells become hydrophilic, thus they completely spread in the cultures and interact with pseudosolubilised and/or emulsified dispersed hydrocarbons. The SAC-induced hydrophilic surface allows the microorganisms to attach the hydrophilic outer layer of the emulsion, stabilised by the bioemulsan(s), and to interact with hydrocarbons. As recently stated by Ron and Rosenberg (2001), there are conceptual difficulties in understanding the evolutionary advantages of producing extracellular emulsifying agents, since it is impossible to obtain an oil emulsion available only for emulsifier-producing strain in open system. However, if the emulsifier-producing strains had evolved also a specific mechanism of interaction with the microemulsion, thus these microorganisms could take advantage of the emulsion over the other microbial populations.

2.4. EXPERIMENTAL PROCEDURES

2.4.1. Sampling, enrichment and isolation of hydrocarbon degraders

Capillary fringe soil samples were obtained from a site chronically contaminated by diesel. Non-aqueous phase liquid (NAPL) samples were collected from the underlying groundwater. An aliquot of NAPL was sterilised by filtration. The recovery of soil bacteria was performed by suspending 2.0 g of capillary fringe soil sample in 18 ml of sterile 0.8% saline solution and vigorously shaking the mixture for 2 min.

Selective enrichment cultures were prepared in Bushnell-Haas broth (Difco) and the sterile NAPL hydrocarbons were added as the only carbon source. Alternatively, the enrichment cultures were prepared using the non-sterile NAPL both as *inoculum* and carbon source. NAPL hydrocarbons were added at concentration equal to 20.0 g/l (w/v). Samples of the enrichment cultures were plated onto Bushnell-Haas medium solidified with agar, and supplemented with 20 g/l of sterile NAPL. The medium was vigorously shaken for 2 min to emulsify the oil prior to pour the plates.

Colonies were obtained in pure culture by repeated streaks on Bushnell-Haas agar plates. The carbon source was offered as a vapour phase by placing a filter disc with 90 mg of sterile NAPL in the lid of each plate and wrapping the plates in Parafilm.

2.4.2. 16S rRNA gene sequence analysis

DNA purification and PCR reactions were carried out as previously described (Tamburini et al., 2003). The determination of 16S rRNA gene nucleotide sequences was performed with a Perkin-Elmer ABI 310 sequence analyser. The 16S rRNA gene sequences were compared with the prokaryotic small subunit rRNA sequence database of the Ribosomal Database Project II (Cole et al., 2005). The 16S rRNA gene sequences of the isolates and the related sequences (retrieved from Ribosomal Database Project II database) were aligned with the MULTALIN software (Corpet, 1988). The resulting alignments were checked manually and corrected if necessary. Phylogenetic trees were inferred using the neighbour-joining method and the software MEGA version 3.1 (Saitou and Nei, 1987; Kumar et al., 2004). Bootstrap analysis (1000 replicates) was used to test the topology of the neighbour-joining method data.

2.4.3. Growth conditions and culture preparation

Liquid cultures were prepared in LD (per litre): yeast extract 5 g, tryptone 10 g, NaCl 5 g. The cells were removed by centrifugation, washed twice and suspended in M1 medium (per litre): K_2HPO_4 1.32 g, KH_2PO_4 1 g, NH_4Cl 0.81 g, $NaNO_3$ 0.84 g, $FeSO_4 \cdot 7H_2O$ 0.01 g, $MgSO_4$ 0.20 g, $CaCl_2$ 0.02 g). Cultures were grown at 30°C in a rotary shaker at 250 rpm.

2.4.4. SAC production

The cultures were prepared in 100 mlErlenmeyer flasks containing 20 mlM1 medium and inoculated to an initial optical density at 600 nm (OD_{600}) of 0.050. Each different carbon and energy source was supplied at an initial concentration of 20.0 g/l (w/v). After seven days growth, emulsification activity, surface tension and critical micelle dilution (CMD) were determined. The measures of bacterial growth on different carbon and energy sources were thus performed as follow: cell collection by filtration through 0.2 µm filters, detachment of cells from filters by vigorously

shaking for 5 min, suspension on saline solution and determination of OD_{600}. All determinations were performed at least in duplicate.

2.4.5. Biodegradation kinetic experiments

In degradation test, the cultures were prepared in ten replicates with 20 mlM1 medium in 100 ml Erlenmeyer flasks. The cultures were inoculated to an initial OD_{600} of 1.00. Each different carbon and energy source was supplied at an initial concentration of 1.0 g/l (w/v). At each fixed kinetic time, two flasks were sacrificed: the first was used for residual hydrocarbon determination and the second one for OD_{600}, emulsification activity, cell surface hydrophobicity determination and for oil spreading technique (OST).

2.4.6. Kinetics of SAC production

The cultures were prepared in 500 mlErlenmeyer flasks containing 100 mlM1 medium and inoculated to an initial OD_{600} of 0.050. *n*-hexadecane was supplied at initial concentration of 20.0 g/l (w/v). At each kinetic time, the following parameters were determined: total DNA content, emulsification activity, surface tension, CMD, cell surface hydrophobicity, Ziehl-Neelsen stain reaction and the distribution of microorganisms by Confocal Laser Scanning Microscopy (CLSM).

Total DNA quantification was performed according to Burton (1956), using calf thymus DNA as standard. The cells were previously collected on glass fibre filters, as described by Bipatnath et al. (1998). All determinations were performed at least in duplicate.

2.4.7. OST, surface tension, interfacial tension and CMD

OST was performed according to Morikawa et al. (2000). Briefly, 50 mlof distilled water were added to a Petri dish (20 cm diameter) followed by addition of 20 µl of crude oil on the water surface. 10 µl of whole culture broths were added to the oil surface and the diameter of the clear zone was measured. Interfacial tension against diesel and surface tension were determined at room temperature. CMD was determined by dilution method as previously described by Pagilla et al. (2002). Analyses were

carried out with De Nouy ring method using K-8 tensiometer (Kruss, Hamburg, Germany). All determinations were performed at least in duplicate.

2.4.8. Emulsification assay

Samples of whole culture broths (with cells) or culture filtrates (without cells) were used. The cells were removed from the culture by filtration through 0.2 µm filters. 3 mlof sample was vortexed while the same amount of hydrocarbon was added drop by drop over 30 seconds in glass graduated tubes. After this, the tube was vortexed for additional 2 minutes. The mixture was allowed to settle for 24 h. The emulsification activity (E24%) is given as percentage of middle emulsion phase normalized to the total volume (Cooper and Goldenberg, 1987). All determinations were performed at least in duplicate.

2.4.9. Residual hydrocarbon analysis

The extraction for residual hydrocarbon determination was carried out adding to the flask 20 ml*o*-terphenyl (Internal Standard = 500 mg/l) solution in methylene chloride and 1 µL of organic fraction was analyzed without any further treatment. The analyses were performed with a HP 5890 gas chromatograph coupled to a Flame Ionization Detector with a HP 5MS column (0.25 mm i.d., 30 m length, 0.25 µm film thickness). The temperature program was 2 min at 40°C, then increasing at 40°C min^{-1} up to 320°C and 15 min at 320°C. The injector and detector temperatures were respectively 280°C and 320°C. Reproducibility of the entire analytical procedure for hydrocarbon quantification was about 10%.

2.4.10. Cell surface hydrophobicity

Cell surface hydrophobicity was determined by bacterial adhesion to hydrocarbons (BATH) test (Rosenberg et al., 1980). Cultures were transferred to a 50 mL screw-top plastic tube and mildly sonicated to disperse clumped cells. An aliquot of culture was washed and suspended in salt medium (16.9 g/l K_2HPO_4 , 7.3 g/l KH_2PO_4) to obtain a final OD_{600} equal to 1 (A_i). 1.5 ml of cell suspension was transferred to a vial to which 150 µl of *n*-heptadecane were added. The culture was vortexed for 2 min and OD_{600} (A_f) of the aqueous phase was measured after 30 min; CH% was expressed as $(1-A_i/A_f) \times 100$.

2.4.11. Acid-fast staining

The acid-fast staining allows to identifying mycolic acid-containing bacteria. Basic fuchsin dye complexes with mycolic acids and complexes resist to the following destaining with an acid-alcohol solution. Smears of sample bacteria were flame fixed and covered with a hot carbol fuchsin solution (SIGMA) for 30 min. The slides were washed with running tap water and bleached for 100 s with 0.4% HCl in 70% ethanol until no more red colour left the preparation. The slides were rinsed with tap water once and counterstained for 5 min with methylene blue 3 g/l. The slides were washed with water and dried in air before microscopic examination (Sokolovska et al., 2003).

2.4.12. CSLM analyses

A Leica TCS SP2 confocal system coupled to a Leica DMIRE2 inverted microscope was used to non-destructively obtain images of cell distribution in the culture. Samples were prepared in 1 mm-depth well slides using Syber green as nucleic acid stain. The microscope was equipped with a 40x PL FLUOTAR oil immersion objective (N.A.= 1.0) and was operated as follow. The fluorescent probe was excited with the Ar laser line at 488 nm and the fluorescence emission was detected between 500 and 680 nm. Z-series were acquired to allow 3D reconstruction of the sample with a z-step of 0.3 μm. The three-dimensional reconstructions were obtained by using the Leica TCS software.

2.4.13. Chemical characterization

The cultures were prepared in M1 medium and inoculated to an initial OD_{600} of 0.050. The carbon source was supplied at initial concentration of 20.0 g/l (w/v).

The bacterial cells were centrifuged at 6000 rpm for 15 min and the cell layer, located on the aqueous surface, was extracted with chloroform - methanol (2:1). The extract was dried and suspended in chloroform.

The residual culture broth was filtered through 0.45 μm filters. The cell-free filtrate was split into two aliquots and separately extracted with solvents with different polarity (chloroform - methanol (2:1) or *n*-butanol). The

extracts prepared with chloroform - methanol (2:1) or *n*-butanol were dried and suspended in chloroform or *n*-butanol, respectively.

The presence of biosurfactants and bioemulsans in the solvent extracts was evaluated by OST test or emulsification assay, respectively.

Samples were then spotted on Thin Layer Chromatography (TLC) silica gel plates (60 F_{254} Merck). Plates were developed in chloroform/glacial acetic acid/methanol/water (80:15:12:4) or chloroform/methanol/water (65:20:2). Silica plates were analysed under UV light and sprayed with α-naphthol solution (Molish reagent) to detect glycolipids (Arino et al., 1998). The chromatographs of the extracts were compared with the TLC pattern of a standard mixture of rhamnolipis by *Pseudomonas aeruginosa* (R1: α- L-Rhamnopyranosil- β - Hydroxydecanoyl- β -Hydroxydecanoate; R2: 2-O- α -L- Rhamnopyranosil - α -L- Rhamnopyranosil - β -idrossicanoil- β - Hydroxydecanoate) (IDRABEL Italia S.r.l., Arenzano, Italy). The rhamnolipids R1 and R2 showed R_f values of 0.46 and 0.13 with chloroform/glacial acetic acid/methanol/water as running solvents and R_f values of 0.85 and 0.57 with chloroform/methanol/water.

Πάντα ῥεῖ καὶ οὐδὲν μένει

Everything flows, nothing stands still.

Ηράκλειτος (Herakleitos; Heraclitus) of Ephesus (535 BC - 475 BC)

CHAPTER 3. FEASIBILITY STUDIES FOR APPLICATION OF GORDONIA SP. BS29 IN ENVIRONMENTAL REMEDIATION

3.1. INTRODUCTION

Due to their properties, microbial surface active compounds (SACs) have been exploited in environmental remediation techniques. Rhamnolipids and surfactin have been investigated for application both in biological and chemico-physical technologies for the remediation of soil and water contaminated by hydrocarbons and heavy metals.

3.1.1. Bioremediation

In recent years, bioavailability is considered one of the most important limiting factors of the biodegradation rate in soil environment. The presence of fraction of the contaminants, strongly bound to the soil particles, often, does not allow achieving remediation goals and can lead to long remediation time. Several studies have shown that the mass transfer from ab/adsorbed phase to liquid phase is the controlling mechanism of biodegradation rate (Weber et al., 1999). Several studies have been carried out on the effect of SACs on the biodegradation rate of both aliphatic and aromatic hydrocarbons (Makkar and Rockne, 2003; Mulligan, 2005).

Biosurfactants can enhance the biodegradation rate of hydrocarbons by two mechanisms, (i) enhanced solubility of the substrate for the microbial cells, and (ii) interaction with the cell surface, which increases the hydrophobicity of the surface for an easier access to hydrocarbons (Zhang and Miller, 1992; Shreve et al., 1995;). Zhang and Miller (1992) showed that a concentration of 300 mg/L of rhamnolipids increased the mineralization of octadecane to

20% from 5%. The rhamnolipids increased the solubility of the hexadecane from 1.8 to 22.8 mg/L even if there have been indications that inhibition can also occur. Rahman et al. (2003) demonstrated a beneficial effect of rhamnolipids for high-molecular weight hydrocarbons (C32-C40) in soil microcosms.

Regarding polycyclic aromatic hydrocarbons (PAHs), rhamnolipid increased the solubility of naphthalene by 30 times (Vipulanandan and Ren, 2000). However, the biodegradation rate of naphthalene was lower (10 g/L) in presence of rhamnolipids than Triton X-100 due to the fact that the biosurfactant was used as a carbon source instead of the naphthalene. This phenomenon did not occur in the case of Triton X-100. Deschenes et al. (1994) showed that the rhamnolipids in a bioslurry were more effective than SDS (up to five times) as they could enhance the solubilisation of four-ring PAHs more significantly than three-ring PAHs. There were also the indications of higher levels of toxicity by SDS compared to the biosurfactant as the concentration of the surfactants increased above 100 mg/kg. High molecular weight PAHs were not biodegraded despite surfactant addition.

3.1.2. Soil Washing

The prospects for the use of (bio)surfactants in soil washing remediation depend on the capacity of these compounds to enhance desorption or dissolution of contaminants, and to increase the rate of transport of contaminants in soil. The mechanism involved in the removal of hydrocarbons from soil are related to the mechanisms involved in increasing bioavailability for bioremediation purpose. The properties of stabilising oil/water emulsion and increasing hydrocarbons solubility may enhance, on one hand, the biodegradation rate and, on the other, the removal of hydrocarbon from soil (Ron and Rosenberg, 2002). The mobilization mechanism occurs also at concentrations below the surfactant CMC. Phenomena associated with this mechanism include reduction of surface and interfacial tension, reduction of capillary force, wettability and reduction of contact angle. At concentrations below CMC, surfactants reduce the surface tension and the interfacial tension between air/water, oil/water, and soil/water systems. Surfactants in contact with the soil/oil system increase the contact angle and reduce the capillary force holding together oil and soil due to the reduction of the interfacial force. Due to the

adsorption of surfactants on soils the mobilization mechanism depends on the surfactants ionic charge. Therefore, the adsorption of surfactants on soils may result in the loss and reduction of their concentrations, rendering them less efficient or ineffective in soil treatments. Above the surfactants CMC, the solubility of oil increases dramatically due to the aggregation of surfactants micelles. The hydrophobic end of the surfactant molecules cluster together inside the micelle structure with the hydrophilic end exposed to the aqueous phase on the exterior. Consequently, the interior of a micelle constitutes a compatible environment for hydrophobic organic molecules; the process of incorporation of these molecules into a micelle is known as solubilisation (Urum and Pekdemir, 2004). Surfactants have been applied for stimulating the dissolution of non-aqueous phase liquids initially present in soil (Bai et al., 1997; Fortin et al., 1997), the dissolution of solid contaminants (Mulder et al., 1998), and the desorption and transport of soil-sorbed contaminants (Edwards et al., 1994).

Rhamnolipids have been used since long time to remove oil from porous matrixes. They released three times as much oil as water alone from the beaches in Alaska after the Exxon Valdez tanker spill (Harvey et al., 1990). Van Dyke et al. (1993) had, previously, found that the same strain could remove at a concentration of 5 g/l, approximately 10% more hydrocarbons from a sandy loam soil than a silt loam soil and that sodium dodecyl sulfate (SDS) was less effective than the biosurfactants in removing hydrocarbons.

3.2. RESULTS

3.2.1. Bioremediation of aliphatic hydrocarbon contaminated soil

Triplicate soil microcosm experiments have been set up with the following conditions: (1) without any addition (TQ), (2) *Gordonia* BS29 addition (10^7 UFC/g) (BS), (3) *Gordonia* M22 addition (10^7 UFC/g) (M22), (4) addition of crude bioemulsan at high concentration (2000 mg/kg) (BEH), (5) addition of crude bioemulsan at low concentration (200 mg/kg) (BEL), (6) addition of rhamnolipids mixture (40 mg/kg) (R). Each microcosm consisted in 50 g of aliphatic hydrocarbon contaminated soil in a 125 ml-glass bottle. Soil was preliminary contaminated with the following compounds at a concentration of 300 mg/kg (each contaminant): n-hexadecane, n-heptadecane, pristane, n-eicosane, n-octacosane, squalene. Over time the

humidity was kept constant (25%) and the analyses of residual hydrocarbons, total microorganisms and hydrocarbon-degrading microorganisms were carried out at the beginning of the experiments and after 8, 19 and 53 days.

Hydrocarbon biodegradation data were analysed by Analysis of Variance (ANOVA) (Montgomery, 2005) to compare the tested condition compared to the condition without any addition. The dependent variables for the comparison were the biodegradation rate (k) and the residual concentration of each contaminant after 53 days of experiments. The biodegradation rate was computed as the first order kinetic constant of the exponential equation best fitting experimental data. In Table 3.2—1 and Table 3.2—2 the mean and the standard deviation of residual concentration and biodegradation rate, for each contaminant for each treatment are reported.

Table 3.2—1 *Means and standard deviations of residual concentration of contaminants (mg/kg) in biodegradation experiments with aliphatic hydrocarbons. <dl: below detection limit; nd: not determined*

Treatment	**TQ**		**BS**		**M22**	
Contaminants	**Mean**	**St. dev.**	**Mean**	**St. dev.**	**Mean**	**St. dev.**
n-hexadecane	< dl	nd	< dl	< dl	< dl	nd
n-heptadecane	< dl	nd	< dl	< dl	< dl	nd
pristane	4.3	0.7	5.6	0.7	4.3	0.6
n-eicosane	3.8	0.4	3.2	0.4	5.6	0.8
n-octacosane	< dl	nd	2	2	< dl	nd
squalene	< dl	nd	< dl	< dl	< dl	nd

	BEH		**BEL**		**R**	
Contaminants	**Mean**	**St. dev.**	**Mean**	**Mean**	**St. dev.**	**Mean**
n-hexadecane	< dl	nd	< dl	< dl	nd	< dl
n-heptadecane	< dl	nd	< dl	< dl	nd	< dl
pristane	2.8	0.4	6.0	2.8	0.4	6.0
n-eicosane	9	2	7	9	2	7
n-octacosane	< dl	nd	< dl	< dl	nd	< dl
squalene	< dl	nd	< dl	< dl	nd	< dl

Table 3.2—2 *Means and standard deviations of biodegradation rate (k) of contaminants (days^{-1}) in biodegradation experiments with aliphatic hydrocarbons*

Treatment	TQ		BS		M22	
Contaminants	**Mean**	**St. dev.**	**Mean**	**St. dev.**	**Mean**	**St. dev.**
n-hexadecane	-0.279	0.004	-0.3	0.1	-0.219	0.003
n-heptadecane	-0.264	0.005	-0.3	0.1	-0.208	0.002
pristane	-0.137	0.004	-0.12	0.02	-0.128	0.002
n-eicosane	-0.256	0.008	-0.19	0.01	-0.223	0.002
n-octacosane	-0.20	0.01	-0.18	0.01	-0.200	0.006
squalene	-0.206	0.002	-0.20	0.02	-0.202	0.004

	BEH		BEL		R	
Contaminants	**Mean**	**St. dev.**	**Mean**	**St. dev.**	**Mean**	**St. dev.**
n-hexadecane	-0.24	0.04	-0.151	0.003	-0.26	0.04
n-heptadecane	-0.250	0.007	-0.146	0.003	-0.25	0.03
pristane	-0.146	0.001	-0.093	0.005	-0.12	0.01
n-eicosane	-0.17	0.02	-0.153	0.003	-0.225	0.006
n-octacosane	-0.201	0.001	-0.189	0.007	-0.190	0.007
squalene	-0.142	0.022	-0.155	0.004	-0.193	0.011

Data analysis

ANOVA tests were carried out to evaluate the significance of the differences of the mean between each treatment and the control TQ both for residual concentration and biodegradation rate. The F Test, used as diagnostic test for ANOVA, allowed us to evaluate whether the treatment led to a significant enhancement of the rate and/or the extent of biodegradation of the tested compounds. Since the significance of the test was fixed at 0.05 and the sums of squares were calculated on three values, thus the critical F value is $F_{0.05,1,4} = 7.70$. If calculated F values are greater than critical F values, the difference between the means can be consider significant.

Table 3.2—3 and Table 3.2—4 present the means and the calculated F values of ANOVA test for residual concentration and biodegradation rate respectively.

Table 3.2—3 *Results of ANOVA test for residual concentration (mg/kg) in biodegradation experiments with aliphatic hydrocarbons. Each treatment is compared with the control TQ. F value in bold indicates significant differences of the means (Fcrit $_{0.05,1,4}$=7.7).*

	n-hexadecane		**n-heptadecane**		**pristane**	
	TQ mean= <dl		**TQ mean = <dl**		**TQ mean = 4.3**	
	mean (mg/kg)	**F**	**mean (mg/kg)**	**F**	**mean (mg/kg)**	**F**
BS	<dl	nd	<dl	nd	5.6	4.19
M22	<dl	nd	<dl	nd	4.3	<0,05
BEH	<dl	nd	<dl	nd	2.8	**10,7**
BEL	<dl	nd	<dl	nd	6.0	**9,0**
R	<dl	nd	<dl	nd	1.3	**45,5**

	n-eicosane		**n-octacosane**		**squalene**	
	TQ mean = 3.8		**TQ mean = <dl**		**TQ mean = <dl**	
	mean (mg/kg)	**F**	**mean (mg/kg)**	**F**	**mean (mg/kg)**	**F**
BS	0.4	3.92	<dl	nd	2.3	0.44
M22	5.6	6,9	<dl	nd	<dl	nd
BEH	4.6	8.3	<dl	nd	<dl	nd
BEL	5,1	0,5	<dl	nd	<dl	nd
R	2.07	0.5	1	nd	<dl	nd

Table 3.2—4 *Results of ANOVA test for biodegradation rate (days^{-1}) in biodegradation experiments with aliphatic hydrocarbons. Each treatment is compared with the control TQ. F value in bold indicates significant differences of the means (Fcrit $_{0.05.\ 1.4}$=7.7).*

	n-hexadecane		**n-heptadecane**		**pristane**	
	TQ mean= - 0.279		**TQ mean = - 0.264**		**TQ mean = - 0.137**	
	mean (mg/kg)	**F**	**mean (mg/kg)**	**F**	**mean (mg/kg)**	**F**
BS	-0.3	<0.05	-0.3	<0.05	-0.12	2.1
M22	-0.219	**362.0**	-0.208	**367.1**	-0.128	**11.1**
BEH	-0.24	1.9	-0.250	8.0	-0.146	**13.5**
BEL	-0.151	**1725.2**	-0.146	**1218.0**	-0.093	**131.4**
R	-0.26	0.8	-0.25	0.8	-0.12	1.3

	n-eicosane		**n-octacosane**		**squalene**	
	TQ mean = -0.256		**TQ mean = -0.20**		**TQ mean = -0.206**	
	mean (mg/kg)	**F**	**mean (mg/kg)**	**F**	**mean (mg/kg)**	**F**
BS	**-0.19**	**47.2**	-0.18	1.9	-0.20	0.2
M22	**-0.223**	**54.8**	-0.200	0.1	-0.202	1.4
BEH	**-0.17**	**53.1**	-0.201	0.3	**-0.142**	**25.3**
BEL	**-0.153**	**464.1**	-0.189	1.0	**-0.155**	**355.4**
R	**-0.225**	**30.6**	-0.190	0.8	-0.193	3.8

It is evident that both bioaugmentation treatments (BS and M22) did not lead neither to an increase of biodegradation rate nor to a reduction of residual concentration for any contaminants. In some cases the addition of exogenous bacteria led to a reduction of biodegradative capacity of the entire community. For most of the contaminants even the addition of rhamnolipids, did not result in the enhancement of biodegradation. However, ANOVA test showed that both the addition of rhamnolipids and bioemulsan reduced the final concentration of pristane in respect to the control. Pristane can be considered a model compounds for highly branched and recalcitrant compounds. It is known as a residual contaminants in several bioremediation application of diesel fuel contaminated soil. Furthermore, the bioemulsan increased also the biodegradation rate of pristane. Summarising the results from data analysis, we found that the extracellular bioemulsan seems to have positive effect on the rate of biodegradation and residual concentration on branched aliphatic hydrocarbons in contaminated soil.

3.2.2. Bioremediation of polycyclic aromatic hydrocarbon contaminated soil

Triplicate soil microcosm experiments have been set up with the following conditions: (1) without any addition (TQ), (2) addition of crude bioemulsan at high concentration (2000 mg/kg) (BEH), (3) addition of crude bioemulsan at low concentration (200 mg/kg) (BEL), (6) addition of rhamnolipids mixture (40 mg/kg) (R). Each microcosm consisted in 50 g of polycyclic aromatic hydrocarbon contaminated soil in a 125 ml-glass bottle. Soil was preliminary contaminated with a total concentration of 800 mg/kg with the following compounds: phenanthrene, anthracene, pyrene and benzo[a]pyrene. Over time the humidity was kept constant (25%) and analyses of residual hydrocarbons were carried out at the beginning of the experiments and after 35, and 75 days.

Hydrocarbon biodegradation data were analysed by Analysis of Variance (ANOVA) (Montgomery, 2005) to compare the tested conditions in respect to the condition without any addition. The dependent variable for the comparison was the final residual concentrations of each contaminant. In Table 3.2—5 the mean and standard deviation of residual concentrations, for each contaminant for each treatment are reported.

Table 3.2—5 *Means and standard deviations of residual concentration of contaminants (mg/kg) in biodegradation experiments with polycyclic aromatic hydrocarbons.*

Treatment	**TQ**		**BEH**		**BEL**		**R**	
Contaminants	**Mean**	**St. dev.**	**Mean**	**St. dev.**	**Mean**	**St. dev.**	**Mean**	**St. dev.**
Phenanthrene	7	5	7,3	0,8	6	5	9,5	0,9
Anthracene	135	5	209	24	189	98	291	23
Pyrene	4	1	76	8	99	60	180	54
Benzo[a]pyrene	33	8	31	7	34	29	64	18

Data analysis

ANOVA tests were carried out to evaluate the significance of mean difference between each treatment and the control TQ for residual

concentration. The F Test, used as diagnostic test for ANOVA, allowed us to evaluate whether the treatment led to a significant enhancement of the extent of biodegradation of the tested compounds. Since the significance of the test was fixed at 0.05 and the sums of squares were calculated on three values, the critical F value is $F_{0.05,1,4} = 7.70$. If calculated F values are greater than critical F values, the difference between the means can be consider significant.

Table 3.2—6 shows the means and the calculated F values of ANOVA test for residual concentration.

Table 3.2—6 *Results of ANOVA test for residual concentration (mg/kg) in biodegradation experiments with polycyclic aromatic hydrocarbons. Each treatment is compared with the control TQ. F value in bold indicates significant differences of the means (Fcrit $_{0.05, ,1,4}$=7.7).*

	Phenanthrene		**Antrhacene**		**Pyrene**		**Benzo[a]pyrene**	
	TQ mean= 7.2		**TQ mean = 189**		**TQ mean = 3.9**		**TQ mean = 33.48**	
	mean (mg/kg)	**F**	**mean (mg/kg)**	**F**	**mean (mg/kg)**	**F**	**mean (mg/kg)**	**F**
BEH	7.3	0.00065	209	16.25	75	**230**	93	0.114
BEL	6.1	0.06	189	0.54	99	4.5	33.68	0.000074
R	9.5	0.76	291	**81**	179	**19**	64	4.7

It is evident that the addition of the bioemulsan did not result in a significant reduction of the contaminants compared to the control. In particular, the bioemulsan effectively inhibited the biodegradation of pyrene. In the BEH treatment the final concentration of this contaminant was 75 mg/kg while in BEL was 99 mg/kg (the high variability in the triplicate results led to a negative F-test) while in the control it was 3.9 mg/kg. Actually, also the rhamnolipid treatment did not increase the degradation of the target contaminants and in the cases of anthracene and pyrene it led to an increase of residual concentration compared to the control.

3.2.3. Washing of crude oil contaminated soil

To evaluate the applicability of extracellular bioemulsan in soil washing treatment a set of experiments was designed. Soil was artificially

contaminated with crude oil (10% w : w) and batch washing experiments were carried out in different conditions. A $3^{(3)}$ three level full factorial design (FFD) with six central points (33 experiments) was chosen with one qualitative variable and two quantitative ones. The qualitative variable was the washing solution while the two quantitative ones were the ratio between soil and washing solution, and the time of washing. Table 3.2—7 shows the variables and the levels chosen for this experimentation.

Table 3.2—7 *Variables and values of the levels in $3^{(3)}$ FFD*

VARIABLE/LEVEL	LOW	MEDIUM	HIGH
Washing solution	Water	Bioemulsan (2% w:v)	Rhamnolipid mixture (2% w:v)
Ratio soil:washing solution (w:v)	10	15	20
Time of washing (min)	30	50	70

Residual crude oil concentration after washing was measured by spectrophotometric analysis of n-hexane extract from soil and the percentage of crude oil removed was calculated for each conditions. A complete list of the crude oil removal in the experiments is reported in Annex 1.

Data analysis

ANOVA tests were used to evaluate the significance of the effect of the bioemulsan and rhamnolipids on crude oil removal compared to water. Furthermore ANOVA was used within the experiments with the same washing solution to individuate the factors between soil:washing solution ratio and time of washing that significantly affect the removal.

Table 3.2—8 and Table 3.2—9 show the results of ANOVA test for the comparison of bioemulsan and rhamnolipids with only water tratment.

Table 3.2—8 *ANOVA results for bioemulsan washing of oil contaminated soil*

Groups	*n*	*Sum*	*Mean*	*Variance*		
Water	9	175.523	**19.50261**	99.33818		
Bioemulsan	9	387.343	**43.0382**	53.97843		
ANOVA						
	SS	*df*	*MS*	*F*	*p*	*F crit*
Between groups	2492.6	1	2492.6	**32.51**	3.3E-05	4.493998
Within groups	1226.5	16	76.6			
Total	3719.1	17				

Table 3.2—9 *ANOVA results for rhamnolipid washing of oil contaminated soil*

Groups	*n*	*Sum*	*Mean*	*Variance*		
Water	9	175.523	**19.5026**	99.33818		
Rhamnolipids	9	456.889	**50.7655**	76.04042		
ANOVA						
	SS	*df*	*MS*	*F*	*p*	*F crit*
Between groups	4398.1	1	4398.16	**50.15618**	2.6E-06	4.493998
Within groups	1403.0	16	87.6893			
Total	5801.1	17				

As indicated by F values, the means of removal for bioemulsan (43.01%) and rhamnolipids (50.76%) are significantly different from the mean of the removal in the experiments with only water (19.5%). These result mean that both products are effective in the removal of crude oil from contaminated soil. Comparing with ANOVA test, the means of bioemulsan and rhamnolipids treatments the difference of the means did not result significant with a level of significance of 0.05 (F=4.13; p=0.58).

Furthermore, ANOVA test was used for the experiments of bioemulsan and rhamnolipids treatment to individuate the whether soil:washing solution ratio and time of washing, significantly, affect the oil removal. The test showed that for the bioemulsan a positive correlation was found between time of washing and oil removal. Figure 3.2-1 shows the second order surface that best fit experimental data.

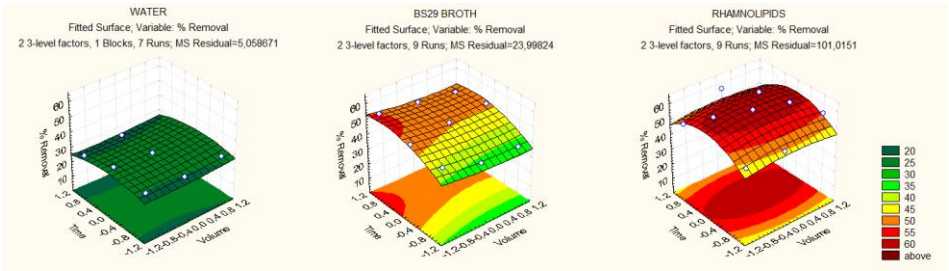

Figure 3.2-1 – *Removal of crude oil for different washing solutions in relation to time of washing and volume of washing solution.*

3.3. DISCUSSION

The results reported in the above paragraphs allow us to evaluate the applicability of SACs produced by *Gordonia* sp. BS29 in environmental remediation techniques for hydrocarbon contaminated soil. Results from bioremediation experiments are very contrasting. Rhamnolipids are known to be effective as enhancers in biodegradation of both aliphatic and aromatic hydrocarbons (Zhang and Miller, 1992; Deschenes et al., 1994). However, in our experimental systems rhamnolipids did not succeeded in increasing neither the rate nor the extent of biodegradation for most of the individual contaminants. In the case of pyrene in PAH experiments the final concentration in rhamnolipid-treated microcosms was two-folds than in the control. Actually, cases of inhibitions by biosurfactants are reported as well in the literature and the reasons of these failures have been put forward. The surfactant micelles can provide a barrier between bacteria and the contaminants (Laha et al., 1991; Yuan, 2000). The possibility for microorganisms to access the micelle-contaminant complex depends on the hydrophobicity of the cell surface (Bouchez-Naitali, 1999). Furthermore the surfactants can inhibit hydrocarbon degradation due to competitive biodegradation (Goudar et al., 1999). Like rhamnolipids, BS29 bioemulsan did not result in enhancing rate and extent of biodegradation. Since the treated control of the experimentation resulted negative, on the basis of the results, we cannot definitely conclude that BS29 bioemulsan is not effective. Other experimentations are needed in different soils or microbial community to definitely assess its applicability.

On the other hand, results from soil-washing experiments demonstrated that the BS29 bioemulsan effectively removes crude oil from soil. The removal efficiencies are comparable to those of rhamnolipids. Moreover, considering that the bioemulsan was used as crude lyophilized product at the same concentration of rhamnolipids as pure active principle and that for the bioemulsan a positive correlation was found between time of washing and oil removal, there is the potential to further increase the extent of contaminant removal. Furthermore, ongoing experiments (data not shown) are indicating a potential for the bioemulsan also in removing heavy metals from contaminated soil.

3.4. EXPERIMENTAL PROCEDURES

3.4.1. Preparation of crude bioemulsan

For preparation of bioemulsan *Gordonia* sp. BS29 cultures were prepared in M1 medium at initial OD of 0.05 and n-hexadecane (2%) as carbon sources. After incubation (200 rpm) at 30° C cultures were centrifuged (7000 rpm for 15 min) and the biomass was mechanically removed. The cultures were then filtered on sterile membrane (0.45 μm) to remove residual bacteria. The filtrate was subsequently lyophilized in order to obtain a solid residue (crude bioemulsan).

3.4.2. Bioremediation experiments

Two different experimental sets of soil microcosm have been designed. Uncontaminated garden soil was amended with inorganic nutrients (N, P) and contaminated with single aliphatic hydrocarbons in experimental set 1 and with single aromatic hydrocarbons in experimental set 2. Soil was contaminated dissolving the contaminants in n-hexane and adequately mixing the solvent and the soil (0.5 ml of n-hexane per gram of soil). After solvent evaporation soil samples, were analyzed for their hydrocarbon concentrations. In Annex 1 initial concentrations of contaminants in all the experiments are reported.

Triplicate microcosms were prepared for each condition. Each microcosm consists in 50 g of contaminated soil in an aerated serum bottle. Bottles were kept al laboratory temperature and soil humidity was maintained constant by de-ionized water addition (25%). At fixed kinetic time soil

samples were removed and the residual hydrocarbon concentration was determined.

3.4.2.1. Residual hydrocarbons determination

2 g of soil samples were added with 30 ml n-hexane containing *o*-terphenil as internal standard. The bottles were sealed with a Teflon stopper and held for 30 min in an ultrasonic bath at 47 kHz frequency; the extracts were filtered on anhydrous sodium sulphate, dried, dissolved in hexane, analysed and quantified by internal calibration.

The analyses were performed with an Agilent 5890 gas chromatograph coupled to a FID detector with HP5 column (30 m length, 0.32 mm i.d., 0.25 µm film thickness). The temperature program was 2 min at 40°C, then 12°C/min to 300°C, and 5 min at 300°C. Injector and detector temperatures were set to 250°C.

3.4.3. Soil washing

Garden soil was artificially contaminated dissolving crude oil in n-hexane and adequately mixing the solvent and the soil to reach the concentration of crude oil in soil of 10% w:w. Soil samples were left uncovered for 48 hours to permit evaporation of the solvent and volatile components of the crude oil.

Batch soil washing experiments were carried out in sealed bottles containing 2 g of soil and the suitable washing solution, according to the experimental design. Bottles were shaken at 200 rpm for the suitable washing time.

3.4.3.1. Residual crude oil determination

After the washing, the bottles were centrifuged at 2000 rpm for 5 minutes, the supernatant solution was discarded, the soil was rinsed with water and centrifuged again. After discarding the supernatant solution, the soil was dried with anhydrous sodium sulphate and extracted four times with 10 ml of n-hexadecane. The extracted crude oil was evaluated by absorbance measurement (400 nm). The crude oil removal was determined by difference between the absorbances of washed and unwashed soil.

3.4.4. Data Analysis

ANOVA analyses of experimental design results and regression analyses have been made using STATISTICA software.

The physicist's problem is the problem of ultimate origins and ultimate natural laws. The biologist's problem is the problem of complexity.

Richard Dawkins (1941) – The Blind Watchmaker (1986)

CHAPTER 4. OPTIMIZATION OF SAC PRODUCTION BY GORDONIA SP. BS29

4.1. INTRODUCTION

So far, microbial SACs have not found wide industrial application due to the high production costs, particularly for extraction and purification. It is also known that microbial SACs production is strongly influenced by medium composition and cultural conditions (Desai and Banat, 1997). Nevertheless, the optimisation of these conditions has not been extensively studied using proper statistic tools of experimental design. The use of these tools for the optimisation can represent a way to sort out the industrial constrains for the economy of the whole process. The classical method of medium optimisation consists in changing one variable at time, keeping the others at fixed level. This leads to neglect the interaction among factors and, often, this time-consuming method does not guarantee the actual determination of the optimal conditions.

4.1.1. Towards a commercial application of microbial SACs

Despite their characteristics of lower toxicity, higher biodegradability, better foaming properties than the synthetic counterparts and stable activity at extremes of pH, salinity and temperature, biosurfactants did not achieve a wide commercial distribution.

The economization of a biotechnological process depends on: (1) initial raw material costs, (2) availability of suitable and economic production and recovery procedures, (3) product yields of the producer microorganisms.

Different strategies have been adopted in order to make the process of the biosurfactant production cost-competitive: (1) use of cheap and waste substrates (2) development of efficient bioprocesses, including optimization

of fermentative condition and recovery process, (3) development of overproducing strains (Mukherjee et al., 2006).

The first two strategies have been explored to a greater extent and have been reported to be effective in substantially increasing the production of biosurfactants. However, the third approach using recombinant overproducing strains, has still not been properly tested, despite the fact that the overproducers have been reported to increase yields several fold.

4.2. RESULTS

The aim of the work reported in this chapter was to find out the optimal cultivation conditions for the production of the cell-bound glycolipid biosurfactant(s) by *Gordonia* sp. BS29 strain. The optimisation process involved three consecutive steps (Montgomery, 2005). In the first step a two level $2^{(8-2)}$ Fractional Factorial Design (FFD) was used to identify cultural factors having a significant influence on biosurfactant(s) biosynthesis. Then, on the selected factors, a steepest ascent procedure and a Central Composite Design (CCD) were applied to obtain a second order polynomial function which fitted the experimental data in the vicinity of the optimum. The factors taken in account were inorganic nutrients, such as phosphorous, ammonium and micronutrients, the carbon sources and the time of growth.

4.2.1. Design of experiments

In each experimental design, the independent variables were coded according to the following equation:

$$C_i = 2 \times \left[\frac{x_i - x_{\min}}{x_{\max} - x_{\min}} \right] - 1$$

Eq. 4.2-1

where C_i is the coded value of i^{th} variable, x_i is the i^{th} actual value of i^{th} variable, x_{max} and x_{min} are higher and lower limit values of the range chosen for the i^{th} variable.

4.2.1.1. Selection of significant factors by Fractional Factorial Design (FFD)

To determine the cultural factors having significant influence on biosurfactant(s) production, on the basis of previous literature, six major inorganic components, the carbon source and the time of growth were selected to be evaluated (Table 4.2—1). According to full factorial design at two levels, 2^8 experiments should have been carried out. The number of experiments was reduced by using a one-quarter fractional design assuming that the three- and higher-order interactions among factors are neglectable. The selected design was so a two level fractional factorial design with a resolution of V ($2^{(8-2)}$ R=V) composed by 64 different experiments which other 4 centre point experiments were added to. Each factor was present in the experimental design at two different levels and the values of the levels have been chosen on the basis of the previous studies (Table 4.2—1).

Table 4.2—1 *Values of higher and lower levels for each variable in $2^{(8-2)}$ FFD.*

		CODED VARIABLE	
		-1	**+1**
ID	**FACTORS**	x^{min}	x^{max}
x_1	$NaNO_3$ (mg/l)	425	4250
x_2	$(NH_4)_2SO_4$ (mg/l)	570	5700
x_3	K_2HPO_4 : KH_2PO_4 (2:1 weight ratio) (mg/l)	364	3640
x_4	$MgSO_4$ (mg/l)	363	3630
x_5	$FeSO_4$ $7H_2O$ (mg/l)	270	2700
x_6	NaCl (mg/l)	126	1260
$x_{7/1}$	Carbon source	Glycerol (2% w/v)	n-hexadecane (2% w/v)
x_8	Time of growth (d)	5	10

The FFD allowed us to select, among the eight tested cultural conditions, three medium components, that significantly affect the amount of biosurfactant(s) produced by *Gordonia* sp. BS29 strain. Among the several cultural factors which can potentially affect the production of biosurfactant(s) we chose six inorganic nutrients and the time of growth of

the cultures. For each of these factors a wide range of concentration has been selected with a 1:10 ratio between the lower and the higher value. Furthermore, the type of carbon source was added to the design as a qualitative variable. n-hexadecane and glycerol were chosen as the best unsoluble and soluble substrates. For each run the CMD of the crude broth was measured as a parameter proportional to biosurfactant(s) concentration. A complete list of the results is reported in Annex 1. Then, results were analysed by ANOVA test to determine which of the factors, significantly, affected the biosynthesis. The output of the ANOVA test (α = 0.05) is shown in Table 4.2—2. In the ANOVA test three- and higher-order interactions have not been considered and in the Table 4.2—2 only most significant factors and interactions are presented.

Table 4.2—2: *ANOVA test results for $2^{(8-2)}$ FFD.*

FACTOR/INTERACTION	SS	df	MS	F	p
$x_{7/1}$	15735.05	1	15735.05	44.49	<0.00001
x_3	6488.30	1	6488.30	18.34	0.00016
x_3 *by* $x_{7/1}$	5825.51	1	5825.51	16.46	0.00031
x_6	3965.85	1	3965.85	11.21	0.00214
x_6 *by* x_7	3588.01	1	3588.01	10.14	0.00328
x_3 *by* x_6	2748.38	1	2748.38	7.77	0.00898
....	....	....	....	....	....
ERROR	10963.16	31	353.65		
TOTAL SS	60084.58	67			

Being $F_{0.05,\ 1\ ,\ 31}$ = 4.16, the factors and the interactions that resulted significant after the test were: the qualitative variable of the carbon source ($x_{7/1}$), the phosphates (x_3), sodium chloride (x_6) and the interactions among these factors ($x_{7/1}$byx_3 ; $x_{7/1}$byx_6 ; $x_3$byx_6). The values of estimated effects (Table 4.2—3) allowed us to evaluate the positive or negative correlations between the significant factors and the response; particularly, the positive correlation of the qualitative variable carbon source (the most important factor) means that the value of the variable coded by +1 (n-hexadecane) results to be better than the other (glycerol).

Table 4.2—3: *Estimated effects of significant factors and interactions in $2^{(8-2)}$ FFD*

FACTOR/INTERACTION	ESTIMATED EFFECT	-95% CONFIDENCE INTERVAL	+95% CONFIDENCE INTERVAL
Mean factor	16.75	12.10	21.41
$x_{7/1}$	30.42	21.12	39.72
x_3	20.13	10.54	29.72
x_3 ***by*** $x_{7/1}$	19.08	9.49	28.66
x_6	-15.74	-25.53	-6.15
x_6 ***by*** x_7	-14.97	-24.56	-5.39
x_3 ***by*** x_6	-13.10	-22.69	-3.51

Ignoring the unsignificant factors and interactions the first-order model best fiting the experimental data was the following:

$$CMD^{pred} = 16.76 + 10.07x_3 - 7.87x_6 + 15.21x_{7/1} - 6.55x_3x_6 + 9.54x_3x_7 - 7.48x_6x_7$$

Eq. 4.2-2

Where CMD^{pred} is the model predicted value of CMD.

Substituting the values of the variables that gave the maximum predicted value (x_3 = 1; x_6 = -1 ; $x_{7/1}$ = 1), a value of CMD^{pred} equal to 73.49 (90% confidence interval: 63.15 – 83.49) was obtained. Even if this was only the factor-screening step, we obtained a maximum CMD^{pred} value among the best ones in the current literature. It is almost 10-fold higher than the one obtained culturing *Gordonia* sp. BS29 strain cultured in BH mineral medium (see Chapter 2).

4.2.1.2. Optimisation of cultural conditions by Step Ascendant Method (SAM) and Response Surface Analysis (RSA)

The Step Ascendant Method (SAM) is an efficient tool to rapidly move towards the optimum region of the experimental field. To detect the direction in which the response increases most rapidly, a full factorial design with three quantitative variables (x_3 ; x_6 ; $x_{7/2}$) has been applied

around the best condition found in the previous design, i.e. $x_1 = x_2 = x_4 = x_5 = x_8 = 0$; $x_3 = 1$; $x_6 = -1$. In this case, n-hexadecane was chosen as quantitative variable ($x_{7/2}$) as showed in Table 4.2—4. This design was built with the significant factors NaCl and phosphates, testing also two different concentrations of n-hexadecane as carbon source. Table 4.2—4 shows the tested range for this design.

Table 4.2—4: *Values of higher and lower levels for each variable in $2^{(3)}$ Full Factorial Design.*

		CODED VARIABLE	
		-1	**+1**
ID	**FACTOR**	$\mathbf{x^{min}}$	$\mathbf{x^{max}}$
x_3	K_2HPO_4 : KH_2PO_4 (2:1 weight ratio) (ppm)	1822	5465
x_6	NaCl (ppm)	36	189
$x_{7/2}$	n-hexadecane (% w/v)	0.5	3.5

ANOVA test for this design showed that n-hexadecane is the only significant factor in these conditions. Even if NaCl did not result significant with this level of significance, a first order model, considering only main effects and ignoring the variable x_3, was applied to find the Eq. 4.2-3 of the plane best fiting the experimental data.

$$CMD^{pred} = 51.59 - 12.25x_6 + 37.30x_{7/2}$$

Eq. 4.2-3

Then, starting from the centre of the full factorial design, eight experiments were conducted along the linear steepest ascent on the plane represented by Eq. 4.2-3. Table 4.2—5 shows the conducted experiments. Both in full factorial design and SAM all the non investigated factors were kept constant at coded value of 0 according to Eq. 4.2-1and ranges of Table 4.2—1.

***Table 4.2—5**: Experiments of the Steepest Ascendant Method.*

FACTOR	n-hexadecane (% w/v)	NaCl (ppm)
Origin	2.00	126
Step1	2.75	122
Step2	3.50	118
Step3	4.25	114
Step4	5.00	110
Step5	5.75	106
Step6	6.25	102
Step7	7.25	98

Results allowed us to detect a maximum along this path that coincided with the step 3. In these conditions, the concentration of n-hexadecane and NaCl were 4.25 % and 114 ppm respectively. We observed a significant increase of the CMD value from the origin to the step 3 (128.3). This confirmed another time the importance of the amount of n-hexadecane on the biosurfactant production.

Then, a Central Composite Design (CCD) was then designed using as centre the best conditions found in SAM. The independent variables were NaCl, phosphates and n-hexadecane. Table 4.2—6 shows the values of the four levels for each factor and their coded values. The design is composed by a two level $2^{(3)}$ FFD (eight experiments), six axial experiments ($\alpha = 1.682$) and two centre point experiments. The aim of this experimental set was to develop an empirical model to quantify the production of biosurfactant(s) and maximise it in the selected operating region for the most significant factors. This approach is called response surface methodology.

Table 4.2—6: *Values of the levels for each variable in CCD.*

		CODED VARIABLE			
		-1.682	**-1.000**	**+1.000**	**+1.682**
ID	**FACTOR**	**$\alpha\ x^{min}$**	**x^{min}**	**x^{max}**	**$\alpha\ x^{max}$**
x_3	K_2HPO_4 : KH_2PO_4 (2:1 weight ratio) (ppm)	1893	2600	4680	5387
x_6	NaCl (ppm)	94	101	127	134
$x_{7/2}$	n-hexadecane (% w/v)	2.57	3.25	5.25	5.93

The chosen variables were x_3, x_6 and $x_{7/2}$, coded according to both Eq. 4.2-1 and ranges shown in Table 4.2—6. ANOVA test (α = 0.05) for significance of the factors is shown in Table 4.2—7. It is evident that there are no significant factors ($F_{0.05,\ 1,\ 6}$= 5.99) in this region of the experimental field. This did not permit to calculate a second order model for identification of the best conditions, being the mean coefficient the only important. The mean coefficient value was 129.43 (95% confidence interval: 119.47 – 139.40). This is one of the best values of CMD in the current literature and it is very far from both the starting value in BH medium (26.00) and the calculated highest value of FFD (73.49). This result demonstrates that there is a quite wide region of the experimental field in which the response could be considered highest and virtually constant. This characteristic of robustness can have a very positive impact on the scaling up of process to industrial scale in which the starting conditions can be controlled with less efficiency than at laboratory scale.

***Table 4.2 – 7**: ANOVA test results for CCD.*

FACTOR/INTERACTION	SS	df	MS	F	P
X3 (L)	48.02	1	48.02	1.44	0.27542
x3 (Q)	65.44	1	65.44	1.96	0.21084
x6 (L)	10.54	1	10.54	0.31	0.59432
x6 (Q)	52.25	1	52.25	1.56	0.25729
x7 (L)	55.74	1	55.74	1.67	0.24366
x7(Q)	46.21	1	46.21	1.38	0.28373
x3(L) by x6(L)	15.68	1	15.68	0.47	0.51855
x3(L) byx7(L)	14.04	1	14.04	0.42	0.54044
x6(L) by x7(L)	160.20	1	160.20	4.8	0.07092
ERROR	200.14	6	33.35		
TOTAL SS	594.39	15			

4.3. DISCUSSION

Factors affecting the biosurfactant biosynthesis have been extensively studied in the last years, but few of these deal with the optimisation of the cultural conditions for biosurfactant production. Biosurfactant production has often been found under growth-limiting conditions. Nitrogen limitation caused an increase in biosurfactant production in *P. aeruginosa* (Ramana and Karanth, 1989), *C. tropicalis* IIP-4 (Singh et al., 1990), and *Nocardia* strain SFC-D (Kosaric et al., 1990). Syldatk et al. (1985) (Syldatk et al., 1985) showed that nitrogen limitation does not only cause overproduction of biosurfactant but also changes the composition of the produced biosurfactant. Guerra-Santos et al. (1984, 1986) showed the maximum rhamnolipid production after nitrogen limitation at a C:N ratio of 16:1 to 18:1 and no surfactant production below a C:N ratio of 11:1, where the culture was not nitrogen limited. On the contrary, Espuny et al. (1996) found a growth-dependent production of biosurfactant by *Rhodococcus* sp.. n-tridecane was identified as the best carbon source for the biosurfactant production while the best medium composition contained sodium nitrate and phosphates at concentrations of 2.5 g l^{-1} and 2.0 g l^{-1} respectively. As previously demonstrated (see chapter 2), *Gordonia* sp. BS29 biosurfactant biosynthesis starts during the exponential phase, but a significant amount of the product (above the CMC) can be found only after the exponential phase. Furthermore, we did not observe any significant influence of nitrate concentration on biosurfactant concentration in cultural broth. In our experiments, the inorganic component of the cultural medium that

significantly affects the biosynthesis was phosphorous. Jacques at al. (1999) found the same influence for lipopeptide production by *Bacillus subtilis* S499 while $FeSO_4$ and $MnSO_4$ affect surfactin production by *Bacillus subtilis* DSM 3256 more that the concentration of nitrogen source (Sen, 1997).

Recently, experimental design techniques are applied for optimisation of biosurfactant production. Jacques et al. (1999) optimised cultural and fermentative conditions for lipopetide (surfactin) production by *B. subtilis*. Cunha et al. (2004) isolated different *Serratia* spp. strains and evaluated the effects of the fermentative conditions and carbon source concentration. Albuquerque et al. (2006) firstly optimised medium components for bioemulsan production by *Candida lipolytica*.

So our study can be considered the first attempt to identify the cultural parameters that influence biosurfactant production in *Gordonia* spp.. The use of different experimental design techniques resulted in a straightforward and time-saving process which allowed us to individuate the most important parameters and interactions and then identify the best values of these parameters in order to maximise the concentration of biosurfactant in the cultural broth. Carrying out 98 batch experiments we could evaluate at the same time eight different cultural parameters. The results showed how statistic techniques of design and analysis of experiments can be very useful for optimisation problems in the biotechnological field. With the optimised broth composition we obtained a more than 16-fold increase in the biosurfactant concentration compared to the normal M1 broth, reaching a CMD value (129.43 (95% confidence interval: 119.47 – 139.40)) among the highest in literature. These results will be the starting point for the optimisation of the fermentative process in a lab-scale fermentor.

4.4. EXPERIMENTAL PROCEDURES

4.4.1. Growth conditions and culture preparation

The cultures of *Gordonia* sp. BS29 were incubated in a rotary shaker at 30°C for 24 h in LD medium (Maniatis et al., 1982). The cells were removed by centrifugation, washed twice and suspended in mineral medium. Cultures were prepared with 20 ml of mineral medium in 100 ml Erlenmeyer flasks

inoculating the cultures to an initial optical density at 600 nm (OD_{600}) equal to 0.1 and adding the necessary amount of carbon source. A part from the investigated components, the basal composition of the mineral medium was as follow: $CaCO_3$ (ppm): 0.40; $ZnSO_4 \cdot 7H_2O$ (ppm): 0.28; $MnSO_4 \cdot H_2O$ (ppm): 0.22; $CuSO_4 \cdot 5H_2O$ (ppm): 0.05; $CoSO_4 \cdot 7H_2O$ (ppm): 0.06; H_3BO_3 (ppm): 0.012.

4.4.2. Quantification of biosurfactant(s)

For each experimental design the dependent variable was the Critical Micelle Dilution (CMD) that is determined as the reciprocal of the dilution factor to achieve Critical Micelle Concentration (CMC) i.e. the minimal concentration of biosurfactant at which the formation of micelles occurs. This parameter is proportional to the amount of produced biosurfactant. CMD was measured on the crude broth and obtained by dilution method as previously described by Pagilla et al. (2002). Analyses were carried out with De Nouy ring method using K-8 tensiometer (Kruss, Hamburg, Germany). All determinations were performed at least in duplicate.

4.4.3. Design of experiments

4.4.3.1. Fractional Factorial Design (FFD)

To determine the cultural factors that have significant influence on biosurfactant(s) production, on the basis of previous literature, six major inorganic components, the carbon source and time of growth were selected to be evaluated (Table 4.2—1). According to full factorial design at two levels, 2^8 experiments should have been carried out. The number of experiments was reduced by using a one-quarter fractional design assuming that the three-order and higher interactions among factors are neglectable. The selected design was so a two level fractional factorial design with a resolution of V ($2^{(8-2)}$ R=V) composed by 64 different experiments which other 4 centre point experiments were added to. Each factor was present in the experimental design at two different levels and the values of the levels have been chosen on the basis of previous studies.

4.4.3.2. Steepest Ascendant Method (SAM)

A two level Full Factorial Design ($2^{(3)}$) with three centre points was then applied centred on the best conditions found in the previous design. This design was built with the significant factors NaCl and phosphates, testing also two different concentrations of n-hexadecane as carbon source. Table 4.2—4 shows the tested range for this design. All the other factors were kept constant at coded value of 0 according to both Eq. 4.2-1 and ranges shown in Table 4.2—1. The aim of this design was to explore the response in the region of the current optimum and find the direction in the experimental field which the response increases most rapidly in.

Then, starting from the centre of the full factorial design, eight experiments were conducted along the linear steepest ascent calculated by the first order function best fitting the Full Factorial Design data using NaCl and n-hexadecane as independent variables. Table 4.2—5 shows the conducted experiments. Both in full factorial design and in SAM all the non investigated factors were kept constant at coded value of 0 according to both Eq. 4.2-1 and ranges of Table 4.2—1.

4.4.3.3. Central Composite Design (CCD)

The aim of this experimental set was to develop an empirical model to quantify the production of biosurfactant(s) and maximise it in the selected operating region, for the most significant factors. This approach is called response surface methodology. A CCD was designed using as centre the best conditions found in SAM. The independent variables were NaCl, phosphates and n-hexadecane. Table 4.2—6 shows the values of the four levels for each factor together with coded values. The design is composed by a two level $2^{(3)}$ FFD (eight experiments), six axial experiments ($\alpha = 1.682$) and two centre point experiments.

4.4.3.4. Data Analysis

ANOVA analyses of experimental design results and regression analyses have been made using STATISTICA software.

"I've spent more time than many will believe, but I've done them with joy, and I've taken no notice those who have said why take so much trouble and what good is it?"

Antonie van Leeuwenhoek (1632 – 1723) - First microbiologist

CHAPTER 5. CONCLUSIONS

Despite their characteristics of lower toxicity, higher biodegradability, better foaming properties than the synthetic counterparts and stable activity at extremes of pH, salinity and temperature, in a very recent review "Towards commercial production of microbial surfactants" (Mukherjee et al., 2006) the authors clearly stated that a wide commercialization has not been accomplished so far. This happeneds ten years later than the potential of application of surfactants have been firstly reviewed (Desai and Banat, 1997).

Microbial surfactants share with the other biotechnological products the close link between the commercial success and efficiency of the entire biotechnological process. The production economy of every microbial metabolite is governed by three basic factors: (1) initial raw material costs, (2) availability of suitable and economic production and recovery procedures, (3) product yields of the producer microorganisms.

Mukherjee et al (2006) exhaustively reported the latest experimentations that adopted different strategies in order to make the process of biosurfactant production cost-competitive: (1) use of cheap and waste substrates (2) development of efficient bioprocesses, including optimization of fermentative condition and recovery process, (3) development of overproducing strains (Mukherjee et al., 2006). They also stated that, although a large number of biosurfactant producers have been reported in the literature, biosurfactant research (particularly the ones related to production enhancement and economics) has been confined, mostly, to a few genera of microorganisms, such as *Bacillus*, *Pseudomonas* and *Candida*. A large group of biosurfactant producers belonging to the genera *Rhodococcus*, *Gordonia*, *Candida* and *Acinetobacter* have not been exploited properly for the

economical. Biosurfactants can be effective in a wide range of applications and their possible application in drugs and medicines can lead to an extensive commercialization. In particular, the positive impacts on the environment of an extensive use of biosurfactants in remediation techniques are multiple. Besides the obvious advantage of remediating polluted environment, the release of eco-compatible molecules results in a minor impact on the environment. Furthermore, the production processes of biosurfactants are less energy-consuming and does not involve hazardous chemical.

The experiments described in this thesis have been planned according to these remarks. We in depth investigated an effective SAC producing *Gordonia* strains, tried to evaluate their products in three different environmental applications and optimized at lab-scale the cultural conditions for the biosynthesis.

The isolated *Gordonia* strains were able to grow using a wide range of straight and branched aliphatic hydrocarbons as carbon and energy source and produce at least two classes of surface-active compounds. Emulsifying agent(s) was released in the culture medium when bacteria grew both on hydrocarbons and water-soluble substrates. Cell-bound biosurfactant(s), reducing the surface tension, was produced on hydrocarbons; however, its production was significantly lower on water soluble substrates. Solvent extraction and Thin Layer Chromatography analyses allowed us to classify the cell-bound biosurfactants as glycolipids and confirm that the chemical nature of the extracellular bioemulsans is different. The relation among growth phase, surface-active compound production and cell-surface properties was analysed in kinetic experiments on hydrocarbons. *Gordonia* sp. BS29 synthesised and released extracellularly, bioemulsan(s) during the exponential phase with *n*-hexadecane as carbon and energy source. The production of biosurfactant(s) started in the exponential phase and its concentration increased during the following linear growth. Furthermore, the adhesion of bacterial cells to hydrocarbons decreased during the growth. Our results let to hypothesize a change in the mode by which *Gordonia* cells access the substrate during growth on hydrocarbons.

The applicability of our strains and SACs was evaluated for the following applications: bioremediation of (i) aliphatic and (ii) aromatic hydrocarbon

contaminated soil and (iii) washing of soil contaminated by crude oil. Bioremediation results showed that the bioemulsan is able to effectively reduce the final concentration of the recalcitrant branched hydrocarbons even if not at the same extent of rhamnolipids.

Bach soil washing experiments were carried and we compared the effect of the BS29 bioemulsans with those of the sole water and of a mixture of rhamnolipids (biosurfactant already experimented and used in soil washing techniques) on the removal of pollutants from soil. The use of a crude solution of the BS29 bioemulsans in different conditions resulted in a mean of crude oil removal of 43.1%, while the mean removal in rhamnolipid experiments was 50.8%. ANOVA test showed that both of them are, significantly, different (α=0.05) from the mean of sole water removal (19.5%). Furthermore, in bioemulsan experiments, a significant effect of time of washing was observed with a positive correlation with the extent of the oil removal. On the contrary, in the tested experimental conditions, neither the solution:soil ratio nor the time of washing significantly affects the removal in rhamolipid experiments.

Regarding the optimization of biosurfactant production, the concentrations of inorganic phosphorous and sodium chloride were found as the most important factors affecting the yield of biosurfactant biosynthesis. By a Central Composite Design (CCD), a second order polynomial function was calculated fitting the experimental data in the vicinity of the optimum. With the optimised broth composition we obtained a more than 16-fold increase in the biosurfactant concentration copared to the BH broth, reaching a CMD value (129.43 (95% confidence interval: 119.47 – 139.40)) among the highest in literature. Critically considering overall results, the most promising products that we characterized and evaluated are the extracellular bioemulsans. We demonstrated that they are highly effective in removing the crude oil from soil, even when used in crude solution. This allows us to reduce the entire cost of production being not necessary the extraction and purification steps. Furthermore, ongoing experiments are demonstrating that they can be produced on renewable and low-cost substrates and their yields of production can be increased by the optimization of cultural conditions. We believe that the information gathered up to now is an important and promising base for a future development of a microbial

bioemulsan potentialy achieving the requirements of a wide applicability and cost-competitiveness.

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"Everything is everywhere, the environment selects"
Lourens Baas-Becking (1895–1963) - Biologist

CHAPTER 6. THERMOPHILIC BACTERIA IN COOL TEMPERATE SOIL ENVIRONMENTS: ARE THEY METABOLICALLY ACTIVE OR CONTINUALLY ADDED BY GLOBAL ATMOSPHERIC TRANSPORT?

6.1. INTRODUCTION

The biogeography of plants and animals, ie. the distribution of biodiversity over space and time, has been studied for many years, however, the question whether microorganisms display similar biogeographical patterns remains unanswered (Fenchel et al., 1997). A recent review (Hughes-Martiny et al., 2006) has drawn attention to the two components of biogeographical distribution, the province and the habitat, where the province represents the legacy of historical events and the habitat the existing environment for the organism. In this review the writers have identified four possible hypotheses to describe microbial biogeography and have attempted to differentiate between the possible alternatives. They conclude that free living microorganisms do show biogeographic patterns and that, as proposed by Baas-Becking (1934) (Hughes-Martiny et al., 2006), it is the environment that selects, although the ancillary portion of the theory that 'everything is everywhere' is now disputed. If the environment is indeed responsible for 'selecting' the organisms in a particular habitat then we should expect to be able to identify the specific controlling factors for particular organisms.

Although the presence of thermophilic bacteria in cold environments has been known for many years, few investigations have been carried out to determine the physiology and the ecological roles of these organisms in

such environments. Rahman et al. (2004) found a ubiquitous presence of thermophilic bacteria in soil samples in the cool temperate soil environment of Northern Ireland; 84.6 % of the isolates were associated with the *Geobacillus* genus (Nazina et al., 2001). Using standard culture methods and assessment of aerobic growth and biomass production none of these geobacilli appears capable of growth at ambient soil temperatures. It is possible that they show very slow growth and cell division rates in the soil. The hypothesis for very slow growth, however, has not been supported so far by more sensitive molecular methods to detect activity; no aerobic physiological activity was detected either in synthetic medium or in soil microcosms below 40° C (Marchant et al., 2002). This activity was measured using expression of the inducible gene for alkane monooxygenase (using RT-PCR) as an indicator of activity, together with direct measurement of degradative activity. One of the most frequent species of *Geobacillus* recovered in the studies carried out in our laboratory has been *G. thermoleovorans*, which has also been recovered from heavy clay soils at a depth of 60cm where conditions were probably anoxic (Rahman et al., 2004). *G. thermoleovorans* has been described as having denitrifying activity (Zarilla et al., 1987) and it is possible, although unlikely, that the organism may be able to grow anaerobically using this pathway at ambient temperatures. Alternatively since these microorganisms are present in cold regions, but so far have not been shown to have any activity, it is possible that they are not autochthonous in that region, but have been transported from other environments. So far, only speculative hypotheses have been put forward. They could be residual organisms that lived in current cold regions when these regions had a different climate and now only grow in limited niches when the temperature becomes appropriate eg. in composting situations, or could be continually brought from warmer regions transported by wind or clouds. Marchant et al. (Marchant et al., 2002) reported the presence of thermophilic aerobic bacilli from soils around the world, including tropical India. The presence of bacteria in fog droplets (Fuzzi et al., 1997), and rainwater (Casareto et al., 1996; Bauer et al., 2002) has already been established although these investigations have not involved any search for thermophiles. Sattler et al. (2001) have additionally demonstrated that bacterial growth is possible in supercooled cloud droplets. The physiological characteristics of the thermophilic

geobacilli, like a Gram-positive cell wall and spore formation, support both hypotheses. It is known that spore-producing bacteria can survive for extremely long periods in the resting state and are able to resist extreme conditions like desiccation, cold and UV radiation (Saffary et al., 2002). Furthermore, it has been reported that airborne bacteria are ubiquitous in the upper atmosphere and can be found up to a height of 77 Km above the earth (Imshenetzky et al., 1978). Microorganisms can also be transported for thousands of kilometres by wind and in clouds (Bovallius et al., 1980) and could cross the oceans attached to dust (Griffin et al., 2002).

In this work thermophilic bacterial isolates were collected from rainwater samples and others from air samples and the collection times were correlated with weather conditions. The numbers of thermophiles in rainwater samples were also determined to establish whether they varied in different rainfall events. The 16S rRNA gene sequences of the isolates were then determined and compared with the already published diversity from soil (Marchant et al., 2002). The aim of the work was to try to determine whether continual long distance transport of thermophilic bacteria provides a possible mechanism for maintenance of the viable cell populations in the soil. As a final step we have examined the survival fate of geobacilli when exposed to different temperatures in the soil and in culture, again using *G. thermoleovorans* T80 as a representative strain.

6.2. RESULTS AND DISCUSSION

6.2.1. Air and Rainwater Sampling

Fifty rainwater samples for thermophilic bacteria counting were collected from 1st of February 2004 until 14th of May 2004, while eight samples for isolation were collected from 10th of October 2005 until 2nd of November 2005. Wind direction, wind speed and rainfall were further recorded during samplings. For MPN counting, thermophilic bacteria were found in detectable amount only in 9 samples. The mean value of these nine samples was 8.5 MPN/100ml, while considering the whole amount of sampled water the mean was 1.1 MPN/100ml. Furthermore, from rainfall data and MPN counting, it is possible to calculate the number of thermophilic microorganisms that have been transported onto one square meter of soil during the sampling period; giving a value of 9.5×10^3 MPN, with an

average of 140 MPN per mm of rainfall. Considering an average rainfall value in this part of Ireland of 1000 mm per year, the total annual input of thermophilic microorganisms from rainwater to soil could be estimated as 1.4 x 10^5 MPN/m^2 of soil. This value supports the hypothesis that rainfall could maintain a significant viable thermophilic population in soil. Air samplings were carried out from 6th of October 2005 until 30th October 2005 both for counting and isolation of thermophilic microorganisms, and weather conditions were also recorded. Nine air samplings were carried out with a mean value of 1.55 CFU per 1,000 l of air. Data were subsequently analyzed to evaluate correlations between the abundance of thermophilic bacteria and weather conditions. No apparent correlation between wind speed and direction was found both for rainwater and for airborne bacteria. This supports the view that, particularly for rain water, the passage of weather systems locally over land or ocean does not influence the microbial flora. On the graph, mean values of MPN counting were calculated in relation to the wind direction; almost all values are very close to the average (1.1 MPN/100ml), in each direction of the wind. Only the MPN value corresponding to a wind direction between 270° and 300° is significantly different, but the mean is calculated only with three different samplings.

6.2.2. Phylogenetic analyses of the isolates

Fourteen rainwater and fourteen airborne microorganisms, were isolated able to grow at 70° C. Partial 16S rDNA sequences (at least 900 bases) were determined to assess the microbial communities of thermophilic bacteria in air and in rainwater and compare them with the already published characterisation of thermophilic population in soil (Marchant et al.,2002). Figure 2.2-1 shows the phylogenetic tree built with the sequences of all the isolates and the sequences of some type strains that showed more then 97% sequence similarity with the isolates. In the rainwater community, all 14 isolates are assigned to *Geobacillus*; particularly, twelve of them have, as nearest phylogenetic neighbours, *Geobacillus thermodenitrificans* DSM 465^T and *Geobacillus subterraneus* T34^T while two stand very close to *Geobacillus stearothermophilus* DSM 22T^T, *Geobacillus thermocatenulatus* DSM 730^T, *Geobacillus vulcani* 3S-1^T (23), *Geobacillus kaustophilus* NCIMB 8547^T and *Geobacillus thermoleovorans* DSM 5366^T. In the air community, seven isolates

are assigned to *Bacillus*, one to *Ureibacillus* and six to *Geobacillus*. Two air isolates (A9.11 and A9.13) showed very high similarity (>99%) with a thermophilic environmental isolate submitted as *Bacillus aestuarii* (GenBank: AB062696) that was included in the tree. Considering all isolates, four Operational Taxonomic Units (OTUs) have been defined (each OTU comprised sequences that shared >97% sequence identity) by distance analysis. The two communities showed very different distributions of their isolates along the OTUs. All the isolates of the rainwater community belong to OTU 1, while in air community six isolates belong to OTU 1, one to OTU 2 , three to OTU 3 and four to OTU 4. Furthermore, only in the air samples were three microorganisms isolated able to grow at 60°C but not at 70° C, which were morphologically identified as thermotolerant actinomycetales. This distribution leads us to the conclusion that the rainwater community of thermophilic bacteria is characterized by a lower biodiversity than the air one. To confirm this lack of biodiversity in rainwater and to avoid any influence due to seasonality, another two rainwater samplings were carried out in January 2006. Ten microorganisms were isolated and 8 showed very high similarity with *Geobacillus thermodenitrificans* DSM 465^T while two isolates shared >99% sequence similarity with *Ureibacillus thermosphericus* P-11^T .

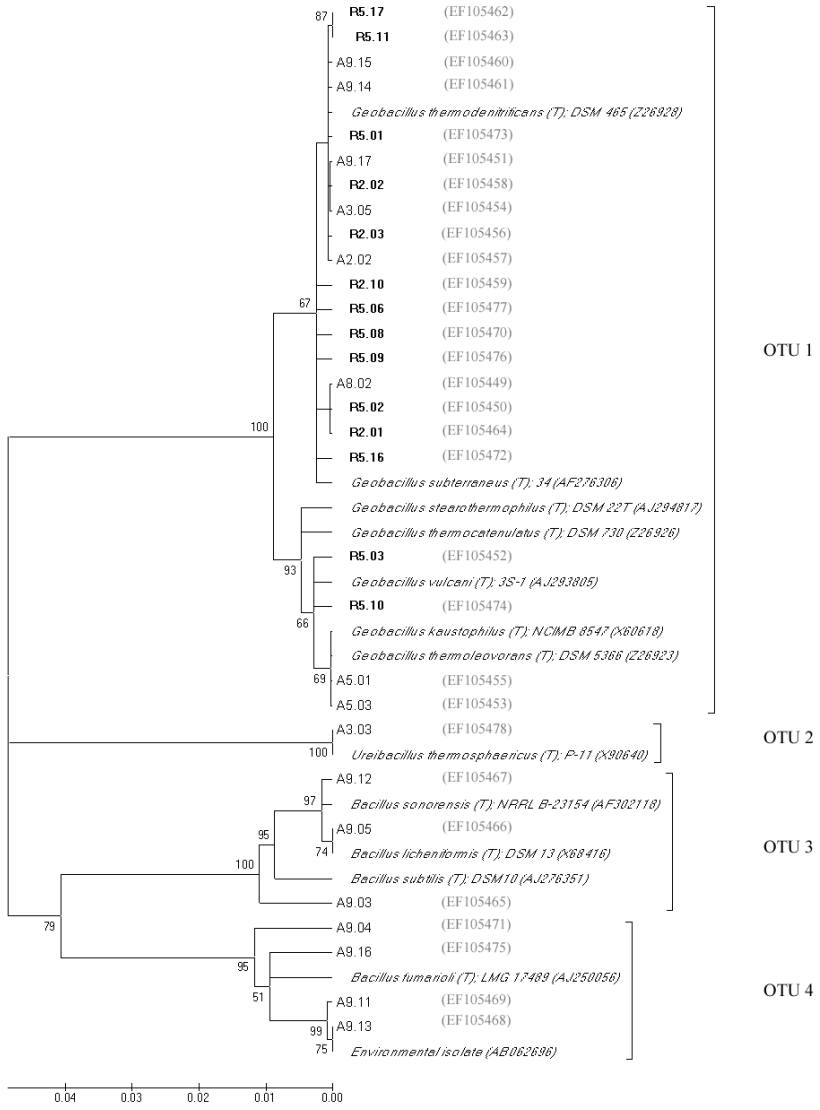

Figure 6.2-1 Unrooted phylogenetic tree based on 16S rRNA gene comparison of thermophilic isolates from rainwater (bold) and air samples Bootstrap probability values that were less than 50% were omitted from the figure. The scale bar indicates substitutions per nucleotide position.The Genbank accession numbers are reported in parenthesis.

6.2.3. Soil microcosms

Soil microcosms were set up in duplicate with one set spiked with *G. thermoleovorans* T80 and incubated at ambient temperature, 25°C, 37°C, 46°C

and 60°C. After 15 days and 4 months a sample was taken from each treatment and each temperature for separation of bacteria and staining with the FISH probes. The numerical data taken from the FISH analyses of soil microcosms maintained at various temperatures show clearly that the initial soil contained small numbers of viable geobacilli. After one week these numbers were too low to be determined by the FISH counting methodology in all but the microcosm kept at 60°C where growth had taken place (Figure 6). Similarly after 4 months, in the unspiked microcosms, geobacillus numbers had increased at 46°C and 60°C indicating that continuing growth had taken place. Even after 4 months geobacilli remained undetectable at the lower temperatures. In the microcosms spiked with *G. thermoleovorans* it is possible to see surviving viable geobacilli at all temperatures after one week but after 4 months populations can only be seen at 60°C and to a lesser extent at 46°C.

6.3. DISCUSSION

The objective of this work was to evaluate whether the long range transport by winds and cloud droplets could produce a viable population of thermophilic bacteria in cold environments. Rahman et al. (2004) reported that 84.6% of the highly thermophilic microorganisms isolated from Irish soil can be assigned to *Geobacillus*. The structure of the rainwater community seems to be more similar to the structure of the soil community than does the air community. Furthermore, Saffary et al. (2002) demonstrated that microorganisms belonging to *Geobacillus* are extremely resistant to space vacuum, UV radiation and gamma-ray exposure and are the only microorganisms selected from hot environments under these extreme conditions. The authors speculated that this kind of organism could be distributed by atmospheric recirculation due to their ability to survive prolonged exposure in the stratosphere. Also Bonjour et al. (1988) hypothesized wind dispersion of thermophilic microorganisms, even over long distances. The low biodiversity and the kinds of bacteria isolated from rainwater samples seem to demonstrate that the transport in cloud droplets and the deposition by rainwater provides a continual supply of thermophilic microorganisms to cold region soils and the low biodiversity

of these populations is due to the high selective pressure of stratospheric environments. Since the geobacilli are spore formers it is possible that spores are the sole means of global transport, however, work in our laboratory (unpublished) has shown that vegetative cells of *Geobacillus* are also highly resistant to adverse conditions. The results from the FISH labelling indicate that at least some of the viable geobacilli in rainwater may be vegetative cells. Isolated individual spores are difficult to visualise due the debris in the rainwater, but they may also be present.

To the best of our knowledge, the only published study dealing with the characterisation of a microbial thermophilic community in the atmospheric environment is by Andersson et al. In a Southern Finland urban air population, the authors found many types of microorganisms able to grow at 50°C similar to the types that we isolated in Northern Ireland; in particularly, *Ureibacillus thermosphericus,* formerly *Bacillus thermosphericus,* (12), *Bacillus licheniformis* and thermotolerant actinomycetales were found as dominant. The ubiquity and the relatively low biodiversity of these populations suggest, for the air environment, mechanisms of distribution at shorter range and with lower selective pressure than rainwater associated transport. Furthermore, airborne thermophilic microorganisms do not seem to be a source for the viable population in soil. Without taking samples directly from cloud droplets at high altitude, something beyond the scope of this study, it is not possible to eliminate the possibility that organisms isolated from rainwater at ground level have not been washed from the air by falling raindrops. However, if this were the case the numbers of organisms in rainwater during periods of prolonged rainfall would diminish. This was not something we observed and coupled with the differences in species diversity support the view that the rainwater bacteria have been transported probably long distances in the upper atmosphere.

The numerical data taken from the FISH analyses of soil microcosms maintained at various temperatures show clearly that the initial soil contained small numbers of viable geobacilli. After one week these numbers were too low to be determined by the FISH counting methodology in all but the microcosm kept at 60^{o}C where growth had taken place. Similarly after 4 months, in the unspiked microcosms, geobacillus numbers had increased at 46^{o}C and 60^{o}C indicating that continuing growth had taken place. Even after 4 months geobacilli remained undetectable at the

lower temperatures. In the microcosms spiked with *G. thermoleovorans* it is possible to see surviving viable geobacilli at all temperatures after one week but after 4 months populations can only be seen at 60°C and to a lesser extent at 46°C.

6.4. CONCLUSIONS

For these thermophilic geobacilli it appears that the precept 'everything is everywhere' does hold since the evidence supports long distance global transport and survival. It also seems that the environment is not specifically selecting these organisms, since the majority of cells are deposited by rainwater into cold environments where they can, at best, grow extremely slowly and survive. There will be infrequent situations, such as composting plant material, where the temperature conditions would be suitable for rapid growth. A number of hypotheses has been advanced to explain the distribution of microorganisms and they should not be treated as mutually exclusive since different mechanisms may apply to different microorganisms.

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soprattutto per quanto sta facendo ora in mia assenza a Milano! Voto: 10. Prezioso!

Grazie moltissimo a Silvia, Valeria e Marco (in rigoroso ordine cronologico) che nel nostro laboratorio hanno lavorato (Marco lo sta ancora facendo) per preparare la loro tesi di laurea. Tutti i loro dati sono in questa tesi che è quindi, in parte, anche loro! Per il loro impegno, serietà, intraprendenza e doti umane…. mi ritengo molto fortunato ad aver lavorato con loro.

In questi anni gli abitanti del laboratorio e del quinto piano sono stati un ambiente rilassante, interessante e … a volte… confortante. Il merito è di Anita, Chiara, Chicca, Claudia, Patrizia, Silvia B., Silvia F., Valeria, Maurizio, Paride. Purtroppo il mero elenco non rende loro giustizia …

And now… let's talk about Ireland! It was a great time! When you are alone, stranger in a country like Ireland… it depends on the people you meet. Another time I was very lucky!!

In university I felt like at home! Thanks to Prof. Banat and Marchant. They provided us all we need. Thank you for your kindness and for every times you were available for us. Thank you also to Sue and Welma… the kindest technicians I've never met!.....and I can't forget my lab-mates, Sarah, Noemi, Olaf and David.

Last but not least….Alì! You were (are) a very good friend!! Thank you very much for all the times we worked, joked, lunched together. (I can assure.. you have never eaten pork!). Thank you very much for every time you trusted me! I hope to meet you soon!

Un ringraziamento particolare va ad Andrea che è stato prima un aiuto, poi un amico che ho molto apprezzato per la disponibilità, la curiosità e per avermi coinvolto nella vita che si era costruito in Irlanda…. e per questo lieve accento romagnolo che molti dicono che mi è rimasto. Thank you to Rachel and Dan. They showed me the strange and fascinating way of living in Northern Ireland!

David… we can't say that you are a perfect landlord… but you are a valuable man.. and you know it. Thank you to you and your beautiful children, Freya and Oliver. You were my Irish family. Thank you for having considered me a friend, not a tenant.

Sembra che questa tesi sia finita… qualche ritocco e verrà spedita… Alessia mi ha aiutato molto per darle la veste grafica che ha oggi. In queste righe la ringrazio solo per questo…..

Nicolò e Carmen mi hanno fatto un sacco di favori. Ultimo di questi… la stampa sul CD… Vi ringrazio qui per tutto!

Ringrazio moltissimo Edi, è stata veramente gentile a rivedere l'intera tesi e correggere gli errori di inglese!

Sullo sfondo di tutto questo c'è la mia famiglia… ultimamente non mi hanno visto molto… ma quando ho avuto bisogno sono sempre stati prontissimi, anche a distanza (quante volte mio fratello ha fatto il corriere tra casa e Milano!?). Il ringraziamento più grande va a loro!

ANNEX 1- ADDITIONAL DATA

In this Annex the crude data of the elaborated results presented in the above chapters are reported.

CHAPTER 2

1. GenBank accession number and partial 16S rRNA sequence of *Gordonia* sp. strains

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partial sequence.
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VERSION   EF064794
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REFERENCE  1 (bases 1 to 1449)
AUTHORS       Tamburini,E.,   Caredda,P.,   La   Colla,P.,
Franzetti,A. and Bestetti,G.
TITLE     Surface-active compounds and hydrocarbon uptake
mechanisms in
      Gordonia strains
JOURNAL  Unpublished
REFERENCE  2 (bases 1 to 1449)
AUTHORS       Tamburini,E.,   Caredda,P.,   La   Colla,P.,
Franzetti,A. and Bestetti,G.
TITLE   Direct Submission
JOURNAL  Submitted (18-OCT-2006) Department of Biomedical
Sciences and
Technologies,   University   of   Cagliari,   Cittadella
Universitaria
 SS554, Monserrato, Cagliari, Italy
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REFERENCE  1 (bases 1 to 1398)
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Franzetti,A. and Bestetti,G.
 TITLE    Surface-active compounds and hydrocarbon uptake
mechanisms in
      Gordonia strains
 JOURNAL  Unpublished
REFERENCE  2 (bases 1 to 1398)
 AUTHORS      Tamburini,E.,   Caredda,P.,   La   Colla,P.,
Franzetti,A. and Bestetti,G.
 TITLE   Direct Submission
 JOURNAL  Submitted (18-OCT-2006) Department of Biomedical
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      Technologies,  University  of  Cagliari,  Cittadella
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REFERENCE  1 (bases 1 to 1421)
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Franzetti,A. and Bestetti,G.
 TITLE    Surface-active compounds and hydrocarbon uptake
mechanisms in
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 JOURNAL  Unpublished
REFERENCE  2 (bases 1 to 1421)
 AUTHORS      Tamburini,E.,   Caredda,P.,   La   Colla,P.,
Franzetti,A. and Bestetti,G.
 TITLE   Direct Submission
 JOURNAL  Submitted (18-OCT-2006) Department of Biomedical
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CHAPTER 3

1. Residual concentrations of aliphatic hydrocarbons in microcosms experiments

	Residual concentration (mg/kg) at time 0					
Treatment	**n-hexadecane**	**n-heptadecane**	**pristane**	**n-eicosane**	**n-octacosane**	**squalene**
TQ 1	256	276	272	274	291	289
TQ 2	261	277	274	275	265	266
TQ 3	232	247	243	243	248	247
Mean	**250**	**267**	**263**	**264**	**268**	**267**
St. Dev	**15**	**17**	**17**	**18**	**22**	**21**
M22 1	171	189	253	221	242	242
M22 2	189	210	271	244	289	291
M22 3	184	201	260	234	300	286
Mean	**182**	**200**	**261**	**233**	**277**	**273**
St. Dev	**9**	**10**	**9**	**11**	**31**	**27**
BS29 1	150	161	261	164	194	255
BS29 2	146	156	259	156	165	225
BS29 3	141	153	252	156	200	263
Mean	**146**	**156**	**258**	**159**	**186**	**247**
St. Dev	**5**	**4**	**5**	**5**	**18**	**20**
R 1	246	260	256	260	274	267
R 2	263	289	284	294	301	302
R 3	276	293	289	287	299	303
Mean	**262**	**281**	**276**	**281**	**291**	**291**
St. Dev	**15**	**18**	**18**	**18**	**15**	**20**
BEL 1	85	95	324	107	315	248
BEL 2	72	80	280	90	285	203
BEL 3	70	78	277	90	302	213
Mean	**76**	**84**	**294**	**96**	**301**	**221**
St. Dev	**8**	**9**	**26**	**10**	**15**	**23**
BEH 1	139	149	251	157	271	208
BEH 2	141	153	266	161	291	208
BEH 3	151	165	274	173	273	216
Mean	**143**	**156**	**264**	**164**	**278**	**211**
St. Dev	**7**	**8**	**11**	**8**	**11**	**5**

	Residual concentration (mg/kg) at time 8 days					
Treatment	**n-hexadecane**	**n-heptadecane**	**pristane**	**n-eicosane**	**n-octacosane**	**squalene**
TQ 1	27.3	32.8	136.3	34.0	62.5	50.1
TQ 2	27.0	32.7	145.8	34.1	70.7	61.2
TQ 3	25.8	31.2	152.2	33.5	74.6	60.2
Mean	**26.7**	**32.2**	**144.8**	**33.9**	**69.3**	**57.1**
St. Dev	**0.8**	**0.9**	**8.0**	**0.3**	**6.2**	**6.1**
M22 1	30.3	36.1	111.7	38.2	80.7	60.4
M22 2	30.2	36.3	110.6	38.5	73.0	56.2
M22 3	31.0	37.6	126.8	40.4	78.1	64.1
Mean	**30.5**	**36.7**	**116.3**	**39.0**	**77.3**	**60.2**
St. Dev	**0.4**	**0.8**	**9.0**	**1.2**	**3.9**	**3.9**
BS29 1	28.2	34.1	124.6	35.1	64.8	56.2
BS29 2	29.5	36.2	131.7	37.4	70.0	60.4
BS29 3	25.0	30.5	126.4	31.0	59.5	49.2
Mean	**27.6**	**33.6**	**127.5**	**34.5**	**64.8**	**55.3**
St. Dev	**2.3**	**2.8**	**3.7**	**3.3**	**5.2**	**5.7**
R 1	31.0	37.5	119.5	41.2	94.5	71.6
R 2	36.6	43.6	140.1	47.9	104.6	79.8
R 3	24.8	30.3	115.9	31.7	79.4	58.7
Mean	**30.8**	**37.1**	**125.2**	**40.3**	**92.8**	**70.0**
St. Dev	**5.9**	**6.7**	**13.1**	**8.1**	**12.7**	**10.7**
BEL 1	27.7	33.7	205.7	36.3	71.7	78.6
BEL 2	24.5	30.0	198.1	32.3	60.8	72.6
BEL 3	22.5	28.0	184.5	30.2	63.7	69.1
Mean	**24.9**	**30.6**	**196.1**	**32.9**	**65.4**	**73.4**
St. Dev	**2.6**	**2.9**	**10.7**	**3.1**	**5.7**	**4.8**
BEH 1	16.5	21.0	78.3	20.4	42.6	32.3
BEH 2	15.7	20.0	69.8	19.1	31.3	27.0
BEH 3	nd	nd	70.6	18.7	31.2	25.5
Mean	**16.1**	**20.5**	**72.9**	**19.4**	**35.0**	**28.3**
St. Dev	**0.6**	**0.7**	**4.7**	**0.9**	**6.6**	**3.6**

	Residual concentration (mg/kg) at time 19 days					
Treatment	**n-hexadecane**	**n-heptadecane**	**pristane**	**n-eicosane**	**n-octacosane**	**squalene**
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TQ 2	<dl	<dl	22.8	<dl	8.4	5.7
TQ 3	<dl	<dl	17.7	<dl	5.5	4.9
Mean	**<dl**	**<dl**	**20.6**	**<dl**	**6.6**	**5.4**
St. Dev	**<dl**	**<dl**	**2.6**	**<dl**	**1.6**	**0.4**
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M22 2	<dl	<dl	21.5	<dl	7.2	5.5
M22 3	<dl	<dl	29.1	4.1	7.8	7.1
Mean	**<dl**	**<dl**	**25.5**	**2.6**	**7.3**	**6.3**
St. Dev	**<dl**	**<dl**	**3.8**	**2.3**	**0.4**	**0.8**
BS29 1	3.5	4.6	33.6	4.7	7.3	7.9
BS29 2	4.0	5.2	40.8	5.2	7.7	9.0
BS29 3	<dl	<dl	23.6	3.9	5.9	4.9
Mean	**2.5**	**3.2**	**32.6**	**4.6**	**7.0**	**7.3**
St. Dev	**2.2**	**2.8**	**8.7**	**0.7**	**0.9**	**2.1**
R 1	2.9	3.9	31.6	4.1	9.0	8.9
R 2	<dl	<dl	22.5	3.7	8.0	7.1
R 3	<dl	<dl	22.4	3.7	7.7	6.8
Mean	**1.0**	**1.3**	**25.5**	**3.8**	**8.2**	**7.6**
St. Dev	**1.7**	**2.3**	**5.3**	**0.2**	**0.7**	**1.2**
BEL 1	5.1	6.4	63.7	6.4	9.3	14.4
BEL 2	4.0	4.8	48.4	4.8	8.6	10.4
BEL 3	3.9	4.9	45.4	4.9	7.2	11.1
Mean	**4.3**	**5.3**	**52.5**	**5.4**	**8.4**	**11.9**
St. Dev	**0.7**	**0.9**	**9.8**	**0.9**	**1.1**	**2.2**
BEH 1	<dl	<dl	17.6	3.9	6.0	8.3
BEH 2	<dl	<dl	16.7	7.4	6.0	17.5
BEH 3	3.2	<dl	16.3	7.5	5.6	15.0
Mean	**1.1**	**<dl**	**16.9**	**6.3**	**5.8**	**13.6**
St. Dev	**1.9**	**<dl**	**0.6**	**2.1**	**0.2**	**4.7**

	Residual concentration (mg/kg) at time 56 days					
Treatment	**n-hexadecane**	**n-heptadecane**	**pristane**	**n-eicosane**	**n-octacosane**	**squalene**
TQ 1	<dl	<dl	4.1	2.4	<dl	0.3
TQ 2	<dl	<dl	5.2	3.0	<dl	3.4
TQ 3	<dl	<dl	3.8	2.5	<dl	0.3
Mean	<dl	<dl	4.4	2.6	<dl	1.3
St. Dev	<dl	<dl	0.7	0.3	<dl	1.8
M22 1	<dl	<dl	3.9	4.5	<dl	3.0
M22 2	<dl	<dl	4.3	3.4	<dl	0.3
M22 3	<dl	<dl	5.0	3.5	<dl	0.3
Mean	<dl	<dl	4.4	3.8	<dl	1.2
St. Dev	<dl	<dl	0.6	0.6	<dl	1.6
BS29 1	<dl	<dl	4.9	3.0	3.1	3.6
BS29 2	<dl	<dl	5.6	3.7	3.2	<dl
BS29 3	<dl	<dl	6.4	2.9	5.7	3.3
Mean	<dl	<dl	5.6	3.2	4.0	2.3
St. Dev	<dl	<dl	0.7	0.4	1.5	2.0
R 1	<dl	<dl	1.3	0.9	<dl	1.2
R 2	<dl	<dl	1.1	3.5	<dl	0.3
R 3	<dl	<dl	1.6	1.8	2.7	3.1
Mean	<dl	<dl	1.4	2.1	0.9	1.5
St. Dev	<dl	<dl	0.2	1.3	1.6	1.5
BEL 1	<dl	<dl	5.5	3.2	<dl	3.0
BEL 2	<dl	<dl	6.6	5.1	<dl	3.2
BEL 3	<dl	<dl	5.9	5.7	<dl	2.8
Mean	<dl	<dl	6.0	4.7	<dl	3.0
St. Dev	<dl	<dl	0.5	1.3	<dl	0.2
BEH 1	<dl	<dl	2.4	3.6	<dl	6.1
BEH 2	<dl	<dl	3.0	5.2	<dl	0.3
BEH 3	<dl	<dl	3.1	6.5	<dl	4.7
Mean	<dl	<dl	2.8	5.1	<dl	3.7
St. Dev	<dl	<dl	0.4	1.5	<dl	3.1

2. Residual concentrations of polycyclic aromatic hydrocarbons in microcosms experiments

	Residual concentration (mg/kg) at time 0			
Treatment	**Phenanthrene**	**Anthracene**	**Pyrene**	**Benzo[a]Pyrene**
TQ 1	196	320	186	52
TQ 2	174	310	179	56
TQ 3	178	306	169	46
Mean	**183**	**312**	**178**	**51**
St. Dev	**12**	**7**	**9**	**5**
R 1	300	366	286	100
R 2	306	377	279	111
R 3	293	362	283	114
Mean	**300**	**368**	**283**	**108**
St. Dev	**7**	**8**	**3**	**8**
BL 1	257	339	229	50
BL 2	257	354	238	62
BL 3	257	354	235	62
Mean	**257**	**349**	**234**	**58**
St. Dev	**0**	**9**	**5**	**7**
BH 1	260	351	252	108
BH 2	253	333	239	88
BH 3	288	378	274	112
Mean	**267**	**354**	**255**	**103**
St. Dev	**19**	**23**	**18**	**13**

	Residual concentration (mg/kg) at time 35 days			
Treatment	**Phenanthrene**	**Anthracene**	**Pyrene**	**Benzo[a]Pyrene**
TQ 1	19.5	325.6	159.2	79.5
TQ 2	24.5	245.2	121.1	51.4
TQ 3	nd	nd	nd	nd
Mean	**14.7**	**190.3**	**93.4**	**43.6**
St. Dev	**12.9**	**169.6**	**83.1**	**40.3**
BEL 1	26.4	377.0	227.2	90.5
BEL 2	24.5	288.1	171.0	58.2
BEL 3	23.0	265.1	147.8	52.6
Mean	**24.6**	**310.1**	**182.0**	**67.1**
St. Dev	**1.7**	**59.1**	**40.9**	**20.4**
BEH 1	25.4	354.8	282.9	91.4
BEH 2	28.7	364.3	304.1	96.5
BEH 3	27.2	370.8	294.9	75.7
Mean	**27.1**	**363.3**	**294.0**	**87.9**
St. Dev	**1.7**	**8.0**	**10.6**	**10.8**

	Residual concentration (mg/kg) at time 75 days			
Treatment	**Phenanthrene**	**Anthracene**	**Pyrene**	**Benzo[a]Pyrene**
TQ 1	10.6	132.0	4.7	28.0
TQ 2	nd	nd	nd	nd
TQ 3	3.9	138.5	3.2	38.9
Mean	**7.2**	**135.2**	**3.9**	**33.5**
St. Dev	**4.8**	**4.6**	**1.0**	**7.7**
R 1	8.5	265.1	122.9	45.4
R 2	10.1	309.0	229.6	82.0
R 3	10.0	299.0	187.4	66.0
Mean	**9.5**	**291.0**	**180.0**	**64.5**
St. Dev	**0.9**	**23.0**	**53.7**	**18.3**
BEL 1	8.6	228.7	112.4	46.0
BEL 2	9.2	261.8	151.8	55.0
BEL 3	0.7	77.3	33.5	0.0
Mean	**6.2**	**189.3**	**99.2**	**33.7**
St. Dev	**4.8**	**98.3**	**60.2**	**29.5**
BEH 1	6.6	189.6	66.4	25.9
BEH 2	8.2	236.4	82.1	39.3
BEH 3	7.0	201.2	78.0	28.4
Mean	**7.3**	**209.1**	**75.5**	**31.2**
St. Dev	**0.8**	**24.4**	**8.1**	**7.1**

3. Percentage of removal in soil-washing experiments of crude oil contaminated soil

Washing solution	Ratio (v:w)	Time (min)	% Removal
Water	10	70	16,4
Water	10	30	20,4
Water	10	50	23,6
Water	15	70	20,2
Water	15	30	19,1
Water	15	50	19,1
Water	20	70	16,6
Water	20	30	21,3
Water	20	50	38,9
Rhamnolipids (2%)	10	70	43,2
Rhamnolipids (2%)	10	30	41,4
Rhamnolipids (2%)	10	50	63,9
Rhamnolipids (2%)	15	70	58,9
Rhamnolipids (2%)	15	30	40,5
Rhamnolipids (2%)	15	50	57,1
Rhamnolipids (2%)	20	70	44,5
Rhamnolipids (2%)	20	30	57,0
Rhamnolipids (2%)	20	50	50,5
Crude bioemulsan (2%)	10	70	51,2
Crude bioemulsan (2%)	10	30	41,9
Crude bioemulsan (2%)	10	50	43,0
Crude bioemulsan (2%)	15	70	48,1
Crude bioemulsan (2%)	15	30	31,7
Crude bioemulsan (2%)	15	50	46,9
Crude bioemulsan (2%)	20	70	44,5
Crude bioemulsan (2%)	20	30	30,7
Crude bioemulsan (2%)	20	50	49,4
Water	15	50	21,2
Water	15	50	17,3
Rhamnolipids (2%)	15	50	52,5
Rhamnolipids (2%)	15	50	48,7
Crude bioemulsan (2%)	15	50	22,8
Crude bioemulsan (2%)	15	50	30,5

CHAPTER 4

1. CMD results in two level fractional design - $2^{(8-2)}$ FFD

$NaNO_3$	$(NH_4)_2SO_4$	Phosphates	$MgSO_4$	$FeSO_4$	NaCl	Carbon source	Time	CMD
-1	-1	-1	-1	-1	-1	n-hexadecane	1	5,3
1	-1	-1	-1	-1	-1	glycerol	-1	< 1
-1	1	-1	-1	-1	-1	glycerol	-1	< 1
1	1	-1	-1	-1	-1	n-hexadecane	1	3,1
-1	-1	1	-1	-1	-1	glycerol	1	< 1
1	-1	1	-1	-1	-1	n-hexadecane	-1	59,7
-1	1	1	-1	-1	-1	n-hexadecane	-1	73,2
1	1	1	-1	-1	-1	glycerol	1	< 1
-1	-1	-1	1	-1	-1	glycerol	1	< 1
1	-1	-1	1	-1	-1	n-hexadecane	-1	70,9
-1	1	-1	1	-1	-1	n-hexadecane	-1	< 1
1	1	-1	1	-1	-1	glycerol	1	< 1
-1	-1	1	1	-1	-1	n-hexadecane	1	88,3
1	-1	1	1	-1	-1	glycerol	-1	< 1
-1	1	1	1	-1	-1	glycerol	-1	5,3
1	1	1	1	-1	-1	n-hexadecane	1	88,9
-1	-1	-1	-1	1	-1	n-hexadecane	-1	< 1
1	-1	-1	-1	1	-1	glycerol	1	< 1
-1	1	-1	-1	1	-1	glycerol	1	< 1
1	1	-1	-1	1	-1	n-hexadecane	-1	28,9
-1	-1	1	-1	1	-1	glycerol	-1	< 1
1	-1	1	-1	1	-1	n-hexadecane	1	141,4
-1	1	1	-1	1	-1	n-hexadecane	1	95,5
1	1	1	-1	1	-1	glycerol	-1	11,4
-1	-1	-1	1	1	-1	glycerol	-1	6,4
1	-1	-1	1	1	-1	n-hexadecane	1	< 1,5
-1	1	-1	1	1	-1	n-hexadecane	1	7,3
1	1	-1	1	1	-1	glycerol	-1	< 1
-1	-1	1	1	1	-1	n-hexadecane	-1	63,9
1	-1	1	1	1	-1	glycerol	1	< 1
-1	1	1	1	1	-1	glycerol	1	< 1
1	1	1	1	1	-1	n-hexadecane	-1	25,3
-1	-1	-1	-1	-1	1	n-hexadecane	-1	< 1
1	-1	-1	-1	-1	1	glycerol	1	< 1
-1	1	-1	-1	-1	1	glycerol	1	< 1
1	1	-1	-1	-1	1	n-hexadecane	-1	< 1
-1	-1	1	-1	-1	1	glycerol	-1	< 1
1	-1	1	-1	-1	1	n-hexadecane	1	23,7
-1	1	1	-1	-1	1	n-hexadecane	1	11,4

1	1	1	-1	-1	1	glycerol	-1	< 1
-1	-1	-1	1	-1	1	glycerol	-1	1,1
1	-1	-1	1	-1	1	n-hexadecane	1	58,5
-1	1	-1	1	-1	1	n-hexadecane	1	7,7
1	1	-1	1	-1	1	glycerol	-1	< 1
-1	-1	1	1	-1	1	n-hexadecane	-1	17,4
1	-1	1	1	-1	1	glycerol	1	3,3
-1	1	1	1	-1	1	glycerol	1	< 1
1	1	1	1	-1	1	n-hexadecane	-1	28,5
-1	-1	-1	-1	1	1	n-hexadecane	1	4
1	-1	-1	-1	1	1	glycerol	-1	< 1
-1	1	-1	-1	1	1	glycerol	-1	< 1
1	1	-1	-1	1	1	n-hexadecane	1	2, 1
-1	-1	1	-1	1	1	glycerol	1	< 1
1	-1	1	-1	1	1	n-hexadecane	-1	4,2
-1	1	1	-1	1	1	n-hexadecane	-1	2
1	1	1	-1	1	1	glycerol	1	< 1
-1	-1	-1	1	1	1	glycerol	1	< 1
1	-1	-1	1	1	1	n-hexadecane	-1	1,2
-1	1	-1	1	1	1	n-hexadecane	-1	1,2
1	1	-1	1	1	1	glycerol	1	< 1
-1	-1	1	1	1	1	n-hexadecane	1	80,6
1	-1	1	1	1	1	glycerol	-1	< 1
-1	1	1	1	1	1	glycerol	-1	< 1
1	1	1	1	1	1	n-hexadecane	1	19,2
0	0	0	0	0	0	glycerol	0	2,5
0	0	0	0	0	0	n-hexadecane	0	38,6
0	0	0	0	0	0	glycerol	0	< 1
0	0	0	0	0	0	n-hexadecane	0	10, 1

CHAPTER 6

1. GenBank accession number and partial 16S rRNA sequence of isolated tehermophiles

AUTHORS Marchant,R., Franzetti,A., Pavlostathis,S., Okutmanas,D., Erdbrugger,I., Unyayar,A. and Banat,I.

DEFINITION Geobacillus sp. A8.02 16S ribosomal RNA gene, partial sequence.
ACCESSION EF105449
ccagcggaagaaagagagcttgctctttcctcggtcagcggcggacgggtgagtaacacgtgggcaacctgcc
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DEFINITION Geobacillus sp. R5.02 16S ribosomal RNA gene, partial sequence.
ACCESSION EF105450
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DEFINITION Geobacillus sp. A9.17 16S ribosomal RNA gene, partial sequence.
ACCESSION EF105451
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DEFINITION Geobacillus sp. R5.03 16S ribosomal RNA gene, partial
sequence.
ACCESSION EF105452
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DEFINITION Geobacillus sp. A5.03 16S ribosomal RNA gene, partial
sequence.
ACCESSION EF105453
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DEFINITION Geobacillus sp. A3.05 16S ribosomal RNA gene, partial
sequence.
ACCESSION EF105454

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DEFINITION Geobacillus sp. A5.01 16S ribosomal RNA gene, partial
sequence.
ACCESSION EF105455
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DEFINITION Geobacillus sp. R2.03 16S ribosomal RNA gene, partial
sequence.
ACCESSION EF105456
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DEFINITION Geobacillus sp. A2.02 16S ribosomal RNA gene, partial
sequence.
ACCESSION EF105457

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DEFINITION Geobacillus sp. R2.10 16S ribosomal RNA gene, partial
sequence.
ACCESSION EF105459
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DEFINITION Geobacillus sp. A9.15 16S ribosomal RNA gene, partial
sequence.
ACCESSION EF105460
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DEFINITION Geobacillus sp. A9.14 16S ribosomal RNA gene, partial
sequence.
ACCESSION EF105461
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DEFINITION Geobacillus sp. R5.17 16S ribosomal RNA gene, partial
sequence.
ACCESSION EF105462
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DEFINITION Geobacillus sp. R5.11 16S ribosomal RNA gene, partial
sequence.
ACCESSION EF105463
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DEFINITION Geobacillus sp. R2.01 16S ribosomal RNA gene, partial
sequence.
ACCESSION EF105464
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DEFINITION Bacillus sp. A9.03 16S ribosomal RNA gene, partial sequence.
ACCESSION EF105465
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DEFINITION Bacillus sp. A9.05 16S ribosomal RNA gene, partial sequence.
ACCESSION EF105466
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DEFINITION Bacillus sp. A9.12 16S ribosomal RNA gene, partial sequence.
ACCESSION EF105467
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DEFINITION Bacillus sp. A9.13 16S ribosomal RNA gene, partial sequence.
ACCESSION EF105468
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acgggtgagtaacacgtgggcaacctgcctgtaagactgggataacttcgggaaaccggagctaataccggat
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g//

DEFINITION Bacillus sp. A9.11 16S ribosomal RNA gene, partial sequence.
ACCESSION EF105469
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cggaggaaggtggggatgacgtcaaatcatcatgcccctatgacctgggctacacacgtgctacaatggatg
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DEFINITION Geobacillus sp. R5.08 16S ribosomal RNA gene, partial sequence.
ACCESSION EF105470
ggtcaggggcggacgggtgagtaacacgtgggcaacctgcccgcaagaccgggataactccgggaaaccggag
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DEFINITION Bacillus sp. A9.04 16S ribosomal RNA gene, partial sequence.
ACCESSION EF105471
gcggcggacgggtgagtaacacgtgggcaacctgcctgtaagactgggataacttcgggaaaccggagctaat
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DEFINITION Geobacillus sp. R5.16 16S ribosomal RNA gene, partial sequence.
ACCESSION EF105472
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DEFINITION Geobacillus sp. R5.01 16S ribosomal RNA gene, partial sequence.
ACCESSION EF105473
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DEFINITION Geobacillus sp. R5.10 16S ribosomal RNA gene, partial
sequence.
ACCESSION EF105474
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DEFINITION Bacillus sp. A9.16 16S ribosomal RNA gene, partial sequence.
ACCESSION EF105475
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DEFINITION Geobacillus sp. R5.09 16S ribosomal RNA gene, partial
sequence.
ACCESSION EF105476
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DEFINITION Geobacillus sp. R5.06 16S ribosomal RNA gene, partial
sequence.

ACCESSION EF105477
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ccttaccaggtcttgacatcccctgacaacccaagagattgggcgttccccccttcgggggacagggtgacag
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DEFINITION Ureibacillus sp. A3.03 16S ribosomal RNA gene, partial
sequence.
ACCESSION EF105478
VERSION   EF105478
Gcaacgatgggggcccgcttaatacatgcaagtccacccgaccaagagaaagcctagctttcttcaggttagc
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ANNEX 2 – PUBLICATIONS AND PRESENTATIONS

PUBLICATIONS

1. **A. Franzetti**, G. Bestetti, P. Caredda, P. La Colla, E. Tamburini (2007) "Surface-active compounds and their role in bacterial access to hydrocarbons in *Gordonia* strains" FEMS MICROBIOLOGY ECOLOGY – Accepted for publication – 3 October 2007

2. **A. Franzetti**, G. Bestetti, P. Caredda, E. Tamburini *Production of biosurfactants by bacteria belonging to Gordonia and Rhodococcus genera* proccedings of "Third European Bioremediation Conference" 4-7 July 2005 - Chania, Greece

PRESENTATIONS TO CONFERENCES

1. **A. Franzetti**, P. Caredda, E. Tamburini, P. La Colla, G. Bestetti *Application of Surface Active Compounds (SACs) produced by Gordonia sp. BS29 in soil remediation* Poster to IX Conference of Federazione Italiana Scienze della Vita (FISV) 26-29 September 2007 – Riva del Garda

2. E. Tamburini, C. Ruggeri, P. La Colla, **A. Franzetti**, G. Bestetti, *Surface Active Compuonds production and hydrocarbon uptake mechanisms in bacteria belonging to the Gordonia genus.* Oral presentation to the VIII Conference Federazione Italiana Scienze della Vita (FISV) 28 Settembre – 1 Ottobre 2006 – Riva del Garda

3. **A. Franzetti**, G. Bestetti, P. Caredda, E. Tamburini, P. La Colla *Production of biosurfactants with high emulsification activity by bacteria belonging to Gordonia and Rhodococcus genera.* Oral presentation to the " First Mediterranean congress on biotechnology" 25-29 March 2006 – Hammamet, Tunisia,

4. **A. Franzetti**, G. Bestetti, P. Caredda, E. Tamburini, P. La Colla *Effect of cultural broth composition on biosurfactant production by Gordonia sp. strain*

poster " First Mediterranean congress on biotechnology" 25-29 March 2006 – Hammamet,Tunisia

5. **A. Franzetti**, P. Caredda, P. La Colla, C. Ruggeri, E. Tamburini, G. Bestetti *Surface Active Compounds (SACs) produced by Gordonia sp. BS29 as enhancer in bioremediation of hydrocarbon contaminated soils.* Oral presentation to the " II International Conference on Environmental, Industrial and Applied Microbiology (BioMicroWorld2007)" 27 November – 1 December 2007 Seville, Spain

6. **A. Franzetti**, G. Bestetti, P. Caredda, P. La Colla, M. Pintus, C. Ruggeri, MT. Tedde, E. Tamburini *Isolation and screening of SAC producing bacteria on renewable substrates.* Poster to the " II International Conference on Environmental, Industrial and Applied Microbiology (BioMicroWorld2007)" 27 November – 1 December 2007 Seville, Spain

7. **A. Franzetti**, G Bestetti, P. Caredda, P. La Colla *Washing of oil and metal contaminated soils with microbial emulsifiers* "ConSoil 2008" 3-6 June 2008, Milano