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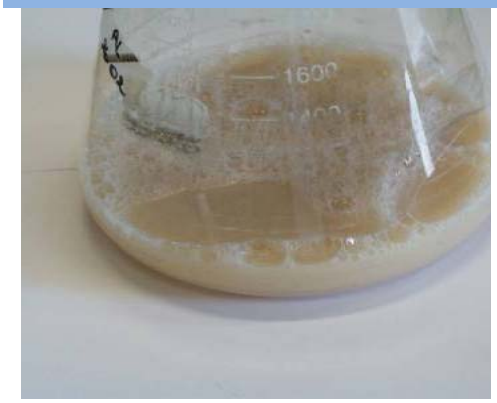
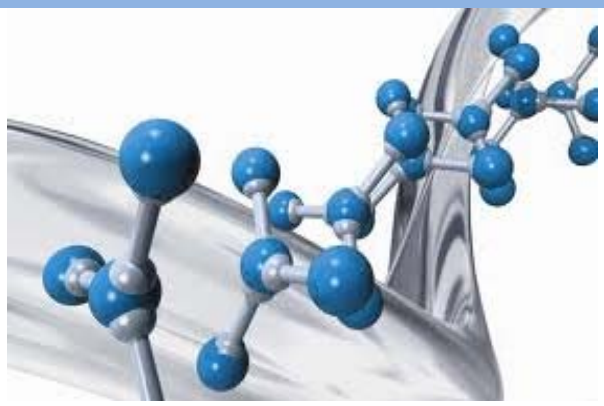
New Surface Active Compounds from bacterial strains: production, characterization and potential application in environmental remediation





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A Irene e Giuseppe,
per avermi sempre aspettato.

CHAPTER 1 *INTRODUCTION*

Many prokaryotic and eukaryotic microorganisms synthesize a wide range of Surface Active Compounds (SACs) which exhibit surface activities at interfaces, including the ability to lower surface and interfacial tension of liquids and to form micelles and microemulsions between different phases [1,2,3].

SACs exist in a wide variety of structurally different amphiphilic molecules containing both hydrophilic and hydrophobic units. Neu [4] divided microbial surface active compounds into low molecular weight SACs, also named biosurfactants, and high molecular weight SACs including amphiphilic and polyphilic polymers. The biosurfactants lower the surface and interfacial tension, whereas the high molecular weight SACs, also called bioemulsifiers, are more effective in stabilizing oil-in-water emulsions [5]. The most extensively studied bioemulsifiers are Emulsan, a lipopolysaccharide isolated from *Acinetobacter calcoaceticus* RAG-1 (now *Acinetobacter venetianus* RAG-1) [6,7], and Alasan, a complex consisting of an anionic polysaccharide and proteins produced by *Acinetobacter radioresistens* KA53 [8].

Many SACs have the advantages of higher biodegradability, biocompatibility and lower toxicity in comparison with chemically synthesized surfactants, as well as higher specific activity at extreme temperatures, pH level, and salinity [9,10,11]. They can be used as emulsifiers, de-emulsifiers, wetting and foaming agents, functional food ingredients and as detergents in petroleum, petrochemicals, environmental management

procedures, agrochemicals, foods and beverages, cosmetics and pharmaceuticals, commercial laundry detergents and in the mining and metallurgical industries [12-16].

These characteristics result in greater acceptability, especially in applications that cause the dispersion of surfactants in the environment.

In the last few years, both low and high molecular weight SACs have been studied extensively as possible alternatives to the use of chemical surfactants to increase the rate of oil and heavy metals removal in soil and water in the field of bioremediation technologies [17, 18]. They have found potential application in various technologies for treating sites contaminated by petroleum derivates, including chemical, physical and biological means. One of the feasible ways is bioremediation, which utilizes the natural degradative ability of plants or microorganisms, usually fungi and/or bacteria, to convert contaminants into less toxic compounds (ideally, carbon dioxide and water only). Bioremediation is both effective and environmentally friendly, though often requiring time and is being not cost-effective when treating large volumes of polluted materials. The use of surfactants in washing crude oil contaminated soil is a new area of application. The *soil washing* method is cost-effective and relatively fast, thereby having potential to be applied in treating and removing large amounts of pollutants [19]. Furthermore, Franzetti et al.[20] claim that the results from soil washing bench scale experiments are more reliable and less uncertain than biodegradation microcosm experiments to evaluate the potential application of a new bioemulsifier. In effect, the removal efficiency of pollutants from soil is determined by the chemico-physical interactions between surfactant and pollutant while the biodegradation is also affected by the effects of surfactants on the cell surface properties and microbial metabolism.

In recent years, applications of surfactants in enhancing ex-situ soil washing have been studied [17, 21]. In particular, recent studies have shown that biosurfactants are able to effectively solubilize and mobilize organic compounds adsorbed by soil constituents [22]. Nevertheless, the soil washing literature available on the remediation of hydrocarbon-compound contaminations by using biosurfactant solutions is limited. Particularly, where high molecular weight biosurfactants are concerned.

For these reasons, the aims of this research project were (1) to identify new SACs-producing microorganisms, (2) to recover and purify the biosynthesized molecules, (3) to evaluate the surface and emulsifying properties ,(4) to determine the chemical

composition and molecular weight of the surface active compound(s) and (5) to evaluate the applicability of the novel SACs for soil washing.

This thesis reports the results of an experimental carried out in collaboration between the Microbiology Laboratory of Environmental Characterization, Prevention and Recovery Unit of ENEA-Casaccia (Rome, Italy) and Agro-Food and Forestry, Biological systems and Innovation Department of the University of Viterbo (Tuscia, Italy). Moreover, a further collaboration between the Laboratory of Environmental Characterization, Prevention and Recovery Unit of ENEA-Casaccia and the group of Prof. E. Tamburini of the Biomedical Science and Technology Department of the University of Cagliari (Italy) concerned the measurement of the surface tension and critical micellar concentration. Furthermore, a further collaboration between Laboratory of Environmental Characterization, Prevention and Recovery Unit of ENEA-Casaccia and the group of Prof. D.O. Cicero of the Department of Science and Chemical Technology, Tor Vergata University (Rome, Italy) involved the study of the hydrodynamic behaviour of the bioemulsifiers. The results of this collaboration are also reported here.

1.1 SUMMARY OF THE THESIS

This section outlines the results presented in the next chapters:

CHAPTER 2 describes the screening procedure used to identify new bacterial strains, able to produce biopolymers with strong superficial and interfacial properties (Surface Active Compounds, SACs). The eleven bacterial strains belonging to six different classes (*Alphaproteobacteria, Actinobacteria, Bacilli, Betaproteobacteria, Flavobacteria, Gammaproteobacteria*), were previously isolated from different environments (contaminated sites, industrial wastewaters, farm soils and archaeological sites) and belong to the ENEA-Lilith Strain Collection of the Microbiology Laboratory of Environmental Characterization, Prevention and Recovery Unit of ENEA-Casaccia (Rome). Furthermore, two type-strains were used as positive controls: *Pseudomonas aeruginosa* (1128 DSMZ) and *Bacillus subtilis* (3257 DSMZ).

The screening for biosurfactant/bioemulsifier production was based on the use of two different culture media and conditions which were considered optimal for biosurfactant production for *Pseudomonas aeruginosa* [23] and *Bacillus subtilis* [24] respectively.

The presence of SACs in the culture media was evaluated using the oil spreading technique [25] and the emulsifying assay [26], in both whole culture broths and cell-free supernatant. Among these strains, *Pedobacter* sp. MCC-Z strain appeared to be particularly interesting, because it belongs to the *Shingobacteriaceae* family and the *Pedobacter* genus, a genus not yet described as a bioemulsifier producer. It showed an emulsification activity in the Cooper medium, both in whole culture (E_{24} 68%) and in cell-free supernatant (E_{24} 56%).

In order to reduce production cost and to simplify the recuperation and purification phases of the bioemulsifier from the culture medium a study in a mineral salts medium (MSM) [27] with different concentrations of glucose was carried out. The maximum emulsifying activity was obtained with 0.5% glucose after 96h of inoculation. The relation among growth phase, surface-active compound production and cell-surface properties was analysed in kinetic experiments with 0.5% of glucose as a carbon source. *Pedobacter* sp., MCC-Z strain synthesised and released extracellularly bioemulsifier(s) during the exponential growth phase. Furthermore, the adhesion of the bioemulsifier to the bacterial strains decreased during growth.

The results reported in **CHAPTER 3** deals with the recovery, the purification procedure and the main physicochemical properties of the purified biopolymers.

In order to purify the active compound(s), the culture supernatant was separated from the cells by centrifugation and then the supernatant was filtered. The filtrate was dialyzed using 12 kDa cut off dialysis membrane. The dialyzed was concentrated using heat evaporation and the concentrate was extracted with hexane. The white emulsion was separated from the water phase and then washed with water. Hexane was removed by rotary evaporation and the residue was then freeze-dried and weighed. For purification, the crude water-soluble bioemulsifier(s) was applied to a Sephadex GC-25. Fractions having major emulsifying activity (64%) were concentrated and lyophilized for further characterizations. The purified emulsifier was called Pdb-Z.

The measurements of surface tension showed that Pdb-Z reduces the surface tension of water to 41 mN/m with a Critical Micelle Concentration (CMC) value of about 2.6 mg/mL. This indicates a good surfactant activity.

Stability studies were performed to investigate the effect of several environmental parameters (temperature, pH and ionic strength tolerance) on emulsifying activity of Pdb-Z. Results demonstrated that Pdb-Z was thermostable showing a slight reduction of

E_{24}% with increasing temperatures. In addition, it gave stable E_{24} values over pH range 3-11 and the addition of NaCl did not influence its activity. Our results led to the hypothesis that Pdb-Z can be used for formulation of emulsions in the areas of food and cosmetics and in a variety of industrial sectors that include extreme environmental conditions.

The ability of the Pdb-Z to form stable emulsions with aliphatic, aromatic and petroleum compounds was compared with that of synthetic surfactants. Our results demonstrated that Pdb-Z exhibits a higher emulsifying ability on all hydrophobic substrates, except diesel fuel, in comparison with the chemical surfactants tested. The broad range of substrate specificity of the bioemulsifier Pdb-Z suggests that it could be a good candidate for application in hydrocarbon remediation and oil recovery.

The results obtained in order to chemically characterize the Pdb-Z by analytical tools (namely, HPLC/MS^n, GC/MS, ^{1}HNMR and FT-IR), are reported in **CHAPTER 4.** Pdb-Z was composed of 67% carbohydrates containing galactose, xylose, N-acetyl glucosamine, galacturonic acid and talose monomeric units. Galactose was the main constituent. Protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad). SDS-PAGE analysis of Pdb-Z indicated the existence of two major proteins with apparent molecular masses of 23 and 8 kDa. The N-terminal amino acid sequences of the two proteins were determined. The first amino acids of the 23-kDa and 8-kDa proteins showed high similarity to two proteins from *Pedobacter agri.*

Pdb-Z was submitted to FT-IR and ^{1}HNMR spectroscopy analyses for identification of the main functional groups present in the bioemulsifier(s). Overall, the FT-IR spectrum suggested that Pdb-Z is predominantly a polysaccharide, although proteins are also present. Proton NMR study in DMSO and D_2O confirmed the presence of sugars and aliphatic chains whereas no signals associated to proteins were evident. These results supported the previous conclusion of GC/MS analysis of Pdb-Z.

In order to obtain insights into the dimension of the molecules constituting Pdb-Z, we conducted a diffusion study using NMR. Dimethyl sulfoxide (DMSO) is known to break the inter- and intra-molecular hydrogen bonds of polysaccharides, leading to the dispersion of aggregates and making it possible to study individual polymer chains. Variable-gradient ^{1}H-NMR experiments permitted the measurement of the hydrodynamic radius R_h of the different components of the mixture, through the

measurement of diffusion coefficient, D. The calculated R_h values are similar to those found for other carbohydrate-containing polymers, such as cellulose fibers, amylase, glycogen and amylopectins. For rabbit glycogen, Dinadalaya and coworkers [28] reported the hydrodynamic radius and molecular mass values as being about 23 nm and $7x10^6$ Da, respectively. Therefore, we can infer that the molecular weight of Pdb-Z is about 10^6 Da. Overall, our results suggested that Pdb-Z is lipopolysaccharaides, polysaccharaides and lipoproteins, or a complex mixture of these biopolymers, containing galactose as the major sugar constituent at unusually high levels (51%) and two proteins from *Pedobacter agri*, containing an outer membrane protein β-barrel domain. Pdb-Z molecular weight is approximately 10^6 Da.

The results reported in **CHAPTER 5** deals with the evaluation of the applicability of Pdb-Z in *soil washing* treatment. Soil was spiked with road diesel and the effects of several environmental parameters were investigated (concentrations of washing solutions, washing time and volume/mass ratio of washing solutions) using Tween-20 as a reference. The results indicate that Pdb-Z was able to remove more than 75% of the road diesel, whereas Tween-20 removed 73%. Therefore, results suggest that Pdb-Z has interesting properties for applications in remediation of hydrocarbon-contaminated environments.

In **CHAPTER 6** the whole conclusions of the project are drawn and the perspectives for the future are put forward.

E’ una caratteristica propria del nostro spirito immaginare disordine e oscurità là dove non sappiamo nulla di certo.

Johann Wolfgang Goethe dal libro“I dolori del giovane Werther”

CHAPTER 2 *SCREENING OF BACTERIAL STRAINS ABLE TO SYNTHESISE SURFACE ACTIVE COMPOUNDS*

2.1 INTRODUCTION

2.1.1 Microbial surfactants

Surfactants and emulsifiers are amphiphilic compounds with both lipophilic and hydrophilic structural moieties in their molecules. Surfactants are soluble compound that reduce the surface tension of liquids, or reduce interfacial tension between two liquids or a liquid and a solid, while an emulsifier does not necessary reduce the surface tension or interfacial tension. However, emulsifiers may only bind water insoluble substrates together to form an emulsion.

They find applications in an wide variety of industrial processes involving emulsification, such as detergency, foaming, wetting, dispersing or solubilisation [29]. Most of these compounds are chemically synthesized and are a potential danger to the environment due to their toxicity [30].

Microorganisms, including bacteria, yeast and fungi, synthesize a wide variety of amphiphilic molecules and they have very different chemical structures and surface properties. These compounds have been the subject of increased interest as potential replacements for synthetic surfactants, and are expected to have many potential industrial and environmental applications in the future [31].

During the last 2-3 decades, much research on bioemulsifiers has been carried out due to their environmentally friendly nature and lower toxicity in comparison to synthetic surfactants [32,33].

Furthermore, since they are produced by a wide variety of diverse microorganisms, they have very different chemical structures and surface properties. Diverse functional properties, namely emulsification, wetting, foaming cleansing, phase separation, surface activity and reduction in viscosity of crude oil, make their use feasible for many application purposes [34].

2.1.2 Biosurfactants classifications and their microbial origin

Biosurfactants are amphiphilic compounds produced on living surfaces, mostly microbial cell surfaces, or excreted extracellularly and contain hydrophobic and hydrophilic moieties. These compounds can be classified as low and high molecular mass bioemulsifiers [5]. The former lower surface and interfacial tensions, whereas the second are more effective at stabilizing oil in water emulsions.

2.1.2.1 Low and high molecular weight biosurfactants

The low molecular mass bioemulsifiers are generally glycolipids, such as treahalose lipids, sophorolipids and rhamnolipids, or lipopeptides like surfactin (Table 2.1). The most commonly known glicolipid bioemulsifiers, rhamnolipids, trehalolipids and sophorolipids, are disaccharides that are carbohydrates in combination with long-chain aliphatic acids or hydroxyaliphatic acids. A large number of microorganisms produce glycolipids.

For example, certain species of *Pseudomonas* are known to produce large amounts of a glycolipid, called rhamnolipids, [35], *Rhodococcus erythropolis* produces trehalose lipids when the bacterium is grown on *n*-alkanes [36, 37], while, different species of yeast *Torulopis* produce extracellular sophorolipids [38,39].

Several bacteria produce large quantities of cyclic liopeptides. *Bacillus subtilis* produces a cyclic lipopeptide, called surfactin, one of the most effective biosurfactants [40, 41] while, *Bacillus brevis* produces the cyclosymmetric decapeptide antibiotic gramicidin S that forms a stable coordination complex [42].

Phospholipids biosurfactants have been synthesized by various researchers using several bacterial and yeast during growth on *n*-alkanes. For example, *Acinetobacter* sp.

produces extracellular membrane vesicles able to form microemulsions of alkanes in water [43].

A large number of bacterial species from different genera produce exocellular polymeric surfactants composed of polysaccharides, protein, lipopolysaccharides, lipoproteins or complex mixtures of high molecular mass. The most thoroughly studied polymeric biosurfactants are Emulsan, liposan, mannoprotein, and other polysaccharide-protein complexes (Table 2.1).

Rosenberg and Ron [44] studied the production of a potent polyanionic amphipatic hetereopolysacharide bioemulsifier by different species of *Acinetobacter*. *Acinetobacter calcoaceticus* RAG-1 produces Emulsan one of the most powerful emulsion stabilizers known today [6]. Cirigliano and Carman [45] reported the production of Liposan by *Candida lipolytica*; this is an extracellular water-soluble emulsifier composed of 83% carbohydrate and 17% protein. *Acinetobacter radioresistens* produces Alasan, a complex of an anionic polysaccharide and protein with a molecular mass of approximately 1MDa [8].

Table 2.1: Major types of biosurfactant produced by microorganisms.

Biosurfactant	Producing microorganisms	Reference
Low Molecular mass		
Glycolipids		
Trehalose lipids	*Pseudomonas* spp.	Parra et al.1989
	R. erythropolis	Ristau and Wagner 1983; Kim et al.1990; Li et al 1984
	Arthrobacter sp.	Li et al. 1984
Rhamnolipids	*Mycobacterium* sp.	Cooper et al.1989
Sophorolipids	*P.aeruginosa*	Rendell et al.1990; Sim et al.1997
	T. bombicola (yeast)	Inoue and Itoh 1982; Davila 1997
	C. borgoriensis (yeast)	Cutler and light 1979
Aminoacid-lipids		
Lipopeptides and lipoprotein		
Viscosin	*P. fluorescens*	Neu and Poralla 1990
Surfactin	*B. subtilis*	Arima et al.1968; wei and Chu 1998
Gramicidin S	*B. brevis*	Katz and Demain 1977
Peptide-lipid	*B. licheniformis*	Horowitz and Griffen 1991
Serrawettin	*S. marcescens*	Matsuyama et al.1991
Polimyxins	*B. polymyxa*	Suzuki et al. 1965
Fatty acids and phospholipids		
Corynomycolic acid	*N. erythropolis*	MacDonald et al. 1981
Phospholipids	*T. thiooxidans*	Beebe and Umbreit 1971
	Acinetobacter spp.	Kaeppeli and Finnerty 1980
High molecular mass		
Rag-1 Emulsan	*A. calcoaceticus* RAG-1	Rosenberg et al. 1979
Liposan	*C. lipolytica*	Cirigliano and Carmen 1984
Alasan	*A. radioreresistens* KA53	Navon-Venezia et al. 1985
Emulsan 378	*P. fluorescens*	Persson et al. 1988
Mannan-lipid-protein	*C .tropicalis*	Kaeppeli et al. 1984
Biodispersan	*A. calcoaceticus* A2	Rosenberg 1993
Food emulsifier	*C. utilis*	Shephered et al. 1995
BD4 Emulsan	*A. calcoaceticus* BD413	Kaplan and Rosenberg 1982
Sulfated polysaccharide	*H. eurihalinia*	Calvo et al. 1998
Thermophilic emulsifier	*B. stearothermophilus*	Gunjar et al. 1995

2.1.3 Factors affecting biosurfactant production

During recently years, there have been numerous reviews that have demonstrated that the composition and surface properties of the biosurfactant depend not only on the producer strains, but also on the culture conditions. Thus, the nature of the carbon and nitrogen sources the related C:N ratio, nutritional limitations, chemical and physical parameters such as, temperature, aeration rate, divalent cations and pH, influence not only the amount of biosurfactant produced but also the type of polymer produced [46]

The quality and quantity of biosurfactant production is dependent on *the nature of the carbon source* [47]. Desai and Banat [29] reported that diesel, crude oil, glucose and sucrose are good substrates for biosurfactant production. Furthermore, there are some microorganisms that produce biosurfactants only by using a hydrophobic carbon source, hydrocarbon or vegetable oil, while others use only carbohydrates in combination or individually [48].

Different *nitrogen* compounds have been used for the production of biosurfactants such as urea, peptone, yeast extract, ammonium sulphate, ammonium nitrate, malt extract and meat extract. Syldatk et al. [49] reported that nitrogen limitation causes overproduction of biosurfactant. Ammonium salts and urea are preferred nitrogen sources for biosurfactant production by *Arthrobacter paraffineus*, whereas nitrate ensures maximum surfactant production in *P. aeruginosa* [50].

Environmental factors and growth conditions such as pH, temperature, agitation and aeration are extremely important for the yield and characteristics of the biosurfctant produced. The pH of the medium plays an important role in the rhamnolipids production by *Pseudomonas* sp.: its optimum is at a pH range from 6 to 6.5 and a sharp decrease is observed above pH 7 [51]. Most biosurfactant productions are reported to be performed within a temperature range of 25-30°C. In *A. paraffineus* and *Pseudomonas* sp. strain DSM-2874, this temperature range caused alteration in the composition of biosurfactant production [29].

Aeration and agitation significantly influence the production of biosurfactants, as well as facilitating the transference of oxygen from the gas phase to the liquid phase. Margaritis and co-workers [52] when evaluating the influence of aeration on the biosurfactant biosynthesis by *Nocardia erythropolis* observed a marked reduction of biosurfactant yield when agitation speed was increased.

The production of biosurfactant also depends on the concentration of salt, which effects cellular activity. However, some biosurfactants were not influenced by concentrations up to 10% (w/v) NaCl, although slight and insignificant reductions in CMC were detected [29].

2.1.4 Towards commercial production of microbial surfactants

Successful commercialization of every biotechnological product depends largely on its bioprocess economics. Despite their multifarious advantages and diverse potential applications at present, the prices of microbial surfactants are not competitive with those of the chemical surfactants, due to their high production costs and low yields [53]. However, different strategies have been adopted to make the process economically competitive: these include the use of inexpensive raw material; the development of economical engineering processes, including optimization of culture conditions and the recovery process; and the development of overproducing strains. Although a large number of biosurfactant producers have been reported in literature, biosurfactant research, particularly 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, Torulopsis* and *Acinetobacter* have not been adequately exploited for the economical production of biosurfactants.

One of the primary approaches applied for obtaining increased yields in fermentative production is the medium optimization. Different elements, such as nitrogen, iron and manganese, are reported to affect the yield of biosurfactants, for example, the limitation of nitrogen is reported to enhance biosurfactant production in *Pseudomonas aeruginosa* strain BS-2 [54]. Similarly, the addition of iron and manganese to the culture medium was reported to increase the production of biosurfactant by *Bacillus subtilis* [55]. The most effective method used for the optimization of factors is the statistical approach [56]. There are considerably sources of literature available on medium optimization using different statistical methods. These include experimental designs such as Plackett-Burman [57], response surface methodology [56, 58] and factorial designs [59].

The choice of raw materials is very important to the overall economics of the process. Raw materials can make up 10%-30% of the final product cost in most biotechnological processes [60]. Thus, to reduce this cost it is desirable to use low-cost raw materials. Some organisms produce biosurfactants only in hydrocarbons, others only in carbohydrates, and others utilize several substrates, in combination or separately. In the last few years most biosurfactant production experiments have been aimed at the development of economical methods for higher yields of biosurfactant by suggesting the use of low-cost raw materials. Rhaman et al. [61] showed the maximum rhamnolipid

production of 4.31, 2.98, and 1.77 g/L using soybean oil, safflower oil, and glycerol, respectively. Nitschke and Pastore [62] showed that cassava flour wastewater is an alternative substrate for surfactant production by *Bacillus subtilis* and reduces the surface tension of the medium to 26.6 mN/m, giving a crude biosurfactant concentration of 3.0 g/L. Attention must be paid, however, to the fact that different carbon sources can influence the composition of the biosurfactant formed and how it is produced. For example, *Arthrobacter* produces 75% extracellular biosurfactant when grown on ethanol or acetate but with hydrocarbons, it is totally extracellular [63]. The trehalose lipids produced by *Arthrobacter*, *Nocardia*, and *Corynebacterium* were replaced by sucrose and fructose lipids when grown on sucrose or fructose [64].

The third approach, using recombinant hyperproducing strains, has still to be adequately tested, despite the fact that the hyperproducers have been reported to increase yields several fold. For example, recombinants of *Bacillus subtilis* MI 113 have been developed by expressing foreign genes related to surfactin production, resulting in high production of surfactin on soybean crud residue [65]. Moreover, recombinant strains often give rise to better product characteristics. For example, *Pseudomonas aeruginosa* is an opportunistic pathogen in humans and is, therefore, not suitable as an industrial strain. To overcome the problem of the pathogenicity of *P. aeruginosa*, recombinant *Pseudomonas putida* and *Pseudomonas fluorescens* were developed that produced rhamnolipids by *P. aeruginosa* considerable amounts [66]. The incorporation of these hyper-producing strains will boost the industrial biosurfactant production process and make it possible to commercialize biosurfactants by making the production process cheaper and safer.

2.1.5 Functional properties of bioemulsifier/biosurfactant

The composition and distribution of the hydrophilic and hydrophobic functional groups determine the property of an bioemulsifier/biosurfactant. Consequently, it is possible to select their suitable application in various industrial fields such as, e.g. cosmetics, food, pharmaceutics, agriculture, mining and oil recovery [29, 67]. Some functional properties are reported below:

Surface and Interfacial tension is the most important property of surfactant. The molecules of water are held together by cohesive forces and surfactants reduce surface

tension. Surfactin produced by *Bacillus* sp. is the most effective biosurfactant, reducing the surface tension of water from 72 mN/m to 27 mN/m [26, 68].

Emulsification is the dispersion degree of one liquid into another leading to the mixing of two immiscible liquids.

The *de-emulsification* action breaks up emulsion through the disruption of the stable surface between the bulk phase and the internal phase. Several industries, such as mining, food, nuclear fuel reprocessing, cosmetics and pharmaceutical need molecules able to determine de-emulsification [34].

Wetting is the spreading and penetrating power toward a substance that lowers the surface tension when added to a liquid.

In the process of *foaming*, the surfactants become concentrated at a gas-liquid interface leading to the formation of bubbles through the liquid and on the interface resulting in foam formation. Surfactin exhibits excellent foaming properties when compared with SDS [69].

Several biosurfactants/bioemulsifiers are stable at various *temperatures, pH and ionic strength*. *Bacillus licheniformis* produces a biosurfactant, the lichenysis, that is stable up to 50°C, pH of 4.5-9.0 and at NaCl (50g/L), Ca (25 g/L) concentrations [70]. Similarly, surfactin from *B. subtilis* is highly stable at 121°C for 20 min and remains so for 6 months at room temperature at pH range 5-11 and 20 % NaCl [62].

2.1.6 Screening methods for detection of biosurfactant/bioemulsifier producers

The methods used for a screening of surface-active compounds producing strains are based on the physical effects of surfactants. Satpute and co-workers [71] reported 11 main methods used to screen, detect or evaluate potential bioemulsifier/biosurfactant producing microorganisms. These methods can give qualitative and/or quantitative results, the use of which has advantages and disadvantages. Listed below, are only two methods that have been used in the present work and that have been considered the most appropriate for selecting new bacterial strains able to produce bioemulsifier/biosurfactant.

2.1.6.1 Oil Spreading Assay

The oil spreading assay was developed by Morikawa et al. [25] and is based on the observation of the putative biosurfactant or sample containing it that is put in contact with distilled water added with crude oil. If biosurfactant is present, the oil is displaced and a clearing zone is formed. The diameter of this clearing zone on the oil surface correlates to surfactant activity, also called oil displacement activity. For pure biosurfactant a linear correlation between the quantity of surfactant and the clearing zone diameter is given. It is one of the most effective methods for detecting the presence of biosurfactant producers.

2.1.6.2 Emulsification index

Another popular assay based on the emulsification capacity of biosurfactants was developed by Cooper and Goldenberg [26]. Kerosene is added directly to the culture broth (1:2 v/v), vortexed for 2 minutes and allowed to stand for 24 h. The height of emulsion is measured by taking the layer formed between the aqueous and kerosene layers. A number of modifications reported by some authors propose to substitute kerosene by other pure hydrocarbons (*n*-hexadecane, *iso*-octane, cyclohexane, toluene, xylene). Thus, it is possible to quantify the emulsion index E_{24} (see below in "Experimental procedures") and to asses the emulsification index stability over time in order to designate the strength of a surfactant.

2.2 EXPERIMENTAL PROCEDURES

2.2.1 Bacterial strains

Bacterial strains used in the present work were previously isolated from different environments such *i.e.*, polluted sites [72], industrial wastewaters [73], archaeological sites [74] and abandoned soil [75], or simply obtained from the ENEA-Lilith Strain Collection of the Microbiology Laboratory of Environmental Characterization, Prevention, and Recovery Unit of ENEA-Casaccia (Rome). The strains were identified (rDNA 16S sequencing) and characterized for biotechnology potential in previous

works (see Tab.2.2). The rDNA 16S sequences are deposited in GenBank (http://www.ncbi.nlm.nhi.gov/BLAST).

The eleven bacterial strains were screened to verify their ability to produce biopolymers with strong superficial and interfacial properties (Surface Active Compounds, SACs). The strains were selected on the basis of two essential features: they should be non pathogenic and non spore forming bacteria in order to protect the environment and the safely of the operators.

Table2.2: List of the tested bacterial strains and their main features.

Strain	Phylogenetic affiliation (%similarity of rDNA 16S)	GenBanck Accession Number[a]	Source	References
TSNRS-4	*Ochrobactrum* sp. (100)	EU249585	Mercareccia Tomb of Tarquinia	Sprocati et al.2008
MCC-A5	*Aeromicrobium erythreum* (98)	JF279932	Soil from an abandoned field located in Piana di Monte Verna (Naples, Italy)	Sprocati et al 2012
MCC-SL5	*Duganella nigrescens* (99)	JF279923		
MCC-Z	*Pedobacter* sp.(99)	JF279930		
MCC-X	*Gordonia* sp.(99)	JF279928		
MCC-S	*Massilia* sp. (99)	JF279920		
MCC-E	*Micromonospora* sp. (99)	JF279912		
MCC-G	*Nocardia* sp. (99)	JF279914		
MCC-T	*Porphyrobacter donghaensis* (99)	JF279925		
AGL17	*Acinetobacter calcoaceticus* (100)	EU118781	Abandoned contaminated site of Italsider Bagnoli	Sprocati et al.2006
CONC18	*Achromobacter xylosoxidans* (99)	EU275351	Sludge from a tannery depuration system (Ariston-Naples, Italy)	Tasso et al.2008

[a]: the GenBank accession number of the tested strains. The strains were identified by 16S rDNA sequence similarity with GenBank data bank (http://www.ncbi.nlm.nhi.gov/BLAST)

2.2.2 Control strains

Two strains of the DSMZ collection (Leibniz –Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zelkulturen GmbH) were used as positive controls for biosurfactant production: *Bacillus subtilis* (DSMZ 3257) and *Pseudomonas aeruginosa* (DSMZ 1128).

Cooper and co-workers [24] and Zhang-Miller [23] reported that the *Bacillus subtilis* and *Pseudomonas aeruginosa* strains produce surfactin and rhamnolipids, respectively

when cultivated in defined conditions. The values of the OST and emulsification activity performed on the samples confirmed the presence of surface active compounds. The GC/MS analysis of the products showed the effectiveness of the methods used.

2.2.3 Growth conditions and culture preparation

The bacterial strains were inoculated in Tryptic Soy Broth (TSB) medium composed of (g/L): Bacto Tryptone (Pancreatic Digest of Casein), 17; Bacto Sortone (Papaic Digest of Soyben Meal) 3.0; Bacto Dextrose 2.5; NaCl 5.0; K_2HPO_4 2.5. The medium pH was adjusted before sterilization (121°C for 15 min) to pH 7.3. The pre-culture was incubated in a rotary shaker at 250 rpm at 37°C for 24 h and then 2 mL of culture broth were used to inoculate 200 mL of two different production media described by Zhang-Miller [23] and Cooper [24] to induce biosurfactant production in *Pseudomonas aeruginosa* and *Bacillus subtilis*, respectively.

The Zhang-Miller medium was composed of NH_4Cl (0.02 M), KCl (0.02 M), Tris-HCl (0.12 M), glucose (0.5%), proteose peptone (1%) and $MgSO_4$ (0.0016 M); the pH was adjusted to 7.2. The production culture was incubated at 37°C in an orbital shaker at 250 rpm.

The Cooper medium was composed of NH_4NO_3 (0.05 M), KH_2PO_4 (0.03 M), Na_2HPO_4 (0.04 M), $MgSO_4$ ($8.0 \cdot 10^{-4}$M), $CaCl_2$ (7.0 10^{-6}M), $FeSO_4$ ($4.0 \cdot 10^{-6}$M) and Na_2 ethylenediaminetetracetic acid ($4.0 \cdot 10^{-6}$M). In this case, the production culture was incubated at 30°C in an orbital shaker at 200 rpm.

Only in the case of *Pedobacter* sp. MCC-Z strain, the preculture was prepared by inoculating MCC-Z into 25 mL of Mineral Salts Medium (MSM) [27] amended with 1% glucose as the sole carbon source in a 125 mL flasks.

The MSM was composed of a solution A to which 1 mL of solution B was added. Solution A contained per liter: $NaNO_3$, 2.5 g; $MgSO_4 \cdot 7H_2O$, 0.4 g; NaCl, 1.0 g; KCl, 1.0 g; $CaCl_2 \cdot 2H_2O$, 0.05 g; H_3PO_4 (85%), 10 mL; the pH was adjusted to pH 7.2 with KOH. Solution B contained (g/L): $FeSO_4 \cdot 7H_2O$, 0.5; $ZnSO_4 \cdot 7H_2O$, 1.5; $MnSO_4 \cdot H_2O$, 1.5; H_3BO_3, 0.3; $CoCl_2 \cdot 6H_2O$, 0.15; $CuSO_4 \cdot 5H_2O$ 0.15; $NaMo_2O_4 \cdot 2H_2O$, 0.1. The preculture was incubated at 200 rpm for 36 h at 26°C, and then 3 mL of culture broth

were used to inoculate 300 mL of MSM with 2% glucose in a 1-liter flask. This flask was incubated under the same culture conditions for 4 days.

2.2.4 Screening for SACs production

Pre-inocula of the strains were inoculated in 200 mL of two different production media described above (Zhang Miller and Cooper), to which 0.5 and 4% (w/v) of glucose were added, respectively, as the sole carbon source. After aerobic incubation at 37°C and 30°C respectively (see above) the surface activity of whole culture broths (with cells) and culture free supernatant (without cells) was tested every 24 h for a period of 4days. The cells were removed from the culture broth by centrifugation at 12500 rpm for 90 s and the emulsification activity (EA) and oil spreading test (OST) was determined as described below.

2.2.4.1 Oil Spreading Test (OST)

The OST was performed according to Morikawa et al. [25]. Briefly, 50 mL of distilled water were added to a Petri dish (20 cm diameter) followed by addition of 10 µL of crude oil on the water surface. Ten µL sample culture or culture supernatant were added to the oil surface and the diameter of the clear zone was measured. All determinations were performed at least in triplicate.

2.2.4.2 Emulsification activity (EA)

Two millilitres of whole culture broths or culture supernatant were vigorously mixed with equal volume of *n*-hexane in glass test tube (15mm h x 2mm Ø) using a vortex mixer for 2 min. After 24 hours, the height of the stable emulsion was measured and the emulsifying activity calculated as the ratio of the height of the emulsion layer and the total height of the liquid:

$$E_{24}\% = \frac{H_{emulsion}}{H_{total}} \times 100$$

Equation 2.1

The emulsification index stability designates the strength of a surfactant. All determinations were performed at least in triplicate.

2.2.5 Pre-optimization of culture conditions

During the production phase of *Pedobacter* sp. bioemulsifier a medium of MSM soil was used [27] to which different glucose concentrations (0.25-0.5-1.0- 2.0 %, w/v), as the sole carbon source, were added. Cultures were grown at 26°C in a rotary shaker at 200 rpm. The emulsifying activity was performed every 24 hours for 7 days. Each emulsification experiment was in triplicate (three independent experiments). Bacterial growth was determined in terms of colony forming units (CFU) by viable cell count (using TSB as growth) medium at 28°C.

2.3 RESULTS AND DISCUSSION

2.3.1 Production of surface active compounds

The screening was aimed at identifying bacterial strains able to produce biopolymers with strong superficial and interfacial properties (Surface Active Compounds, SACs).
Of the eleven bacterial strains, belonging to six different classes (Alphaproteobacteria, Actinobacteria, Bacilli, Betaproteobacteria, Flavobacteria, Gammaproteobacteria), three, and namely *Duganella nigrescens* MCC-SL5, *Massilia* sp. MCC-S and *Porphyrobacter donghaensis* MCC-T, did not grow on both production media (Tab. 2.4). The highest values obtained every 24h by OST and E_{24} are shown in Tab.2.3. *Pseudomonas aeruginosa* and *Bacillus subtilis*, used as positive controls, have validated the OST and emulsification assay, by confirming the data found in literature [76-78] (see Fig. 2.1). Four bacterial strains, appeared to be of potential interest: in particular *Pedobacter* sp. MCC-Z and *Gordonia* sp. MCC-X, produced SACs on Cooper culture media, while *Acinetobacter calcoaceticus* AGL17 and *Achromobacter xylosoxidans* CONC18 produced active molecules on both culture media.

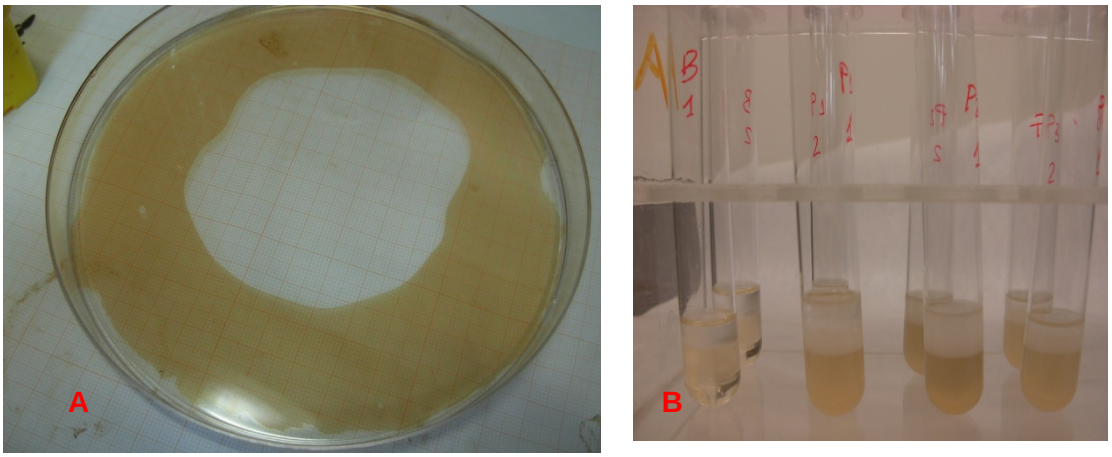

Fig.2.1:Oil spreading test (A) and emulsification assay (B) of *Pseudomonas aeruginosa* (1128 DSMZ) in Miller culture media.

Among these strains, *Pedobacter* sp. MCC-Z is of particular interest because it belongs to the *Sphingobacteriaceae* family and the *Pedobacter* genus [79], a genus not yet described as a bioemulsifier producer and not yet used in remediation technology.

It showed a higher emulsification activity on the Cooper medium both in whole culture (E_{24} 68%) and in cell-free supernatant (E_{24} 56%) (see Fig. 2.2) than that obtained from the type-strain *Bacillus subtilis* (E_{24} 36%), that was able to produce SACs only on Cooper's medium. The presence of activity in cell-free supernatant is important, since it would allow the use of cell-free product, thus reducing significantly the potential costs of downstream and purification processes. None of the selected strains showed similar OST results to those recorded with reference strains (see Tab.2.3).

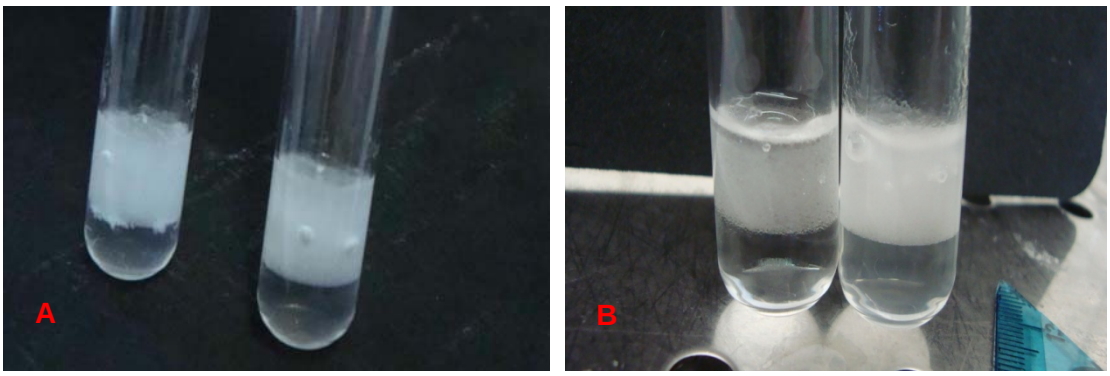

Fig.2.2:Emulsification assay of bioemulsifier MCCZ strain in Cooper culture media whole cells (A), supernatant free of cells (B).

Hence, on the basis of the screening results *Pedobacter* sp., MCC-Z was selected for further study. This bacteria is a Gram negative and non-flagellated heterotrophic bacterium; it is a non pathogen and non spore-forming organism, characterized by rod shaped and pink-colored colonies [79].

Table 2.3: Evaluation of SACs production by selected strains, compared with type-strains, in two different culture media, by emulsification assay (E_{24} %) and Oil Spreading Test (OST). Data are means of three determinations.

Strain code	Identification (%similarity 16S rDNA)	Zhang-Miller culture media				Cooper culture media			
		E_{24}% [a]		**OST[b]**		**E_{24}% [a]**		**OST[b]**	
		Whole culture	Cell-free supernatant	Whole culture	Cell-free supernatant	Whole culture	Cell-free supernatant	Whole culture	Cell-free supernatant
MCC-A5	*Aeromicrobium erythreum* (98)	35.3	3.7	+	+	22.3	17.3	+	+
MCC-SL5	*Duganella nigrescens* (99)	**No**				**No**			
MCC Z	*Pedobacter* sp.*(99)*	No	No	+	+	**67.7**	**56.3**	+	+
MCC-X	*Gordonia* sp.(99)	24.7	4.3	No		**52.3**	**28.7**	+	+
MCC-S	*Massilia* sp. (99)	**No**				**No**			
MCC-E	*Micromonospora* sp.*(*99)	No	No	No	No	9.0	No	No	No
MCC-G	*Nocardia* sp.*(99)*	27.0	No	+	No	3.7	No	No	No
MCC-T	*Porphyrobacter donghaensis* (99)	**No**				**No**			
AGL17	*Acinetobacter calcoaceticus* (100)	**47.7**	**12.0**	+++	++	**42.7**	**10.3**	+	+
TSNR 4	*Ochrobactrum* sp. *(100)*	**No**				12.0	16.3	+	+
CONC18	*Achromobacter xylosoxidans (*99)	**31.7**	**48.0**	+	+	**48.3**	**51.3**	+	+
Type strain DSMZ 3257	*Bacillus subtilis*					**34.3**	**4.3**	**++++**	**++++**
Type strain DSMZ 1128	*Pseudomonas aeruginosa*	43.7	45.0	+++++	+++++				

2.3.2 Pre-optimization of culture medium

During the last few years, much research has demonstrated that the composition and surface properties of the biosurfactant depends not only on the producer strains, but also on the culture conditions, such as, e.g. the nature of the carbon source, the nitrogen source and the chemical and physical parameters [46].

Recently, the statistical approach has been applied for the optimization of biosurfactant production. Kiran et al [80] optimized the production of biosurfactant by *Brevibacterium aureum* MSA13 using industrial and agro-industrial solid waste residues as substrates in solid state culture. Rodrigues et al. [59] optimized medium for biosurfactants production by *Lactobacillus lactis* 53 and *Streptococcus thermophilus* A

using multifactorial analysis. Jacques et al. [81] optimized cultural conditions for surfactin production by *Bacillu subtilis* S499. Franzetti et al. [82] identified the cultural parameters that influence biosurfactant production by *Gordonia* sp.BS29 and found the optimal composition of growth medium for the production.

However, the principal objective of this study was to chemically characterize and determine the physico-chemical and surface properties of a new bioemulsifier produced by a microorganism (of which there is no trace in literature) in order to evaluate its potential application in environmental areas.

For this reason, MSM medium was used in this phase since it has been considered a suitable medium for the production of biosurfactants from *Flavobacteria* [27] and for recovery and purification phase of biosurfactant.

Using the medium it has been possible to assess the effect of glucose concentrations on biosurfactant production with two reasons in mind: 1) to lower production cost; 2) to simplify the recovery and purification phases of the bioemulsifier from the culture medium.

Figure 2.3 shows the time course of extracellular emulsifier production, in terms of emulsification index E_{24}%, by *Pedobacter* sp. MCC-Z grown on MSM medium added of increasing concentrations of glucose, as the only carbon source (0.25, 0.5, 1.0 and 2.0 %, w/v).

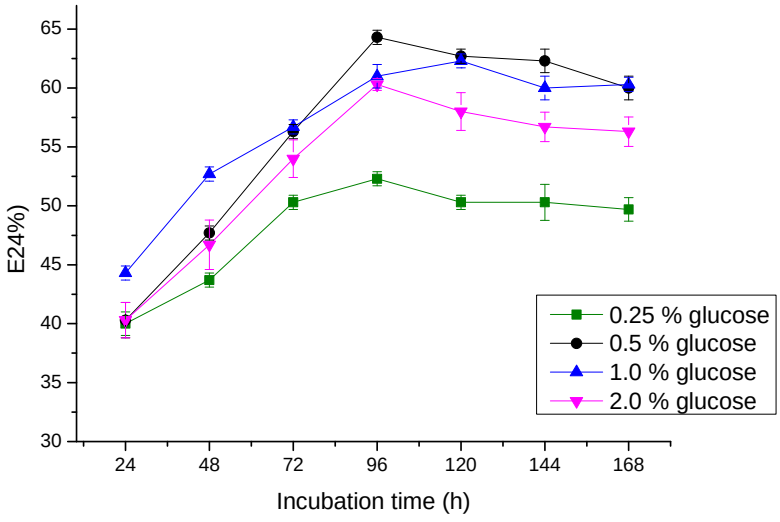

Figure 2.3: E_{24}% values of cell-free supernatants of *Pedobacter* sp. MCC-Z cultures in MSM with different glucose concentrations. Samples were taken at 24 h intervals and the values reported are averages of three replicates ±the standard deviation.

The emulsification index E_{24}% was maximum when 0.5% glucose was added to MSM and reached the peak (64%) at 96 h of inoculation. Furthermore increases of glucose addition resulted to ineffective in term of both production and productivities.

To characterize the relationship between bacterial growth and SACs production, the time course of bacterial cell density was monitored. The growth curve and the emulsifying activity of *Pedobacter* sp. MCC-Z strain in MSM with 0.5% (w/v) glucose, as the only carbon source, are shown in Fig. 2.4.

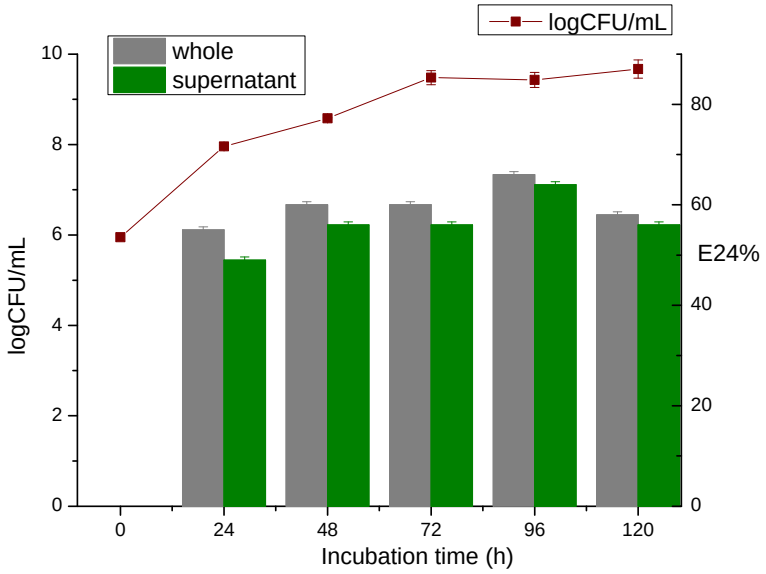

Figure 2.4: Time course of bacterial growth and emulsifying activity of bioemulsifiers produced by *Pedobacter* sp. MCC-Z on whole culture broths and cell-free supernatant. Samples were taken at 24 h intervals. Samples were taken at 24 h intervals and the values reported are averages of three replicates ± the standard deviation.

Results showed that the strain excreted the biopolymers in the medium during the exponential phase (24-48 h) and its concentration reached the maximum during the late stationary phase of the growth (96 hours). Interestingly, the emulsifying activity of the supernatants was slightly lower that observed in the whole culture broth; moreover, the differences decreased during the fermentation process, and was the lowest at 96 and 120 h incubation.

In physical science the first essential step in the direction of learning any subject is to find principles of numerical reckoning and practicable methods for measuring some quality connected with it. I often say that when you can measure what you are speaking about, and express it in numbers, you know something about it; but when you cannot measure it, when you cannot express it in numbers, your knowledge is of a meagre and unsatisfactory kind; it may be the beginning of knowledge, but you have scarcely in your thoughts advanced too the state of Science, whatever the matter may be.

Lord Kelvin

CHAPTER 3 *RECOVERY, PURIFICATION AND PHYSICO-CHEMICAL CHARACTERIZATION OF THE BIOEMULSIFIER PRODUCTION BY PEDOBACTER sp. STRAIN MCC-Z*

3.1 INTRODUCTION

3.1.1 Recovery and purification of SACs

The most important step in the production of biotechnological products are the recovery and purification processes. Generally, these step cost approximately 60% of the total production amount [71]. Thus, the price of microbial surfactants is quite expensive and they are not competitive with chemical surfactants. This cost can be reduced through the use of inexpensive and renewable substrates, by improving product yield and combining steps [29, 31, 83].
On the other hand, the biosurfactant required for the MEOR (Microbial Enhanced Oil Recovery) does not necessarily need to be as pure as required in pharmaceutical preparation, especially in cosmetics and medicine.

The most common SACs recovery methods include extraction, precipitation, and crystallization. The cells must first be separated and either the cell mass or the supernatant is extracted for biosurfactants. Settling, flotation, centrifugation, or rotary vacuum filtration are used for this step. A variety of solvents can be used for product recovery from the culture broth, such as chloroform-methanol, dichloromethane-methanol, butanol, ethyl acetate, pentene, hexane, acetic acid and ether. During the whole of this process the risk of contamination with undesired compounds exist. Further

purification must be carried out by column chromatography, thin layer chromatography, and/or crystallization.

Several conventional methods known for recovery of SACs are mentioned in Table 3.1. The choice is dependent on cost and effectiveness.

For example, precipitation of biosurfactant by ammonium sulphate has been reported for high molecular weight bioemulsifier/biosurfactant, such as Emulsan from *Arthrobacter* [6] and extracellular emulsifier from *Acinetobacter calcoaceticus* [84]. The pellet obtained after centrifugation is dissolved in water and extracted with an equal volume of hexane for the removal of residues. The product is further purified by a dialysis procedure, and lyophilized.

Surfactin, glycolipids and lipopeptides can be obtained by acidification of the supernatants [39]. This method is simple inexpensive and readily available for the recovery of crude bioemulsifier/biosurfactants. The method of precipitation with ethanol and acetone, to purify bioemulsifier/biosurfactant has been used by many different researchers. An example is the isolation of the emulsifier from *Pseudomonas*, *Acinetobacter* and *Bacillus*. Product recovery yields from the culture broth can be quite low. Product losses are an important factor to be considered when selecting an appropriate recovery process. Yields of 30-50% from the recovery steps can effectively double the cost of the previous steps.

Table 3.1: Downstream processes for recovery/purification of biosurfactants/bioemulsifiers

Recovery/purification process	Biosurfactant source	References
Ammonium sulphate precipitation	*Arthrobacter* RAG 1	Rosenberg et al. 1979
	Acinetobacter calcoaceticus	Kaplan and Rosenberg 1982
	A. venetianus RAG-1	Bach et al.2003
Acid precipitation	*Bacillus subtilis*	Lee et al. 2006
Solvent precipitation:		
• Ethanol	*Acinetobacter calcoaceticus*	Phetrong et al. 2008
• Acetone	*Pseudomonas* PG-1	Cameotra et al. 1990
• Methyl tertiary butyl ether	*Rhodococcus*	Kuyukina et al. 2001
Ultrafiltration	*Bacillus subtilis*	Lin 1997
	B. licheniformis	Lin 1998
Adsorption and elution on ion exchange chromatography	*Pseudomonas* sp.	Matsufuji, Nakata 1997
Dyalisis and liophilization	*A. calcoaceticus* BD4	Kaplan and Rosenberg 1982
Filtration and precipitation	*P. aeruginosa*	Turkovskaya, 2001

Rhamnolipid from *Pseudomonas* sp. was purified by ion exchange chromatography [85]. Ultrafiltration membranes have been used mostly to concentrate and purify biosurfactant, such as surfactin or rhamnolipids, in one step [86]. This method is fast, easy and yields highly pure biosurfactant/bioemulsifier. Seamless cellulose tubing dialysis bags are also used for the purification of bioemulsifier. Kaplan and Rosenberg [84] reported production of bioemulsifier from *A. calcoaceticus* BD4, while, Shah and Prabhune [87] reported a simple method for resolution of sophorolipids using dialysis tubing. This method allows easy and rapid purification of bioemulsifier at a low cost. Dialysis and ultrafiltration techniques are commonly used to enhance the purity of bioemulsifier.

The ability to obtain low and high molecular weight bioemulsifier which is reasonably pure, requires several extraction and purification steps. These steps are made simpler by the use of a pure carbon source but, the use of these pure products is extremely expensive. Indeed, recent research has reported the use of low cost sources such as frying oils and other waste oils as a main carbon source, which would also be an environmentally-friendly solution for the recycling of these waste products. Therefore, future prospects should be focused on the production of SACs using inexpensive carbon substrates. In this way, the microbial surface active compounds would become a reasonably attractive alternative to commercial surfactants.

3.1.2 Characteristics and properties of SACs

The term “surfactant” covers a wide diversity of surface active compounds, both synthetic and biological, which concentrate and alter the conditions at interfaces (air-water, oil-water and solid-liquid). Accumulation of surfactants at interfaces or surfaces imply the reduction of the surface tension (air-liquid) or interfaces tension (liquid-liquid). The surface tension is a contractive tendency of the surface of a liquid that allows it to resist an external force. The net effect is an inward force at its surface that causes water to behave as if its surface were covered with stretched elastic. Water has a high surface tension, 72.8 mN/m at 20°C, compared to that of most other liquids. The physicochemical characteristics that define an effective surfactant are its ability to reduce the surface tension of water from 72 to 35 mN/m and to enhance the apparent water solubility of hydrophobic compounds to form water emulsions (interfacial

tension, IT, of water/hexadecane from 40 to 1 mN/m) [27]. Surfactin from *Bacillus subtilis* can reduce the surface tension of water to 25 mN/m and the interfacial tension of water/hexadecane to <1 mN/m [88]. Rhamnolipids from *P. aeruginosa* decrease the surface tension of water to 26 mN/m, and the interfacial tension of water/hexadecane to <1 mN/m [89].

The most important property of bioemulsifier is the ability to form a stable emulsion for months and, in some cases, years. Higher molecular weight SACs are in general better emulsifiers than low molecular weight SACs. For example, the Sophorolipids from *T. bombicola* have been shown to reduce surface tension, but are not good emulsifiers. Instead, Liposan does not reduce surface tension, but has been used successfully to emulsify edible oils. This property is especially useful for application in the cosmetics and food industries.

Surfactants are characterized by properties such as critical micelle concentration (CMC), hydrophilic-lipophilic balance (HLB), chemical structure and charge. Surfactants exist in aqueous solution, at low concentration, as monomers or single molecules; at the CMC, the surfactant molecules begin to spontaneously associate into structured aggregates such as micelles and vesicles and a drastic change occurs in many physico-chemical properties, such as surface tension, turbidity or conductivity [90,91]. These aggregates are capable of dissolving hydrophobic contaminants in their hydrophobic core. The net effect is an increase of apparent aqueous solubility of the hydrophobic compounds [92, 93]. The CMC depends on surfactant structure, composition, temperature, ionic strength and the presence of organic additives in the solutions [94].

Another important parameter is the HLB number of surfactant, it is specific for each surfactant and indicates the types of oil that it can emulsify. This number is determined by the relationship of the hydrophilic and the hydrophobic parts of the surfactant molecule [95]. Surfactants with a low HLB are lipophilic whereas a high HLB is indicative of better water solubility [96].

Microbial surfactants are not generally affected by environmental conditions such as temperature, pH and ionic strength. McInerney et al.[70] reported that lichenysin from

B. licheniformis JF-2 was not affected by temperature up to 50°C, pH 4.5-9.0 and ionic strength of NaCl up to 50 g/L.

3.2 EXPERIMENTAL PROCEDURES

3.2.1 Recovery of the extracellular crude bioemulsifier

The culture supernatant containing the crude bioemulsifier was separated from the cells by centrifugation at 9,000 x g at 25°C for 35 min. The supernatant was filtered through a 0.45-µm Millipore membrane (Milford, MA, USA), and the filtrate was dialyzed using 12 kDa cut off dialysis membrane (Sigma-Aldrich, Steinheim, Germany) in order to separate molecules of less than 12 kDa. The dialyzed solution was concentrated by evaporation and the concentrate was extracted with *n*-hexane (4:1, v/v) in a separatory funnel at 25°C. The white emulsion was separated from the water phase and then washed four times with additional water. Hexane was removed by rotary evaporation at 50°C under reduced pressure and the residue was then freeze-dried and weighed.

3.2.2 Purification of crude bioemulsifier and emulsifying activity

In order to investigate the nature of the compound, the crude extract was purified. For purification, the crude water-soluble bioemulsifier was applied to a Sephadex GC-25 (Pharmacia) column. The column was pre-equilibrated and eluted with deionized water with the flow rate maintained at 1.0 mL/min. Fractions having major emulsifying activity (>60%) were concentrated and lyophilized for further studies.

The emulsification index (E_{24}%) was employed to quantify the emulsifying activity and the standard emulsification assay was conducted as previously described [2.2.4.2]. All measurements were mean values from three independent experiments.

3.2.3 Physico-chemical characterization of Pdb-Z

3.2.3.1 Surface tension measurements

Surface tension measurements were performed by the du Nöuy ring method using a 3S tensiometer (GBX, Romans sur Isère, France) on Pdb-Z solubilized in Ultra pure MilliQ

water at concentrations ranging from 0 to 5.0 mg/mL. All determinations were performed in three replicates, in two separate experiments.

3.2.3.2 Emulsifying activity with various hydrophobic substrates

The hydrocarbon substrate specificity of Pdb-Z was determined by the emulsification assay as described in Cooper and Goldenberg [26]. However, the standard hydrocarbon substrate, *n*-hexane, was substituted by other pure hydrocarbons (*n*-hexadecane, *iso*-octane, cyclohexane, toluene, xylene) or diesel fuel.

The emulsification assay was performed on Pdb-Z samples diluted in distilled water at different concentrations (0.25-0.5-0.75-1.0 mg/mL). The synthetic surfactants Tween-20, Tween-80 and Triton X-100 (Sigma Aldrich) at 0.75% w/v were used as reference compounds. All measurements were mean values from three independent experiments.

3.2.3.3 Stability study: temperature, pH and ionic strength tolerance

Stability studies were conducted to investigate the effect of several physico-chemical parameters on the emulsifying activity of the bioemulsifier, as described elsewhere [97]. All the tests were carried out on three replicates.

- The effect of the temperature on the bioemulsifier activity was evaluated by keeping a 1mg/mL solution of Pdb-Z at different temperatures (-80, -20, +7, +25, +37, +70, +121°C) for 30 min, and then bringing it to room temperature before the emulsification assays.
- The pH of bioemulsifier solutions was adjusted from 2 to 12 to test the effect of pH on the emulsification capability using 1 N HCl or 1 N NaOH at a final bioemulsifier concentration of 1 mg/mL. After 30 minutes, the E_{24} test for each pH condition was carried out.
- The effect of the addition of NaCl (5.0-20.0 %, w/v) on emulsion stability was investigated. After addition of the salt to a 1 mg/mL solution of bioemulsifier crude extract in deionized water, the emulsifying activity was assessed by the emulsification index method (E_{24}%) as described earlier.

3.3 RESULTS AND DISCUSSION

3.3.1 Purification and emulsifying activity

The dialyzed solution of the crude bioemulsifier was separated by gel filtration chromatography on Sephadex GC-25. Four fractions separated from the column, named fraction I - IV, were tested for their emulsifying activity. Fractions I and IV possessed no emulsifying activity, whereas fractions II and III showed an E_{24}% approximately equal to 64%. Respective recovery rates for the fractions were found to be 64.5% and 25.7%. Fractions II and III, namely Pdb-Z, were collected, concentrated and characterized further. An established criterion for emulsion-stabilizing capacity is the ability of an emulsifier to maintain at least 50% of the original emulsion volume 24 h after its formation [98]. Therefore our results indicate that Pdb-Z is an efficient bioemulsifier.

3.3.2 Surface properties of Pdb-Z: Surface tension and CMC value

In order to evaluate the surface properties of Pdb-Z, the surface tension of increasing Pdb-Z concentrations was determined. Concentrations from 0 to 2 mg/mL reduced the surface tension from 73.7 ±0.1 to 41.4±0.6 mN/m (n=6) whereas no further decrease was observed when the concentrations were increased up to 5 mg/mL. A plot of surface tension versus the log of Pdb-Z concentration is presented in Figure 3.1. The CMC value for Pdb-Z, calculated as the intersection between two regression lines describing the curve, was equal to 2.6 mg/mL.

The CMC is the concentration above which Pdb-Z reaches saturation, forming supra-molecular aggregates. In order to compare Pdb-Z performance with well-characterized high molecular weight SACs, the surface tension was used to measure the Pdb-Z effectively whereas the CMC value was used as a measurement of its efficiency as previously established by Neu [4]. Pdb-Z exhibited comparable ability to reduce the surface tension with Alasan by *A. radioresistens* [99] and superior performance when compared with Emulsan by *A. venetianus* RAG-1 [7] even though a higher CMC value was obtained for Pdb-Z in both cases. Nevertheless, Pdb-Z presents CMC values comparable with Arabic gum (1.7 mg/mL) [100], a commercial emulsifier extensively used in the food industry, indicating similar efficiency. Recently, Gutiérrez et al. [101] have characterized the emulsifying properties of a glycoprotein extract produced by a

marine bacterium belonging to the Bacteroidetes phylum, *Flexibacter* sp. strain TG382. However, its surface properties have still to be determined.

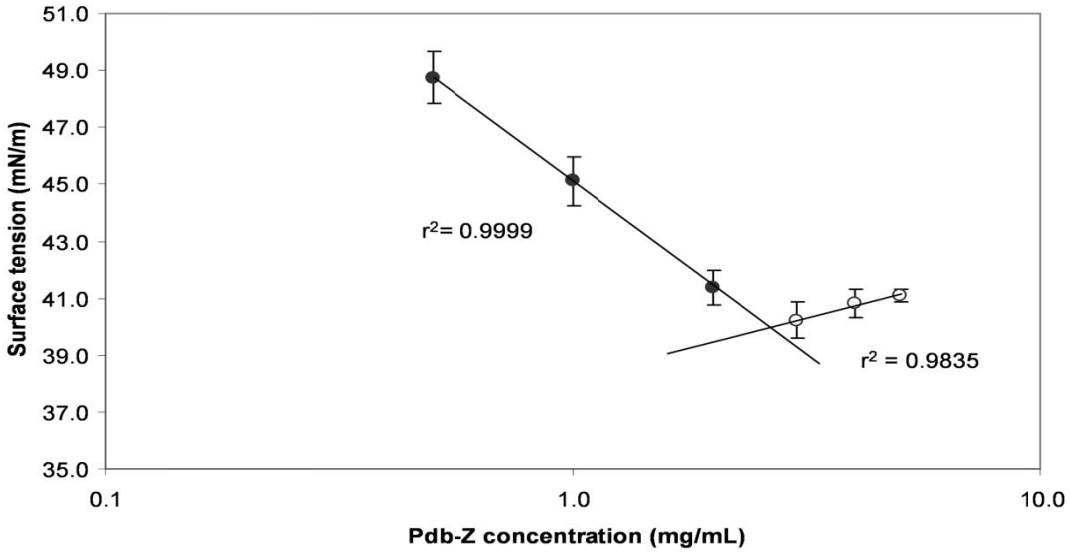

Figure 3.1: The surface tension of Pdb-Z. solutions at different concentrations. The values are averages based on three replicates in two separate experiments (n=6) ± the standard deviation.

3.3.3 Emulsifying property

The substrate specificity of Pdb-Z was evaluated on different hydrophobic substrates and the results are presented in Table 3.2. Pdb-Z formed stable emulsions with alkanes, aromatic hydrocarbons and diesel. The highest emulsifying activity was obtained with aromatic compounds, xylene (68%) and toluene (66%). Among aliphatic compounds, *iso*-octane served as the best substrate, whereas the lowest E_{24}% value (21%) was obtained with *n*-hexadecane and cyclohexane at a Pdb-Z concentration of 0.25 mg/mL (Figure 3.2). Synthetic commercial surfactants Tween-20, Tween-80 and Triton X-100 at 0.75 mg/mL were used to evaluate Pdb-Z performance as a hydrocarbon emulsifier. Pdb-Z exhibited higher emulsifying activity on all hydrophobic substrates, except diesel fuel (40±1.5), in comparison with the synthetic surfactants (Table 3.2 and Figure 3.3).
All water-in-oil emulsions formed during the experiment showed a good stability, maintaining about 90% of the original emulsion volume over a period of 90 days at room temperature. Comparable extended stability was previously observed for the glycoprotein bioemulsifiers produced by a marine *Antarctobacter* [102].

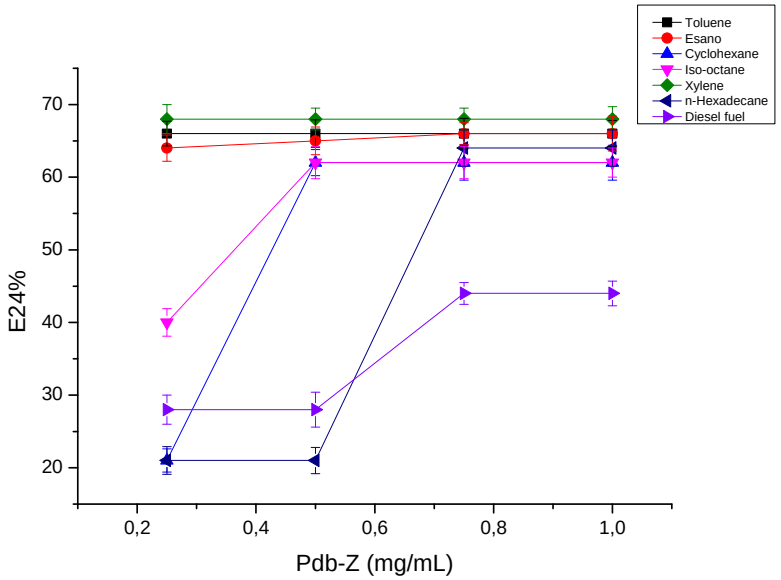

Figure 3.2: Emulsifying activity of Pdb-Z at different concentrations (0.25-0.5-0.75-1.0 mg/mL) with several hydrocarbon substrates.

Table 3.2: Hydrocarbon substrate specificity of Pdb-Z and commercial synthetic surfactants[a].

Hydrocarbon/oil	Emulsifying index (E_{24}%)						
	Pdb-Z(mg/mL)				Tween-20	Tween-80	Triton X-100
	0.25	0.5	0.75	1.0	0.75 (mg/mL)		
n-Hexane	64.2±1.8	65.4±1.9	66.2±1.5	66.1±2.4	53.3±1.8	55.2±2.3	58.5±1.4
n-Hexadecane	21.4±1.9	21.4±1.8	64.1±2.5	64.5±2.5	57.2±1.4	59.4±1.6	60.1±2.1
Cyclohexane	21.2±1.6	62.6±1.8	62.3±2.4	62.3±2.4	48.2±2.1	44.1±1.9	48.0±1.8
iso-octane	40.4±1.9	62.0±2.2	62.6±2.2	62.1±2.0	58.1±2.4	54.0±1.8	54.3±1.9
Toluene	66.3±1.7	66.5±1.9	66.4±2.1	66.1±1.8	28.4±1.5	32.3±1.8	35.0±1.6
Xylene	68.2±2.0	68.5±1.5	68.3±1.5	68.4±1.7	62.3±2.5	59.3±1.6	61.7±1.7
Diesel fuel	28.5±2.0	28.1±2.4	44.4±1.5	44.1±1.7	61.0±2.6	59.6±1.9	63.7±1.5

[a] The values are the means±S.D.(n=3).

The results indicated that the bioemulsifier was capable of effectively emulsifying both aromatic and aliphatic hydrocarbons, suggesting that it could be used for hydrocarbon remediation and oil recovery [103]. Similar results have also been previously described in another bioemulsifier capable of forming stable emulsion with hydrocarbons. For example *Pseudomonas nitroreducens* TSB MJ10 produced a lipopeptide that formed stable emulsions with aliphatic, aromatic and petroleum compounds. It exhibited a maximum emulsification activity with weathered crude oil (97%) [104]. M.A. Luna-Velasco and co-workers produced a biosurfactant by *Penicillum* sp. that was able to

emulsify the pure aromatic, aliphatic hydrocarbons and the hydrocarbons mixtures [105].

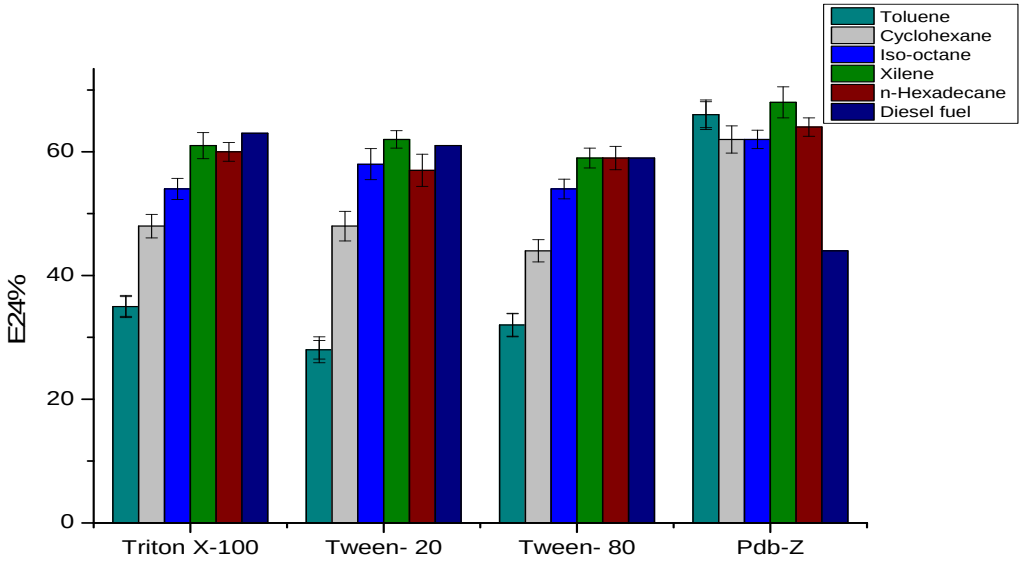

Figure 3.3: Emulsifying activity of Pdb-Z (0.75 mg/mL) with several hydrocarbon substrates in comparison with synthetic surfactants (Triton X-100,Tween-20, Tween-80).

3.3.4 Stability study of Pdb-Z: temperature, pH and ionic strength tolerance

To explore the possibilities of the surfactant applications, a thermal stability analysis of the crude bioemulsifier was carried out with temperatures between 0°C and 120°C, revealing that the properties of the surfactants were maintained with the temperature increase and only a small decrease in emulsifying activity was observed after a thermal treatment of 80°C (Fig.3.4). The product is thermostable, showing a slight reduction of E_{24}% with increasing temperatures. Heat stability of surface active compounds has been reported by Anyanwu et al. [105] (30-100°C), Ilori et al.[102] (20-50°C) and Maneerat and Phetrong [106] (30-121°C).

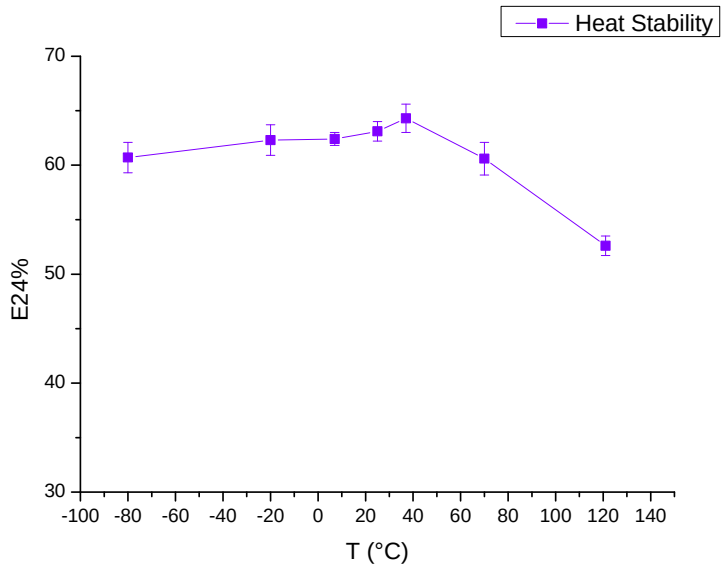

Figure 3.4: Heat stability on emulsifying activity.

The effect of pH on biosurfactant activity is shown in Fig.3.5. The bioemulsifier demonstrated a stable E_{24} over the range of pH 3-11. Such an effect of pH on surface active compounds has been reported earlier [105, 106], as have pH sensitive biosurfactants [102].

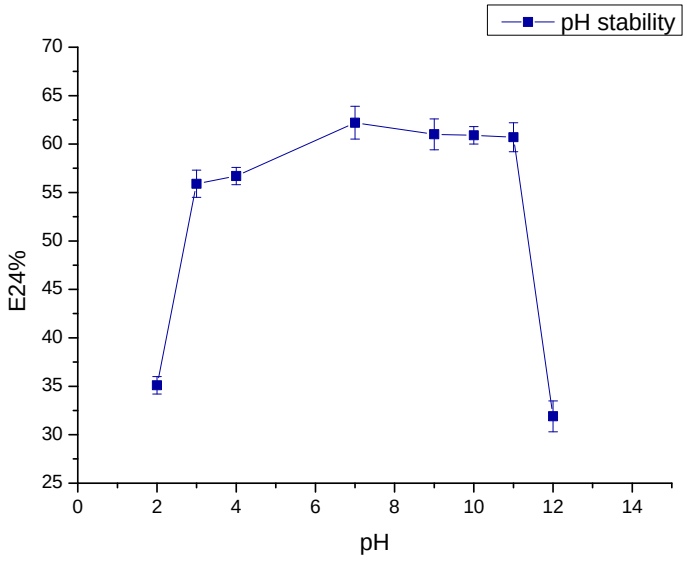

Figure 3.5: Influence of pH on emulsifying activity.

While the effect of added NaCl (5-20%) on the stability of bioemulsifier from MCC-Z is shown in Fig.3.6. The addition of NaCl was ineffective in influencing the activity of bioemulsifiers. Similar results were previously with *Pseudomonas nitroreducens*

TSB.MJJ10 bioemulsifier, whereas SDS used as a comparison showed no activity beyond a 10% NaCl concentration [103]. The tolerance of bioemulsifier to NaCl has already been shown as being restricted to 5% [103], 9% [106], 12% [105] and 15% [107] NaCl.

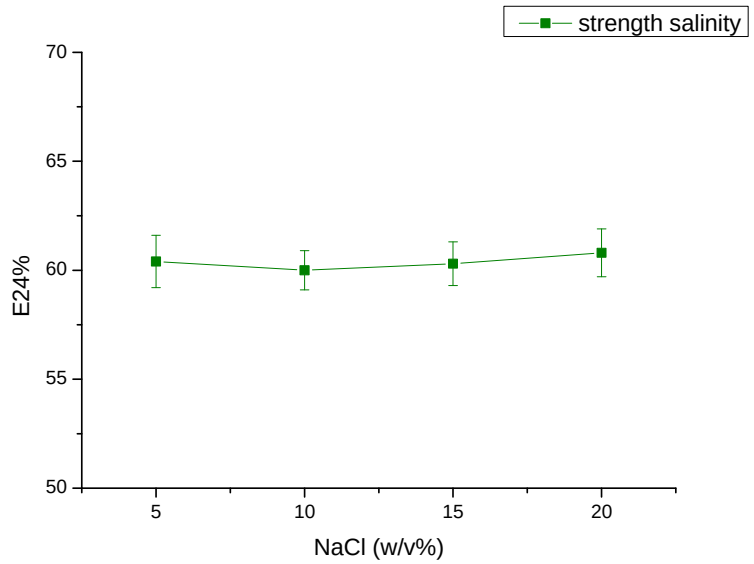

Figure 3.6: Effect of the salt concentration on emulsifying activity.

Emulsion stability in time was verified up to 16 weeks (E_{24}% test, see Fig.3.7). This performance is comparable to examples of bioemulsifiers produced by strains that can be found in literature. Lotfabad et al. [108] described a bioemulsifier produced by *Pseudomonas aeruginosa* MR01 that showed a maximum emulsifying activity of about 70%, which remained stable for more than 5 months. This extended stability has also been previously observed for glycoprotein bioemulsifiers produced by a marine *Antarctobacter* [97].

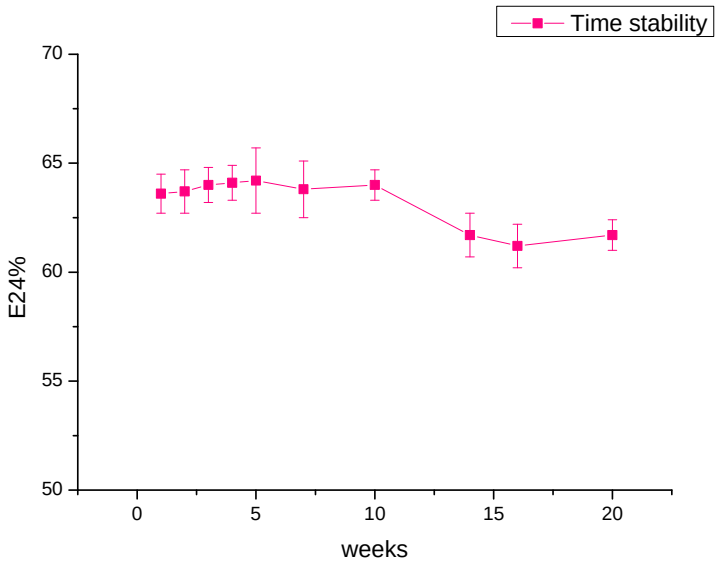

Figure 3.7: Emulsion time stability of Pdb-Z

Wir sehen nur das, was wir wissen.

Johann Wolfgang Goethe

CHAPTER 4 CHEMICAL CHARACTERIZATION OF Pdb-Z

4.1 INTRODUCTION

Microorganisms synthesize an enormous variety of amphipathic molecules that typically concentrate at the interfaces between hydrophobic and hydrophilic phases or on gas/liquid or solid/liquid surfaces. As with chemical surfactants, they are able to reduce surface or interfacial tensions and they have the ability to form molecular aggregates. These compounds exemplify immense structural/functional diversity and consequently possess remarkable applications in a wide range of fields. In the last few years, a growing number of new SAC-producing microorganisms have been described although their products often remain uncharacterized in terms of their chemical structures.

Most research on microbial SACs has been confined to few well-characterized molecules produced by a small number of microbial genera, such as *Acinetobacter, Pseudomonas, Candida, Bacillus, Serratia*. Consequently, our understanding of the diversity, physiological roles and potential applications of microbial SACs is limited to a relatively narrow spectrum of microbial metabolites and biological systems.

4.1.1 Microbial surfactants and their structures

The most extensively studied biosurfactants are **rhamnolipids** produced by several *Pseudomonas* species. These compound were found to be glycolipids, disaccharides acylated with long chain fatty acids or hydroxyl fatty acids. **Sophorolipids,** synthesized

by different species of the yeast *Candida* (formerly *Torulopsis*) [110] are composed of sophorose disaccharide glycosidically linked to a hydroxyl fatty acid.

Trehalolipids contain carbohydrates and long chain aliphatic acid/hydroxyl aliphatic acids and are the most effective bioemulsifiers produced by *Micobacteria, Corynebacteria* and *Rhodococcus* species [111]. Most of the biosurfactants produced by rhodococci are trehalose mycolates consisting of a trehalose residue linked by an ester bond to mycolic acids, long α-alkyl β-hydroxy fatty acids [112].A variety of structurally different variants is produced by several *Bacillus* species. *Bacillus subtilis* produces a cyclic lipopeptide called **surfactin or subtilisin** which has been reported as the most active biosurfactant discovered to date [113]. The most extensively studied bioemulsifiers are the ones produced by different *Acinetobacter* species [5]. An example of a well-characterized high molecular weight SAC is **Emulsan,** an effective emulsifier produced by the *Acinetobacter lwoffii* strain RAG-1 (formerly *Acinetobacter calcoaceticus*). Emulsan is a complex polysaccharide that presents a polyphilic structure being composed of fatty acids attached over the entire molecule to the polysaccharidic backbone [114, 6]. Different species of *Acinetobacter* are known to produce protein polysaccharide complexes. Another well characterized bioemulsifier is **Alasan** produced by *A. radioresistens* KA53, that finds significant application in bioremediation [99]. Alasan is an alanine containing complex heteropolysaccharide and protein polymer that stabilizes oil/water emulsions with *n*-alkanes [8].

Followed by *Acinetobacter*, *Pseudomonas* and *Bacillus* strains, *Serratia* is one of the most well-studied bacterium in terms of molecular genetic studies of bioemulsifier production. *Serratia* is known to produce extracellular surface active bioemulsifier [115]. *Serratia marcescens* produces a cyclic lipopeptide bioemulsifier **Serrawettin** which contains 3-hydroxy-C10 fatty acids side chains [116].

Bodour and co-workers reported a new glycolipid class, the **flavolipids,** produced by a *Flavobacterium* strain isolated from soil. Flavolipids exhibit a unique polar moiety which features citric acid and two cadaverine molecules and display strong surfactant and emulsifying activities [27].

4.2 EXPERIMENTAL PROCEDURES

4.2.1 Chemical composition: proteins, lipids and carbohydrates

Protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad). After the addition of 2x Laemmli buffer (SIGMA), the samples were boiled at 100°C for 5 min and resolved using SDS-polyacrylamide gel electrophoresis. Analysis was performed on the Coomassie blue-stained spots excised from the gels. The spots were selected for mass spectral identification through the merging of images analysis.

Proteins excised from the gel were reduced, alkylated and digested in situ with trypsin, as described by Di Luccia et al.[117]. The peptide mixtures were analysed using a CHIP MS 6520 QTOF equipped with a capillary 1200 HPLC system and a chip cube (Agilent Technologies). The sample was then fractionated on a C18 reverse-phase capillary column (75 μmx43mm in the Agilent Technologies chip) at flow rate of 400 nl min^{-1} with a linear gradient of eluent B (0.1 formic acid in 95% acetonitrile) in A (0.1% formic acid in 2% acetonitrile) from 7 to 60% in 50 min. Spectra were scanned over the range of 400-2000 m/z. Analysis software and MSMS spectra were compared with non-redundant protein databases (NCBInr 20090924, 9760158 sequences) and UniprotSwissprot (2011, 167910 sequences), with the taxonomy restriction to Homo sapiens, using MASCOT 2.1 software (Matrix Science, Boston, USA).

The lipid content of Pdb-Z was determined by GC-MS analysis as described by B. Di Luccia et al.[117]. After an alkaline digestion, the extracted mixture of species was directly analysed by GC-MS as TMS derivates. For lipid analysis the oven temperature was increased from 25°C to 90°C in 1 min and held at 90°C for 1 min before increasing to 140°C at 25°C/min, to 200°C at 5°C/min and finally to 300°C at 10°C/min. Each species was univocally identified on the basis of retention times and electron impact fragmentation spectra (NIST library).

Neutral sugars were determined by GC-MS analysis after hydrolysis with methanolic-HCl at 80°C for 16 h. After neutralization by adding Ag_2CO_3, the re-N-acetylation was achieved with 50 μL acetic anhydride and incubating at room temperature overnight. The trimethylsilylation was carried out in 500 μL SIGMA-SIL-A at 80°C for 20 min. The sample was dried down under nitrogen, dissolved in 50 μL hexane, and centrifuged to remove the excess of solid reagents. The hexane supernatant was used for the GC-MS analysis. GC-MS analyses were performed on a Agilent 7890 GC/5975 MS system

(Agilent technologies) equipped with DB-5MS fused silica capillary column (30 m, 0.25 mm ID, 0.25 μm ft) from J&W.

4.2.2 Fourier Transform Infrared spectroscopy

The main functional groups of the purified bioemulsifier were assigned using Fourier-transformed infrared (FT-IR) spectroscopy. Pellets for infrared analysis were prepared by pressing the purified product. FT-IR spectra were recorded covering an area of 600-4600 cm^{-1}, with 45 accumulated scans and resolution of 8 cm^{-1},using a IR Affinity-1 with ATR Miracle 10 Shimadzu spectrometer.

4.2.3 ^{1}HNMR spectroscopy and Hydrodynamic study of Pdb-Z

The ^{1}H NMR spectra of Pdb-Z were obtained at 600 MHz in D_2O and DMSO-d6 solution on a Brucker Avance 600 MHz, equipped with a 5 mm inverse broadband probe with z-axis gradients. All data were processed with TopSpin software (Brucker). ^{1}H NMR diffusion experiments were performed using the LED sequence with bipolar gradients [118]. The attenuation measured with this sequence is given by:

$$I = I_0 exp^{[-D\left(2\pi\gamma_H G\delta\Delta - \frac{\delta}{3}\right)]}$$

Equation 4.1

where I/I_0 is the normalized signal intensity, D is the diffusion coefficient, δ is the duration of the gradient pulse, γ_H is the gyromagnetic ratio of ^{1}H, G is the gradient strength, Δ is the diffusion time and τ is eddy current delay. Typical acquisition parameters were: recycle delay time between diffusion experiments 5 s; Δ, 1 s (DMSO) or 3 s (D_2O); δ, 4 ms; τ, 5 ms. For the DMSO sample, hydrodynamic radius (R_h) of an equivalent spherical particle was calculated using the Stokes-Einstein equation:

$$D = \frac{K_B T}{6\pi\eta r}$$

Equation 4.1

Where η is the macroscopic viscosity value of the solvent, T the absolute temperature and k_B the Boltzmann constant. When dealing with the D_2O sample, the hydrodynamic radius was calculated using dioxane as internal standard [119]. The Rh of the particle is calculated through the following relationship:

$$R_{h,eff}^{part} = R_h^{dioxane} \left(\frac{D_{dioxane}}{D_{part}}\right)$$

Equation 4.2

where $R_h^{dioxane}$ is the hydrodynamic radius of dioxane (0.21 nm) and $D_{dioxane}$ and D_{part} are the measured diffusion coefficients for dioxane and the particle, respectively.

4.3 RESULTS AND DISCUSSION

4.3.1 Chemical composition of Pdb-Z

The composition of Pdb-Z was 67% of carbohydrates and was composed of galactose, xylose, N-acetyl glucosamine, galacturonic acid and talose monomer units. In terms of peak area, galactose was present as major constituent. A similar percentage of galactose has been described for two surfactants produced by *Trichosporon loubieri* CLV20 and *Geotrichum* sp. CLOA40, which showed a predominance of galactose [120]. However, although a number of polysaccharides and oligosaccharides from various bacteria and yeast have been chemically and structurally characterized to reveal the presence of unusual sugars [121], this is the first study describing a biopolymer from *Pedobacter* sp. containing galactose at significantly high levels.

Therefore, Pdb-Z can be considered as similar to galactan polymers. Pdb-Z showed a lipid content of about 30%, pentadecanoic acid being the major constituent, and 12-methyl-tridecanoic acid and adipic acid the minor constituents. The weight percentages of monosaccharides and lipids of Pdb-Z are shown in Table 4.1.

Table 4.1: Monosaccharide and fatty acid composition of Pdb-Z produced of *Pedobacter* sp.MCC-Z.

Compound	Monosaccharides	
	Molecular formula	**Wt%**
Xylose	$C_5H_{10}O_5$	11.54
Galactose	$C_6H_{12}O_6$	51.17
N-acetylglucosamine	$C_8H_{15}NO_6$	4.73
Talose	$C_6H_{12}O_6$	0.14
Galacturonic acid	$C_6H_{10}O_7$	0.96
Compound	**Fatty acids**	
	Molecular formula	**Wt%**
12-methyl-tridecanoic acid	$C_{14}H_{28}O_2$	2.43
Pentadecanoic acid	$C_{15}H_{30}O_2$	10.97
2-methoxy-myristic acid	$C_{14}H_{28}O_2$	6.07
Palmitic acid	$C_{16}H_{32}O_2$	4.47
Stearic acid	$C_{18}H_{32}O_2$	2.54
3-hydroxy-stearic acid	$C_{18}H_{36}O_3$	4.75
Adipic acid	$C_6H_{10}O_4$	0.21

SDS-PAGE analysis of Pdb-Z indicated the existence of two major proteins with apparent molecular masses of 23 and 8 kDa respectively. The N-terminal amino acid sequences of the two proteins were determined (Table 4.2). The first amino acids of the 23-kDa and 8-kDa proteins showed high similarity to two proteins from *Pedobacter agri:* the first one (Accession number WP_010600298) containing an outer membrane protein β-barrel domain and OmpA domain, and the second one (Accession number WP_010601798) containing a conserved domain of putative periplasmatic proteins. The secretion of OmpA-like proteins with emulsifying activity has recently been demonstrated to be a general property of the oil degrading *Acinetobacter* strains [122], the activity being due to hydrophobic amino acids in a β-barrel region [123]. Moreover, the first evidence is provided of an involvement of OmpA-like proteins in the emulsifying activity by bacteria other than *Proteobacteria.*

Table 4.2: List of proteins identified by LC-MS/MS.

	Proteins identified by LC-MS/MS	PROTEIN ID NCBI
BAND 1	OmpA/MotB domain-containing protein [Pedobacter agri PB92]	gi|409098154
BAND 2	OmpA/MotB domain-containing protein [Pedobacter agri PB92]	gi|409098154
BAND 3	OmpA/MotB domain-containing protein [Pedobacter agri PB92]	gi|409098154
BAND 4	OmpA/MotB domain-containing protein [Pedobacter agri PB92]	gi|409098154
BAND 5	Hypothetical protein PagrP_15035, partial [Pedobacter agri PB92] OMP_b-brl_2outer membrane protein beta-barrel domain; pfam13568" OMP CHANNELS SUPERFAMILY	gi|409099655
BAND 6	Hypothetical protein PagrP_15035, partial [Pedobacter agri PB92] OMP_b-brl_2outer membrane protein beta-barrel domain; pfam13568" OMP CHANNELS SUPERFAMILY	gi|409099655

The molecular composition of these biopolymers may have influenced their emulsifying activity. Kim and co-workers [124, 125] have suggested that both the composition and the distribution of fatty acids, carbohydrates and proteins in an biosurfactant play an important role in its emulsifying activity. An example are the bioemulsans produced by different species of *Acinetobacter* RAG-1; Emulsan is a complex of an anionic heteropolysaccharide and protein [126]. Its surface activity is due to the presence of fatty acids, comprising 15% of the Emulsan dry weight.

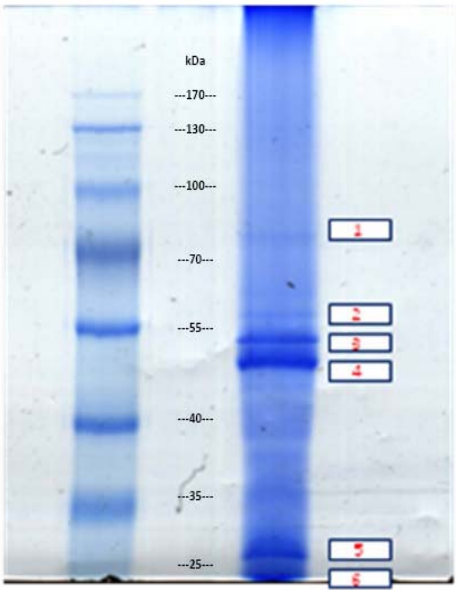

Figure 4.1: SDS-PAGE of Pdb-Z

4.3.2 Fourier Transform Infrared and ^{1}HNMR spectroscopy

Pdb-Z was submitted to FT-IR and ^{1}HNMR analyses for identification of the main functional groups present in the bioemulsifier. In the FT-IR spectrum (Figure 4.2) the presence of a large broad band at 3300 cm^{-1} assigned to O-H stretching, was indicative of significant water and O-H content, typical of polysaccharides. The spectrum also showed a band at 1654 cm^{-1} (υ C=O, amide) and another intense band at 1060 cm^{-1} (υ C-O-C, ethers). The attribution of the carbonyl band to an amide group was supported by the presence of bands at 1550 cm^{-1} (υ N-H, amide). Considering the carbohydrate structure, a small band at 900 cm^{-1} can be seen in the spectra. This band is related to anomers in polysaccharides since the region between 950 and 700 cm^{-1} is strongly dependent on the anomeric carbon [127]. Other important absorption bands which can

be seen in FT-IR spectra of Pdb-Z are the ones at 1740 cm^{-1}, assigned to C=O stretching of acetyl ester bonds, two bands at 2970 and 2880 cm^{-1}, assigned to C-H asymmetric stretch of CH_2 and CH_3 groups, respectively [128]. Overall, the FT-IR spectrum suggested Pdb-Z is predominantly a polysaccharide, although proteins are also present. Similar infrared spectra were reported for the polysaccharide isolated from *Yarrowia lipolytica* IMUFRJ50682 [101] and a water-soluble acidic EPS produced by *Gordonia polyisoprenivorans* CCT 7137 [129].

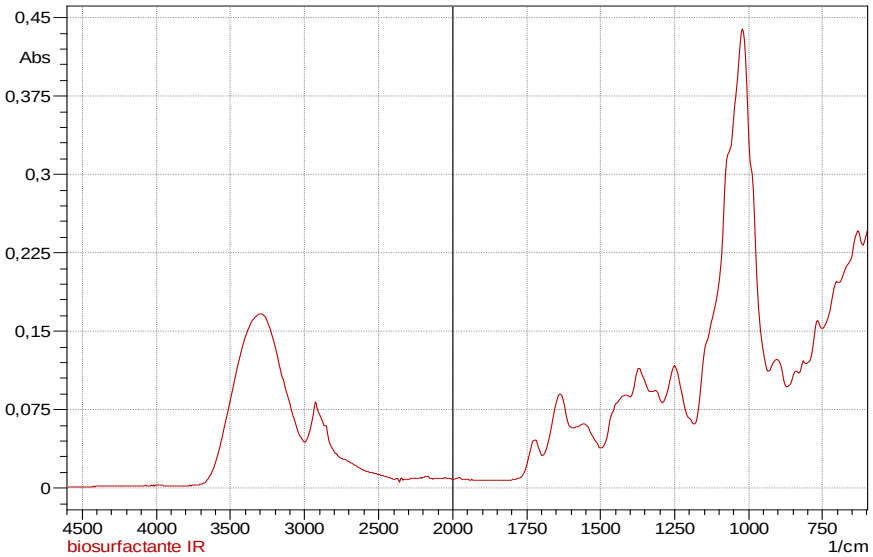

Figure 4.2: FT-IR spectra of bioemulsifier produced of *Pedobacter* sp.MCC-Z strain

Proton NMR study in DMSO and D_2O (Figures 4.3 and 4.4) also confirmed the presence of carboxyl, alkyl, methyl and keto groups (5.01 ppm, 4.88 ppm, 4.975 ppm, ether/ester at 3.477 ppm, alkanes at 1.626 ppm, 1.241 ppm and 0.853 ppm). These results also supported the previous conclusions of GC/MS analysis of Pdb-Z.

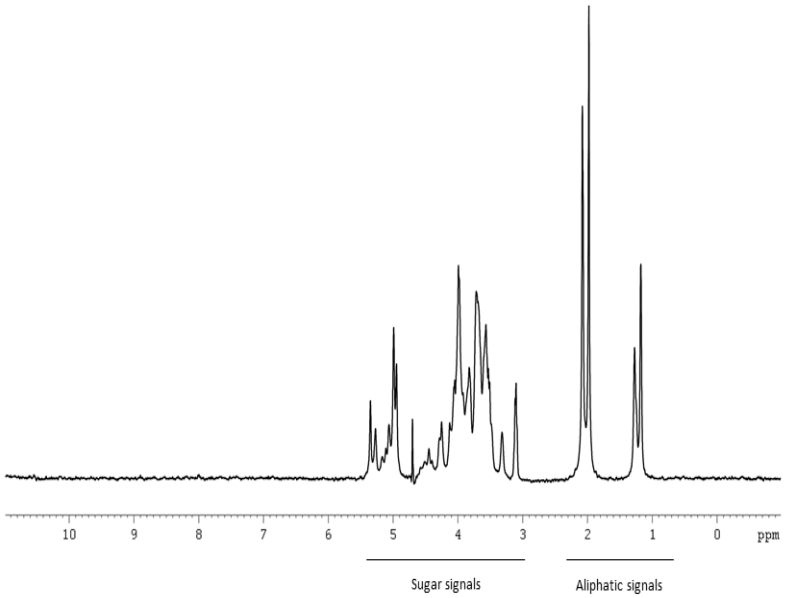

Figure 4.3: ^{1}H spectrum NMR in D_2O and of Pdb-Z

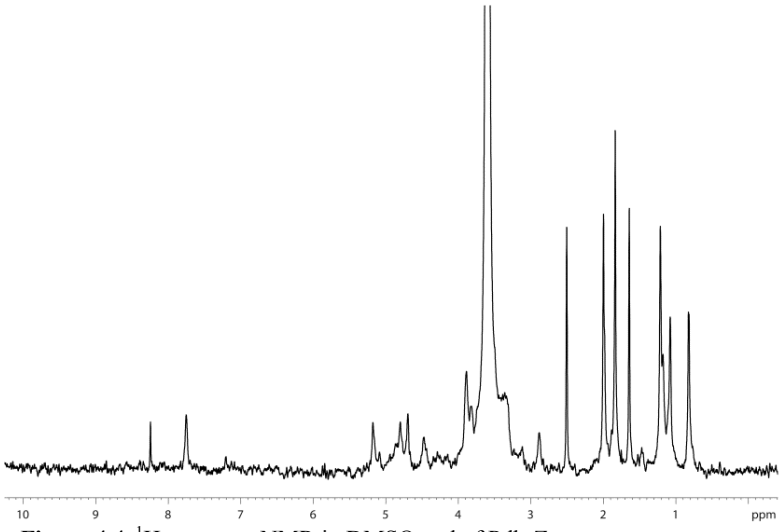

Figure 4.4: ^{1}H spectrum NMR in DMSO and of Pdb-Z

4.3.3 Hydrodynamic behaviour

In order to get insights into the dimension of the molecules that are present in the sample, we conducted a diffusion study using NMR. DMSO is known to break the inter- and intra-molecular hydrogen bonds of polysaccharides, leading to the dispersion of aggregates and making it possible to study individual polymer chains. Variable-gradient ^{1}H-NMR experiments allowed the measurement of the R_h of the different components of the mixture, through the measurement of D, the diffusion coefficient. In Table 4.3, the apparent D values for different signals are reported, together with the

calculated R_h using equation (4.2). From a hydrodynamic point of view, the mixture appears to be heterogeneous. R_h values smaller than 0.8 nm are typical of small molecules. In the sugar region we find two values of around 5 nm, whereas larger values are observed for two signals in the aliphatic region (in the 11-13 nm range).

Table4.3: Self-diffusion coefficients, D, measured for Pdb-Z and the calculated hydrodynamic radius (R_h) for different signals observed in the DMSO spectrum.

δ (ppm)	$D/10^{-10}$ (m^2s^{-1})	R_h (nm)
8.25	6.19	0.19
7.75	0.14	8.34
5.18	0.22	5.30
4.80	1.93	0.60
4.70	0.23	5.09
3.89	0.69	1.68
3.60	6.22	0.19
2.00	0.10	10.99
1.84	0.09	13.43
1.64	4.80	0.24
1.22	1.70	0.68

A different situation was observed in D_2O. D measurements for all the signals in the spectrum appeared to be homogeneous. Table 4.4 shows the D measurements for the bioemulsifier (D^{part}) and dioxane (D^{diox}) used as internal standard [119] and the calculated R_h for the bioemulsifier, using equation (4.3).

A second measurement was performed on a sample diluted 1:10 diluted sample, in order to determine the effect of concentration on particle dimensions.

Table4.4: Self-diffusion coefficients, D, measured at 300K in D_2O for the bioemulsifier (D^{part})

Dilution	$D^{part}/10^{-10}$ (m^2s^{-1})	$D^{diox}/10^{-10}$ (m^2s^{-1})	R_h (nm)
1:1	0.088	9.77	23.5
1:10	0.096	9.88	21.8

The behavior in D_2O differs from that in DMSO in two main aspects: i) the signals present a homogeneous D; ii) the particle size is largely increased, giving R_h values in the order of 20 nm. The calculated R_h values are similar to those found for other

carbohydrate-containing polymers such as cellulose fibers [129], amylose [130], glycogen [131] and amylopectins [132]. Dinadayala and coworkers [28] reported the values of the hydrodynamic radius and of molecular mass for rabbit glycogen of approximately 23 nm and $7x10^6$ Da, respectively. Consequently, we can deduce that Pdb-Z has an molecular weight of approximately 10^6 Da.

At the concentrations used in this study, the aggregation state of the molecules did not change significantly.

Ninguna ciencia, en cuanto a cencia, engagña; el engagño está en quien no sabe

Miguel de Cervantes Saavedra

CHAPTER 5 FEASIBILITY STUDY FOR APPLICATION OF Pdb-Z IN SOIL WASHING REMEDIATION

5.1 INTRODUCTION

5.1.1 Remediation technologies

A variety of in situ and ex situ remediation technologies exist to manage contaminated environmental sites. The ex situ techniques consist of excavation, contaminant fixation or isolation, incineration or vitrification, washing and biological treatment processes, while in situ processes include: bioremediation, extraction methods for soluble components, chemical treatments for oxidation or detoxification and stabilization/solidification for contaminant matrices containing heavy metals [133].

The addition of SACs can be used in bioremediation technologies to improve the biodegradation rate of organic compounds and also for remediation of both organic and metal contaminations, such as in situ soil flushing and ex situ soil washing for remediation of unsaturated areas and pump and treat for aquifer remediation [99, 134, 135]. Biosurfactants have also been found to be useful in oil spill remediation and for dispersing oil slicks into fine droplets and converting mousse oil into an oil-in-water emulsion [136].

For example, there are bioemulsifiers that are capable of increasing the bioavailability of poorly soluble organic compounds, such as polycyclic aromatics. One such emulsifier is Alasan [8], which increases the solubility of several polycyclic aromatic

hydrocarbons, such as phenanthrene, fluorene and pyrene, and significantly accelerates the rate of their mineralization [99].

Shin et al. [137] used a rhamnolipid from *Pseudomonas* to remediate soil contaminated with phenanthrene, using the combined solubilisation-biodegradation process. They reported a high percentage of removal in the solubilisation step and a significant decrease of phenanthrene in the soil sample taken during the biodegradation. Rhamnolipids, due to their anionic nature and to their complexation ability are able to remove metals and ions such as cadmium, cooper, lanthanum, lead and zinc. [135].

Many low molecular weight SACs have shown a high capacity for the removal of crude oil and PAHs from soil, while the information about the removal efficiency of hydrocarbons by high molecular weight SACs is limited to few cases [138]. These compounds are efficient emulsifying agents and are often applied as an additive to stimulate bioremediation and removal of oil substances from environments.

Franzetti et al.[82] evaluated the application of the surface active compound produced by *Gordonia* sp. strain BS29 in various soil remediation technologies, namely bioremediation of soils contaminated by aliphatic and aromatic hydrocarbons, and washing of soils contaminated by crude oil, PAHs and heavy metals. Bioremediation results showed that the bioemulsans produced by *Gordonia* sp. strain BS29 were able to slightly enhance the biodegradation of hydrocarbons. Indeed, in soil washing experiments the crude oil removal with a solution of bioemulsans was 33%. Therefore, bioemulsans produced by *Gordonia* sp. strain BS29 are a promising washing agent for the remediation of hydrocarbon-contaminated soils. In addition, BS29 bioemulsans were also able to remove metals (Cu, Cd, Pb, Zn, Ni) but their potential in the soil washing process was lower than rhamnolipids.

Examples of biosurfactant/bioemulsifier, their producers and applications in environmental technologies are presented in Table 5.1.

Table 5.1: Microbial SACs and their use in environmental remediation.

Surface Active Compounds		Producing Microorganisms	Applications in Environmental Biotechnology	References
Low Molecular Weight				
Group	**Class**			
Glycolipids	Rhamnolipids	*Pseudomonas aeruginosa*, *Pseudomonas* sp.	Enhancement of the degradation and dispersion of different classes of hydrocarbons; emulsification of hydrocarbons and vegetables oils; removal of metals from soil.	Sifour et al. 2007 Whang et al. 2008 Herman et al. 1995 Maier et al.2000
	Trehalolipids	*Micobacterium tuberculosis*, *Rhodococcus erythropolis*, *Arthrobacter* sp., *Nocardia* sp., *Corynebacterium* sp.	Enhancement of the bioavailability of hydrocarbons.	Franzetti et al. 2010
	Sophorolipids	*Torulopsis bombicola*, *Torulopsis petrophilum*, *Torulopsis apicola*	Recovery of hydrocarbons from dregs and muds; removal of heavy metals from sediments; enhancement of oil recovery.	Whang et al. 2008 Pesce et al. 2002 Baviere et al. 1994
Fatty acids, phospholipids and neutral lipids	Corynomycolic acid	*Corynebacterium lepus*	Enhancement of bitumen recovery	Gerson et al. 1978
	Spiculisporic acid	*Penicillium spiculisporum*	Removal of metal ions from aqueous solution; dispersion action for hydrophilic pigments; preparation of new emulsion-type organogels, superfine microcapsules, heavy metals sequestrants.	Ishigami et al. 1983 Ishigami et al. 2000 Hong et al. 1998
	Phosphati-dylethanolamine	*Acinetobacter* sp., *Rhodococcus erythropolis*	Increasing the tolerance of bacteria to heavy metals.	Appanna et al. 1995
Lipopeptides	Surfactin	*Bacillus subtilis*	Enhancement of the biodegradation of hydrocarbons and chlorinated pesticides; removal of heavy metals from a contaminated soil, sediment and water; increasing the effectiveness of phytoextraction.	Awashti et al.1999 Arima et al. 1968 Jennema et al. 1983
	Lichenysin	*Bacillus licheniformis*	Enhancement of oil recovery.	Thomas et al. 1993
High Molecular Weight				
Polymeric biosurfactants	Emulsan	*Acinetobacter calcoaceticus* RAG-1	Stabilization of the hydrocarbon in water emulsions.	Zosim et al. 1982
	Alasan	*Acinetobacter radioresistens* KA-53		Toren et al. 2001
	Biodispersans	*Acinetobacter calcoaceticus* A2	Dispersion of limestone in water.	Rosenberg et al. 1988
	Liposan	*Candida lipolytica*	Stabilization of hydrocarbon in water emulsions.	Cirigliano et al. 1984
	Mannoprotein	*Saccharomyces cerevisiae*		Cameron et al. 1988

5.1.2 Overview of soil washing

Soil washing is a treatment technology used for removing contaminants from polluted soils. Largely based on the use of aqueous surfactant solutions, it exploits both the solubilisation capabilities of micelles and the lowering of the interface tension between the washing liquid and the soil particles, thus facilitating the solutes desorption and their removal from the soil pores [93, 139, 140]. With this process, the washing solution extracts and separates the contaminants from the soil, thereby reducing the quantity of contaminant for further treatment. Figure 5.1 illustrates a typical soil washing process. Surfactants may be added to the washing water, which should be recycled, or treated prior to disposal.

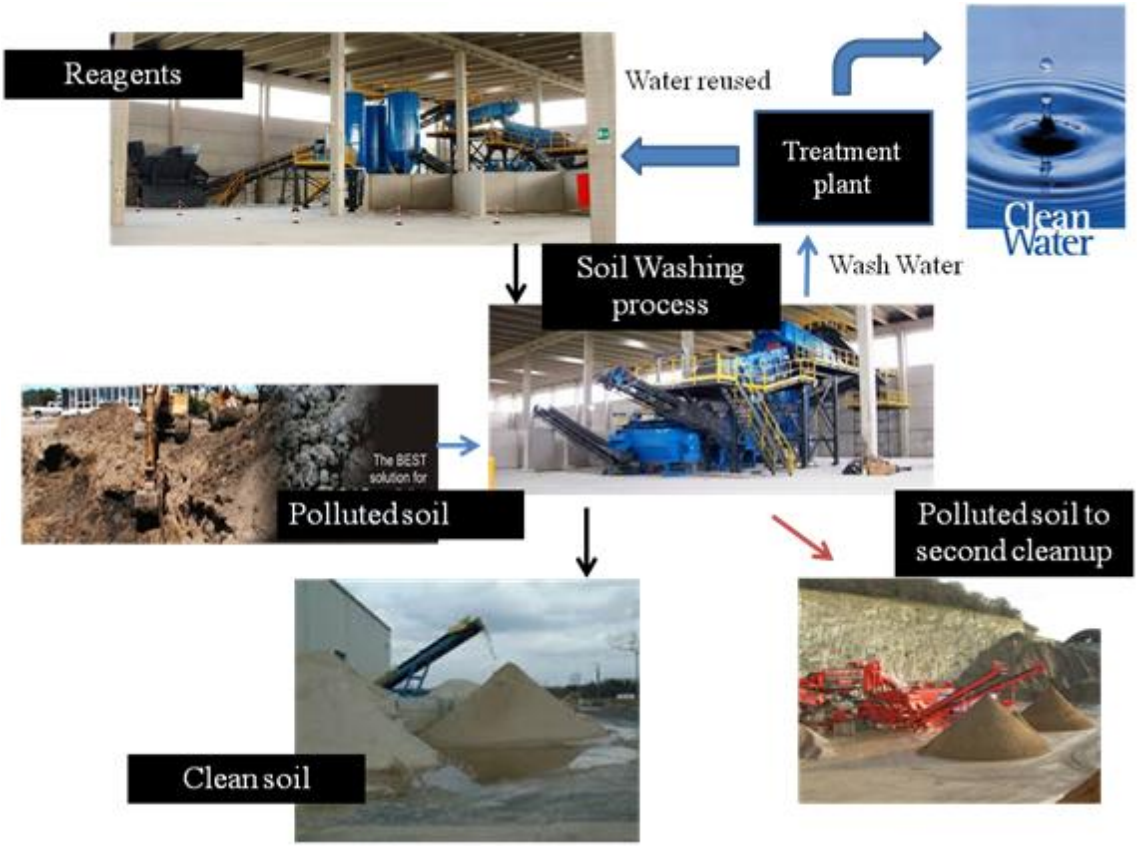

Figure 5.1: Scheme of typical soil washing process.

SACs applications in enhancing ex-situ soil washing have been studied [17]. These studies suggested that the removal of organic pollutants using surface agents occurs at concentration both above and below the surfactant CMC. Thus, the mechanisms suggested involved two steps: mobilization and solubilization [17, 21, 141]. The mobilization occurs at concentrations below the biosurfactant CMC. At such concentrations, the biosurfactant reduces the surface and interfacial tension between air/water and soil/water systems. Due to the reduction of the interfacial tension, contact of biosurfactants with soil/oil systems increases the contact angle and furthermore, causes the reduction in the capillary force that hold the oil and soil together. Above the surfactants CMC, the solubility of organic compounds increases dramatically due to the formation of dynamic aggregates known as micelles. The hydrophobic ends of the surfactant molecules connect together inside the micelle structure, while the hydrophilic ends are exposed to the aqueous phase on the exterior. Therefore, the interior of a micelle constitutes a suitable site for organic compounds. This process is known as solubilization.

Lai et al. [142] have studied the ability of removing total petroleum hydrocarbons (TPH) from soil by using four surfactants: rhamnolipid, surfactin, Tween-80 and Triton X-100. The TPH removal efficiency was studied for low TPH contaminated (LTC) and high TPH contaminated (HTC) soils by washing them with the surfactant solutions. The results obtained demonstrated that using a washing solution containing 0.2% w/w of surfactant to LTC soil the TPH removal was of 23%, 14%, 6% and 4% respectively, while for HTC soil a significantly higher TPH removal efficiency of 63%, 62%, 40% and 35% respectively was observed. These results indicated that among the four surfactants, rhamnolipid and surfactin showed superior performance on TPH removal, compared to synthetic surfactants.

Van Dyke et al. [143] found that *Pseudomonas aeruginosa* UG2 at a concentration of 5 g/L removed approximately 10% more hydrocarbons from a sandy loam soil than from a silty loam soil and that sodium dodecyl sulphate (SDS) was less effective than the biosurfactants in removing hydrocarbons.

Urum et al.[18] investigated the efficiency of several surfactant solutions in removing crude oil from contaminated soil by soil washing techniques. They demonstrated higher crude oil removal by synthetic surfactant (SDS) and rhamnolipids biosurfactants (46% and 44% respectively) than saponins, natural surfactants (27%).

5.2 EXPERIMENTAL PROCEDURES

5.2.1 Soil and its contamination

The soil used for this study was collected from Malagrotta-Valle Galeria site (Italy, Rome). The soil was classified according to Wentworth scale of soil siltiness (Table 5.2). Properties of the soil used are indicated in Table 5.1.

A fixed mass (2.0 g) of the soil was artificially contaminated (2.5%, w/w) with road diesel (Q8 Quaser, http://www.q8quaser.it/pdf/Gasolio_Autotrazione_Quaser.pdf) and adequately shaken at 20°C for 24 hours using an overhead mixer at a constant speed (20 rpm), ensuring uniform distribution of the road diesel in the soil

Table 5. 2: Properties of soil samples.

Properties	Value
Particle size distribution	
Silty (≤0.06 mm)	4%
Sandy (0.06-2mm)	96%
Gravel (≥2mm)	---

5.2.2 Soil washing experiments

Batch washing experiments were carried out in several conditions (washing time, concentration of solution and ratio soil/washing solution ratio) along the experimental design reported in Table 5.3. After washing, the road diesel removal was determined by GC/MS analysis.

Table 5.3: Experimental plan and parameter levels.

Studies	Parameters levels tested					
	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3
Washing solution		**Pdb-Z**			**Tween-20**	
Concentration of solution (% w/v)	0.75	1.5	2.5	0.75	1.5	2.5
Washing time (min)	30	60	90	30	60	90
Ratio soil/washing solution (w:v)	10	20	30	10	20	30

In each investigation, soil washing was conducted with 2 g of contaminated soil, which was poured into sealed 25 mL bottles. The experiment was conducted to study the removal of road diesel from soil first with distilled water, secondly with solutions at different concentrations of Pdb-Z and Tween-20 (Sigma Aldrich). The experimental plans and different parameters levels outlined in Table 5.3 were followed during the experiment. After the introduction of the suitable solution according to the experimental design, the bottles were shaken at 200 rpm and at 20°C.

5.2.3 Analysis of TPH in soil

After 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 left for 24 h. The Total Petroleum Hydrocarbon (TPH) in soil was determined after n-hexane extraction, as already described in Urum and co-workers[144]. Ten cm^3 of n-hexane was added to 2 g of contaminated soil, shaken for 5 min and the n-hexane/oil extract was then removed. This process was repeated four times. All the *n*-hexane/oil extract was collected into one volumetric flask and made up to 50 cm^3 with *n*-hexane. A sample from the 50 cm^3 extract was centrifuged for 20 minutes at a speed of 2000 rpm. This process separates suspended particles that may cause problems during subsequent measurement.

The quantity of total petroleum hydrocarbons (TPH) in the extract was determined using a gas chromatography mass spectrometry analysis using the Agilent 7890 GC/5975 MS system (Agilent technologies) equipped with DB-5MS fused silica capillary column (30 m, 0.25 mm ID, 0.25 µm ft) from J&W. Electron impact ionisation, electron energy 70 eV Scan rate of sample was 40-600 amu at 2 scans s^{-1}. Helium at flow rate of 1 mL/min was used as a carrier gas. The temperature was first held at 40°C for 5 minutes, then increased from 40°C to 300°C (for 10 minutes) at a rate of 10°C/min. One µL of the TPH/*n*-hexane extract, containing α-androstane, as internal standard, was injected into the equipment for each investigation

The TPH removal efficiency was estimated as below:

$$TPH\ removal\ efficiency\ (\%) = \frac{TPHi - TPHr}{TPHi} \times 100\%$$

Equation 5.1

Where TPH_i is the initial oil in the soil before washing and TPH_r the oil remaining in the soil after washing. This experiment was repeated in duplicate and the average of the results is presented. Statistical analysis was carried out using analysis of variance (ANOVA) followed by least significant difference (LSD) method. In this method, a difference is calculated that is judged to be the smallest difference that is significant. The difference between each pair of means is then compared with the least significant difference to determine which means are significantly different.

$$LSD = t\sqrt{\frac{2 \times MSE}{N_g}}$$

Equation 5.2

Where MSE is the mean square error, N_g is the number of replicates in each group and t is the Student's t. The LSD method was applied to all the experimental data, to investigate whether the difference between the experimental values obtained under different conditions could be considered significant.

5.3 RESULTS AND DISCUSSION

5.3.1 Washing of road diesel contaminated soil

In order to evaluate the applicability of Pdb-Z in soil washing treatment a set of experiments was performed. ANOVA tests were used for the experiments of Pdb-Z, Tween-20 and water-only treatment. The aim was individuate the significant differences between the TPH removal capability of the washing solution at different experimental conditions. The estimated relative errors were obtained from the calculated pooled standard deviation, which represents an accurate estimation of the true standard deviation of the experimental method. Tables 5.4-5.6 show the mean, the relative errors of the TPH removal (%) and the results of ANOVA tests such as confidence level (CL) and p-value. Tabled values refer to each washing solution at several experimental conditions.

As reported in Tables 5.4-5.6, the means of TPH removal for all washing solutions of Pdb-Z and Tween-20 are significantly different, with a confidence level higher than 95%, from the mean of the TPH removal in the experiments in which was used just water. These results demonstrated that both (bio)surfactants were able to remove road diesel from contaminated soil and the performance of Pdb-Z in TPH removal was comparable to that of Tween-20. In particular, Pdb-Z shows a better performance than Tween-20, with a suitable confidence level of 90% at 30 minutes, by using a concentration of washing solution of 0.75%(w/w) and volume/mass ratio of 30%.

Table 5.4: Means, relative error and ANOVA results in soil washing experiment at different concentrations.

Concentration (%, w/w)	Washing solution			
	Tween-20		Pdb-Z	
	Mean (%)	E_r (%)	Mean (%)	E_r (%)
0.75 (1)	60.5	5.2	68.0	4.6
1.5 (2)	67.5	4.6	72.5	4.3
2.5 (3)	73.0	4.3	75.0	4.2
ANOVA	C.L.(%)	p	C.L.(%)	p
mean1-mean2	85	0.15	60	0.4
mean1-mean3	99	0,01	85	0.15
mean2-mean3	70	0.3	45	0.55
ANOVA Tween-20-Pdb-Z	C.L.(%)	p		
0.75	90	0.1		
1.5	70	0.3		
2.5	40	0.6		

Table 5.5: Means, relative error and ANOVA results in soil washing experiment at different washing time.

Washing time (minutes)	Washing solution					
	Water		Tween-20		Pdb-Z	
	Mean (%)	E_r (%)	Mean (%)	E_r (%)	Mean (%)	E_r (%)
30 (1)	40.0	7.8	60.5	5.2	68.0	4.6
60 (2)	49.0	6.4	65.0	4.8	70.0	4.5
90 (3)	60.0	5.2	70.0	4.5	73.0	4.3
ANOVA	C.L.(%)	p	C.L.(%)	p	C.L.(%)	P
mean1-mean2	95	0.05	60	0.4	40	0.6
mean1-mean3	99.99	0.0001	95	0.05	70	0.3
mean2-mean3	95	0.05	70	0.3	50	0.5
ANOVA Water-Tween-20						
Washing time (minutes)	**30**		**60**		**90**	
	C.L.(%)	p	C.L.(%)	p	C.L.(%)	P
	99.99	0.0001	99.9	0.001	95	0.05
ANOVA Water-Pdb-Z						
Washing time (minutes)	**30**		**60**		90	
	C.L.(%)	p	C.L.(%)	p	C.L.(%)	P
	99.9999	1E-06	99.99	0.0001	99	0.01
ANOVA Tween-20-Pdb-Z						
Washing time (minutes)	**30**		**60**		**90**	
	C.L.(%)	p	C.L.(%)	p	C.L.(%)	P
	90	0.1	70	0.3	50	0.5

Table 5.6: Means, relative error and ANOVA results in soil washing experiment at different volume/mass ratio.

Volume/mass ratio (v/w)	Washing solution					
	Water		Tween-20		Pdb-Z	
	Mean (%)	E_r (%)	Mean	E_r (%)	Mean (%)	E_r (%)
10 (1)	40.0	7.8	56.5	5.5	64.0	4.9
20 (2)	40.0	7.8	60.0	5.2	66.0	4.7
30 (3)	40.0	7.8	60.5	5.2	68.0	4.6
ANOVA	**C.L.(%)**	**p**	**C.L.(%)**	**p**	**C.L.(%)**	**p**
mean1-mean2	0	1	50	0.5	40	0.6
mean1-mean3	0	1	60	0.4	60	0.4
mean2-mean3	0	1	10	0.9	40	0.6
ANOVA Water-Tween-20						
Volume/mass ratio (v/w, %)	10		20		30	
	C.L.(%)	**p**	**C.L.(%)**	**p**	**C.L.(%)**	**p**
	99.9	0.001	99.99	0.0001	99.99	0.0001
ANOVA Water-Pdb-Z						
Volume/mass ratio (v/w, %)	10		20		30	
	C.L.(%)	**p**	**C.L.(%)**	**p**	**C.L.(%)**	**p**
	99.999	1E-05	99.999	1E-05	99.9999	1E-06
ANOVA Tween-20-Pdb-Z						
Volume/mass ratio (v/w, %)	10		20		30	
	C.L.(%)	**p**	**C.L.(%)**	**p**	**C.L.(%)**	**p**
	90	0.1	70	0.3	40	0.6

The effects of different parameters studied in road diesel removal from soil are reported in the next paragraphs.

5.3.2 Effect of surfactant concentrations

Biosurfactant concentration is usually a critical factor for the removal of hydrocarbons from soil. To evaluate the performance of Pdb-Z and Tween-20 in removing TPH from contaminated soil, three (bio)surfactant concentrations (0.75-1.5-2.5 w/w%) were applied to wash the contaminated soil. It was observed that increasing the concentration of (bio)surfactant both Pdb-Z and Tween-20 appeared to enhance TPH removal from soil (Fig. 5.2). The maximum TPH removal efficiency for Pdb-Z and Tween-20 both occurred at 2.5%w/v giving a removal percentage of 75% and 73% respectively

(Fig.5.2). TPH removed from soil by distilled water was about 40% (Fig. 5.2). This trend was expected, since the force of attraction between soil and road diesel would be reduced by the increase in contact angle in the presence of the (bio)surfactant. However, all the washing solutions of (bio)surfactants showed a slight rise in TPH removal between 0.75 and 2.5%, w/v. Using the ANOVA test, the comparison between the means of TPH removal of the two (bio)surfactants shows that the significant differences decrease with increasing concentration. At low concentration the TPH removal increases from 60.5% to 67.5% for Tween-20, and from 68% to 72.5% for Pdb-Z, while at high concentration the removals are practically constant with a p-value of 0.3 for Tween-20 and 0.55 for Pdb-Z. However, increasing the (bio)surfactant concentration beyond 0.75% w/v may not be economically feasible.

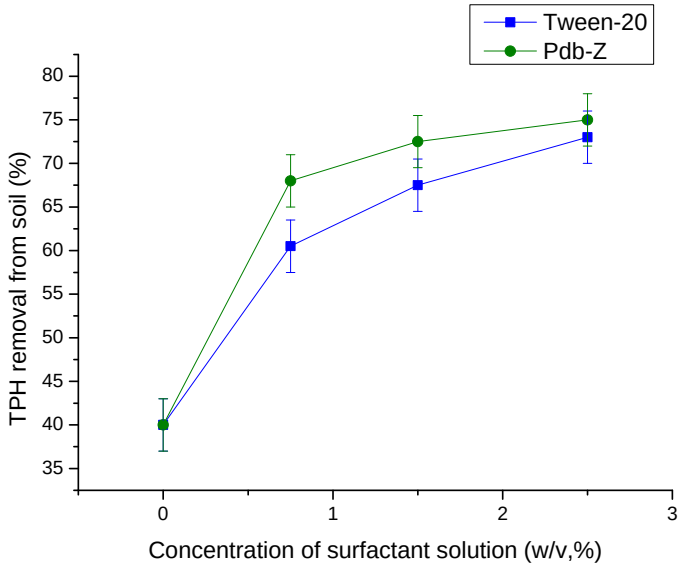

Figure 5.2: Crude oil removal *vs* concentration of surfactant solutions (washed with 60 mL for 30 minutes).

5.3.3 Effect of washing time

In order to find out the TPH removal effectiveness of the PdbZ solutions, experiments of soil washing at different time intervals between 30 and 90 minutes were performed. As can be seen from Fig. 5.3, oil removal increased as time rose from 30 to 90 minutes. All the washing solutions of (bio)surfactants showed a slight rise in TPH removal between 30 and 90 minutes. The increased TPH removal may be attributed to the greater contact time of the surfactants Pdb-Z and Tween-20 with the contaminated soil.

However, the water samples showed that maximum TPH removal could be attained within 60 minutes of washing. The comparison using the ANOVA test, between the means of TPH removal provided by every (bio)surfactant shows that the significant differences increase while increasing the washing time. From 30 to 60 minutes the TPH removal increases while increasing from 60.5% to 65.0% for Tween-20 and from 68% to 70.0% for Pdb-Z, while at 90 minutes the removal is practically constant with a p-value of 0.3 for Tween-20 and 0.5 for Pdb-Z. Therefore, increasing washing time beyond 30 minutes may not be practical, nor economically feasible and cost effective.

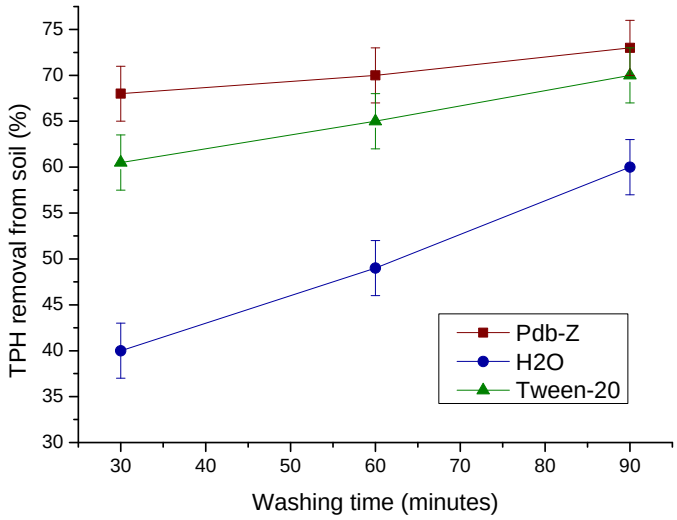

Figure 5.3: Crude oil removal ***vs*** washing time (washed with 60 mL solution 0.75 %w/v).

5.3.4 Effect of volume/mass ratio

To understand the effects of the volume of washing solutions variation on TPH removal, a set of experiments were carried out. Results obtained from this study are shown in Fig. 5.4. TPH removal was slightly enhanced between 10 and 30% v/w for the (bio)surfactants solutions and the divergence between Pdb-Z and Tween-20 was negligible. However, for the water samples, removal of TPH remains constant in the range 10-30% v/w. All the washing solutions of (bio)surfactants showed slight increases in TPH removal within the range tested. The comparison, using the ANOVA test, between the means of TPH removal of Pdb-Z and Tween-20 shows that the significant differences decrease while increasing the volume /mass ratio. From 10 to 30%, v/w the TPH removal increases from 56.5% to 60.0% for Tween-20 and from 64% to 66% for Pdb-Z. At 30% v/w the removal is practically constant with a p-value of 0.9 for Tween-

20 and 0.6 for Pdb-Z. Therefore, the removal of TPH from the soil samples would be effective with 20 mL solutions with a mass of contaminated soil of 2 g.

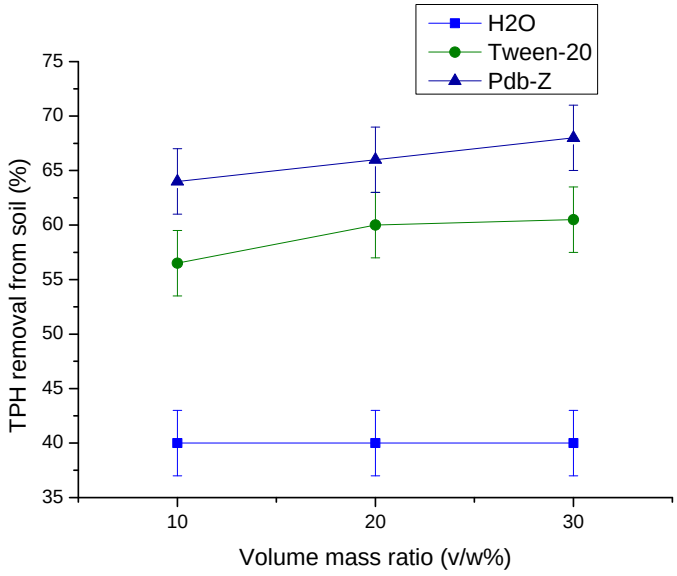

Figure 5.4: Crude oil removal *vs* volume/mass ratio (washed with 0.75% w/v for 30 minutes).

Regardless of the TPH concentration, Pdb-Z showed excellent effectiveness on TPH removal from contaminated sandy soil, thereby being suitable for future application in environmental remediation. In order to evaluate, the potential application of Pdb-Z, further experiments may be performed using historically contaminated soils which are characterized by a reduced availability of organic pollutants.

Meravegliarsi di tutto è il primo passo della ragione verso la scoperta

Louis Pasteur

CHAPTER 6 CONCLUSIONS

Environmental biotechnology, using scientific and engineering knowhow as well as microorganisms and their products in the prevention of environmental pollution through biotreatment of solid, liquid and gaseous bioremediation of polluted environments, and biomonitoring of the environment and treatment processes, have begun to expand particularly in the last few decades [145].

Low solubility and high hydrophobicity of certain hydrophobic soil contaminants limit the remediation process [145]. Addition of surface active compounds can contribute positively to the remediation process by improving the mobilization, solubilization, or emulsification and, consequently SACs removal from contaminated soils [146]. However, the relative high toxicity, low biodegradability, and limited efficiency at low concentrations limit their utilization in bioremediation technology. Microbial surfactants, produced by a wide variety of microorganisms may be a valid alternative in order to avoid the use of chemical surfactants in bioremediation technology [17].

They posses many advantages over synthetic surfactants: lower toxicity, biodegradability, ecological acceptability and effectiveness at a wide range of pH, temperature, and salinity values [147]. In addition, biosurfactants could easily be produced from renewable resources, making their use an additional advantage over chemically synthetic surfactants. Furthermore, the production processes of biosurfactants are less energy-consuming and do not involve hazardous chemicals.

Many reports have described the efficacy of microbial surfactants in improving the solubility of hydrocarbons [148, 149]; other studies have reported the washing ability of biosurfactants of hydrocarbon-contaminated soils [150, 151]; several studies have been

carried out on the effect of SACs on the biodegradation rate of both aliphatic and aromatic hydrocarbons [82, 30].

In the last few years, a growing number of new SACs-producing microorganisms have been described, although their active products have often remained uncharacterized in their chemical structure. Consequently, our understanding of the physiological roles, the surface properties and potential applications has been generally limited to few well-characterized molecules produced by a small number of microbial genera (*Pseudomonas, Candida, Bacillus, Acinetobacter*).

Several research groups have presented intriguing data suggesting that biosurfactants are important for the growth and survival of those microorganisms in the environment [113]. However, the reason behind the production of biosurfactants by microorganisms is not always so obvious. Some suggested physiological roles of biosurfactants include the increase of surface area and the bioavailability of hydrophobic and water-insoluble substrates, heavy metal binding, bacterial pathogenesis, quorum sensing and biofilm formation [113].

The experiments described in this thesis have been planned according to these remarks.

In the **first part** of the thesis work we have developed a screening method to identify novel bacterial strains able to produce bioemulsifiers that show high emulsion-stabilizing capacity after grown in mineral salt medium supplemented with glucose.

Pseudomonas aeruginosa (1128 DSMZ) and *Bacillus subtilis* (3257 DSMZ) were used as positive controls and to validate the oil spreading technique and emulsifying assay; in fact, a confirmation of the data found in literature for both strains was obtained.

Out of eleven bacterial strains submitted to the screening procedure, *Pedobacter* sp. strain MCC-Z showed the highest emulsifying activity and was therefore selected as a potential bioemulsifier producing microorganism. It is especially interesting, because it belongs to a genera not yet described as a bioemulsifier producer. It shows an emulsification activity in the Cooper medium, both in whole culture (E_{24} 68%) and in cell-free supernatant (E_{24} 56%). The presence of activity in cell-free supernatant is important, since it would permit the product to be used cell-free.

The relationship among growth phase, SACs production and cell-surface properties was determined in kinetic experiments by using mineral salt media with glucose as a carbon source. *Pedobacter* sp. strain MCC-Z synthesized and released extracellularly bioemulsifier(s) during the exponential phase and their concentration increased during

the subsequent stationary phase. The maximum emulsifying activity was observed at 96 h after the inoculation using the minimum amount of glucose 0.5%w/v (tested as the only source of carbon and energy).

In the **second part** of the thesis a novel biopolymer in the form of extracellular polysaccharide (EPS) was extracted, purified and characterized, and its surface and emulsifying properties were evaluated. The purified bioemulsifier, named Pdb-Z, showed high emulsifying activity (E_{24}%=64%) and reduced the surface tension of water up to 41 mN/m with a critical micelle concentration value of 2.6 mg/mL. Nevertheless, Pdb-Z presents CMC values comparable with Arabic gum (1.7 mg/mL) [100] a commercial emulsifier extensively used in the food industry, indicating similar efficiency.

Pdb-Z maintained its emulsifying properties over a wide range of pH (3-11), high salinity (20% NaCl), and elevated temperatures (up to 80°C). The water-in-oil emulsions formed by Pdb-Z showed good stability at room temperature, maintaining 62% of the original emulsion volume over a period of 20 weeks. Our results clearly demonstrate that Pdb-Z can be used for potential applications that involve extreme environmental conditions.

The substrate specificity of Pdb-Z was evaluated on different hydrophobic substrates. The results show that Pdb-Z formed stable emulsions with saturated and aromatic hydrocarbons, as well as with diesel fuel. The highest emulsifying activity was obtained with the aromatic compounds xylene (68%) and toluene (66%). Among aliphatic compounds, *iso*-octane served as the best substrate whereas the lowest E_{24}% value (21%) was obtained with *n*-hexadecane and cyclohexane at Pdb-Z concentration of 0.25 mg/mL. The broad range of substrate specificity of the bioemulsifier Pdb-Z suggests that it could be applied in hydrocarbon remediation and oil recovery. Synthetic commercial surfactants like Tween-20, Tween-80 and Triton X-100 were used to evaluate Pdb-Z performance as hydrocarbon emulsifier. Pdb-Z exhibited higher emulsifying activity on all hydrophobic substrates, except diesel fuel (40±1.5), in comparison with the synthetic surfactants. The emulsions formed by Pdb-Z remained stable for four months at room temperature.

Pdb-Z was chemically characterized by using ^{1}HNMR, FT-IR, HPLC/MS^{n} and GC/MS. Pdb-Z was found to be composed of 67% of carbohydrates, consisting mainly of galactose and minor quantities of talose, 30% lipids, being pentadecanoic acid the major lipidic constituent, and 3% proteins. Therefore, Pdb-Z contains lipopolysaccharides,

lipoproteins or complex mixtures of these polymers and galactose as the major sugar constituent, at unusually high levels (51%).

The SDS-PAGE analysis of Pdb-Z indicated the existence of two major proteins with apparent molecular masses of 23 and 8 kDa.

The N-terminal amino acid sequences of the two proteins showed high similarity to two proteins from *Pedobacter agri:* the first one containing an outer membrane protein β-barrel domain and OmpA domain, and the second one containing a conserved domain of putative periplasmatic proteins.

Recently, the secretion of OmpA-like proteins with emulsifying activity has been demonstrated to be a general property of the oil degrading *Acinetobacter* strains [122], the activity being due to hydrophobic amino acids in a β-barrel region [123]. However, secretion of an OmpA with emulsifying ability could be of physiological importance in the utilization of hydrophobic substrates as carbon sources. *Acinetobacter* strains secrete a variety of emulsifiers which are efficient in producing and stabilizing oil-in-water emulsions.

Moreover, the first evidence of an involvement of OmpA-like proteins in the emulsifying activity by bacteria other than *Proteobacteria* is provided. Nevertheless, it is not clear how the secretion of an outer membrane protein can occur. Our results led to hypothesis that the OmpA proteins have additional functions, and are not merely structural outer membrane components.

Variable-gradient ^{1}H-NMR experiments were carried out by measuring the R_h of the different components of the mixture in both D_2O and deuterated DMSO through the measurement of the diffusion coefficient D. The behavior in D_2O differs from that in deuterated DMSO in two main aspects: i) the signals present a homogeneous D; ii) the particle size is largely increased, giving R_h values in the order of 20 nm. Such large particles display very broad/not detectable signals for the core, and only sugar and aliphatic chains that have mobile tails can be detected. At the concentrations used in this study, the aggregation state of the molecules did not change significantly.

The calculated R_h values are similar to those found for other carbohydrate-containing polymers such as cellulose fibers, amylose, glycogen and amylopectins. For rabbit glycogen, Dinadayala and co-workers [28] reported the hydrodynamic radius and molecular mass values as being about 23 nm and $7x10^6$ Da, respectively. Therefore, we can infer that the molecular weight of Pdb-Z is about 10^6 Da.

Pdb-Z underwent FT-IR and ^{1}HNMR analyses in order to identify functional groups present in the bioemulsifier. Overall, the FT-IR spectrum and ^{1}HNMR analyses supported the previous conclusion of GC/MS analysis of Pdb-Z.

In the **third part** we evaluate the applicability of Pdb-Z for the washing of soil contaminated by road diesel.

Batch experiments soil washing were carried out and we compared the effects of Pdb-Z with those of the neat water and of Tween-20 on the removal of road diesel from soil.

The results showed that Pdb-Z is able to effectively reduce the final concentration of the total petroleum hydrocarbon, TPH, by more than 75% whereas Tween-20 removed 73%, consequently the performance of Pdb-Z in TPH removal was comparable to that of Tween-20. However, under the tested experimental conditions, the results show that neither solutions significantly vary the TPH removal by increasing the mass/volume ratio, the washing time and the surfactant concentration.

We believe this work is a promising base for future applications of Pdb-Z in the soil-washing of hydrocarbon-contaminated environments and in bioremediation processes.

In conclusion, the high molecular weight, the stable physicochemical behaviour and the ability of Pdb-Z to form stable emulsions with hydrocarbons and oils opens up potential applications in various industrial sectors, such as food, cosmetics, emulsion formulation, in environmental remediation such as oil-removal processes from tanks and ducts.

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ANNEX - ADDITIONAL DATA

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

CHAPTER 2

1 Screening of bacterial strains for SACs production by emulsification assay (E_{24}%) and Oil Spreading Technique (OST) at time 24 hours.

Strain code	Replicate	Zhang-Miller culture media E_{24}%[a,b]		Cooper culture media E_{24}%[a,b]		Zhang-Miller culture media OST[a,b]		Cooper culture media OST[a,b]	
		Whole culture[c]	Cell-free supernatant[c]	Whole culture[c]	Cell-free supernatant[c]	Whole culture[c]	Cell-free supernatant[c]	Whole culture[c]	Cell-free supernatant[c]
MCC-A5	1	----	----	22.0	17.0	----	----	----	----
	2	----	----	21.0	16.0	----	----	----	----
	3	----	----	24.0	19.0	----	----	----	----
Mean				**22.3**	**17.3**				
S. D.				**±1.5**	**±1.5**				
MCC-SL5	1								
	2								
	3					**No**			
Mean									
S. D.									
MCC-Z	1	----	----	50.0	41.0	----	----	----	----
	2	----	----	50.0	41.0	----	----	----	----
	3	----	----	52.0	45.0	----	----	----	----
Mean				**50.7**	**42.3**				
S. D.				**±1.1**	**±2.3**				
MCC-X	1	----	----	22.0	14.0	----	----	----	----
	2	----	----	24.0	16.0	----	----	----	----
	3	----	----	21.0	13.0	----	----	----	----
Mean				**22.3**	**14.3**				
S. D.				**±1.5**	**±1.5**				
MCC-S	1								
	2								
	3					**No**			
Mean									
S. D.									
MCC-E	1	----	----	14.0	----	----	----	----	----
	2	----	----	11.0	----	----	----	----	----
	3	----	----	12.0	----	----	----	----	----
Mean				**12.3**					
S. D.				**±1.5**					

MCC-G	1	----	----	----	----	----	----	----	----
	2	----	----	----	----	----	----	----	----
Mean	3	----	----			----	----	----	----
S. D.									
	1								
MCC-T	2								
	3					**No**			
Mean									
S. D.									
	1	----	----	41.0	11.0	----	----	----	----
AGL17	2	----	----	43.0	10.0	----	----	----	----
	3	----	----	44.0	10.0	----	----	----	----
Mean				**42.7**	**10.3**				
S. D.				**±1.5**	**±0.6**				
	1			12.0	15.0				
TSNR 4	2			11.0	18.0				
	3		**No**	13.0	16.0		**No**		
Mean				**12.0**	**16.3**				
S. D.				**±1.0**	**±1.5**				
	1	----	----	48.0	51.0	----	----	----	----
CONC18	2	----	----	48.0	52.0	----	----	----	----
	3	----	----	49.0	52.0	----	----	----	----
Mean				**48.3**	**51.3**				
S. D.				**±0.6**	**±0.6**				
Type strain	1			34.0	5.0			++++	++++
DSMZ 3257	2			33.0	4.0			++++	++++
	3		**No test**	36.0	4.0		**No test**	++++	++++
Mean				**34.3**	**4.3**				
S. D.				**±1.5**	**±0.6**				
Type strain	1	45.0	47.0			+++++	+++++		
DSMZ 1128	2	44.0	43.0			+++++	+++++		
	3	42.0	45.0		**No test**	+++++	+++++	**No test**	
Mean		**43.7**	**45.0**						
S. D.		**±1.5**	**±2.0**						

2 Screening of bacterial strains for SACs production by emulsification assay (E_{24}%) and Oil Spreading Technique (OST) at time 48 hours.

Strain code	Replicate	Zhang-Miller culture media E_{24}%[a,b]		Cooper culture media E_{24}%[a,b]		Zhang-Miller culture media OST[a,b]		Cooper culture media OST[a,b]	
		Whole culture[c]	Cell-free supernatant[c]	Whole culture[c]	Cell-free supernatant[c]	Whole culture[c]	Cell-free supernatant[c]	Whole culture[c]	Cell-free supernatant[c]
MCC-A5	1	36.0	3.0	22.0	12.0	----	----	----	----
	2	34.0	4.0	18.0	14.0	----	----	----	----
	3	36.0	4.0	21.0	12.0	----	----	----	----
Mean		**35.3**	**3.7**	**20.3**	**12.7**				
S. D.		**±0.6**	**±0.6**	**±2.1**	**±1.1**				
MCC-Z	1	----	----	67.0	57.0	+	----	+	+
	2	----	----	68.0	56.0	+	----	+	+
	3	----	----	68.0	56.0	+	----	+	+
Mean				**67.7**	**56.3**				
S. D.				**±0.6**	**±0.6**				
MCC-X	1	12.0	5.0	53.0	30.0	----	----	+	+
	2	11.0	3.0	51.0	28.0	----	----	+	+
	3	12.0	5.0	53.0	28.0	----	----	+	+
Mean		**11.7**	**4.3**	**52.3**	**28.7**				
S. D.		**±0.6**	**±1.5**	**±1.1**	**±1.1**				
MCC-E	1	----	----	----	----	----	----	----	----
	2	----	----	----	----	----	----	----	----
	3	----	----	----	----	----	----	----	----
Mean									
S. D.									
MCC-G	1	12.0	----	----	----		----	----	----
	2	11.0	----	----	----		----	----	----
	3	12.0	----	----	----		----	----	----
Mean		11.7							
S. D.		**±0.6**							
AGL17	1	48.0	12.0	40.0	11.0	+++	++	+	+
	2	47.0	12.0	38.0	10.0	+++	++	+	+
	3	48.0	12.0	40.0	8.0	+++	++	+	+
Mean		**47.7**	**12.0**	**39.3**	**9.7**				
S. D.		**±0.6**	**0**	**±0.6**	**±1.5**				
CONC18	1	10.0	24.0	25.0	48.0	+	+	+	+
	2	12.0	22.0	22.0.	48.0	+	+	+	+
	3	12.0	23.0	24.0	47.0	+	+	+	+
Mean		**11.3**	**23.0**	**23.7**	**47.7**				

S. D.		**±0.6**	**±1.0**	**±1.5**	**±0.6**			
Type strain DSMZ 3257	1			38.0	----		++++	++++
	2			36.0	----		++++	++++
	3		**No test**	36.0	-----	**No test**	++++	++++
Mean				**36.7**				
S. D.				**±0.6**				
Type strain DSMZ 1128	1	22.0	12.0					
	2	18.0	11.0					
	3	20.0	12.0		**No test**		**No test**	
Mean		**20.0**	**11.7**					
S. D.		**±2.0**	**±0.6**					

3 Screening of bacterial strains for SACs production by emulsification assay (E_{24}%) and Oil Spreading Technique (OST) at time 72 hours

Strain code	Replicate	Zhang-Miller culture media E_{24}%[a,b]		Cooper culture media E_{24}%[a,b]		Zhang-Miller culture media OST[a,b]		Cooper culture media OST[a,b]	
		Whole culture[c]	Cell-free supernatant[c]	Whole culture[c]	Cell-free supernatant[c]	Whole culture[c]	Cell-free supernatant[c]	Whole culture[c]	Cell-free supernatant[c]
MCC-A5	1	12.0	12.0	33.0	----	----	----	----	----
	2	10.0	13.0	35.0	----	----	----	----	----
	3	12.0	12.0	31.0	----	----	----	----	----
Mean		**11.3**	**12.3**	**33.0**					
S. D.		**±0.6**	**±0.6**	**±2.0**					
MCC-Z	1	----	----	58.0	54.0	----	----	+	+
	2	----	----	56.0	56.0	----	----	+	+
	3	----	----	56.0	54.0	----	----	+	+
Mean				**56.7**	**54.7**				
S. D.				**±0.6**	**±1.1**				
MCC-X	1	24.0	5.0	49.0	----	----	----	+	----
	2	25.0	4.0	50.0	----	----	----	+	----
	3	25.0	3.0	48.0	----	----	----	+	----
Mean		**24.7**	**4.3**	**49.0**					
S. D.		**±0.6**	**±1.5**	**±1.1**					
MCC-E	1	----	----	----	----	----	----	----	----
	2	----	----	----	----	----	----	----	----
	3	----	----	----	----	----	----	----	----
Mean									
S. D.									
MCC-G	1	26.0	----	4.0	----	----	----	----	----
	2	26.0	----	4.0	----	----	----	----	----
	3	29.0	----	3.0	----	----	----	----	----
Mean		**27.0**		**3.7**					
S. D.		**±1.7**		**±0.6**					
AGL17	1	26.0	----	26.0	----	+	+	+	+
	2	23.0	----	30.0	----	+	+	+	+
	3	24.0	----	28.0	----	+	+	+	+
Mean		**24.3**		**28.0**					
S. D.		**±1.5**		**±2.0**					
CONC18	1	31.0	48.0	40.0	22.0	----	----	+	----
	2	32.0	49.0	40.0	25.0	----	----	+	----
	3	32.0	48.0	42.0	20.0	----	----	+	----
Mean		**31.7**	**48.3**	**40.7**	**22.3**				

S. D.		**±0.6**	**±0.6**	**±1.1**	**±2.5**			+	+
Type strain DSMZ 3257	1			15.0	----			+	+
	2			18.0	----			+	+
	3		**No test**	19.0	-----		**No test**		
Mean				**17.3**					
S. D.				**±2.0**					
Type strain DSMZ 1128	1	41.0	43.0			+++++	+++++		
	2	38.0	44.0			+++++	+++++		
	3	36.0	44.0		**No test**	+++++	+++++		**No test**
Mean		**38.3**	**43.7**						
S. D.		**±2.5**	**±0.6**						

4 Screening of bacterial strains for SACs production by emulsification assay (E_{24}%) and Oil Spreading Technique (OST) at time 96 hours.

Strain code	Replicate	Zhang-Miller culture media E_{24}%[a,b]		Cooper culture media E_{24}%[a,b]		Zhang-Miller culture media OST[a,b]		Cooper culture media OST[a,b]	
		Whole culture[c]	Cell-free supernatant[c]	Whole culture[c]	Cell-free supernatant[c]	Whole culture[c]	Cell-free supernatant[c]	Whole culture[c]	Cell-free supernatant[c]
MCC-A5	1	12.0	12.0	35.0	----	+	----	----	----
	2	12.0	10.0	30.0	----	+	----	----	----
	3	11.0	9.0	31.0	----	+	----	----	----
Mean		**11.7**	**10.3**	**32.0**					
S. D.		**±0.6**	**±1.5**	**±2.6**					
MCC-Z	1	----	----	60.0	58.0	----	----	+	+
	2	----	----	58.0	60.0	----	----	+	+
	3	----	----	60.0	56.0	----	----	+	+
Mean				**59.3**	**58.0**				
S. D.				**±1.5**	**±2**				
MCC-X	1	----	----	----	----	----	----	----	----
	2	----	----	----	----	----	----	----	----
	3	----	----	----	----	----	----	----	----
Mean									
S. D.									
MCC-E	1	----	----	----	----	----	----	----	----
	2	----	----	----	----	----	----	----	----
	3	----	----	----	----	----	----	----	----
Mean									
S. D.									
MCC-G	1	----	----	----	----	----	----	----	----
	2	----	----	----	----	----	----	----	----
	3	----	----	----	----	----	----	----	----
Mean									
S. D.									
AGL17	1	----	----	----	----	----	----	----	----
	2	----	----	----	----	----	----	----	----
	3	----	----	----	----	----	----	----	----
Mean									
S. D.									
CONC18	1	20.0	24.0	----	----	----	----		----
	2	19.0	24.0	----	----	----	----		----
	3	19.0	23.0	----	----	----	----		----
Mean		**19.3**	**23.7**						

S. D.		**±0.6**	**±0.6**				+	+
Type strain DSMZ 3257	1			----			+	+
	2			----			+	+
	3		**No test**	----		**No test**		
Mean								
S. D.								
Type strain DSMZ 1128	1	44.0	40		+++++	+++++		
	2	42.0	36		+++++	+++++		
	3	44.0	42	**No test**	+++++	+++++	**No test**	
Mean		**43.3**	**39.3**					
S. D.		**±1.1**	**±3.0**					

a: emulsifying activity was expressed as the percentage of the total height occupied by the emulsion.

b: diameter of OST; “+”: 0- 5 mm,; “++”: 6 -10 mm; “+++”: 11- 20 mm; “++++”: 21 -50 mm,;“+++++”: > 51 mm

c: average of three replicates

dNO: none emulsifying activity

5 Emulsifying activity (E_{24}%) values of *Pedobacter* sp. strain, MCC-Z at different glucose concentrations.

% Glucose	E_{24}% (24h)[a]	E_{24}% (48h)[a]	E_{24}% (72h)[a]	E_{24}% (96h)[a]	E_{24}% (120h)[a]	E_{24}% (144h)[a]	E_{24}% (168h)[a]
0.25 ($\bar{a}$)	41.0	43.0	50.0	53.0	50.0	50.0	51.0
0.25 ($\bar{b}$)	40.0	44.0	50.0	52.0	51.0	52.0	50.0
0.25 ($\bar{c}$)	39.0	44.0	51.0	52.0	50.0	49.0	48.0
Mean	**40.0**	**43.7**	**50.3**	**52.3**	**50.3**	**50.3**	**49.7**
S.E.	**±1.0**	**±0.6**	**±0.6**	**±0.6**	**±0.6**	**±1.53**	**±1.0**
0.5 ($\bar{a}$)	40.0	47.0	56.0	64.0	63.0	63.0	59.0
0.5 ($\bar{b}$)	42.0	48.0	56.0	64.0	63.0	61.0	60.0
0.5 ($\bar{c}$)	39.0	48.0	57.0	65.0	62.0	62.0	61.0
Mean	**40.3**	**47.7**	**56.3**	**64.3**	**62.7**	**62.0**	**60.0**
S.E.	**±1.5**	**±0.6**	**±0.6**	**±0.6**	**±0.6**	**±1.0**	**±1.0**
1.0 ($\bar{a}$)	44.0	53.0	57.0	60.0	62.0	61.0	60.0
1.0 ($\bar{b}$)	45.0	53.0	57.0	61.0	63.0	59.0	60.0
1.0 ($\bar{c}$)	44.0	52.0	56.0	62.0	62.0	60.0	61.0
Mean	**44.3**	**52.7**	**56.7**	**61.0**	**62.3**	**60.0**	**60.3**
S.E.	**±0.6**	**±0.6**	**±0.6**	**±1.0**	**±0.6**	**±1.0**	**±0.6**
2.0 ($\bar{a}$)	40.0	46.0	54.0	61.0	56.0	55.0	56.0
2.0 ($\bar{b}$)	42.0	45.0	56.0	60.0	58.0	58.0	58.0
3.0 ($\bar{c}$)	39.0	49.0	52.0	60.0	60.0	57.0	55.0
Mean	**40.3**	**46.7**	**54.0**	**60.3**	**58.0**	**56.7**	**56.3**
S.E.	**±1.5**	**±2.1**	**±1.6**	**±0.5**	**±1.6**	**±1.25**	**±1.25**

[a]: average of three replicates

6 Growth curve of *Pedobacter* sp. strain, MCC-Z in MSM with 0.5% glucose concentrations.

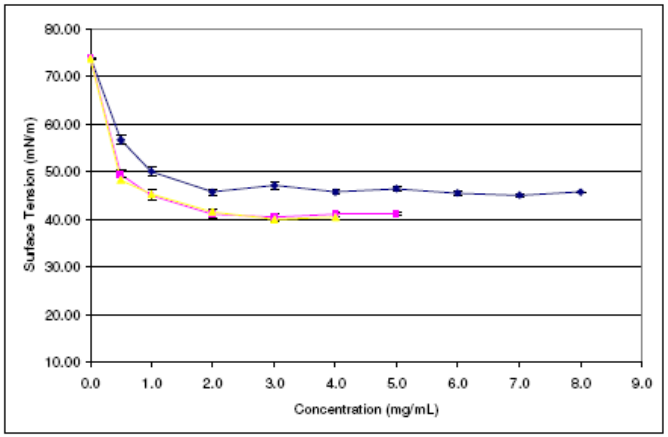
logCFU/mL						
Replicate	**0 (h)**	**24(h)**	**48(8h)**	**72(h)**	**96(h)**	**120(h)**
1	5.95	7.89	8.50	9.46	9.58	9.41
2	5.77	8.02	8.75	9.76	9.26	9.01
3	6.14	7.97	8.48	9.68	9.44	9.15
Mean	**5.95**	**7.96**	**8.58**	**9.48**	**9.43**	**9.67**
S. D.	**±0.18**	**±0.07**	**±0.15**	**±0.16**	**±0.17**	**±0.20**

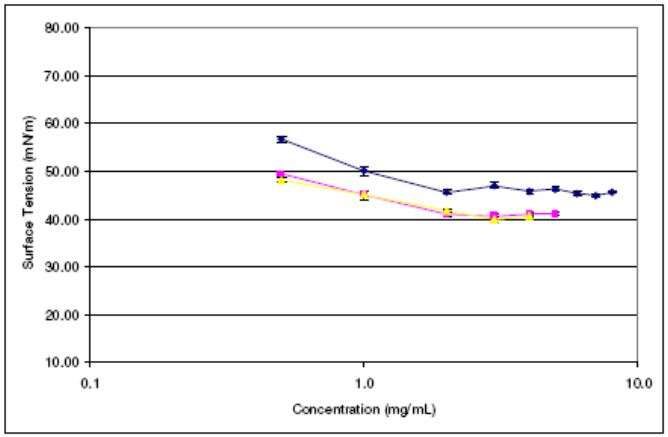
CHAPTER 3

1 Surface tension of Pdb-Z solutions at different concentrations

Concentration (mg/mL)	ST (mN/m)			Mean	S.D.
	a	b	c		
8.00	**45.48**	**45.63**	**45.66**	**45.59**	**0.10**
7.00	**44.70**	**44.82**	**45.23**	**44.92**	**0.28**
6.00	**44.84**	**45.41**	**45.83**	**45.36**	**0.50**
5.00	**45.74**	**46.58**	**46.62**	**46.31**	**0.50**
4.00	**45.20**	**45.73**	**46.20**	**45.71**	**0.50**
3.00	**46.33**	**47.13**	**47.64**	**47.03**	**0.66**
2.00	**45.02**	**45.62**	**46.30**	**45.65**	**0.64**
1.00	49.06	50.20	50.88	50.05	0.92
0.50	56.04	57.51	56.54	56.70	0.75
0.00	73.95	73.97	73.96	73.96	0.01

Aliquota 1					
Concentration (mg/mL)	ST (mN/m)			Mean	**S.D.**
	a	b	c		
5.00	**40.86**	**41.20**	**41.28**	**41.11**	**0.22**
4.00	**40.67**	**41.05**	**41.62**	**41.11**	**0.48**
3.00	**39.83**	**40.54**	**41.09**	**40.49**	**0.63**
2.00	**40.22**	**41.25**	**41.92**	**41.13**	**0.86**
1.00	44.03	44.93	46.09	45.02	1.03
0.50	48.66	49.25	50.17	49.36	0.76
0.00	73.62	73.67	73.74	73.68	0.06
Aliquota 2					
Concentration (mg/mL)	ST (mN/m)			Mean	**S.D.**
	a	b	c		
4.00	**40.15**	**40.66**	**40.79**	**40.53**	**0.34**
3.00	**39.40**	**39.83**	**40.60**	**39.94**	**0.61**
2.00	**41.57**	**41.91**	**41.36**	**41.61**	**0.28**
1.00	44.47	45.03	46.09	45.20	0.82
0.50	47.51	48.13	48.68	48.11	0.59
0.00	73.62	73.67	73.74	73.68	0.06





2 Emulsifying activity of Pdb-Z with several hydrocarbon at different concentrations.

Hydrocarbon	Emulsifying activity (E_{24}%) Pdb-Z (mg/mL)																			
		0.25					0.5					0.75					1.0			
	$\bar{a}$	$\bar{b}$	$\bar{c}$	$\overline{Mean}$	S.E.	$\bar{a}$	$\bar{b}$	$\bar{c}$	$\overline{Mean}$	S.E.	$\bar{a}$	$\bar{b}$	$\bar{c}$	$\overline{Mean}$	S.E..	$\bar{a}$	$\bar{b}$	$\bar{c}$	$\overline{Mean}$	S.E.
n-Hexane	65.8	64.6	62.2	64.2	±1.8	65.8	67.1	63.4	65.4	±1.9	66.8	67.2	66.2	66.2	±1.5	63.7	66.2	68.4	66.1	±2.4
n-hexadecane	23.1	21.8	19.4	21.4	±1.9	23.0	21.8	19.4	21.4	±1.8	66.8	64.9	61.8	64.5	±2.5	64.9	61.8	66.8	64.5	±2.5
Cyclohexane	23.0	21.3	19.8	21.4	±1.6	64.2	60.6	63.0	62.6	±1.8	59.9	62.4	64.6	62.3	±2.4	59.9	62.4	64.6	62.3	±2.4
Iso-octane	42.1	40.8	38.4	40.4	±1.9	60.1	64.1	61.6	62.0	±2.2	60.7	64.7	61.2	62.6	±2.2	64.4	60.7	61.3	62.1	±2.0
Toluene	65.1	68.2	65.4	66.2	±1.7	66.8	68.2	64.5	66.5	±1.9	65.0	66.3	69.1	66.8	±2.1	66.8	64.1	67.50	66.1	±1.8
Xylene	66.80	70.50	67.40	68.2	±2.0	68.1	67.2	70.2	68.5	±1.5	67.9	69.9	67.0	68.3	±1.5	67.3	67.4	70.3	68.3	±1.7
Diesel fuel	30.8	27.1	27.7	28.5	±2.0	25.7	28.2	30.4	28.1	±2.4	43.1	46	44.0	44.4	±1.5	43.1	46.1	43.1	44.1	±1.7

3 Emulsifying activity of several synthetic surfactants (0.75 mg/L) with hydrocarbon substrates.

Hydrocarbon	Emulsifying activity (E_{24}%)													
	Synthetic surfactants (0.75 mg/mL)													
	Tween-20					Tween-80					Triton X-100			
	$\bar{a}$	$\bar{b}$	$\bar{c}$	$\overline{Mean}$	S.E.	$\bar{a}$	$\bar{b}$	$\bar{c}$	$\overline{Mean}$	S.E.	$\bar{a}$	$\bar{b}$	$\bar{c}$	$\overline{Mean}$	S.E.
n-Hexane	53.97	51.27	54.67	53.3	±1.8	53.2	54.4	57.6	55.1	±2.3	59.2	59.4	57.0	58.5	±1.3
n-hexadecane	58.1	57.9	55.7	57.2	±1.3	57.8	59.3	61	59.4	±1.6	58.3	59.6	62.4	60.1	±2.1
Cyclohexane	46.4	47.3	50.9	48.2	±2.4	45.8	44.5	42.1	44.1	±1.9	48,7	46.0	48.0	48.0	±1.8
Iso-octane	55.7	58.0	60.4	58.1	±2.4	52.0	54.7	55.4	54.0	±1.8	56.0	52.3	54.7	54.3	±1.9
Toluene	27.1	30.0	28.0	28.4	±1.5	33.0	30.3	33.7	32.3	±1.8	36.6	34.9	33.4	35.0	±1.6
Xylene	64.6	62.7	59.6	62.3	±2.5	60.9	57.7	59.2	59.3	±1.6	60.7	60.8	63.7	61.7	±1.7
Diesel fuel	59.6	64.0	59.4	61.0	±2.6	60.0	57.6	61.3	59.6	±1.9	62.4	63.3	65.3	63.7	±1.5

4 Heat stability on emulsifying activity of Pdb-Z

Temperature (°C)	Replicate	Emulsifying activity (E_{24}%)					
		X_1	X_2	X_3	Mean	S.D.	S.E.
-80	a	58.0	60.0	59.0	59.0	±1.0	±0.6
	b	61.0	60.0	63.0	61.3	±1.5	±0.9
	c	63.0	62.0	60.0	61.7	±1.5	±1.0
Mean	60.7						
S.E.	±1.4						
-20	a	64.0	61.0	62.0	62.3	±1.5	±0.9
	b	63.0	63.0	61.0	62.3	±1.2	±0.7
	c	64.0	62.0	61.0	62.3	±1.5	±1.0
Mean	62.3						
S.E.	±1.4						
+7	a	63.0	64.0	64.0	63.7	±0.6	±0.3
	b	63.0	63.0	62.0	62.7	±0.6	±0.3
	c	62.0	63.0	63.0	62.7	±0.6	0
Mean	62.4						
S.E.	±0.6						
+25	a	65.0	63.0	65.0	64.3	±1.1	±0.7
	b	63.0	65.0	64.0	64.0	±1.0	±0.6
	c	62.0	63.0	63.0	62.7	±0.6	0
Mean	63,1						
S.E.	±0.9						
+37	a	64.0	66.0	63.0	64.3	±1.5	±0.9
	b	62.0	65.0	63.0	63.3	±1.5	±0.9
	c	65.0	64.0	64.0	64.3	±0.6	0
Mean	64.3						
S.E.	±1.3						
+70	a	61.0	62.0	60.0	61.0	±1.0	±0.6
	b	57.0	60.0	61.0	59.3	±2.1	±1.2
	c	62.0	60.0	62.0	61.3	±1.1	±1.0
Mean	60.6						
S.E.	±1.5						
+121	a	53.0	52.0	52.0	52.3	±0.6	±0.3
	b	54.0	54.0	52.0	53.3	±1.1	±0.7
	c	51.0	53.0	52.0	52.0	±1.0	±1.0
Mean	52.6						
S.E.	±0.9						

5 Influence of pH on emulsifying activity of Pdb-Z

pH	Replicate	Emulsifying activity (E_{24}%)					
		X_1	X_2	X_3	Mean	S.D.	S.E.
2	a	36.0	37.0	35.0	36.0	±1.0	±0.6
	b	34.0	35.0	34.0	34.3	±0.6	±0.3
	c	35.0	36.0	34.0	35.0	±1.0	±1.0
Mean	35.1						
S.E.	±0.9						
3	a	53.0	54.0	56.0	54.3	±1.5	±0.9
	b	57.0	55.0	54.0	55.3	±1.5	±0.9
	c	59.0	57.0	58.0	58.0	±1.0	±1.0
Mean	55.9						
S.E.	±1.4						
4	a	57.0	58.0	57.0	57.3	±0.6	±0.3
	b	54.0	56.0	54.0	55.0	±1.1	±0.7
	c	55.0	54.0	55.0	55.0	±1.0	±1.0
Mean	56.7						
S.E.	±0.9						
7	a	60.0	63.0	65.0	62.7	±2.5	±1.5
	b	58.0	59.0	61.0	59.3	±1.5	±0.9
	c	64.0	65.0	65.0	64.7	±0.6	±0
Mean	62.2						
S.E.	±1.7						
9	a	61.0	60.0	63.0	61.3	±1.5	±0.9
	b	62.0	61.0	58.0	60.3	±2.1	±1.2
	c	62.0	62.0	60.0	61.3	±1.1	±1.0
Mean	61.0						
S.E.	±1.6						
10	a	61.0	62.0	60.0	61.0	±1.0	±0.6
	b	60.0	61.0	60.0	60.3	±0.6	±0.3
	c	60.0	62.0	62.0	61.3	±1.1	±1.0
Mean	60.9						
S.E.	±0.9						
11	a	64.0	62.0	62.0	62.7	±1.1	±0.7
	b	58.0	56.0	60.0	58.0	±2.0	±1.2
	c	60.0	62.0	62.0	61.3	±1.1	±1.0
Mean	60.7						
S.E.	±1.5						
12	a	36.0	33.0	34.0	34.3	±1.5	±0.9
	b	30.0	28.0	30.0	29.3	±1.1	±0.7
	c	34.0	32.0	30.0	32.0	±2.0	±1.0
Mean	31.9						
S.E.	±1.6						

6 Effect of the salt concentration on emulsifying activity of Pdb-Z

NaCl (w/v %)	Replicate	Emulsifying activity (E_{24}%)					
		X_1	X_2	X_3	Mean	S.D.	S.E.
5	a	58.0	60.0	60.0	59.3	±1.2	±0.7
	b	62.0	60.0	60.0	60.7	±1.2	±0.7
	c	60.0	62.0	62.0	61.3	±1.2	±1.0
$\overline{Mean}$	60.4						
S.E.	±1.2						
10	a	60.0	60.0	58.0	59.0	±1.2	±0.7
	b	62.0	60.0	60.0	61.0	±1.2	±0.7
	c	60.0	60.0	60.0	60.0	0	±0
$\overline{Mean}$	60.0						
S.E.	±0.9						
15	a	62.0	60.0	62.0	61.3	±1.2	±0.7
	b	61.0	60.0	60.0	60.3	±0.6	±0.3
	c	58.0	60.0	63.0	60.3	±2.5	±1.0
$\overline{Mean}$	60.3						
S.E.	±1.0						
20	a	60.0	60.0	60.0	60.0	0	0
	b	61.0	60.0	60.0	60.3	±0.6	±0.3
	c	63.0	60.0	60.0	61.0	±1.7	±1.0
$\overline{Mean}$	60.8						
S.E.	±1.1						

7 Emulsion time stability of Pdb-Z

Time (weeks)	Replicate	Emulsifying activity (E_{24}%)					
		X_1	X_2	X_3	Mean	S.D.	S.E.
	a	62.0	62.0	62.0	62.0	0	0
1	b	62.0	64.0	64.0	63.3	±1.2	±0.7
	c	64.0	66.0	66.0	65.3	±1.2	±1.0
Mean	63.6						
S.E.	±0.9						
	a	64.0	65.0	64.0	64.3	±0.6	±0.3
2	b	66.0	64.0	66.0	65.3	±1.2	±0.7
	c	62.0	60.0	62.0	61.3	±1.2	±1.0
Mean	63.7						
S.E.	±1.0						
	a	64.0	65.0	64.0	64.3	±0.6	±0.3
3	b	65.0	64.0	65.0	64.7	±0.6	±0.3
	c	64.0	64.0	62.0	63.3	±1.2	±1.0
Mean	64.1						
S.E.	±0.8						
	a	64.0	66.0	66.0	65.3	±1.2	±0.7
4	b	62.0	64.0	64.0	63.3	±1.2	±0.7
	c	64.0	66.0	62.0	64.0	±2.0	±1.0
Mean	64.2						
S.E.	±1.5						
	a	66.0	66.0	66.0	66.0	0	0
5	b	60.0	58.0	62.0	60.0	±2.0	±1.2
	c	66.0	66.0	64.0	65.3	±1.2	±1.0
Mean	63.8						
S.E.	±1.3						
	a	64.0	65.0	63.0	64.0	±1.0	±0.6
7	b	66.0	63.0	62.0	63.7	±2.1	±1.2
	c	66.0	64.0	63.0	64.3	±1.5	±1.0
Mean	64.0						
S.E.	±1.6						
	a	64.0	64.0	63.0	63.7	±0.6	±0.3
10	b	61.0	63.0	63.0	62.3	±1.2	±0.7
	c	66.0	66.0	65.0	66.0	0	0
Mean	64.0						
S.E.	±0.7						
	a	64.0	62.0	62.0	62.7	±1.2	±0.7
14	b	60.0	62.0	60.0	60.7	±1.2	±0.7
	c	62.0	61.0	62.0	61.7	±0.6	±0
Mean	61.7						
S.E.	±1.0						
	a	61.0	64.0	60.0	61.7	±2.1	±1.2
16	b	58.0	60.0	62.0	60.0	±2.0	±1.2
	c	60.0	64.0	62.0	62.0	±2.0	±1.0
Mean	61.2						
S.E.	±1.0						
	a	61.0	61.0	62.0	61.3	±0.6	±0.3
20	b	62.0	61.0	60.0	61.0	±1.0	±0.6
	c	63.0	63.0	62.0	62.7	±0.6	0
Mean	61.7						
S.E.	±0.7						

CHAPTER 5

1 Percentage of TPH removal *vs* concentration of surfactant solutions (washed with 60 mL for 30 minutes).

TPH removal (%)							
Washing solution	Water		Pdb-Z		Tween-20		Concentration of washing solution (%w/w)
	1	2	1	2	1	2	
	42.0	38.0	67.0	69.0	64.0	57.0	0.75
Mean	40.0		68.0		60.5		
E_r %			4.6		5.2		
	1	2	1	2	1	2	1.5
			68.0	77.0	70.0	65.0	
Mean			72.5		67.5		
E_r %			4.3		4.6		
	1	2	1	2	1	2	2.5
			72.0	78.0	71.0	75.0	
Mean			75.0		73.0		
E_r %			4.2		4.3		

2 Percentage of TPH removal *vs* volume/mass ratio (washed with 0.75 %w/v for 30 minutes).

TPH removal (%)							
Washing solution	Water		Pdb-Z		Tween-20		Ratio soil:washing solution (v:w)
	1	2	1	2	1	2	
	42.0	38.0	60.0	68.0	52.0	61.0	10
Mean	40.0		64.0		56.5		
E_r %	7.8		4.9		5.5		
	1	2	1	2	1	2	
Mean	41.0	39.0	62.0	70.0	62.0	58.0	20
E_r %	40.0		66.0		60.0		
	7.8		4.7		5.2		
	1	2	1	2	1	2	
Mean	38.0	42.0	67	69	57.0	64.0	30
	40.0		68.0		60.5		
E_r %	7.8		4.6		5.2		

3 Percentage of TPH removal *vs* washing time (washed with 60 mL solution 0.75% w/v).

TPH removal (%)							
Washing solution	**Water**		**Pdb-Z**		**Tween-20**		**Washing Time (min)**
	1	2	1	2	1	2	
	42.0	38.0	67.0	69.0	64	57	30
Mean	40.0		68.0		60.5		
E_r %	7.8		4.6		5.2		
	1	2	1	2	1	2	
Mean	46.0	52.0	65.0	75.0	62.0	68.0	60
E_r %	49.0		70.0		65.0		
	6.4		4.5		4.8		
	1	2	1	2	1	2	
Mean	66.0	54.0	71.0	75.0	68.0	72.0	90
E_r %	60.0		73.0		70.0		
	5.2		4.3		4.5		

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Tiziana