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Exploiting the Biocatalytic Potential of Marine Bacteria

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Abstract

Marine bacteria are often under extreme conditions of temperature, pressure, salinity and depletion of nutrients. To survive, these microorganisms developed mechanisms of adaptation and produce biologically active compounds. This work aimed to assess and exploit the potential of marine bacteria to produce interesting compounds for biotechnological processes.

Strains isolated from samples harvested in harbour of Ponta Delgada (Azores) and Aveiros beach in Albufeira (Algarve) were identified by their fatty acid profile using the Sherlock® Microbial ID System.

The identified isolates from Azores were screened to produce biosurfactants and lipases. Among the tested strains, *Bacillus pumilus* and *Micrococcus luteus* were found to be potential biosurfactant and lipase producers, respectively. *B. pumilus* produced biosurfactants and exopolysaccharides able to reduce superficial tension of supernatant until 21.3 mN/m and 12.4 mN/m by using *n*-hexadecane and raffinose as carbon source, respectively. Furthermore, *B. pumilus* degraded *n*-alkanes while changing the cell membrane to overcome substrate toxicity, which suggests that this bacterium is suitable for bioremediation. *M. luteus* produced extracellular lipases with enzymatic activities of 0.09 and 0.06 U/mL in presence and absence of the tributyrin, respectively.

The adaptation of marine bacteria from Aveiros beach to different NaCl concentrations (5, 35, 50, 100 and 150 g/L) was studied. The results showed that almost all bacteria could grow until 150 g/L by adapting their fatty acids of cell membrane, especially in the presence of 100 and 150 g/L of salt.

Keywords: marine bacteria, screening, biosurfactants, lipases, fatty acids, bacterial adaptation

Resumo

As bactérias marinhas estão frequentemente sob condições extremas de temperatura, pressão, salinidade e escassez de nutrientes. Para sobreviver, estes microrganismos desenvolveram mecanismos de adaptação e produzem compostos biologicamente ativos. Este trabalho teve como objetivo avaliar e explorar o potencial das bactérias marinhas para produzir compostos com interesse em processos biotecnológicos.

As estirpes isoladas a partir de amostras recolhidas no porto de Ponta Delgada (Açores) e na praia de Aveiros, em Albufeira (Algarve) foram identificadas pelo seu perfil de ácidos gordos, utilizando o sistema Sherlock® Microbial ID.

Foi avaliada a capacidade dos isolados identificados dos Açores para produzir biossurfactantes e lipases. Entre as estirpes testadas, o *Bacillus pumilus* produziu biossurfactantes e exopolissacáridos com capacidade de reduzir a tensão superficial até 21.3 mN/m e 12.4 mN/m, usando hexadecano e rafinose como fonte de carbono, respetivamente. Para além disso, o *B. pumilus* degradou *n*-alcanos enquanto alterava a sua membrana celular para superar a toxicidade destes substratos, o que sugere que esta bactéria é adequada para biorremediação. O *Micrococcus luteus* produziu lipases extracelulares com atividades enzimáticas de 0.09 U/mL e 0.06 U/mL na presença e na ausência de tributirina, respetivamente.

Foi estudada a adaptação das bactérias marinhas da praia de Aveiros a diferentes concentrações de NaCl (5, 35, 50, 100 and 150 g/L). Os resultados mostraram que quase todas as bactérias conseguiram crescer até 150 g/L, adaptando os ácidos gordos da sua membrana celular, especialmente nas concentrações de 100 e 150 g/L de sal.

Palavras-chave: bactérias marinhas, rastreio, biossurfactantes, lipases, ácidos gordos, adaptação bacteriana

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List of Abbreviations

ABS – Alkyl Benzene Sulfonates

API – Analytical Profile Index

BCFAs – Branched Chain Fatty Acids

BSA – Bovine Serum Albumin

CMC – Critical Micelle Concentration

ED – Evolutionary Distance

EPS – Exopolysaccharide Substance

FA – Fatty Acid

FAME – Fatty Acid Methyl Esters

GC – Gas Chromatography

LAS – Linear Alkylbenzene Sulfonate

MIS – Sherlock ® Microbial Identification System

MB – Marine Broth

MEOR – Microbial Enhanced Oil Recovery

MM – Mineral Medium

MH – Mueller-Hinton broth

MTBE – Methyl *tert-butyl* ether

MUFAs – Monounsaturated Fatty Acids

PCA – Principal Component Analysis

PCR – Polymerase Chain Reaction

PFLAs – Phospholipid-derived fatty acids

p-NP – p-nitrophenol

p-NPB – 4-nitrophenyl butyrate

PUFAs – Polyunsaturated Fatty Acids

SACs – Surface-Active Compounds

SEM – Scanning Electron Microscopy

SSFAs – Saturated Straight Fatty Acids

SSGLU – Sea salts with glucose

T – Temperature

Thio – Thioglycollate Broth with Resazurine

TSA – Tryptic Soy Agar

TSB – Tryptic Soy Broth

UI – Unsaturation index

1. Introduction

The oceans represent a source of a varied type of organisms due to the several environments offered by the different oceanic zones [1]. The ecological resources offered by the seas have been explored since ancient times by man, from the use of marine animals such as fish, and algae preparations for medicine, to compounds with suitable properties to be used in pharmaceutical products [1]. However, the oceans and seas, that occupy around 70% of Earth, continue largely undiscovered [2]. It is estimated that there are 10^9 taxa of bacteria on the Earth with 10^6 of bacterial taxa from the oceans [3].

With the advancement and improvement of techniques, like culturing technique, small ribosomal RNA (rRNA) analysis and metagenomics approach, it is possible to study marine microbial diversity [4].

Marine bacteria can be found in all oceans, e.g. from the seafloor to fish stomachs, and they are present in a wide variety of habitats, like deep-sea sediments, seamounts and sometimes outside of algal cells [5], and they can survive on more diverse environmental conditions than terrestrial bacterial owing to ocean currents that can transport these organisms substantial distances [2]. Marine environments are considered one of the most adverse due to fluctuations in pH, salinity, temperature, sea surface temperature, pressure, radiation, tides, precipitation regimes and sea level rise [2, 6]. The ability of marine bacteria to endure and utilize the resources found in these habitats has an important role on the distribution of bacteria in the sea [6].

Relatively to the functions performed by marine microorganisms: they are responsible for the uptake of large part of the carbon dioxide that human society emits into the atmosphere by fixation; they are the basis of the ocean food web due to fix some of the dissolved inorganic carbon and provide the particulate and dissolved organic carbon [7, 8]. Nevertheless, the survival mechanisms developed by the marine bacteria have made them a promise source for new biologically active substances with potential uses in industrial processes, medicine, food and bioremediation [5].

Researchers started to explore the diversity of the oceans by identifying and isolating the biocatalysts and secondary metabolites produced by marine organisms [9]. Examples include the green fluorescent protein produced by the jellyfish *Aequorea Victoria* and its use in diverse types of clinical diagnostics and therapeutics [10, 11], antiviral and anticancer compounds isolated from marine sponges [12], biocatalysts with potential application on the production of pharmaceutical building blocks like solketal [13] and trabectedin as an example for chemotherapeutic substance produced by putative endosymbiotic bacteria of the sea squirt *Ecteinascidia turbinate* [14].

Furthermore, some reports refer that many heterotrophic bacteria are known to carry genetic and metabolic potential to synthesize and control extracellular enzymes, which can modify and degrade many natural polymers in water basins [2]. Other important bioactive compounds produced by marine microbes include exopolysaccharides (EPS), biosurfactants and antibacterial compounds [2].

1.1. Isolation and identification of microorganisms

Metagenomics is a culture-independent technique that applies a suite of genomic technologies and bioinformatics tools to study and analyses genomic DNA from a whole community [15, 16]. In recent years, metagenomics allowed substantial advances in microbial ecology, evolution and diversity studies by providing information about the functional gene composition of microbial communities and a much broader description than phylogenetic surveys, that are based in the diversity of only one gene, like 16S rRNA gene sequencing [16]. Therefore, this technique provides genetic information on potentially novel biocatalysts or enzymes, genomic linkages between function and phylogeny for uncultured organism, discover of new antibiotics, personalized medicine and bioremediation [16, 17].

Metagenomics can be divided in two research areas driven by technological application: (i) environmental single-gene surveys and (ii) random shotgun of all environmental genes [18]. Sample processing is the first step and consists in the extraction of DNA from the microbial community. Single targets are amplified using polymerase chain reaction (PCR) and then, the products are sequenced followed by the assembly and annotation of the genes. Other type of metagenomics is random shotgun, where total DNA is isolated from a sample and then sequenced, resulting in a profile with all genes of the community. The choice of approach depends on the number of fragments obtained during the sequencing step, i.e. of the sequencing technique [16].

With the increase of interest on the enormous biodiversity of marine microorganisms and owing to the majority of these microorganisms still being uncultivable, metagenomics tools are required to explore them [19]. In 2011, marine microbial databases comprised around 400 billion base pairs of DNA, where only ≈ 3% of that it was found in 1 mL of seawater [18]. These numbers reflect the influence of metagenomics on understanding the marine biodiversity. For instance, in genomics analysis of an arctic sediment bacterium it was found that *Colwellia psychrerythraea* has the ability to produce polyhydroxyalkanoates and polyamides, which is a psychrophilic behaviour against to the limitation of carbon and nitrogen uptake caused by cold temperatures [20]. Furthermore, until 2017, it was only described that about 2100 different locations worldwide were explored using metagenomic techniques, 60% of which correspond to marine samples and in only approximately 11% of them was identified new enzymes [21]. An example is the discovery made by Hånderman and Sjöling, who isolated a novel low temperature active lipase from uncultured bacteria from marine sediment. The lipases produced by these bacteria present conserved regions, including the putative active site and catalytic triad, that are similar to the culturable lipases Metagenomics have the advantage to lead to the development of bioprocess production of enzymes or other products with high value. Although, metagenomics presents some limitations, as the high costs and the fact that it produces a large amount of uninterpretable data [16] due to the difficulties in functional screening of useful genes, in cloning sequences of genes in suitable hosts and in screening for enzymes [22]. So, it is necessary another complementary approach, such as the culturomics [23].

Microbiologists have been using a culture-dependent technique, culturomics, to study the diversity of bacteria in natural habitats. This technique has been applied by selective enrichment and plating on agar with a variety of selective media [24]. After the growth, each colony is secondarily isolated, and identified by Matrix Assisted Laser Desorption Ionisation – Time of flight (MALDI-TOF) mass spectrometry or analysed by 16S RNA amplification and sequenced, providing its taxonomic denomination [23]. Culturomics also allow the study of the microbial physiology, the full taxonomic characterization of the microbe and the understanding of the role of the microorganism in the environment [25]. Besides that, culturomics results in a pure culture of the organism, allowing the study of its application for bioprospection purposes [23]. The differences, advantages and disadvantages of using metagenomics and culturomics are presented in the table 1.

Table 1 – Comparison between metagenomics and culturomics (Adapted from [26]).

	Metagenomics	**Culturomics**
Definition	Allows the description of microorganisms by high-throughput sequencing	Allows the description of the microorganisms by high-throughput culture
Methods	Pyrosequencing of 16S rRNA amplicons and/or direct metagenomics without amplification step	Use of various selective and/or enrichment culture conditions combined to MALDI-TOF MS identification
Advantages	Detects non-cultivable microorganisms	Easier to implement Detects minority populations Open approach
Disadvantages	Does not provide a strain for further studies; Does not detect minority populations Does not provide information of enzymatic activities	Difficulties to be applied to non-cultivable microorganisms Detects only viable bacteria Major workload Does not provide information on enzymatic activity
Possible future developments	Increase quality of sequencing because of new technology Combine pyrosequencing with direct metagenomics	Automated detection of microbial growth Automated identification Other innovative culture conditions

1.2. Techniques for identifying microorganisms

In the past, the most common techniques used to identify microorganisms were based on the culture of microorganisms and the determination of their phenotypic characteristics. Nevertheless, these procedures are labour-intensive, time-consuming, and sometimes not adequate when the species are phenotypically similar, which brings some limitations, especially in clinical microbiology. For example, when some pathogenic agent cause a disease, it is necessary to do implementation of a quickly treatment [27]. The identification of microorganisms is also important in areas such as the pharmaceutical industry, food quality control and environmental research [27].

Nowadays, methods with high sensitivity and reproducibility are used in the taxonomy of microorganisms and molecular biology methods: (i) 16S ribosomal RNA (rRNA) gene sequencing, (ii)

polymerase chain reaction (PCR), (iii) other related PCR-based methods and (iv) MALDI-TOF MS [27]. Biochemical tests are also used to identify many microorganisms in the present because have some advantages, such as, short analysis time (2-4 h) and good accuracy. Some of these tests are Vitek 2 compact and analytical profile index (API) 20 E [27]. Another method with a good accuracy that is used to identify microorganisms is the *Sherlock ® Microbial Identification System* (MIS) that analyses the fatty methyl ester (FAME) composition of bacteria [28].

1.2.1. 16S ribossomal RNA gene sequencing

The use of marker 16S rRNA gene sequences to study bacterial phylogeny and taxonomy has been used extensively for many reasons: (i) is present in almost all bacteria, often existing as multigene family or operons; (ii) 16S rRNA gene presents a 1,500 base pair gene coding for a catalytic RNA which belongs to the 30S ribosomal subunit and for that is large enough for informatic purposes; (iii) 16S rRNA gene has conserved and variable sequence regions at very different rates, being that the variable sequencing regions may be a result of evolution [29, 30]. This is critical for the concurrent universal amplification and measurement of both close and distant phylogenetic relationships [30]. Another very important characteristic of this marker is that its function does not change over time, leading to the belief that random changes in their sequence are due to the evolution [29].

To use the marker 16S rRNA gene in identification processes, it is needed to begin with PCR, that is used to amplify the 16S rRNA gene sequence from the genomic DNA. After that, the PCR product is sequenced by the dideoxy DNA sequencing method, through use of PCR primers complementary to the conserved sequences in the small unit of rRNAs. Thus, with sequencing step done, it is necessary to align the raw data with the previous aligned sequences, using a sequence editor [31].

As not all RNAs are exactly the same length, during alignment, gaps can be inserted wherever necessary in regions where one sequence can be shorter than the other. Finally, the aligned sequences are imported into a treeing programme and comparative analysis is performed [31].

The main advantage of using this technique is that it can identify strains difficult to growth. Although 16S rRNA gene is highly conserved at the species level, subspecies and strain level differences are not shown, the costs per sample and equipment are high and requires a high level of technical proficiency [32].

1.2.2. FAME analysis

The microorganisms may be identified based on composition of their fatty acids (FAs) because FAs act as a biomarker to identify the organism. Since the FA may be saturated, unsaturated, linear, branched, containing hydroxyl or methyl groups or cyclopropane groups, each bacterium has a specific profile that acts as a fingerprint. When the growth conditions are standardized in terms of culture medium composition, incubation, temperature and physiological age of the cell population, cellular FA profiles are reproducible and the accuracy of identification increases, allowing the use of automated systems such as the Microbial Identification System (MIS). Furthermore, this method is useful when members of different species have high rDNA similarity [33].

One of the commercial methods that can be used to identify microorganisms based on their fatty acid profile is the Sherlock ® Microbial Identification System (MIS) [28]. This system was introduced in the 1990s and is able to identify over 1500 microbial species by gas chromatography (GC) analysis of extracted microbial fatty acid methyl esters (FAMEs) [32, 34]. Microbial fatty acid profiles are unique from one microorganism to another, and his allowed the creation of a large library [32]. The Sherlock system is an accurate, high-throughput method with low cost per sample [32]. However, this system has some disadvantages: minor changes at the quantification of peak height can distort the results; the quantification of peaks when two peaks not are totally separated may give erroneous peak areas, and therefore, the bacterial identification is not possible [35]. Nevertheless, the system allows a fast identification of microorganisms and to study the proximity of species even those not present in the database.

1.3. Screening for compounds produced by marine bacteria

In the last decades, the exploration of oceans has allowed the discovery of multiple habitats characterized by extreme conditions [36]. In these environments, several marine organisms adapted to these conditions are present and may produce a wide range of active biomolecules with useful properties, such as drugs, biosurfactants, antibiotics, enzymes, and vitamins [36, 37].

1.3.1. Biological surface-active compounds

Surface-active compounds (SACs) are produced by microorganisms including bacteria, yeast and fungi and can be classified according to their physico-chemical properties, molecular weights and localizations [38, 39]. These compounds can be divided in (i) biosurfactants that are molecules constituted by low molecular weight compounds and (ii) bioemulsifiers that include molecules with high molecular weight [40]. Both types of SACs are amphiphilic molecules which consist in two parts, a polar (hydrophilic) group and non-polar (hydrophobic) group (figure 1) [39]. The structural characteristics of SACs allow to change the conditions at interfaces (solid/liquid, liquid/liquid and gas/liquid) [38], through the solubilisation of hydrophobic compounds so that microorganisms may use them as substrate [41]. Moreover, SACs may be present on cell membrane, which increases the hydrophobicity of cell and help to protect it in hydrophobic environments, but they also have the capability to control their adherence property to water-insoluble substrates (emulsification) [39]. Other functional properties, such as wetting, cleansing, phase separation and reduction in viscosity of crude oil are also showed by SACs [42]. The production of biosurfactants and bioemulsifiers is motivated by similar conditions as presented above, although in different degrees [43].

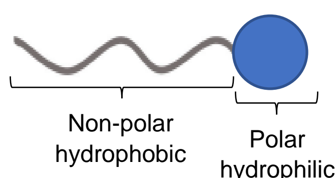


Figure 1 – General structure of a surfactant molecule with apolar (hydrophobic) and polar (hydrophilic) parts.

The metabolic pathways to produce SACs depend of carbon source and are thus diverse. If the microorganisms use hydrophilic substrates, they use this substrate for cell metabolism and synthesis of the polar moiety of a SAC, while if the microorganisms use hydrophobic substrates, they apply these substrates in the production of the hydrocarbon portion of the SAC [44].

SACs have a wide range of applications due to their properties (table 2). They can be produced from waste and renewable substrates such as vegetable oils, hydrocarbon wastes, and crude oil. Besides that, SACs are biodegradable and non-toxic [45].

Table 2 – Potential applications of SACs (Adapted from [46, 47]).

Industry	Application	Biosurfactant function
Food industry	Emulsification and breaking the emulsions, functional compounds	Emulsifier, stabilizer, moisturizer, foaming agent, antiadhesive and antimicrobial activities
Cosmetic	Lotions, creams, gels, sprays, among others	Emulsifier, moisturizer, foaming agent, dissolver, antimicrobial agents, improver of enzyme activity
Agriculture	Biological control	Insecticide, antibiotics for parasites
Biotechnology	Pharmaceutical and therapeutic	Antibacterial, antifungal, antiviruses and anti-adhesive agents, vaccine, gene therapy
Oil industry	Oil recovery	Microbial enhanced oil recovery (MEOR), wetting agent for solid surfaces, reducing superficial tension, pour point, viscosity reducer agent
	Emulsion	Crude oil emulsion breaking, reducing viscosity, moisturizer
Environment	Bioremediation and soil leaching	Reducing superficial tension, emulsification of hydrocarbons, removal of heavy metal, foaming agent
Bioprocessing	Downstream processes	Biocatalysts in micro emulsions and aquatic phase system, intracellular product recovery, improving of extracellular enzyme and fermentative products

Synthetic surfactants are used to produce several bulk commercial products, especially for cosmetics products and household cleaners [45]. It is estimated that the consumption of surfactants reaches 13 million tonnes per year worldwide [48], being that the problem of these compounds lies essentially in the use of non-renewable sources to produce them [45], like petrochemical and oleochemical sources [49]. In addition, the most used synthetic surfactants, such as alkyl benzene sulfonates (ABS) used in detergents, are not biodegradable and cause environmental impact. So, the replacement of synthetic surfactants by SACs of biological origin has increased to overcome the problems associated to the toxicity of synthetic surfactants [39]. For example, manufacturers from detergents industry replaced the ABS for linear alkylbenzene sulfonate (LAS) which do not present toxicity for the environment and has the same characteristics as ABS [50]. Moreover, SACs need to

compete with chemical surfactants in terms of cost, functionality, and production capacity to match the demands [47].

The extreme conditions of marine habitats make the metabolic and physiological capabilities of marine microorganisms unique and give them the ability to produce novel metabolites, such SACs [51]. Some of the features that characterize SACs from marine resources and other resources, such as, soil, fresh water, among others are presented in table 3.

Table 3 – Comparison between SACs produced by microorganisms from marine and other habitats (Adapted from [39]).

Feature	Marine resources	Other resources
Place of isolation	Oceans, sea, salty brine and Antarctic areas	Soil, fresh water, waste water, industrial effluents, sewage, refineries
Ability to cultivate	Difficult to cultivate under laboratorial conditions	Easy to culture under laboratorial conditions
Functional properties	Diverse biological and functional properties	Similar type of functional properties
Toxicity nature of SACs	Relatively high toxicity	Less toxic and more biodegradable
Probability of contamination in culture	Difficult of contamination due to extreme environment conditions (salinity, temperature, pH)	Easily contaminated

1.3.1.1. Biosurfactants

Biosurfactants are a vast group of SACs produced by microorganisms, especially when grown on hydrophobic substrates [52, 53]. These SACs are composed by sugars, amino acids, FAs and functional groups such as carboxylic acids [54], which can be classified according to the different chemical structures that exhibit: glycolipids, lipopeptides, phospholipids, FAs, neutral lipids and polymeric biosurfactants [37]. Glycolipids and lipopeptides are the most common biosurfactants [55]. Glycolipids are structurally composed by a mono or disaccharides acylated with long chain FAs or hydroxyl FAs [55] and can be subdivided into rhamnose lipids, sophorose lipids, trehalose lipids, cellobiose lipids, mannosylerythritol lipids, lipomannosyl-mannitols, lipomannans and lipoarabinomannanes [56]. Lipopeptides consist in linear or cyclic amino acid chain (peptide) linked to fatty acid. These FAs may differ in length and structure and usually are composed by 13-16 carbon atoms that may be branched [57]. Phospholipids, neutral lipids and some FAs are present in cell structures and have surface activity [57]. The polymeric surfactants are composed by a polysaccharide backbone covalently linked to fatty acid side chains [58]. Furthermore, biosurfactants are secondary metabolites because their production occur essentially during stationary phase of microbial growth [57], although they may have a survival role of biosurfactants microorganisms producers by improving the nutrient transport, interactions between microbe and host and by acting as biocide agents [59].

Due to having amphiphilic properties, biosurfactants have the ability to dissolve in polar and non-polar solvents [43, 60]. In addition, these molecules have an excellent surface activity, which allow to reduce the superficial and interfacial tensions between different phases, such as solid/liquid, liquid/liquid and gas/liquid, form stable emulsions and present low critical micelle concentration (CMC) [43], being that some cases is even lower than most of the traditional chemical surfactants [61].

When there is an interface between two immiscible fluids or between a fluid and a gas, biosurfactants can reduce the interfacial tension (liquid-liquid) or superficial tension (liquid-air), by decreasing the repulsive forces between two dissimilar phases and allow that these two phases are mixed and interact between them more easily. [57] For example, the surfactin produced by *Bacillus subtilis* can reduce the superficial tension of water to 25 mN/m and the interfacial tension of water/hexadecane until to less than 1 mN/m [62]. Moreover, biosurfactants have the capacity to disperse hydrocarbons in aqueous phase, when they are placed on an aqueous solution with an immiscible compound like a non-polar hydrocarbon, thus forming an emulsion [57]. This happens because the hydrophilic and hydrophobic portions of surfactant molecule orient to water and hydrocarbon, respectively [57]. Another example where biosurfactant has a crucial role is in degradation of hydrophobic pollutants or insoluble compounds [54]. In this case, the biosurfactants help to form micelles that enable to reduce the surface and interfacial tension which increase solubility and bioavailability of hydrophobic organic compounds [57] and, the access of microorganisms to degrade these polluted compounds is easier. However, there is a concentration above which no changes in interfacial properties occur (figure 2) [57].

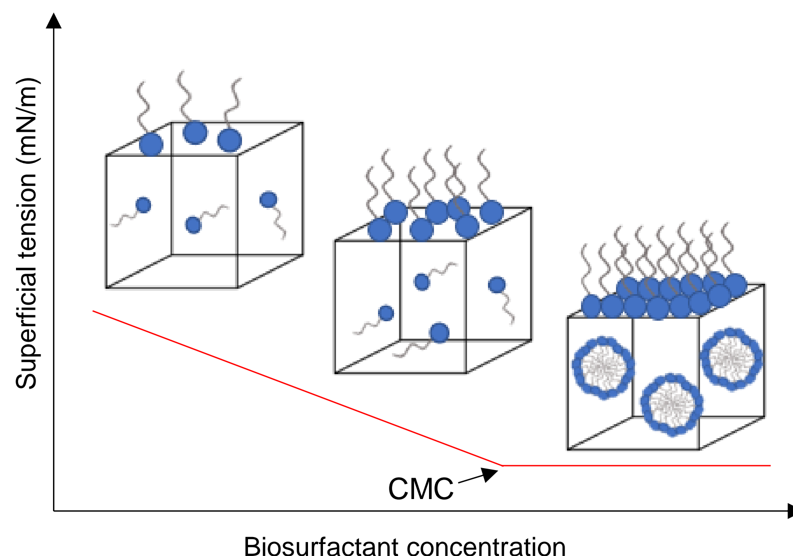


Figure 2 – Relationship between superficial tension and biosurfactant concentration and illustration of region where micelles occur (Adapted from [44]).

There are several applications of biosurfactants (table 4): in industry, such as agriculture, cosmetic, pharmaceutical, chemistry and food production [52], bioremediation, anti-adhesive action against pathogens and biomedical applications, such as antimicrobial activity, [63], immune-modulative and antitumoral activity [64].

Table 4 – Examples of biosurfactants produced by different microorganisms and their application/function.

Biosurfactant class	Biosurfactant type (structure)	Microbial strain	Application/Function	Reference
Glycolipids	Rhamnolipids	*Pseudomonas aeruginosa* DAUPE 614	Environmental pollution control	[65]
	Sophorolipids	*Candida bombicola*	Pre-treatment of high fats and oils contained in food industry wastewaters	[66]
	Trehalolipids	*Rhodococcus erythropolis*	Environmental remediation	[67]
Lipopeptides	Surfactin	*Bacillus subtilis*	Antimicrobial, antibacterial and antiviral activities	[68]
	Viscosin	*Pseudomonas viscosa*	Antiviral activity	[69]
	Lichenysin	*Bacillus licheniformis*	Oil recovery	[70, 71]
Phospholipids	-	*Thiobacillus thiooxidans*	Oxidation of elemental sulphur, through wetting	[72]
Neutral lipids	-	*Nocardia erythropolis*	High surface activity	[73]
Fatty acids	-	*Corynebacterium lepus*	Can alter superficial tension, contact angles, emulsification and de-emulsification	[74]

The use of biosurfactants has many advantages when compared to chemically synthetized equivalents, being that the biosurfactants are biodegradable, less toxic and non-hazardous. Furthermore, biosurfactants have greater foaming activities and higher selectivity to a number of metal ions and organic compounds [52, 75]. They can be produced from industrial wastes and bio-products, which can reduce the pollution effect and can be active under extreme conditions, like temperature, pH and salinity [52]. The disadvantages of using these SACs are associated with the substrates used because sometimes substrates contain undesired compounds and require purification in large quantities to industry, which may prove difficult to get the continuous supply for the industrial process [76]. Therefore, the costs associated to the production of biosurfactants increase. The use of organic solvents for biosurfactant recovery may involve large quantities of solvent, which also increase the production costs owing to the high prices of solvents and problems associated with the environment [44].

As previously mentioned, with increasing demand for new biosurfactants, marine-derived biosurfactants have gained more attention due to their properties and chemical diversity [64], particularly for bioremediation of the sea polluted by crude oil, but marine biosurfactants have also other applications (table 5).

Table 5 – Biosurfactants produced by marine bacteria and their applications.

Microorganism (origin)	Biosurfactant type (structure)	Application/Function	Reference
Bacillus megaterium	Glycolipid	Emulsification activity against different hydrocarbon pollution	[77]
***Rhodococcus erythropolis* 3C-9**	Glycolipid	Potential for oil spill clean-up operations through solubilization of oil	[78]
***Nesterenkonia* sp.**	Lipopeptide	Emulsifier, antioxidant activity, protect agent against *Staphylococcus aureus* in food industry	[79]
Bacillus circulans	Lipopeptide	Antimicrobial activity against Gram-positive and Gram-negative pathogenic and semi-pathogenic microbial strains, including multidrug resistant strains; Not present haemolytic activity	[80]
***Aneurinibacillus aneurinilycticus* SBP-11**	Lipopeptide	Antimicrobial activity and enhanced crude oil recovery due to excellent surface activity reduction, CMC and stable emulsification displayed	[81]
***Myroides* sp.**	Phospholipids and fatty acids	Good surface-active agent	[82]
Corynebacterium kutscheri	Glycolipopeptide	Emulsification of different hydrocarbons; Can be used in bioremediation of pollutants compounds	[83]

1.3.1.2. Bioemulsifiers

Bioemulsifiers are complex biopolymers, polysaccharides, proteins, lipopolysaccharides, lipoproteins, and a mixture of these compounds [43]. Lipopolysaccharide is a surface unique molecule constituted by a lipid and a polysaccharide, composed of O-specific or O-antigen and are present in the outer membrane of Gram-negative bacteria [84]. Lipoproteins are a complex of particles with a central core containing cholesterol esters and triglycerides surrounded by free cholesterol, phospholipids and apolipoproteins, which help lipoprotein formation and function [85]. Although, the bioemulsifiers have similar functions when compared with biosurfactants, they cannot reduce the superficial tension [54]. Bioemulsifiers are able to emulsify two immiscible liquids such as hydrocarbons or hydrophobic substrates even at low concentrations and, can do the solubilization of poorly-soluble substrates and therefore increasing the access and availability for biodegradation [43]. In addition, bioemulsifiers have the capability to stabilize emulsions due to the high number of reactive groups exposed, present in their molecules, that binding to the hydrocarbons and oil dispersed in polluted environment, forming a boundary which avoids the drop of coalescence [43, 54]. Bioemulsifiers can also increase the stability of emulsions by increasing their kinetic stability. The emulsifying activity has interest and applicability in food, cosmetic, pharmaceutical and petroleum industries [54]. Numerous bioemulsifiers have been described, being some examples presented in the table 6.

Table 6 – Examples of bioemulsifiers produced by different microorganisms and their application/function.

Bioemulsifier class	Bioemulsifier type (structure)	Microbial strain	Application/Function	Reference
Polysaccharide	Polysaccharide	*Trichosporon loubieri* CLV20, *Geotrichum* sp. CLOA40 and *Galactomyces* sp.	Formulation of emulsions and food and cosmetic industries; Removal of oils in tanks and ducts	[86]
Lipopolysaccharide	Emulsan	*Acinetobacter calcoaceticus* RAG-1	MEOR and cleaning oil spills	[87]
Lipoprotein	Mycrobactan (glycolipoprotein)	*Microbacterium* sp. MC3B-10	Emulsification of aromatic hydrocarbons and oils	[88]
Proteins	Liposan	*Candida lipolytica*	Stabilize a variety of oil-water emulsions	[89]
Polymer	Alasan (polysaccharide and protein)	*Acinetobacter radioresistens* KA53	Stabilize a variety of oil-water emulsions, including, n-alkanes with long chain, liquid paraffin, among others	[90, 91]
	Mannoproteins (Polysaccharide and proteins)	*Kluyveromyces marxianus*	Bioemulsifier in the food industry	[92]

Despite the potential applications of bioemulsifiers, these compounds present some advantages which are similar to biosurfactants and some disadvantages, such as: the high production costs when large-scale production is envisaged. The prices of bioemulsifiers are supported if the products use low amounts of these compounds, like cosmetic industry, but are unaffordable for applications that need large amounts to produce the products. When compared with synthetic surfactants, bioemulsifiers can be used in bioremediation, they are produced *in situ* by selected microorganisms that use inexpensive substrates, which lowest the production costs. Therefore, the search for new microorganisms capable of producing cost-effectively bioemulsifiers and development of processes that use renewable substrates has been increasing [93]. The microorganisms from marine habitats have been studied because they can degrade hydrocarbons, which is important for the environment, such in cases of oil spill. Moreover, they have applications in food industry. Some examples are presented in table 7.

Table 7 – Bioemulsifiers produced by marine bacteria and their applications.

Microorganism (origin)	Bioemulsifier type (structure)	Application/Function	Reference
Yarrowia lipolytica	Yansan (glycolipopeptide)	Formulation of perfluorocarbon based emulsions and degradation of hydrocarbons	[94]
***Antarctobacter* sp. TG22**	Glycoprotein	Biotechnological applications: emulsan-stabilizing agent (e.g.: food oils)	[95]
Halomonas TE39 Halomonas TE67	Glycoproteins HE39, HE67	Bioremediation of toxic metals	[96]
***Streptomyces* sp. S1**	Bioemulsifier: protein (82%), reducing sugar (1%) and polysaccharide (17%)	Degradation of hydrocarbons	[97]
***Bacillus* sp., *Pseudomonas* sp. and *Brevibacterium* sp.**	Biopolymer	Degradation of hydrocarbons	[98]

1.3.1.3. Exopolysaccharides

Exopolysaccharides (EPS) are high molecular weight (10 a 30 kDa) carbohydrate polymers that are composed of sugar residues [99] and exist in three forms: (i) associated as a capsule to the cell surface, (ii) secreted by microorganisms into the surrounding environment or (iii) remain weakly attached to the cell surface [100] . EPS have different compositions and might be linear or branched with side chains of other compounds linked to the polymeric chain. Based on the composition, these compounds can be divided in two types of molecules (i) homopolysaccharides and (ii) heteropolysaccharides [101]. Homopolysaccharides consist in a single type of monosaccharide usually α-D-glucans, β-D-glucans, fructans and other polygalactan, while heteropolysaccharides may be composed by different types of monosaccharides namely, D-glucose, D-galactose, L-rhamnose and their derivates [102].

The biosynthesis of EPS includes three phases: assimilation of carbon source, intracellular synthesis of polysaccharide and the release of this compound out of the cell [103]. Many microorganisms, such as species of Gram-positive and Gram-negative bacteria, archaea, fungi and some alga [104], produce EPS as a response to biotic stress (e.g., competition), abiotic stress factors (e.g., temperature, pH, salinity, light intensity) or as a strategy of adaptation of cells to extreme environments [103]. The production of EPS in adaptation to extreme environments support microorganisms to survive in the following conditions: high or low temperatures, salinity and limitations of nutrients availability that surrounds the cells or that are present in the environment. Besides that, EPS are involved in some cell functions, such as, adhesion to surface or to other organisms, in biofilm production, as storage of reserve carbon sources or as a support to pathogenic and virulence mechanisms to protect the cells [103]. EPS also present the capability to form gel, emulsification, adsorption and flocculation [101].

Due to the physico, chemical and rheological properties presented by EPS, these compounds have many applications, namely in industry, such as, pharmaceutical, cosmetic, detergents, food additives,

textile, adhesives and can do microbial enhanced oil recovery (MEOR) and wastewater treatment [101]. The production of EPS requires energy waste up to 70% by bacterial cells to produce them, representing a significant carbon investment for microorganisms, but the benefit given by the EPS compensate the carbon and energy costs [104, 105]. Furthermore, the microbial EPS do not contain toxic compounds, can be synthesized from renewable substrates by fermentation and they are biodegradable [106]. So, the EPS can be considered a "green" compound.

To meet the demands of EPS for industry, microorganisms isolated from extreme environments, have been explored because the EPS produced in these habitats present a great diversity of physical and chemical properties and stability when compared with EPS from another sources [107].

Marine bacteria produce essentially heteropolysaccharides with three or four different monosaccharides (e.g. pentoses, hexoses, amino sugars or uronic acids) organized in groups of ten or less repeating units [105]. In the studies with marine bacteria, it was observed that EPS are produced in large amounts during stationary phase, in response to an environmental condition or as a result of lack of nutrients (e.g. nitrogen, phosphorous, sulphur and potassium) [105].

In a natural aquatic environment, the EPS may support the microbial growth because it facilitates the rate of nutrient uptake by cells and concentrate dissolved organic compounds, through the contact with the nutrients available [104]. Besides, the strains isolated from deep-sea hydrothermal vents characterized by having high temperature and pressure, high level of sulphur and heavy metals, also produce EPS. The properties of these EPS have been used for tissue regeneration, treatment of cardiovascular and oncological diseases [105]. EPS may also act as cryoprotective agent in brine channels of sea ice, where the low temperature and high salinity conditions the microbial growth [39]. Other applications of marine microorganisms are listed in table 8.

Table 8 – Exopolysaccharides produced by marine bacteria and their applications.

Microorganism (origin)	Exopolysaccharide type (structure)	Application/Function	Reference
***Halomonas* sp. TG39**	-	Biosurfactant; biodegradation of hydrocarbons	[108]
Pseudoalteromonas tunicata	Polysaccharide	Antifouling activity	[109]
Thermococcus litoralis	Homopolysaccharide	Biofilm formation	[110]
Bacillus licheniformis B3-15	Homopolysaccharide	Antiviral activity	[111]
S*treptomyces carpaticus*	Heteropolysaccharide	Free radical scavenging activity and antitumor activity against breast and colon cell lines	[112]

1.3.2. Biocatalysts

Enzymes are biological catalysts or biocatalysts involved in different biochemical reactions in living organisms. These substances are characterized by two fundamental properties: (i) ability to increase the rate of chemical reactions without being consumed or altered by the reaction and (ii) increase the reactions rate without altering the chemical equilibrium between reacts and products, i.e., enzymes can reduce the activation energy and therefore, increase the rate of reaction [113].

The catalytic activity of enzymes involves the binding of substrate (S) to the active site of the enzyme (E) in order to form the enzyme-substrate complex (ES). This binding is a very specific interaction between the active site of enzyme and substrate and is usually described by the lock-and-key model. The ES complex is responsible for reducing the activation energy and to convert the substrate in the product (P) of reaction. The enzyme-catalysed reaction can be written by (expression (1)) [113]:

$$\mathrm{S} + \mathrm{E} \rightleftharpoons \mathrm{ES} \rightleftharpoons \mathrm{E} + \mathrm{P} \qquad (1)$$

Enzymes have an important role in living organisms and can be divided in six groups depending on their classification as described in table 9. They catalyse the biochemical reactions that are the basis of metabolism of all organisms, they are involved in process of DNA replication and transcription, protein synthesis, signal transduction, *etc* [114, 115]. In addition, enzymes have the ability to perform very specific chemical transformations in industrial segments, such as food, cosmetic, pharmaceutical and animal nutrition [114].

Table 9 – Classification of enzymes (Adapted from [116]).

Enzyme classification	Representative subclasses
Oxidoreductases	Oxidases, oxygenases, peroxidades, dehydrogenases
Hydrolases	Lipases, esterases, proteases, glycosidases, phosphatases, aminoacylases, endo- and exo-nucleases, halohydrolases
Transferases	Glycosyltransferases, methyltransferases, transaminases, sulfotransferases, phosphotransferases, transaldolases, transkelotases, acyltransferases, alkyltransferases, nucleotidyltransferases
Ligases	Synthethases, carboxylases
Lyases	Decarboxylases, aldolases, hydratases, dehydratases, kelotases, polysaccharide lyases, ammonia lyases
Isomerases	Racemases, epimerases, isomerases

The advantages of using biocatalysts instead of catalysts are associated to high productivity, specificity, catalytic efficiency, the ability of enzymes to discriminate between similar parts of molecules (regiospecificity) or optical isomers (stereospecificity) [117], present good activity in synthetic substrates [118] and present enzymatic activities in mild reaction conditions (temperature and physiological pH) [119]. Furthermore, biocatalysts derived from renewable resources and are biodegradable, biocompatible and non-toxic [120]. However, there are some disadvantages in using enzymes such as high costs, low stability and in some industrial processes, biocatalysts have some limitations like instability under high temperatures, in turbulent flow regimes or in presence of toxic solvents [117, 121].

In 2012, almost 4000 enzymes were known, and of these, approximately 200 microbial original types are used commercially [114]. It was expected that market of enzyme biocatalysts in industrial applications will grow, from about $4.8 billion in 2014 to $7.1 billion in 2018 and $10 billion in 2020. In the food/beverage and cosmetic markets, it was expected to reach $1.7 billion and $1.8 billion in 2018, respectively [122].

The enzymes from microbial origin are in higher demand when compared to the animal and plant sources due to the high growth rate of microorganisms, availability in a variety of environments and inexpensive media [123]. Enzyme bioprospecting for novel biocatalysts has been increasing, especially for marine habitats. In addition, the enzyme bioprospecting activity has been improved in the last years with the incorporation of new techniques, for instance, metagenomics [124].

Enzymes from marine source from extremophiles exhibit diverse characteristics useful in bioprocesses (table 10), such as salt tolerance (halophiles), high pressure (piezophiles), high thermal (thermophiles) and cold adaptivity (psychrophiles) due to environmental conditions present in the marine habitats [124]. Furthermore, enzymes from marine sources present novel chemical and stereochemical properties when compared with terrestrial counterparts, which include substrate specificity and enantioselectivity [122]. These properties have high potential for pharmaceutical industry, where the enzymes from marine sources can be used in organic synthesis and in the resolution of racemic mixtures [122].

Table 10 – Examples of applications of enzymes from marine sources [125-127].

Enzyme (type)	Enzyme	Application/Function
Polysaccharide-Degrading Enzymes	Amylase Cellulase Xylanase	Food industry: baking, brewery and natural sweeteners
	Carrageenolytic enzymes	Liquefaction of carrageenan for food, pharmaceutical and cosmetic industries
	Agarases	Emulsifying, gelling and stabilizing agent in food processing
	Cellulase and lignocellulase	Production of biofuels
Proteases	-	Food processing, detergents, leather and pharmaceutical industries; Bioremediation and waste management
Lipases	-	Detergents, paper production, food flavouring and wastewater treatment

One of the most studied and used type of enzymes are lipases [128]. Lipases, triacylglycerol acylhydrolases, are serine hydrolases that catalyse the hydrolysis of tri-, di-, and monoglycerides to glycerol and FAs, in the presence of excess water, while in water-limiting conditions they promote ester synthesis [128, 129]. These enzymes often express other activities, like phospholipase or esterase activities.

Lipases and esterases (carboxylesterases) can hydrolyse carboxylic esters, being that lipases are more active towards long-chain FAs, particularly those containing acyl chains of more than ten carbon atoms [128]. Lipases can be differentiated from esterases because: (i) esterases prefer to hydrolyse shorter chain FAs, while lipases prefer to hydrolyse long chain FAs [126], (ii) esterases display higher activity towards the soluble state of its substrate and lipases show high activity towards the aggregates state of its substrate [130]. Moreover, lipases and esterases present differences in their 3D-structures [130].

Lipases display high stability in organic solvents, show broad substrate specificity and act under mild conditions [127]. Besides, lipases from marine bacteria exhibit other valuable features, including tolerance to salt, low temperature and slightly acidic or alkaline pH [128]. Esterases from marine microorganism present biocatalytic potential in the presence of high salinity, organic solvents, and low water activity environments, such as stereospecific and polymerization reactions, esterification, and transesterification [131] . Some examples of the applicability of marine lipases and esterases are the sectors of food, pharmaceuticals, environment, cleaning and textile (table 11) [128].

Table 11 – Lipases and esterases produced by marine microorganisms and their applications.

Microorganism (origin)	Lipase	Application/Function	Reference
***Aspergillus awamori* BTMFW032**	Lipase	Bioremediation of oil laden effluent	[132]
***Oceanobacillus* sp. PUMB02**	Lipase	Inhibit biofilm formation in food processing environment	[133]
***Halobacillus trueperi* RSK CAS9**	Lipase	Ionic, non-ionic and commercial detergents	[134]
***Aeromonas* sp. EBB-1**	Lipase	Industrial applications due to stability at high temperatures and the hydrolysing of long length esters	[135]
***Erythrobacter seohaensis* SW-135**	Esterase	Industrial applications	[131]

1.3.3. Screening methods for different microbial compounds

The screening methods consist in the verification if target analytes are present above or below a threshold and in fast acquisition of semi-quantitative data about all components present in sample [136]. In general, these methods have some characteristics, such as, a qualitative analysis and not quantitative, they do not usually involve the preparation of sample, produce fast results and require low investment [136].

The main goal in screening new surface active compounds is finding new strains that produce new structures with strong interfacial activity, good solubility, low critical micelle concentration, high emulsion and that are active in broad range of pHs [137]. In addition, SACs should be produced in high yields [137]. The ability of strains to interfere with hydrophobic interfaces is generally evaluated [138]. In the screening for enzymes, such as, lipases and esterases, the aim is to found strains that produce these enzymes with high lipolytic activity. Thus, to find new strains which produce compounds of interest, screening methods should be applied (table 12).

Table 12 – Screening methods for different compounds produced by microorganisms.

Compound	Screening methods	Reference
Biosurfactants	Du-Nouy-Ring method; Stalagmometric method; Pendant drop shape technique; Axisymmetric drop shape analysis by profile; Drop collapse assay; Microplate assay; Penetration assay; Oil spreading assay; Emulsification capacity assay; Solubilization of crystalline anthracene; Bacterial adhesion to hydrocarbons assay; Salt aggregation assay	[138]
Bioemulsifier	Drop collapse assay; Microplate assay; Penetration assay; Oil spreading assay; Emulsification capacity assay	[138]
Exopolysaccharides	Detection of EPS producing phenotype through colony morphology; Agar plate with dyes; Precipitation with alcohols; Microhaematocrift capillaries; Uronic acid determination with m-hydroxydiphenyl; Colorimetric assay phenol-sulfuric acid method	[139]
Lipases and esterases	Gel diffusion assay using different substrates (tributyrin, tween 80, olive oil, triolein); Gel diffusion assay with indicator dyes (victoria blue, nile blue sulfate, night blue, phenol red)	[140]

1.4. Bioremediation of compounds by marine bacteria

In day-to-day life, petroleum-based products are the major source of energy to daily life and industry [141]. However, the rapid growth of industries, leaks and accidental spills that sometimes occur during the exploration, production, refining, transport and storage of petroleum and petroleum products lead to the pollution of water and soils and other hazards [141, 142].

One of the most common accidents is the pollution of oceans due to oil spills that affect the aquatic living organisms, especially the microbial population. Crude oil is a complex mixture of hydrocarbons, including more than 70% of *n*-alkanes along with aromatics, resins and naphthalenes. Due to long chain alkanes, crude oil remains persistent because of its non-volatile nature and therefore, represents a threat to terrestrial and marine ecosystems [143]. The technological methods usually used to remove the pollutants are from chemical origin, which include dispersants and agents to catalyse photo-oxidation [144]. However, these chemicals are toxic [142] and they may not decompose completely the contaminants and are expensive [141].

Bioremediation is an alternative to chemical approaches because using microorganisms that produce natural compounds, to clean the contaminated environment (soil and water) [6], being that the cells may use the contaminants as energy source. The diverse catabolic activities presented by microorganisms are due to the presence of catabolic genes and enzymes. Moreover, microorganisms have the ability to modify the cell membrane in order to maintain the biological functions and use the

efflux pumps to decrease the concentration of toxic compounds inside the cells. All these mechanisms and metabolic abilities make the microorganisms an interesting tool in bioremediation [145].

There are two approaches associated to bioremediation: bioaugmentation and biostimulation. In bioaugmentation, bacteria are added to the existing population in order to increase the microbial population that degrade oil spill [141] This approach can be applied in diverse forms, such as, the addition of a pre-adapted pure bacterial strain, addition of a pre-adapted consortium, addition of biodegradation relevant genes packaged in a vector to be transferred by conjugation into indigenous microorganisms or introduction of genetically engineered bacteria [145]. This technique is useful for sites that not have sufficient microbial cells, or the native population does not possess the metabolic routes necessary to metabolize the compounds under concern. The biostimulation consists in stimulation of growth of microbial population by the addition of nutrients or other substrates [141]. This technique involves the identification and adjustment of factors such as nutrients that are approaching of the biodegradation rate of contaminants by the indigenous microorganism of the place affected [145].

In the case of marine environments, the addition of biostimulants has some problems, such as they should target the microbes near the oil droplets in the seawater and then be washed out or diluted by the wave action, higher concentrations of N and P sources can cause eutrification and therefore, increase the algal growth and reduce the dissolved oxygen concentration in the water. Besides that, bioaugmentation also presents some disadvantages, such as, insufficiency of substrate, use of other organic substrates in preference to pollutant and competition between introduced and indigenous biomass. So, due to the limitations presented by bioaugmentation and biostimulation, these approaches should be used simultaneously to complement each other [145].

Furthermore, bioremediation is considered the best way of restoring the ecological environment of the sea and oceans owing to be environmentally friendly, do not present secondary pollution and to be low cost [146].

Marine microorganisms are a group of microorganisms that are exposed to extreme natural conditions. For this reason, these microorganisms are considered an excellent candidate to do bioremediation of polluted extreme habitats because they have the ability to degrade, transform and accumulate toxic compounds, such as, hydrocarbons, toxic metals, heterocyclic compounds, pharmaceutical substances and radionuclides [6].

Marine bacteria produce SACs with the ability to form stable emulsions, which degrade the oil slicks that float on the surface of water through the dispersion of oil in water, and thereby, increase the available area to biodegradation of polluted compounds. The degradation of hydrocarbons can also be enhanced by using them as nutrients [142]. An example of application of SACs in bioremediation at large scale was reported on the Exxon Valdez oil spill. In this case, the rhamnolipid from *Pseudomonas aeruginosa* exhibited high removal capacitie for oil from contaminated Alaskan gravel [147]. Other example is the exopolysaccharide that marine *Halomonas* species produce to degrade hydrocarbons. This strain can create oil aggregates and emulsify the oil [108].

The release of heavy metals into the marine ecosystems is also other environmental problem because of their unique charateristics. These heavy metals may be provenient from industrial, municipal and agricultural wastewaters or from natural different geoochemical processes. Marine bacteria can

remove the heavy metal even in low concentrations, without producing any bioproduts. One of the mechanims used by marine bacteria is the bioaccumulation of heavy metals that is dependent of catabolic and anabolic energy of bacteria. One example of a bacterium that perform this process is *Psychrobacter* strains [148].

1.5. Adaptation of marine bacteria to salt environments

Extremophiles, which include thermophiles, psychrophiles, halophiles, among others, are microorganisms that grow well in the presence of harsh environmental conditions, such as temperature, pressure, salinity and pH. These conditions lead to the production of new enzymes and metabolites with potential [149] to be used in biotechnological applications such as the food, detergents and pharmaceutical industries and in bioremediation [150]. Moreover, extremophiles present unique membrane lipid composition and thermostable membrane proteins [150].

Salinity is a condition which varies along the seas and oceans. Therefore, two types of microorganisms can be found in these places, (i) halophiles which are microorganisms that require salt to survive and (ii) halotolerant which are able to grow in the absence as well in the presence of salt [151]. The advantage of using halophilic bacteria is related to the fact that high salt concentrations allow non-sterile conditions and therefore, low-cost processes [149]. On other hand, halotolerant can survive in high salt concentrations, but grow at higher rates under moderate salt concentrations [149].

Marine bacteria are often under extreme conditions of temperature, pressure, salinity and nutrient concentration. In order to survive in these environments, the marine bacteria developed some mechanisms of adaptation to maintain their biological functions, such as, production of biosurfactants, siderophores, terpenoids and specialized FAs [149]. To adapt to high salinities, marine bacteria change their membrane to keep the same level of fluidity. The high salinity concentration usually causes an increase in the content of negatively charges phospholipids from of neutral phospholipids. Gram-negative bacteria change polar lipid composition by increasing the proportion of the negatively charged phosphatidylglycerol and/or diphosphatidylglycerol (cardiolipin), while decreasing the proportion of zwitterionic phosphatidylethanolamine in the membrane. These changes preserve the membrane bilayer structure, because phosphatidylglycerol forms bilayers whilst, phosphatidylethanolamine that contain unsaturated FAs tends to form non-bilayer phases. In Gram-positive bacteria, in the presence of higher salinities, there is an increase of anionic lipid fraction due to the percentage of cardiolipin increase rather than phosphatidylglycerol [105].

1.6. Objectives

Marine bacteria have been shown to be a source of biological active compounds with useful physical and chemical properties due to the exposure to extreme conditions of oceanic environments, such as temperature, pH and salinity. So, the main goal of this Thesis was to assess and exploit the potential of marine bacteria to produce interesting compounds for biotechnological processes.

To achieve this goal, the work included the following tasks:

1. Identification of strains present in the bacterial collection AZ1-C16 from samples collected in the harbour of Ponta Delgada (Azores) with the ability to degrade n-alkanes and paraffin.
2. Evaluation and quantification of the production of biosurfactants by the AZ1-C16 cells.
3. Screening for lipases and/or esterases activities in the bacterial collection AZ1-C16.
4. Identification of strains present in the bacterial collection AV-SALT from Aveiros beach in Albufeira (Algarve) and determination of the salt tolerance of these cells.

2. Materials and Methods

2.1. Identification of marine bacteria

The strains used in this work were isolated from samples collected (i) at the harbour of Ponta Delgada, Azores (AZ1-C16), as described by de *Rodrigues et al.* (2017) [22], (ii) and at Aveiros beach, Albufeira, Algarve (AV-SALT). The sample from Aveiros beach used in the present study was collected in a small pool way from the seawater where the water had evaporated (figure 3), and a salt deposited could be collected. The samples were diluted in water and then plated under different conditions to promote the isolation of cultures in a previous study performed in the IBB-IST.

Figure 3 – Sampling site in Aveiros beach, Albufeira (Algarve).

The cells from the sampling site of the Azores, were isolated following an enrichment culture approach by using hexadecane as carbon source, and the cells from the Algarve were isolated using for selection the media tryptic soy agar (TSA; Sigma-Aldrich®) and sea salts with glucose as carbon source (SSGLU). The conditions of each place in the moment of sampling are present in table 13. The strains used in the present study were already isolated and deposited in a bacterial library at IBB-IST.

Table 13 – Sample identification, location and physical parameters measured.

Sample ID	Location	T(ºC), H_2O	T(ºC), Air	pH	Identification Method
AZ1–C16	Harbour of Ponta Delgada	23	18	8.53	Sherlock
AV-SALT	Aveiros beach - Albufeira	-	23	-	Sherlock

The cells were identified using the Sherlock® Microbial ID System (MIS) from MIDI (Newark, USA). Each isolated colony grown on TSA plate was incubated in a Memmert incubator at 30ºC for 24 ± 1 hours. After this time, cells were harvested to a 1.5 mL Eppendorf tubes (Eppendorf, Hamburg, Germany) and the FAs of the cells were extracted and simultaneously methylated to fatty acid methyl esters (FAMEs) using the Instant FAME method from MIDI.

The FAMEs were analysed on a 6890N gas chromatograph from Agilent Technologies (Palo Alto CA), with a flame ionisation detector and a 7683 B series injector, equipped with a 25 m long Agilent

J&W Ultra 2 capillary column. The Sherlock® software package (version 6.2) allowed bacterial identification based on the FAME profile of each bacterium using the ITSA1 method. Principal component analysis (PCA) of the FAMEs profiles using the 2D plot function of the software was used to study relationships between the large number of isolated strains [22] (tables 14 and 15).

Table 14 – Identification of microorganisms from Ponta Delgada harbour by Sherlock® MIS from MIDI.

Sample ID (AZ1-C16 collection)	Identification
2, 3, 4, 5, 14, 16	*Bacillus pumilus*
7, 18, 21	*Bacillus* sp.
8,12	*Micrococcus luteus*
13	*Staphylococcus* sp.
19	*Bacillus subtilis*
20	*Bacillus licheniformis*
26B	*Staphylococcus conhii*
1, 6, 9, 10, 11, 15, 17, 22A, 22B, 22C, 25, 26A, 26C	No match

Table 15 – Identification of microorganism from Aveiros beach – Algarve by Sherlock® MIS from MIDI.

Sample ID (AV-SALT collection)	Identification
32 - AVSALT-TSA1 - 3	*Bacillus licheniformis*
40 - AVSALT-SSGLU100 - 2 41 - AVSALT - SSGLU10 - 1 42 - AVSAL-TSA1 - 1	No match
53 - AVSALT – TSA1 - 2	*Staphylococcus aureus*

Through a PCA analysis of unidentified AV-SALT bacteria, it was possible to verify that strains 40 and 42 AV-SALT are close to the *Bacillus* cluster, and strain 41 AV-SALT appeared closer to *Bacillus gibsonii*.

2.2. Screening for compounds of industrial interest

2.2.1. Production of biosurfactants

The cells identified as 2, 3, 4, 5, 14, 16, 18, 19, 20 and 21 of collection AZ1-C16 were harvested from agar plates to 1.5 mL Eppendorf tubes and resuspended in 1 mL of mineral medium (MM). The MM includes the following compounds per litre of demineralised water [152] : 0.01 g EDTA, 0.002 g $ZnSO_4{\cdot}7H_2O$, 0.001 g $CaCl_2{\cdot}2H_2O$, 0.005 g $FeSO_4{\cdot}7H_2O$, 0.0002 g $Na_2MoO_4{\cdot}2H_2O$, 0.0002 g $CuSO_4{\cdot}5H_2O$, 0.0004 g $CoCl_2{\cdot}6H_2O$, 0.001 g $MnCl_2{\cdot}4H_2O$, 0.1 g $MgCl_2{\cdot}6H_2O$, 2 g $(NH_4)_2SO_4$ and 1.55 g K_2HPO_4 and 0.85 g $NaH_2PO_4{\cdot}H_2O$ for buffering (in order to obtain a buffer concentration of 50 mM and a final pH of 7.0). All chemicals were from Sigma-Aldrich®.

To each well of a 96 deep-well microplate (Thermo Fisher Scientific, USA), 500 µL of MM, 50 µL of cell suspension and a given amount of hexadecane were added. On rows A, B and C, *n*-hexadecane (Merck-Schuchardt, Germany) was added to reach a concentration of 0.25 %(v/v), while on rows D, E and F, the amount was added to reach 0.50% (v/v). The last two rows, G and H, contained *n*-hexadecane at a concentration of 1% (v/v). The microplate was incubated at 30ºC and 200 rpm in an Agitorb 200 incubator (Aralab, Portugal).

To assess the production of biosurfactants, 100 µL of supernatant of each culture was removed after 3 days of cell growth. The cell-free supernatant was obtained by centrifugation of 96 deep-well microplate at 3,500 rpm for 5 min (Eppendorf centrifuge 5810 R). The supernatant was transferred to a new 96-well microtiter plate (Sarstedt, UK) and optical distortion was evaluated as described by de Walter et al (2010) [138]. To help this evaluation, millimetric paper was placed under the microtiter plate.

2.2.2. Production of lipases and esterases

To assess the production of lipases and/or esterases, the cells were grown on 12 cm x 12 cm squared agar plates using as principal carbon and energy source tributyrin (Carl Roth, Germany) or tween 80. The composition of the agar medium was the following (per litre): 20 g of agar, 5 g of NaCl (Carl Roth, Germany), 3.5 g of yeast extract, 10 mL of 100x concentrated mineral medium and 10 mL of 100x concentrated phosphate buffer (to reach the concentrations mentioned in 2.2.1). Regarding the agar plate with tributyrin, a concentration of 10 mL/L was used and in the case of tween 80, 20 g/L was used. The plates were inoculated using sterile toothpicks and incubated at 30ºC during seven days. At least 28 isolates were tested per plate. Lipase/esterase activities were determined by visualization of "halos" surrounding colonies with active enzymes.

2.3. Exploring the potential of *Bacillus pumilus*

2.3.1. Bacterial strain

One of the isolates from Ponta Delgada's harbour (*vide* 2.1) was identified by the Sherlock® MIS as *Bacillus pumilus* (strain 3 AZ1-C16). Since this species produce biosurfactants [153] and presented enzymatic activities [154], it was selected for further studies.

2.3.2. Growth in different broths

Cells were grown in 100 mL Erlenmeyer flasks containing 20 mL of growth medium. The media tested were: Thioglycollate Broth with Resazurine (Thio; Sigma-Aldrich®), Marine Broth (MB; Pronadisa) and Mueller-Hinton Broth medium (MH, Fluka). The flasks were inoculated with an exponential growing inoculum to reach an initial optical density of ca. 0.2 and incubated at 30ºC and 200 rpm in an Agitorb 200 incubator during ca. 56 hours. Cell growth was monitored by optical density measurements at 600 nm using a T70 UV/VIS Spectrophotomer (PG Instruments Ltd.). All assays were carried out at least in duplicate.

2.3.3. Screening for biosurfactant production

Cells grown in two 100 mL Erlenmeyer flasks, containing 20 mL of MM with 35 g/L NaCl and 0.25% (v/v) of *n*-hexadecane. In one of the flasks, MTBE (Fluka) was added. The flasks were inoculated with cells previously grown under the same conditions but with MM without salt. After 24 hours of growth, 15 mL from each Erlenmeyer were transferred to two 15 mL Falcon tubes (Thermo Fisher Scientific, USA), and the tubes were centrifuged at 4,500 rpm for 10 min in a Sigma Laborzentrifugen 2-15 centrifuge (B. Braun) to harvest the supernatant.

To assess the presence of biosurfactants, the superficial tension of ca. 15 mL of cell-free culture supernatants were measured in a K8 tensiometer from Krüss GmbH (Hamburg, Germany), using the ring method and superficial tension of distilled water was used as reference value.

2.3.4. Degradation of *n*-alkanes

2.3.4.1. Growth conditions

To evaluate the growth of *Bacillus pumilus* on *n*-alkane, dry weight of the cells was determined and the cell adaptations at the lipid level were assessed by FAME analysis.

Pre-inocula were prepared by growing cells in 20 mL of MB at 30ºC and 200 rpm in 100 mL Erlenmeyer flasks. Assays were carried out at least in duplicate. After ca. 24 hours of growth, optical density of pre-inocula was measured, and all volume of one Erlenmeyer was collected and divided to two 15 mL Falcon tubes. The tubes were centrifuged at 1,500 rpm for 10 min (Sigma Laborzentrifugen 2-15 centrifuge) and supernatant was discharged. The pellet was resuspended in 3 mL of MM with 35 g/L NaCl, to wash the cells and remove the nutrients present in MB and centrifuged again under the same conditions. The supernatant was removed, and the pellet was resuspended in 3 mL of MM with 35 g/L NaCl. Growth on C5 to C17 *n*-alkanes was carried out in twenty-six 15 mL Falcon tubes (thirteen tubes for dry weight and another thirteen tubes for FAMEs analysis) containing 3 mL of MM with salt and 10 µL of one of the *n*-alkanes tested. The tubes were inoculated with an initial optical density of ca. 0.13 (100 µL of pre-inocula) and incubated at 30ºC and 200 rpm at Agitorb 200 incubator.

The *n*-alkanes used were the following: *n*-pentane (99%; Merck), *n*-hexane (>99%, Fisher Scientific, UK), *n*-heptane (99%, Panreac), *n*-octane (>99%; Merck-Schuchardt), *n*-nonane (99%; Acrós Organics), *n*-decane (>99%; Merck-Schuchardt), *n*-undecane (99%; Sigma-Aldrich®), *n*-dodecane

(99%; Acrós Organics), *n*-tridecane (>99%; Sigma-Aldrich®), *n*-tetradecane (99%; Sigma-Aldrich®), *n*-pentadecane (≥98%; Sigma-Aldrich®), *n*-hexadecane and *n*-heptadecane (99%; Sigma-Aldrich®).

2.3.4.2. Fatty Acid analysis

Cells were harvested after seven days of growth on each alkane by centrifugation at 5,000 rpm for 5 min (Eppendorf centrifuge 5810 R). The supernatant was discharged, the pellet was resuspended in 1 mL of distilled water, to wash the cells, and centrifuged again under the same conditions. The supernatant was removed. The cell lipids were extracted, and the FAs were simultaneously methylated to FAMEs using the Instant FAME method from MIDI as described in 2.1. The FAMEs were analysed on a 6890N gas chromatograph from Agilent Technologies and identified by the PLFAD1 method of Sherlock® software v. 6.2. The degree of saturation of FAs of the cell membrane was defined by the ratio between the saturated straight chain FAs and mono-unsaturated FAs.

2.3.4.3. Dry weight cell measurements

At the end of the growth, 3.30 mL of culture were transferred to 15 mL Falcon tubes previously weighted. The tubes were centrifuged for 5 min at 5,000 rpm (Eppendorf centrifuge 5810 R). The supernatant was removed and the falcon tubes were dried at 65ºC for 24 hours (Memmert incubator). After cooling to room temperature, the Falcon tubes containing the cells were weighted to determine the weight of the dried cell biomass.

2.3.5. Degradation of paraffin

2.3.5.1. Preparation of paraffin solution

In a 100 mL Erlenmeyer flask a suspension of paraffin was prepared, containing 0.5016 g of paraffin in 65 mL of MM. The suspension was autoclaved during 20 min at 121ºC. After cooling, and in sterile environment, 194.6 mg of surfactant produced by *Rhodococcus erythropolis* DCL14 were added, and the suspension was homogenized with the help of agitation.

Afterwards, the suspension was sonicated (Bandelin, Sonopuls, Labometer) with the following conditions: power = 50 W, working 9 s on and 3 s off during 15 min. To help the homogenization of paraffin in the suspension, the suspension was heated in a microwave.

To precipitate paraffin particles with the form of spheres, the solution was stirred in an ice bath for 15 min. The morphology of the particles were observed in an optical microscope (Olympus CX40) and the particle size was measured by dynamic light scattering (Zetasizer Nano ZS, version 7.12, Malvern Instruments Ltd.) using a plastic cuvette with 2 mL of Milli-Q water and 20 µL of suspension.

2.3.5.2. Growth conditions

Pre-inocula were prepared by growing cells in 20 mL of MB at 30ºC and 200 rpm in the Agitorb 200 incubator. Once the cells reached the exponential phase, which was determined by monitoring the optical density at 600 nm in spectrophotometer, 5 mL of each pre-inoculum were added to two 15 mL Falcon Tubes under sterile condition to recover the cells. The tubes were centrifuged at 6,000 rpm for 10 min (Eppendorf centrifuge 5810 R). The supernatant was discharged, the pellet was resuspended in

MM to wash the cells and remove the nutrients present in MB and the cell suspension was centrifuged again at 6,000 rpm for 5 min. This procedure was repeated one more time under the same conditions. After the last removal of supernatant, the pellet was resuspended in MM and the optical density of cell suspension was measured at 600 nm.

To each well of 6-well plates (Sarstedt, UK), MM, paraffin to reach a concentration of 5%, 10% and 20% (v/v), and cell suspension to reach an initial optical density of ca. 0.2 were added. The total volume for each well was 4 mL and in each 6-well plates there were an assay for control and another for cells for each concentration of paraffin. Four 6-well plates were prepared: one to collect samples at 0 h (only controls) and another three plates for collection at 24, 48 and 120 hours. The 6-well plates were incubated at 30ºC and 200 rpm in an Agitorb 200 incubator.

2.3.5.3. Determination of paraffin consumption

To determine the concentration of paraffin consumed, paraffin was extracted from suicidal samples with *n*-hexane and the paraffin consumption over time was evaluated by gas chromatography-mass spectrometry. At 0 hours, all the volume of the control wells was removed to 5 mL Eppendorf tubes. Paraffin was extracted through the addition of ca. 2 mL of *n*-hexane to each well (to wash wells) whose volume was transferred to Eppendorfs. To promote the extraction, Eppendorfs were vortexed. About 500 µL of organic phase were transferred to glass vials. For the remaining samples that were taken at 24, 48 and 120 hours, the procedure was the same.

Peak identification of paraffin hydrocarbons was confirmed by injecting both standard and selected samples on a gas chromatograph Agilent 7820A equipped with an autosampler and an Agilent 5977E quadrupole mass spectrometer. The capillary column was an Ultra-2 (25 m x 200 µm x 0.33 µm) from Agilent which worked at a constant flow of 1.5 mL/min. The mass detector was at 200ºC and the mass transfer line was set at 280ºC. The GC injector was set at 1:20 split mode and 250ºC.

2.3.5.4. Fatty acid analysis

The extraction of bacterial lipids of pre-inoculum was made according to the steps described in 2.3.4.2. For the remaining samples, after 24, 48 and 120 hours of growth, the Eppendorfs were centrifuged during 5 min at 10,000 rpm (Eppendorf centrifuge 5810 R), the supernatant was removed, and the pellet was resuspended in 1 mL of Milli-Q water to wash the cells. The Eppendorfs were centrifuged again under the same conditions, the supernatant was discharged and the extraction of FAs from the cells was performed as described in section 2.1. The degree of saturation of FAs of the cell membrane was defined as the ratio between the saturated FAs and the unsaturated FAs.

2.3.6. Observation of *Bacillus pumilus* cells over paraffin by SEM

2.3.6.1. Growth conditions

R. erythropolis DCL14 used in this work was isolated from a sample collected on a ditch in Reeuwijk, The Netherlands, and is currently stored and maintained at the iBB – Institute for Bioengineering and Biosciences, Instituto Superior Técnico, Lisbon, Portugal. This bacterium was used as a comparison because in a previous study, the bacterium showed to degrade *n*-alkanes [155].

Pre-inocula of *B. pumilus* and *R. erythropolis* DCL14 were prepared by growing cells in 20 mL of MM containing 10 g/L of glucose and 0.25% (v/v) of absolute ethanol (Panreac), respectively, at 30ºC and 200 rpm, in 100 mL Erlenmeyer flasks. Optical density measurements at 600 nm were used to monitor the cell growth. When the cells of each pre-inoculum reached the exponential phase, 4 mL of cell suspension was harvested into four Eppendorf tubes (Daslab, Barcelona, Spain). To avoid the presence of glucose in cell suspension of *B. pumilus*, Eppendorfs were centrifuged during 5 min at 10,000 rpm (Sigma 1-15 P sartorius), the supernatant was removed, and the pellet was resuspended in 1 mL of MM to wash the cells and centrifuged under the same conditions. The supernatant was discharged, and the cells were resuspended in 1 mL of MM.

To prepare the observation of cells and paraffin in the SEM, silicon pieces were embedded in liquid paraffin (melted with the help of a hotplate) over coverslips. The coverslips were removed from the hotplate and paraffin solidified at room temperature. To each well of a 6-well plate, MM, coverslips with silicon embedded in solidified paraffin, and cell suspension to reach an initial optical density of approximately 0.2 were added to make a total volume of 4 mL per well. In two wells were just placed MM in order to do the control, in other two wells cell suspension of *B.pumilus* was added, and the last two wells contained cell suspension of *R. erythropolis* DCL14. The plate was incubated at 30ºC at a stirring of 150 rpm in an Agitorb 200 incubator during seven days.

2.3.6.2. Analysis of samples by SEM, light and fluorescence microscopy

Cells and paraffin over coverslips of each condition were harvested after seven days of growth to visualize the cells of *B. pumilus* and *R. erythropolis* DCL14 over paraffin. This analysis was done by Scanning Electron Microscopy (SEM, JEOL 7001F with Oxford light elements EDS detector and EBSD detector).

To be able to observe the cells in SEM, they had to undergo a process of dehydration. First, the medium was discharged, and 4 mL of distilled water were added with 0.25% (v/v) of glutaraldehyde in order to do the primary fixation of the cells. This stage lasted 1 h. Secondly, the volume of the wells was removed and coverslips were washed with 4 mL of distilled water during 20 min for removing the remaining glutaraldehyde. After this time, the volume was discharged, and the cells were subjected to successive increases in absolute ethanol concentrations, which were 25%, 50%, 75%, 95% and 100% (v/v). Each step lasted 10 min. The last addition of 100% (v/v) of absolute ethanol, was repeated two times for 10 min. Finally, the last volume of ethanol was discharged, and the coverslips were dried. All phases of this work were performed at room temperature.

The samples were coated with a gold palladium alloy (to a thickness of ~ 20 nm; Q150T ES) and observed under SEM. On SEM, it was also possible to observe the bacteria size and morphology. Other coverslips were observed by light and fluorescence microscopy in order to complement the information given by SEM. The microscope was an Olympus CX40 equipped with an Olympus U-RFL-T burner, an U-MWB mirror cube unit (excitation filter: BP450-480; barrier filter: BA515), and an U-MWG mirror cube unit (excitation filter: BP510-550; barrier filter: BA590). The cells were observed by fluorescence microscopy after staining the cells with SYTO®9 which stains nucleic acids, and Nile Red which stains

neutral lipids. Images were captured by Evolution™MP5.1 CCD colour camera using software Image-Pro Plus (Media Cybernetics, Inc. (USA)).

2.3.7. Production of EPS using sugars as carbon sources

2.3.7.1. Growth conditions

The cells of pre-inocula were grown in 100 Erlenmeyer flasks containing 20 mL MH broth at 30ºC and 200 rpm in an Agitorb 200 incubator. The growth on inocula was carried out in cylindrical 200 mL closed flasks containing 40 mL of MH broth and 10 g/L of sugar. The initial optical density was ca. 0.2. The extent of growth was monitored by measuring of the optical density at 600 nm using a spectrophotometer until 120 hours of growth.

The sugars used in this work were glucose (Fisher Chemical), galactose (Sigma-Aldrich®), arabinose (Sigma-Aldrich®), sucrose (Fisher Chemical), raffinose (Sigma-Aldrich®), mannose (Sigma-Aldrich®) and xylose (Sigma-Aldrich®).

2.3.7.2. Extraction of EPS

To extract the EPS from the different cultures, 5 mL of medium culture were collected to 5 mL Eppendorf tubes and they were centrifuged at 8,000 rpm for 10 min (Eppendorf centrifuge 5810 R). The supernatant was placed on pre-weighted 15 mL Falcon tubes. The falcon tubes were placed on ice vessel, and 5 mL of absolute ethanol was added in order to precipitate the EPS. The tubes stayed overnight at 4ºC.

To recover the EPS, the tubes were centrifuged at 6,000 rpm for 10 min and the supernatant was discharged. To determine the dry weight of EPS, the tubes were incubated at 65ºC. This procedure was repeated until 96 hours of growth.

2.3.7.3. Cell dry weight measurements

At the same time as the samples of point 2.3.7.2 were collected, 1 mL of medium culture was harvested for dry cell weight to pre-weighted 1.5 mL Eppendorf tubes. The sample was centrifuged at 10,000 rpm for 5 min (Sigma 1-15 P sartorius). The supernatant was then removed, the pellet was resuspended in 1 mL of Milli-Q water to wash the cells and centrifuged under same conditions. The supernatant was discharged and the eppendorfs were dried at 65ºC for 24 hours. After cooling to room temperature, the eppendorf containing the cells were weighted to determine the weight of the dried cell biomass.

2.3.7.4. Superficial tension measurements

The superficial tension of cell-free supernatants (samples of 120 hours) was measured in a K8 tensiometer from Krüss GmbH. The superficial tension of distilled water was used as reference value.

2.4. Exploring the potential of *Micrococcus luteus*

2.4.1. Bacterial strain

One of the isolates from Ponta Delgada's harbour (*vide* 2.1) was identified by the Sherlock® MIS as *Micrococcus luteus*. Since this species usually present interesting enzymatic activities [156, 157], the strain was selected for further studies.

2.4.2. Impact of media composition on the production of extracellular lipase/ esterase activity

Pre-inocula was carried out in 100 mL Erlenmeyer flasks containing 20 mL of MH broth. The flasks were inoculated with 1 mL of exponentially growing cells and incubated at 30ºC and 200 rpm (Agitorb 200 incubator). The media used were MH, MB, Thio and Tryptic Soy Broth (TSB). Growth was monitored by measuring the optical density at 600 nm in a spectrophotomer. All assays were carried out in duplicate.

After about 24 hours, all volume of each flask was collected into a 50 mL Falcon tube (Sarstedt, UK), and they were centrifuged at 8,600 rpm for 10 min (Eppendorf centrifuge 5810 R). 10 mL of supernatant were transferred to 15 mL Falcon Tubes and analysed to assess the extracellular enzymatic activity using 4-nitrophenyl butyrate (*p*-NPB) as substrate. The activity of free lipases/esterases was determined by their capability to hydrolyse *p*-NPB into *p*-nitrophenol (*p*-NP) and butyric acid. In a cuvette of 3 mL, 200 µL of supernatant, 15 µL of substrate and 1,285 µL of 20 mM Tris HCl buffer, pH 8, were added to reach a final volume of 1.5 mL [158]. The enzymatic activity was measured at 410 nm in an HITACHI U-200 spectrophotometer. A mixture of 20 mM Tris HCl buffer (1,485 µL) and *p*-NPB (15 µL) was used as negative control to assess the existence of a chemical reaction (figure A.1). One enzyme unit (U) was defined as the amount of enzyme that converts 1 µmol of *p*-NP per min.

2.4.3. Inducing extracellular lipase/esterase activity by addition of tributyrin

To assess the influence of tributyrin in extracellular lipase/esterase activity, bacterial growth was promoted on 100 mL Erlenmeyer flasks, containing 20 mL of MH broth with tributyrin and without tributyrin. The flasks were inoculated with an exponential growing culture to an initial optical density of ca. 0.2 and incubated at 30ºC and 200 rpm. All assays were carried out at least in duplicate. After 24 hours of growth, the supernatant of both cultures was collected to determine the extracellular enzymatic activity as described in 2.4.2. Protein quantifications in the extract was done using the Bradford method [159]. The quantification was carried out in a 96-well microtiter plate by adding 150 µL of the Bradford reagent (Alfa Aesar) to 150 µL of sample. Control was done with distilled water. The reaction mixture was incubated for 10 min at room temperature, and after that, the absorbance of the solution was measured at 595 nm in a microplate spectrophotometer reader (SPECTRAmax Plus 384, Molecular Devices). Bovine serum albumin (BSA, Rockford, IL, USA) was used as the standard for the calibration curve (figure A.2).

2.5. Tolerance of marine bacteria from Aveiros-Algarve to salt

2.5.1. Bacterial strain

All strains from AV-SALT, identified by Sherlock® MIS, were used in this test. Some of them were *Bacillus licheniformis* and *Staphylococcus aureus (vide* 2.1).

2.5.2. Growth conditions

Cell growth was carried out in 100 mL Erlenmeyer flask containing 20 mL of TSB. The flasks containing TSB at different concentrations of salt (5, 35, 50, 100, and 150 g/L) which were inoculated to reach an initial optical density of ca. 0.2 and incubated at 30ºC and 200 rpm for 120 hours. Growth was monitored by measuring the optical density at 600 nm in a spectrophotomer.

2.5.3. Fatty acid analysis

To assess the effects of different concentrations of salt in cell membrane, 1 mL was taken from medium culture to do FA analysis. The steps of this procedure were described in 2.1. The unsaturation index (UI) of cell membrane was defined as the sum of the percentage of each unsaturated fatty acid multiplied by the number of double bonds in the molecule [160].

3. Results and Discussion

3.1. Screening for biosurfactant production

To assess if bacteria from the harbour of Ponta Delgada produced biosurfactants, a microplate assay was applied, where the optical distortion of the grid caused by altered superficial tension of culture supernatants was evaluated. The bacteria used in this test were selected according to results presented in section 2.1., i.e. only bacteria identified by Sherlock® MIS from MIDI were used in this screening test. Therefore strains 2, 3, 4, 5, 14, 16, 18, 19, 20, 21 from AZ1-C16 were used and grown in MM with 0.25, 0.50 and 1.00% (v/v) of *n*-hexadecane as carbon source. The MM with *n*-hexadecane (0.25, 0.50 and 1.00% (v/v)) and without *n*-hexadecane were used as control. The results can be observed in figures 4 and 5.

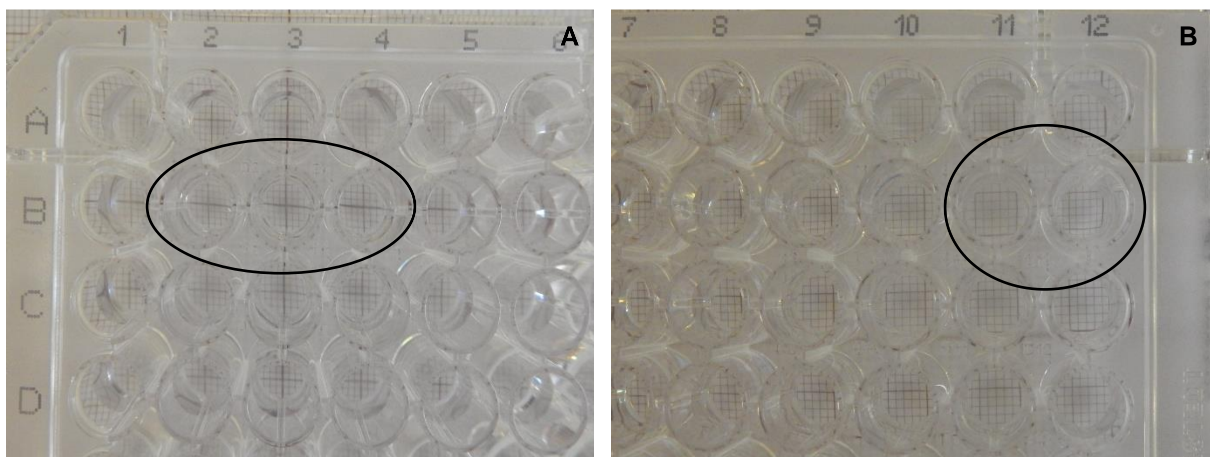


Figure 4 – Microplate assay to asses biosurfactant producers. Supernatant analysis (A): the columns of wells 2, 3 and 4 correspond to MM with 0.25% (v/v) hexadecane and cell-free supernatant from the culture strains 3 AZ1-C16 (B2), 4 AZ1-C16 (B3) and 5 AZ1-C16 (B4), respectively. Controls (B): the column 11 correspond to MM with 0.25% (v/v) hexadecane and column 12 had only MM.

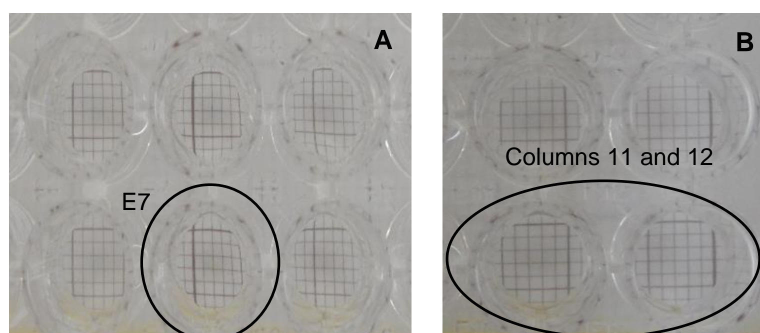


Figure 5 – Microplate assay to asses biosurfactant producers. Supernatant analysis (A): well E7 correspond to MM with 0.50% (v/v) hexadecane and cell-free supernatant from the culture strain 18 AZ1-C16. Controls (B): the column 11 correspond to MM with 0.50% (v/v) hexadecane and column 12 had only MM.

By analysing the grid assay, it was verified that wells B2, B3 and E7 presented some distortion. This distortion can indicate that these bacteria may produce surfactants with possible potential use for some fields like bioremediation or industrial processes. So, with these results, it was chosen strain 3 AZ1-C16

that was previously identified as *Bacillus pumilus* (*vide* 2.1) for further studies. Furthermore, in the literature was reported that the marine strain *B. pumilus* KMM 1364, isolated from the surface of ascidian *Halocynthia aurantium*, produced a mixture of lipopeptide surfactin in the presence of medium with salts, yeast extract, $FeSO_4$, Tris buffer, artificial seawater and distilled water [161]. Another study with the strain B. pumilus AAS3 isolated from the Mediterranean sponge Acanthella acuta, synthesized a diglucosyl-glycerolipid when grown on artificial seawater medium supplemented with yeast extract, glucose and nitrogen or phosphate sources [162].

3.2. Exploring the potential of *Bacillus pumilus*

3.2.1. Cultivability in Different Media: growth rate and duplication time

B. pumilus cells were grown in different media cultures in order to evaluate in which medium this bacterium grew better. For this purpose, the growth of *B. pumilus* was monitored by measuring the optical density at 600 nm along time (figure 6) and the growth rate and duplication time were determined (table 16).

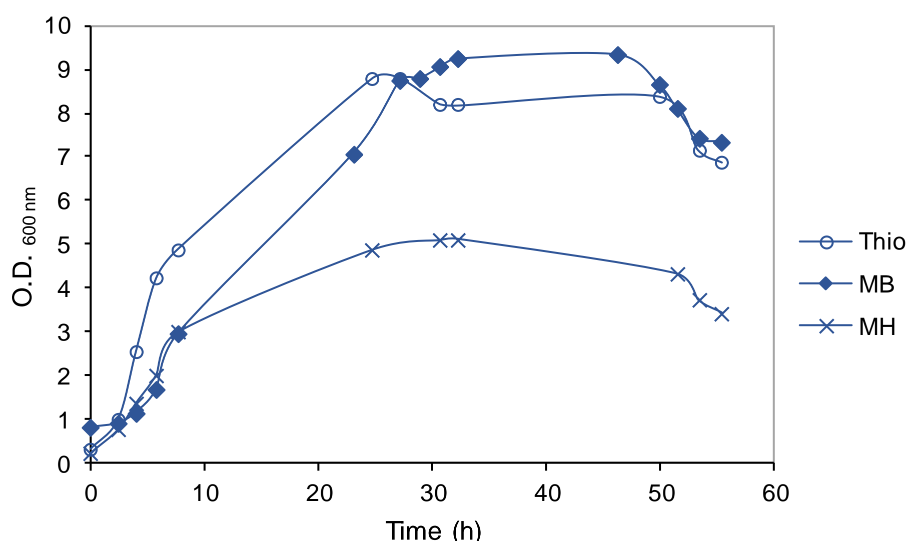


Figure 6 – Growth curves of *B. pumilus* in Thio, MB and MH broth.

Table 16 – Growth rate and duplication time of *B. pumilus* in different growth media.

Medium	Growth rate (h^{-1})	Duplication time (h)
Thio	0.53	1.31
MH	0.44	1.58
MB	0.26	2.71

From the results present in table 16, *B. pumilus* grew ca. 1.2 and 2.1 times faster in Thio when compared to MH and MB, respectively. One reason for these results may be the composition of the medium. Thio is composed by (per litre): 0.75 g agar, 15.00 g casein enzymic hydrolysate, 0.50 g L-cystine, 5.50 g dextrose, 0.001 g resazurine, 2.50 g NaCl, 0.50 g sodium thioglycolate and 5.00 g yeast extract [163]; MH is composed by (per litre): 2.0 g beef infusion solids, 17.5 g casein hydrolysate and

1.5 g starch [164]; MB is composed by (per litre): 19.40 g NaCl, 8.80 g $MgCl_2$, 5.00 g bacteriological peptone, 3.24 g Na_2SO_4, 1.80 g $CaCl_2$, 1.00 g yeast extract, 0.55 g KCl, 0.16 g $NaHCO_3$, 0.10 g $C_6H_5FeO_7$, 0.08 g KBr, 0.034 g $SrCl_2$, 0.022 g H_3BO_3, 0.008 g Na_2HPO_4, 0.004 g sodium silicate, 0.0024 g NaF and 0.0016 g NH_4NO_3 [165]. Therefore, one of the compounds that may have contributed and possibly influenced the growth of *B. pumilus* was the carbon source, as casein or dextrose.

According to the literature, *B. pumilus* presented presented a doubling time of ca. 1.5 h [166].

3.2.2. Biosurfactant production

B. pumilus was identified in previous works as promising biosurfactant producer [161, 162]. To confirm this ability, *B. pumilus* was grown in two different media (*vide* 2.3.3.) and after 24 hours of growth, superficial tension of supernatant was measured.

Table 17 – Superficial tension for different cultures of *B. pumilus* after 24 hours of growth and water as control.

Supernatant	Superficial tension (mN/m)
Water	77.0
MM with 35 g/L NaCl + 0.25% (v/v) C16	21.3
MM with 35 g/L NaCl + 0.25% (v/v) C16 + 0.25 % (v/v) MTBE	55.8

Table 17 shows that *B. pumilus* produced biosurfactants because the superficial tension of the supernatants was lower than the control (water). Besides that, effect of MTBE on biosurfactant production was also evaluated, and it was verified that MTBE did not favour extracellular production of the active surface compound. In another investigation with *B. pumilus* (CCT 2487) from the Tropical Foundation of Research and Technology, André Tosello, was reported that this bacterium could produce biosurfactants in the presence of 0.01 g/L of medium rich in mineral salts with 0.1 g/L yeast extract and 1% (v/v) of diesel. This medium was also supplemented with 1% to 5% total solids of vinasse and waste frying oil [153]. The lowest superficial tension for both substrates reached the value of 45 mN/m [153]. Production of biosurfactants by another *Bacillus* sp. was also observed. *B. licheniformis* ATCC 10716 was incubated in MM with salt with a trace element. In that medium was also added glucose and yeast extract to reach a final concentration of 2% and 3%, respectively [167]. After 24 hours of growth, the supernatant of the culture reached a superficial tension of 46 mN/m [167]. *B. megaterium* and *B. subtilis* from Tuticorin harbour, India, were isolated from sea water samples and after 7 days of growth in mineral salt medium with 1.0% (w/v) of crude oil, cell free culture broth of both bacteria presented superficial tensions of 30.8 mN/m and 38.75 mN/m, respectively [53].

3.2.3. Degradation of *n*-alkanes

To assess if *B. pumilus* could use alkanes as carbon and energy sources, this strain was grown in MM with 35 g/L NaCl and in the presence of *n*-alkanes, from *n*-pentane (C5) to *n*-heptadecane (C17), during seven days. The cell growth in each *n*-alkane was evaluated through biomass dry weight and the adaptations in cell membrane were verified by FA analysis.

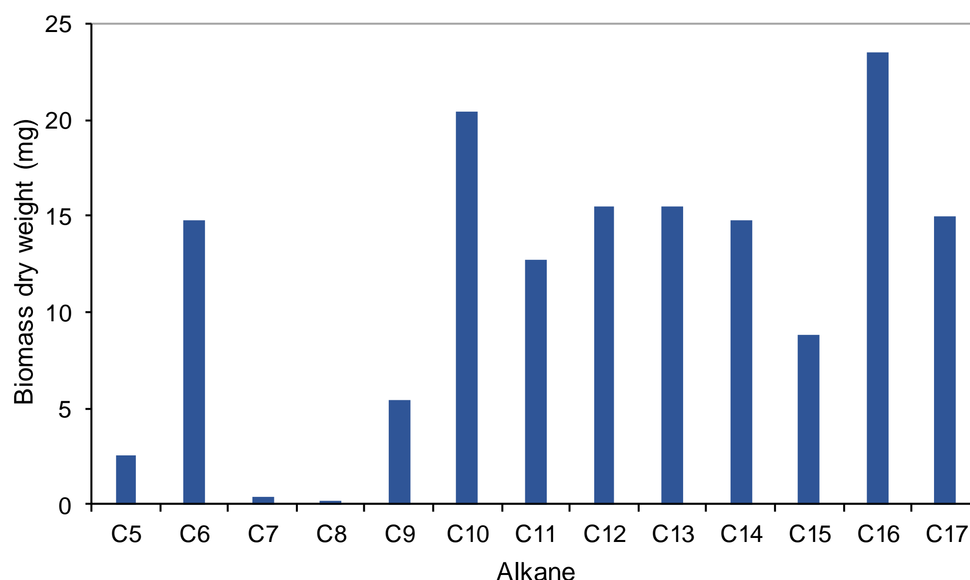


Figure 7 – Biomass dry weight (mg) of *B. pumilus* in the presence of C5-C17 *n*-alkanes after seven days of growth.

Analysing the dry weight of biomass (figure 7), it was possible to verify that *B. pumilus* grew better in the presence of alkanes with long chain, especially in C16. The reason of this growth in C16 may be due to the fact that *B. pumilus* (3 AZ1-C16) has been isolated in *n*-hexadecane. In the C7 and C8 *n*-alkanes did not appear any bar due to an error of weighing, although there was a small amount of biomass in the Eppendorf tubes.

With the aim of understanding the differences observed in the lipid composition of the cells in presence of *n*-alkanes, the FA were analysed. FA were grouped into the different classes according to their chemical structure: saturated straight chain (SSFAs), mono-unsaturated (MUFAs), poly-unsaturated (PUFAs), saturated *iso*- and *anteiso*-methyl branched (BCFAs), 10-metyl branched (10MBFAs), saturated cyclopropyl-branched (SCBFAs) and dimethylacetals (DMAFAs).

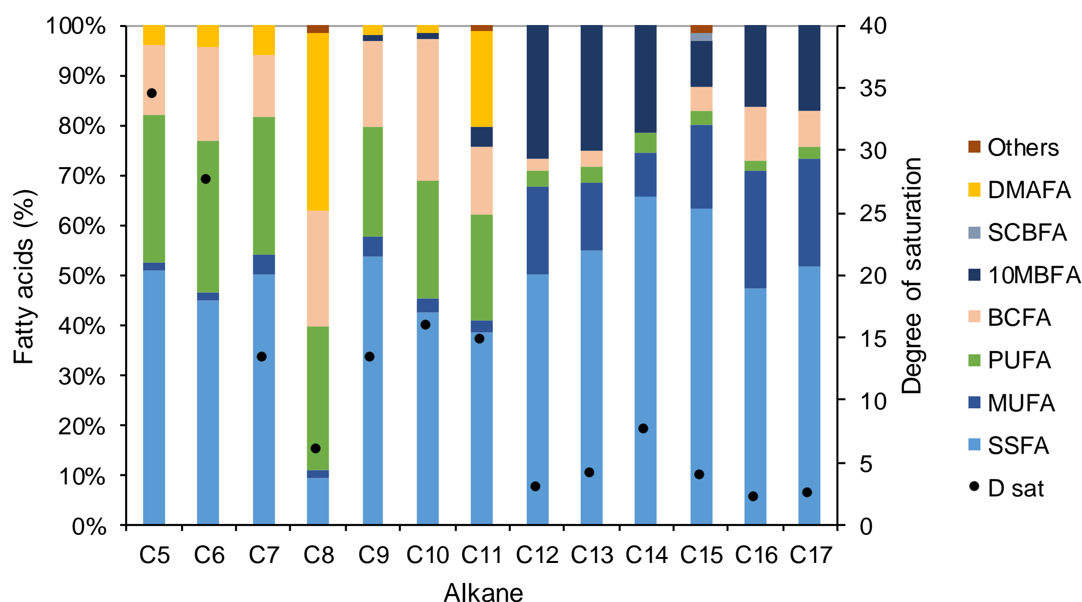


Figure 8 – Fatty acid composition of *B. pumilus* cells in the presence of C5-C17 *n*-alkanes after seven days of growth.

The composition of the cell membrane changed significantly from alkanes with small carbon chain (C5-C11) to long chain alkanes (C12-C17). The group of SSFAs constituted between 41.1% (C10) and 59.9% (C14) of the cell membrane, except for *n*-octane that reached the value of 9.26%. On other hand, MUFAs increased for alkanes with long chain (ca. 2.5% to 15.6%), which leads to a decrease in the degree of saturation. The degree of saturation decreased considerably from alkanes with small carbon chain to long chain alkanes, except in case of C8 alkane. In the C8 alkane, the percentage of SSFAs in cell membrane decreased significantly and then the degree of saturation also decreased.

The PUFAs decreased from ca. 25.9% in *n*-alkanes with small carbon chain to ca. 2.7% in long chain alkanes. The presence of PUFAs in greater amounts in C5-C11 alkanes, may be a form of the cells to maintain a suitable membrane fluidity [160]. Furthermore, the presence of BCFAs on cell membrane was higher in *n*-alkanes with small carbon chain (ca. 18.0%) than in long chain alkanes (ca. 4.4%), while the amount of 10MBFAs, presented an increase from ca. 0.9% in small carbon chains to ca. 17.6% in long chain alkanes. These results suggest that BCFAs and 10MBFAs promote the fluidity of cell membrane when cells grow in *n*-alkanes with small chain and long chain, respectively [33]. Other reason to verify these differences between the groups of alkanes C5-C11 to C12-C17 is that some bacteria are able to accumulate saturated and unsaturated straight long-chain FAs into cellular lipids during the cultivation under nitrogen-limiting conditions and, as well to other FAs derived from β-oxidation pathway [168]. In this case, when the cells grew on C12 up to C17 *n*-alkanes incorporated C14:0, C15:0 and C16:0 into their membrane phospholipids with a high percentage compared to other FAs. The results showed that these FAs had 45.3% in *n*-tetradecane, 45.6% in *n*-pentadecane and 33.2% in *n*-hexadecane of the cell membrane, respectively. It was reported in a study with *Rhodococcus erythropolis* that this bacterium grew on C13-C16 *n*-alkanes and then usually incorporate saturated FAs into their cell membrane, corresponding to the chain length of the substrate used for cell growth [155].

Other study with *Alcanivorax borkumesis* SK2 showed that generally FAs with a chain length of C14 to C18 are incorporated into the membrane, specially the C16:0 (palmitic acid) [169].

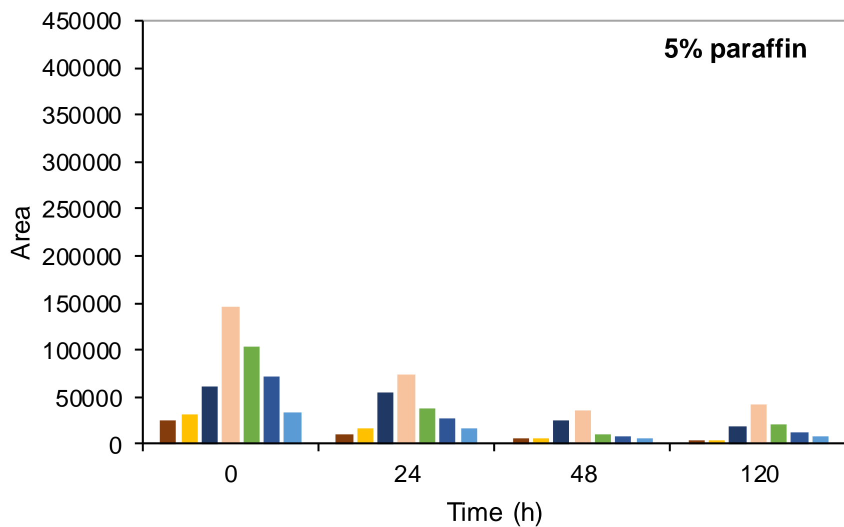
The results presented in figure 8 also suggest that the bacterium has changed its lipid composition as response to *n*-alkane, being visible the difference between C5-C11 to C12-C17 *n*-alkanes. In presence of *n*-octane, cell membrane changed significantly when comparing with other *n*-alkanes with small chain.) Chen, Janssen and Witholt (1995) reported that during growth on *n*-octane (C8), the accumulation of 1-octanol, a metabolite of the alkane monooxygenase, can be toxic to the cells [170], which means that C8 could have caused a toxic effect in the cells and to contrast this effect, cell membrane triggered some mechanisms of adaptation [155].

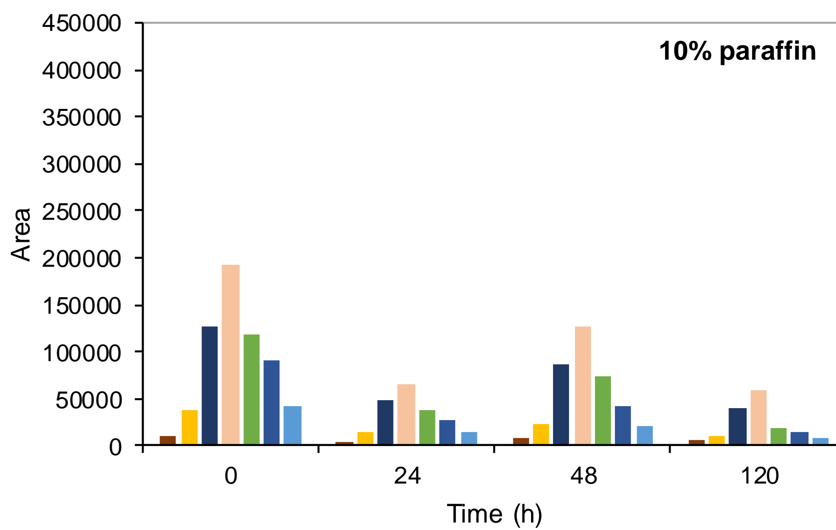
3.2.4. Degradation of paraffin

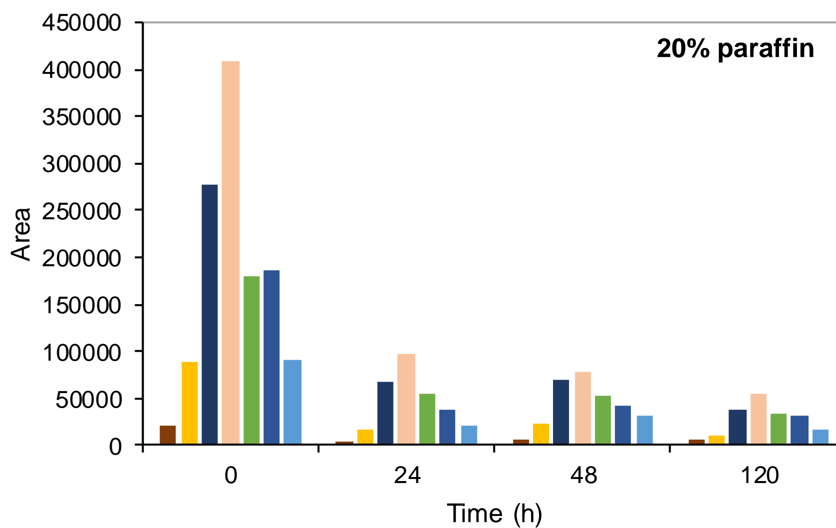
The microbial degradation of paraffin wax has been studied under the scope of the problems associated to oil industry and as an alternative to mechanical and chemical methods of paraffin degradation that are environmentally problematic [171].

In the present study, the efficiency of *B. pumilus* for degrading paraffin wax in different concentrations (5, 10 and 20%) was evaluated in terms of alkane degradation because this bacterium showed to produce biosurfactants that possibly solubilize hydrocarbons of alkanes. The adaptation of the cell membrane to paraffin was also assessed. The particle size of paraffin used in this work was measured by dynamic light scattering with at an angle 173º, having obtained an average size of 289 nm after sonication.

The analysis of consumption of paraffin wax (figure 9) was performed by gas chromatography, where the peak area of each *n*-alkane was determined for the samples collected at 0 hours (control) and also after 24, 48 and 120 hours (cells).







■ Docosane ■ Heneicosane ■ Tetracosane ■ Pentacosane ■ Tetratriacontane ■ Triacontane ■ Hexacosane

Figure 9 – Peak area of each *n*-alkane in paraffin consumed over time.

Figure 9 shows that the paraffin consumption was higher when the concentration that was placed on medium culture was higher, except on the concentration of 10%, where it was verified more paraffin in 48 hours than 24 hours or 120 hours. The reason for this was that suspension of paraffin is not totally well homogenised and the concentration present in medium culture could be more than expected. Therefore, experimental errors may have occurred when the paraffin suspension was placed in the culture media.

At 5% paraffin, almost the same quantity of paraffin was observed after 48 hours and 120 hours of growth, which can suggests mass transfer limitation between cells and particles of paraffin. Velankar *et al.* (1975) reported that when the cell surface is adjacent to the hydrocarbons, the transport of hydrocarbons to the cell surface is more quickly [172]. This concentration of paraffin can be considered relatively low and hydrocarbon surface area can be limiting, especially because *B. pumilus* grows in suspension and forms aggregates in MM. At a concentration of 10% paraffin, the same situation also happened.

The consumption of paraffin at a concentration of 20% was ca. 1.3 times faster than at 10%, if the analysis is done until 120 hours, because at the end of this time the peak area was similar for these two concentrations. This result suggests that the cell growth might be limited by the amount of substrate available.

However, the monitoring time at 10% and 20% paraffin, should had been extended to verify if the degradation of paraffin could be complete. In conclusion, *B. pumilus* can degrade paraffin, so it could be a potential candidate to do the biodegradation of long chain alkanes.

To assess the influence of paraffin on cell membrane during growth, the FA composition was analysed at 0 hours (control) and 24, 48 and 120 hours (cells).

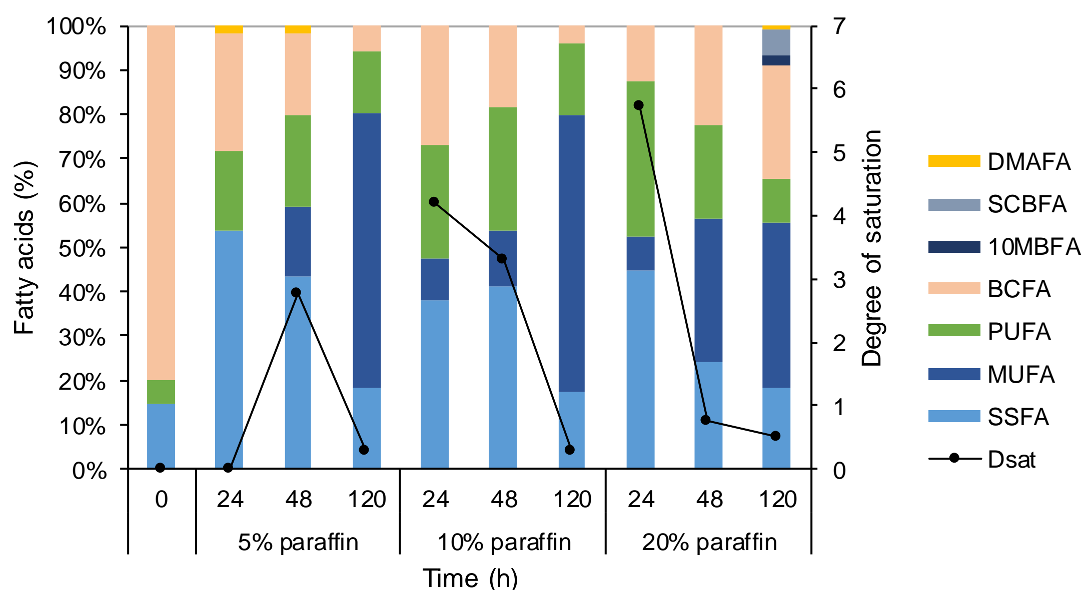


Figure 10 – Fatty acid composition of *B. pumilus* cells in the presence of different concentrations of paraffin after 24, 48 and 120 hours.

The FAs of cell membrane changed along time of growth. After 24 hours of growth, the cells produced more ca. 32.3% of SSFAs and ca. 28.0% of PUFAs for all concentrations of paraffin tested. Relatively to MUFAs, cells produced ca. 9.1% and 7.8% for the 10% and 20% concentrations of paraffin when comparing to the control (0 hours). From 24 hours to 120 hours, SSFAs decreased ca. 36.3, 21.5 and 18.0% for the paraffin concentrations of 5, 10 and 20%, respectively, and increased the MUFAs in ca. 59.5, 51.8 and 46.0% for the paraffin concentrations of 5, 10 and 20%, respectively. These results led to a decrease of degree of saturation, which means that the cell membrane became more flexible with time [173].

Furthermore, the PUFAs decreased ca. 4.3, 10.0 and 20.3% from 24 hours to 120 hours of growth for the paraffin concentrations of 5, 10 and 20%, respectively, especially the C18:3ω6c FA.

Regarding BCFAs, this class of FAs decreased ca. 21.3 and 23.2% until 120 hours of growth for 5% and 10% paraffin concentrations and increased ca. 24.7% from 24 hours until 120 hours in the paraffin concentration of 20%. The FAs that triggered these results was C15:0 iso and C15:0 anteiso. BCFAs affect the membrane fluidity due to the disruptive effect of the methyl group on acyl-chain packing, being that anteiso FAs promote a more fluid membrane [150].

After 120 hours of growth in paraffin concentration of 20%, 10MBFAs constituted ca. 3.1% of cell membrane, and SCBFA and DMAFA represented 8.1% and 1.5% of cell membrane, respectively.

3.2.5. Observation of paraffin degradation by *Bacillus pumilus* cells on SEM

To assess the interaction between cells of *B. pumilus* and paraffin as well the interaction between *R. erythropolis* DCL14 and paraffin, the cover slides where the cells grew were visualized by SEM. Before that, some photos of paraffin on a cover slide after seven days of growth were taken.

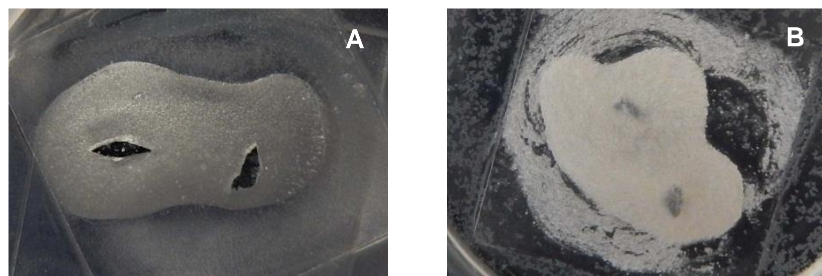


Figure 11 – Paraffin on a cover slide after seven days of growth: (A) *Bacillus pumilus* and (B) *Rhodococcus erythropolis* DCL14.

Both strains grew in 6-well plates, but they had different behaviours during growth. *B. pumilus* grew in suspension and formed aggregates, while R. *erythropolis* DCL14 formed a biofilm over paraffin.

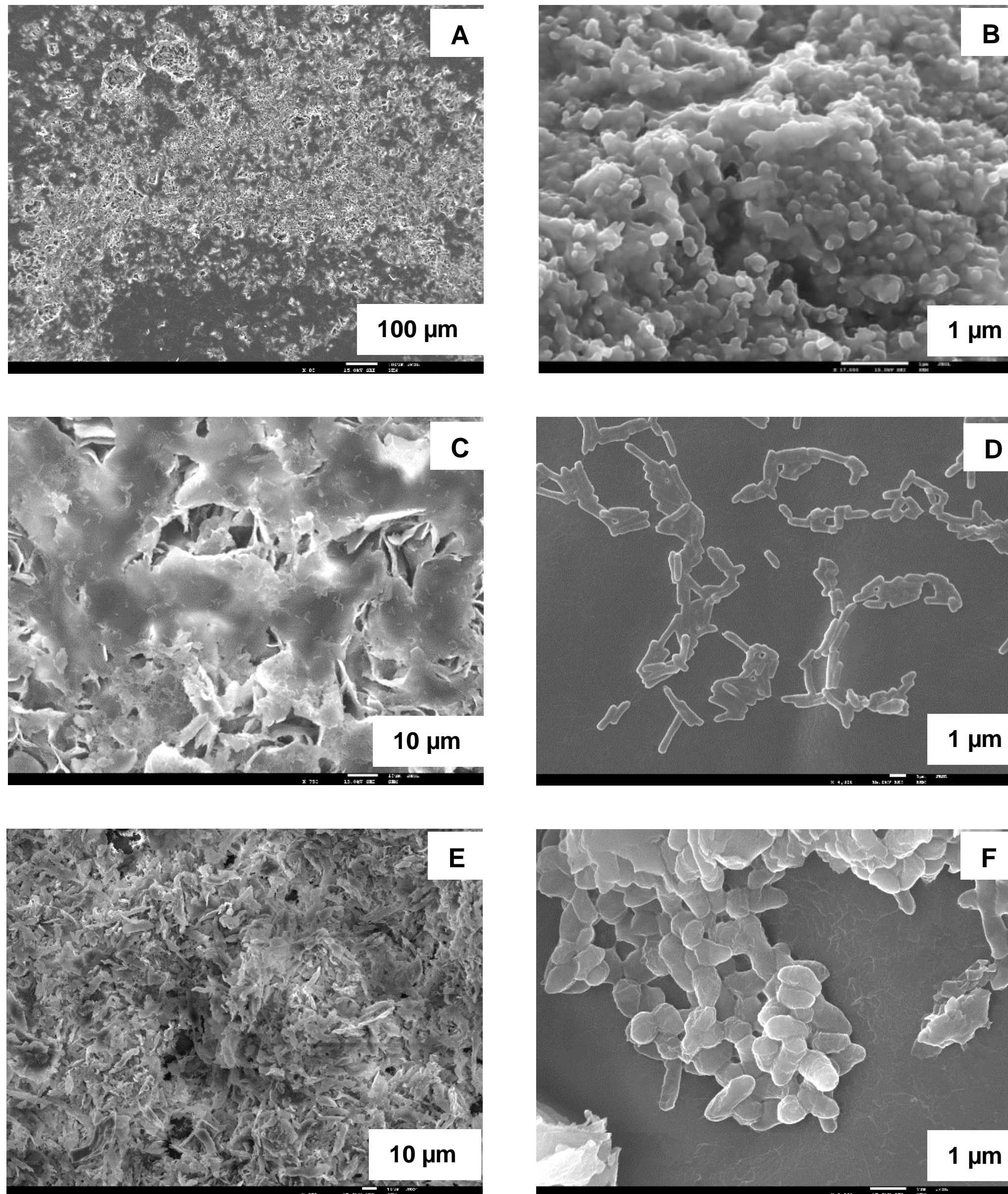


Figure 12 – SEM images of paraffin on a cover slip: Control (A, B), *B. pumilus* (C, D) and *R. erythropolis* DCL14 (E, F). Amplifications = 80x (A); 17,000x (B); 750x (C); 4,000x (D); 370x (E); 9,000x (F).

On figure 12, it was possible to observe the differences between the *B. pumilus* and *R. erythropolis* DCL14 growth on paraffin. Both *B. pumilus* and *R. erythropolis* DCL14 covered the paraffin surface (figures 12C and 12E, respectively), and formed a biofilm. However, the bacteria presented different behaviour on the surface of paraffin. *R. erythropolis* DCL14 degraded paraffin at higher rate than *B. pumilus* probably because of the scale-like structures and the size of paraffin particles observed in the figures 12C and 12E. Besides that, it was possible to observe the morphology of cells: *B. pumilus* were long-rod shaped cells (figure 12D) whilst *R. erythropolis* DCL14 were short-rod and spherical shaped cells (figure12F).

The strains over paraffin were also observed in an optical microscope with brightfield and fluorescent light.

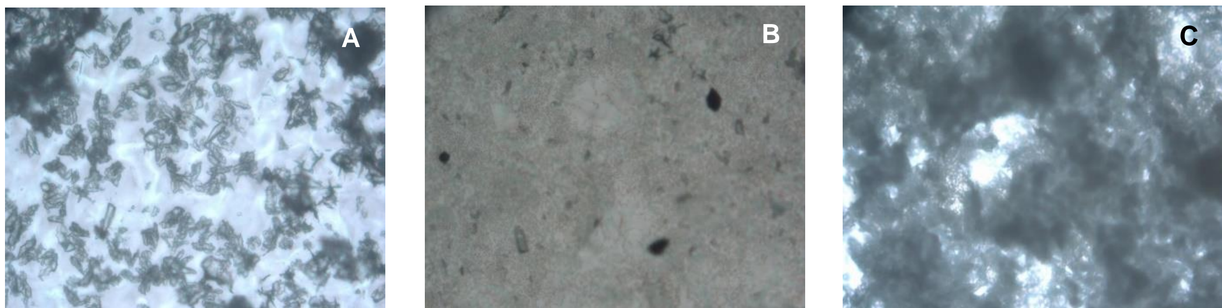


Figure 13 – Visualization of bacterial cells on the surface of paraffin under bright field: Control (A), *B. pumilus* (B) and *R. erythropolis* DCL14 (C). Amplification = 40x.

Under the bright field of microscope with an amplification of 40x, the structure of paraffin (figure 13A), the cells of *B. pumilus* covering the paraffin which was close to silicon pieces (figure 13B) and the cells of *R. erythropolys* DCL14 covering the whole paraffin surface (figure 13C) were observed.

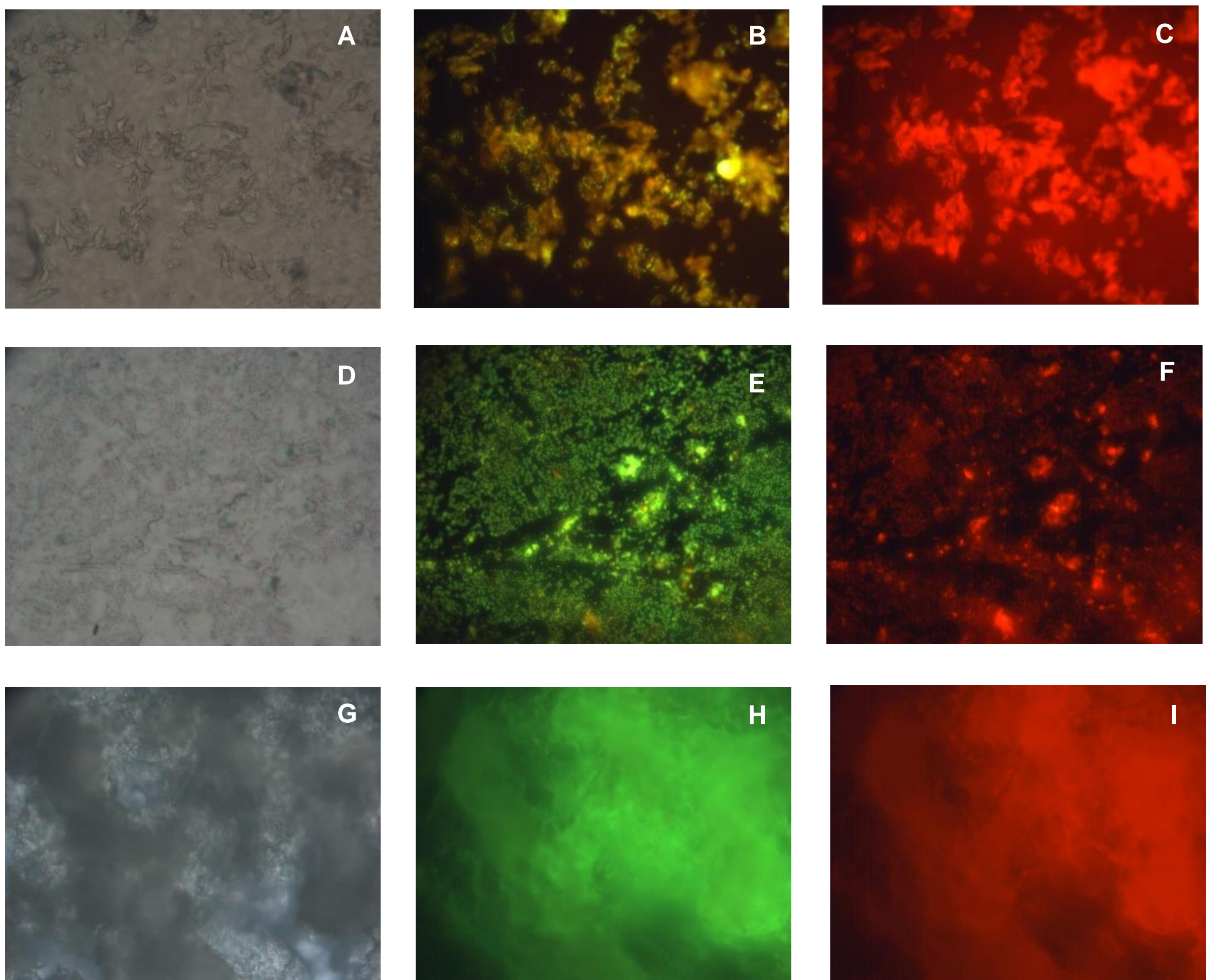


Figure 14 – Bacterial cells on the surface of paraffin observed by microscopy: Control (A, B, C), *B. pumilus* (D, E, F) and *R. erythropolis* DCL14 (G, H, I). (B, E, H) - cells were stained with the green fluorescent dye SYTO®9; (C, F, I) – lipids of the cells were stained with Nile red. Amplification = 40x.

Using fluorescence microscopy, the coverage of paraffin by cells of *B. pumilus* and *R. erythropolis* DCL14 was observed, through the staining with green fluorescent dye SYTO®9 and Nile red. In both strains, the viability was observed, because the fluorescent dyes stained the cells (figures 14E and 14H) and lipids (figures 14F and 14I), respectively. As previously noted, the *B. pumilus* did not form biofilm over the paraffin surface, while *R. erythropolis* DCL14 formed. So, with these results, it was possible to confirm that *B. pumilus* grew mainly in suspension and *R. erythropolis* DCL14 formed a biofilm over the paraffin surface.

3.2.6. Production of EPS using sugars as carbon source

Exopolymeric substances (EPS) produced by marine microorganisms, in diverse conditions have been sought and studied [103] due to their importance in several fields, like industry and bioremediation [102]. Some marine *Bacillus* sp., namely, *B. licheniformis* [111], *B. marinus* [174] and *B. thermoantarcticus* [99] were described as EPS producers. To assess the production of EPS by *B. pumilus*, this bacterium was grown in different carbon sources: glucose, galactose, arabinose, sucrose, raffinose, manose and xylose. The effect of carbon source in the production of biomass and EPS along time was evaluated (figures 15 and 16) and product/substrate ($Y_{EPS/S}$), product/biomass ($Y_{EPS/X}$) and biomass/substrate ($Y_{X/S}$) yields were determined (table 18). The potential of EPS to reduce the superficial tension of the supernatant was analysed by a tensiometer (table 19).

The growth of *B. pumilus* was monitored by measuring the absorbance for each carbon source. The growth curves showed that the exponential phase of each growth ended after ca. 20 h for all carbon sources, except for xylose, and after this time, the growth curves tended to stationary phase. In case of xylose, the end of stationary phase was verified after ca. 48 hours of growth.

The production of EPS occurred along time in all carbon sources tested. These compounds were quantified by dry weight.

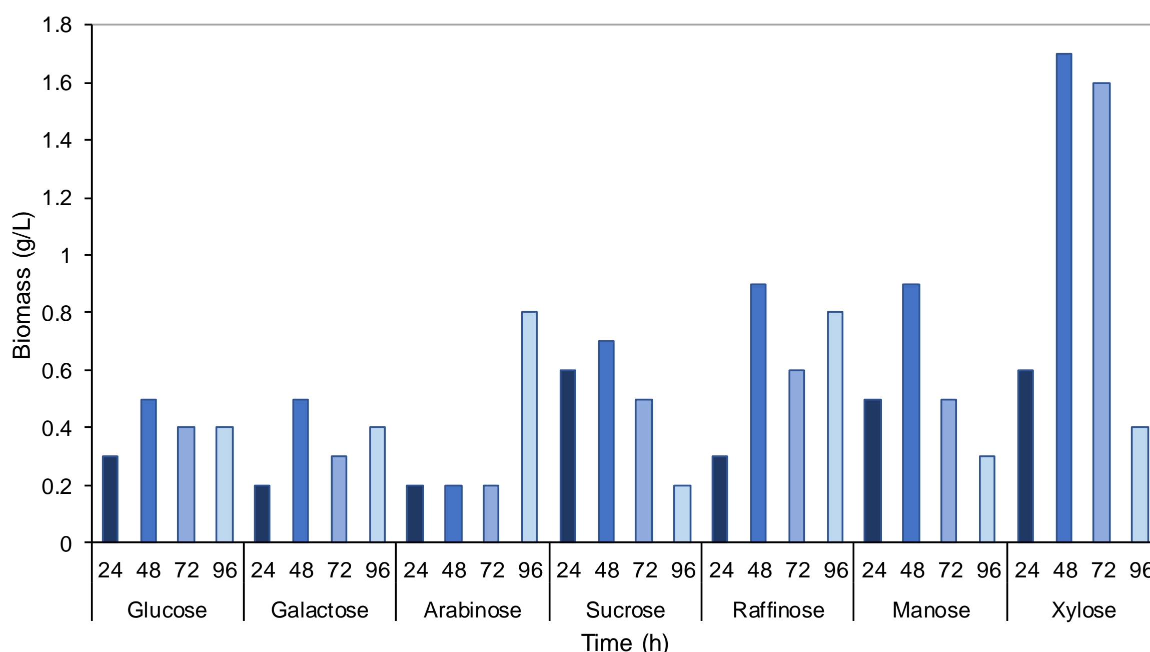


Figure 15 – Effect of carbon source on biomass dry weight of *B. pumilus* along time.

The production of biomass depended significantly of carbon source used, but in general reached a maximum concentration after 48 hours of growth.

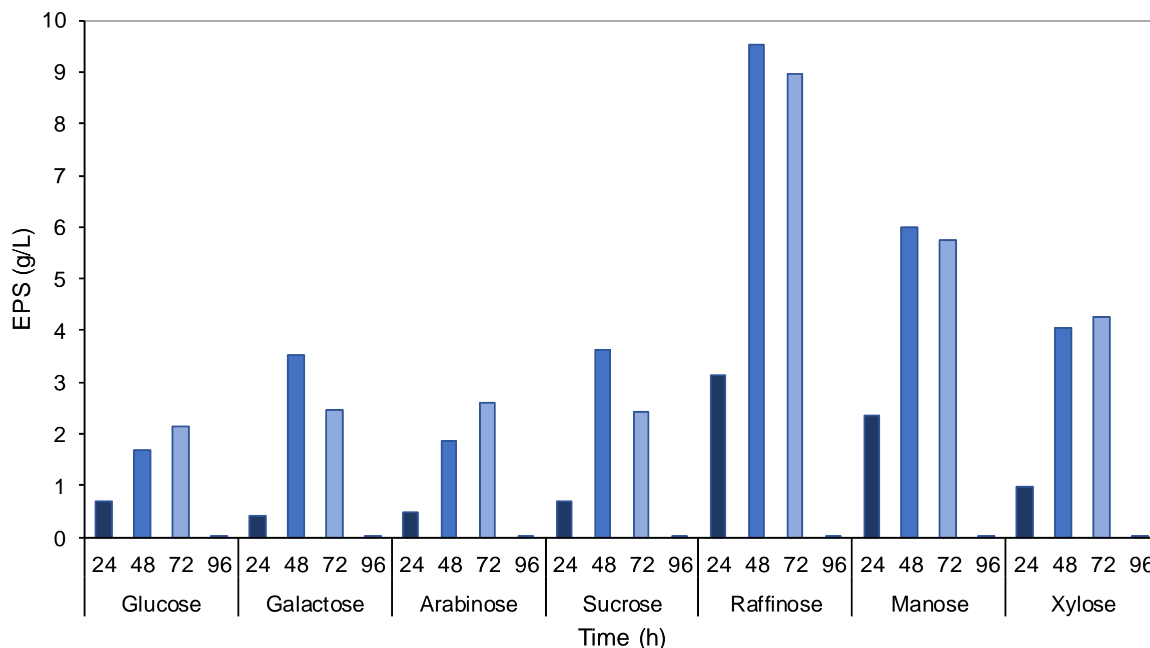


Figure 16 – Effect of carbon source on EPS production by *B. pumilus* along time.

The maximum production of EPS was registered between 48 hours and 72 hours of growth, depending on the carbon source. This result showed that the production of EPS occurred essentially in stationary phase. After 96 hours of growth, the production of EPS was lower than 0.1 g/L, which may suggest that in the absence of substrate, the cells consume the EPS produced by them. This time coincided with the stationary phase of growth or with the final of stationary phase depending on the carbon source used. The highest production of EPS (9.52 g/L) was noted when raffinose was used as carbon source and after 48 hours of growth. This value represented almost 5.6 more times higher than the lowest production registered that was for glucose (1.70 g/L).

On the literature, marine bacterium *Saccharophagus degradans* was reported to have EPS productions of 1.5 g/L for glucose, 0.75 g/L for galactose, 0.9 g/L for sucrose and 0.8 g/L for xylose after 48 hours of growth and using a concentration of 200 g/L of carbon source solution [175]. On the other hand, deep-sea bacterium *Zunongwangia profunda* SM-A8 showed the EPS production in presence of 30 g/L of carbon source, like glucose and sucrose [176]. The results obtained for EPS production was 1.3 g/L for glucose and 1.6 g/L for sucrose after 144 h of growth [176]. Comparing these values with the obtained in this study, *B. pumilus* produced higher amounts of EPS with lower concentration of carbon source.

Table 18 – Product and biomass formation yields obtained while growing B. *pumilus* in different carbon sources.

Carbon source	$Y_{EPS/S}$ (g/g) [a]			$Y_{EPS/X}$ (g/g) [b]			$Y_{X/S}$ (g/g) [c]		
	24 h	**48 h**	**96 h**	**24 h**	**48 h**	**72 h**	**24 h**	**48 h**	**72 h**
Glucose	0.07	0.17	0.21	2.33	3.40	5.35	0.03	0.05	0.04
Galactose	0.04	0.35	0.25	2.00	7.04	8.20	0.02	0.05	0.03
Arabinose	0.048	0.19	0.26	2.40	9.30	13.10	0.02	0.02	0.02
Sucrose	0.07	0.36	0.24	1.17	5.20	4.88	0.06	0.07	0.05
Raffinose	0.314	0.95	0.90	10.47	10.58	14.93	0.03	0.09	0.06
Mannose	0.236	0.60	0.58	4.72	6.64	11.52	0.05	0.09	0.05
Xylose	0.096	0.40	0.43	1.60	2.38	2.66	0.06	0.17	0.16

[a] $Y_{EPS/S}$ = g EPS produced/ g carbon source consumed.

[b] $Y_{EPS/X}$ = g EPS produced/ g biomass produced.

[c] $Y_{X/S}$ = g biomass produced/ g carbon source consumed.

Analysing the yields, the g of EPS produced per g of substrate consumed ($Y_{EPS/S}$) or per g of biomass ($Y_{EPS/X}$) were highest when using raffinose and were lowest for glucose and xylose, respectively. The obtained yield values for biomass production on substrate ($Y_{X/S}$) were similar for all carbon sources tested (around 0.06 g biomass/g substrate at 48 hours), except for xylose.

In other studies, some yields were described for the same carbon sources used in this work. One of them, was the study of biosynthesis of EPS by the marine bacterium *Saccharophagus degradans* that presented the same $Y_{EPS/S}$ for glucose (0.17 ± 0.06 g/g) and lower $Y_{EPS/S}$ for galactose, sucrose and xylose (0.10 ± 0.04 g/g, 0.09 ± 0.02 g/g and 0.12 ± 0.01 g/g, respectively) after 48 hours of growth [175]. Relatively to the $Y_{EPS/X}$, this work presented better results than the paper (in some cases 10 fold higher) for the same carbons sources, but worst $Y_{X/S}$ than paper (ca. 10 fold less).

Table 19 – The effect of different substrates on superficial tension of EPS produced by *B. pumilus*.

Supernatant	Superficial tension (mN/m)
Water	77.0
Glucose	48.9
Galactose	27.8
Arabinose	14.5
Sucrose	33.3
Raffinose	12.4
Mannose	24.7
Xylose	23.9

In this study, a decrease in superficial tension of cell free culture was observed for all carbon sources tested after 120 hours of culture. When comparing with the control, there are reductions in superficial tension of supernatant in ca. of 36.5% and 83.9% when cells used glucose and raffinose as carbon sources, respectively. Results also revealed that superficial tension reduction was greater with arabinose and raffinose. So, all carbon sources favoured the extracellular production of active surface agent by *B. pumilus*.

In the literature, it was reported a study with *Bacillus subtilis* HOB2 that produced EPS with the ability to reduce the superficial tension to 28 mN/m and 27 mN/m, using as carbon sources, glucose (10 g/L) and sucrose (10 g/L), respectively [177]. These results was registered after 48 hours of growth [177]. Other study with isolates from terrestrial and marine samples collected in areas contaminated with crude oil or its byproducts, presented cell free-cultures with a superficial tension between 37 and 42 mN/m after growth in 20 g/L of glucose for 120 hours [178]. With these results, it can be concluded that EPS produced by *B. pumilus* did not reduce the superficial tension as other EPS produced by other microorganisms when compared with the same carbon source. However, *B. pumilus* was able to reduce the superficial tension below the values described using another substrate. The study with *R. erythropolis* that grew on glucose as carbon source, exhibited a superficial tension of 67 mN/m on the supernatant [155].

3.3. Screening for lipases/ esterases activities

In the present study, lipase/ esterase producing bacteria were screened from Harbour of Ponta Delgada, Azores. This screening was done using agar plates with medium containing as principal carbon and energy source the substrate, tributyrin or tween 80. The formation of opaque zones around the colonies, denominated by halos, is an indicator of the hydrolytic activity of the of the enzymes present in the cells. The diameter of colony formed after 24 hours and after seven days was considered, since diameter is indicative of the activity of the enzymes present in the cells. Higher enzymatic activities should lead to bigger halos surrounding colony of bacteria.

After 24 hours of growth, visible halos surrounding colony of bacteria were observed. In tributyrin agar plate, the growth of the strains 6, 8, 11, 12, 15, 17, 19, 21, 26A and 26C AZ1-C16 were visualized, but halos were bigger and more visible in 12, 19, 21 and 26A AZ1-C16 bacteria (figure 17). In case of tween 80 agar plate, the growths of 2, 8, 14, 17, 18 and 19 AZ1-C16 bacteria were observed but the halos were not visible. After seven days of growth, the highest lipolytic activities in tributyrin agar plate were observed for 8, 12, 15 and 26C AZ1-C16 bacteria, while for tween 80 agar plate, were observed for the 2, 18 and 19 AZ1-C16 bacteria.

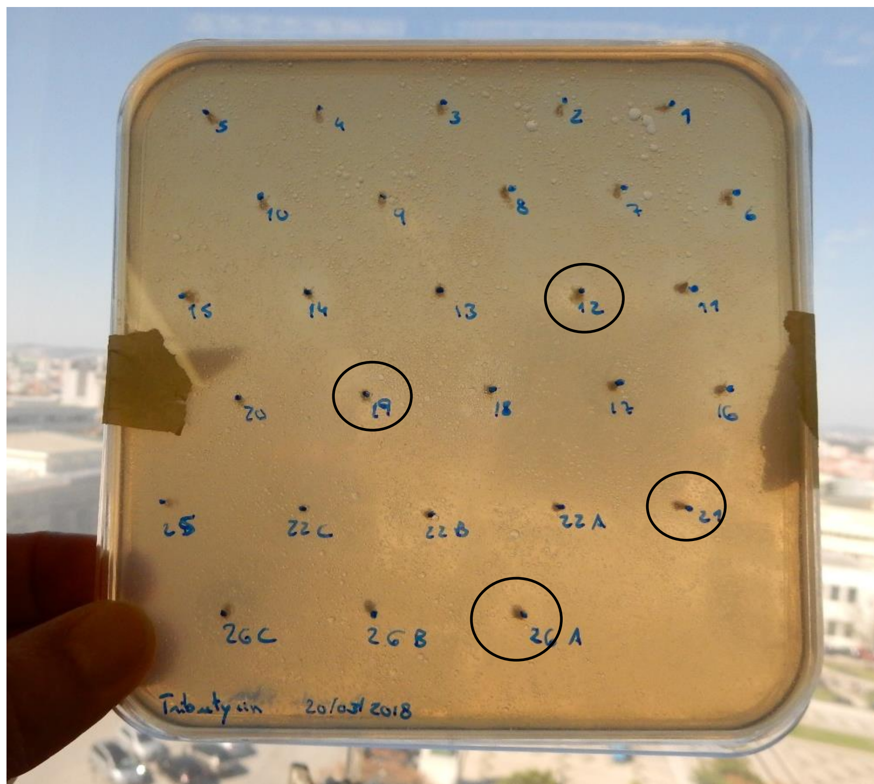


Figure 17 – Visible halos surrounding colonies of bacteria after 24 hours of growth on a tributyrin agar plate.

For further studies, the strain 12 AZ1-C16 was selected because of the growth that this bacterium presented in tributyrin agar plates and the halo surrounding this colony. Besides that, this isolate was identified by Sherlock® MIS (*vide* 2.1) as *Micrococcus luteus*.

3.4. Exploring the potential of *Micrococcus luteus*

3.4.1. Analysis of enzymatic activity of the supernatant of cultures grown in different culture media

Micrococcus luteus from Ponta Delgada harbour, Azores, was cultivated in different media cultures in order to do an evaluation of the presence of lipases/esterases in the supernatant and to assess which medium induced the production of larger amounts of extracellular lipases/esterases activities. The activity of free lipases/esterases was determined spectrophotometrically using *p*-NPB as substrate [158].

Table 20 – Enzymatic activity of extracellular lipase/esterase of *M. luteus* grown on different culture media after 24 hours of growth.

Medium	Lipase/esterase activity (U/mL)
MH	0.06 ± 0.004
Thio	0.03 ± 0.005
MB	0.03 ± 0.006
TSB	0.04 ± 0.001

In this study, the supernatants of all culture medium of *M. luteus* presented extracellular lipase/esterase activity. The highest enzymatic activity was verified in MH culture medium, with 0.06 U/mL. A study carried out with strains of *Halobacteria* from an Algerian culture displayed diverse lipolytic activities with *p*-NPB, being that the highest lipolytic activity obtained was 0.02 U/mL and the lowest was 0.0018 U/mL when this strain grew in Gibbons medium [179]. In other study, halophilic archaeal isolates that grew in SG medium presented lipase activities between 0.02 and 0.09 U/mL depending on isolate and pH of medium [180]. Other study reported that lipases found in strain LAMA 582 (*Bacillus aerophilus*) from deep-sea have extracellular activities of ca. 0.03 and 0.04 U/mL when the strain grew in marine broth 2216 [181]. So, the extracellular activity achieved with *M. luteus* is in the same range than other microorganism studied.

3.4.2. Production of lipases/esterases in presence and absence of tributyrin

To assess if the extracellular lipases/esterases activity could be induced by tributyrin, *M. luteus* was cultivated in MH with and without tributyrin. MH medium was chosen to this study, because in the previous point (3.4.1.), the extracellular lipase activity was higher in this medium.

Table 21 – Enzymatic activity of extracellular lipase/esterase of *M. luteus* grown on MH media with and without tributyrin after 24 hours of growth.

Medium	Lipase/esterase activity (U/mL)
MH with tributyrin	0.09 ± 0.01
MH without tributyrin	0.06 ± 0.01

In the presence of tributyrin, *M. luteus* increase the extracellular lipase/esterase activity. In literature, it was reported that the extracellular lipase from *Pseudomonas aeruginosa* KM110 from wastewater of an oil processing plant located in Tehran, presented an enzymatic activity in presence of tributyrin of ca. 0.06 U/mL [182]. However, this strain displayed greater results when using other carbons sources, like olive oil (0.46 U/mL) [182]. These results suggest that extracellular lipase/esterase activity of *M. luteus* may be improved using other substrates.

In this study, was also performed the Bradford test, where 0.03 mg of total protein per mL of extract and 0.01 mg of total per mL of extract was obtained for medium with tributyrin and without tributyrin, respectively. However, these values included the enzymes produced by *M. luteus* and the proteins of MH broth, so is not possible to know exactly the enzymatic lipase activity produced per mL of extract.

3.5. Tolerance of marine bacteria from Aveiros-Algarve to salt

AV-SALT bacteria were cultivated under different salinity (5, 35, 50, 100 and 150 g/L) in TSB medium, to assess the influence of different NaCl concentrations on bacteria growth and cell membrane. The growth was followed by optical density measurements and the adaptation of cell membrane was evaluated by analysis of fatty acid profile.

3.5.1. Effect of salinity on cell growth

The effect of salinity on the cell growth and growth rate is shown in figures 18-22, for each bacterium from AV-SALT collection.

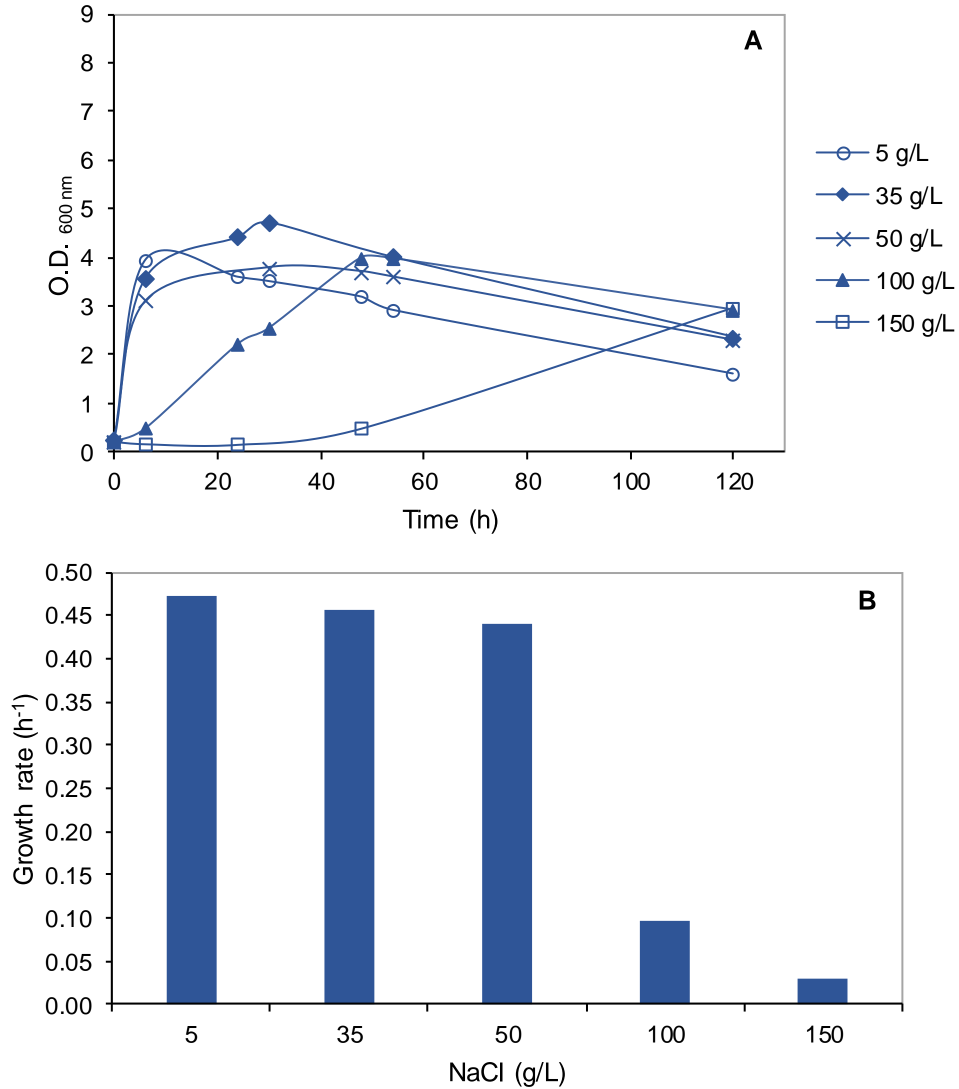


Figure 18 – Growth curve of isolate 32 AV-SALT in presence of 5, 35, 50, 100 and 150 g/L of NaCl (**A**). Effect of different NaCl concentrations on growth rates (**B**).

In the study with the bacterium 32 AV-SALT, the maximum growth rate (0.47 h^{-1}) was verified for the lowest concentration of NaCl. However, the growth rate just decreased when the salt concentration reached 100 g/L, which means that this bacterium is halotolerant to higher concentrations of salt.

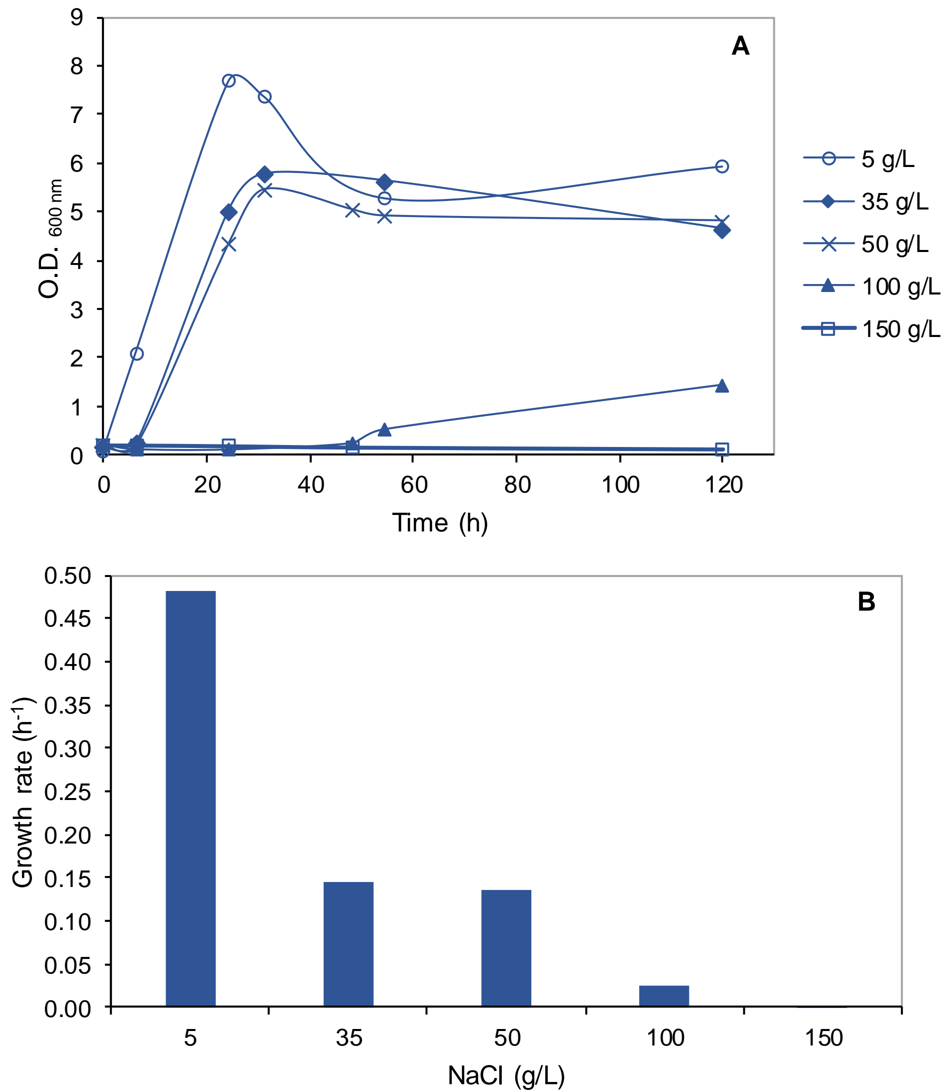


Figure 19 – Growth curve of isolate 40 AV-SALT in presence of 5, 35, 50, 100 and 150 g/L of NaCl (**A**). Effect of different NaCl concentrations on growth rates (**B**).

The study with the bacterium 40 AV-SALT showed that this microorganism presented the highest growth rate for the lowest concentration of sodium chloride (5 g/L), and after that, the growth rate decreased around 70% for concentrations of 35 g/L and 50 g/L of salt. From the concentration of 50 g/L to 100 g/L, the growth rate decreased 82% and for concentration of 150 g/L, the bacterium could not grow. So, with these results, it can be concluded, that bacteria 40 AV-SALT are not halotolerant for high concentrations of salt.

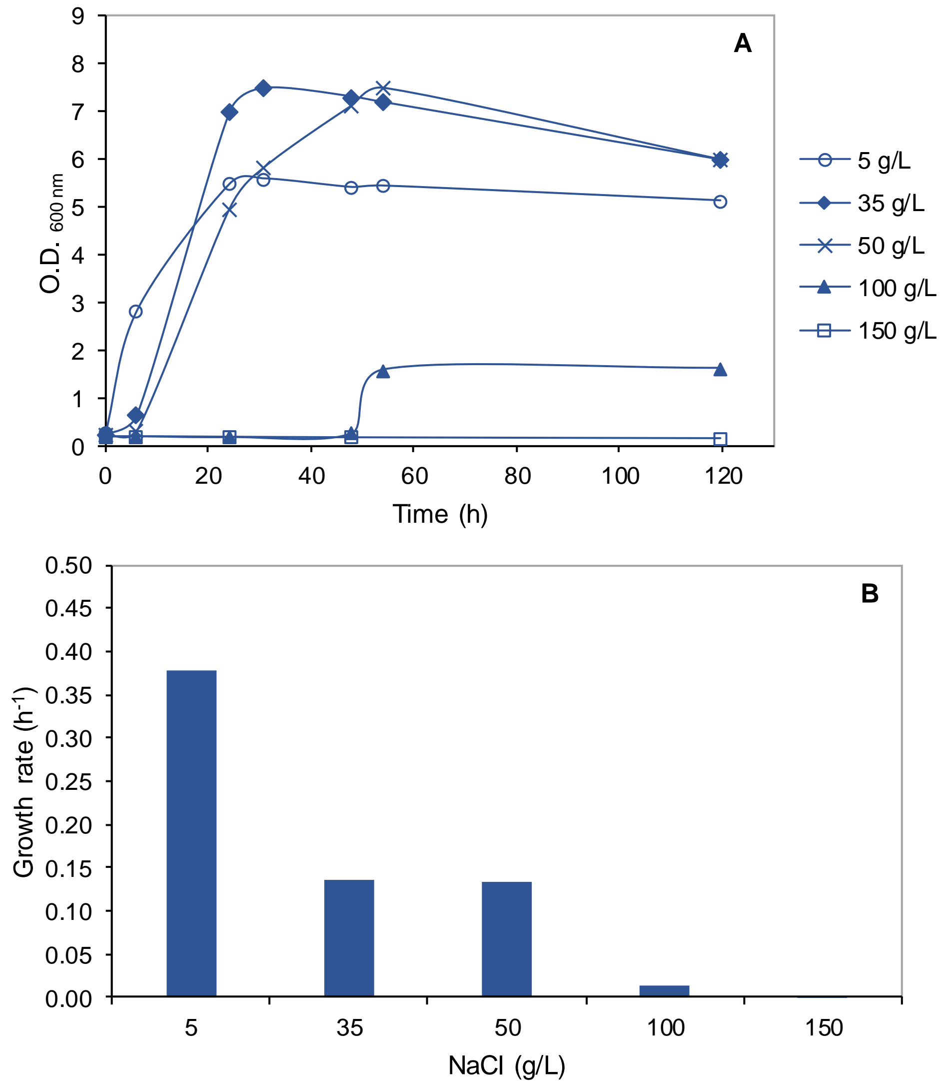


Figure 20 – Growth curve of isolate 41 AV-SALT in presence of 5, 35, 50, 100 and 150 g/L of NaCl (**A**). Effect of different NaCl concentrations on growth rates (**B**).

In the case of 41 AV-SALT, this bacterium just could growth until a concentration of 100 g/L of sodium chloride. The results showed a higher growth rate for the lowest concentration of salt (0.38 h^{-1}), that decreased ca. 64% for the 35 g/L and 50 g/L of salt. Therefore, it can be concluded that this bacterium is not halotolerant for high concentrations of salt.

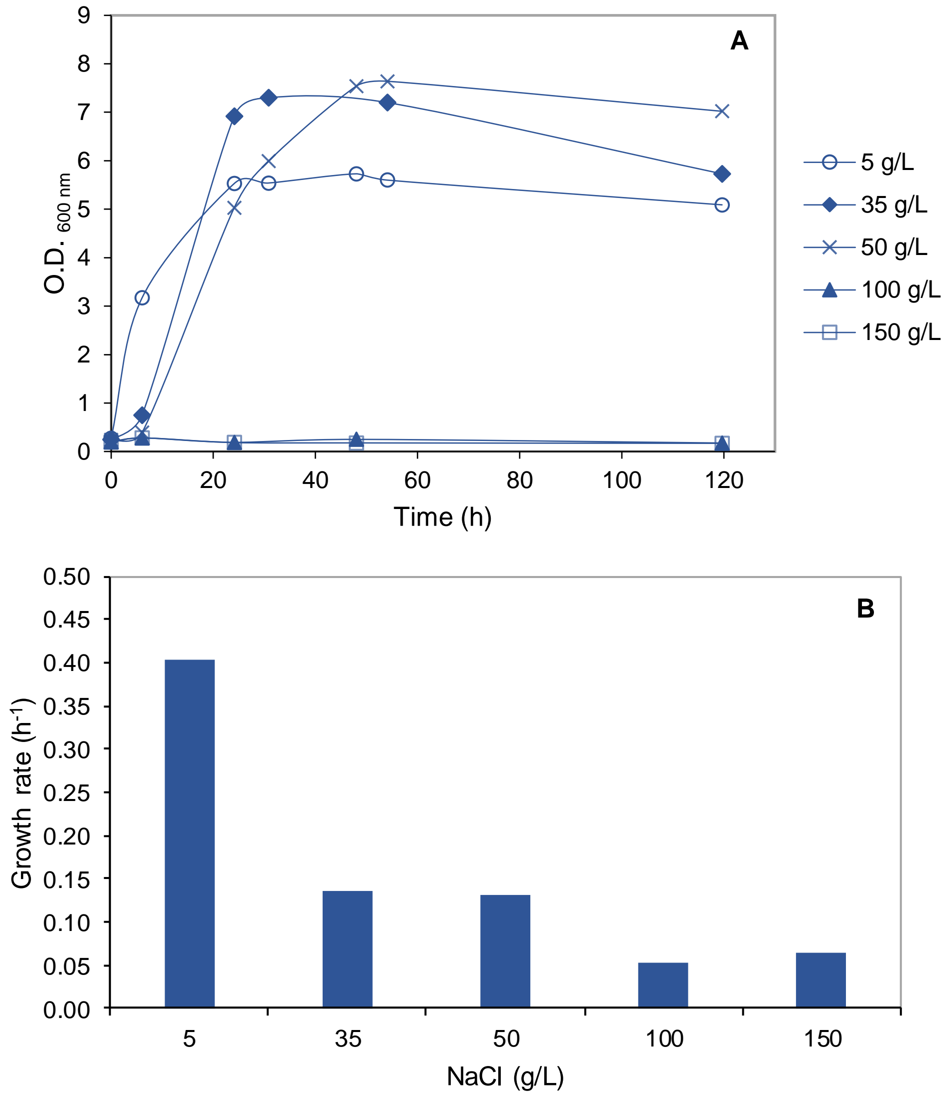


Figure 21 – Growth curve of isolate 42 AV-SALT in presence of 5, 35, 50, 100 and 150 g/L of NaCl (**A**). Effect of different NaCl concentrations on growth rates (**B**).

The bacterium 42 AV-SALT presented growths for all concentrations of salt tested, especially for the lowest concentrations of salt. The higher growth rate was obtained for 5 g/L of salt, being that is value decreased to 0.14 h^{-1} and 0.13 h^{-1} for the salt concentrations of 35 g/L and 50 g/L, respectively. After that, growth rate decreased ca. 59% to the lowest concentrations. Therefore, 42 AV-SALT can be considered a halotolerant bacterium.

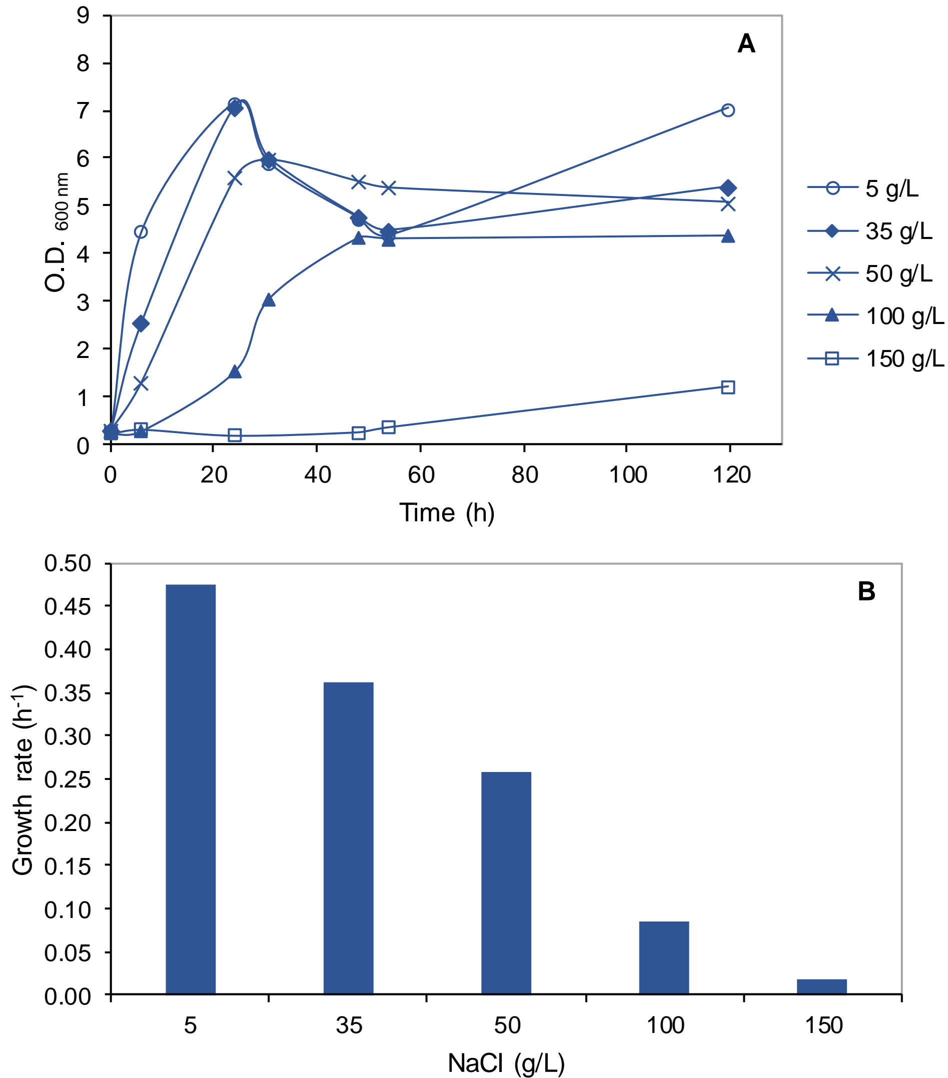


Figure 22 – Growth curve of isolate 53 AV-SALT in presence of 5, 35, 50, 100 and 150 g/L of NaCl (**A**). Effect of different NaCl concentrations on growth rates (**B**).

The bacterium 53 AV-SALT showed the highest growth rate to the lowest concentration of salt, with 0.47 h^{-1}. From the 5 g/L to 150 g/L of salt, growth rate was decreasing ca. 96%. However, this bacterium can be considered halotolerant, because can grow in higher concentrations of salt.

Aveiros beach bacteria showed a maximum growth rate in the presence of 5 g/L of sodium chloride due to the pre-inoculum of all bacteria have grown in the presence of 5 g/L of this salt. Then the adaptation to the new medium was easier for this concentration when compared with others. However, cells were able to grow in the presence of higher concentrations of sodium chloride, like 35 g/L, 50 g/L and 100 g/L and in some cases, cells were able to grow in 150 g/L.

In concentrations of 35 g/L and 50 g/L, almost all bacteria showed similar growth rates among themselves, followed by a large decrease when salinity was increased to concentrations of 100 g/L and 150 g/L because in these last two concentrations, the cells had more difficulty of adapting.

The strains 32, 42 and 53 AV-SALT showed the highest salinity tolerance and grown even up to a concentration of 150 g/L. The other strains could not grow in 150 g/L. In addition, the bacterium 41 AV-SALT presented the lowest salinity tolerance because could not grow in 150 g/L of salt and present the lowest growth rates when compared with other bacteria. In fact, all bacteria demonstrated to be able to grow better in the salt concentration of 5 g/L, which is lower than the average concentration of salt in sea water (around 35 g/L, [183]). This result suggest that the bacteria present in this study may not be halophilic.

3.5.2. Effect of salinity on cell membrane

To investigate the adaptative response of the cells to different concentrations of sodium chloride, the fatty acid compositions were analysed, and UI was calculated (figure 23 – figure 27).

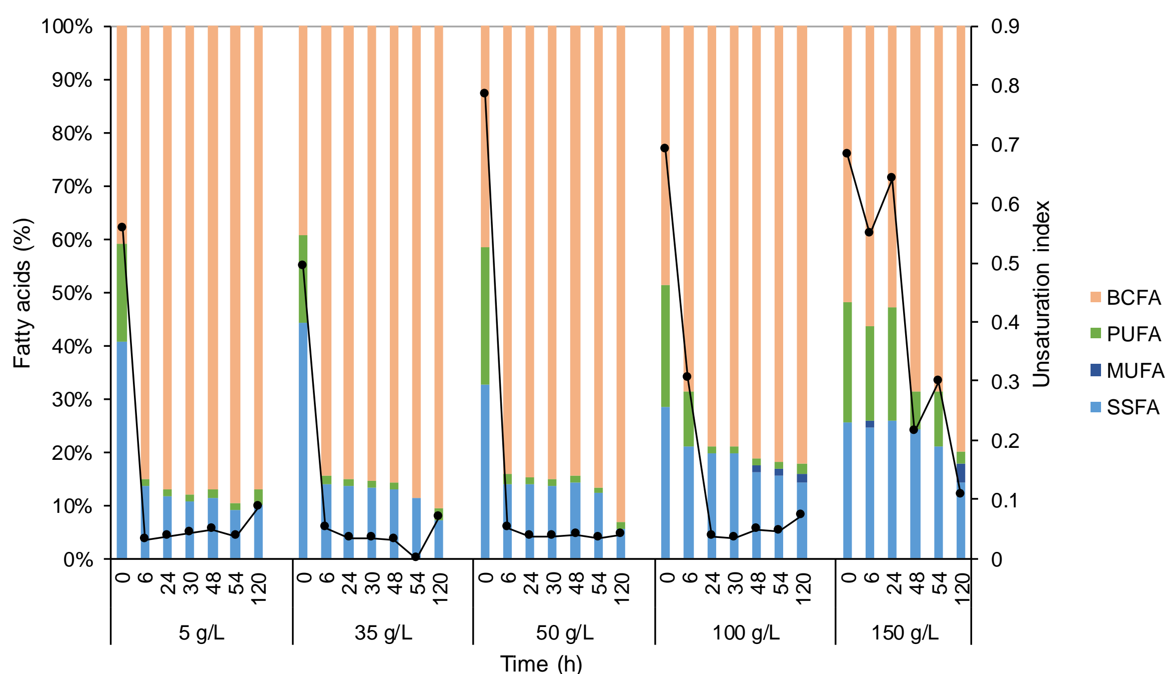


Figure 23 – Fatty acid composition of 32 AV-SALT cells exposed to different concentrations of NaCl (bars) and corresponding calculated unsaturation index (line).

The concentrations of NaCl that allowed the highest growth rates of 32 AV-SALT cells (5, 35 and 50 g/L), induced a lower extent of changes in cell membrane than higher concentrations (100 and 150 g/L). The higher the salt concentration, the lower the percentage of BCFAs produced by 32 AV-SALT cells, such as C15:0 anteiso. This percentage decreased from ca. 85.3% in 5 g/L to ca.64.3% in 150 g/L of NaCl.

The sodium chloride concentrations tested induced modifications in the fatty acid profile after ca. 6 hours of growth, where in the lowest concentrations of salt, cell membrane stabilized at the end of this time. For the highest concentrations of salt, fatty acid profile changed until 24 hours of growth in case of 100 g/L, but in case of concentration of 150 g/L, cell membrane presented changes until 120 hours. In the presence of 100 g/L NaCl, an increase of SSFAs and MUFAs, especially of C18:0 and C18:1ω9c,

respectively, were observed. When compared with the lowest concentrations of NaCl (5, 35 and 50 g/L of NaCl), SSFAs increased from ca. 11.5% to 17.2% and MUFAs decreased from ca. 0% to 0.7%, respectively. The SSFAs, as C12:0 and C16:1ω9c, also increased from ca. 11.5% in the lowest concentrations of NaCl to ca. 21.7% in 150 g/L of NaCl. An increase of SSFAs and decrease of BCFAs, from 50 g/L to 150 g/L, means a reduction of membrane fluidity and permeability [150]. These changes occur because cell membrane has the ability to prevent the release of solutes in the presence of high salinity in order to control the osmotic pressure [184] and they were observed until the end of exponential phase (*vide* 3.5.1.) Other group of FAs that changed along time was PUFAs. These FAs increased in cell membrane from ca. 17.5% at 0 hours for the salt concentrations of 5 and 35 g/L to ca. 24% for the remaining concentrations and for the same time. However, for the concentrations 100 and 150 g/L of salt, PUFAs did not decrease as in the lowest concentrations of NaCl. The presence of PUFAs in great amounts at higher salinities probably allowed the maintenance of cytoplasmatic membrane fluidity [160] and represented one of the most important adaptations under salt stress.

Furthermore, UI decreased after 6 hours of growth in presence of 5, 35 and 50 g/L of salt to ca. 0.3 and maintained this value in the remaining time of study. For the higher concentrations of salt, the stabilization of UI was observed after 24 hours of growth for 100 g/L, but in the concentration of 150 g/L, UI decreased until 120 hours. These results also showed a slower reduction of UI for higher concentrations of salt, which indicates that cell membrane had more difficulties to adapt and to overcome the problem, increased unsaturated FAs [160].

In the literature, there are some reported cases of bacteria that adapted their cell membrane with the increase of salinity, such as genus *Micrococcus* from Antarctica, which increased the amount of long FAs while decreased the iso chain and increased straight chain fatty acids [150] and *Rhodococcus erythropolis* which synthetized PUFAs, being this response is very uncommon for mesophilic bacteria [160].

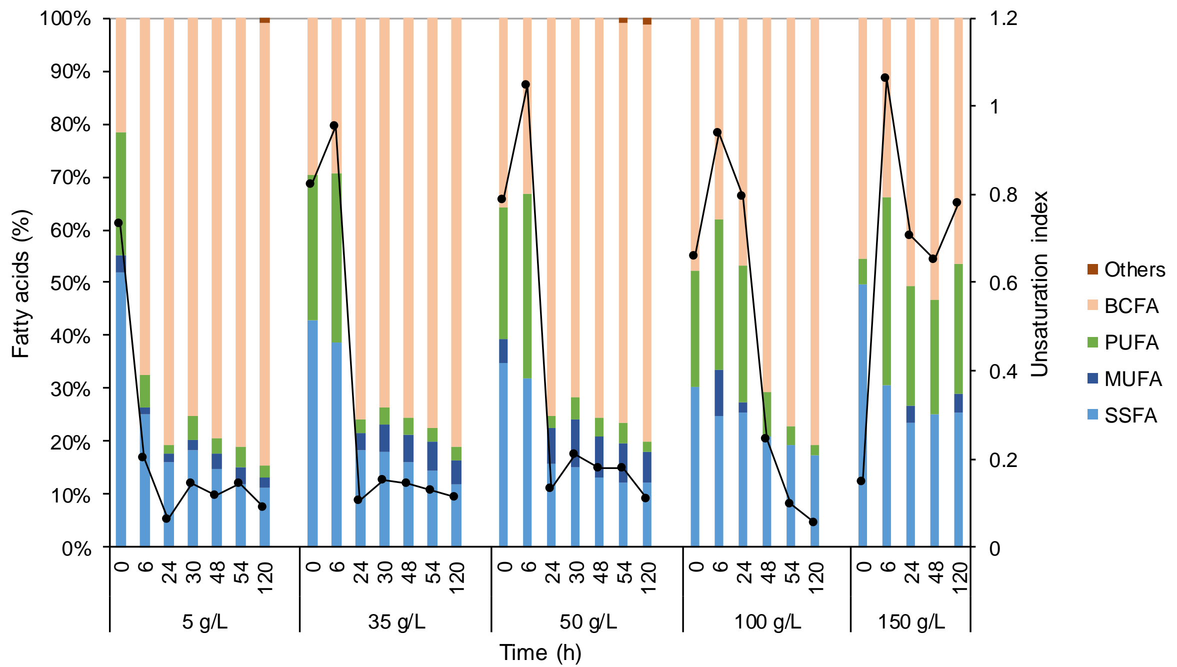


Figure 24 – Fatty acid composition of 40 AV-SALT cells exposed to different concentrations of NaCl (bars) and corresponding calculated unsaturation index (line).

In 40 AV-SALT cells, the same behaviour of 32 AV-SALT was observed, but the stabilization of FA composition after 6 hours of growth did not happen for the salt concentrations of 35 and 50 g/L. For these concentrations, the amount of PUFAs was similar relatively with 0 hours, and after this time decreased ca. 31.1%. In the salt concentration of 100 g/L, cell membrane presented a high amount of PUFAs up to 24 hours, and in the salt concentration of 150 g/L, this amount appeared until 120 hours. Besides that, UI decreased with time for all concentrations of salt tested, but in 150 g/L, did not stabilize for any hour. This result is according with data shown in the previous point, because 40 AV-SALT did not grow in 150 g/L due to a possible inadaptation to this concentration. So, the 40 AV-SALT cells presented more differences of fatty acid composition along the increase of salt concentration and the time, which means that this bacterium needed more time to adapt to higher salinities and can be considered halotolerant until 100 g/L of chloride sodium. Others FAs occurred in the cell membrane for some concentrations at 54 hours and 120 hours. This suggests that cells were old because after that time of growth, the cells were in the stationary phase of growth [185].

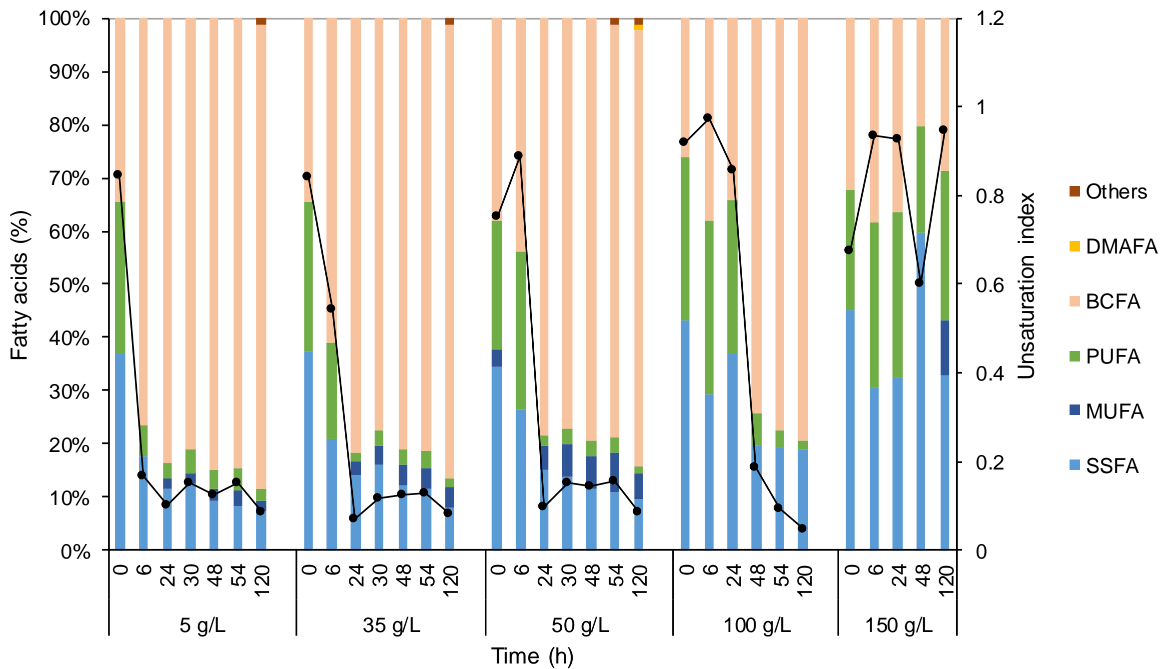


Figure 25 – Fatty acid composition of 41 AV-SALT cells exposed to different concentrations of NaCl (bars) and corresponding calculated unsaturation index (line).

The results of bacterium 41 AV-SALT showed similar changes in fatty acid composition along time and salinity tested when compared with bacterium 40 AV-SALT.

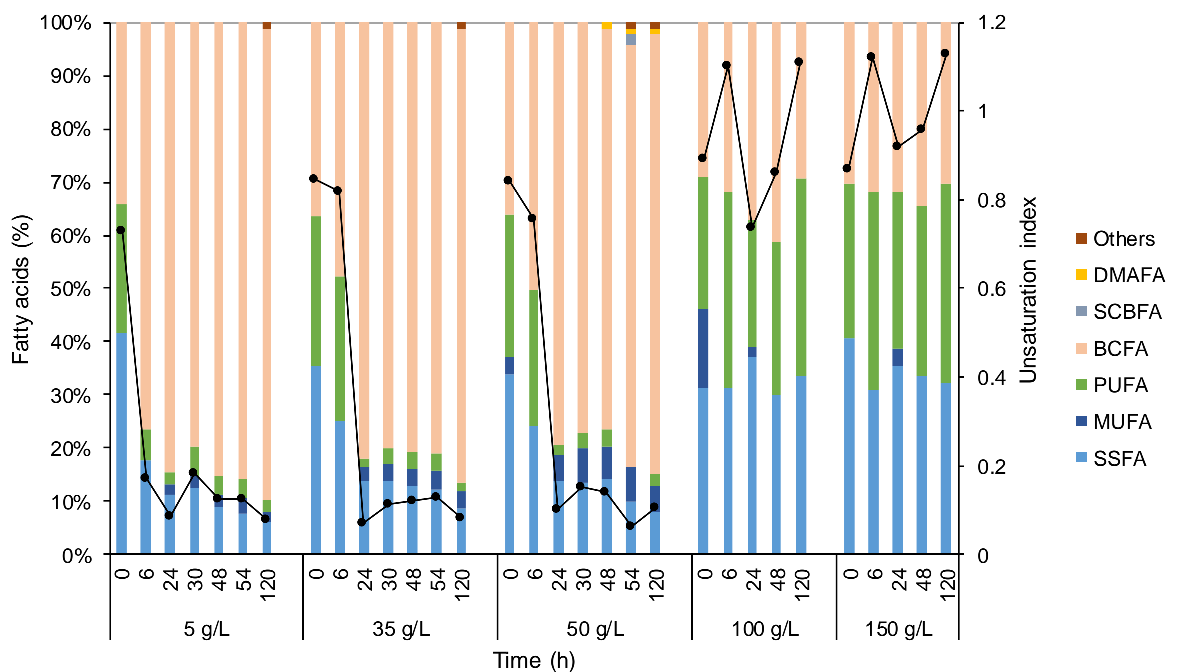


Figure 26 – Fatty acid composition of 42 AV-SALT cells exposed to different concentrations of NaCl (bars) and corresponding calculated unsaturation index (line).

In the study with the bacterium 42 AV-SALT, for the lowest concentrations of salt (until 50 g/L), cell membrane presented the same behaviour when compared to 40 AV-SALT and 41 AV-SALT bacteria. For the highest concentrations of salt (100 g/L and 150 g/L), cell membrane had a big percentage of

PUFAs (ca. 30%), which indicates that did not occur adaptation of cells for this concentration, although cells had grown, and the UI is unstable over time.

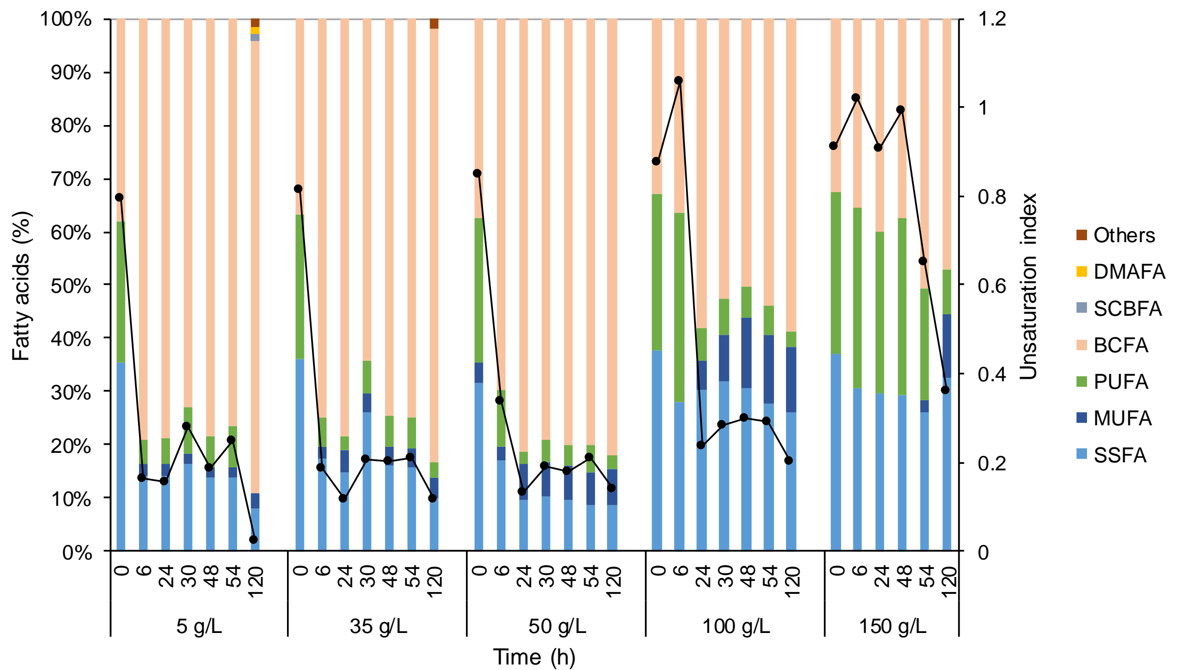


Figure 27 – Fatty acid composition of 53 AV-SALT cells exposed to different concentrations of NaCl (bars) and corresponding calculated unsaturation index (line).

In the study with bacterium 53 AV-SALT, the results were similar to bacterium 32 AV-SALT, except, that these cells produced more MUFAs and PUFAs, especially in higher concentrations. Other FAs occurred in the cell membrane at 120 hours for the concentrations of 5 and 35 g/L due the ageing of the cells. These bacteria can be considered halotolerant since they can grow at 100 g/L.

4. Conclusions and future work

The screening methods allowed the selection of two marine strains from the harbour of Ponta Delgada, Azores, with suitable properties to produce compounds to be used in biotechnological processes: the strains #3 AZ1-C16 and #12 AZ1-C16, previously identified as *B. pumilus* as *M. luteus*, respectively.

B. pumilus presented a growth rate of 0.53 h^{-1} and a duplication time of ca. 1.31 h when grown in Thio medium. The strain was also able to grow in media with less nutrients as mineral medium. In the presence of mineral medium with 35 g/L of NaCl and 0.25% of *n*-hexadecane, *B. pumilus* produced a biosurfactant with the ability to reduce the superficial tension to 21.3 mN/m.

B. pumilus also produced EPS using sugars as carbon sources. These EPS presented the ability to reduce the superficial tension (14.5-48.9 mN/m), suggesting they could be applied in biotechnological applications of a day-to-day life, such as, in cosmetic and food industry.

The biodegradation of *n*-alkanes and paraffin wax could be also performed by *B. pumilus*. The results showed that this marine microorganism can degrade hydrocarbon compounds, especially *n*-alkanes with long carbon chain (C12-C17) and paraffin at concentrations between 5 and 20% (v/v) in mineral medium. Furthermore, *B. pumilus* adapted its cell membrane to the composition of the medium, by changing the FAs, especially the MUFAs, PUFAs and BCFAs. In the study with *n*-alkanes, the MUFAs increased when the carbon chain of alkane had C12-C17, which led to a decrease of degree of saturation and therefore, to an increase of the fluidity of the membrane. The adaptation of the cell membrane in the presence of paraffin wax was similar to that presented in the study with *n*-alkanes, but the changes were observed over time. By SEM, it was observed that cells of *B. pumilus* and *R. erythropolis* covered the paraffin surface during the growth.

Since *B. pumilus* could degrade hydrocarbons, this strain could be applied in processes of bioremediation, such as, of crude oil.

Relatively to *M. luteus*, this microorganism showed extracellular lipase activity in different media, but in larger quantities in MH broth. This lipase activity can be enhanced by the addition of tributyrin to the medium culture that induced the enzymatic activity of lipases from 0.06 U/mL to 0.09 U/mL.

In the study with AV-SALT strains, from Albufeira – Algarve, all marine bacteria showed to be halotolerant until 100 g/L, although with growth rates less than 0.05 h^{-1} for strains 40 and 41 AV-SALT. The bacteria 32, 42 and 53 AV-SALT even grew in the presence of 150 g/L of NaCl. These bacterial strains could grow in high concentrations of salt due to adaptations of their cell membrane. Moreover, these bacteria may not be strictly halophilic because the cells grew better in the lowest concentrations of salt, especially in the concentration of 5 g/L.

In future works, the marine bacteria from harbour of Ponta Delgada as well as those from Aveiros beach should be identified by 16 sRNA sequencing to confirm the results obtained by the Sherlock® Microbial ID System and to identify other isolates which could not be identified.

The biosurfactant produced by *B. pumilus* should be characterized in order to understand the potential applications of these compounds, and lipase production by strain *M. luteus* can be enhanced in further studies, by optimization of the conditions of culture.

The study of degradation of paraffin should be extended in order to monitor the consumption over time (sampling in shorter intervals of time) and therefore, determine the degradation rate of paraffin in the tested strains.

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Appendix

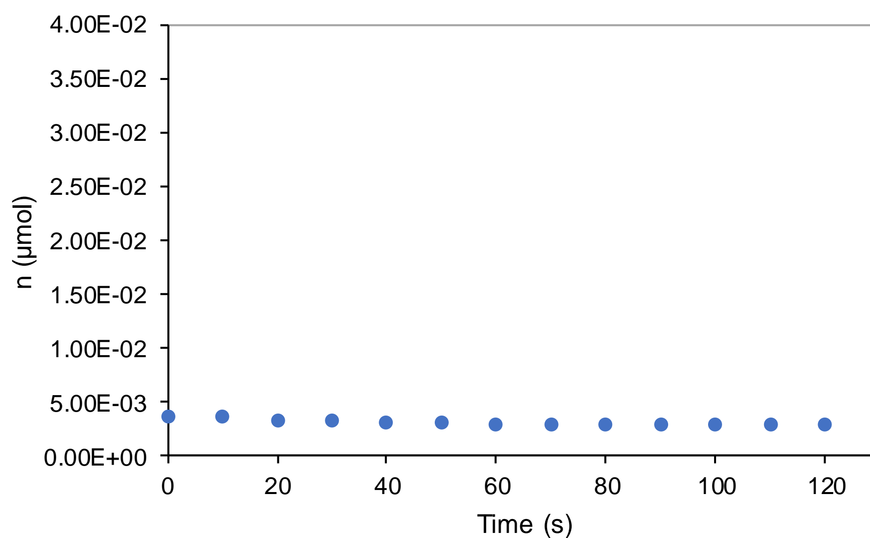

Figure A. 1 – Chemical reaction control of *p*-NPB test.

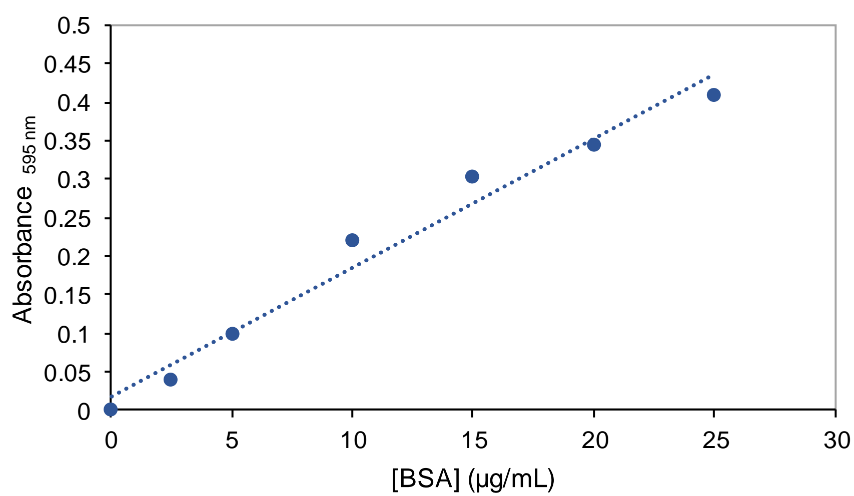

Figure A. 2 – Calibration line of BSA Bradford test: Abs = 0.0168 [BSA] + 0.0167.