TÉCNICO LISBOA

New approach for marine oils spill responses using mannosylerythritol lipids and their physicochemical characterization

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Thesis to obtain the Master of Science Degree in

Chemical Engineering

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May 2019

I declare that this document is an original work of my own authorship and that it fulfills all the requirements of the Code of Conduct and Good Practices of the Universidade de Lisboa.

Acknowledgments

I would like to thank my advisor, Dr. Nuno Faria, for the support and attention provided throughout this thesis. Also, I want to thank Prof. Frederico Ferreira for his guidance and knowledge.

I would also like to express my gratitude to all of the IBB members for their help, especially Rita Valente for the important assistance at the beginning of my research, Petar Keković for all the support and helpful ideas as well as Dona Rosa for all the help and all-around niceness.

I would like to thank my family and friends for the support and motivation.

Finally, I would like to thank Ricardo Neves without whom none of this would be possible, as well as my constant source of happiness, Monsieur Jacquês.

This thesis was supported by Miguel Caetano from IPMA and by Margarida Romão and Georgina Sarmento from LAIST.

Funding from Fundação para Ciência e Tecnologia, through the projects:

MERO: Marine oil spill: green biological emulsifier agents production and autonomous robotic tools for response optimization (MIT-EXPL/ISF/0115/2017)

CRUISE: Moesziomyces spp. based biorefinery: Membrane bioreactor for production of aviation fuel and biosurfactants (PTDC/AAG-TEC/0696/2014)

Resumo

Esta tese foca-se em investigar o potencial dos Manosileritritolípidos (MEL) na assistência à biorremediação de crude derramado em ambientes marinhos.

O MEL foi produzido por *Moesziomyces antarcticus* alimentada com glucose e óleo de soja. Com 1 g/L de MEL o índice de emulsificação de parafina foi 61.9%. Foi testada a estabilidade da solução de MEL quando submetida a diferentes temperaturas, salinidades e pH medindo o índice de emulsificação. Obteve-se emulsões estáveis em água com até 4% de salinidade, pH entre 7 e 12, e após ter sido submetida a temperaturas inferiores a 40°C. Os mesmos ensaios foram realizados para SDS e os resultados comparados.

O efeito do MEL na degradação de crude foi inconclusivo nos testes de biorremediação em água do mar. Em meio sintético com microrganismos isolados da água do mar houve um aumento de degradação de hidrocarbonetos por parte da estirpe MS1 quando suplementada com MEL, mas não se verificou variações significativas com a estirpe MS2. Para as diferentes concentrações de MEL testadas não foi observada qualquer inibição no crescimento das estirpes isoladas.

Keywords: Biosurfactantes; Manosileritritolípidos, Degradação de crude, Bioremediação, Dispersantes

Abstract

This thesis focused on investigating the possibility of mannosylerythritol lipids (MEL) assisted bioremediation of crude oil spilled in a marine environment.

MEL was produced from *Moesziomyces antarcticus* fed with glucose and soybean oil. It was found 1 g/L of MEL obtained an emulsification index in kerosene of 61.9%. This solution was tested for its stability over a wide range of pH, temperature and salinity by measuring its emulsification index for kerosene. Stable emulsions were obtained in up to 4% salinity, from pH 7 to 12, and after being submitted to temperatures below 40°C. SDS was also tested under the same conditions and results were compared.

Bioremediation tests in seawater were overall inconclusive concerning MEL's effect in assisting crude degradation by natural microorganisms. Tests in synthetic medium supplemented with isolated marine microorganisms showed an increase in hydrocarbon degradation by isolate MS1 when MEL was added, but no significant effect in degradation by isolate MS2. For the different concentrations of MEL tested, no inhibition of growth of the marine isolates was found.

Keywords: Biosurfactants, Mannosylerythritol lipids, Crude oil degradation, Bioremediation, Dispersants

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List of Abbreviations

BH – Bushnell Hass

CDW – Cell dry weight

CMC – Critical micelle concentration

DOR – Dispersant to oil ratio

EI_{24} – Emulsification index at 24 hours

GC – Gas chromatography

Glu – D-Glucose

HPLC – High performance liquid chromatography

M – Molar concentration (mol/L)

M. antarcticus – *Moesziomyces antarcticus*

MB – Marine broth

MEL – Mannosylerythritol lipids

MS – Marine strain (isolate)

OD – Optical density

PAH - polycyclic aromatic hydrocarbons

rpm – Revolutions per minute

SDS – Sodium dodecyl sulphate

TPH – Total petroleum hydrocarbons

1 Introduction

1.1 Overview and Motivation

Currently, water and soil pollution caused by petroleum and its derivatives is one of the most widespread problems affecting the environment [1]. Oil spills in the ocean can have a devastating impact in aquatic life, particularly when oil slicks are formed at the water surface since they can impregnate marine macro fauna. Several approaches have been tested in order to minimize these effects. One of them is the application of chemical dispersants to oil slicks in order to increase the rate of dispersion of these contaminants from the sea surface into the water column. This diminishes oil concentration and increases its bioavailability for naturally occurring oil-degrading microorganisms [2]. However, the use of chemical dispersants has been controversial due to their toxicity to aquatic organisms [3].

Another approach is enhanced bioremediation, also referred as bioaugmentation or biostimulation, which aims to increase the degradation of contaminants by microorganisms. Bioaugmentation can be applied either through in situ use of biosurfactant producing microorganisms or through supplementation of ex situ produced biosurfactants. The first method often fails when applied in the field due to the fact that laboratory grown microorganisms usually do not adapt well to the oil spill site. Moreover, carries the risk of introducing foreign species to the environment with unknown effects on the rest of the aquatic organisms [4][5], and thus many countries legislation limited the introduction of microorganisms in marine environments.

Bioavailability of the hydrocarbons is one of the limiting factors controlling biodegradation rates since they have very low solubility in water [6]. Surfactants can help emulsify and lower the surface tension of the organic compounds and thus increase the access to the hydrocarbons by the oil-degrading microorganisms. Many marine microbes produce biosurfactants in order to make hydrocarbons more available to be used as a carbon source [7].

The supplementation of biosurfactants to oil slicks has already been tested in site with promising results [8]. Various studies done ex situ have shown that many different biosurfactants can enhance bioremediation of contaminated water or soil [9][10][11]. However, failure to replicate laboratory results when applying the biosurfactants to the field shows the need to design experiments with conditions as close as possible to the actual site [4]. That includes the use of crude oil and it's derivates instead of pure hydrocarbons and the use of marine microorganisms or seawater samples in biodegradation tests.

The biosurfactant must be capable of withstanding the harsh conditions of the marine environment (pH, salinity, temperature) as well as have a low impact on the species that inhabit it. Low production costs, high yields and small environmental impact are very important characteristics for market adoption and currently are some of the limiting factors for a more wide-spread use of biosurfactants [12].

This thesis focused on the application of the biosurfactant Mannosylerythritol lipids since it (i) is potentially more biodegradable and less toxic than chemical surfactants [13], (ii) has excellent interfacial properties [14]; (iii) has one of the highest biosurfactant's titres achieved so far (165 g/L) [15]; and (iv) can be produced from renewable sources and industrial wastes [16].

1.2 Objectives

The purpose of this thesis is to preliminary assess one of the many proposed applications of biosurfactants: bioremediation. The biosurfactant of focus is mannosylerythritol lipids (MEL) which can be produced from the yeast *M. antarcticus* using sugars or vegetable oils as the carbon sources.

Studying MEL solutions stability in different physicochemical conditions is of importance to its possible uses.

Since one of the problems affecting bioremediation tests done in laboratory is their lack of reproducibility on the field, this study intended to use both seawater samples and isolated marine microorganisms in its experiments in order simulate near to actual in situ conditions.

As such, the main objectives of this thesis are:

- to assess the viability of using MEL to augment the rate of bioremediation of oil spills occurring in marine environments;
- to study MEL's toxicity to marine microorganisms; and
- to characterize MEL properties as a biosurfactant and their ability to disperse oil.

1.3 Research questions

In order to attain the main objectives of this thesis the following questions were asked:

- What is the ability and stability of MEL in emulsifying water/kerosene mixtures in a range of pH, temperature and salinity?
- What is the impact of different concentrations of MEL on the growth kinetics on isolated marine microorganism?
- Can the supplementation of MEL increase the rates of bioremediation of crude oil by marine microorganisms?

1.4 Research strategy

With the objectives of this thesis and the previous research questions in mind the following strategy was outlined:

- Use the yeast *M. antarcticus* PYCC 5048T to produce MEL, following methods previously established at iBB/IST to maximize MEL production and substrate use.
- Create a MEL solutions and assess its stability, toxicity and its potential in aiding bioremediation.
- Characterize MEL physicochemical properties, namely estimating emulsification index in comparison with the chemical surfactant Sodium Dodecyl Sulphate (SDS).
- Isolate hydrocarbon-degrading marine strains from seawater samples and develop a growth protocol for them; aiming further use of these strains in MEL toxicity assessments and in crude oil/hydrocarbon biodegradation.
- Study the effects on growth kinetics of different concentrations of MELand synthetic dispersant Corexit 9500 to assess surfactants toxicity on the isolated marine microorganisms.
- Assess the capacity of different concentrations of MELand Corexit 9500 to increase the rate of bioremediation of either crude oil or an alkane mixture in experiments made on both seawater samples and on a synthetic medium with the marine isolates.

2 Literature Review

2.1 Surfactants

In our current industrial society, it is hard for one to not interact with a surface-active agent (Surfactant) of some sort on a daily basis. That has to do with its extensive usage for a wide range of applications from cosmetics and detergents to pharmaceutical products.

Surfactants are compounds capable of reducing the surface and interfacial tensions. These molecules possess an amphipathic structure exhibiting at least one hydrophilic head group and at least one hydrophobic tail group, as depicted in Figure 1

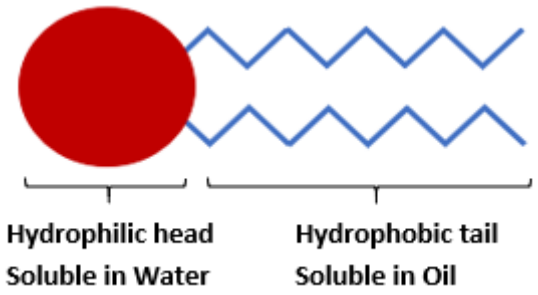


Figure 1 – Surfactant molecule structure

Given their structure, these compounds tend to migrate to interfaces or surfaces and orientate themselves in an attempt to lay the head group in a polar environment, e.g., water; and the tail group in a nonpolar environment, e.g., oil. This behaviour denotes Surfactants' fundamental property, i.e., the tendency to accumulate at interfaces and form tightly packed structures [17].

Note that, the term interface refers to the boundary between two immiscible phases. In total, there exist five different interfaces [18]:

- Solid–vapor;
- Solid–liquid;
- Solid–solid;
- Liquid–vapor;
- Liquid–liquid.

The effectiveness of a surfactant depends on the interface. Therefore, there is no universally best surfactant independent of the use case [17].

Depending on the interface, different structures might be formed. E.g., monolayers are formed at the air-water and the oil-water interface, and monolayers and aggregates at the solid-water interface [17].

In the presence of water, a surfactant tends to minimize the contact between their hydrophobic tail group and the water by positioning itself at the water-air interface. Increasing the surfactant concentration lowers the surface tension of the solution until the surface is fully occupied, at which point the surfactants will start to form micelles and/or vesicles. Such is referred to as critical micelle concentration (CMC) and its one of the most important characteristics of a surfactant [19]. After reaching the CMC increasing the surfactant concentration will result in almost no change of the surface tension. This process is depicted in Figure 2.

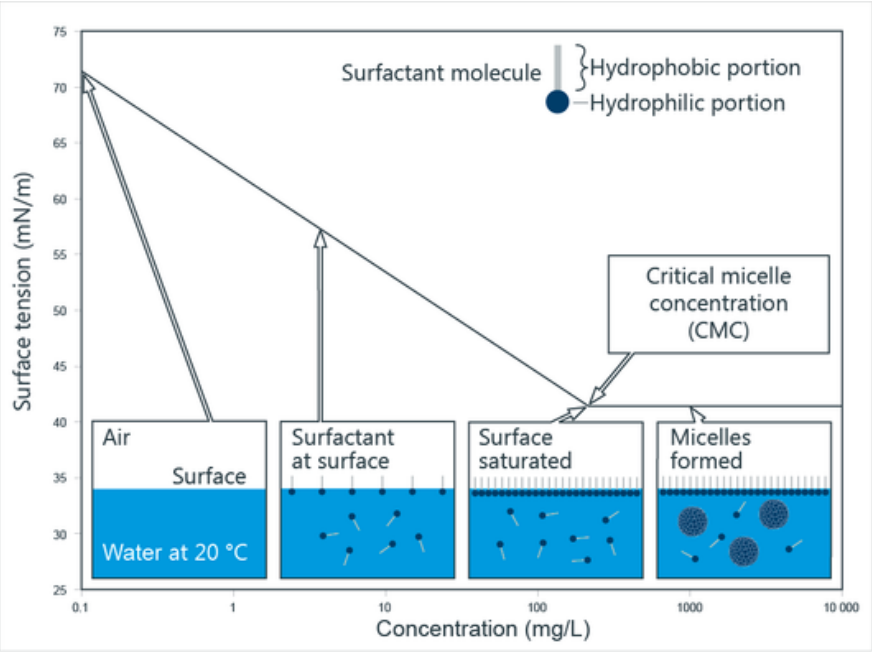


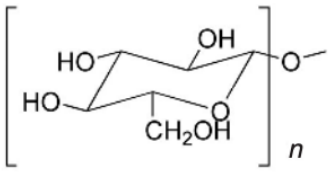
Figure 2 – Decreasing surface tension of a solution with increasing surfactant concentration until CMC and micelle formation. Retrieved from [20]

The CMC varies with temperature as well as the surfactant and the solution properties. Some rules of thumb for these changes are:

- Increasing temperature generally lowers the CMC. There is also a minimum temperature below which no micelles are formed, known as Krafft temperature [21].
- At constant temperature, for increasing surfactants chain length the CMC decreases due to increased hydrophobic character of the molecules.
- Increase in lipidic chains or branching results in higher CMC values due to a more significant decrease of free energy from micelle formation [22].
- The CMC of ionic surfactants is typically higher than non-ionic surfactants due to the increase in electrical work needed to form micelles.

There exists a wide variety of surfactants, each with different properties, structure, and applications. The classification of a surfactant is related to their hydrophilic group structure and water dissociation [23]. Surfactants can be classified as ionic or non-ionic. Ionic surfactants are further divided into anionic, cationic and amphoteric [19]. Table 1 provides examples of hydrophilic groups for each category along with their respective structure.

Table 1 - Common hydrophilic groups, retrieved from [19]

Ionic type	Example	Structure
Anionic	Sulphate	$-OSO_2O^-$
	Sulphonate	$-SO_2O^-$
	Ether sulphate	$-(OCH_2CH_2)_nOSO_2O^-$
	Ether phosphate	$-(CH_2CH_2O)_nP(O)O^-$
	Ether carboxylate	$-(CH_2CH_2O)_nCO_2^-$
	Carboxylate	$-C(O)O^-$
Cationic	Primary ammonium	$-N^+H_3$
	Secondary ammonium	$-N^+(R)H_2$
	Tertiary ammonium	$-N^+(R)_2H$
	Quaternary ammonium	$-N^+(R)_3$
Amphoteric	Amine oxide	$-N^+(R)_3O^-$
	Betaine	$-N^+(R)_3(CH_2)_nC(O)O^-$
	Aminocarboxylates	$-N^+H(R)_2(CH_2)_nC(O)O^-$
Non-ionic	Polyoxyethylene (an 'ethoxylate')	$-(OCH_2CH_2)_nOH$
	Acetylenic	$-CH(OH)C{\equiv}CH(OH)-$
	Monoethanolamine	$-NHCH_2CH_2OH$
	Diethanolamine	$-N(CH_2CH_2OH)_2$
	Polyglucoside	

There are also different kinds of hydrophobic groups. Table 2 contains some examples along with their respective structure.

Table 2 - Common hydrophobic groups, retrieved from [19]

Group	Example	Structure
Alkylbenzene	Linear dodecyl-benzene	$CH_3(CH_2)_5CH(C_6H_4)(CH_2)_4CH_3$ [a]
Linear alkyl[b] (saturated)	n-dodecyl	$CH_3(CH_2)_{10}CH_2-$
Branched alkyl[b] (saturated)	2-ethyl hexyl	$CH_3(CH_2)_3CH(-CH_2-CH_2CH_3)$
Linear alkyl[b] (unsaturated)	Oleyl	$(cis-)CH_3(CH_2)_7{=}CH(CH_2)CH_2-$
Alkylphenyl (branched)	Nonylphenyl	$C_9H_{19(\text{branched isomers})}C_6H_4-$
Polyoxypropylene		$-[OCH_2CH(CH_3)]_n-$
Polysiloxane		$(CH_3)_3Si[OSi(CH_3)]_nOSi(CH_3)_3$ |

From the few examples provided by Table 1 and Table 2 one can perceive that there exists a plethora of possible combinations between hydrophilic and hydrophobic groups. This variety is what enables surfactants to provide a rich set of capabilities like wetting, foaming, emulsification, aggregation of solids, solubility and solubilization, adsorption, micellization, and detergency [19].

As a result, surfactants applications are vast, and they are used in the production of textiles, agricultural, chemicals, and paints. Still, despite their broad scope of applications, from a revenue's perspective, today's global major applications are household detergents and personal care [24]. Such is also expected to stay true in 2024. Figure 3 - Surfactants Global Market: Revenue (%) by application, retrieved from **[24]**Figure 3 clearly illustrates this statement.

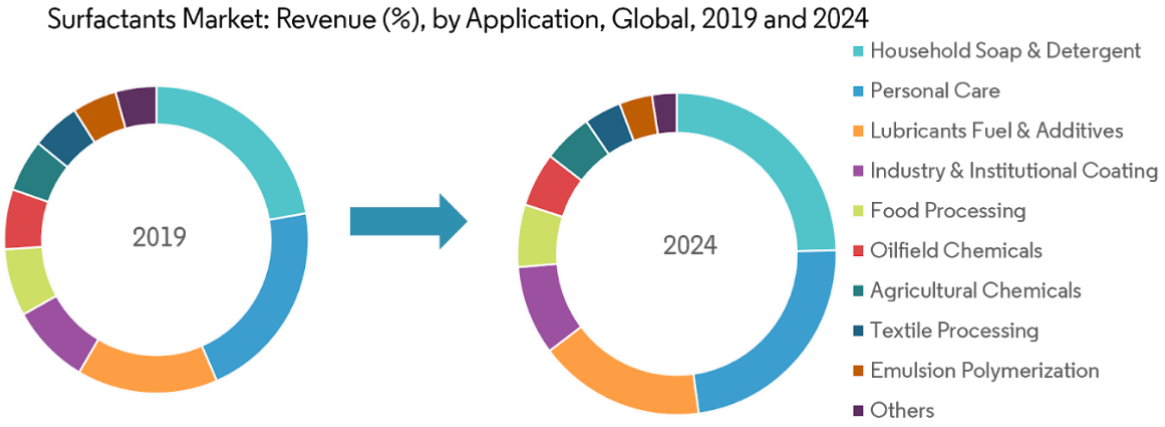


Figure 3 - Surfactants Global Market: Revenue (%) by application, retrieved from [24]

Large scale industries such as detergent production have been relying on chemically synthesized surfactants since their inception. These compounds originate from petrochemical or oleochemical sources [13].

Recently, there has been an active effort towards achieving industrial sustainability. Therefore, an interest in pursuing alternatives for chemical surfactants has sparked. Biosurfactants emerged as a promising alternative and have been considered one of the most important technologies for development in the current century [7].

In comparison with chemical surfactants, biosurfactants main strengths are their biodegradability and low toxicity. There is also their tolerance to elevated salt concentrations, which makes them appealing for many industrial applications [25].

Such properties have led to a proliferation of research studies, mainly, in the fields of bioremediation and petroleum [13]. The Biodegradability subject will be further explored in the bioremediation section.

The chapter that follows presents a state of the art for biosurfactants.

2.2 Biosurfactants

Biosurfactants are a class of surfactants that have been looked upon as a sustainable option for replacing chemical surfactants in large scale commercial applications like detergents and house cleaners [13].

These surfactants have been subject of increasing interest and research since surfactin, the first microbial biosurfactant, was purified and characterized in 1968 [26]. Surfactin is commonly used as an antibiotic due to its antibacterial and antiviral properties [27].

These compounds are produced from biological sources like plants and animals as well as microorganisms. In particular, there has been an increased focus from the research community in biosurfactants produced specifically from microorganisms like bacteria, yeast, and fungi. One reason for this interest is that microbial biosurfactants are typically considered more environmentally sustainable since they are biodegradable, have low toxicity and their production requires less resources comparatively with other biological surfactants [28][29][13].

Microorganisms are capable of using a variety of organic compounds as a source of carbon and energy for their growth. The mix of carbon sources with insoluble substrates eases their diffusion into the cell and production of different substances, the biosurfactants [30][31]. Researchers from the field have been accessing a wide range of carbon sources for production like pyruvate, alkanes, simple sugars, succinate, citrate, glycerol, mannitol, n - paraffin, and hexadecane supplemented with different nitrogen sources

Different microorganisms can produce different biosurfactants with different molecular structures [32]. Figure 4 shows the structural differences in the most studied microbial biosurfactants.

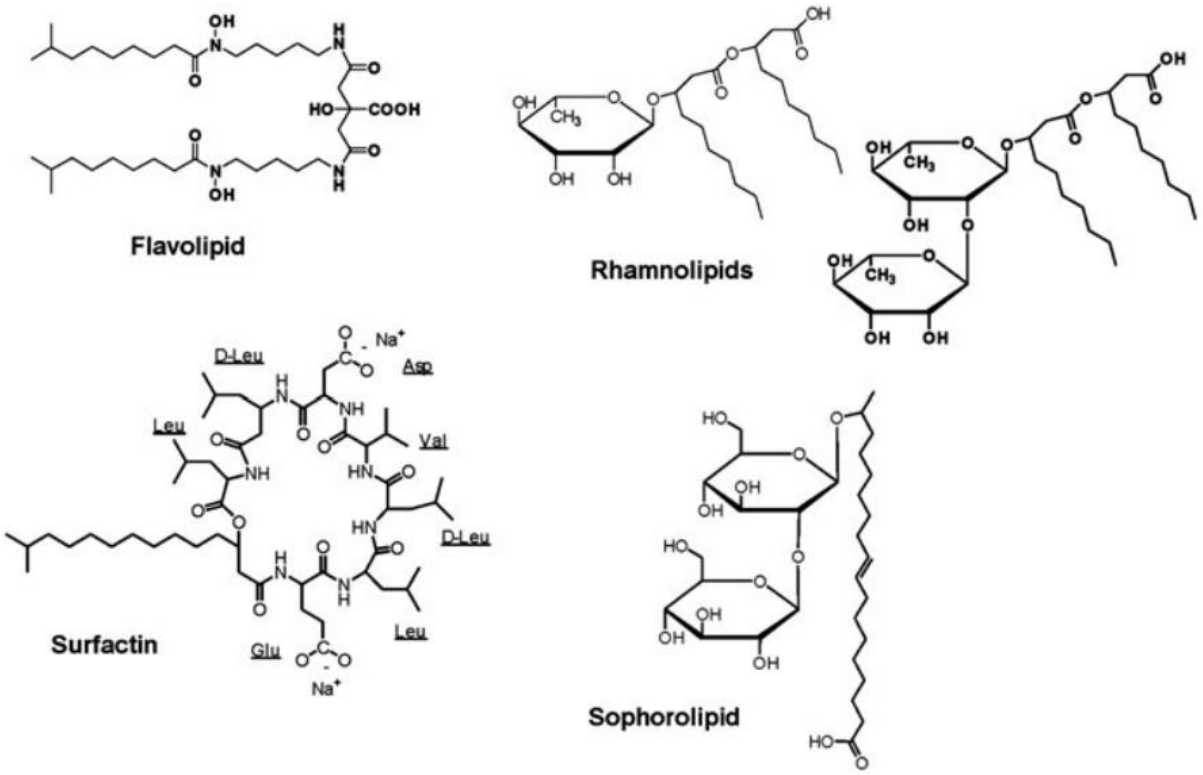


Figure 4 - Chemical structure of most studied microbial biosurfactants, retrieved from [13]

The majority of biosurfactants are anionic or neutral, but there exist cationic biosurfactants as well. Biosurfactants possess an amphipathic structure. In this type of surfactants, the hydrophobic moiety possesses long-chain fatty acids and the hydrophilic part can be a carbohydrate, cyclic peptide, amino acid, phosphate carboxyl acid or alcohol. Their molar mass can range between 500 to 1500 Da [33][34].

While chemical surfactants are usually classified by their polarity, microbial biosurfactants are categorized according to their origin microorganism and chemical composition [35]. Table 3 shows a variety of common biosurfactants along with their origin and head group.

Table 3 - Common microbial biosurfactants, adapted from [28]

Head Group	**Biosurfactant**	**Microorganism**
Fatty acids	Fatty acid	*Corynebacterium lepus*
Neutral lipids	Neutral lipid	*Nocardia erythropolis*
Phospholipids	Phospholipid	*Thiobacillus thiooxidans*
Lipopeptides	Surfactin	*Bacillus subtilis, Bacillus pumilus A*
Glycolipids	Viscosin	*Moesziomyces fluorescens, Moesziomyces libanensis*
	Serrawettin	*Serratia marcescens*
	Mannosylerythritol lipids	Genus *Moesziomyces* (yeast), *Candida antarctica, Ustilago maydis*
	Sophorolipids	*C. batistae, T. bombicola, C. lypolytica, C. bombicola, T. apicola, T. petrophilum, C. bogoriensis*
	Rhamnolipids	*Moesziomyces sp., M. aeruginosa*
	Trehaloselipids	*Rhodococcus sp., Arthrobacter sp., R. erythropolis, N. erythropolis*
	Cellobiolipids	*Ustilago zeae, Ustilago maydis*
Polymeric	Emulsan	*Acinetobacter calcoaceticus*
	Biodispersant	*A. calcoaceticus*
	Mannan lipid protein	*C. tropicalis*
	Alasan	*A. radioresistens*
Siderophore	Flavolipids	*Flavobacterium*

The head groups that have seen more focus from the research community are lipopeptides, glycolipids, fatty acids, and polymeric [36].

Glycolipids have low molecular mass and represent the majority of the existing biosurfactants. They are carbohydrates linked to long-chain aliphatic acids or hydroxyaliphatic acids by an ester group [35].

Within glycolipids, the biosurfactants that have been more widely studied are rhamnolipids. Rhamnolipids have low toxicity, strong emulsifier properties, and capability to help in the remediation of both inorganic and organic micropollutants from the soils and the sediments [18][19]. These biosurfactants have direct application in disparate domains like bioremediation, pharmaceuticals and therapeutics, cosmetics, agriculture, and detergents [38].

Despite the advantages that biosurfactants possess over chemical ones, there still exists a major problem to overcome related to the absence of large scale and low-cost production support. Such a gap has led to increasing efforts on reducing the production costs with the goal of promoting commercial adoption.

Researchers believe that the main barrier for its adoption has to do with the usage of expensive substrates, limited product concentrations, low yields and the formation of product mixtures rather than pure compounds [12].

Currently, the microbial biosurfactants available for commercial use are rhamnolipids and surfactin; and more recently MEL has been applied in cosmetics [36]. MEL is also a biosurfactant within the glycolipids head group and it is the focus of this thesis. The next chapter further describes this compound.

2.2.1 Mannosylerythritol lipids

2.2.1.1 Overview

Mannosylerythritol lipids (MEL) is a biosurfactant that belongs to the glycolipids head group. These biosurfactants are mainly produced by yeasts such as the genus *Moesziomyces,* which was formerly known as *Pseudozyma* [37]. MELs contain 4-O-β-D-mannopyranosyl-erythritol or 1-O-β-D-mannopyranosylerythritol as a hydrophilic headgroup and fatty acids as the hydrophobic chain [38]. Like other microbial biosurfactants, MEL produced by *Moesziomyces* is a mixture of congeners instead of a single predominant molecule [13].

Different types of MEL exist, and they can have different chemical structures as shown in Figure 5. MELs that are produced naturally have acylated structures possessing either two acyl groups, MEL-A, or one acyl group, MEL-B, and MEL-C [13]. There is also a non-acylated MEL designated as MEL-D, which was produced by modifying an existent biosurfactant by removing the acyl group through a lipase catalysed hydrolysis [39]. Fukuoka et al. have also reported that *Moesziomyces antarctica* can produce a mono-acylated MEL when using glucose as a carbon source [40].

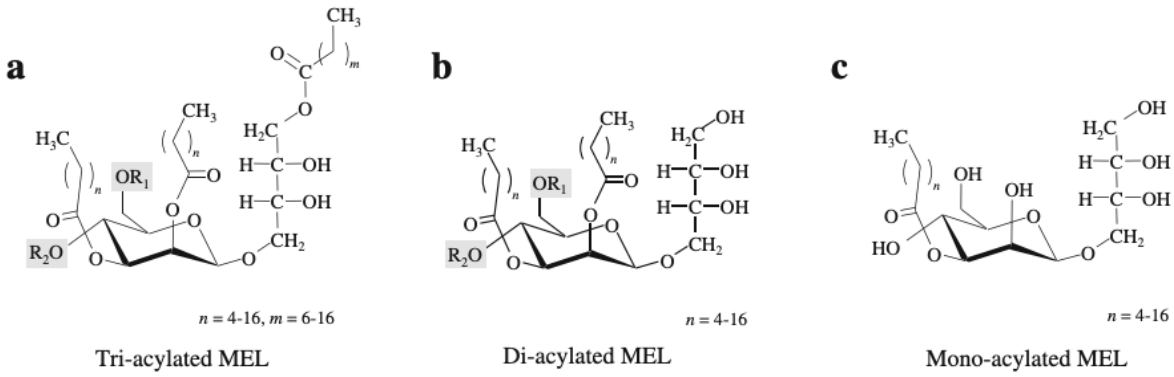


Figure 5 - Chemical structure of mannosylerythritol lipids: a - triacylated type of MEL, b - diacylated MEL, c- mono-acylated MEL. MEL-A: R1=Ac, R2= Ac; MEL-B: R1=Ac, R2=H; MEL-C: R1=H, R2=Ac; MEL-D: R1=H, R2=H; retrieved from [36]**.**

Still, the variation present in MELs is not only tied to the variation in the different levels of acylation, since their fatty acid chains can also vary both in terms of length and saturation [13].

Table 4 contains a list of existing MEL producers, respective products, yields and their interfacial properties.

Table 4 - MEL producers and their products, retrieved from [41]**.**

Microbial producers	Carbon sources	Glycolipids	Yield (g/L)	cmc (M)	gcmc (mN/m)
Pseudozyma aphidis	Soybean oil and glucose	MEL-A (main), MEL-B, MEL-C	165[a,b,c]	ND	26.2
Pseudozyma antarctica	Soybean oil	MEL-A (main), MEL-B, MEL-C	40[a]	2.7×10^{-6}	28.4
	Soybean oil	MEL-B (purified)	10	4.5×10^{-6}	28.2
	n-Alkane	MEL-A (main), MEL-B, MEL-C	140[a,b]	ND	ND
	Glucose	Mono-acylated MEL (purified)	1.3	3.6×10^{-4}	33.8
	Soybean oil	Try-acylated MEL (purified)	ND	ND	ND
Pseudozyma churashimaensis	Soybean oil	Mono-acylated/tri-acetylated MEL (purified)	3.8[a]	1.7×10^{-6}	29.2
Pseudozyma crassa	Oliec acid and glucose	Diastereomer MEL-A (main), MEL-B, MEL-C	4.6[a]	5.2×10^{-6}	26.5
Pseudozyma fujiiformata	Soybean oil	MEL-A (main), MEL-B, MEL-C	4[a]	ND	ND
Pseudozyma graminicola	Soybean oil	MEL-A, MEL-B, MEL-C (main)	9.6[a]	4.0×10^{-6}	24.2
Pseudozyma hubeiensis	Soybean oil	MEL-A, MEL-B, MEL-C (main)	76.3[a,b,c]	6.0×10^{-6}	25.1
Pseudozyma parantarctica	Soybean oil	MEL-A (main), MEL-B, MEL-C	106.7[a,b]	ND	ND
	Glucose	Mono-acylated MEL (purified)	1.2	ND	ND
	Soybean oil	Try-acylated MEL (purified)	22.7	ND	ND
	Olive oil and mannitol	Mannosyl-mannitol lipids (purified)	18.2	2.6×10^{-6}	24.2
	Olive oil and arabitol	Mannosyl-arabitol lipids (purified)	ND	1.5×10^{-6}	24.2
	Olive oil and ribitol	Mannosyl-ribitol lipids (purified)	ND	1.2×10^{-6}	23.7
Pseudozyma rugulosa	Soybean oil and erythritol	MEL-A (main), MEL-B, MEL-C	142[a,b]	ND	ND
	Soybean oil	Try-acylated MEL (purified)	ND	ND	ND
Pseudozyma shanxiensis	Soybean oil	MEL-C	2.72	ND	ND
Pseudozyma siamensis	Safflower oil	MEL-B, MEL-C (main)	18.5[a]	4.5×10^{-6}	30.7
Pseudozyma tsukubaensis	Soybean oil	Diastereomer MEL-B	73.1[b,c]	3.1×10^{-6}	26.1
	Castor oil	Diastereomer MEL-B containing a hydroxy fatty acid	22	2.2×10^{-5}	28.5
Ustilago cynodontis	Soybean oil	MEL-C	1.4	ND	ND
Ustilago maydis	Sunflower oil	MELs and cellobiose lipids	30[d]	ND	ND
Ustilago scitaminea	Sugarcane juice	MEL-B	25.1	3.7×10^{-6}	25.2

[a] As a mixture of MELs
[b] Feeding using resting cells
[c] Large scale production with jer-fermenter
[d] As a mixture of MELs and cellobiose lipids
ND: no data

Recently, MEL has experienced increasing traction, due to its high biodegradability, mild production conditions, and variety of functions. MEL's applications include food, cosmetic, and pharmaceutical industries; environmental protection, and energy-saving technologies [42].

It is important to highlight that apart from rhamnolipids and surfactin, MEL is the third biosurfactant available for commercial use, namely in cosmetics [36].

2.2.1.2 Biosynthesis

The identification of the genes responsible for MEL synthesis was first performed on a fungi microorganism designated by *Ustilago maydis* [36].

This fungus has the ability to secrete large amounts of MEL under certain conditions of nitrogen starvation. The synthetization process of MEL in this microorganism is an enzymatic reaction performed in four distinct steps by four distinct genes: emt1, mac1, mac2, and mat1 [43].

MEL can also be produced by the *Moesziomyces genus*, e.g., *M. antarctica*, *M. rugulosa*, and *M. aphidis*.

There exist three mandatory molecular blocks for MEL assembly: mannose, erythritol and medium-chain fatty acids. Figure 6 illustrates the two possible pathways for MEL's biosynthesis: 1 - MEL produced using vegetable oil as carbon source; and 2- MEL produced using glucose as a carbon source.

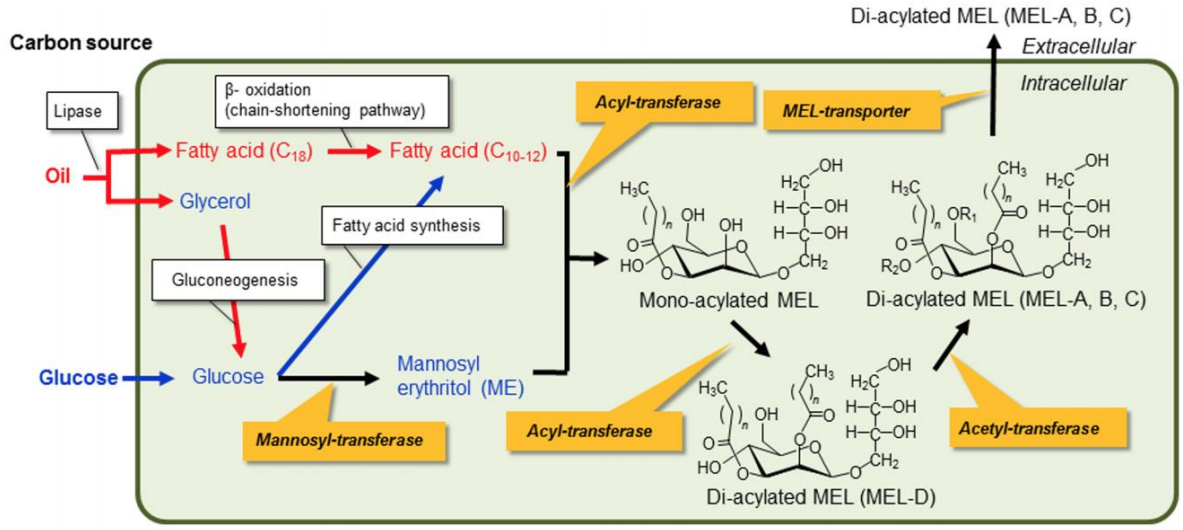


Figure 6 - MEL biosynthesis, retrieved from [44]**.**

When glucose is used as a carbon source the process is as follows. The glucose ring is phosphorylated with the help of hexokinase enzyme resulting in a molecule designated by glucose-6-phospate. This molecule is then converted to fructose-6-phospate through isomerization reactions with the help of phosphoglucose isomerase enzyme. The fructose-6-phospate produced from the glycolytic pathway is then converted into mannose-6-phospate by enzyme phosphomannose isomerase, followed by a conversion to mannose-1-phospate by phosphomannomutase enzyme and addition of guanosine diphosphate (GDP) by GDP-mannose pyrophosphorylase. Erythritol is synthesized from glucose through the pentose phosphate pathway. After production of erythritol and mannose follows the mannosylation of erythritol via mannosyl-transferase (emt1). Emt1 is critical for formation of MEL and is highly induced by nitrogen starvation [43]. There also occurs the production of fatty acids through fatty acid synthesis process [45]. After these two steps, the mannosylerythritol is then acylated with the produced fatty acids by the acyl transferases (mac1 and mac2) at C-2 and C-3. In the last phase, acetylation by acetyl transferases (mat1) occurs, and finally MEL is secreted by a MEL-transporter (mmf1).

When vegetable oil is used as carbon source the process is as follows. In a first phase, vegetable oils are cleaved by lipases forming fatty acid (C18) and glycerol. After this first stage beta-oxidation occurs forming fatty acid (C10-C12). The glycerol formed in the initial phase, i.e., after the lipases cleaving, is transformed into glucose through gluconeogenesis. After this, the remaining process is the same as previously described.

2.2.1.3 Applications

The potential set of applications for MEL are several thanks to its surface activity, high yield (above 100 g/L), biocompatibility, self-assembling properties, antimicrobial activities, and biochemical functions [45][46].

MEL can be used as an antimicrobial agent. Both MEL-A and MEL-B have demonstrated strong activity against gram positive bacteria, weak against gram negative and neutral towards fungi [47]. Still, Kitamoto et al. reported that it should not be hard to improve MEL's current antimicrobial activity capabilities through chemical modification of the sugar moiety [14].

MEL can induce cell differentiation and apoptosis. Both MEL-A and MEL-B can be used for differentiation–induction against human leukemia [48], rat pheochromocytoma [49] and mouse melanoma cells [49].

Another MEL's application is for the purification of glycoprotein. Studies demonstrate that MEL-A, MEL-B, and MEL-C have a high binding affinity towards different immunoglobulins, e.g., human immunoglobulin [50].

MEL's capability to form thermodynamically stable vesicles with the ability to fuse with the membrane of cells was found to be useful for acting as a vehicle for gene and drug delivery [51]. MEL-A was reported as the biosurfactant better suited for gene transfection [52].

MEL has proven to possess anti-inflammatory action by inhibiting the secretion of inflammatory mediators from mast cells [53].

MEL has also been seen as a strong candidate for inhibiting the aggregation of ice for ice slurry systems, which are "green" cold thermal storage units, e.g., air conditioners [54].

In recent years, an important milestone was achieved for MEL. Thanks in part to improvements performed in the production of MEL [36], a Japanese company named Toyobo Co., Ltd., has developed SurfMellow®, which is a cosmetic product that contains MEL-A in its composition. This fact puts MEL as the third biosurfactant available for commercial use.

2.3 Oil spills

2.3.1 Overview

Water pollution is the result of mixing or dissolving hazardous substances in the surface water. Hydrocarbon contamination is a major source of pollution in today's industrialized world. It is mainly related with oil spills, and consequently the oil industry, which already contributed to a significant contamination of the marine environment [58].

The release of hydrocarbons into the water can happen in a variety of situations like accidents during transportation of fuel by ships, oil spills from underground tanks, or during extraction and processing of oil. While oil spills may correspond to a small percentage of the oil released in the sea [59], the large ones, despite rare, attract public concern [60], especially since these larger accidents can drastically affect ocean and shorelines as well as the wildlife therein contained.

To have a better sense of the scale of large oil spill events, let's look into the numbers of two incidents: the Amoco Cadiz and Exxon Valdez. During Amoco Cadiz oil spill incident, 0.2 megatons of crude oil were released along the Brittany coast in 1978. As for the Exxon Valdez event, around 0.04 megatons were discharged into the Prince William Sound in 1989. Some 2800 sea otters, 300 harbor seals and about 250000 seabirds died in the aftermath of the spill and oil can still be found on the beaches 30 years after the disaster [61].

Figure 7 – Effect of Exxon Valdez oil spill in wildlife. From left to right: (i) cormorant soaked in oil on the beach of Green Island in Prince William Sound, Alaska by Bob Hallinen; (ii) oil-soaked sea otters in Green Island by Chris Wilkins.

There are also deliberate releases of oil, which can cause considerable damage in the environment. An example of a large discharge of this kind, is the release of 0.82 megatons of oil in Kuwait, during the Gulf War in 1991, which threatened the Gulf's desalination plants and coastal ecosystems [62].

The aforementioned incidents have triggered and motivated the research and development of new technologies to deal with oil pollution. Consequently, bioremediation emerged as a technology to counter oil spill pollution, and it was initially defined as: the use of microorganisms to accelerate the degradation of oil or other environmental contaminants [63].

Another important definition to elucidate is that of biodegradation. This term refers to the natural process of transforming or removing non-volatile fractions of oil from the environment through the biological action of microorganisms.

2.3.2 Oil composition

Hydrocarbons are organic compounds containing different configurations of only hydrogen and carbon atoms. Crude oil is mainly composed by hydrocarbons. Usually, two thirds of its composition are alkanes and cycloalkanes, and their derivatives like methylcyclohexane. The remaining third is

composed by polycyclic aromatic hydrocarbons (PAHs), benzene, naphthalene, phenanthrene and their alkylated derivatives [64]. Table 5 shows the composition of some common oils.

Table 5 - Composition of common oils, adapted from [65]

	(%—except for metals)				
Group	Compound class	Gasoline	Diesel	Light crude	Heavy crude
Saturates		50–60	65–95	55–90	25–80
	Alkanes	45–55	35–45	40–85	20–60
	Cycloalkanes	5	25–50	5–35	0–10
Olefins		5–10	0–10		
Aromatics		25–40	5–25	10–35	15–40
	BTEX	15–25	0.5–2.0	0.1–2.5	0.01–2.0
	PAHs		0–5	10–35	15–40
Polar compounds			0–2	1–15	5–40
	Resins		0–2	0–10	2–25
	Asphaltenes			0–10	0–20
Sulfur		0.02	0.1–0.5	0–2	0–5
Metals (in parts per million—ppm)				30–250	100–500

Alkanes are a class of hydrocarbons that do not have double bonds or aromaticity. They are “saturated” with hydrogen, and they can be straight chain, branched, or cyclic.

The Aromatics group comprehend cyclic organic compounds that are stabilized by pi electrons forming a circle. They include compounds such as benzene, toluene, ethylbenzene, and the three xylene isomers (BTEX), and PAHs like naphthalene, and phenanthrene.

An aromatic hydrocarbon possesses at least one benzene ring of six carbons. The three carbon-to-carbon double bonds float around the ring, thus, providing high stability to the compound. This stability makes the benzene rings persistent, and therefore potentially toxic for the environment. PAHs are hydrocarbons that are formed by at least two benzene rings, which makes them highly toxic compounds.

2.3.3 Dispersants

In 2003, it was reported that in every year 700 million litres of crude oil were entering the marine environment through natural hydrocarbon seeps [66]. Oil tanks and pipeline spills contribute to these numbers by releasing around 131 million litres of oil per year, as reported in the same year.

As an emergency response to the oil spill problem, chemical dispersants have been applied on contaminated waters.

Dispersants are a mixture of solvents and surfactants. These compounds allow the oil to dissolve into the water, reducing oil aggregation at the surface, and the contact of hydrocarbons with shorelines. Still, the complete extend of the impact of its usage in water’s indigenous microorganisms remains in part unknown [2].

One of the major reasons for this has to do with reproducibility challenges when attempting to replicate the complexity of the natural environment in laboratory. As so, there is a lack of experimental research performed, considering that experiments can only be performed after a real oil spill or by acquiring permission to perform a controlled release of oil in the environment [67].

Corexit EC9500A, Corexit EC9500B and Corexit EC9527A, are examples of commercial dispersants that were approved by the existing US Environmental Protection Agency (EPA) to be used as emergency response to oil spill. Recommended dispersant to oil ratio (DOR) varies according to the dispersant, and its upper limits must be respected.

Both Corexit EC9500A and Corexit EC9527A contain hazardous compounds for the marine environment like sulfonic acid salt and propylene glycol, and both have a recommended DOR of 1:50 to 1:10. Additionally, Corexit EC9500A also includes hydrotreated light petroleum distillates.

When dispersants interact with oil in the aqueous media, the hydrophilic part of the surfactant orientates towards the sea water, while the hydrophobic moiety migrates towards the oil. Such leads to the formation of micelles containing oil droplets with size ranging from microns to millimetres. Consequently, the oil becomes emulsified, the stabilized oil droplets are dispersed, hence, reducing the interfacial tension, and increasing the surface area. Figure 8 illustrates the aforementioned phenomena.

The future of dispersants use in oil spills depends on several factors like efficiency of the dispersion of oil droplets, interaction of the dispersed oil with organic matter, biodegradation, evaporation, and emulsification. The effectiveness of the dispersant depends not only in the composition of the oil, but also on external factors like temperature, salinity, and light.

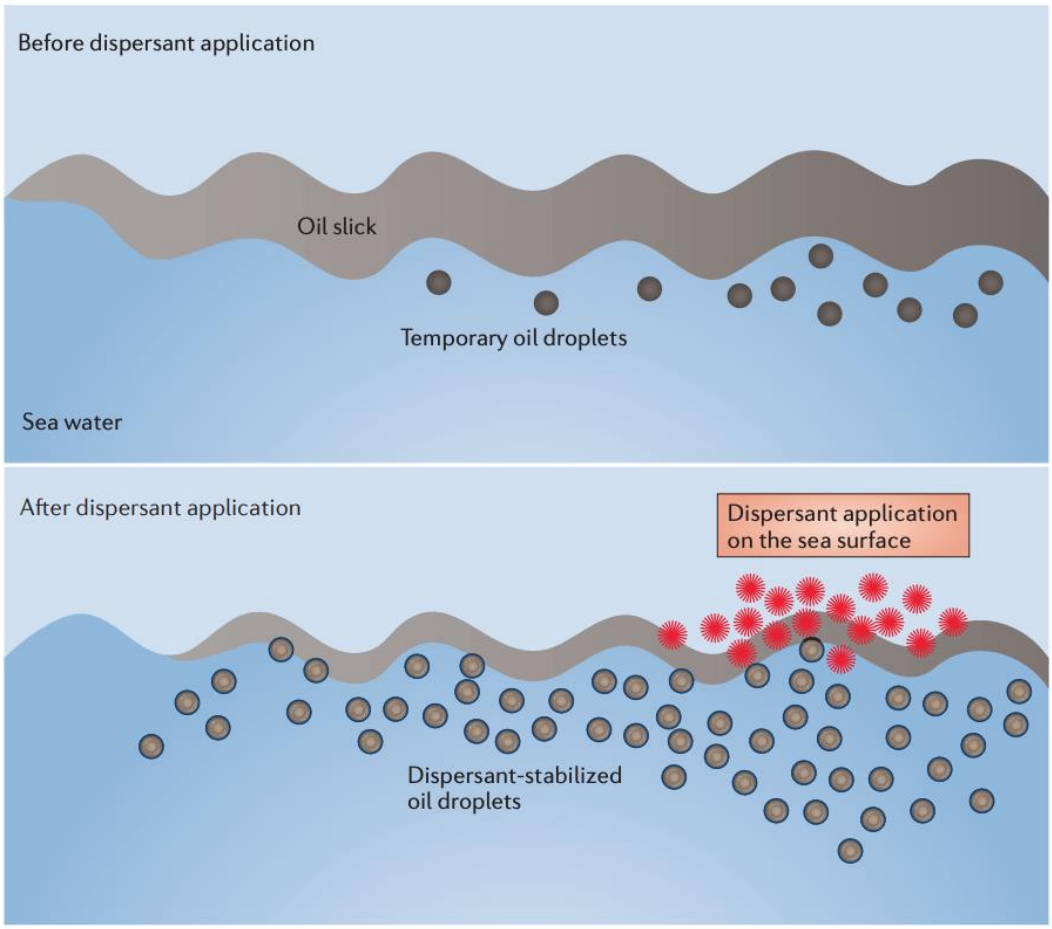


Figure 8 - Formation of oil droplets before and after dispersant application, retrieved from [2].

Recently, biosurfactants have been seen as the natural sustainable solution for countering oil spill effects [10][68]. Biosurfactants present many advantages over chemical dispersants like lower toxicity, and biodegradability. These compounds are natural dispersants that allow oil emulsification in water, and facilitate microbial degradation of crude oil contamination [10].

In 2010, the explosion and sinking of the Deepwater Horizon (DWH) drilling platform in the Gulf of Mexico resulted in the first release of dispersants to deep waters [69]. Figure 9 illustrates the hydrocarbon degradation phenomenon that followed the event.

When the DWH event took place, the oil that was released from the well head led to the formation of a hydrocarbon column at a depth of 1000 m to 1300 m [70][71]. The oil on the water surface was treated with dispersants. In the seawater, oil coagulated with bacterial marine oil snow and other microorganism, then sinking down to the sediment surface. Such event is designated as marine oil snow sedimentation [72]. Oil components were transported both horizontally and vertically. Volatile hydrocarbons evaporated at the water surface. The hydrocarbons in the contaminated water column were degraded by pelagic microorganism in the upper ocean, leaving oil components into the higher trophic levels of the food web. Persistent oil components sank to the sea floor, and were consumed by benthic microorganisms that leveraged them as carbon and energy sources [73][74].

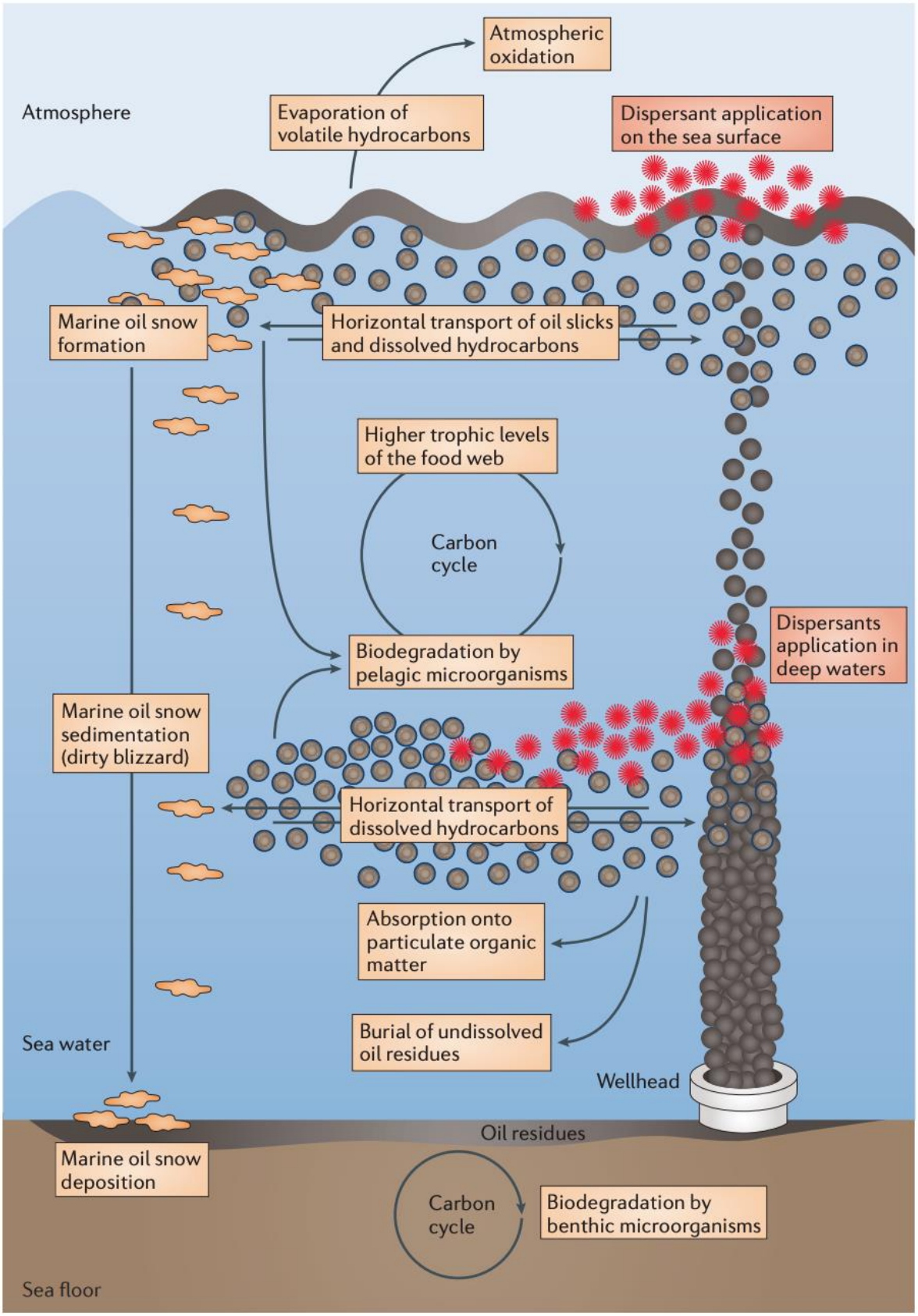


Figure 9 - Hydrocarbon degradation following the Deepwater Horizon oil spill, retrieved from [2].

2.3.4 Bioremediation

Water contamination with petroleum hydrocarbons raises global awareness due to environmental and health concerns. Consequently, bioremediation emerged has an effective, sustainable, and “green” technology to counter this problem.

Bioremediation can be described as the use of microorganisms to accelerate the degradation of oil or other environmental toxic compounds in an attempt to completely eliminate or convert those into non-toxic compounds [75][10]. This technology is not only cost effective considering that the process uses a microbial culture, but also environmentally friendly considering that the end product is mostly not harmful to the environment [76].

The success of bioremediation in a contaminated environment depends on the presence of indigenous microorganisms capable of degrading the existing hydrocarbons. Previous studies in the field delved into understanding the possible factors that would inhibit the growth of these types of microorganisms in seawater. A pioneering study reported that the concentrations of nitrogen and phosphorus in the water environment could constrain their growth [77]. Hence, it can be expected that the addition of nitrogen and phosphorus fertilizers in a given environment would improve the biodegradation of oil.

Bioavailability refers to the rate of substrate mass transfer into the microbial cells relative to their intrinsic catabolism and excretion [78]. Since it varies with individual oil components further studies using complex mixtures of hydrocarbons are a priority in order to increase the efficiency of bioremediation [79].

When surfactants are applied in concentrations above their CMC values, they hold the capability to reduce interfacial tensions, increase the emulsification of hydrophobic pollutants, and improve the solubility of hydrocarbons. Thus, enhancing the bioavailability of hydrophobic organic compounds facilitating their biodegradation [80][81].

Although, chemical surfactants can increase both bioavailability and biodegradation [80] they also have negative impact on the environment, which led to the increasing interest in bioremediation strategies using biosurfactants instead [82][83][76].

A study on the effectiveness of biosurfactants also revealed that natural surfactants such as rhamnolipids and sophorolipids are more effective in facilitating hydrocarbon degradation than chemical surfactants [84].

In 2008, Whang et al. have investigated the effectiveness of rhamnolipids and surfactin on enhancing diesel solubility, biomass growth, and biodegradation efficiency. In this study, it was proved that these biosurfactants improved both solubility and biodegradation of phenanthrene, which promotes these compounds as useful tools for bioremediation in sites polluted by PAHs [10].

In 2010, Sponza et al. has also reported 90% improvement on the efficiency of PAH removal using rhamnolipids at 15 mg/L, which is a positive indicator towards the potential of these biosurfactants for waste water treatment [85].

In 2015, Sajna et al. conducted an experiment with the purpose of understanding the impact that biosurfactants produced from a *Pseudozyma* isolate would have on the crude oil degradation process performed by a microbial culture designated by *Pseudomonas putida* MTCC 1194 (*P. putida*) [86]. In this study, it was verified that the MEL produced by the *Pseudozyma* has improved the crude oil degradation by *P. putida*. Such findings led to the conclusion that supplementing *P. putida* culture with a biosurfactant like MEL can be a cost-effective strategy for improving biodegradation of oil.

Even though biosurfactants can benefit bioremediation, when used at high concentrations they can have an adverse effect by performing antimicrobial activity. Hence, finding of the optimal concentration or DOR should be a concern when attempting to leverage biosurfactants for enhancing hydrocarbon degradation [86].

2.3.5 Culturing marine bacteria

The accuracy and generalizability of an experiment is strictly tied to how close the experimental environment models the real world. In the context of bioremediation, the accuracy of its experiments heavily relies in the availability of marine bacteria for experimentation in laboratory. The culturing of marine bacteria is therefore a topic of paramount importance since it can provide both an accessible and accurate way for evaluating bioremediation performance. Without it researchers would be stuck with either laboratory testing using standard microorganism stocks or field trials. Both these approaches are far from ideal, considering that field trials are costly and might have downside effects in the environment, and standard stocks produce inaccurate experimental results that usually do not generalize well when applied in the real environment [87].

The term “uncultured” is used to refer to bacteria that have not yet been cultured in laboratory. Once these uncultured strains are successfully isolated in laboratory, they can be referred as “previous uncultured” bacteria [88].

It is know that there exist more bacteria in the surface ocean than what can be cultured by the traditional approach of plating a sample into a selective media [89]. In 1987, all recognized bacterial divisions possessed cultured representatives. 20 years have passed and another 100 divisions were proposed but only 30 had a cultivated representative. In 2008, it was reported that there are only 7031 microbial species whose taxonomy has been completely validated [90].

Morris et al. suggested that, on average, 35% of the cells in the ocean surface layer are SAR11, and in some samples it can reach densities of 450 000 cells ml^{-1} [87]. SAR11 is a clear example of the problem that has been haunting researchers from this field for decades, i.e., the impossibility of culturing even the most abundant microbes in the sea through standard procedures.

Thus, despite new bacteria still being cultured with success by only variating the media and growth conditions, the novel techniques clearly diverge from traditional methodologies by employing single cell and high-throughput strategies, with the goal of better simulating their natural environment, increasing the length of incubation and reducing the concentration of nutrients [91][92][93].

It appears that establishing culture representatives for all bacterial divisions is still a major challenge, and such hinders the development of marine biodiscovery [94]. There is also the difficulty of accessing the genetic information of uncultured organisms, and from a research perspective, it is not as efficient as having the target organism available for experimentation in laboratory [95].

3 Materials and Methods

3.1 Seawater samples

Seawater (SW) was collected from coordinates 38º24.977N, 8º58.073W at a depth of 4 meters by Instituto Português do Mar e da Atmosfera (IPMA). This sampling station is situated at a distance of 3 to 5 NM from the coast of Setubal, Portugal and the water column has a depth of 74 m.

The samples were kept in a refrigerated chamber at 4°C for a maximum of 14 days before being used.

3.2 Surfactants, dispersants and biosurfactant formulation

All the bioremediation experiments were tested against the chemical dispersant Corexit 9500 (Nalco Holding Company) which was used in a dispersant to oil ratio (DOR) of 1%.

The emulsification capability and stability experiments were tested against the well-known chemical surfactant Sodium dodecyl sulphate (≥98,5%, Sigma-Aldrich).

3.3 MEL fermentation

3.3.1 Microorganism

The microorganism used to produce mannosylerythritol lipids was *Moesziomyces antarcticus* PYCC 5048^T provided by the Portuguese Yeast Culture Collection (PYCC), CREM, FCT/UNL, Caparica, Portugal. The strain was plated in yeast malt agar (yeast extract 3 g/L, malt extract 3 g/L, peptone 5 g/L, D-glucose 10 g/L and agar 20 g/L) and grown for 3 days at 30°C. Stocks were then made by growing the yeast in liquid medium and storing it in 20% v/v glycerol at -80°C.

3.3.2 Medium and growth conditions

In order to produce MEL an inoculum was prepared according to the procedure referred in [96]. Briefly, *M. antarcticus* stock was transferred to a mineral medium consisting of 3 g/L $NaNO_3$, 0.3 g/L $MgSO_4$, 0.3 g/L KH_2PO_4, 1 g/L yeast extract (OXOID) and 40 g/L D-glucose, that was previously

sterilized in an autoclave at 121 °C and 1 bar for 20 minutes. The inoculum was then incubated in an orbital (Agitorb 200, Aralab) for 2 days at 250 rpm and 27°C.

After, 10% (v/v) of the inoculum was used to start the fermentation in fresh media. Two conditions were tested, one with 60 g/L of D-glucose added at day 0 and 40 g/L at day 4 and the other with 60 g/L of D-glucose added at day 0 and 21 g/L of soybean oil added at day 4. The fermentations were maintained in an orbital at 250 rpm and 27°C for 10 days.

Both the inoculum and the fermentations were conducted in 250 mL Erlenmeyer flasks, with a working volume of 50 mL. Duplicates were made for each experiment.

3.3.3 Extraction of MEL

To recover MEL from the fermentation broth a liquid-liquid extraction was performed with ethyl acetate. The fermentation broth was first centrifuged for 10 min at 6000 rpm. The supernatant was transferred to a separator funnel and an equal amount of ethyl acetate was added to extract the MEL (this procedure was repeated twice). The organic phase was then transferred to a round bottom flask and the sample was concentrated using a rotary evaporator (Rotavapor R-3, BUCHI). The aqueous phase was discarded. The pellet was washed with equal amount of ethyl acetate, and the MEL in the organic phase was concentrated using the same procedure.

3.3.4 Biomass growth

Biomass growth was analysed by measuring the cell dry weight (CDW) of the samples taken at day 2, 4, 7 and 10. The 1 mL samples were centrifuged (Sartorius 1-15P, Sigma) at 10000 rpm for 5 min resulting in the formation of a pellet, containing the biomass, and a supernatant, which was then removed and stored. The pellet was then washed twice with Milli-q water and left to dry at 60°C for 48 hours. After, the dry biomass was weighted and the CDW was calculated.

3.3.5 D-glucose and nitrate quantification

The samples D-glucose and nitrate content was determined using high performance liquid chromatography (HPLC). The previously collected supernatant was first diluted with H_2SO_4 0.05 M, in a proportion of 1:2, and centrifuged to precipitate any cellular content still in the sample. This supernatant was then further diluted with a proportion of 1:10 and transferred to an HPLC vial. Samples were then analysed in an HPLC system (Merck Hitachi, Darmstadt, Germany) equipped with a refractive index detector (L-2490, Merck Hitachi, Darmstadt, Germany) and a Rezex ROA Organic Acid H^+ (8%) column (300 mm× 7.8 mm, Phenomenex) at 65°C, using sulfuric acid 0.005 M as mobile phase at 0.5 ml/min.

3.3.6 MEL and fatty acids quantification

MEL and fatty acids contents of the samples taken at day 4, 7 and 10 were determined through methanolysis and gas chromatography (GC) of methyl esters [87] [88] [98].

For the transesterification reaction a mixture of pure methanol (cooled to 0°C) and acetyl chloride was prepared in a 20:1 v/v proportion. An internal standard of heptanoic acid (4% in hexane) was also prepared. Since transesterification only works in the absence of water, samples were lyophilized (Alpha 1-2 LD plus, Christ) for 48 hours. Afterwards, the samples were weighted and 2 mL of the methanol/acetyl chloride mixture was added as well as 100 µL of the internal standard. The samples were incubated for 1h at 80 °C for the reaction into methyl esters. The resulting product was extracted with 1 mL of hexane and 1 µL of the organic phase was injected in a GC system (HP5890, Hewlett-Packard), equipped with a FID detector and an HP-Ultra 2 column with the following set-up. The oven initial temperature was 140 °C and it was increased at a rate of 15°C/min until reaching 170°C, then at 40 °C/min until reaching 210°C and at 50 °C/min until the final temperature of 310 °C. Nitrogen gas was used at a flow rate of 25 ml/min.

MEL concentration was obtained through the amount of C8, C10 and C12 while fatty acids concentration was obtained through the amount of C14, C16 and C18.

3.4 Physicochemical characterization of MEL

3.4.1 Emulsification Index (EI_{24}) Assay

Emulsification index was measured using an adaptation of the method referred in [99]. 4 mL of kerosene was added to 4 mL of milli-Q water containing either MEL (0.01, 0.1, 1 g/L) or SDS (1 g/L) in a glass test tube and vortexed at high speed for 2 min. After 24 hours the height of the emulsion was measured, and the emulsification index was calculated using the equation 1.

$$EI_{24} = \frac{Height\ of\ emulsion\ layer}{Height\ of\ liquid\ column} \times 100 \quad (1)$$

Triplicates were made for every tested condition and results were presented with the average ± standard deviation.

3.4.2 Stability testing

MELand SDS solutions were tested for the effect of pH, salinity and temperature in their emulsification capability (E_{24}) and their surface tension. Two concentrations of MEL (0.1 and 1 g/L) and one concentration of SDS (1 g/L) were analysed.

The pH of the MEL and the SDS solutions (in Milli-q water) was adjusted between 2 and 12 by addition of H_2SO_4 (0.5 M) or NaOH (1M) [100]. The thermal stability was analysed by submitting each sample to temperatures of -20, 20, 40 and 80 °C for one hour and then leaving it to rest a room temperature for 2 hours prior to testing. The effect of salinity was analysed by adding the surfactants to solutions with different concentrations of NaCl: 5 g/L, 10 g/L, 20 g/L and 40 g/L.

3.5 Marine Isolates

3.5.1 Isolation and screening of hydrocarbon degrading microorganisms

1 mL of the seawater sample from Setubal was added to a 250 mL shake-flask filled to 1/5 of its volume with marine broth medium (PanReac AppliChem) and incubated for 48 h at 27°C and 250 rpm. Subsequently samples of 20 uL were each used to streak six marine broth agar (PanReac AppliChem) plates which were then incubated for 48 h at 30°C. Morphologically different colonies in the plates were picked and streaked into MB agar plates to obtain pure cultures. The cultures were incubated at 30°C for 48 h, and afterwards stored until needed at 4°C.

Selection of hydrocarbon degrading strains was made by growing the previously plated microorganisms in BH media supplemented with 1% (v/v) of hexadecane as the sole carbon source. The cultures were incubated for 9 days (27°C and 250 rpm) and optical density at 600 nm was measured at regular intervals in order to quantify growth. The hexadecane remaining at the end of the experiment was extracted with hexane, its volume reduced to 0.9 mL using a rotary evaporator (Rotavapor R-3, BUCHI) and liquid nitrogen and 0.1 mL of hexane with 1 mg/mL dodecane was added. The hexadecane was then quantified through GC-FID analysis using the same settings as previously described for MEL and lipids quantification. Three strains (MS1, MS2 and MS8) were selected for further testing based on measured growth and hexadecane degradation.

Seed cultures stocks were prepared by inoculating from the culture plates of the previous selected strains into 100 mL Erlenmeyer flasks filled to 1/5 of its volume with marine broth and 1% of D-glucose as the carbon source. The inoculums were then incubated in an orbital at 250 rpm and 27°C for 1 day for MS2 and 2 days for MS1 and MS8. Stocks were prepared using equal volumes of seed culture and glycerol and stored at -80°C.

3.5.2 Medium and growth conditions

Marine isolates growth tests were performed in 100 mL Erlenmeyer's containing 20 mL of growth medium. The two following media were used for growth tests: Marine Broth (PanReac AppliChem); Bushnell-Haas broth composed of 0.2 g/L $MgSO_4$, 0.02 g/L $CaCl_2$, 1 g/L KH_2PO_4, 1g/L $(NH_4)_2HPO_4$, 1g/L KNO_3, 0.05 g/L $FeCl_3$.

In order to optimize growth different temperatures (27°C, 37°C), inoculum incubation times (24h, 48h, 72h), seed culture volumes (2%, 4%, 6%, 12% v/v) and carbon sources (10 g/L glucose, 20 g/L glucose, 20 g/L glucose + 1g/L yeast extract) were tested.

Bacteria cells growth was determined by measuring the optical density at 600 nm in a spectrophotometer (Multiskan Go, Thermo Scientific) of samples taken at regular intervals.

3.5.3 Growth inhibition of marine isolates by MEL and Corexit 9500

The effects on the growth of the isolates by different MEL concentrations (1, 5, 10, 20, 100, 500 ppm), Corexit 9500 (2.5 ppm) and Crude Oil (50 ppm) was studied.

Cell growth was measured by analysing the optical density at 600 nm of samples taken at regular intervals.

3.6 Bioremediation

3.6.1 Procedure for mixing of dispersant/MEL and crude oil

For the bioremediation experiments using crude oil the procedure mentioned in [101] was followed for the mixing of dispersant/MEL in crude oil prior to application. Corexit 9500 (5% DOR) and MEL (12.5% DOR) were added to individual recipients containing oil which were then:

- Heated at 50 °C for 5 min
- Shaken vigorously for 1 min
- Sonicated in an ultrasonic bath for 5 min
- Shaken vigorously for 1 minute

The oil-dispersant/MEL mixture was then used in the respective amounts in the bioremediation experiments.

3.6.2 Enhanced bioremediation tests

Bioremediation was tested in seawater and in BH medium supplemented with 10 % v/v of two different marine isolates (MS1 and MS2).

Two different carbon sources were used for the bioremediation experiments: crude oil sample (MC-2016-204-S5 fresh, Statfjord, SINTEF) and a mixture of seven linear alkanes. The alkanes used were decane (99+%), dodecane (99%), n-tridecane (99+%), tetradecane (99%), n-hexadecane (99%), n-heptadecane (99%) and octadecane (99+%), each corresponding to 14.3% v/v.

The following table contains the conditions tested in the four sets of bioremediation experiments.

Table 6 – Summary of the bioremediation tested conditions

Crude oil 50 ppm	**Crude oil 50 ppm, 500 ppm**	**Crude oil 50 ppm**	**Alkane mixture 50 ppm**
4, 7 days	7 days	7 days	7 days
SW (sterile)	SW (sterile)	BH	BH
SW	SW	BH + MS	BH + MS
SW + Corexit (5% DOR)	SW + Corexit (5% DOR)	BH + MS + Corexit (5% DOR)	BH + MS + Corexit (5% DOR)
SW + MEL (12.5% DOR)	SW + MEL (12.5% DOR)	BH + MS + MEL (12.5% DOR)	BH + MS + MEL (12.5% DOR)
SW + supernatant (1.7 g/L MEL, 12.5% DOR)		BH + MS + MEL (25% DOR)	BH + MS + MEL (25% DOR)

Duplicates were made for each condition of the seawater bioremediation experiments to analyse the degradation of hydrocarbons through GC-FID and through FTIR (LAIST internal method).

3.6.3 Hydrocarbon extractions

At the end of each experiment 10 mL of HCl (15 % v/v) was added to the samples in order to prevent further hydrocarbon degradation by microorganisms. In order to later quantify extraction losses 0,1 mL of pristane (10 g/L in n-hexane) was also added as an internal standard. Samples were then stored at 4 °C to avoid loss of hydrocarbons due to volatilization and degradation while awaiting extraction. No sample was stored for more than 3 days.

Extractions of the hydrocarbon contents were performed using n-hexane (≥95%, HPLC grade, Fisher Chemical) as the separation solvent. Each shake-flask content was first transferred to falcon tubes and a total 50 mL of hexane was added. Another 25 mL of hexane was used to rinse each flask and then added to a falcon tube. The contents were then centrifuged at 5000 rpm for 5 min to precipitate

any formed biomass and help the transfer of the hydrocarbons to the organic phase. The upper phases (organic) were transferred to a round bottom flask and the solvent, n-hexane, was evaporated using a rotary evaporator until the volume was roughly 2 mL. The concentrated sample was filtered with a Pasteur pipette containing cotton, silica gel and anhydrous sodium sulphate and transferred to a glass vial. Its volume was further reduced to 0,9 mL using liquid nitrogen and the samples were then completed with the addition of 0.1 mL of 5-α-androstane (10 g/L in n-hexane) as a second internal standard. Samples were stored at -20 °C prior to GC-FID analysis.

3.6.4 GC-FID analysis of total hydrocarbon concentration (THC)

The bioremediation samples THC was analysed by gas chromatography (Hewlett-Packard, HP5890) using the following set-up. Initial oven temperature was 60°C and held for 2 min. Temperature was then increased at a rate of 6°C/min until reaching 310°C, where it was held for 5 min. Injector and detector temperature was 300°C and 310°C respectively. Purge was set to turn on after 0,66 min. Injected sample volume was 1 µL.

Calibration curves were made for both crude oil and alkane mixture using a series of dilutions (5 g/L, 2 g/L, 1 g/L, 0.5 g/L, 0.2 g/L in n-hexane) with 5-α-androstane as an internal standard. A relative response factor (RRF) was then calculated using equation 2.

$$RRF = \frac{A_{std} \times C_{andr}}{A_{andr} \times C_{std}} \quad (2)$$

For the crude oil samples, the total area (A_{total}) was obtained by automatic integration of peaks with a minimum area to height ratio of 1 from 6 min to 50 min of the GC-FID spectrum. The THC area (A_{THC}) was then calculated by subtracting the area of the internal standards (A_{andr}, A_{pris}) to the total area (equation 3).

$$A_{THC} = A_{total} - A_{andr} - A_{pris} \quad (3)$$

For the alkane mixture, the seven hydrocarbon areas were individually integrated and A_{THC} is calculated as the sum of those areas.

Internal standards of pristane and 5-α-androstane were used to quantify the percentage of hydrocarbons recovered in the extraction using equation 4.

$$Recovery\ (\%) = \frac{A_{prist}}{A_{andr}} \times 100 \quad (4)$$

The concentration of hydrocarbons in each sample (C_{THC}) was calculated using the equation 5.

$$C_{THC} = \frac{A_{THC} \times C_{andr}}{A_{andr} \times RRF \times Recovery} \quad (5)$$

The degradation of crude oil/alkanes was expressed as the percentage of the THC in the sample ($C_{THC\,i}$) in relation to the THC in the appropriate control made at day 0 ($C_{THC\,0}$), before the extractions.

$$Degradation\ (\%) = \frac{C_{THC\,0} - C_{THC\,i}}{C_{THC\,0}} \times 100 \quad \textbf{(6)}$$

4 Results and discussion

4.1 MEL production by *M. antarcticus*

With the objective of producing MEL for use in later experiences, *M. antarcticus* was cultivated for 10 days using two different sets of carbon sources, soluble and insoluble. The first one used D-glucose as the only substrate with an initial concentration of 60 g/L and a second addition of 40 g/L at day 4 [Glu60,0;Glu40,4]. The second one had 60 g/L of D-glucose as the initial substrate and 21 g/L of soybean oil (SBO) added at day 4 [Glu60,0;SBO21,4]. The reasoning behind this last condition was that using oils as the sole carbon source is reported to produce high titres of MEL with high fatty acid content, which increases the complexity of purification process, and using soluble carbon sources typically yields low titres of MEL but with smaller fatty acids content [102][41].

As such, the glucose only fermentations were expected to produce both small titres of MEL and fatty acids content, while the glucose and SBO fermentations were expected to produce higher titres of MEL with only a small increase in fatty acids content. Nitrate concentration during the fermentation was monitored since it is essential for cell growth. Fermentation profiles are presented in Figure 10 for [Glu60,0;Glu40,4] and Figure 11 for [Glu60,0;SBO21,4].

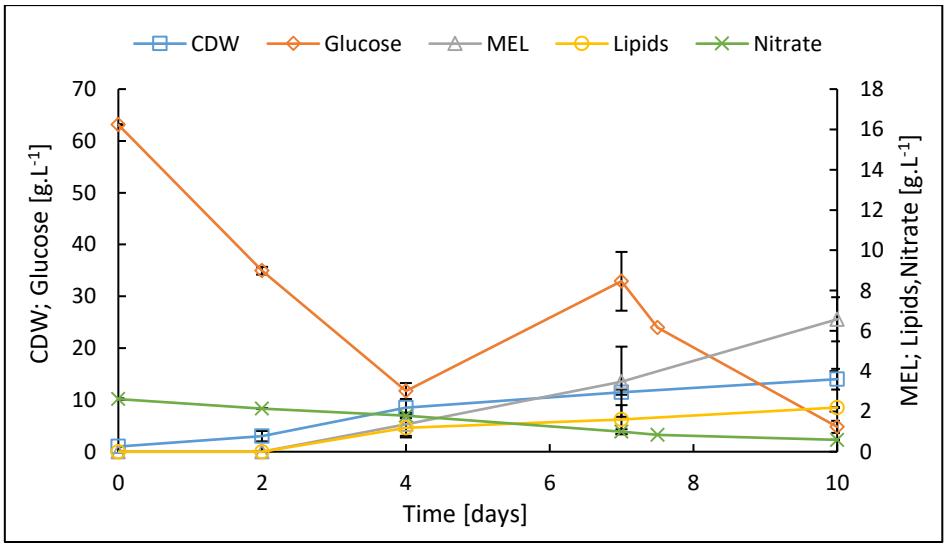


Figure 10 – Fermentation profile of *M. antarcticus* fed with D-glucose at day 0 (60 g/L) and 4 (40 g/L). Results are presented as the average of duplicates.

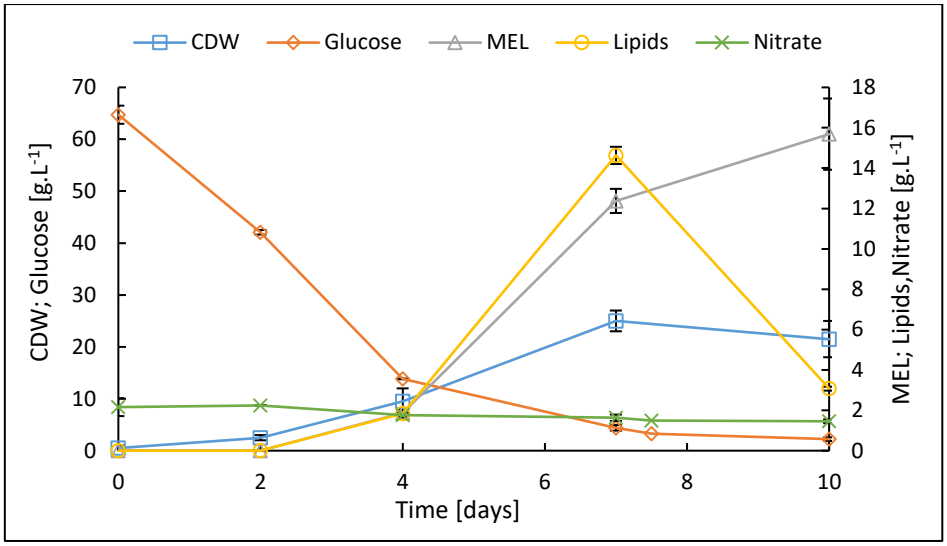


Figure 11 - Fermentation profile of *M. antarcticus* fed with D-glucose at day 0 (60 g/L) and soybean oil (21 g/L) at day 4. Results are presented as the average of duplicates.

Cell growth on [Glu60,0;SBO21,4] was significantly faster than on [Glu60,0;Glu40,4], after the addition of the different substrates at day 4, which shows a faster incorporation of the lipids into the cells than of glucose. In average, biomass had a 22% increase from day 7 to day 10 in [Glu60,0;Glu40,4] compared to a 14% decrease at the same time in [Glu60,0;SBO21,4]. This likely happened due to consumption of the previously incorporated lipids because of the low concentration of carbon sources at that time in [Glu60,0;SBO21,4], with only 19.0 g/L remaining at day 7 and 5.3 g/L remaining at day 10, in average. For comparison, [Glu60,0;Glu40,4] still had an average of 34.5 g/L at day 7 and 7.0 g/L at day 10 of carbon sources.

As expected, average MEL titres for [Glu60,0;SBO21,4] at 15.7 g/L were way higher than those of [Glu60,0;Glu40,4], at a mere 6.6 g/L. However, the average purity ratio of the obtained MEL was higher for [Glu60,0;SBO21,4] at 84% compared to [Glu60,0;Glu40,4] at 75%, which was better than expected. This purity is defined as the amount of MEL in the final organic product assuming that it only contains lipids and MEL.

The composition of MEL lipidic chains for the two conditions tested is shown in Table 7. As was previously reported in [15], the MEL produced by *M. antarcticus* using soybean oil as a carbon source has mainly C10 lipidic moieties. With glucose as the only fed carbon source, MEL's lipidic profile changes to C12 as the highest contributor. These profiles affect the Hydrophilic–Lipophilic Balance of MEL which in turn will affect its solubility in aqueous or in organic solutions and determine its emulsification capabilities [103].

Table 7 - Fatty acid profile of extracted MEL from *M. antarcticus* cultivation.

	C8	C10	C12
[Glu60,0;Glu40,4]	3.6 ± 0.6%	45.4 ± 0.6%	51.0 ± 0%
[Glu60,0;SBO21,4]	14.0 ± 0.3%	80.8 ± 2.5%	5.2 ± 2.2%

The MEL chosen for the stability, toxicity and bioremediation assays was the one produced from [Glu60,0;SBO21,4].

4.2 Stability of MEL determined by Emulsification Index

The usefulness of surfactants depends on their ability to retain their properties over a wide range of temperatures, pH and salinity. As such, the effects of those factors on the emulsification index was tested. Concentrations above the critical micellar concentration (CMC) were used for MEL (CMC = 0.02 g/L [55]) and below for SDS (CMC = 2.3 g/L [104]). Kerosene was used as the organic phase.

The major limiting factor in bioremediation of oil is the hydrocarbons solubility, which can be increased with resource to surfactants that help lower the interfacial tension and increase emulsification [105]. It is then of particular interest to MEL's application in bioremediation the effects that seawater's pH and salinity have in its emulsification capability.

4.2.1 MEL concentration effect

The emulsifying capabilities of MEL in kerosene at pH 7 and room temperature, were tested for different MEL solutions: one concentration below CMC (0.01 g/L) and two above (0.1 g/L, 1 g/L) were also tested. The results can be seen in Figure 12. Blanks of water/kerosene were also tested and no emulsion was obtained.

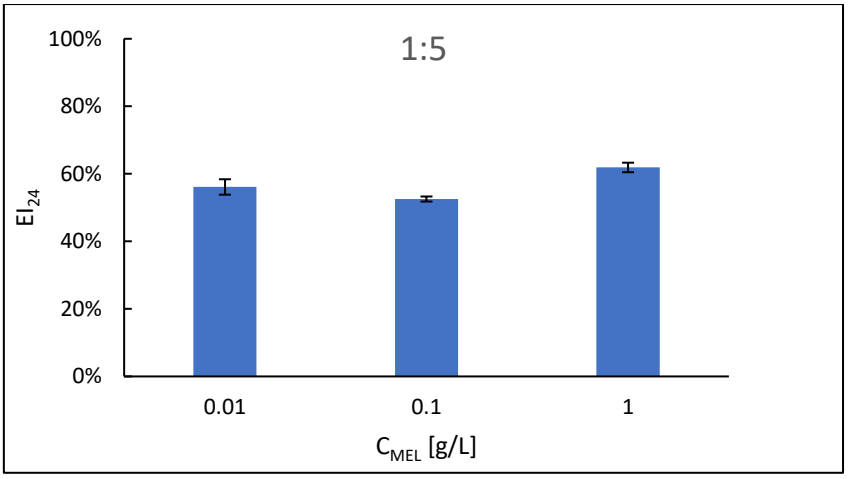


Figure 12 – Effect on the emulsification capability of MEL for different concentrations.

Since the highest emulsification index of 61.9 % ± 1.9 % for kerosene was achieved for a concentration of 1 g/L of MEL, this was the solutionused for the following emulsification stability tests.

The surface tension of 0.1 g/L of MEL solution was 26.8 mN m^{-1}, 5% higher than previously reported for the same concentration of MEL in water [55].

4.2.2 pH effect

The effect of pH on the surfactants emulsification index (in kerosene) was tested for pH of 2, 4, 7, 10 and 12 and the results are shown in Figure 13 and **Error! Reference source not found.**, respectively.

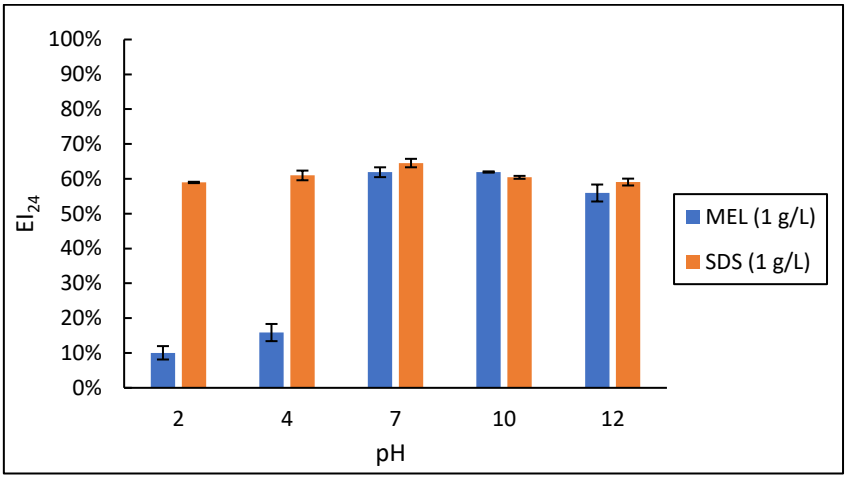


Figure 13 – Influence of pH on the emulsification capability of MEL and SDS.

The formulation of MEL showed high emulsification stability of kerosene at high pH values, but significantly reduced stability at low pH. Stable emulsification occurred for the full range of pH tested for SDS. For the use of MEL in bioremediation this lack of emulsification capability at low pH is not

problematic since seawater typically has a pH range of 7.5 to 8.4, however it may limit the relevance of MEL in other fields.

4.2.3 Temperature effect

The thermal stability of a biosurfactant is of high interest since many purification or sterilization steps as well as end applications require the use high temperatures. As such, the effect of submitting the surfactants to temperatures of -20, 20, 40 and 80 °C for 1 hour was tested and the results are presented in Figure 14 for emulsifying capability.

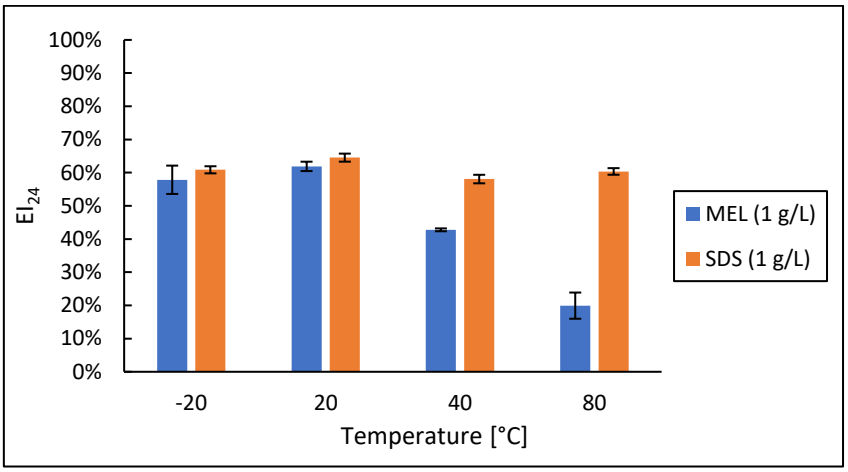


Figure 14 - Influence of temperature on the emulsification capability of MEL and SDS.

Emulsion stability of the biosurfactant formulation seems to steeply decrease for temperatures higher than 40 °C, while SDS shows stable emulsification at all tested temperatures. The length of time (1 hour) which the surfactants were submitted to these temperatures may have played a key role in the decrease of emulsification capability of MEL that may not be seen at smaller exposure times. The solvent effect may also play a part in the loss of stability.

4.2.4 Salinity effect

The effect of salinity on the surfactants was tested for NaCl concentrations of 0, 5, 10, 20 and 40 g/L and the results are shown in Figure 15 for emulsification index.

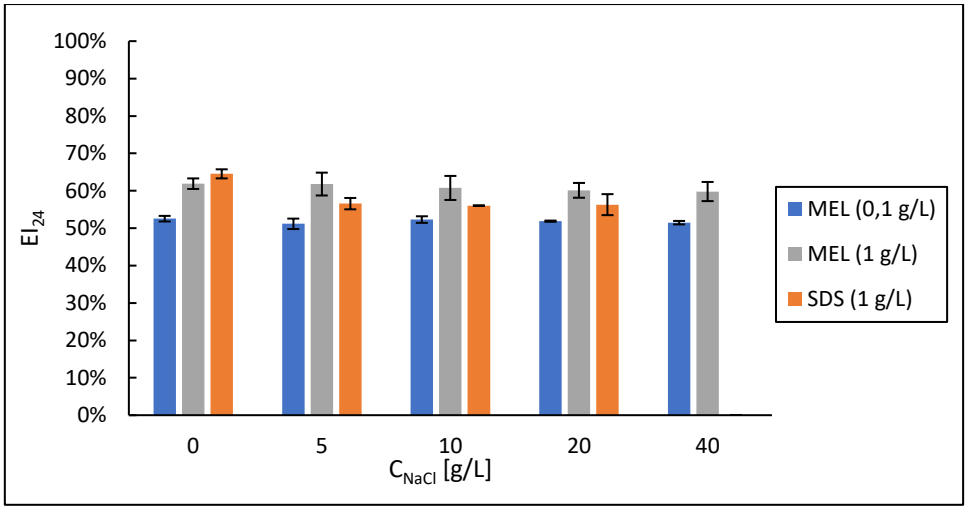


Figure 15 - Influence of salinity on the emulsification capability of MEL and SDS.

Little change to emulsification capability of the MEL formulation can be seen up to 40 g/L of NaCl. This suggests that MEL can maintain a stable emulsion in marine environments were salt concentration is typically 35 g/L. Other research [106] seems to indicate the stability of biosurfactants emulsification capabilities at high salinity. SDS emulsification capability slightly decreases with salinity up to 20 g/L but at concentration of 40 g/L of NaCl no emulsion had persisted after 24h.

4.3 Effect of MEL on the growth of hydrocarbon degrading marine bacteria

4.3.1 Isolation of marine strains

From the seawater samples obtained of the coast of Setubal, Portugal, 2% v/v was added to MB medium and incubated as previously described in Materials and Methods. The intent was to isolate a number of strains capable of growing or at least surviving in a nutrient deficient medium for later use in bioremediation experiments.

After two days of growth, samples were taken and spread plated in MB agar and incubated. A variety of cultures grew on these plates from which ten morphologically different colonies were selected and streaked into individual MB agar plates. These isolates were identified with the prefix MB and a number.

After incubation and growth of these isolates, a preliminary screening was done by eliminating morphologically similar colonies (MS3, MS4, MS5 and MS7). The remaining colonies were then screened for the ability to degrade hexadecane, as described in the following section. Figure 16 shows the plated cultures of the six remaining isolates.

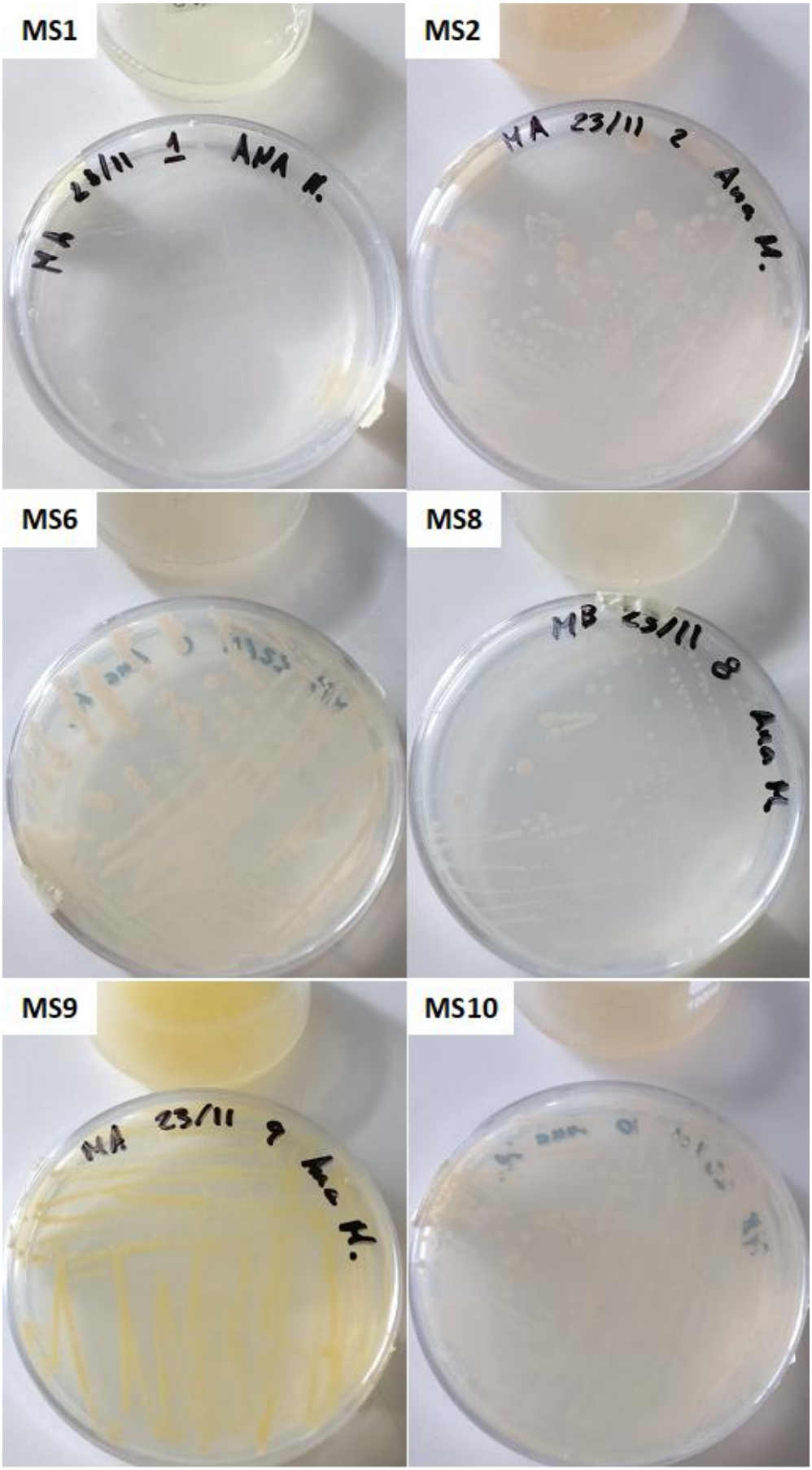


Figure 16 – Marine isolates in MB agar plates after 48h incubation at 30°C.

4.3.2 Selection of hydrocarbon degrading strains

In order to select marine isolates capable of degrading hydrocarbons, seed cultures of strains MS1, 2, 6, 8, 9 and 10 were added to BH medium supplemented with 1% (v/v) of hexadecane as the sole carbon source and incubated for 9 days. Culture growth was analysed through optical density measurements at 600 nm (OD_{600}) taken throughout the experience. Hexadecane was extracted in the end and its concentration determined through GC-FID in order to quantify its consumption.

Growth kinetics of the isolates are shown in Figure 17. We can see that some strains had a rapid growth in the first 3 days (MS1, MS9 and MS10), some had a slow but steady growth (MS2) while others only grew ever so slightly (MS6 and MS8).

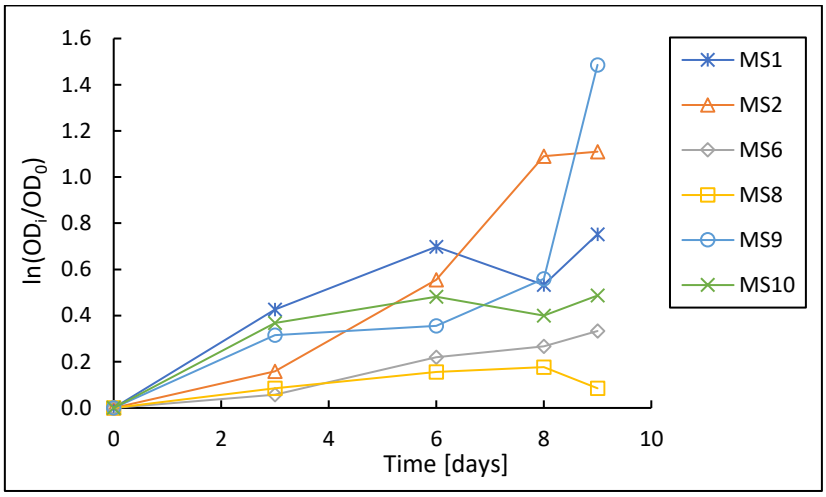


Figure 17 – Growth kinetics of marine isolates in BH medium with hexadecane as the carbon source incubated at 27 °C and 250 rpm.

Hexadecane consumption by each strain can be seen in Figure 18. MS8 and MS9 were the strains that degraded the most hexadecane, with 43% and 49% respectively. MS2 and MS6 were the ones that had the highest concentration of hexadecane after 9 days, with only 31% and 26% of degradation, respectively.

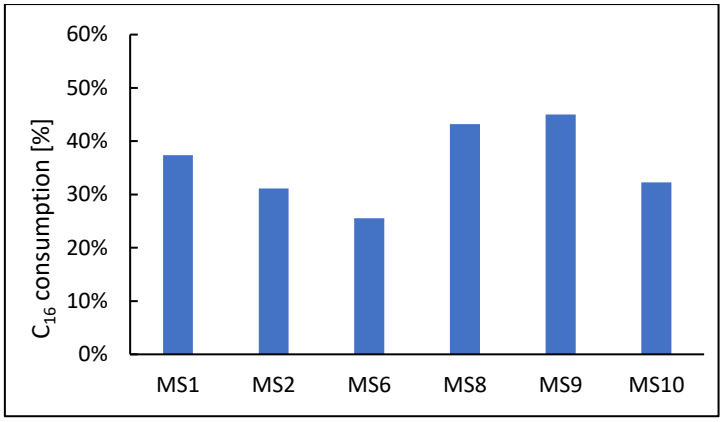


Figure 18 – Hexadecane consumption of marine isolates in BH media after 9 days incubation at 27 °C and 250 rpm.

MS9 had the highest hexadecane consumption, but it was not selected for use in the following experiments due to suspicion of being a contamination (based on its morphology and exponential growth kinetics). MS2 had the second highest growth rate so, although it had a low hexadecane consumption, was selected for further studies. Both MS8 and MS1, with the second and third highest hexadecane degradation values, respectively, were also chosen for further studies. It should be noted that MS8 had a very low growth rate in the tested conditions, which would constitute a problem in the analysis of its growth kinetics causing it to be dropped from bioremediation experiences.

4.3.3 Testing and optimization of growth methodology

In order to understand the effect of temperature on the growth of the isolates, the three previously selected strains were incubated at 27°C and 37°C for 2 days and OD_{600} measurements were taken at day 0 and 2. Growth rates are shown in Figure 19.

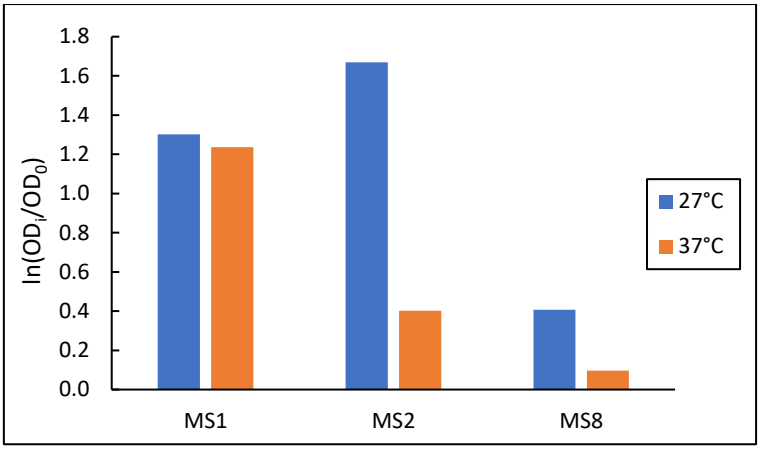


Figure 19 – Temperature effect on growth of marine isolates in MB medium after 48h incubation at 250 rpm.

All isolated strains showed maximum growth at 27°C. Temperature had a significant impact on strains MS2 and MS8 with growth at the lowest temperature being four times higher than at 37°C. For strain MS1 differences in growth between temperatures were not significant.

A second experiment was made in order to asses D-glucose consumption and the growth of biomass through time. The isolates MS1, MS2 and MS8 were incubated for 3 days at 27°C and 250 rpm and results are presented in Table 8. Isolate MS1 achieved maximum growth after 24 hours at an OD_{600} of 0.461 and CDW of 0.6 g/L, with no further increase in biomass between day 1 and day 3. Glucose consumption was also not detected after day 1. Isolate MS2 also reached maximum growth at day 1 with an OD_{600} of 1.558 and CDW of 3.1 g/L, and its biomass declined between day 1 and day 3. However, this decline could have been an autophagic degradation of the cells due to lack of carbon source since no glucose could be detected at day 3. As such, further supplementation of glucose could potentially increase the biomass of MS2. Isolate MS8 achieved its highest biomass values at day 3, with an OD_{600} of 0.548 and CDW of 0.6 g/L. In another short assay it was observed that MS8's OD_{600} after 2 days was 0.538. Note that this value is approximately the same that was obtained at day 3 in the previous experiment.

Table 8 – Cell dry weight, optical density at 600 nm and glucose concentration after 0, 1 and 3 days of growth in MB medium of marine isolates MS1, MS2 and MS8.

	Time [days]	0	1	3
MS1	CDW [g/L]	-	0.6	0.6
	OD (600 nm)	0.227	0.461	0.446
	Glucose [g/L]	20.0	16.5	17.0
MS2	CDW [g/L]	-	3.1	2.8
	OD (600 nm)	0.23	1.558	1.136
	Glucose [g/L]	20.0	4.5	0.0
MS8	CDW [g/L]	-	0.3	0.6
	OD (600 nm)	0.231	0.493	0.548
	Glucose [g/L]	20.0	13.3	15.3

Based on the previous results an inoculum growth period was established of 24 h for strain MS1 and MS2 and of 48h for strain MS8.

4.3.4 Assessing MEL impact on growth kinetics of selected strains

The influence of MEL in the growth kinetics of the marine isolates was assessed by growing the strains in both a rich (MB) and a minimal (BH) medium supplemented with D-glucose and different concentrations of the biosurfactant. For comparison purposes, conditions with Corexit 9500 and crude oil were also tested.

Growth curves of MS1 in MB medium can be seen in Figure 20. Most of the growth occurred in the first 24 h with results almost identical for all tested conditions. The condition with 50 ppm of crude oil had the highest activity. Considering the high concentrations of MEL used (100 ppm) these are very positive results.

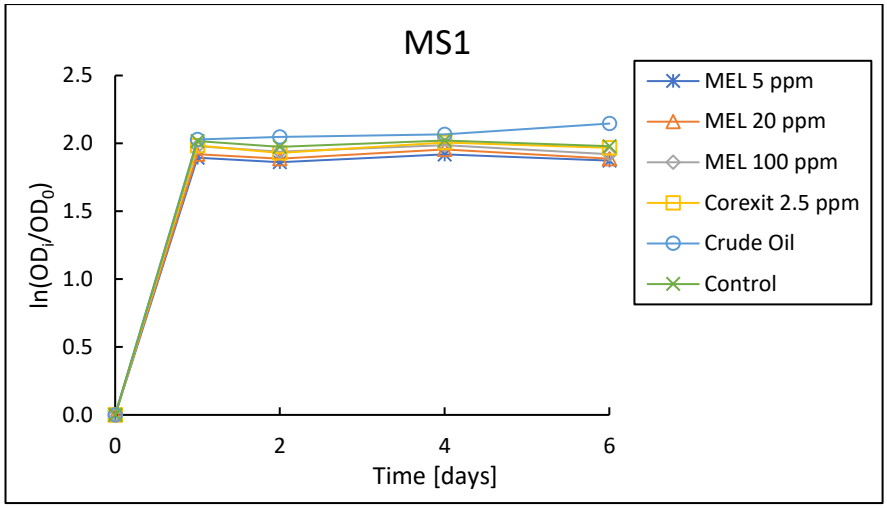


Figure 20 - Growth kinetics of MS1 in MB medium with 20 g/L of D-glucose and 1 g/L of yeast extract for different conditions.

Figure 21 shows MS2 growth kinetics in MB media. Again, all the conditions gave very similar values of growth which indicates that at the values tested none significantly inhibited the growth of MS2. It can also be noted that both Corexit 2.5 ppm and MEL 100 ppm gave the smaller growth curves, however these values are not very different from the control. This shows that the use of rich media provides nutrients enough to compensate for any inhibitory effect that could be observed on more stringent environments.

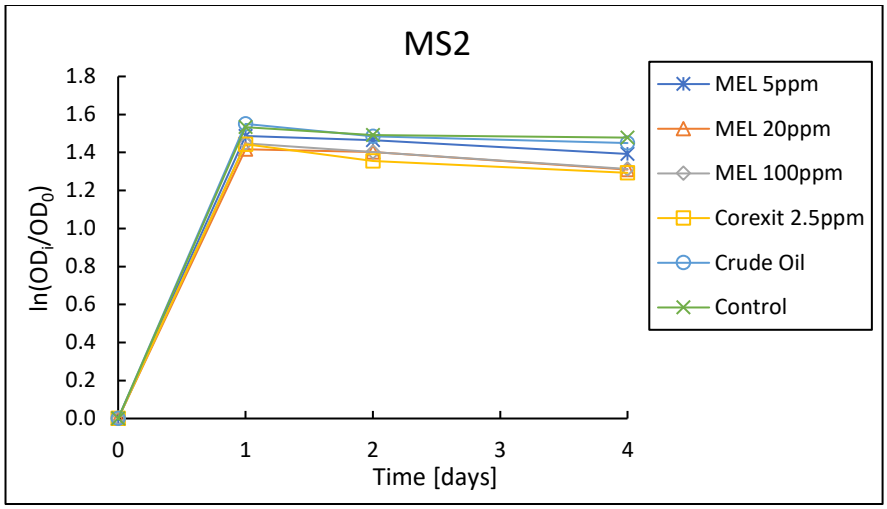


Figure 21 - Growth kinetics of MS2 in MB medium with 20 g/L of D-glucose and 1 g/L of yeast extract for different conditions.

Growth experiments of MS1, MS2 and MS8 in BH medium were highly inconsistent in terms of results. In a first set of experiments where MS2 and MS8 where streaked directly from the plates to the

inoculums and after which were grown in BH medium supplemented with 10 g/L of D-glucose the growth rates, of the tested conditions, were as seen in Figure 22 and Figure 23, respectively. Consistent growth was obtained for both strains as well as differentiation in rate depending on the conditions tested. The two isolates showed the smallest growth in the medium containing 2.5 ppm of Corexit. MS2 had the highest growth for the condition with crude oil, followed closely by the ones with MEL (1, 5, 10 and 20 ppm). On the other hand, crude oil seemed to have an adverse effect on MS8 while all tested MEL concentrations yielded growths either similar or higher than the control.

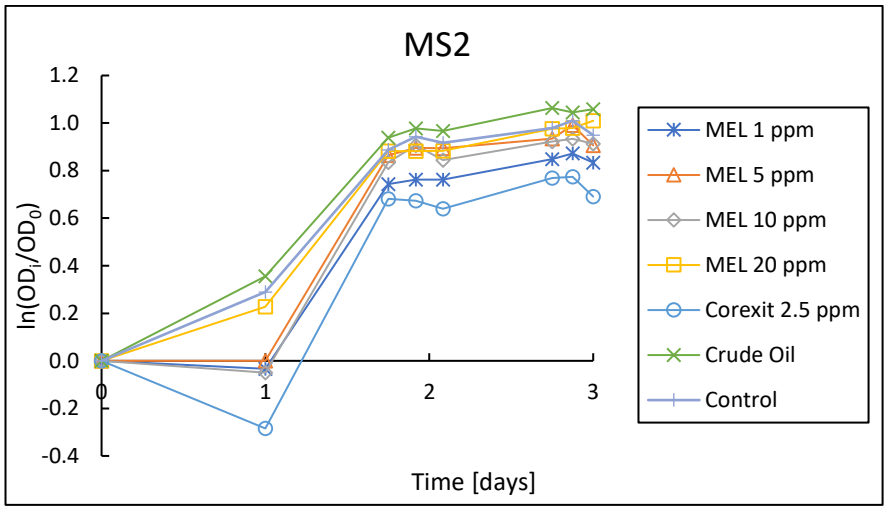


Figure 22 – Growth kinetics of MS2 in BH medium with 10 g/L of D-glucose for different conditions.

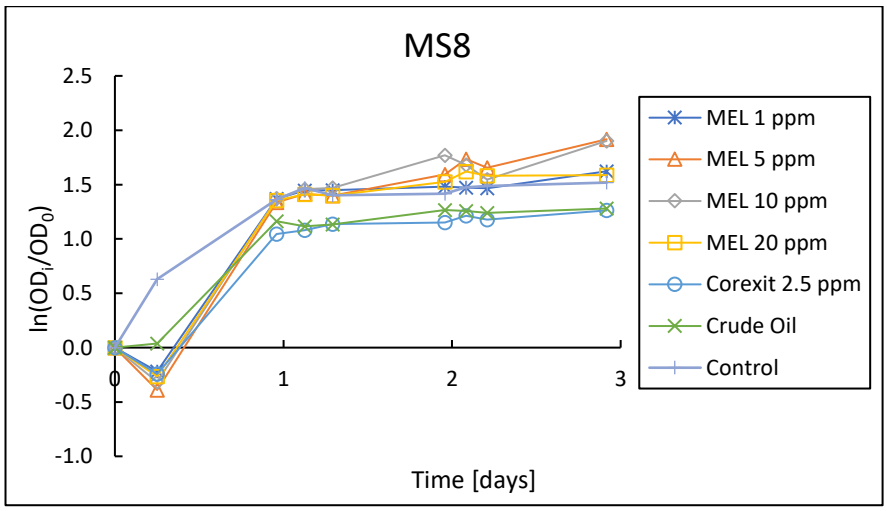


Figure 23 - Growth kinetics of MS8 in BH medium with 10 g/L of D-glucose for different conditions.

When the same experiment was tested in MS1 the culture didn't grow as can be seen in Figure 24. This is likely due to the difficulty of this strain in growing in poor medium. It could also be due to the time

difference between experiments, since this one was done from culture plates that were more than 2 months old, while the previous experiment was done when the culture plates had 10 days. However, the inoculum from which this experiment was started had grown to an OD_{600} of 0.464 which is coherent with the value previously obtained in the first cultivation of this strain.

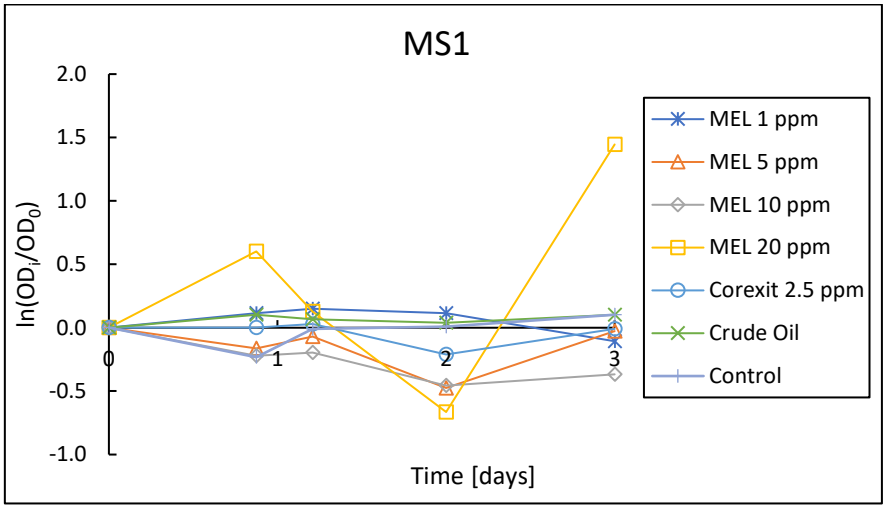


Figure 24 – Growth kinetics of MS1 in BH medium with 10 g/L of D-glucose for different conditions

A repeat of the first experiments was also attempted, this time with duplicates, and the average results can be seen in Appendix A1 for MS2 and MS8. None of the conditions tested for MS8 grew, and for MS2 the only growth occurred for the highest concentrations of MEL (100 and 500 ppm). This could have been either a contamination or the species may be feeding on MEL. As for the inability to reproduce previous results, it could be due to a number of factors, like a smaller starting seed volume was used (2% for MS2 and 4% for MS8, v/v) compared to the previous experiment (6% v/v). A lot of flocculation could also be seen in the flasks which interfered with the optical measurements.

4.4 MEL effect on bioremediation

4.4.1 Seawater samples

Two sets of bioremediation experiments were carried out in natural seawater obtained near Setubal, Portugal. The first intended to study the degradation of crude oil through time (analyses done after 3 and 7 days) in seawater, in seawater with MEL (13% DOR), in seawater with Corexit (5% DOR) and in seawater with the supernatant of *M. antarcticus* fermentation broth with a concentration of MEL of 1.7 g/L (13% DOR). The second studied how the initial concentration of crude oil affects bioremediation in seawater by adding either 50 ppm or 500 ppm of crude oil. In this case the conditions tested were seawater, seawater with MEL (13% DOR), seawater with Corexit (5% DOR) and seawater with MEL

(13% DOR) plus Corexit (5% DOR). This study also intended to understand the effect of surfactants and biosurfactants on assisting marine microorganism's degradation of crude oil.

All data is expressed as the percentage of hydrocarbons remaining in each sample compared to the amount in the control made a time zero, i.e., right before the extractions, to account for evaporation losses during the experiments.

4.4.1.1 Effect of time on bioremediation

The concentration of crude oil in seawater diminishes through time due to evaporation losses and through the degradation of hydrocarbons by some microorganisms. It is relevant to investigate the rate at which these losses occur and apply that knowledge to the methodology of following bioremediation experiments.

Analysis by GC-FID of crude oil consumption trough time can be seen in Figure 25. The highest crude oil degradation occurred for the seawater only samples, both at day 3 and 7. Degradation only slightly increased through time, except for the supernatant samples, where it showed a decrease. Since no additional hydrocarbons were supplemented, the only explanation for this occurrence is errors in either the extractions or sample preparation. Indeed, the recovery values had significant variation between samples from as high as 34% [Supernatant, t7] to as little as 7%. All the samples supplemented with dispersant/biosurfactant showed lower amounts of degradation than the sterile control, which seems to confirm the inadequacy of the followed procedure, at least without a higher number of replicas of the tested conditions. This seriously affects the usefulness of the obtained data and is also a problem that persists in further experiments and for different analytical methods.

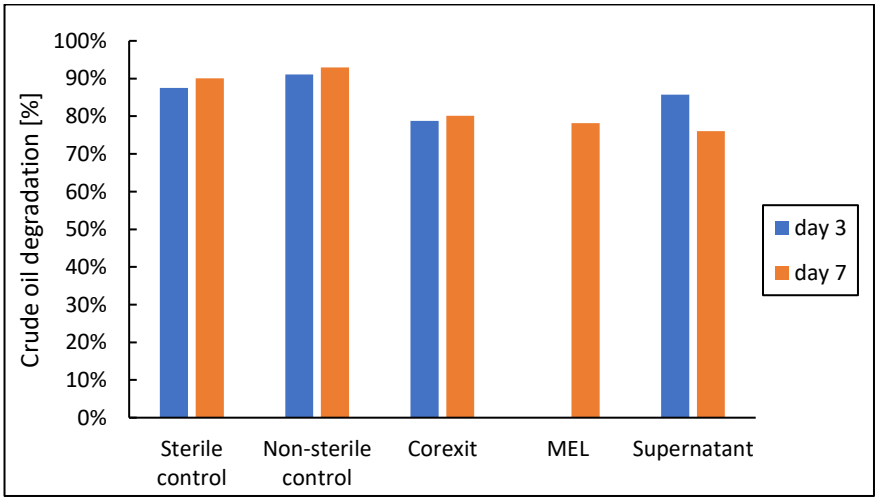


Figure 25 - Crude oil percent degradation after 3 and 7 days for various conditions and 50 ppm starting concentration. Results obtained through CG-FID.

Duplicates of the samples were analysed through FTIR by LAIST using an internal method and results are shown in Figure 26. There's an increase in crude oil degradation with time only for MEL and Supernatant samples, with the rest showing a decrease. As previously stated, this can only be explained by a significant margin of error in the methodology applied.

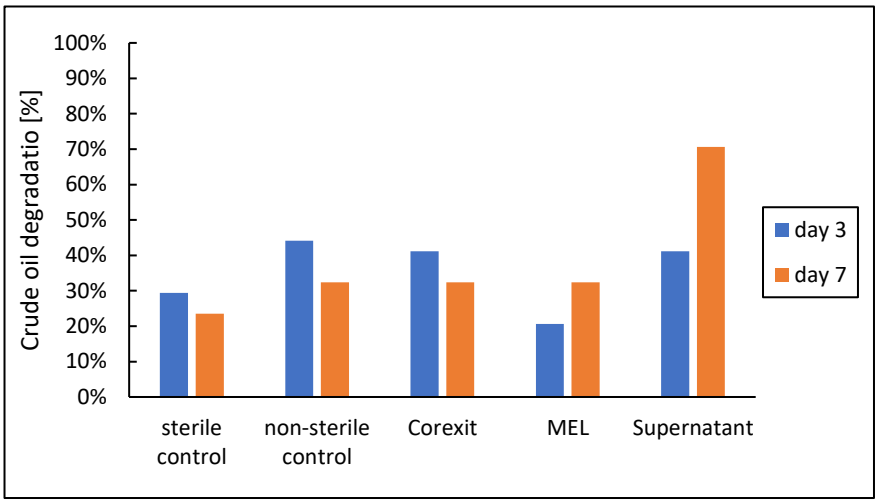


Figure 26 - Crude oil percent degradation after 3 and 7 days for various conditions and 50 ppm starting concentration. Results obtained through FTIR by LAIST.

It was decided that an experiment duration of 7 days would yield better results and help reduce the influence of the technical problems on the overall crude oil consumption values, since it allowed for further degradation to occur and thus a widening of differences between conditions.

4.4.1.2 Effect of initial crude oil concentration on bioremediation

Crude oil concentration in seawater heavily impacts the rate at which bioremediation occurs since at higher concentrations its toxicity increases even for hydrocarbon degrading microorganisms. Dispersants like Corexit 9500 and MEL can help decrease the crude oil concentration and thus aid in bioremediation. This effect is particularly difficult to test in a laboratory environment, due to the experiments being done in containers which limits the dispersion of oils.

The results of CG-FID analysis of hydrocarbon content in each sample are shown in Figure 27. Higher degradation was achieved for samples containing MEL, especially for the ones with the higher initial concentration of crude oil. Possibly the bioavailability of the hydrocarbons is increased with MEL's aid without compromising the viability of degrading microorganisms. The chemical dispersant, Corexit, does not show the same effect for higher concentrations of crude oil, with a steep decrease in degradation occurring (from 80% to 50%). This could be caused by higher toxicity to the microorganisms due to increased concentration of Corexit in the seawater (DOR is kept constant).

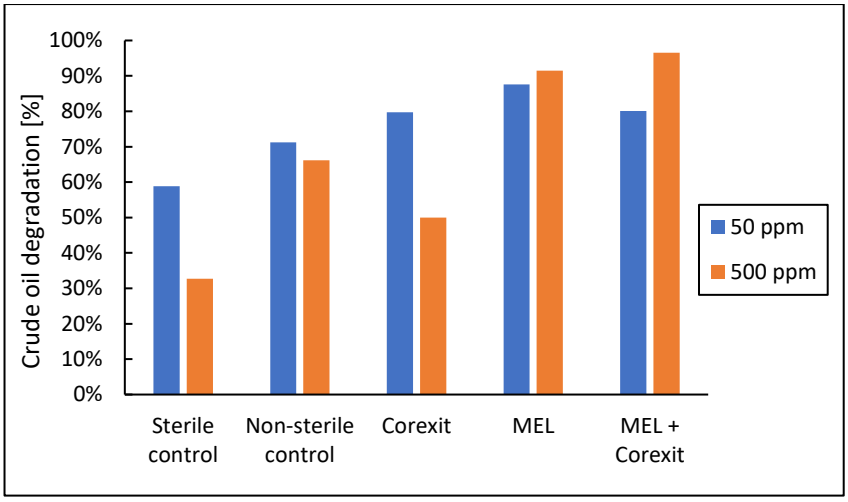


Figure 27 - Crude oil percent degradation after 7 days for various conditions and two starting concentrations (50 ppm, 500 ppm). Results obtained through GC-FID.

The FTIR analysis results, seen in Figure 28, show a slightly different degradation profile for most conditions, with the exceptions being the sterile controls and the Corexit samples. This once again confirms the need for both an increased number of replicas and better hydrocarbon extraction procedures to obtain higher quality data in future experiments.

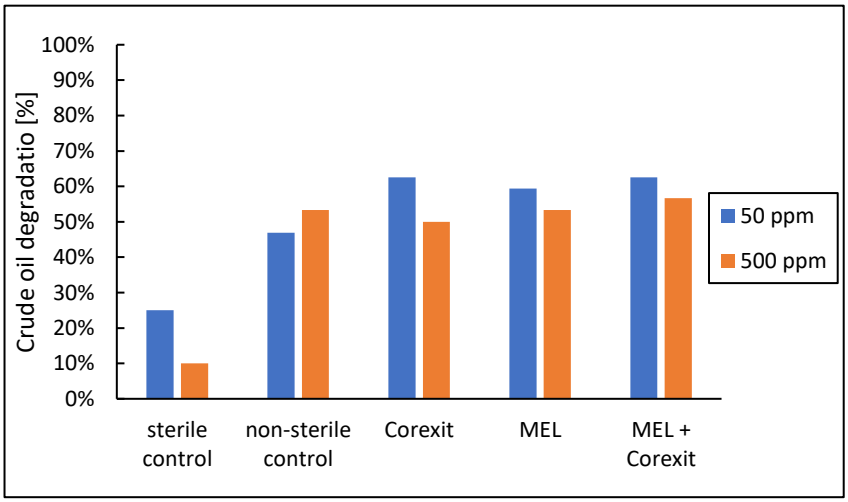


Figure 28 – Crude oil percent degradation after 7 days for various conditions and two starting concentrations (50 ppm, 500 ppm). Results obtained through FTIR by LAIST.

Looking at the sterile control's degradation values obtained using both analyses, it seems that the higher concentration of crude oil helps decrease the losses during either the experiment or the extraction process. Nevertheless, since it appears to have a heavier impact on the marine organisms'

survivability, the lower concentration of 50 ppm was chosen for use in further experiments where the main objective was to access the effects of MEL and Corexit in bioremediation.

4.4.2 Marine isolates in BH media

The previously isolated microorganisms were used for bioremediation experiments which purpose is twofold. Firstly, to examine the effects of different concentrations and formulations of MEL in the bioremediation of hydrocarbons by the isolated marine species and compare them to the effects of Corexit 9500. Secondly, to test procedures and microorganisms for current and future bioremediation experiments and assist in the establishment of new methodology for in-water bioremediation analysis. With that in mind, strains MS1 and MS2 were grown in Bushnell-Haas broth since this medium is recommended for studying microbial hydrocarbon deterioration. Two hydrocarbon sources were also tested, namely crude oil and a mixture of alkanes (C10, C12, C14, C16, C17, C18).

4.4.2.1 Crude oil degradation

The isolated strains bioremediation capabilities were analysed by quantifying their consumption of crude oil, after 7 days in a medium where it's their only carbon source. Optical density at 600 nm was also measured throughout the duration of the experience in order to quantify cell growth. Three additional conditions were tested for each strain, specifically, [MEL 13% DOR], [MEL 25% DOR] and [Corexit]. Growth kinetics are shown in Figure 29 for MS1 and Figure 30 for MS2.

With strain MS1 a substantial increase in cell density is seen for both MEL conditions between day 2 and 3, and good growth occurs through all 7 days. A similar trend can be seen for [MEL 25% DOR] in MS2, however for the [MEL 13% DOR] a cell density barely grows throughout the experience. Similar results had already been observed in previous growth kinetics tests in BH medium, with difficulty of obtaining consistent growth of the isolates. For the strain only conditions, cell density never reaches high values, with growth being small but consistent for MS1 and initially decreasing until day 3 for MS2 after which a sharp increase was seen. The Corexit conditions showed barely any cell growth for MS1 and some small growth for MS2.

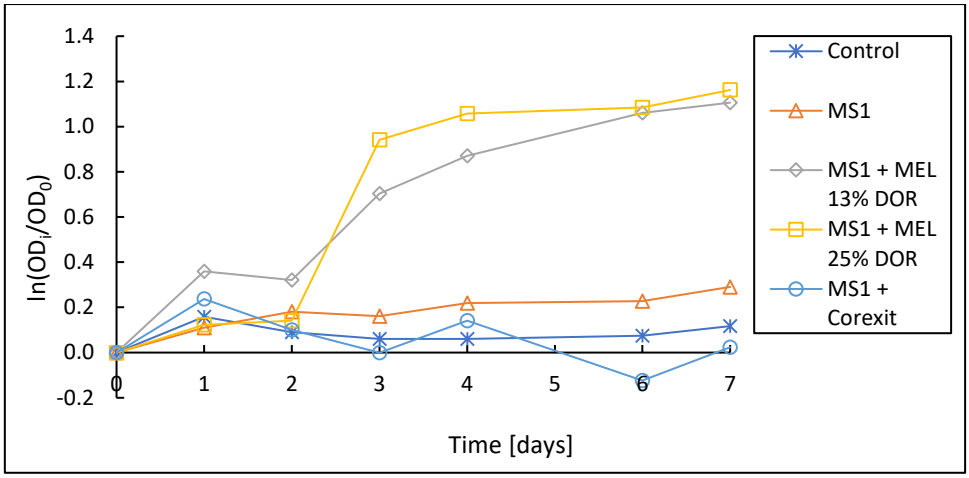


Figure 29 – Growth kinetics of MS1 in BH medium with 50 ppm of crude oil and different conditions.

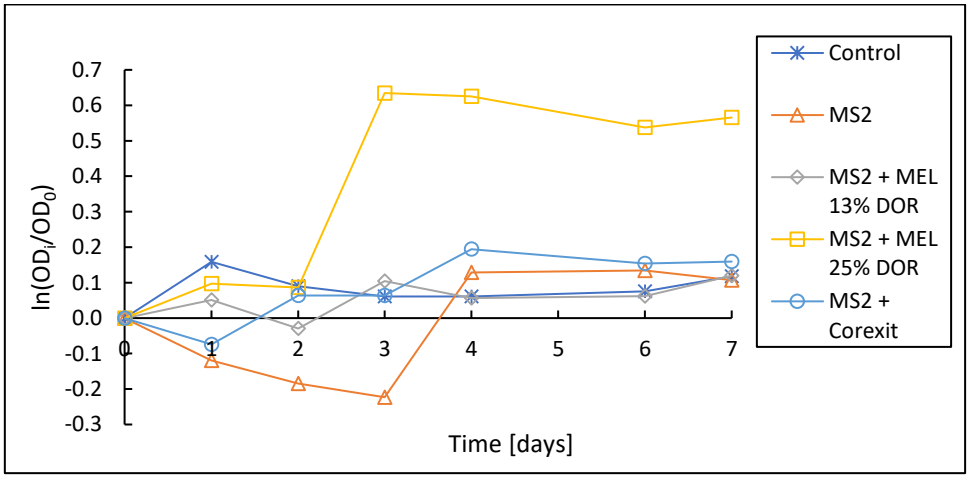


Figure 30 - Growth kinetics of MS2 in BH medium with 50 ppm of crude oil and different conditions.

Strain MS1 had the highest consumption of oil (91%) by itself, closely followed by the condition [MEL 25% DOR] with 90%. For strain MS2 the highest degradation occurred in [MEL 13% DOR] but the higher MEL concentration, [MEL 25% DOR], showed relatively low degradation, in contrast with the observed cell growth. The conditions with [Corexit] showed the least degradation for both strains, which is not surprising since they had barely any cell growth. The sterile control shows a rather high crude oil degradation, that could have been caused by losses during the extraction process.

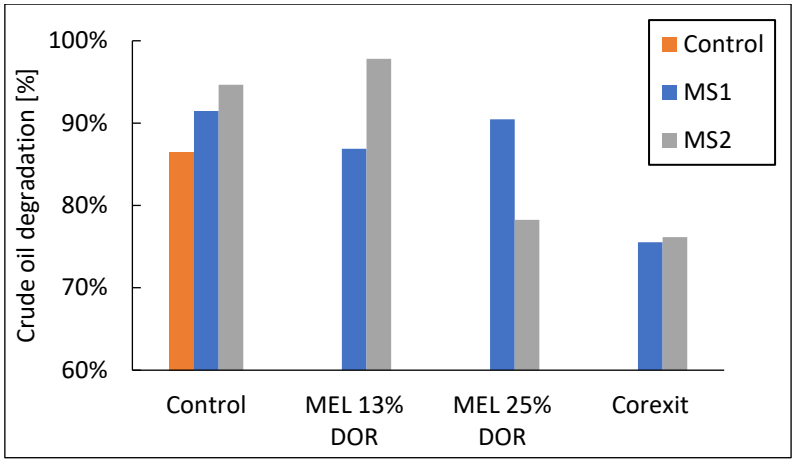


Figure 31 – Crude oil percent degradation after 7 days for strains MS1 and MS2 in BH medium and a 50 ppm starting concentration.

4.4.2.2 Alkane mixture degradation

With the intent to have a better understanding of how the hydrocarbons are being consumed by the marine isolates, a mixture of linear alkanes was used instead of crude oil in bioremediation tests.

It was noticed that foam was present in most of the conditions, with the ones containing the strain MS2 seemingly producing the most foam. In particular, the condition with [MEL 25% DOR] and MS2 produced a huge amount as can be seen in Figure 32.

Figure 32 -Foam presence in some of the alkane bioremediation shake-flasks at day 6.

For strain MS1 the highest alkane degradation occurred for [MEL 25% DOR] while the lowest was for [Corexit] which is in line with the results obtained with crude oil in the previous section. The degradation was higher for all three MEL conditions in comparison to the value obtained for the control with only the strain [MS1], suggesting that MEL is aiding the strain by increasing hydrocarbon bioavailability, thus helping bioremediation. MS1, in particular, seems to be unable to consume the hydrocarbons without this aid, as evidenced by the similar values of alkane degradation in both [Sterile] control and [MS1].

Alkane degradation was overall higher for strain MS2 than for strain MS1. Interestingly, the two conditions for MS2 with lower foam formation were also the two were fewer degradation of alkanes occurred, namely, [MEL 13% DOR] . Looking at [MEL 25% DOR], the alkane degradation of 98% is at the same value as the MS2 control and as the Corexit condition, which may indicate that the lower degradation obtained in the other two MEL conditions is not due to the concentration of MEL used having a toxic effect on the strain. Finally, errors in the process cannot be excluded and conclusions should only be made after further testing.

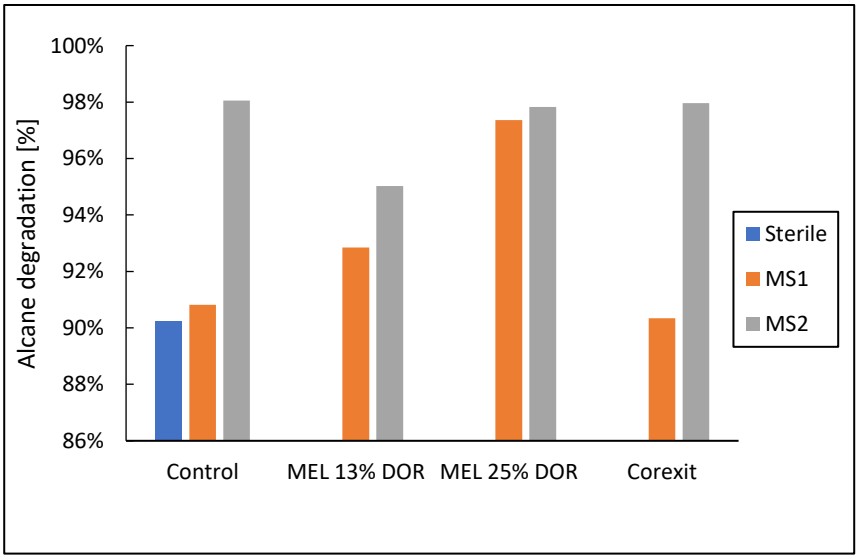


Figure 33 – Alkane percent degradation after 7 days for strains MS1 and MS2 in BH medium and a 50 ppm starting concentration.

The alkanes profile after bioremediation for each condition is presented in Figure 34 for strain MS1 and Figure 35 for strain MS2. By looking at the profile of alkanes in the [Sterile] sample it can be concluded that a large amount of alkanes are lost either through evaporation during the experiment or in the extraction process, especially the smaller alkanes. For both strains and for the [Sterile] control heptadecane seems to be heavily depleted in comparison to hexadecane and octadecane remaining percentages, and evaporation losses do not explain these results since its boiling point (302°C) is higher

than that of C16 (287°C). For the rest of alkanes, no other preferential consumption by both strains can be detected, with concentrations increasing with increasing chain length.

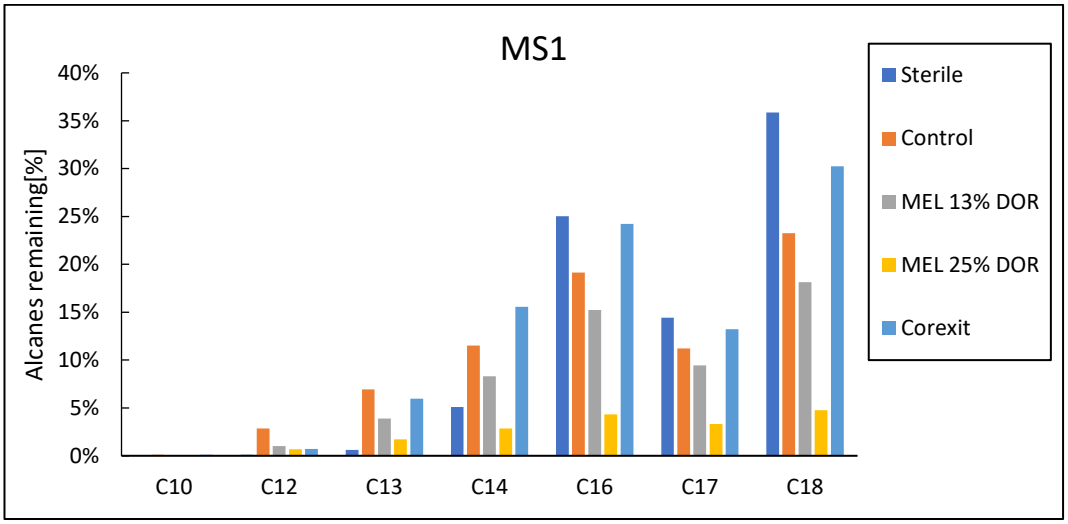


Figure 34 – Alkane profile after 7 days of strain MS1 bioremediation in BH medium.

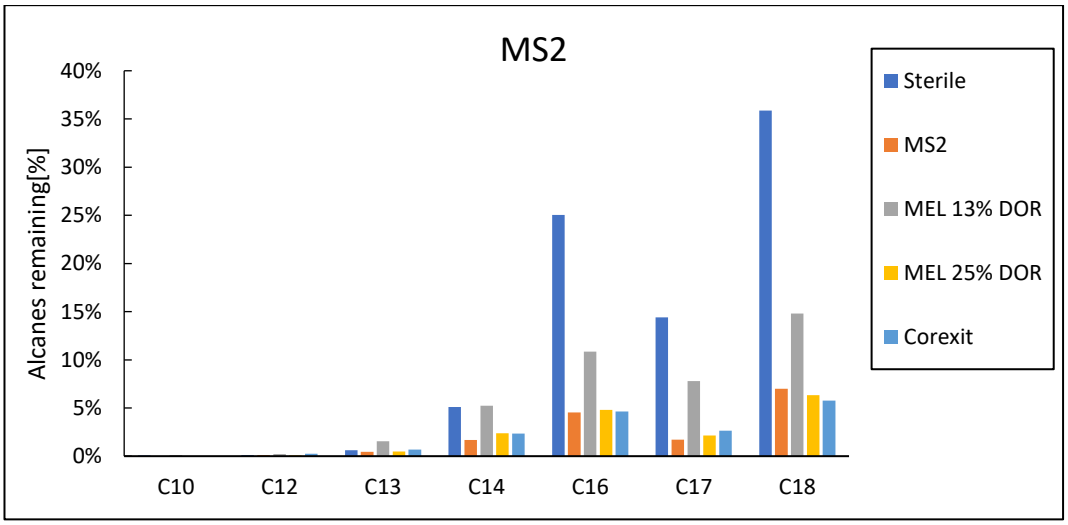


Figure 35 – Alkane profile after 7 days of strain MS2 bioremediation in BH medium

5 Conclusions

Mannosylerythritol lipids were produced via fermentation of *M. antarcticus*. In order to obtain high purity MEL two different substrates were used: D-glucose and soybean oil. The first fermentation used a sole hydrophilic carbon source (D-glucose) and obtained a low MEL titre of 6.6 g/L and 75% purity. The second fermentation used both hydrophobic (soybean oil) and hydrophilic (D-glucose) substrates and resulted in much higher MEL titres of 15.7 g/L with a purity of 84%. These results are in line with previously obtained values for similar fermentation conditions [102][41].

MEL solutions were created and tested for its emulsification activity. The best emulsification activity in kerosene (62%) was found for

Since one of the purposes of this solutions is to be used in marine oil spill responses, its stability over a wide range of pH, temperature and salinity was investigated. It was found to produce stable emulsions and maintain its surface tension around 27 mN m^{-1} in water with NaCl concentrations up to 40 g/L. It formed stable emulsions at high pH but not at acidic.

Overall these results show great stability of the MEL formulation in marine environments. However, the decline of its emulsifying ability after being subject to high temperatures for 1 hour may limit the possible routes of the production and purification processes. It could be that shorter exposure times avoid the degradation of its emulsifying capabilities, and further testing is needed to clarify MEL's limitations.

From seawater samples, three hydrocarbon degrading microorganisms were successfully isolated and used in toxicity and bioremediation experiments.

The effects of different concentrations of MEL on the growth of isolated marine microorganisms were assessed. No conclusive evidence was obtained for either assistance in microbial growth or an inhibitory concentration in BH medium. The difficulty of replicating results when using a nutrient deficient medium (BH) due to low survivability of the isolates was a problem that plagued these experiments. Yet, when growth inhibition of the isolates was tested in rich medium (MB) all strains grew but showed little differences in growth rates between the tested conditions. We can then conclude that in a rich medium and for concentrations of MEL up to 100 ppm no inhibitory effects occur on the growth of isolated strains MS1 and MS2.

The application of MEL in enhanced biodegradation of crude oil in seawater had very inconsistent results, in part because a significant amount of hydrocarbon lost due to evaporation, sticking to the walls of the baffled shake-flasks and during the extraction processes. Different methods were used to extract and analyse the remaining TPH concentrations, but they failed to have consistent results either between or within them.

Due to the difficulty of obtaining seawater samples in winter months, bioremediation was also tested in synthetic medium (BH) containing the previously isolated marine strains (MS1 and MS2). The first

experiment saw that crude oil degradation of the tested conditions varied with each strain. We can then infer that strain MS1 and MS2 have different sensibilities to the presence of MEL and of Corexit 9500, with MEL even augmenting MS1 hydrocarbon degradation rate.

To have a better view of individual hydrocarbon degradation, the bioremediation of an alkane mixture was tried. The results confirmed the improvement of MS1 bioremediation rates when MEL was supplemented. Once again, MS2 showed no significant difference in consumption rates between the sole strain and the surfactant aided (either MEL or Corexit 9500) conditions. Looking into individual hydrocarbon concentrations, the smaller alkanes have all but been depleted, possibly due to evaporation during the experiment and the extraction. Concentration of the alkanes appears to have a linear decrease with their decreasing chain length, except for heptadecane which was preferentially consumed.

It would be interesting to include BTEX and PAH's in future experiments since these are some of the most toxic and difficult to degrade of the oil components. Another possibility would be to use a consortium of hydrocarbon degrading marine isolates instead of single strains, in order to create a better simulation of marine conditions.

6 References

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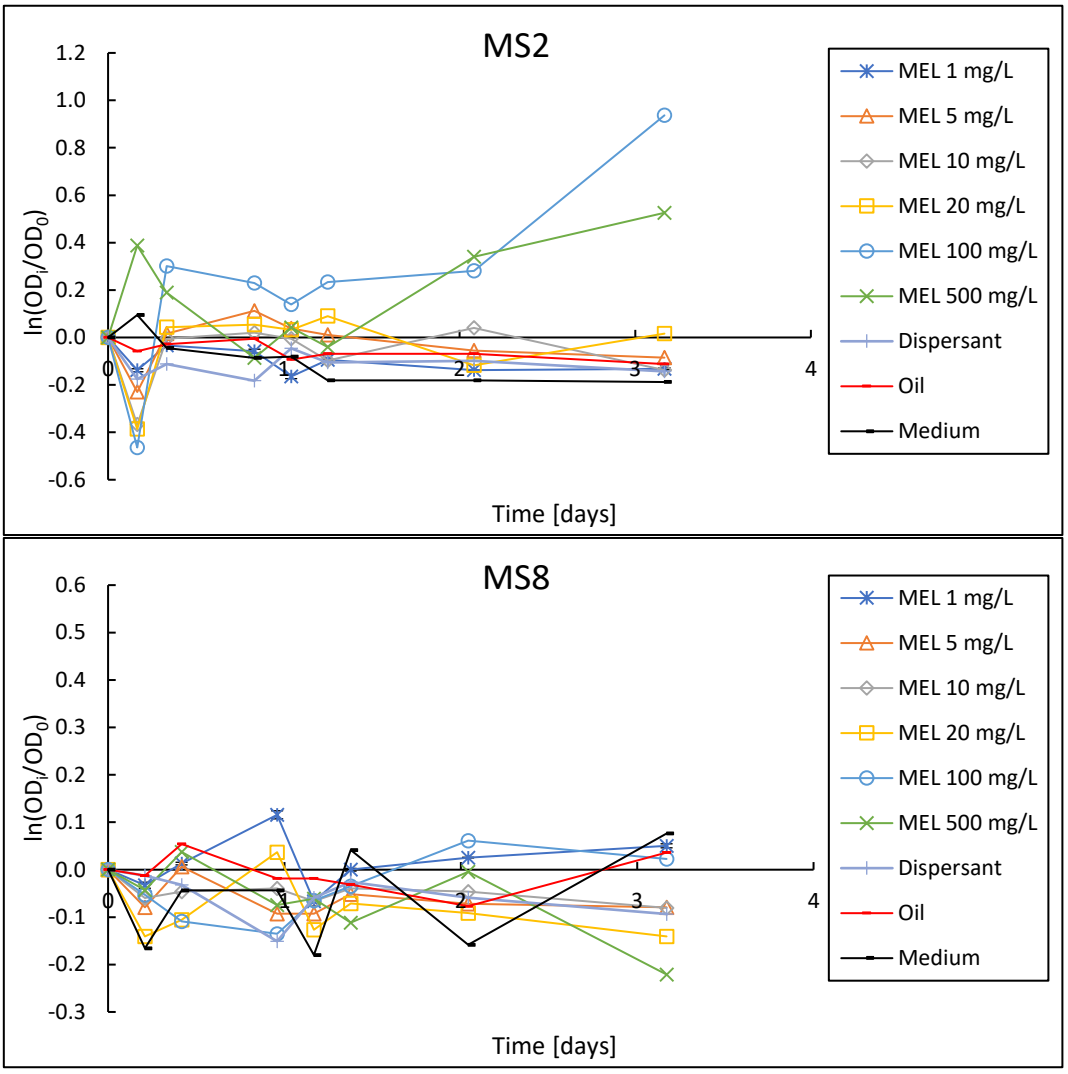
Appendix 1

The results shown in A1 are from inoculums started from stock cultures.



A 1 - Growth kinetics of MS2 and MS8 in BH medium with 10 g/L of D-glucose for different conditions