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

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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 that 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 surface tension and 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.

1 Introduction

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 natural dispersal 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 laboratory grown microorganisms not adapting to the site and carries the risk of introducing foreign species to the environment with unknown effects on the rest of the aquatic organisms [4][5].

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 rate of mass transfer to the oil-degrading microorganisms. Many marine microbes produce biosurfactants in order to degrade hydrocarbons and use them 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 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 that it needs to possess 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 (MEL) 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 yields achieved so far (165 g/L) [15]; and (iv) can be produced from renewable sources and industrial wastes [16].

2 Materials and Methods

2.1 Seawater samples

Seawater (SW) was collected from coordinates 38º24.977N, 8º58.073W at a depth of 4 meters by IPMA. Samples were kept in a refrigerated chamber at 4°C for a maximum of 14 days before being used.

2.2 Surfactants, dispersants and biosurfactant

The bioremediation experiments were tested against the dispersant Corexit 9500 (Nalco Holding) which was used in a dispersant to oil ratio (DOR) of 1%. The emulsification capability, surface tension and stability experiments were tested against the well-known chemical surfactant Sodium dodecyl sulphate (≥98,5%, Sigma-Aldrich).

2.3 MEL fermentation

2.3.1 Microorganism

MEL was produced by *Moesziomyces antarcticus* PYCC 5048^T provided by PYCC, CREM, FCT/UNL, 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.

2.3.2 Medium and growth conditions

In order to produce MEL an inoculum was prepared according to the procedure referred in [1]. 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 min. The inoculum was then incubated in an orbital (Agitorb 200, Aralab) for 48h 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 40 g/L of D-glucose added at day 0 and 4 and the other with 40 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.

2.3.3 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.

2.3.4 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 washed twice with Milli-q water and left to dry at 60°C for 48 h. Then the dry biomass was weighted and CDW calculated.

2.3.5 D-glucose and nitrate quantification

The 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. It was then further diluted with a proportion of 1:10 and transferred to an HPLC vial. Samples were 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 (300mm× 7.8mm, Phenomenex) at 65°C, using H_2SO_4 5 mM at 0.5 ml/min.

2.3.6 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] [3].

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 h. Then they 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 to react into methyl esters. The resulting product was extracted with 1 mL of hexane. 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 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 through the amount of C14, C16 and C18.

2.4 Physicochemical characterization of MEL

2.4.1 Emulsification Index (EI_{24}) Assay

EI_{24} was measured using an adaptation of [4]. 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 tube and vortexed at high speed for 2 min. After 24 h the height of the emulsion was measured, and the EI_{24} was calculated using Eq. 1.

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

Triplicates were made for every tested condition. Results are shown as average ± standard deviation.

2.4.2 Stability testing

MEL and 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)[5]. The thermal stability was analysed by submitting each sample to temperatures of -20, 20, 40 and 80 °C for 1 h and then leaving it to rest a room temperature for 2 h prior to testing. Salinity's effect was analysed by adding the surfactants to solutions with different concentrations of NaCl: 5, 10, 20 and 40 g/L.

2.5 Marine Isolates

2.5.1 Isolation and screening of hydrocarbon degrading microorganisms

1 mL of the SW sample 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 then made using equal volumes of seed culture and glycerol and stored in 1 mL Eppendorf's at -80°C.

2.5.2 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 and 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.

2.5.3 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 through optical density at 600 nm of samples taken at regular intervals.

2.6 Bioremediation

2.6.1 Procedure for mixing of dispersant/MEL and crude oil

For the bioremediation experiments using crude oil the procedure mentioned in [6] was followed for the mixing of dispersant/MEL in crude oil prior to application. Corexit 9500 (1% DOR) and MEL (5% DOR) were added to individual recipients containing oil which were then: (1) Heated at 50 °C for 5 min; (2) Shaken vigorously for 1 min; (3) Sonicated in an ultrasonic bath for 5 min; (4) Shaken vigorously for 1 min. The mixture was then used in the respective amounts in the bioremediation experiments.

2.6.2 Enhanced bioremediation tests

Bioremediation was tested in SW and in BH medium supplemented with 10 % v/v of two marine isolates (MS1 and MS2). Two carbon sources were used for these experiments: crude oil (Statfjord, Norway) and a mixture of seven n-alkanes (C10, C12, C13, C14, C16, C17 and C18), each corresponding to 14.3% v/v. The following table contains the conditions tested in the four sets of bioremediation experiments.

Table 1 – 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 SW experiments to analyse the degradation of oil through GC-FID and FTIR (LAIST internal method).

2.6.3 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. 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 hydrocarbon loss 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. The organic phase was 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.

2.6.4 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, 2, 1, 0.5 and 0.2 g/L in n-hexane) with 5-α-androstane as an internal standard. A relative response factor (RRF) was then calculated using Eq. 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 (Eq. 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 were used to quantify the percentage of hydrocarbons recovered in the extraction using Eq. 4.

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

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

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

3 Results and discussion

3.1 MEL production by *M. antarcticus*

M. antarcticus was cultivated for 10 days using two 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 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 [7][8]. 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. Fermentation profiles are presented in Fig. 1.

Cell growth on [Glu60,0;SBO21,4] was significantly faster than on [Glu60,0;Glu40,4], after the addition of the di[illegible]nt substrates at day 4, which shows a faster in[illegible]oration 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.

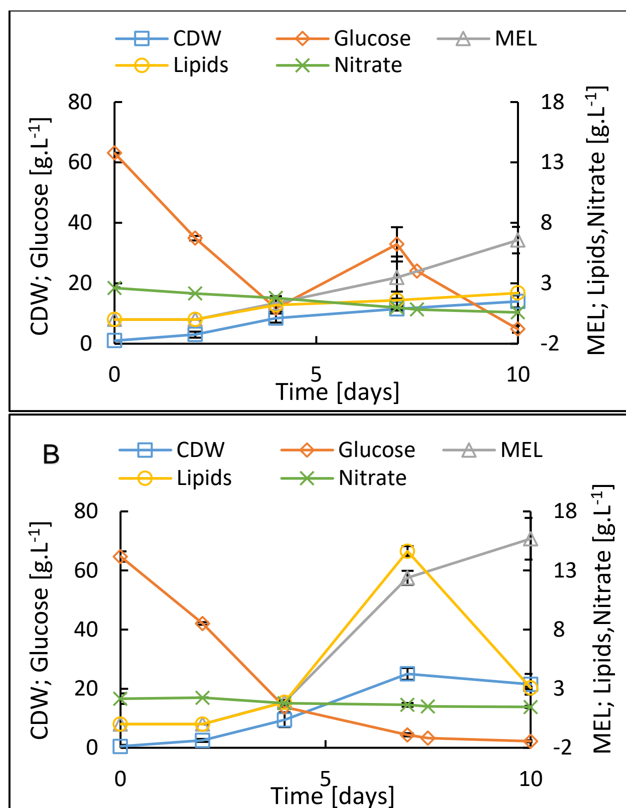

Fig. 1 - Fermentation profile of *M. antarcticus* (A) fed with D-glucose at day 0 and 4; and (B) fed with D-glucose at day 0 and SBO at day 4. Results are shown as the average of duplicates.

As expected, MEL titres for [Glu60,0;SBO21,4] at 15.7 g/L were way higher than the 6.6 g/L of [Glu60,0;Glu40,4]. However, the 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 is shown in Table 2 and is in line with previously reported results [9].

Table 2 - MEL lipidic chains profile. Results are presented as averages.

	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%

3.2 Stability of MEL determined by Emulsification Index and Surface Tension

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 and the surface tension were tested. Concentrations above the critical micellar concentration (CMC) were used for MEL (CMC = 0.02 g/L [10]) and below for SDS (CMC = 2.3 g/L [11]). 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 [12]. It is then of interest to MEL's application in bioremediation the effects that seawater's pH and salinity have in its surface tension and emulsification capability.

3.2.1 Concentration and solvent effect

The emulsifying capabilities of MEL in kerosene were tested. 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 Fig. 2.

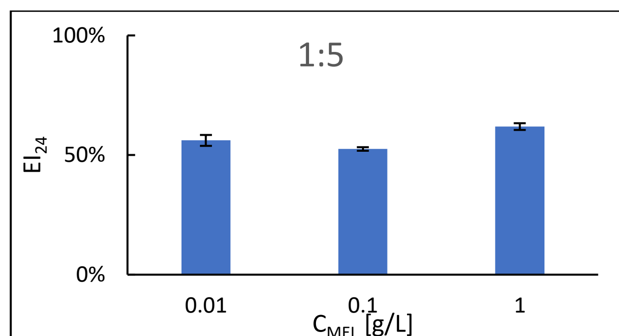

Fig. 2 - Effect on the emulsification capability of MEL for different concentrations.

Since the highest EI_{24} of 61.9 % ± 1.9 % for kerosene was achieved for a concentration of 1 g/L of MEL, this was the solution used for the following EI24 stability tests. The surface tension of 0.1 g/L in water was 26.8 mN/m, 5% higher than previously reported for the same concentration of MEL in water [10]. This result shows that MEL is very effective in lowering the surface tension of water (72 mN m^{-1}).

3.2.2 pH effect

The effect of pH on the surfactants emulsification index was tested for pH of 2, 4, 7, 10 and 12. The results are shown in Fig. 3.

MEL showed high EI_{24} 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 SW typically has a pH range of 7.5 to 8.4. In terms of surface tension, the opposite is observed, with MEL's surface tension having little variation throughout the range of pH tested, while SDS' decreasing for the pH of 2 and 12.

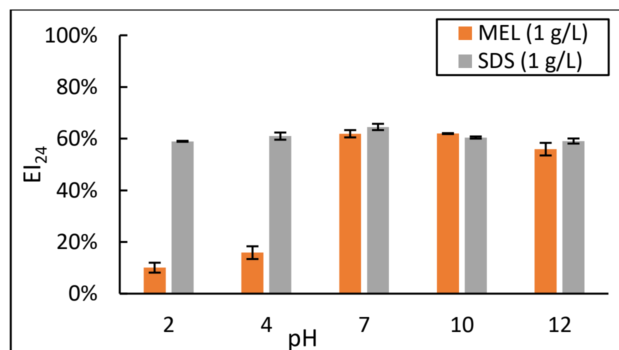

Fig. 3 - Influence of pH on the emulsification capability of MEL and SDS.

3.2.3 Temperature effect

The thermal stability of a biosurfactant is important since many purification or sterilization steps as well as end applications require high temperatures. As such, the effect of submitting the surfactants to temperatures of -20, 20, 40 and 80 °C for 1 h was tested and the results are shown in Fig. 4**Error! Reference source not found.** for EI_{24}.

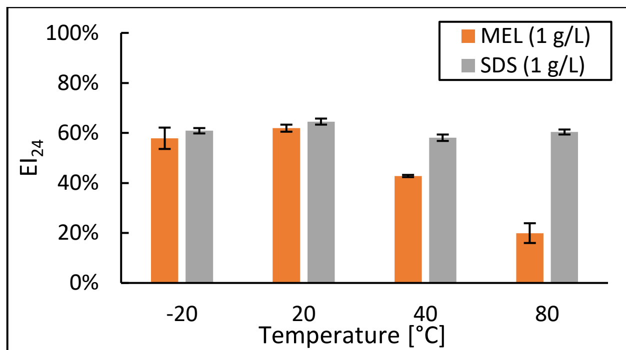

Fig. 4 - Influence of temperature on the emulsification capability of MEL and SDS.

The EI_{24} of the MEL steeply decreased for temperatures higher than 40°C, while SDS had stable emulsification at all tested temperatures. The exposure time (1 h) at these temperatures may have played a key role in this decrease.

3.2.4 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 Fig. 5 for emulsification index and for surface tension.

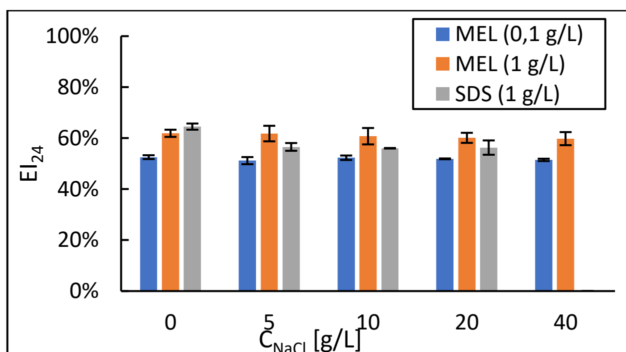

Fig. 5 - Influence of salinity on the emulsification capability of MEL and SDS.

Little change to EI_{24} of the MEL 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. SDS EI_{24} slightly decreases with salinity up to 20 g/L but at concentration of 40 g/L of NaCl no emulsion had persisted after 24h.

3.3 Effect of MEL on the growth of hydrocarbon degrading marine microorganisms

3.3.1 Isolation of marine strains

From the SW samples 2% v/v was added to MB medium and incubated as previously described in Materials and Methods. The intent was to isolate strains for later use in bioremediation experiments. After 2 days, samples were 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 their incubation and growth, morphologically similar colonies were discarded (MS3, MS4, MS5 and MS7). The remaining colonies were screened for the ability to degrade hexadecane, as described in the following section.

3.3.2 Selection of hydrocarbon degrading strains

In order to select 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 measured at 600 nm (OD_{600}). 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 Fig. 6. 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).

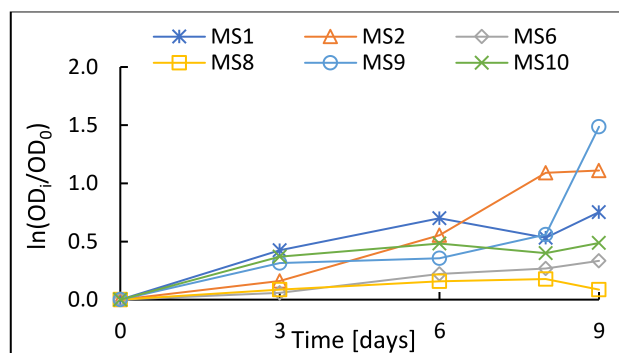

Fig. 6 - Growth kinetics of marine isolates in BH medium with hexadecane incubated at 27 °C and 250 rpm.

Fig. 7 shows hexadecane consumed by each strain. MS8 and MS9 were the strains that degraded the most C16, with 43% and 49% respectively. MS2 and MS6 were the ones that had the highest concentration of C16 after 9 days, with only 31% and 26% of degradation, respectively.

MS9 had the highest C16 consumption, but it was not selected for use in the following experiments due to suspicion of contamination (based on its morphology and exponential growth kinetics). MS2 had the second highest growth rate so, although it had a low C16 consumption, was selected for further studies.

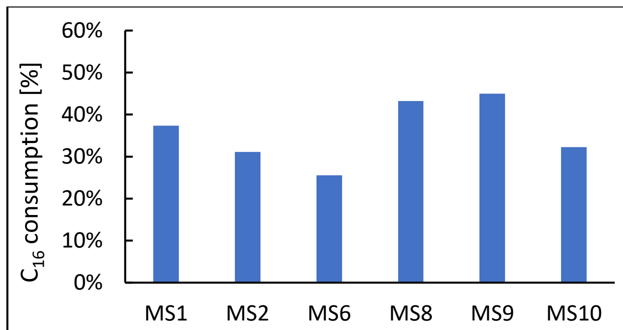

Fig. 7 - Hexadecane consumption of marine isolates in BH media after 9 days incubation at 27 °C and 250 rpm.

Both MS8 and MS1, with the second and third highest degradation values, respectively, were also chosen for further studies.

3.3.3 Testing and optimization of growth methodology

To understand the effect of temperature on the growth of the isolates, the three previously selected strains were incubated at 27 and 37°C for 2 days and OD_{600} measurements were taken at day 0 and 2 (Fig. 8). 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 were not significant.

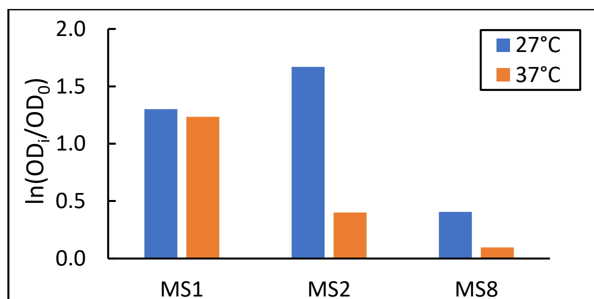

Fig. 8 - Temperature effect on growth of marine isolates in MB medium after 48h incubation at 250 rpm.

A second experiment was made in order to assess glucose consumption and the growth of biomass through time. The isolates MS1, MS2 and MS8 were incubated for 3 days at 27°C and results are shown in Table 3. Isolate MS1 achieved maximum growth after 24 h 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 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 remained at day 3. Isolate MS8 achieved its highest biomass values at day 3. With these results an inoculum growth period was established of 24h for MS1 and MS2 and of 48h for MS8.

Table 3 – 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

3.3.4 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 a rich (MB) and in a minimal (BH) medium supplemented with D-glucose and different concentrations of the biosurfactant. Conditions with Corexit 9500 and crude oil were also tested.

Fig. 9 shows the growth curves of MS1 and MS2 in MB medium. Most of MS1's growth occurred in the first 24 h with results almost identical for all tested conditions. All the conditions gave very similar values of growth which indicates that at the values tested none significantly inhibited the growth of MS2. Considering the high concentrations of MEL used (100 ppm) these results show a low toxicity to marine bacteria.

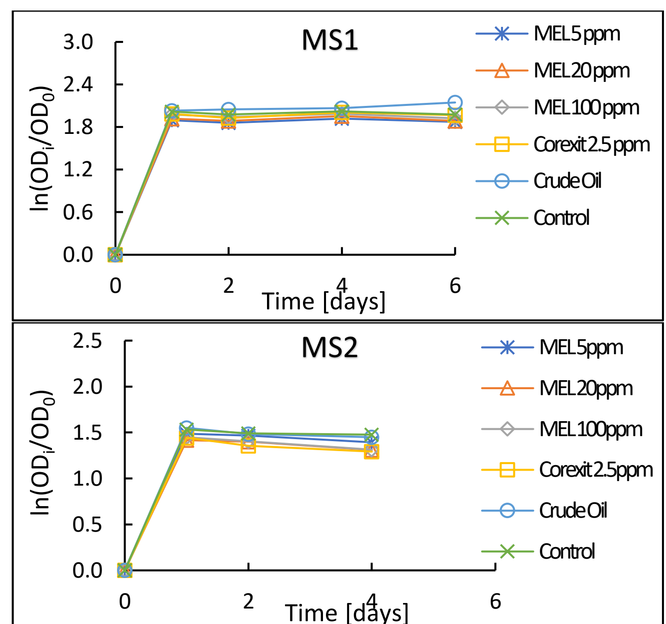

Fig. 9 - Growth kinetics of MS1 and MS2 in MB medium with 20 g/L of D-glucose for different conditions

Growth experiments of MS2 and MS8 in BH medium are shown in Fig. 10. The two isolates showed the smallest growth in the medium containing 2.5 ppm of Corexit. MS2 had the highest growth with crude oil, followed by the ones with MEL (1, 5, 10 and 20 ppm). Crude oil seemed to have an adverse effect on MS8 while all

tested MEL concentrations yielded growths either similar or higher than the control.

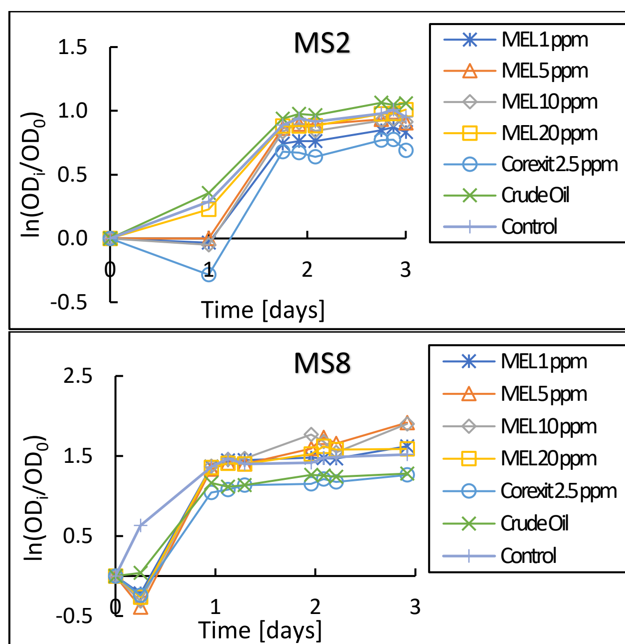

Fig. 10 - Growth kinetics of MS2 and MS8 in BH medium with 10 g/L of D-glucose for different conditions.

Growth experiments of MS2 and MS8 in BH medium are shown in Fig. 10. The two isolates showed the smallest growth in the medium containing 2.5 ppm of Corexit. MS2 had the highest growth with crude oil, followed by the ones with MEL (1, 5, 10 and 20 ppm). Crude oil seemed to have an adverse effect on MS8 while all tested MEL concentrations yielded growths either similar or higher than the control.

When the same experiment was tested in MS1 the culture didn't grow likely due to the difficulty of this strain in growing in poor medium

3.4 MEL effect on bioremediation

3.4.1 Seawater samples

Two sets of bioremediation experiments were carried out in natural seawater. The first intended to study the degradation of crude oil through time (analyses done after 3 and 7 days) in SW, in SW with MEL (13% DOR), in SW with Corexit (5% DOR) and in SW 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 SW, SW with MEL (13% DOR), SW with Corexit (5% DOR) and SW 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.

3.4.1.1 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 **Error! Reference source not found.**. The highest degradation occurred for the SW only samples, both at day 3 and 7. Degradation only slightly increased through time, except for the supernatant samples, where it decreased. The recovery values had significant variation between samples from as high as 34% [Supernatant, t7] to as little as 7%. It was decided that an experiment duration of 7 days would yield better results and reduce the influence of technical problems.

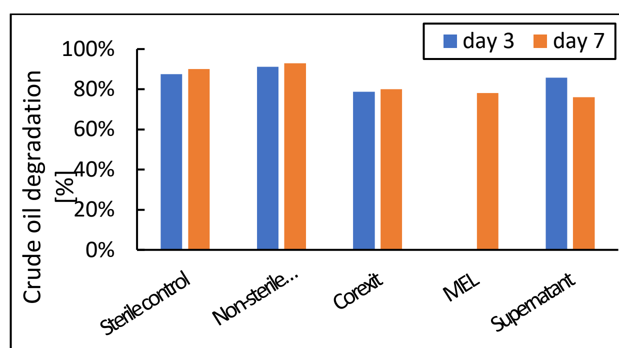

Fig. 11 - Crude oil percent degradation after 3 and 7 days for various conditions and 50 ppm starting concentration. Results obtained through GC-FID.

3.4.1.2 Effect of initial crude oil concentration on bioremediation

Crude concentration in SW impacts the rate at which bioremediation occurs since at higher concentrations its toxicity increases even for hydrocarbon degrading microorganisms.

The results of CG-FID analysis of hydrocarbon content (Fig. 12) show higher degradation in samples containing MEL, especially the ones with higher initial concentration of crude oil. Possibly the bioavailability of the hydrocarbons is increased with MEL's aid without compromising the viability of the isolates. Corexit does not show the same effect in higher concentrations of crude, 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), however the same effect was not seen in the mixture of Corexit and MEL.

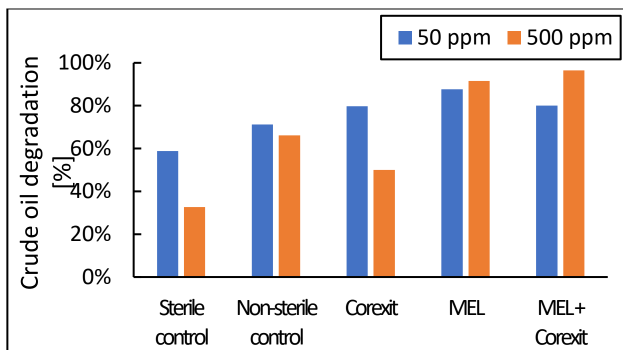

Fig. 12 - Crude oil percent degradation after 7 days for various conditions and two starting concentrations (50 ppm, 500 ppm). Results obtained through GC-FID.

Looking at the sterile control's degradation values 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.

3.4.2 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 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).

3.4.2.1 Crude oil degradation

The isolated microbial strains were used as models for bioremediation of crude oil in synthetic sea water. Three additional conditions were tested for each strain: [MEL 13% DOR], [MEL 25% DOR] and [Corexit]. As seen in Fig. 13 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. The conditions with [Corexit] showed the least degradation for both strains. The sterile control shows a rather high degradation, that could have been caused by losses during the extraction process.

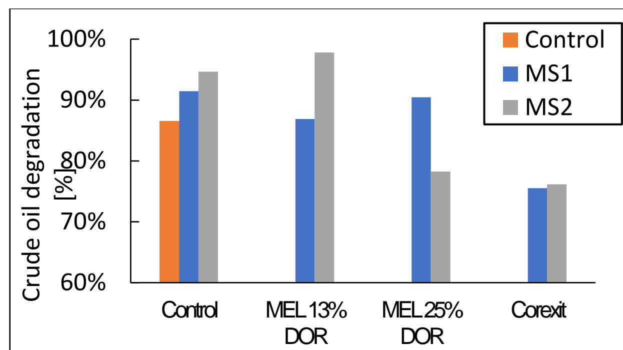

Fig. 13 - Crude oil percent degradation after 7 days for MS1 and MS2 in BH medium and a 50 ppm starting concentration.

3.4.2.2 Alkane mixture degradation

With the intent to have a better understanding of how the hydrocarbons are being consumed, a mixture of n-alkanes was used instead of crude. MS1 had the highest alkane degradation for [MEL 25% DOR] and the lowest for [Corexit] which is in line with previous results obtained with crude oil. The degradation was higher for all MEL conditions in comparison to the control with only [MS1], suggesting that MEL is aiding the strain by increasing hydrocarbon bioavailability. MS1 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 MS2 than for MS1. Looking at [MEL 25% DOR], the alkane degradation of 98% is the same as the MS2 control and the Corexit condition, which implies 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.

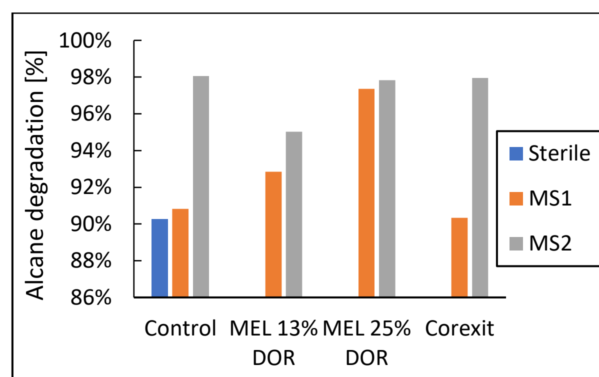

Fig. 14 - Alkane percent degradation after 7 days for MS1 and MS2 in BH medium and a 50 ppm starting concentration

4 Conclusions

MEL was produced via fermentation of *M. antarcticus*. In order to obtain high purity MEL two different substrates were used: glucose and soybean oil. The first fermentation used glucose and obtained a low MEL titre of 6.6 g/L and 75% purity. The second fermentation used both SBO and glucose and resulted in much higher MEL titres of 15.7 g/L with a purity of 84%. These are in line with previously obtained results in [7][8].

MEL solutions were tested for its emulsification activity and capability of lowering water's surface tension. The best emulsification activity in kerosene (62%) was found for a MEL concentration of 1 g/L. This solution was capable of lowering water's surface tension up to 26.8 mN m^{-1}. Since one of the purposes of this solution 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 pH and the surface tension saw a maximum increase to 31 mN m^{-1} for a pH of 12, which is still an excellent value. After being exposed to different temperatures for 1 h, the solution was found to have a consistent surface tension for all tested temperatures while its emulsification activity had a sharp decline at 40°C and at 80°C it was virtually inexistent.

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 MS1 and MS2.

The application of MEL in enhanced biodegradation of crude oil in seawater had inconsistent results even though different methods were used to extract and analyse the remaining TPH concentrations. Tests were also made in synthetic medium (BH) containing 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.

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, to better simulate marine conditions.

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