University of Stavanger

Faculty of Science and Technology

Study program/ Specialization: MSc Environmental Technology	Spring semester, 20..16. Open Access
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Thesis title: Screening for biosurfactant-producing bacteria for crude oil degradation, under cool and saline conditions	
Credits (ECTS): 30	
Key words: Surfactants Biosurfactants Crude oil degradation Screening methods Surface tension MEOR	Pages: ……56…… + enclosure: ……0……

Abstract

Using three sources with different hydrocarbon exposure histories—bacteria grown from Arctic seawater (Arctic), and bacteria from an offshore wastewater treatment system from both the bioreactor (BR) and settling chamber (SC)—biosurfactant production capabilities for crude oil degradation were tested. Isolation for colony purity for later DNA sequencing was also attempted, but was not successful. Traditional techniques, for both cultivating bacteria and biosurfactant production assessment methods were applied.

The majority of Arctic samples demonstrated positive results for the qualitative tests—oil drop collapse and visual emulsion and flocculation test—for biosurfactant screening. Only 15 of the 34 samples showed a reduction of 10-37% of the measured surface tension of seawater liquid media with oil. The presence of the oil (Tyrihans) alone lowered surface tension to of the media to 58.114 mN/m from 73.115 mN/m of seawater media without oil.

All SC and BR samples cultivated with nutrient agar and broths failed to demonstrate significant changes in surface tension. The greatest recorded change was with BR sample 6, which reduced surface tension of the control with oil from 38.843 mN/m to 36.667 mN/m.

The 5 BR cultures from Bushnell Haas (BH) media with oil produced strong emulsions, which were difficult to break, as well as demonstrating complete spreading in the oil drop collapse test. The lowest recorded surface tension produced by sample 2 was 32.829 mN/m, compared to the control liquid BH media with oil of 54.003 mN/m. SC samples provided more possible candidates for biosurfactant production, with 8 samples reducing the surface tension to between 24.245 mN/m and 27.556 mN/m. Based on the results, SC bacteria have a greater potential as candidates for future analysis of biosurfactant production, and the BH media is better suited for cultivation purposes than the nutrient agar and broth media.

Preface

This thesis has been completed and submitted in partial fulfillment of the requirements for the Masters of Science degree

in

ENVIRONMENTAL TECHNOLOGIES

Submitted for the deadline of 15 June 2016

Department of Mathematics and Science

University of Stavanger

The research and work was carried out from January 2016 to submission in June 2016.

I would like to firstly thank my supervisors, Krista Kaster and Andrea Bagi of the department of Mathematics and Science at the University of Stavanger, for support and crucial feedback and direction throughout my study. I would also like to thank the University of Stavanger and it's staff for providing facilities and materials that allowed for the completion of this research project.

Finally, I would like to express my gratitude to my fellow students, my parents and my partner, for providing continuous and unwavering support and encouragement. This accomplishment would not have been possible without them. Thank you all.

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List of Abbreviations and Symbols

γ	Surface tension (mN/m)
Π	Surface pressure
Θ	Contact angle
BATH (MATH)	Bacterial (microbial) adhesion to hydrocarbon
BH	Bushnell Haas
BR	Bioreactor
CMC	Critical micelle concentration
DGGE	Denaturing gel gradient electrophoresis
E24 (48, 72)	Emulsion index at 24 (48, 72) hours
EOR	Enhanced oil recovery
MEOR	Microbial enhanced oil recovery
GEMEOR	Genetically engineered microbial enhanced oil recovery
IFT	Interfacial tension (mN/m) (surface tension)
NA	Nutrient agar
NB	Nutrient broth

MMS	U.S. Minerals Management Service
PAH	Polycyclic aromatic hydrocarbons
PCR	Polymerase chain reaction
RL	Rhamnolipids
SC	Settling chamber
SDS	Sodium dodecyl sulphate
SDV	Standard deviation (±)
TPH	Total petroleum hydrocarbons
UiS	University of Stavanger

1 Introduction

Commercially produced surfactants have applications in many industries. The most common commercially produced examples are detergents, whether for residential or industrial purposes. However, a large portion of the manufacturing is for the oil industry. This is notably for enhanced oil recovery (EOR) and bioremediation purposes. According to eia.gov, global crude oil production peaked at 80 to 80.5 million barrels per day in the latter half of 2015 (tab. 1). Whether to increase well productivity, or to remediate oil spills, surfactants are critical for the oil industry.

Table 1. World Crude Oil Production: Persian Gulf Nations, NON-OPEC, and World (Thousand Barrels per Day) [taken from eia.gov (May, 2016) U.S. Energy Information Administration/Monthly Energy Review (pp 167)].

	Persian Gulf Nations[b]	Selected Non-OPEC[a] Producers									Total Non-OPEC[a]	World
		Canada	China	Egypt	Mexico	Norway	Former U.S.S.R.	Russia	United Kingdom	United States		
2015 January	23,349	3,885	R 4,232	508	2,290	1,579	--	10,231	872	E 9,341	R 46,197	R 79,367
February	23,405	3,906	R 4,218	516	2,370	1,589	--	10,181	812	E 9,451	R 46,205	R 79,362
March	24,010	3,775	R 4,256	525	2,356	1,586	--	10,264	867	E 9,648	R 46,504	R 80,257
April	24,066	3,463	R 4,258	503	2,235	1,614	--	10,111	925	E 9,694	R 45,845	R 79,818
May	24,317	3,212	R 4,271	512	2,263	1,555	--	10,270	1,016	E 9,479	R 45,526	R 79,384
June	24,772	3,457	R 4,408	504	2,283	1,596	--	10,166	870	E 9,315	R 45,501	R 79,897
July	24,872	3,821	R 4,263	524	2,308	1,611	--	10,213	839	RE 9,432	R 45,952	R 80,513
August	24,772	3,922	R 4,278	523	2,291	1,599	--	10,268	788	E 9,407	R 45,999	R 80,472
September	24,872	3,422	R 4,317	501	2,306	1,581	--	10,209	862	RE 9,453	R 45,490	R 80,029
October	24,672	3,582	R 4,259	517	2,314	1,685	--	10,341	912	RE 9,379	R 45,772	R 80,169
November	24,672	3,819	R 4,297	494	2,310	1,644	--	10,361	972	RE 9,329	R 46,230	R 80,630
December	24,517	R 3,866	R 4,275	509	2,308	1,682	--	10,407	979	RE 9,246	R 46,353	R 80,517
Average	**24,363**	**3,677**	**R 4,278**	**511**	**2,302**	**1,610**	**--**	**10,253**	**893**	**RE 9,431**	**R 45,964**	**R 80,039**
2016 January	R 24,707	R 3,877	R 4,166	498	2,294	R 1,667	--	10,485	R 980	RE 9,180	R 46,024	R 80,213
February	24,627	3,797	4,133	497	2,247	1,674	--	10,485	996	E 9,129	45,579	79,653
2-Month Average	**24,668**	**3,838**	**4,150**	**498**	**2,271**	**1,670**	**--**	**10,485**	**988**	**E 9,155**	**45,809**	**79,942**
2015 2-Month Average	**23,376**	**3,895**	**4,225**	**512**	**2,328**	**1,584**	**--**	**10,207**	**844**	**E 9,393**	**46,201**	**79,364**
2014 2-Month Average	**23,531**	**3,573**	**4,198**	**516**	**2,543**	**1,620**	**--**	**10,119**	**875**	**8,040**	**44,160**	**77,497**

Surfactant research and application in oil and petroleum industry in EOR for reservoirs and other production steps has been long established. Typically, EOR requires surfactants that adsorb from solution onto the porous core geology of the reservoir, but also have EOR applications topside (Shramm & Marangoni, 2000). For example, surfactants used in oil production in the demulsification stage of the oil-water systems can use Tweens (non-ioninc polymers), which outcompete and displace natural surfactants, minimising the surface area at the interface (Roodbari, et al, 2011). EOR and other oil production steps using surfactants is a large and well-developed area.

Remediation of oil through biodegradation is limited to the bioavailability—available surface area of the oil for biological degradation. Surfactant application can be used with the aim of increasing the bioavailability of oil for microbial degradation. Along with bioavailability, microorganism properties—of individuals or communities, environmental conditions, and hydrocarbon substrate properties are also noteworthy for parameters that impact biodegradation

success (Abbasnezhad, H., Grey, M., & Foght, J. M. 2011). Optimally, remediation for biodegradation would also be implemented with intense mixing/aeration and supplementation with nutrients to maintain a high quality environmental condition (Alexander, 2009). In order to promote biodegradation by increasing the bioavailability of the oil, surfactants application offers a practical solution.

Corexit is a well-studied synthetically manufactured surfactant for oil dispersion, which aims to increase bioavailability for degradation, as well as to limit damages to marine macro fauna and coastal wildlife and environments. It was used at an unprecedented level, with seven million litres, during Deepwater Horizon blowout in April 2010 (Kleindienst, et al, 2015). However, debates have emerged over its effectiveness, as well as other environmental costs from applications of synthetic surfactants.

An environmental concern over surfactant applications means there is a market for less persistent and less toxic alternatives than that is currently provided by synthetic surfactants today. Biosurfactants can fill that need in offering an environmentally friendlier alternative, with crossover applications for microbial EOR (MEOR) sector. Examples of how MEOR can be accomplished, are by either growing biosurfactant producing microbes *ex situ* in a lab, collecting the biosurfactants, and adding them to water, for applications such as increasing sweep efficiency—displacing the oil towards the producing well; or by simply injecting the microbes directly into the reservoir (Patel, et al., 2015). The former *ex situ* method is more costly and time consuming, but the latter provides less controllable variables as microbes injected directly into a reservoir may have unforeseen consequences during or later steps of the oil recovery process.

Microorganisms, particularly those native to the application sites, are an area of interest for MEOR and remediation studies. They possess important biosurfactant traits for site-specific applications, but present limitations. This inspired research into genetically engineered microbial enhanced oil recovery (GEMEOR), which has crossover application in the remediation sector. A comprehensive review on MEOR by Patel *et al.* (2015) describe how this field uses genetic engineering, such as protoplast fusion, to combine multiple favourable biosurfactant traits into a single engineered microorganism. A possible aim for this line of research would be to place the rhamnolipid (an extensively studied and effective biosurfactant) synthesising gene, from *P. aeruginose*—a pathogenic bacterium—into the more industrial safe *Escherichia coli* (Soberón-Chávez, 2011). The GEMEOR method has not yet been significantly applied in the field, limiting available data.

2 Background

The word surfactant is a compounded word from 'surface active agent'. Surfactants are amphiphilic agents, containing both a hydrophobic tail group and a hydrophilic head group. They are categorised by their hydrophilic group: anionic, cationic, non-ionic and amphoteric (zwitterionic) (Barnes & Gentle, 2005; Pennell 1998). Due to their physiochemical structure, surfactants arrange themselves to surface interfaces, with their moieties oriented to their complementary bulk phase. At a water-air and water-oil interface, the hydrophilic head group will be immersed in water, while the hydrophobic (i.e. lipophilic/oleophilic) tail groups will be mainly located in the air or oil bulk phase. This single layer of surfactants forms a Gibbs monolayer (fig. 1).

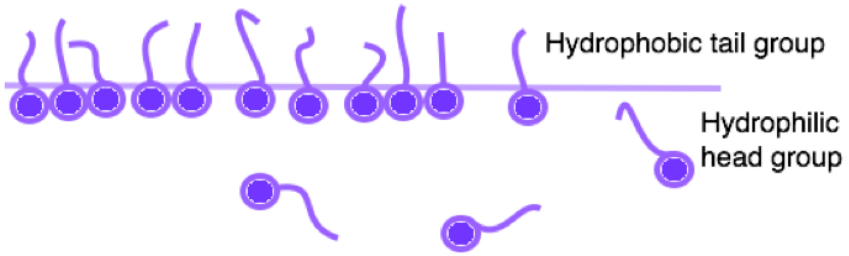


Figure 1. A schematic of Gibbs monolayer formation in water-air/oil interface surfactants, with hydrophilic groups immersed in water.

When an event changes the surface tension (γ), such as the addition of surfactants, the surface pressure (Π) is often used to describe the event.

$$\Pi = \gamma_o + \gamma_f$$

Where γ_o is the initial surface tension and γ_f is the final surface tension after the event (Barnes & Gentle, 2005). The surface tension will normally reduce after surfactant adsorption, creating a positive surface pressure value.

Surfactants can be applied to make two immiscible fluids into an emulsion. Water-oil system is the prime example of two immiscible liquids. Lowering the surface tension of the water will increase the emulsification of the two liquids. As the interfacial surface tension becomes increasingly negative, so does the free energy, leading to spontaneous increase in surface area (Barnes & Gentle, 2005). This creates a more stable emulsion where the reduction to the

minimum surface energy reduces the rate of coalescing droplets that normally occurs to minimise surface area, thus limiting the destabilisation of emulsions.

2.1 Mechanism of surfactant action

Equal attractive forces balance molecular interactions in a pure liquid, however there is an imbalance of these attractive forces at the surface (fig. 2). This creates excess in surface free energy (Sigma 700). Liquids that are polarised, like water, have a higher surface tension (γ) due to the strong intermolecular interactions. The surface tension of polar liquids will be lowered by increasing the temperature or pressure of the system, or by impurities—especially surfactants (Sigma 700, Barnes & Gentle, 2005).

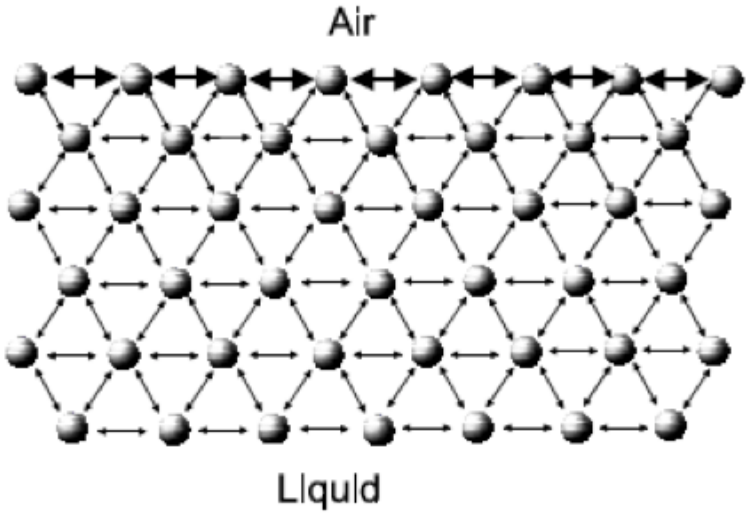


Figure 2. A schematic diagram of attractive forces acting upon molecules (Sigma 700)

2.1.1 Micelles

In some cases, the definition of surfactants is characterised as a molecule capable of associating to form micelles (Schramm and Marangoni, 2000). A micelle is a surfactant aggregate dispersed in liquid as a colloid. The critical micelle concentration (CMC) is the point where micelles are formed in the surfactant-solvent system. Almost all physiochemical properties, such as surface tension, plotted against concentration demonstrate an abrupt change in slope at the CMC point (fig. 3) (Pennel and Abriola, 1998; Schramm and Marangoni, 2000). This is followed by minimal to no significant changes as concentration increases past the CMC point.

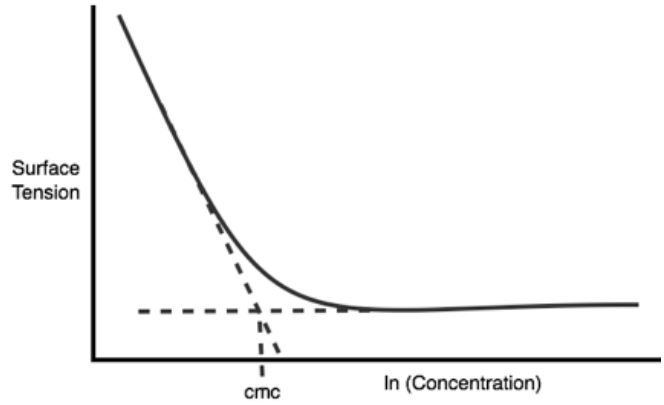


Figure 3. Surface tensions plotted against natural logarithmic (ln) of concentration with critical micelle concentration (CMC) intersection.

Krafft point

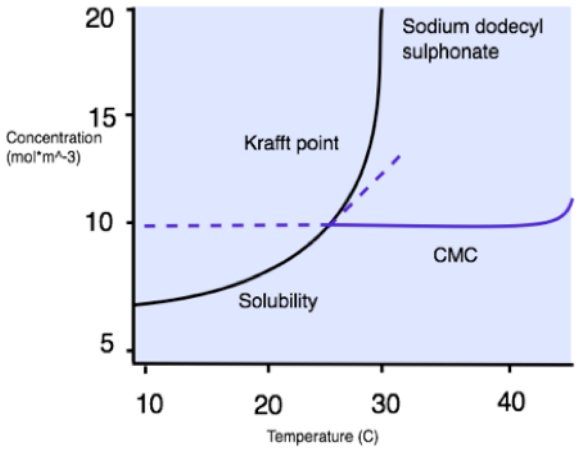


Figure 4. Krafft point for CMC of SDS plotted concentration over temperature taken from Barnes & Gentle (2005) page 79.

Krafft temperature is the minimum temperature required for ionic surfactants to aggregate into micelles. The temperature dependence of the CMC and solubility, given in figure 4, explains the origin of the Krafft temperature, or point (Barnes and Gentle, 2011). While micelles form at and above the Krafft temperature, below this point the ionic surfactant will remain crystalline, rendering to no surface-active function of the surfactant (Cai, Zhang et al. 2014). The Krafft temperature can vary drastically; depending on the surfactant, for example, the anionic surfactant sodium dodecyl sulphate (SDS), a surfactant typically used for laundry detergency, has a Krafft temperature of 9^{o}C. In contrast, sodium palmitate, a common ingredient in napalm, has a Krafft temperature of 48^{o}C (Barnes and Gentle, 2011).

Cloud point

While Krafft temperature is a crucial point for solubility of ionic surfactants, non-ionic surfactants solubility is reversed. Raising the temperature will reduce the solubility of the surfactant, possibly due to the weakening of the hydrogen bonds (Barnes and Gentle, 2011). This temperature is known as cloud point. Once cloud point is reached, the surfactants separate out into a distinct phase, rendering the fluid 'cloudy'. Cloud point temperature is dependent on hydrophilic-hydrophobic structure of the non-ionic surfactant. Increasing the hydrophobic components will lower the cloud point (Barnes and Gentle, 2011). When cloud point occurs, reducing the temperature allows for this reversal of the reaction, to return the system to a clear state.

2.1.2 Bacterial interface adhesion

The production of biosurfactants in a water-oil system can establish and stabilise emulsions. The emulsion increases the oil dispersal by increasing interface surface area between the two liquids, which in bioremediation terms, increases the bioavailability for degradation. Bioavailability can be loosely defined as a degree of interaction amongst substrates and microbial organisms or communities, increasing the uptake readability (Dorobantu, *et al*., 2004). However it is possible to stabilise the water-oil or oil-water systems microbially, without the production of biosurfactants. It is established that fine particles, such as silica beads, can prevent the coalescence and merging of oil drops in water-oil and oil-water emulsions. Some hydrophobic cells without biosurfactant production or active oil uptake—i.e. not altering the surface tension—can act as fine particles, preventing coalescence and thus stabilising the emulsion (Abbasnezhad, Gray & Foght, 2011). It is therefore not distinctly necessary for micro-fauna to produce biosurfactants to increase oil dispersion in a water system.

2.2 Types of surfactants

2.2.1 Synthetic surfactants

Synthetically manufactured surfactants for oil dispersion, like Corexit 9500, are ironically petroleum derived. In fact, nearly all of the 2.7 million tonnes of surfactants produced are petroleum derived (Radmann, et al, 2015). They are known to be more persistent and pose potential higher toxicity. After such an unprecedented use in the Gulf of Mexico from the Deepwater Horizon oil blowout, the effectiveness has been called into question.

The effectiveness for applying synthetic oil dispersants onto oil spill incidents has been an on going academic debate. A study published in 2009 that was funded and conducted by U.S. Minerals Management Service (MMS) to address the effectiveness of Corexit 9500 and Corexit 9527 dispersant in filtered clear cold saltwater (Belore *et al*, 2009). The study concluded 85-99% effectiveness in dispersing the oil, with Corexit 9500 producing smaller oil drops than Corexit 9527, making it marginally more effective.

Conflicting arguments for Corexit, and other surfactants for oil dispersion, are also abundant. A 2015 study by Sara Kleindienst *et al*. established that the application of oil-dispersants, in both simulated and *in situ* in Gulf deep waters, stimulated the growth of oil-dispersant degrading microbes, like *Colwellia*, while supressing the growth of oil-degrading microorganisms, like *Marinobacter*. The study continued by testing hydrocarbon-rich oil addition in simulated deep-waters without dispersants, which enhanced the *Marinobacter* population from 2% to 42% of total microbes. Kleindienst *et al*. (2015) corroborated the deep-water findings with simulated surface marine water results, concluding synthetic dispersants such as Corexit 9500, can have a negative effect on deep ocean water remediation from crude oil contamination. The effectiveness of Corexit 9500 is questionable, especially in terms of environmental implications when biodiversity and abundance is altered in a non-favourable manner.

2.2.2 Biosurfactants

Since the initiation of REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals), further investigation has been stimulated into biosurfactants as alternatives to synthetics surfactants (SAMS, 2016). Research into biosurfactants has also further developed not only for environmentally friendlier reasons, but also for the vast variations in applications for the food industry, such as altering the flow properties to reduce fat content (SAMS, 2016; Radmann, et al., 2015).

Many organisms, such as bacteria or fungi, can produce biosurfactants. The biogenic origins of biosurfactants mean they are considered a separate class to synthetic surfactants, but are still amphiphilic in nature. Biosurfactants are typically less toxic and less persistent than the synthetically and petroleum derived surfactants due to isomer variations, however, this also leaves less certainty in quality of function (Edwards, Lepo & Lewis, 2003; Pennell 1998). Within the class of biosurfactants, various types with differing properties exist. The major classification groups include glycolipids, fatty acid biosurfactants, lipopeptides, emulsifying protein, and particulate biosurfactant (Patel, et al., 2015). Important biosurfactants for MEOR are lipopeptides and a type of glycolipid, known as rhamnolipids (RL), which can reduce

interfacial tension (IFT) of liquid hydrocarbon-water interface to below 0.1 mN/m, making these biosurfactants popular for application and research (Patel et al, 2015).

Lipopeptides

Pseudomonas spp. and *Bacillus subtilis* are notable producers of lipopeptides, which are potent biosurfactants that can decrease the surface tension of distilled water from 72 mN/m to 27.9 mN/m at a CMC of 0.017 g per litre (Ismail, *et al*, 2013; Patel, et al., 2015). Lipopeptides are classified into four groups: surfactins, iturins, fengycins or plipastatins, and kurstakins; with surfactins as established biosurfactants used in MEOR (Soberón-Chávez, 2011).

Rhamnolipids

Most notably derived from *Pseudomonas aeruginose*—an opportunistic pathogen—RL are the most investigated biosurfactants, having been first reported by Bergström *et al.,* (1946). They are glycolipids composed of a glycon group and an aglycon part (fig. 5) (Soberón-Chávez, 2011).

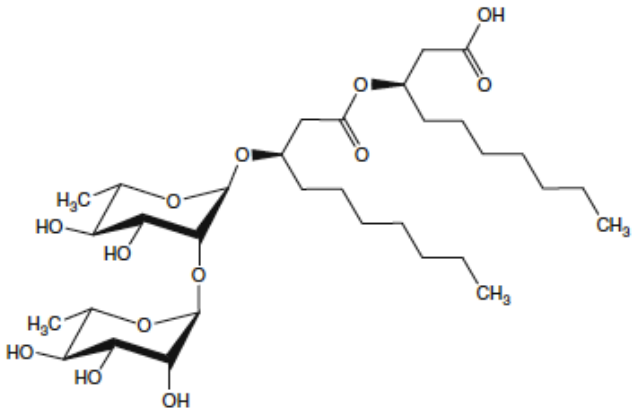


Figure 5. "Chemical structure of the first identified rhamnolipid" (Soberón-Chávez, 2011: pp 17).

RL have also had extensive research for their anti-bacterial, -fungal and –viral properties, as well as reports on RL interactions within the human body to promote the uptake of hydrocarbons with low solubility (Soberón-Chávez, 2011). The well-established hydrocarbon interactions of RL led to research and applications for MEOR. RL are extremely effective for flooding operations to increase the sweep efficiency, but also have crude oil-remediation applications (Patel, et al, 2015).

2.3 Biosurfactant producing microorganisms

2.3.1 Fungi

While mechanical processes can be used to breach interfaces, fungi, such as the filamentous *Schizophyllum commune*, are able to produce stable amphipathic protein containing a hydrophobin (SC3) at a water-air interface, which allows for fruiting in the air phase (Wösten, et al., 1999). Adaptations for biosurfactant production are advantageous for fruiting fungi that would otherwise not be able to exert necessary mechanical force to breach growing substrate-air interfaces.

2.3.2 Algae

According to the Scottish Marine Institute in Oban, Argyll (SAMS, 2016), algal surfactant research requires more development, as it offers functional use within confectionary food industry, such as by reformulating food to contain less fat. Algae, and microalgae, such as cyanobacteria *Arthrospira* sp., produced biosurfactants that also have applications within medical and pharmaceutical industries, and are of a significant area of research, as algae and microalgae are generally certified as safe for toxicity and pathogenicity (Radmann, et al., 2015).

2.3.3 Bacteria

Bacteria studies for biosurfactant production are well developed. In petroleum industry terms, focus is aimed for MEOR and remediation purposes. Many studies have focused on *Pseudomonas* species, as readily available and affordable choices (Rocha e Silva, *et al*. 2014). Researching affordable production is key within bacterial-derived biosurfactant industry, as biosurfactant production is often three to ten times the cost of synthetic surfactant production (Cai, et al, 2014). *Pseudomonas cepecia* is one such studied *Pseudomonas* species for cost effectiveness. Rocha e Silva, *et al*. (2014), demonstrated promising results, with *P. cepecia*, grown with 2% corn steep liquor and 2% soybean (waste) frying oil supplements in a mineral medium, reducing surface tension to 27.57 mN/m, and being able to produce 5.2 g/L of isolated biosurfactant. Rocha e Silva, *et al.* (2014) determined the isolated biosurfactant would disperse approximately 80% of oil droplets in seawater. Crucially, Rocha e Silva, *et al.* (2014) demonstrated that the biosurfactant produced did not have a toxic effect against the bio-indicators used—seeds of *Brassica oleracea* for terrestrial environments and the micro-crustacean *Artemia salina* for salt lakes, inferring *P. cepecia* as a potential candidate for biosurfactants production as a component in oil industry remediation efforts.

Mixed bacteria consortium

The use of a single species or genera for biosurfactant production has a feasible application use for small-scale site, or laboratory exercises. However, for large-scale field applications it is more feasible to consider a mixed consortium of bacteria strains to offer greater coverage and certainty in remediation processes. This validity for this argument can be seen in a study conducted by Rahman *et al.* (2002). Rahman *et al.* (2002) assessed the efficiency of degradation for Bombay High crude oil using single specie strains and mixed bacteria consortium comparatively. They found that at 1% crude oil in a twenty-day test that while *Pseudomonas* sp. DS10-129*, Bacillus s*p. DS6-86, *Micrococcus* sp. GS2-22*, Corynebacterium* sp. GS5-66, *and Flavobacterium* sp. DS5-73, were capable of respective degradations of 66%, 59%, 49%, 43%, and 41%; the mixed consortium exceeded single strain efficiency with 78% degradation. Rahman, *et al*. (2002) were able to further support their findings by repeating the test with 10% crude oil, with the single strain degradation ranging from 16%-32% and the mixed consortium executing 52% crude oil degradation.

2.4 Methods for biosurfactant screening

In order to assure the biosurfactants from the bacteria meet a standard of function, certain characterisations are measured (Hassanshahian 2014). Hassanshahian (2014) selected five screening methods for selecting biosurfactant-producing bacteria: haemolysis in blood agar, oil spreading, drop collapse, liquid surface tension, and emulsification activity and Bacterial Adhesion to Hydrocarbon test (BATH)—also known at microbial adhesion to hydrocarbon test (MATH). These tests are well established in previous research as methods of detection for biosurfactant-producing bacteria.

2.4.1 Physiochemical measurements

Drop collapse test

Drop collapse test can be applied qualitatively or quantitatively when screening for biosurfactants. Small wells or cavity plates are coated in high quality oil and dried (Hassanshahian 2014). Sample drops are placed on the surface and spreading is measured. For qualitative results, the liquid-solid (oil-coated) contact angle (Θ) is measured from a close-up captured image of the drop on the surface. A water droplet on a smooth solid surface has a contact angle of 120^{o} at equilibrium (Barnes & Gentle, 2005).

Emulsification activity—E index

Screening methods for emulsifying biosurfactants can be achieved by testing the emulsification index. This technique consists of adding an amount of refined oil—such as linseed or vegetable oil—to an equal amount of cultured sample. The oil and cultured sample is thoroughly mixed by vortex, and left to rest at 24, 48, and/or 72 hours, with emulsification index being noted as E24, E48 and E72 respectively (Abbasi & Amiri, 2007). The indices are given as a percentage of the total height of the emulsified layer (mm) divided by total height of the liquid column (mm) (Mnif et al., 2011).

IFT—Interfacial tension

Measuring changes in IFT gives a strong indication for presence of biosurfactant production in cultured broths. IFT determination methods can be accomplished with either a Du Noüy Ring or a Wilhelmy plate, to measure surface tension by measuring the force between the probe and the surface of the liquid phase.

Du Noüy ring

This method uses a platinum ring, typically with a diameter of 10 mm, which is submerged into a liquid and raised—pulling up the meniscus disk of the liquid (fig. 6). The assumption of force owing to surface tension with the Du Noüy ring is twice the ring's circumference (Sigma 700; Barnes & Gentle 2005). A mathematical correction procedure is required.

$$F = 2\pi \cdot (r_i + r_a) \cdot \gamma$$

Where F is force, r_i is the radius of the inner ring, and r_a is the radius of the outer ring of the liquid meniscus (Barnes & Genetle, 2005).

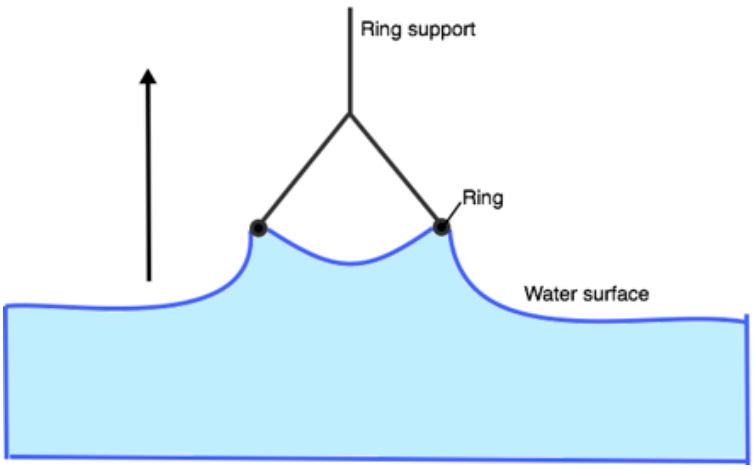


Figure 6. Schematic of meniscus of liquid phase pulled up as ring it withdrawn.

Wilhelmy plate

In this technique, a platinum plate is lowered and raised in the same fashion as the ring technique (fig. 7). Calculations are based on geometry of the fully wetted platinum Wilhelmy plate and require minimal correction. If the plate is hanging vertically—perpendicular to the water—surface tension can be determined by rearranging the equation for force (Barnes & Gentle, 2005), as all variables accept surface tensions are known.

$$F = \gamma 2(x + y)$$

Where x and y are the horizontal length and thickness of the plate respectively.

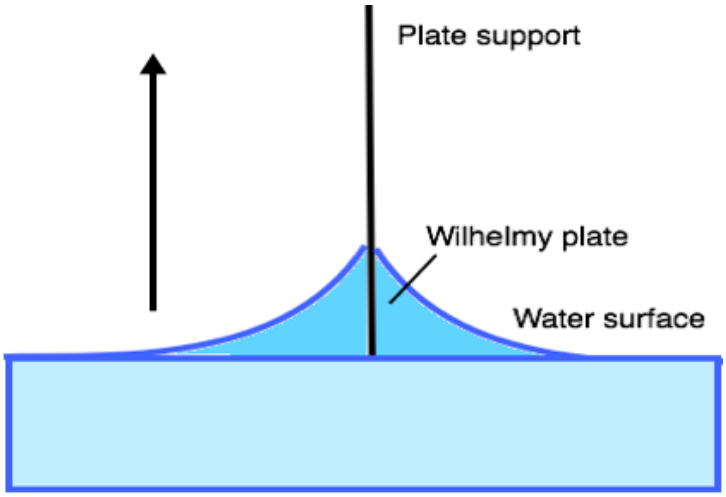


Figure 7. Schematic of a Wilhelmy plate being withdrawn from water, with water cohesion onto the plate.

IFT is method, with either Du Noüy ring or Wilhelmy plate, to detect surface tension of sample, which are compared to controls. It is commonly used as a tool to investigate biosurfactant-producing microorganisms from cultured broths, and to determine isolated biosurfactant efficiency to applied samples.

2.4.2 Enzyme activity based methods

The bioremediation of total petroleum hydrocarbon (TPH) degradation has advanced into screenings for selection refinement, such as the Kumari, Singh and Singh (2012) study comparing the metabolic degradation pathways of two strains. Kumari, Singh and Singh (2012) results demonstrated *Pesudomonas* sp. BP10 catechol 1,2 dioxygenase pathway activity exhibiting higher degradation capabilities than the *Rhodococcus* sp. NJ2 catechol 2,3 dioxygenase pathway, with TPH of 60 and 50%, respectively. The implications from studies

like this are not only for biosurfactant producing selection based on enzyme activity, but also for enzyme expression selection for genetically engineered microbes.

2.5 Objectives

The overall aim of this study was to isolate biosurfactant-producing bacteria from sources with different history of hydrocarbon exposure. The objectives were: (1) to obtain pure cultures of candidates capable of growing on oil as the carbon source and (2) to evaluate the capacity of these candidates for biosurfactant production by using traditional techniques.

3 Methodology

3.1 Samples

Three samples were used to search for crude oil-degrading bacteria that produce biosurfactants. The screening was aimed for crude oil remediation in cold marine environments, so salinity in the media were incorporated for the selection of slight halophiles, and low incubation temperatures were applied for psychrophile selection. Norwegian-Group AS provided the first two samples from an offshore wastewater treatment system. The samples were from the bioreactor (BR) and the settling chamber (SC). The third sample, Arctic, was bacteria grown from seawater on oil coated marine agar plates prior to research commencement; provided by Andrea Bagi, University of Stavanger (UiS). The original sample came from Ny-Ålesund, Svalbard, Norway, near Sverdrup Station. The three samples are from abundant and readily available sources, with the potential to offer novel species for site-specific (i.e. cold marine environments) crude oil remediation purposes.

3.2 Experimental approach

The experimental approach was broken down into three segments. Firstly, samples were prepared by growing and isolating bacteria colonies from sample sources, BR, SC and Arctic, and transferred to liquid media. Secondly, biosurfactant assessments, using qualitative and quantitative screening methods, were applied to determine the presence of biosurfactants, and finally culture-dependent and culture-independent methods were applied to assess culture purity. The latter was preformed in order to determine if successful bacterial colonies could be sequenced.

3.2.1 Bacteria sample preparation

Samples were prepared for biosurfactant producing bacteria screening tests by firstly growing and establishing potential colonies (fig. 8). The 5 steps illustrated in figure 8 are described in detail below:

a) Samples from the BR and the SC were prepared at dilutions of 10^0 (i.e. no dilution) to 10^{-6}, and triplicates were inoculated onto oil-coated nutrient agar (NA) and Bushnell Haas (BH) agar plates. Arctic samples were inoculated onto oil-coated marine agar plates.

b) BR and SC samples were incubated for approximately two weeks at 10˚C, for sufficient growth for selection. Arctic samples were slower growing and were incubated between 3-4˚C for approximately three weeks.

c) Samples with colonies that displayed selection criteria traits were picked and streaked onto new oil-coated media for isolation, in a standard pick and streak method. The streaked colony samples were incubated again for the same amount of time, at the same temperatures.

d) Incubated isolated sample colonies were examined to verify selection criteria traits were still present. If not, they were deemed false positives, and discarded from further treatment. Isolated colonies were again picked, but transferred to liquid media with oil. The same incubation as before was repeated.

e) Incubated bacteria cultures were split into two larger liquid (50ml) media with oil. Incubation was repeated

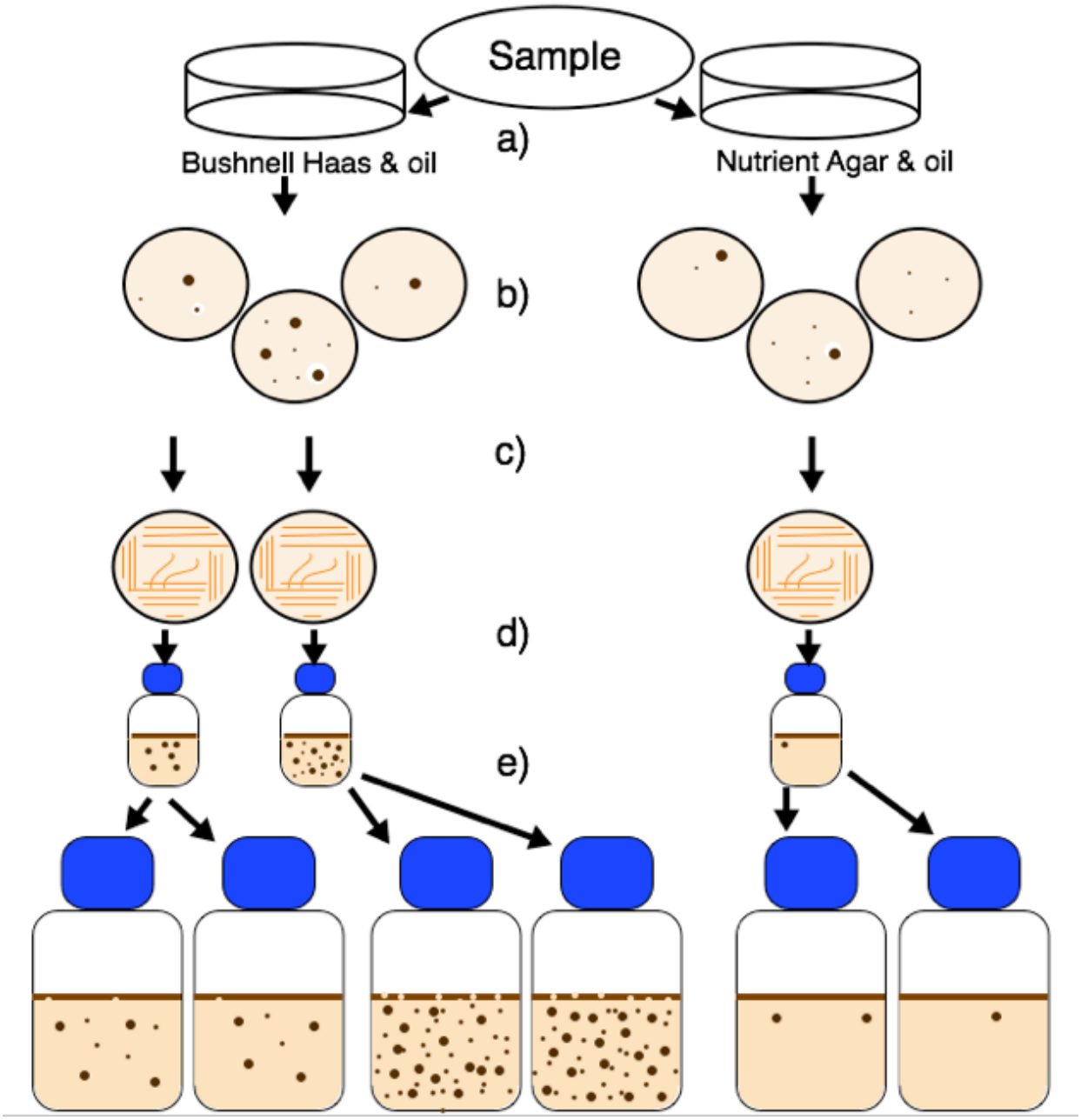


Figure 8. Bacteria sample preparation with oil and growth media, including steps of a) inoculation, b) incubation, c) colony isolation streaking, d) transferring to liquid media and e) up scaling and duplicated bacterial cultures in liquid media.

3.2.2 Biosurfactant screening

After the final incubation period of the 50 ml liquid media, bacterial cultures were screened for biosurfactant production. Biosurfactant production for degradation of crude oil included two qualitative tests: a) oil drop collapse test and b) visual emulsification and flocculation test after sample agitation, and a quantitative test; c) measurements of interfacial tension (IFT) using a Wilhelmy Plate instrument (fig 9).

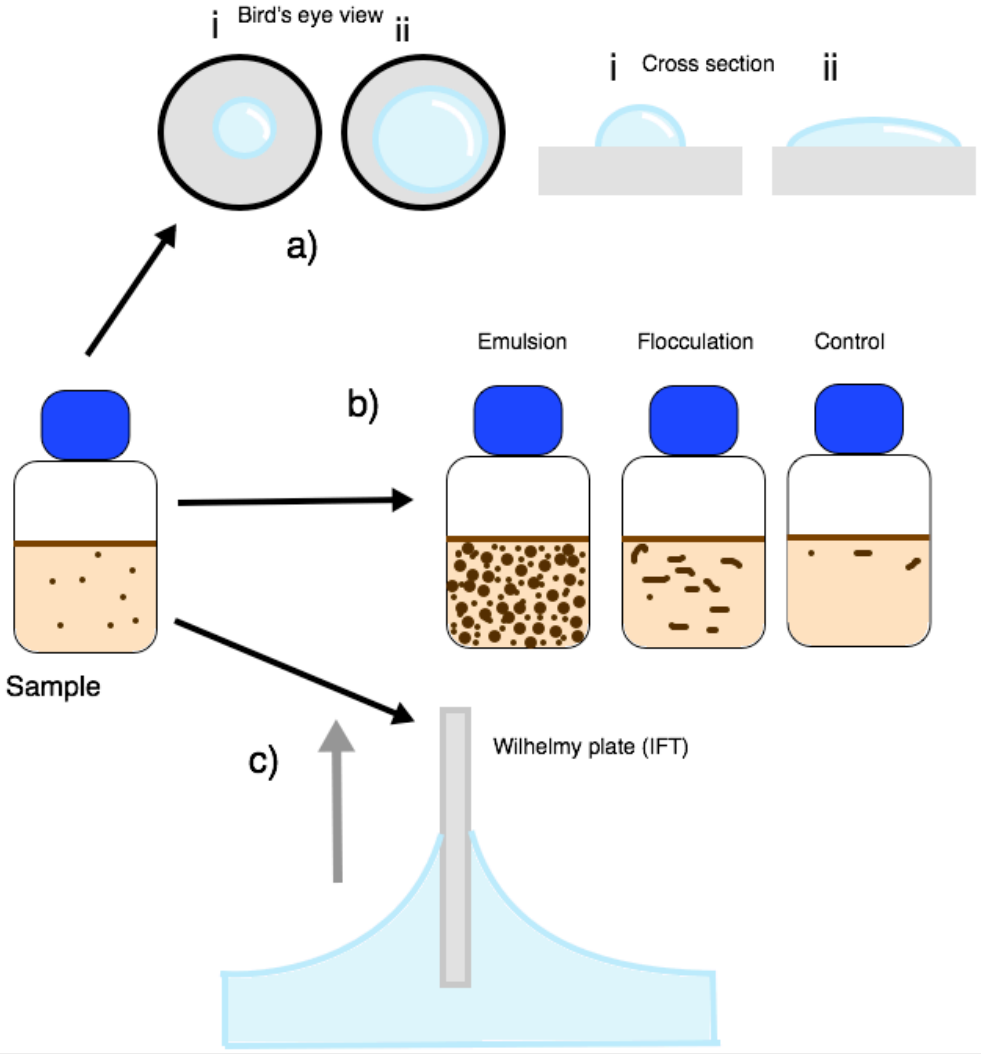


Figure 9. Biosurfactant screening approach using a) oil drop collapse test comparing i) no spreading to ii) drop spreading—i.e. surfactant presence, b) visual emulsification and flocculation after sample agitation compared to a control, and c) IFT measurement using a Wilhelmy plate.

3.2.3 Purity assessment

Bacterial purity was assessed to determine if sequencing would be possible for the successful biosurfactant producing candidate samples (fig. 10). The steps in figure 10 are described in detail below:

a) Morphological assessment was first conducted using a microscope as an inference of purity. Combinations of cocci, bacillus or spiral-shaped bacteria would quickly determine lack of purity, while morphological uniformity would infer probably purity.

b) & c) DNA fingerprinting was conducted using denaturing gel gradient electrophoresis (DGGE) of 16s rRNA gene fragment amplification via polymerase chain reaction (PCR).

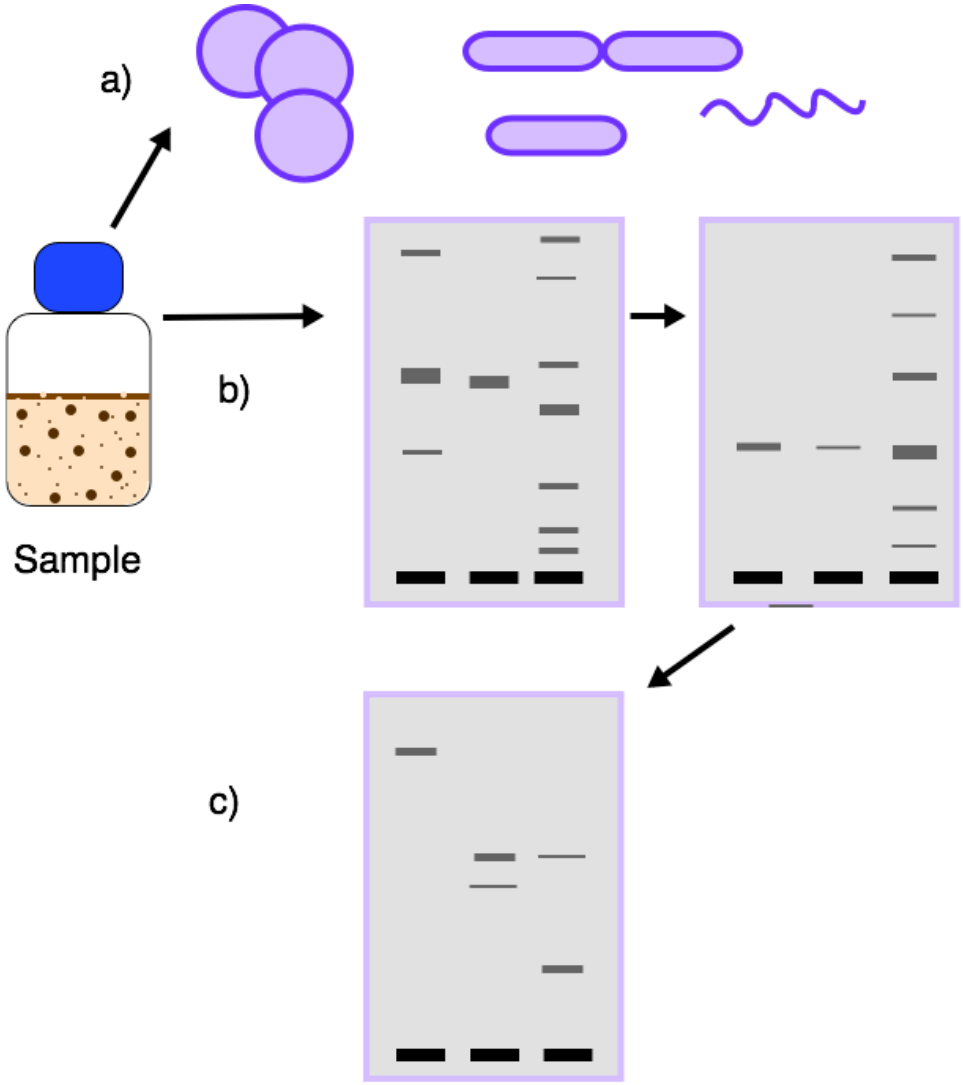


Figure 10. Bacteria purity assessment approach using a) morphological assessment, b) & c) DNA fingerprinting based on 16s rRNA gene using denaturing gel gradient electrophoresis (DGGE)

3.3 Methods for inoculation and incubation

The Arctic samples were incubated on seawater agar with 100 µl of evenly spread filter-sterilised Tyrihans oil, while BR and SC samples were inoculated onto both NA and BH agar media with 100 µl of evenly spread filter-sterilised light Arabian oil. Oil was coated 24 hr before inoculation and left to dry in place. Triplicates were made at each dilution by mixing 100 µl of sample with 900 µl of peptone buffered water (10 g/l peptone: Amresco Peptone; and 5 g/l NaCl: BDH Prolabo). The dilutions were 10^0 (i.e. no dilution: 1000 µl of raw sample), 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}. The initial incubation time and temperature as mentioned were between 3-4^{o}C for three weeks for Arctic samples, 10^0C for approximately two weeks for BR and SC samples.

After the incubation period, colonies were isolated and streaked onto their corresponding agar media (coated with 100 µl oil), using a standard pick and streak method. Criteria for selecting colonies were as followed:

- Clearing of oil around the colony—suggesting interaction with oil by possible biosurfactant release.
- Signs of oil uptake into the colony—suggesting colonies were able to utilise the oil in metabolism
- Coating in oil—suggesting colony attachment, or adhesion, to oil surface

Sub categories to ensure colony diversity:

- Different colony edges—smooth edged or fringed edge
- Different colony structure—circular or amorphic
- Size—small/slow growth or large/fast growth
- Colour or visible differences in internal colony structure.

The Arctic isolation streaked samples were incubated for three weeks between 3-4°C, while the BR and SC samples were incubated for approximately two weeks at 10°C. After incubation, some false positives were easily identified and removed from further investigation. The colonies that continued to express traits of the selection criteria were the picked for the liquid media incubation phase. Although a secondary isolation streaking would have provided greater certainty for colony purity, a time constraint prevented this.

The colonies were picked and transferred to their corresponding 10 ml liquid media with 100 µl filter-sterilised oil. Incubation time and temperatures for BR and SC samples were again 10°C for approximately two weeks and Arctic samples were held between 3-4°C for three weeks. In order to produce enough bacteria culture for testing, a final incubation was repeated after transferring 5 ml of each bacteria culture to new 50 ml liquid media with 500 µl filter-sterilised oil.

3.3.1 Media composition

Solid media

The SC and BR samples had two types of agar media plates, both prepared with 25.0 g/l sodium chloride (BDH Prolabo: NaCl) for saline tolerant bacteria selection. A standard nutrient agar (NA), composed of 20.0 g/l nutrient agar (Merck Chemical) was prepared and a Bushnell Haas

(BH) agar, composed of 3.75 g/l Bushnell Haas nutrient (Flukar Analytical), 15.0 g// agar (Merck Chemical). Further composition of medias provided in Appendix A.

The BH agar and NA media plates were coated with 100 µl light Arabian oil, provided by University of Stavanger (UiS). All oil used in assessments was first filter-sterilised by syringing the oil through a 0.2 µm cellulose acetate filter (Pall Corportation: Acrodisc Syringe Filters).

As mentioned, Arctic samples were inoculated prior to being provided for the research. Incubation had taken place on a seawater agar media. Seawater agar plates were prepared and consisted of one litre sterilised seawater, 15.0 g/l agar (Merck Chemical) and 1 ml/l of inorganic nutrients (16.2 mg/ L K_2HPO_4, 0.8 mg/L KH_2PO_4, 42.0 mg/L $NaNO_3$, 0.05 mg/L $FeCl_3$, 2.5 mg/L $CaCl_2$ and 1.5 mg/L $MgSO_4$) and trace minerals as described by Balch et al (1979). The seawater agar plates were coated in 100 µl filter-sterilised Tyrihans oil.

Liquid media

The liquid media (also referred to as broth) recipes were identical to the solid media, with the exception that no agar was added. As NA recipe had agar incorporated already in the ingredients, a nutrient broth (NB) was instead prepared. The NB was composed of 20.0 g/l nutrient broth (Merck Chemical) as well as the 25.0 g/l NaCl. All liquid media had 0.1 µl/l of filter-sterilised oil added. Initially, colonies were transferred to 10 ml liquid media. After incubation, the liquid culture samples were split into two (5ml each) and added to new 50 ml broth with filter-sterilised oil.

3.4 Methods for biosurfactant screening

After the final incubation period of the 50 ml liquid media, bacterial cultures were screened for biosurfactant production using three tests described in detail below.

3.4.1 Oil drop collapse test

Cavities of a well-plate (Falcon Sterile: Microtest 96), measuring 0.8 cm in diameter, were coated with 2 µl of cold-pressed linseed oil (Biofood: Naturenliga Livsmedel) and dried for 24 hours at room temperature (approximately 21˚C). The control drops were 10 µl of distilled water to signify no spreading, and 10 µl of Light Arabian oil to signify complete spreading. To be able to further assess sample spreading, 10 µl of control liquid media prepared with oil were

also tested. Spreading greater than that of the water and liquid media with oil would suggest positive results for biosurfactant presence.

3.4.2 Visual—Emulsification and flocculation

The second of the qualitative test was used to help determine if biosurfactant production was present. Time constraints did not allow for emulsification indices to be conducted, as described in the background section. Instead, vigorous swirling agitated samples, and a visual comparison was made to the non-inoculated control media with oil before agitation, and at more than 30 sec after agitation. If emulsions or flocculants held in the liquid media for longer, or in apparent greater numbers than the controls, than a positive result for biosurfactant presence was assumed.

3.4.3 IFT—Interfacial tension

A Sigma 700 and platnimum Wilhelmy plate were used to determine the IFT of the samples and controls. Both samples and controls were prepared identically according to the following.

To break any emulsions formed, 30 g of controls and samples were transferred to 50 ml Falcon tubes[1]. They were centrifuged for five minutes at 5000 rpm and held in a water bath at 55˚C for 24 hours. Samples with strong emulsions—i.e. difficult to break—underwent this process twice. After this treatment, 15 ml of the samples were transferred to the acetone-washed small (40ml) Wilhelmy vessel dish using disposable sterile syringes and needles.

Heavy phase was set as water and light phase as air, while speed-up and speed-down were set to 63 mm/min and 60 mm/min, respectively. Specific wetting depth was set to 3 mm. The minimum time of three minutes and minimum number of points were set to five. The platinum Wilhelmy plate was washed with acetone and flamed with a Bunsen burner between tests.

3.5 Methods for purity assessment

3.5.1 Purity assessment using cell morphology

An Olympus BX 61 at 100 times magnification was used to examine cell morphology of bacteria cultivated in oil-amended broths. A volume of 30 μl of each cultured broth was examined under the microscope and consistency in morphology was used to infer colony purity. While cocci species are visually distinctive from spiral-shape and bacilli species, further distinctions within the same morphological class, such as cocci and bacilli species, can be

[1] 30 g were used instead of 30 ml to balance the centrifuge.

further classified by arrangement. Hence morphological characterisation was preformed according to distinctions shown in figure 11.

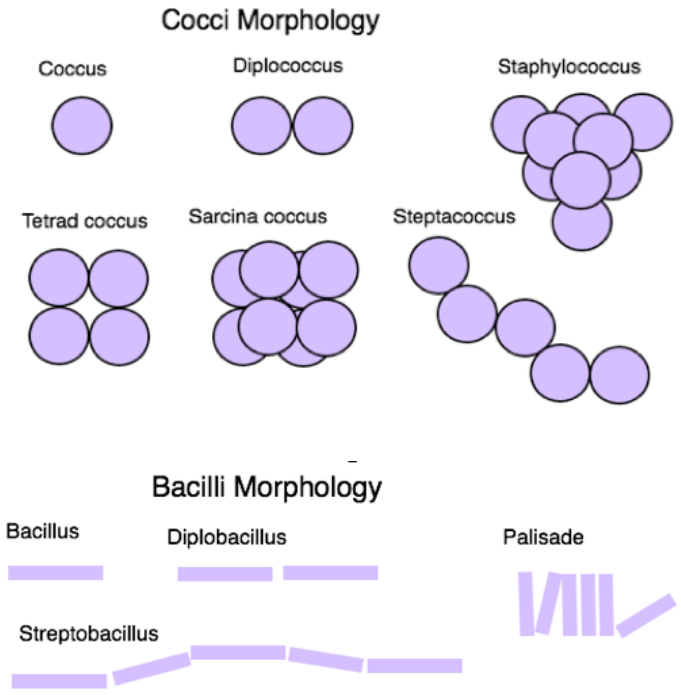


Figure 11. Cocci (top) and bacilli (bottom) morphology classification groups based on cell arrangements.

3.5.2 4.5.2 Purity assessment using PCR-DGGE analysis

Sample selection for DNA analysis

Based on results from biosurfactant screening tests, a selection of two Arctic samples, one BR BH and two SC BH samples were chosen for DNA analysis. Samples for DNA analysis were chosen primarily based on IFT results, complemented by positive results from both qualitative assessments. The morphological assessment was also instrumental in selection to increase likelihood for sample purity.

DNA isolation

The DNA from 20 ml of 50ml liquid media samples was isolated following the protocol for PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc.) without modifications. The isolated DNA was assessed for size and integrity on a 2% agarose gel (1.0 g agarose 50 ml 1 x TAE buffer, 5 µl GelGreen dye) ran for 90 minutes at 80 volts in 1 x TAE buffer (prepared from 50 x TAE, pH 8.3 tris-acetate EDTA buffer, Merck).

PCR of 16s rRNA gene fragments

The isolated DNA samples were prepared for polymerase chain reaction (PCR) as described in Table 2.

Steps	Volume (µl)	Ingredient	
1	39	molecular grade water	(blank at 40µl)
2	5	Taq buffer (with Mg2+)	
3	1	Isolated DNA sample	
4	5	PCR mix	
	PCR Mix for 10 samples		
1	27	molecular grade water	
2	2.5	dNTPs	
3	5	primar forward	
4	5	primar reverse	
5	2.5	enzyme Taq polymerase	

Table 2. Polymerase chain reaction (PCR) sample preparation steps

The PCR programme was described in the following: initialization at 94.0˚C for two min, followed by 25 cycles of denaturation at 94.0˚C for 30 sec, annealing at 55.0˚C for 40 sec and elongation at 72.0˚C for 1 min. A final elongation step at 72.0˚C for 7 min was also preformed. Then the reaction mix was cooled to and held at 4.0˚C. Samples were stored frozen (-20˚C) until further analysis.

DGGE analysis

Denaturing gradient gel electrophoresis (DGGE) solutions of 20 and 80% were prepared according to the description of Table 3.

Table 3. Denaturing solutions of 20 and 80% preparation for a 6% acrylamide gel in DGGE analysis.

	20% Denaturing Solution	80% Denaturing Solution
40% Acrylamide/Bis	30 ml	30 ml
50x TEA buffer	4 ml	4 ml
Formamide	16 ml	64 ml
Urea	16.8 g	67.2 g
dH20	to 200 ml	to 200 ml

On the day of DGGE analysis, a 6% polyacrylamide gel was prepared using 30 ml of each of the denaturing solutions (20 and 80%). Right before pouring the gel via a gradient former, 30 µl of tetramethylethylenediamine (TEMED) and 300 µl freshly prepared 10% ammonia persulfate (Sigma >98% APS) was added to both, and mixed. After being pumped into its cast, the gel was left to set for approximately 3 hr.

Buffer and loading

The buffer tank of the IngenyPhorU DGGE system was filled with 50 x TAE buffer (340 ml) and 17 litres of MilliQ water. The buffer was warmed to 60˚C prior to immersing the gel into it. The wells of the gel were rinsed with buffer solution prior to loading samples. Each well was loaded with 20 µl of sample pre-mixed with 5 µl of 1:5 DGGE dye (total well loading of 25 µl). The DGGE was run for 18 hours at 90 volts and 60˚C. After that, gel was removed and stained in 1 x TAE buffer containing 1 x GelRed (VWR) for 1 hr at room temperature. DNA in the gel was visualised using GelDoc XR (BioRad) gel documentation system.

4 Results

Arctic samples presented 39 colonies that could be picked and had selection criteria traits as described in the methodology section. The 39 colonies were picked for isolation streaking. Of those, 34 plates had colony growth that continued to present selection criteria traits for biosurfactant presence, and individual colonies from each plate were picked and transferred to seawater liquid media with oil.

NA plates with oil were able to cultivate high number of colonies. While 47 colonies were picked based on expression of selection criteria for SC samples, and 22 from the BR samples, 20 and 12 isolated colonies respectively continued to express traits for selection criteria and were picked for NB liquid media with oil. Once in the liquid media, 10 SC samples and 8 BR samples failed to cultivate. The remaining 10 SC and 4 BR samples had exhibited signs of cell death.

BH media with oil cultivated 23 SC colonies 10 BR colonies that expressed selection criteria traits, and were picked and streaked for isolation. Of those, 22 SC isolated colony plates and 5 BR isolated colony plates continued to express selection criteria, and were thus picked and inoculated into liquid media with oil.

4.1 Results for biosurfactant screening

4.1.1 Colony selection

Colony selection was based on selection criteria mentioned in the methodology section. Figure 12 demonstrates examples for the initial selection processes, from a BH plate with BR sample from 10^{-3} dilution. Colony A is a good example of active oil uptake into a larger—faster growing—colony, while colony B is smaller—slower growing—it demonstrates a clearing zone around the colony. The colonies in the area marked C, also have clearing, while D(i) and D(ii) demonstrate colony morphology difference of smooth-edged colony and fringe-edged colony, respectively.

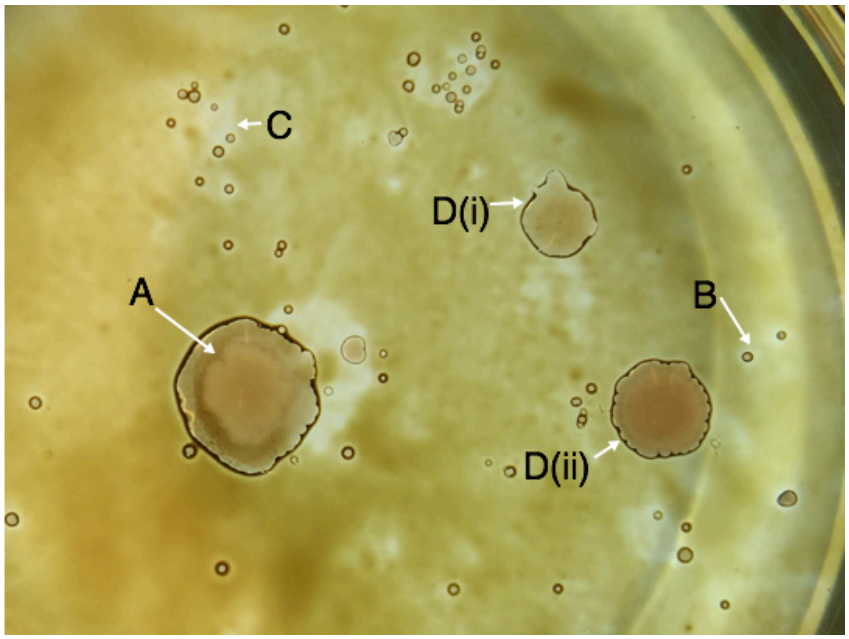


Figure 12. Oil-coated Bushnell Haas agar, inoculated with bioreactor sample, dilution factor 10^{-3}, on plate 1. Some smaller colonies have clearing (B), while others show clearing (C), which could be due to oil having ben moved during inoculation. Larger colonies show active oil uptake (A, D(i) and D(ii).

4.1.2 Visual—Emulsification and flocculation

The seawater liquid media with oil was able to form some flocculation after agitation for more than 30 sec compared to the BH and NB liquid media, making some positive results difficult to infer for larger flocculent formations. The cultured Arctic samples that were able to provide smaller flocculent size with high dispersal were readily inferred as a positive result for biosurfactant presence.

Neither SC nor BR samples cultivated using NB liquid media presented as strong emulsions, but 5 SC samples and 3 BR samples had some large flocculants formed and held for longer than the control, but dispersal remained near the surface.

BH liquid media cultivated high prevalence for emulsification, as well as being the only media to provide emulsion-forming bacteria (fig. 13). For the SC samples, 8 samples provided strong emulsions, and 5 samples with good flocculent formation and dispersion. All 5 BR samples provided strong emulsions

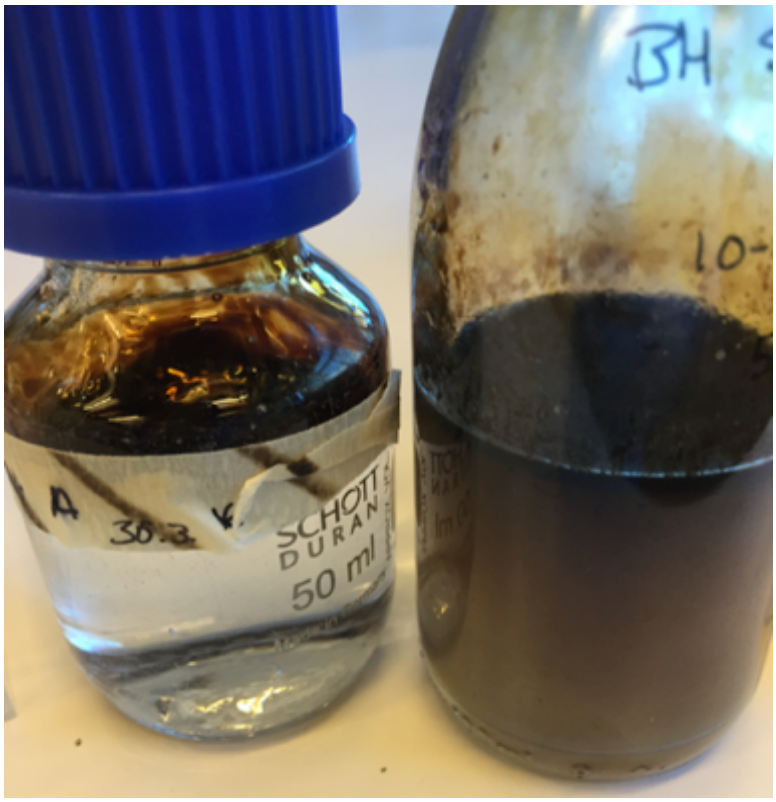


Figure 13: Control (left) Bushnell Haas (BH) with oil compared to (right) cultured settling chamber (SC BH) colony from plate 1 at 10^{-4} dilution. After gentle swirling, visible emulsion is formed, and held for significant time (more than 30 min).

4.1.3 Oil drop collapse test

The oil drop collapse tests indicated positive results that several samples contained biosurfactants. The control liquid media with oil was also tested as a comparison. Due to the thick top layer of oil, a positive result for spreading was observed for the control BH liquid medium with oil. Samples that also had comparatively an observable thick top layer of oil, and that did not form emulsions in the visual test, were tested multiple times, such as SC BH sample 15 referred to as B in figure 14.

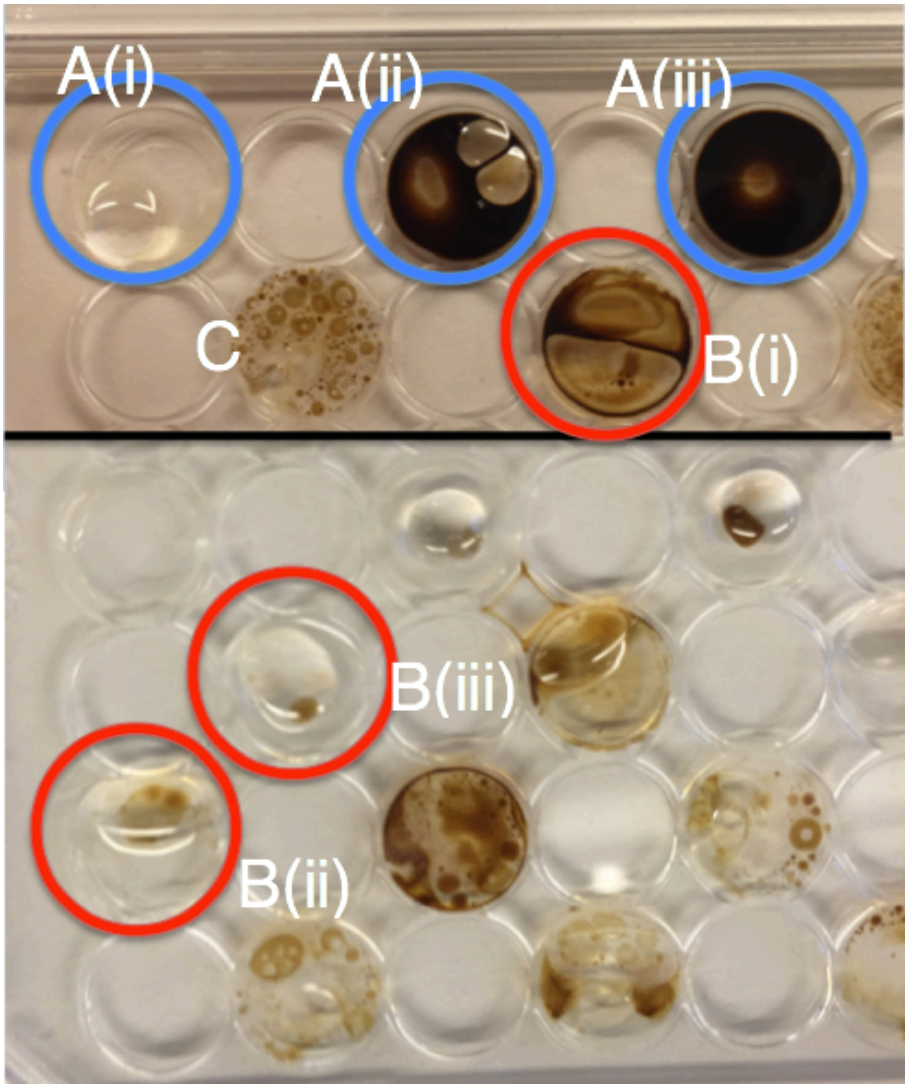


Figure 14. Control sample spreading of 10 ul drops of **A(i)** distilled water, **A(ii)** light Arabian oil, and **A(iii)** Bushnell Haas liquid media with oil; BH SC sample 15 retested to show oil in drop affect on results: **B(i)** first drop test positive results with oil, **B(ii)** first repeated drop test borderline positive result with less oil, and **B(iii)** second repeated drop test negative result. Well **C** demonstrating positive spreading from emulsion positive sample.

As contact angle was not measured, sample results that were borderline positive for spreading were difficult to discern. For example, SC BH samples provided four samples that were negative for the visual test, and were later shown to have an IFT reduction of less that 4%, were false positives for spreading. Samples that were strong positives in the visual test, were also strong positives for spreading with the oil drop collapse test, as can be seen in figure 14 above.

4.1.4 IFT—Interfacial tension

To ensure Wilhelmy plate measurement accuracy, IFT of distilled water to air must first be measured to approximately 72 mN/m at 20˚C before measuring controls and samples (Barnes &

Gentle, 2005). Initially, IFT of the distilled water was measured at 71.113 mN/m, as seen in the results in figure 15, thus extra step of equipment sterilisation was conducted. The final IFT of distilled water to air was 72.478 mN/m, as seen in the results of figure 16.

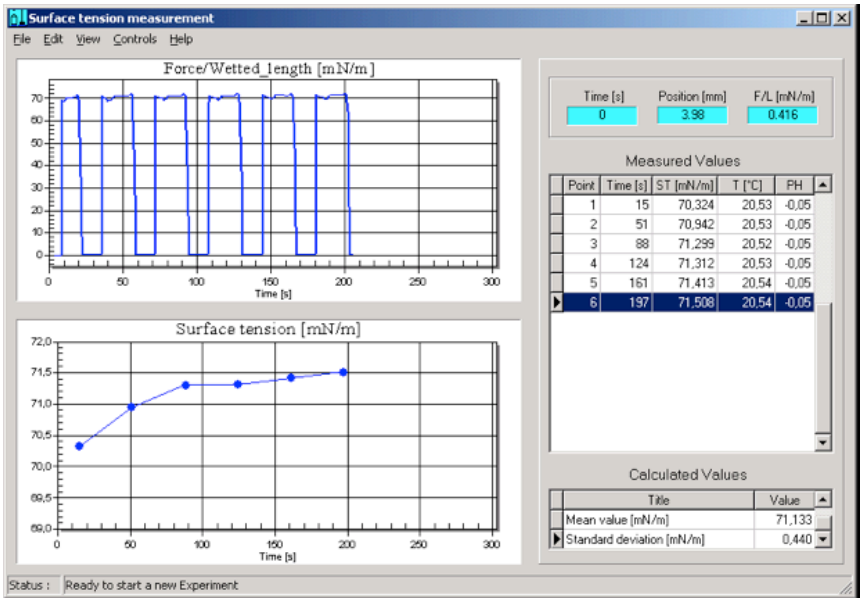


Figure 15. Initial distilled water IFT results lower than expected for room temperature

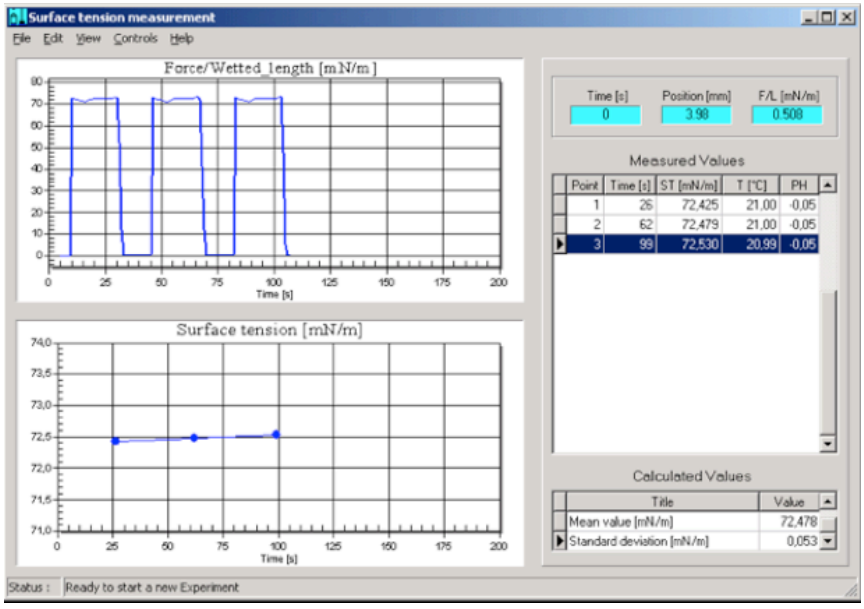


Figure 16. IFT results of sterilised and distilled water with expected values

The Wilhelmy plate test offered the only quantitative results for biosurfactant screening, and can be used to support the two qualitative methods. As with the other tests, NB liquid media results were negligible, while BH samples and Arctic samples presented possible candidates.

Table 4 shows all biosurfactant screening results using SC BH samples assessed for biosurfactant presence. For this sample set, only one negative results for the drop collapse test results was shown for sample 14, when a strong IFT result of 27.556 mN/m and a positive result for the visual emulsion and flocculent dispersal test. SC BH samples provided many positive results for effective biosurfactant presence, with 9 samples that reduced surface tension by more that 41% of the control BH liquid media with oil. BH liquid media with oil control had an IFT of 54 mN/m (64.5 mN/m for BH liquid media control without oil), and SC samples provided the lowered IFT achieved of 24.178 mN/m in sample 6. SC BH samples that reduced surface tension to 27.556 mN/m or less are highlighted in green and samples that are within 4% of the of the control with oil IFT are highlighted in red. The samples highlighted in orange reduced to the surface tensions by 10-20%, and the sample highlighter in yellow reduced surface tension to 31.486 mN/m, and produced a strong emulsion that, like all emulsions formed under this experiment, was difficult to break in preparation for IFT assessment, as seen in figure 17.

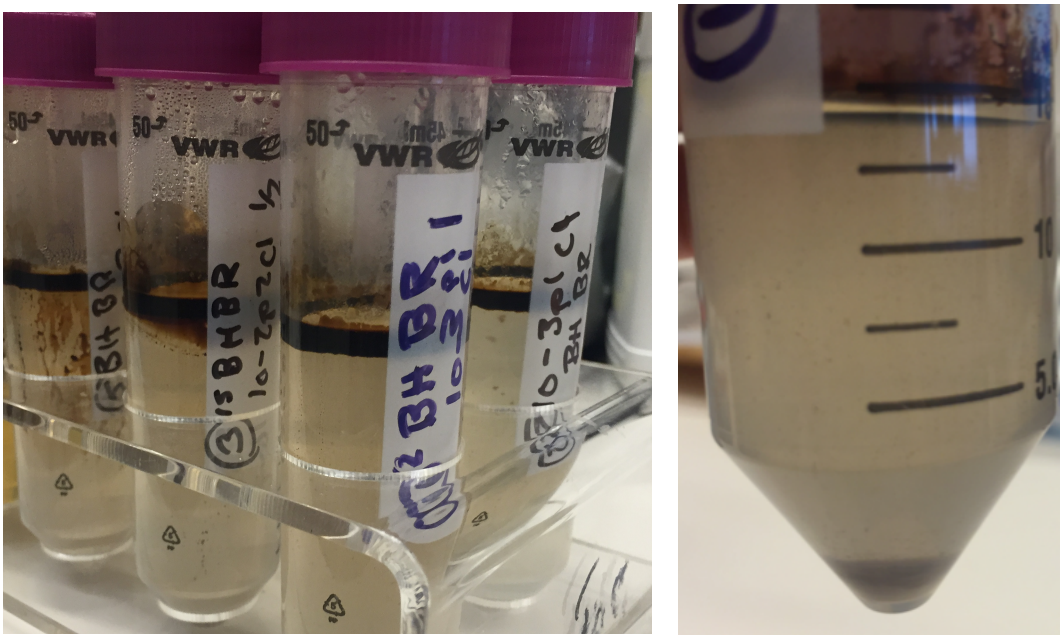


Figure 17. Bushnell Haas bioreactor samples after breaking of emulsion steps (centrifuged 5 min 5000 rpm, 50°C water bath for 24 hours, repeated), cloudy and visible oil particulates seen in bulk broth phase, with some oil at the top and precipitates at the bottom.

Table 4. Biosurfactant screening results for settling chamber samples grown with Bushnell Haas media (SC BH), showing measured surface tension (mN/m) of samples, compared to control Bushnell Haas liquid media with oil, positive (+) and negative (x) results for both oil drop collapse test (Oil Drop) and the visual emulsion and flocculation test (Swirl).

Sample	Sample IFT mN/m	Control with oil IFT mN/m	Oil Drop	Swirl
1	31.486	54.003	+	+
2	24.245	54.003	+	+
3	26.954	54.003	+	+
4	26.865	54.003	+	+
5	25.535	54.003	+	+
6	24.178	54.003	+	+
7	25.132	54.003	+	+
8	25.032	54.003	+	+
9	42.937	54.003	x	x
10	43.87	54.003	+	x
11	42.937	54.003	+	x
12	47.25	54.003	+	x
13	47.598	54.003	x	+
14	27.556	54.003	x	+
15	53.394	54.003	+	x
16	53.481	54.003	+	x
17	53.788	54.003	+	+
18	52.476	54.003	x	x
19	52.991	54.003	x	+
20	53.127	54.003	+	x
21	52.214	54.003	x	+
22	54.032	54.003	x	x

Nearly all BR and SC samples in NB liquid media either failed to cultivate or did not present successful results for biosurfactant presence. However, BH liquid media continued to demonstrate positive results for biosurfactant screening assessments with BR samples (tab. 5). All five samples also received positive results for the oil drop collapse test and formed strong emulsions. IFT changes were not as severe as with SC BH samples, but still ranged between 32.829 to 47.114 mN/m. Results highlighted in green demonstrated reduction of IFT of the control by 40% or more, yellow demonstrates reduction by 25%, and orange by 10-20% reduction.

Table 5. Biosurfactant screening results for bioreactor samples grown with Bushnell Haas media (BR BH), showing measured surface tension (mN/m) of samples, compared to control Bushnell Haas liquid media with oil, positive (+) and negative (x) results for both oil drop collapse test (Oil Drop) and the visual emulsion and flocculation test (Swirl).

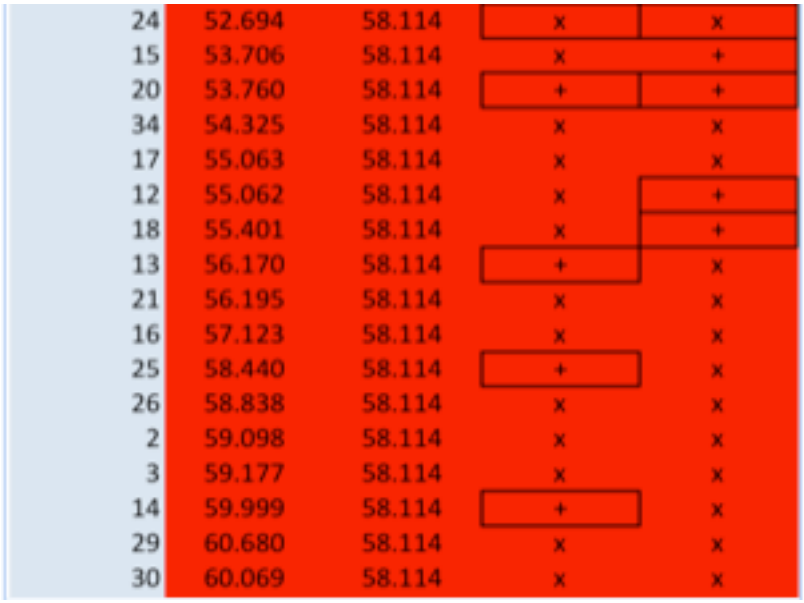
Sample	Sample IFT mN/m	Control with oil IFT mN/m	Oil Drop	Swirl
1	32.939	54.003	+	+
2	32.829	54.003	+	+
3	44.784	54.003	+	+
4	40.627	54.003	+	+
5	47.114	54.003	+	+

Arctic results for IFT changes and emulsions, were not as extreme as with the strong BH broth cultured samples, but present interesting results as the bacteria grew at cooler temperatures—between 3°C to 4°C instead of 10°C, which is more representative of marine temperatures. This can be shown in Table 6, which lists the strongest IFT positive results, which were 63-79% of the control seawater broth with oil. It is also important to note that the presence of the light Tyrrhenian oil with seawater reduced surface tensions from 73.115 mN/m to 58.114 mN/m. Comparing the surface tension chances of cultured broth with oil to sterile seawater, the surface tension would be reduced to 50-63% of the control.

Table 6. Biosurfactant screening results for Arctic samples grown with seawater media in ascending order of IFT results, showing measured surface tension (mN/m) of samples, compared to control Bushnell Haas liquid media with oil, positive (+) and negative (x) results for both oil drop collapse test (Oil Drop) and the visual emulsion and flocculation test (Swirl).

Sample	Sample IFT mN/m	Control with oil IFT mN/m	Oil Drop	Swirl
19	36.616	58.114	+	+
1	41.345	58.114	+	+
27	41.561	58.114	x	+
4	45.329	58.114	+	+
23	45.539	58.114	+	+
11	45.768	58.114	x	+
6	46.789	58.114	x	+
7	46.441	58.114	+	+
22	48.371	58.114	x	+
32	49.280	58.114	+	+
5	49.735	58.114	+	+
9	50.143	58.114	x	+
28	50.495	58.114	x	x
10	50.732	58.114	+	+
8	51.475	58.114	x	x
31	52.525	58.114	x	+
33	51.739	58.114	+	+

Table 6 continued on next page.



24	52.694	58.114	x	x
15	53.706	58.114	x	+
20	53.760	58.114	+	+
34	54.325	58.114	x	x
17	55.063	58.114	x	x
12	55.062	58.114	x	+
18	55.401	58.114	x	+
13	56.170	58.114	+	x
21	56.195	58.114	x	x
16	57.123	58.114	x	x
25	58.440	58.114	+	x
26	58.838	58.114	x	x
2	59.098	58.114	x	x
3	59.177	58.114	x	x
14	59.999	58.114	+	x
29	60.680	58.114	x	x
30	60.069	58.114	x	x

4.2 Results for purity assessment

The sample set chosen for DNA analysis can be seen listed in Table 7. Samples chosen for DNA analysis must have presented positive results for biosurfactant presence in both qualitative tests, as well as IFT measurements that demonstrated a reduction in surface tension from control liquid media with oil by more than 20%. This presented 8 cultures from SCB H samples, 3 cultures from BH BR samples, and 4 cultures from Arctic samples. Due to time constraints, a limit for the sample set was fixed to 6 cultures. Morphological assessments were used to further refine the list that is provided in Table 7.

Table 7. Sample set list for DNA analysis, including 3 Arctic cultures, 1 bioreactor Bushnell Haas media, and 2 settling chamber Bushnell Haas media cultures. Samples are designated new sample numbers for DNA analysis 1-6, with corresponding biosurfactant screening results.

Sample origin	Sample origin	Sample reference	Sample IFT mN/m	Control with oil IFT mN/m	Oil Drop	Swirl
Arctic 4	Dilution 10^{-1}	1	45.329	58.114	+	+
Arctic 23	Dilution 10^{-3}	2	45.539	58.114	+	+
Arctic 19	Dilution 10^{-2}	3	36.616	58.114	+	+
BH BR 2	Dilution 10^{-3} plate 1 colony 4	4	32.829	54.003	+	+
BH SC 2	Dilution 10^{-3} plate 1 colony 3	5	24.245	54.003	+	+
BH SC 3	Dilution 10^{-4} plate 2 colony 5	6	26.954	54.003	+	+

4.2.1 Cell morphology

The bacteria morphology was viewed under a microscope, where uniform morphology would suggest colony purity. The majority of samples were dominated by single bacillus and palisade bacillus morphology. Including cultures that presented bacillus/coccus mixed cultures, the prevalence of single bacillus and palisade bacillus are as follows: 22 of 34 for Arctic samples, 3 of 5 for BR BH samples and 14 of SC BH samples. Many mixed morphology samples, such as Arctic sample 27 (fig. 18), also presented strong IFT results.

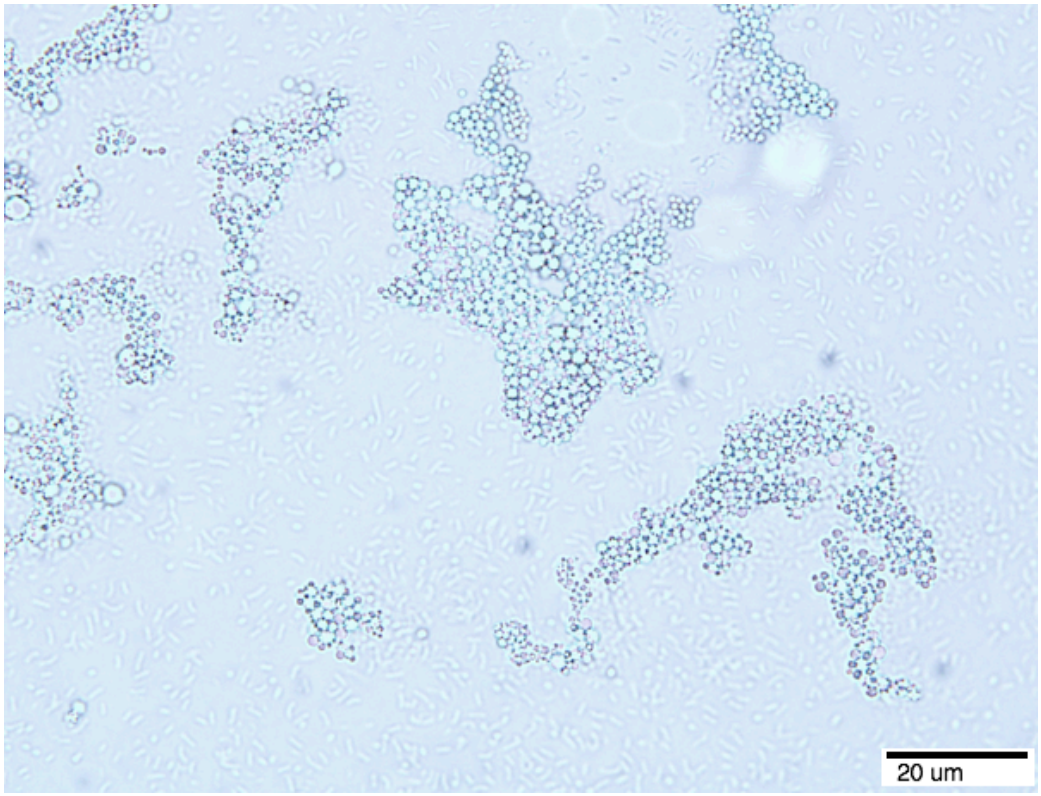


Figure 18. An example of non-pure culture (Arctic sample 27) with two distinct bacteria morphologies—staphylococcus and bacillus—at 100 x magnification.

Arctic cultures

Samples 1 and 3 (formerly Arctic samples 4 and 19, respectively) both showed only single bacillus species present, with strong flocculent formation after the swirl agitation test (fig. 19).

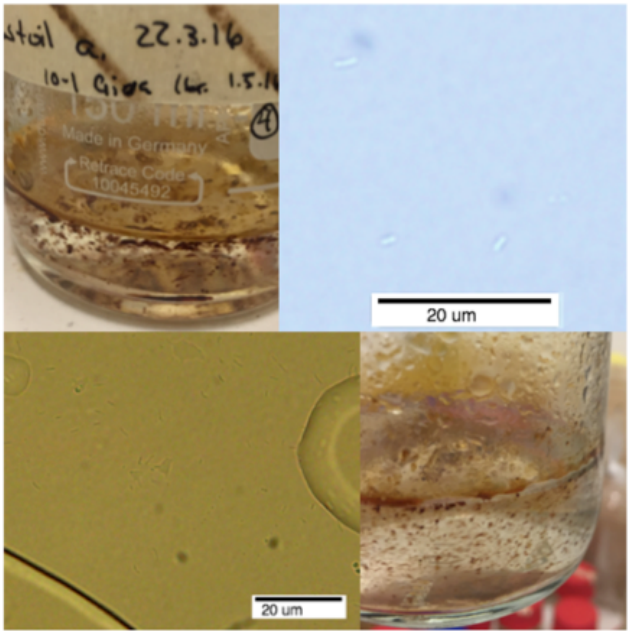


Figure 19. (Top) Sample 1 and (bottom) sample 3 (originally known as Arctic samples 4 and 19) demonstrating good flocculent formation and bacillus at 100x magnification.

Sample 2 (formerly Arctic sample 23) was distinctly not pure, as both staphylococcus and single bacillus species were present (fig**.** 20). Single bacillus species were clearly present in the bulk liquid media phase, while small staphylococcus species were present in the oil phase. The sample was chosen for further analysis to determine whether indeed only two species were present. Co-occurrence of two species could suggest a direct tight symbiotic-like relationship, while presence of more than two species would indicate a more complex scheme of interactions.

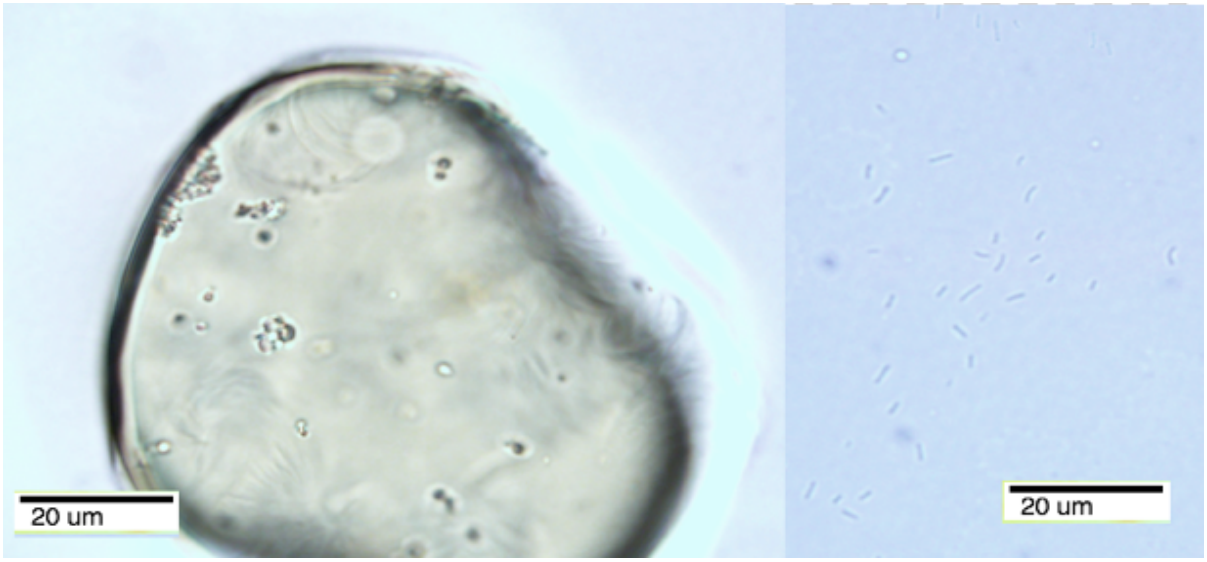


Figure 20. Sample 2 (formally known as Arctic sample 23) at 100 x magnification. Bulk broth phase (right), and oil phase (left).

Bushnell Haas media

Settling chamber

Sample 5 (formerly SC BH sample 2) suggested colony purity based on morphology (fig. 21), while sample 6 (formerly SC BH sample 3) had both streptococcus and bacillus morphologies present (fig. 22). Sample 6 was chosen for the same reason as sample 2.

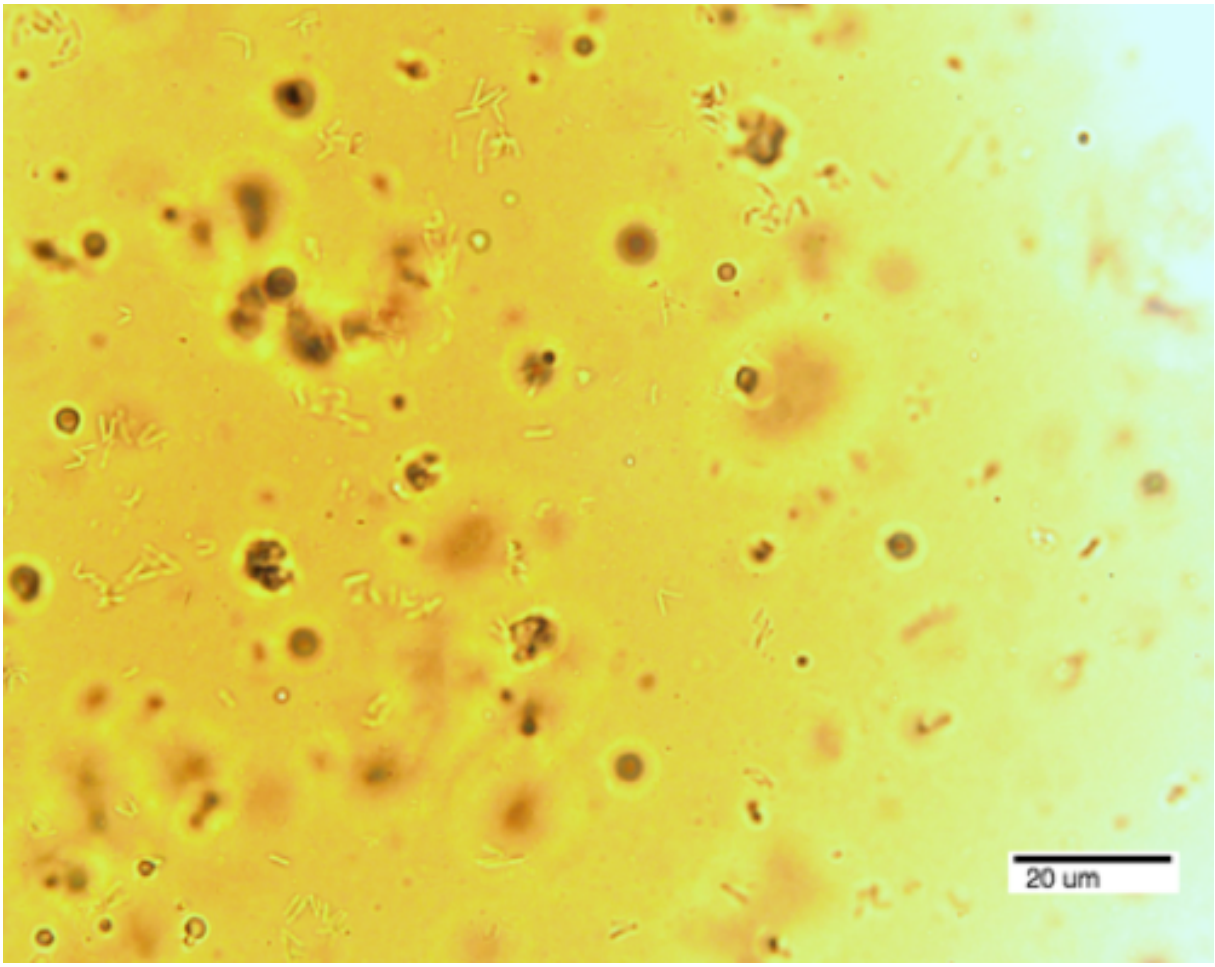


Figure 21. Sample 5 (settling chamber Bushnell Haas media sample 2) at 100 x magnification, with bacillus morphology present.

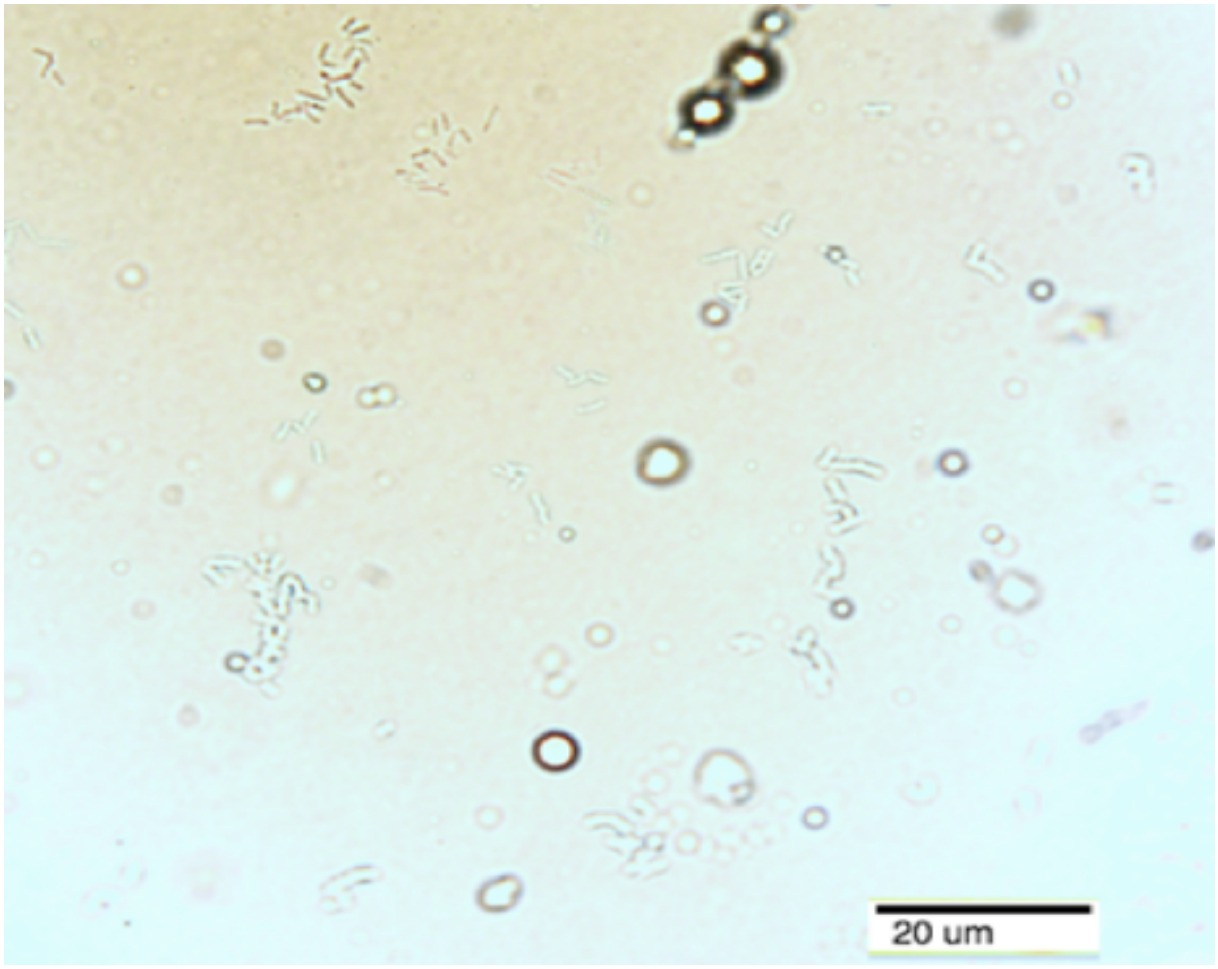


Figure 22. Sample 6 (settling chamber Bushnell Haas media sample 3) at 100 x magnification, with both bacillus and streptococcus morphologies present.

Bioreactor

Sample 4 (formerly BR BH sample 2 had given an indication for colony purity based on coccus morphology present. However, the varying sizes and arrangements could suggest different cocci species present (fig. 23). All other BR BH samples had at least two distinct morphologies present.

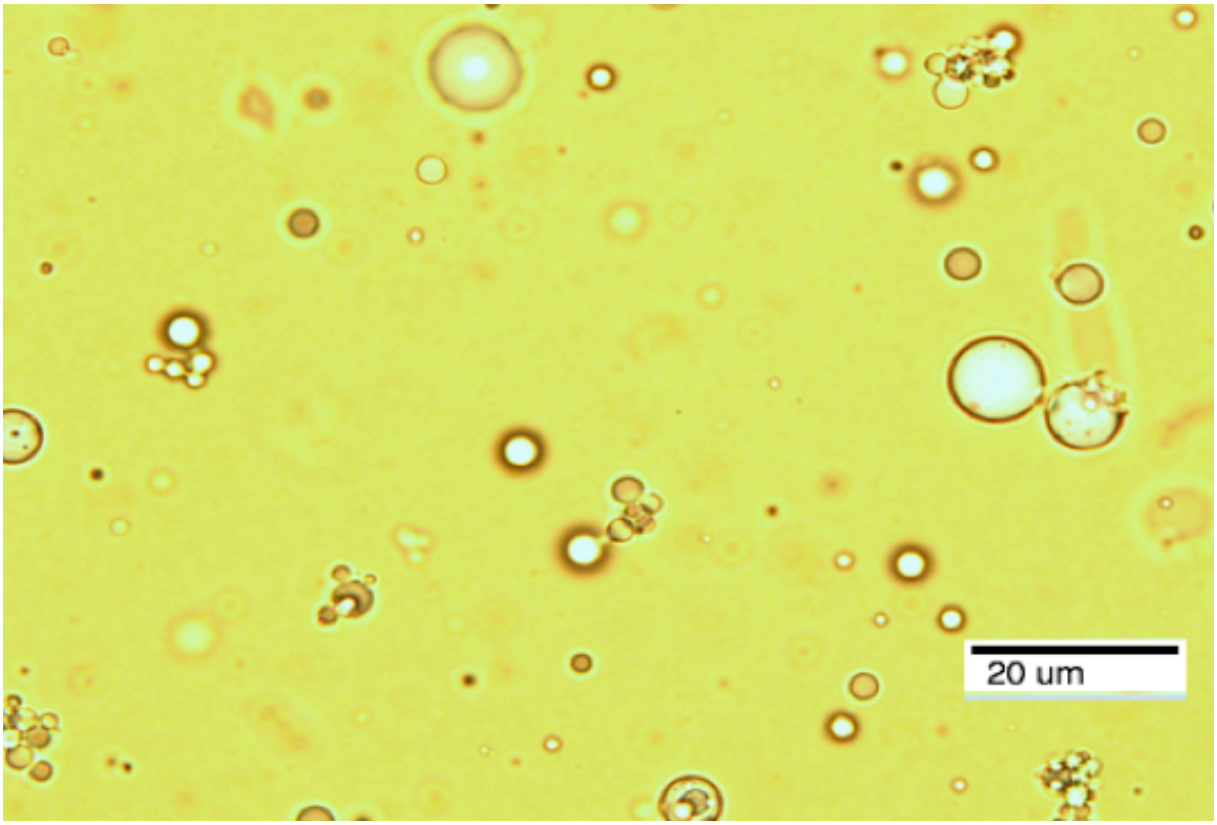


Figure 23. Sample 4 (bioreactor Bushnell Haas media sample 2) at 100 x magnification, with various cocci sizes and arrangements present.

PCR-DGGE analysis

Figure 24 and figure 25 shoes the 2% agarose gel electrophoresis of raw DNA and PCR amplified 16s rRNA gene fragments, respectively.

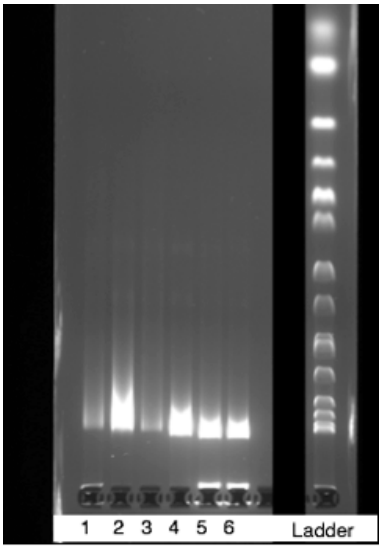


Figure 24. Raw DNA on 2% agarose gel electrophoresis with ladder of samples 1 to 6.

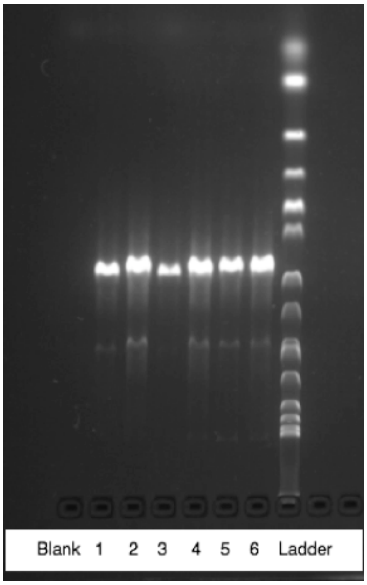


Figure 25. 2% agarose gel electrophoresis gels with ladder and blank of samples 1 to 6 of amplidied 16s rRNA gene fragmentation.

DGGE was used to test for sample purity, based on the amplified 16s rRNA gene fragments. As can be seen in figure 26, samples 3 and 5 demonstrated single and strong band formation. However, the shading around the bands, and especially the faint shading above and below sample 3, suggest some degree of impurities. Sample 6 demonstrates that the purity of two species is to a high degree, but not exclusive enough for further sequencing. Samples 1 and 2 demonstrate long and dark shading, inferring high degree of a species mix, while sample 4 demonstrates dominance, but not exclusive, of three species.

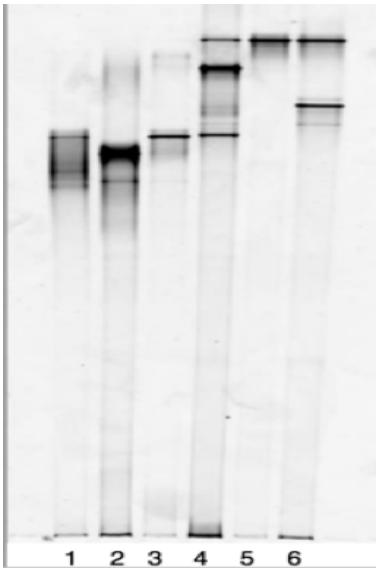


Figure 26. DGGE of amplified 16s rRNA gene fragments in 6% acrylamide gel with denaturing solutions of 20% and 80%.

5 Discussion

The biosurfactant-producing bacteria were cultivated from the three sources—Arctic seawater, offshore wastewater bioreactor (BR) and offshore wastewater settling chamber (SC), using media with limited carbon sources, so that oil was the sole, or major carbon source. Isolation of bacteria into pure cultures for analysis and sequencing was not successful. This could be likely rectified by repeating the streaking on solid media with oil step for isolation, and continuing the later steps.

Only two Arctic samples (sample 8 and 28) had negative visual test results interpreted out of 17 Arctic samples that later presented IFT reduction of 10% or greater of the control with oil, and only 5 results misinterpreted as positive for biosurfactant presence out of 17 Arctic samples that later presented an IFT reduction of less than 10%.

Sample 5 of BR cultivated sample set from Bushnell Haas media with oil formed a strong (difficult to break) emulsion, but with surface tension only reduced to 47.114 mN/m from the control Bushnell Haas liquid media with oil of 54.003 mN/m. Possible applications for this could be for situations that would benefit from forming stable emulsions, but with limited reduction to surface tension.

Settling chamber cultivated with Bushnell Haas media and oil presented significant results for biosurfactant-producing candidates, with the greatest observed surface tension reductions ranging form 24.178 mN/m to 27.556 mN/m. Further studies of the two samples used in DNA analysis also hold promising research with further isolation for purity, with sample 5 for single strain analysis, and sample 6 for investigation into possible symbiotic relationship.

5.1 Techniques

5.1.1 Bacteria cultivation

Cultivation techniques for growing and isolating bacteria applied in this study yielded varying results. Media selection for low carbon source, such as seawater media and Bushnell Haas media, generated (more) candidates for future investigations than the nutrient and carbon rich media of nutrient agar and broth. Nutrient media were probably able to support to the growth of bacteria that were crude oil-tolerant, but unable to actively degrade by either dispersal or active-uptake of the oil. Bushnell Haas and seawater media were only able to provide the added oil as the main carbon source, thus allowing for better selection of biosurfactant-producing bacteria. The low carbon-sourced media allow for better screening selection from a mixed bacteria

combination source, while carbon rich media could be used to enhance the growth of pure cultures for biosurfactant production, as seen in Rocha e Silva, *et al*, (2014) analysis of *P. cepecia* grown with comparably high carbon and nutrient supplements, containing 2% corn steep liquor and 2% soybean (waste) frying oil supplements in a mineral medium.

Isolation streaking required repetition, not only to ensure colony purity, but also to prevent false positives for biosurfactant production making it to the liquid media phase. Drying the oil coat on top of the solid agar media, although limited, did not prevent oil displacement during inoculation. The oil layer was coated evenly, but inoculation, as can be seen in figure 12 from the results section repeated below, varied the thickness of oil, with some oil clearings possibly being attributed to oil displacement, rather than biosurfactant production.

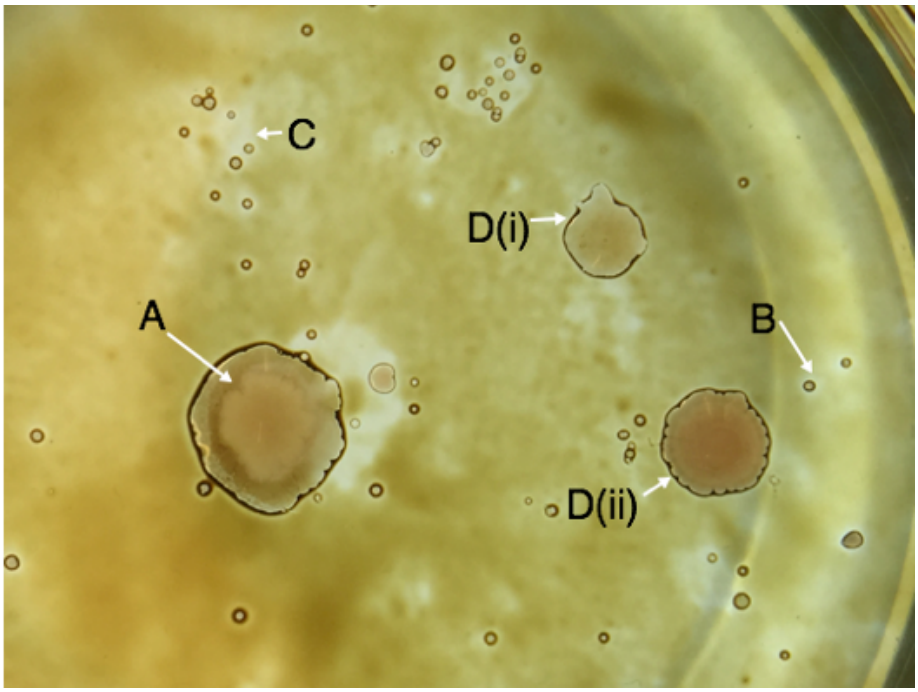


Figure 12 (from Results Section). Dried oil coat was evenly spread, but after inoculation disturbances, different oil thickness layers can be seen.

Incubation times of approximately 2 weeks for the settling chamber and bioreactor samples at 10°C appeared to have been sufficient for adequate colony growth, while the Arctic samples grown between 3-4°C for 3 weeks may have benefited from longer incubation periods to eventually establish emulsions.

Variation in size for selection was implemented in an attempt to increase bacteria diversity amongst the bacteria culture samples, as was also noted in figure 12 above. However, considering Rocha e Silva, *et al.* (2014) investigate *Pseudomonas* spp. for cost effectiveness, the larger, and therefore faster growing colonies that meet other selection criteria for

biosurfactant production, would be better suited candidates for investigation, as they would reduce cultivation time, and therefore costs.

5.1.2 Screening for biosurfactants

False positives and negatives were observed more with the oil drop collapse test conducted quantitatively than the visual emulsion and flocculation test, but both still function as easy detection techniques for pre-screening for biosurfactant production. The visual emulsion and flocculation was simple and provided clear results for positives that formed emulsions. The bacteria cultures that formed flocculants were more difficult to ensure accuracy for positive results, and were helped by the oil drop collapse test. Possible better accuracy as a pre-screening method for biosurfactant production could be achieved if the oil drop-test contact angles were measured. IFT measurements conducted with the Wilhelmy plate provided the most conclusive results for inferring biosurfactant production by using surface tension measurements.

Both qualitative techniques presented limitations. Both tests were limited in discerning samples with marginal surface tension, with false negative and false positives misinterpreted from human error. The visual emulsion and flocculation test presented strong emulsions, which indicated positive for biosurfactant production, but was shown via IFT measurements to not have a major surface tensions reduction, as mentioned previously, possible due to bacteria adhering to the oil and acting like fine particles to stabilise the emulsions, as described by Abbasnezhad, Gray & Foght (2011). These candidates are still of interest as they can provide emulsion stability similar to that achieved by strong surfactant production, but without drastically altering the surface tension.

5.1.3 Bacteria purity assessment

Morphological assessment offers rapid dismissal of bacteria cultures for purity with distinctly contrasting morphological expression, for example bacillus (rod-shaped) species compared to coccus (spherical) species, such as was seen in figures 18 and 22 from results section. Some further discrepancy, such as cell arrangements—e.g. single coccus versus staphylococcus—can also be determined, but DNA analysis was required for confirmation. Morphological distinctions, as seen in figure 20, however, demonstrated two distinct species variety, both occupying different bulk phases. If the two colonies could be isolated for strain purity, they may offer insight into a symbiotic relationship for future investigations.

The assessment of 16s rRNA gene fragmentation using DGGE is a well-established and commonly utilised method that is a valid and significant tool in extrapolating bacteria colony

purity. While it should not have affected the results, the DGGE cast was not set on a level surface, which is white the columns veer to the left. Due to a single isolation phase, colonies were shown to not be pure. Some samples, such as samples 3, 5 and 6, showed strong colouring for bands, but the shading around them insinuated impurities. Samples 3 and 5, with further isolation protocols imposed on the, should be able to provide pure bacteria colony samples for future sequencing. Sample 6, and to an extent 4, provide an interesting opportunity for future studying for sequencing, as well as for potential symbiotic relationship evaluations for mixed bacteria applications, such as with the research by Rhaman*, et al*, (2002).

5.2 Recommendation for future research

Better isolation of biosurfactant-producing species present from the three sources could provide valuable candidates for future research. Future research would benefit from the following steps: research for biosurfactant identification, as seen by Bergström, *et al* (1949); identifying biosurfactant-producing bacteria, for cost-effective production, as researched by Rocha e Silva, *et al,* (2014) with *P. cepecia* on high carbon and nutrient sourced media; and gene biosurfactant-production identification for possible future GEMEOR research or genetically engineered for remediation purposes, or enzymatic degradation pathway identification, as investigated by Kumari, Singh and Singh (2012) for similar applications.

5.3 Conclusion

Further research into the Arctic sample species could offer significant candidates for *in situ* cold marine applications, as samples were cultivated at cold (3-4°C) and saline conditions. Biosurfactant-producing bacteria were cultivation on Bushnell Haas media with salt incorporated for salinity and crude oil for carbon source at cool temperatures. These samples were able to reduce surface tension, which was used as an indicator for biosurfactant production, with results as low as 24.178 mN/m from a liquid media with oil surface tensions of 54.003 mN/m. Arctic seawater bacteria cultivation also presents biosurfactant producing fauna, capable of reducing surface tension to 36.626 mN/m of a seawater liquid media with oil with a occurring surface tensions of 58.114 mN/m, and may have benefited from longer incubation periods. While it could be expected that media high in nutrients, such as carbon sources, like that of nutrient agar and nutrient broth, should promote cultivation, this inevitably promotes all cell growth, including cells with no biosurfactant production capabilities. Single execution of pick and streak approach does not appear to be effective for strain isolation for these sample sources, as was shown in DGGE analysis using 16s rRNA gene fragmentation and

amplification. Repeated pick and streaking for isolation may provide a more pure selection of biosurfactant-producing bacteria.

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7 Appendices

7.1 Appendix A—media compositions

7.1.1 Nutrient Broth (Merck Chemicals)

Composition	Amount (g/l)
Casein Peptone	5.0
Meat Extracts	3.0

7.1.2 Nutrient Agar (Merck Chemicals)

Composition	Amount (g/l)
Casein Peptone	15.0
Yeast Extract	3.0
Sodium Chloride	6.0
Dextrose	1.0
Agar	15.0

7.1.3 Bushnell Haas media (Flukar Analytical)

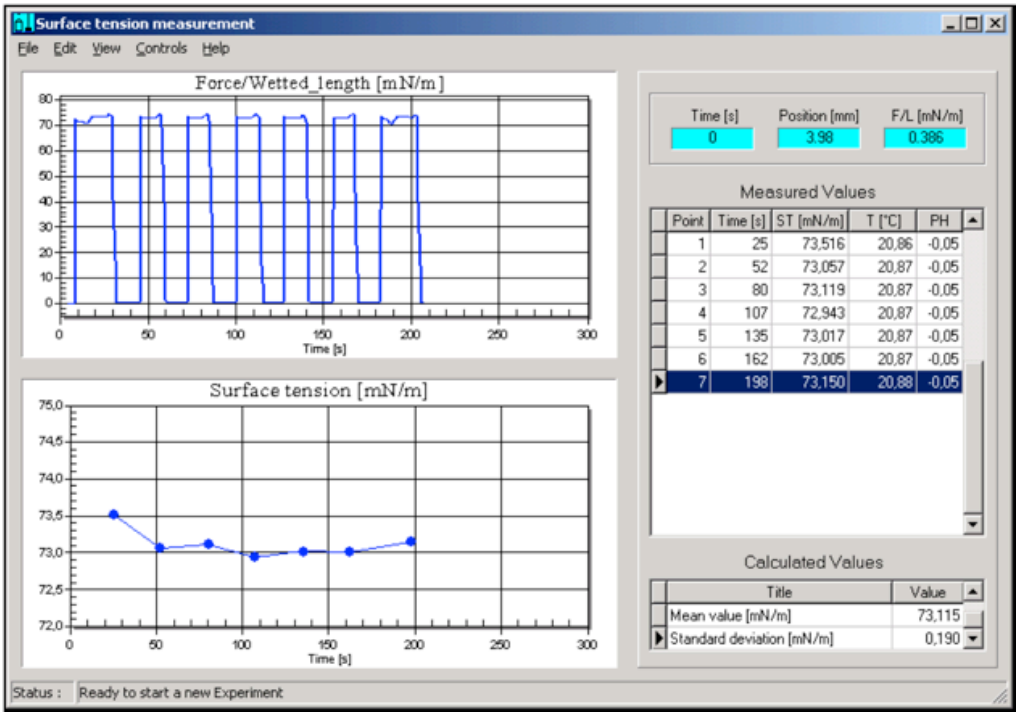
Composition	Amount (g/l)
Magnesium Sulphate	0.20
Calcium Chloride	0.02
Monopotassium Phosphate	1.00
Dipotassium Phosphate	1.00
Ammonium Nitrate	1.00
Ferric Chloride	0.05

7.2 Appendix B—Sample Sources

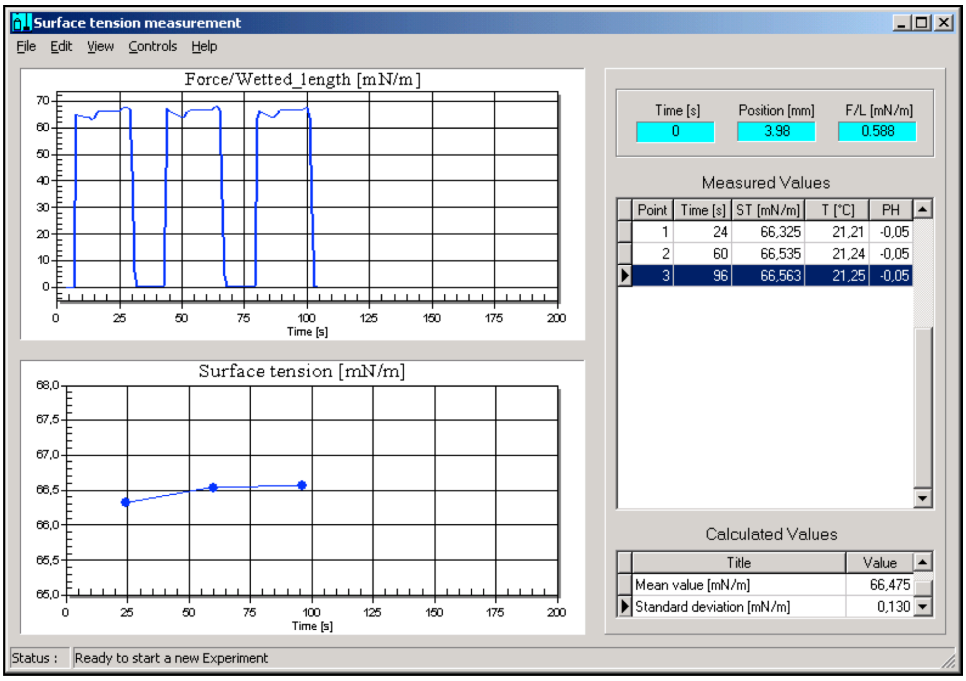
Samples	Bioreactor	Settling Chamber	Arctic
Site	Offshore Wastewater system: Norwegian Group AS	Offshore Wastewater system: Norwegian Group AS	Ny-Ålesund, Svalbard (from the floating dock outside Sverdrup Station)
Date	Arrived at UiS at 2 pm 12 Feb. 2016 (inoculated 15 Feb. 2016)	Arrived at UiS at 2 pm 12 Feb. 2016(inoculated 16 Feb. 2016)	26 October 2015 (arrived to Stavanger at 8 am 28 Oct. 2015)
Type	Offshore Wastewater	Offshore Wastewater	Coastal seawater sample (20 L)
Depth	N/A	N/A	Surface (water depth 3-4 m max at sampling site)
Temperature	10,0 degrees Celsius in incubator	10,0 degrees Celsius in incubator	0,5 degrees Celsius (1,0 dgr. Upon arrival, held at 3,0 dgr. In incubator)

7.3 Appendix C—IFT controls without oil

7.3.1 Seawater



7.3.2 Bushnell Haas media



7.3.3 Nutrient Broth