BIOSURFACTANT PRODUCTION AND APPLICATIONS IN OIL CONTAMINATE CONTROL

by

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ABSTRACT

Surfactants are a versatile group of chemicals. They are amphiphilic compounds that exert impacts at interfaces among air, water, oil, and solid phases. Currently, the prevalent players in the market are chemically synthesized surfactants, which have concerns of considerable toxicity and low biodegradability. Because they are mainly derived from fossil fuels, they are not sustainable in the long run and the production costs are subjected to the price variance of raw materials. In view of these limitations, biosurfactants have been proposed as promising alternatives. They are surfactant molecules produced by microorganisms during their growth. Biosurfactants exist naturally in the environment and play some roles in the ecosystem even without human interferences. In this dissertation we define this virtue as "environmental friendly". They are also renewable and non/less toxic. They have also been found with the intriguing advantages such as enormous structural diversity, lower critical micellar concentrations (CMCs), and the feasibility to use renewable and/or waste streams as the source of production. Biosurfactants are considered as multifunctional biomolecules of the 21st century with a thriving global market share. Their applications in environmental and oil industries are among the top market sectors thanks to their environmental friendly nature.

In order to harness the power of biosurfactants, the economic effectiveness of production of these molecules needs improvements. The inocula/microorganisms are the engine of a production process, which determine the maximum yield potential and the functionality of biosurfactants, yet limited inocula have been reported. The functional

diversity of biosurfactants includes emulsification, dissolution, dispersion, emulsion breaking, reduction in viscosity, and surface activity, which result in a broad spectrum of potential applications in oil contaminate control including soil washing, enhanced bioremediation, oily wastewater treatment, and spilled oil dispersion. However, limited research efforts have been placed into evaluating the application potential of biosurfactants in oil contaminate control.

The objectives of this thesis are to 1) identify novel and robust biosurfactant producing microorganisms and develop hyper-production mutants; 2) examine the functionality of the produced biosurfactants; and 3) investigate the potential of using these produced biosurfactants in diverse applications of oil contaminate control.

The outputs of the thesis include: (1) the successful isolation, identification, characterization and functionality analysis of one-hundred-and-fourteen biosurfactant producing and oil degrading marine bacteria; (2) the discovery of a novel bacterial species, *Alcanivorax atlaticus* for the first time and its proposed type strain with comprehensive genotype and phenotype characterizations; (3) an in-depth characterization, functionality analysis and application demonstration of a novel bioemulsifier (exmulsins) and its bacterium (*Exiguobacterium* sp. N4-1P); (4) reporting of thirty-seven novel oil-in-water emulsion breaking marine bacteria for oily wastewater treatment, and a recommended screening strategy for their identification; (5) the first attempt to genetically modify *Rhodococcus* strains for hyper production of biosurfactant and to investigate the dispersing abilities of the the produced biosurfactants; and (6) a comprehensive investigation of 4

types of biosurfactants produced from selected isolates and mutants as marine oil spill dispersants.

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LIST OF ABBREVIATIONS AND SYMBOLS

1- λ	Simpson's index of diversity
1/ λ	Simpson's reciprocal index
λ	Simpson's index
16S rDNA	16S ribosomal deoxyribonucleic acid
ALC	Arabic light crude oil
ANOVA	analysis of variance
$A_{o/w}$	oil-water interfacial area
BAP	blue agar plate test
BFT	baffled flask test
BLAST	Basic Local Alignment Search Tool
C.V. %	coefficient of variation
CAGR	compound annual growth rate
CCD	central composite design
CH	cell hydrophobicity test
CMC	critical micellar concentrations
CMD	critical micelle dilution
CREAIT	Core Research and Instrument Training Network
DCM	dichloromethane
DF	desirability function
DOSS	dioctylsulfosuccinate, sodium salt

E24	emulsification index
EBR	emulsion breaking ratio
EBRn	normalized emulsion breaking ratio
EI	emulsification index
FAME	fatty acid methyl esters
FT-IR	Fourier transform infrared
GC/FID	gas chromatography/flame ionized dectector
GC/MS	gas chromatography/mass spectrometry detector
H	Shannon-Weaver index
HA	hemolytic activity test
H_{EL}	the height of the emulsion layer
HLB	hydrophilic-lipophilic balance
H_S	the height of the total solution
K_p	partitioning coefficient
J	evenness index
MAF IIC	Micro Analysis Facility
MAOA	modified Atlas oil agar
MATH	microbial adhesion to the hydrocarbon
MTBE	methyl tert-butyl ether
MUN	Memorial University of Newfoundland
O/W	oil-in-water
ODC	oil drop collapsing test

ORP	oil removal percentage
OS	oil spreading test
PCR	polymerase chain reaction
PERMANOVA	permutational analysis of variance
PLFA	phospholipid-derived fatty acid
PRESS	predicted residual error sum of squares
RSM	response surface methodology
$\gamma_{o/w}$	oil-water interfacial tension
S	number of species
SEM	scanning electron microscopy
ST	surface tension
TDS	total dissolved solids
ThOD	theoretical chemical oxygen demand
TLC	thin layer chromatography
TOC	total organic carbon
W/O	water in oil
WANS	weathered Alaska north slope crude oil
W_k	mixing energy

CHAPTER 1 INTRODUCTION AND OVERVIEW

1.1 Background

Surfactants are a versatile group of chemicals with various applications as household detergents, personal care products, pharmatheutical agent agricultural chemicals, oilfield chemicals, food processing agents, industrial additives, environmental remediation agents and so on (Varjani and Upasani, 2017). They are amphiphilic compounds with both hydrophilic and hydrophobic moieties that align themselves accordingly among diverse interfaces of air, water, oil and solid phases, and affect the properties of these phases (Lamichhane et al., 2017). In 2014, the global surfactant market was USD 25.6 billion value, and has been expected to grow at a compound annual growth rate (CAGR) of 4.6% from 2015 to 2020 (Grand view research, 2016). The current dominate players in the market are chemically synthesized surfactants such as Tween 20/80, Triton X-100, and Brij35 (Lamichhane et al., 2017; Santos et al., 2016). However, such chemically synthesized surfactants generally have concerns of toxicity and low biodegradability (Santos et al., 2016). Moreover, as they are derived from fossil fuels, their production is not sustainable in a long run and the production costs are subjected to the price variance of fossil fuels (Otzen, 2017).

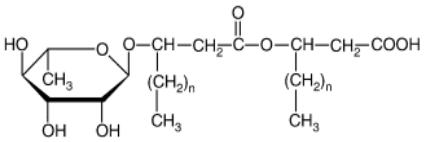
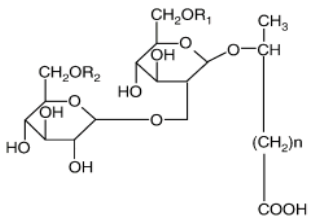
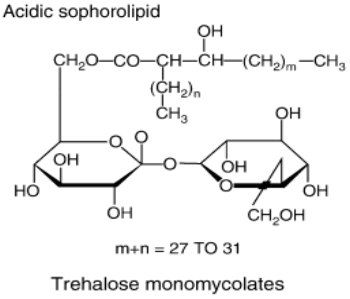
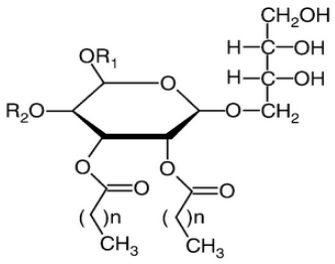
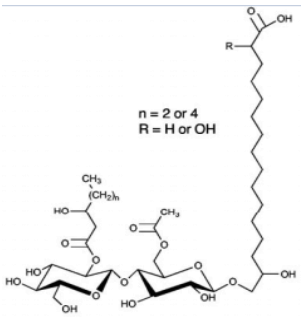
In view of these limitations, in the past decades, extensive research efforts have been placed in the development of environmental friendly, renewable and non/less-toxic alternatives such as biosurfactants, which are surfactant molecules produced by microorganisms during their growth (Vijayakumar and Saravanan, 2015). Such development efforts have resulted in a thriving global biosurfactant market which was estimated at USD 4.2 billion in 2017 and was projected to reach USD 5.52 billion by 2022,

at a CAGR of 5.6% (Markets and Markets, 2017). The CAGR of the biosurfactants market is projected to be significantly higher than the overall surfactant market and is mainly driven by the increasing demand for green products or biologically derived surfactants which exhibit enhanced functionality compared to chemically synthesized surfactants, and are also renewable and biodegradable (Markets and Markets, 2017). In addition, biosurfactants have other intriguing advantages of enormous structural diversity, ability to function in wide ranges of pH, temperature and salinity as well as greater selectivity, lower critical micellar concentrations, and the feasibility to use renewable material, industrial waste streams/by-products and municipal waste streams as the source of production (da Rosa et al., 2015; Li and Yu, 2011; Makkar et al., 2011; Pacwa-Płociniczak et al., 2011). In recent studies, it was revealed that due to the complex structural properties, biosurfactants have a more mosaic distribution of polar and apolar regions than chemically synthesized surfactants (Otzen, 2017). This mosaic distribution gives biosurfactants unique ability to control biofilm formation and leads to milder interactions with proteins/enzymes, which make them promising detergents, pharmatheutical and bioremediation agents (Otzen, 2017). Biosurfactants are considered as multifunctional biomolecules of the 21st century because of these features (Santos et al., 2016). Environmental and oilfield applications are among the primary market sectors due to the environmental friendly nature of bisourfactants (Mulligan, 2009; Pacwa-Płociniczak et al., 2011).

1.2 Structural Properties of Biosurfactants and their Classification

The hydrophilic moieties of biosurfactants can be amino acids, peptides, proteins, mono-/di- or polysaccharides, phosphates, etc. The hydrophobic moieties can be long-chain fatty acids and their derivatives. These functional groups have been used to classify biosurfactants as glycolipids, lipopeptides, lipopolysaccharide, glycoprotein, polysaccharide, etc. Ron and Rosenberg (2001) proposed another structural criterium to broadly classify biosurfactant as low-molecular-weight and high-molecular-weight biosurfactants. Low-molecular-weight biosurfactants, such as many glycolipids, generally excel at lowering the surface/interfacial tension of water/oil; whereas high-molecular-weight biosurfactants, such as polysaccharides, glycoproteins, lipopolysaccharides, proteins and particulate biosurfactants, are normally not as effective with surface/interfacial reduction yet but are better at stabilizing emulsions. These biosurfactants are called bioemulsifiers (Uzoigwe et al., 2015). Many glycolipids (e.g. rhamnolipids and trehalose lipids) and lipopeptides (e.g., surfactin and lichenysin) are low-molecular-weight compounds, but capable of both reducing surface/interfacial tension and stabilizing emulsions (Cai et al., 2017a; Lovaglio et al., 2011). The biosurfactants can also be classified according to the types of the producing microorganisms and can be divided into archaea, bacteria, algae and fungi produced biosurfactants (Cai et al., 2015b; Cai et al., 2017a). The majority of the biosurfactants/bioemulsifiers that have been reported in the literature are produced by bacteria. Tables 1.1 and 1.2 summarize popular low-molecular-weight and high-molecular-weight biosurfactants, respectively.

Table 1.1 Examples of low-molecular-weight biosurfactants

Type	Name	Molecular structure/composition	Microorganism	Ref.
Glycolipids	Rhamolipids	 Monorhamnolipids	*Pseudomonas aeruginosa* **(Bacteria)**	Reiling et al. (1986)
	Sophorolipids	 Acidic sophorolipid	*Corynebacterium batistae, Thiobacillus bombicalo* **(Bacteria)**	Van Bogaert et al. (2007)
	Trehaloselipids	 m+n = 27 TO 31 Trehalose monomycolates	*Rhodococcus* sp., *Arthrobacter* sp., *Nocardia erythropolis* **(Bacteria)**	Lang and Philp (1998)
	Mannosylerythritol lipids		*Pseudozyma* sp., *Candida antartica*, *Ustilago maydis* **(Fungi)**	Kitamoto et al. (2002)
	Cellobiose lipids	 n = 2 or 4 R = H or OH	*Ustilago zeae, Ustilago maydis* **(Fungi)**	Hewald et al. (2005)

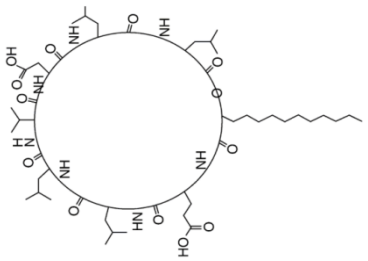
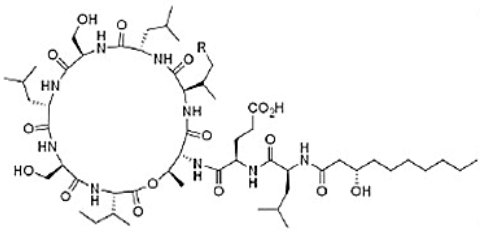
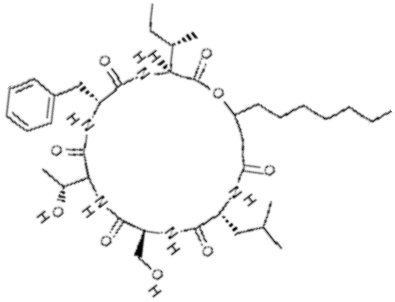
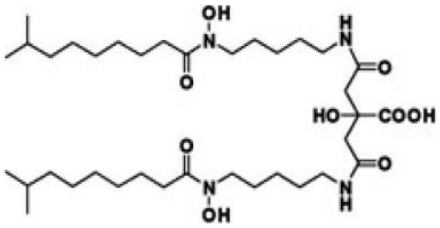
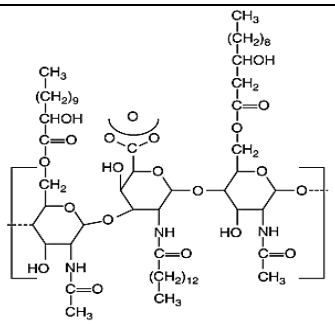
Lipopeptides	Surfactin		*Bacillus subtilis, Bacillus pumilus A* **(Bacteria)**	Seydlová and Svobodová (2008)
	Viscosin		*Pseudomonas fluorescens* **(Bacteria)**	Laycock et al. (1991)
	Serrawettin		*Serratia marcescens* **(Bacteria)**	Matsuyama et al. (1992)
	Flavolipids		*Flavobacterium* **(Bacteria)**	Bodour et al. (2004)

Table 1.2 Examples of high-molecular-weight biosurfactants

Type	Name	Molecular structure/composition	Microorganism	Ref.

Lipopolysaccharide	Emulsan		*Acinetobacter calcoaceticus* **(Bacteria)**	Rosenberg and Ron (1999)
		Polysaccharide fraction contained l-rhamnose,d-galactose,d-glucose, andd-glucuronic acid at a molar ratio of 3:1:1:1; fatty acid content consisted of C16:0, 3-OH C12:0 and C12:0 (molecular weight=1,700-2000 kDa)	*Klebsiella oxytoca* BSF-1 **(Bacteria)**	Kim and Kim (2005
Glycoprotein	Alasan	Protein polysaccharide (1 mDa)	*Acinetobacter radioresistens* **(Bacteria)**	Navon-Venezia et al. (1995)
	Liposan	83% carbohydrate and 17% protein	*Candida lipolytica* **(Fungi)**	Cirigliano and Carman (1985)
	Mannoprotein	90% mannose 5-10% protein	*Saccharomyces cerevisiae* **(Fungi)**	Cameron et al. (1988)
Lipoprotein		Lipid and protein (31.3:68.7)	*Azotobacter Chroococcum* **(Bacteria)**	Toledo et al. (2008)
Carbohydrate protein lipid complex		Carbohydrate:lipid:protein (40:27:29)	*Corynebacterium kutscheri* **(Bacteria)**	Thavasi et al. (2007)

Protein		Cell-associated protein with a molecular weight greater than 5000 Da	*Methanobacterium thermoautotrophicum* **(Archaea)**	Trebbau de Acevedo and McInerney (1996)
		Molecular weight of 200 kDa and containing 30 kDa monomeric subunits flagellin-like protein	*Solibacillus silvestris* AM1 **(Bacteria)**	Markande et al. (2013)
Polysaccharide		Extracellular polysaccharides	*Porphiridium cruentum* **(Alga)**	de Jesús Paniagua-Michel et al. (2014)
Particulate	Vesicles	Phospholipid-rich, lipopolysaccharide-rich particle with a polypeptide composition similar to the outer membrane	*Acinetobacter calcoaceticus* **(Bacteria)**	Käppeli and Finnerty (1979)

1.3 Production of Biosurfactants

The utilization and commercialization of biosurfactants will be highly determined by their economic effectiveness. Currently, the cost of biosurfactants has been estimated to be 3-11 times higher than that of their chemical synthetic counterparts (Mukherjee et al., 2006). In order to compete with chemical synthetic surfactants economically, the cost of production needs to be brought down to around 1.97 USD per liter (Sekhon Randhawa and Rahman, 2014), which is a challenging task. The schematic mechanism of biosurfactant production is illustrated in Figure 1.1. As shown in the figure, the four key components involved in a biosurfactant production process are (1) feedstock, (2) inoculum, (3) fermentation conditions, and (4) downstream processing. Research efforts and technological development have been focused on reducing the production costs while improving the yields. Among the four components, the inoculum/microorganism is the engine of the process. It determines the maximum yield potential and the functionality of a biosurfactant product (Geys et al., 2014). Robust novel microorganisms and their hyper-production mutants/hybrids will be the key driving force to move the biosurfactant industry forward (Geys et al., 2014; Mukherjee et al., 2006). Therefore, the primary focus of the biosurfactant production part of this thesis is on the innovative development of inocula for the fermentation process.

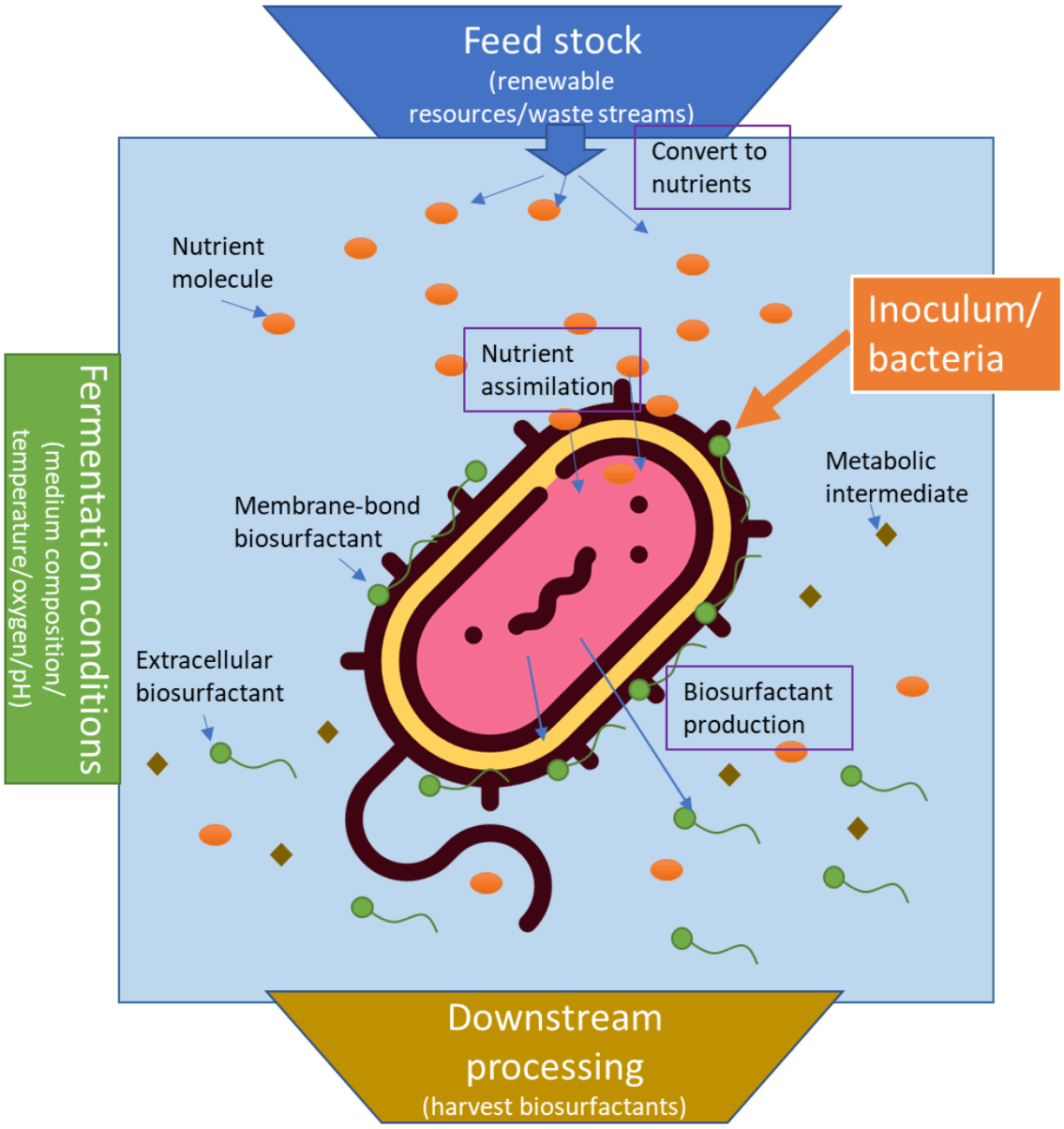


Figure 1.1 Schematic graph of biosurfactant production

1.4 Biosurfactant Functionality and Applications in Oil Contaminate Control

The important functional capabilities of biosurfactants include emulsification, dissolution, dispersion, emulsion breaking, reduction in viscosity, and surface activity, which result in a broad spectrum of potential applications in oil contaminate control (De Almeida et al., 2016; Panjiar et al., 2017). Examples of these functions and applications are illustrated in Figure 1.2.

When oil pollution occurs in a soil system, biosurfactants can exert their pollution control power through enhanced soil washing or bioremediation. During a soil washing process, a biosurfactant can improve the mobilization of hydrocarbons when the biosurfactant concentration is below its critical micelle concentration (CMC) by the reduction of the interfacial force between soil/oil system, which in turn increases the contact angle and reduces the capillary force between soil and oil (Urum and Pekdemir, 2004). When the concentration of a biosurfactant is above its CMC, solubilization occurs with the formation of micelles, which dramatically increases the concentration of oil in the washing solution (Pacwa-Płociniczak et al., 2011). Furthermore, the emulsification ability of a biosurfactant impedes the coalescence of the oil-in-water (O/W) micelles (Pacwa-Płociniczak et al., 2011), while the formation of oil in-water-emulsion reduces the apparent viscosity of heavy oils and improves their transportation in a subsurface porous system (Hasan et al., 2010).

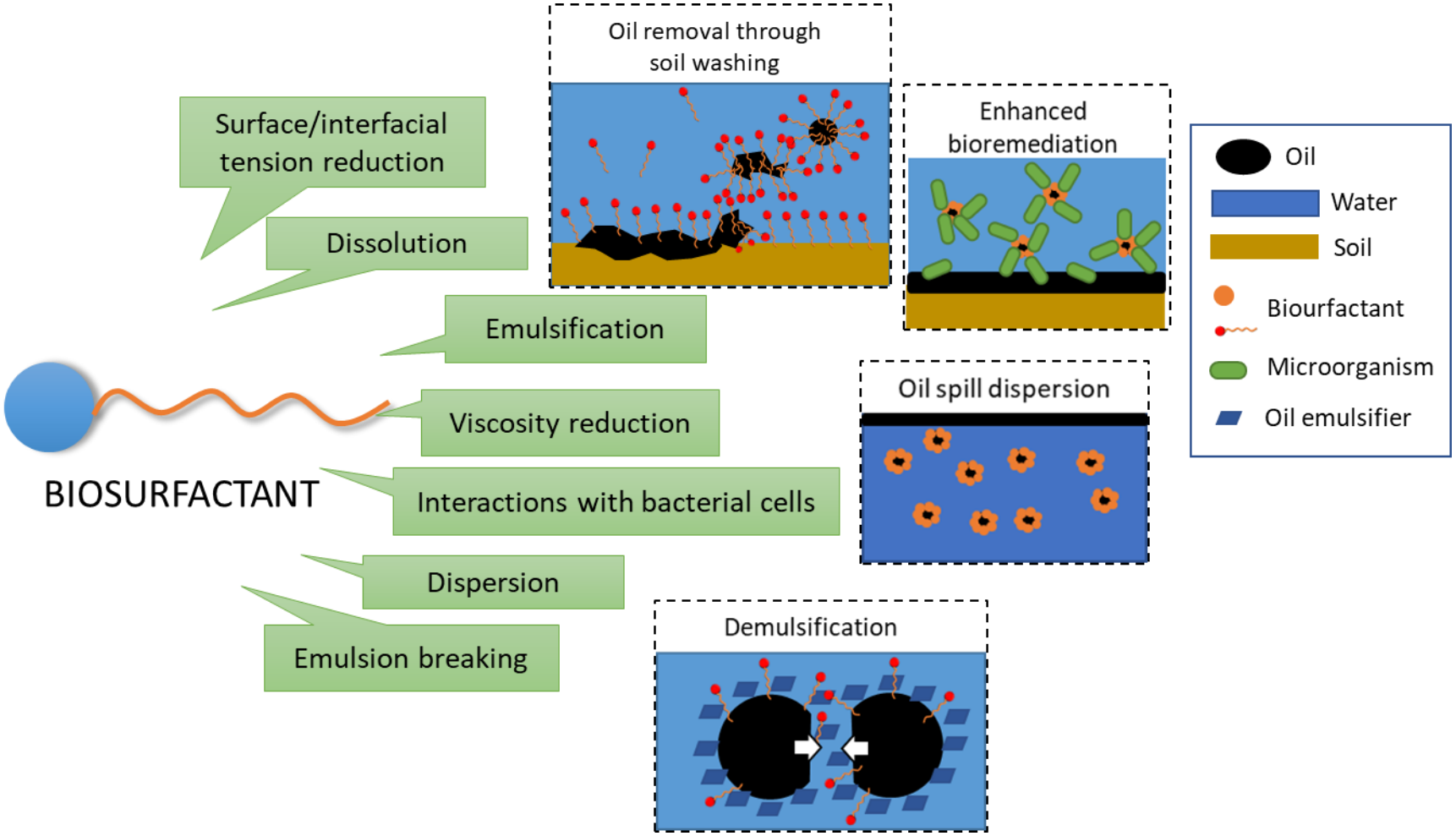


Figure 1.2 Biosurfactant in oil contaminate control

When a biosurfactant is used for enhanced bioremediation, the biosurfactant mainly serve as a "mediator" between the oil/soil system and microorganisms. The above-mentioned mechanisms still work in this case to bring down the oil into small micelles which significantly improve the contact surface areas between oil and microorganisms (Nguyen et al., 2008). The large migrating range of oil in the presence of the biosurfactant enables the involvement of higher amounts of microorganisms (Christofi and Ivshina, 2002). Further, the biosurfactants can interact with the bacterial cells and alter the cell hydrophobicity, and thus facilitate the access of the cells to water-solubilized hydrocarbons, large oil droplets, and pseudo-solubilized or emulsified oils (Al-Tahhan et al., 2000; Franzetti et al., 2009; Franzetti et al., 2010). In addition, biosurfactants were also reported to facilitate direct uptake of micelles inside a cell in a way similar to active pinocytosis for subsequent degradation (Cameotra and Singh, 2009).

Treatment of oily wastewater can be a challenge when a stable O/W emulsion forms from various sources such as oil and gas platforms, petroleum refineries, and chemical processing and manufacturing plants (Al-Anzi and Siang, 2017; Yang et al., 2016; Zhang et al., 2014). A demulsification process is thus required to achieve effective water/oil separation and water purification (Jamaly et al., 2015; Zolfaghari et al., 2016). Biosurfactants and their producing microbes can serve as biological demulsification agents by disturbing or replacing the oil emulsifiers (Hou et al., 2014; Huang et al., 2014). However, only a handful of studies have attempted to develop demulsifying bacteria targeting oily wastewater (O/W emulsion) (Coutinho et al., 2013; Das, 2001; Li et al., 2012;

Park et al., 2000). Thus, the further exploration of novel demulsiftying bacteria for O/W emulsion and investigation of the demulsifying effectiveness and mechnisms is needed.

When oil pollution occurs in a water body (e.g., during offshore oil spills), biosurfactants can be used as dispersants. Dispersants are chemically synthesized and can be defined as a blend of surfactants, solvents, and additives (Kujawinski et al., 2011). The biosurfactant can reduce interfacial tension between oil and water, and with enough mixing energy, result in formation fine oil droplets that remain entrained in the water column (<70 μm D.I.) (Lee et al., 2013; National Research Council, 2005). Moreover, oil dispersion is essentially an emulsification process, and the most effective dispersants blends have the structural compatibility of surfactants that offer a stable arrangement at the interface which impede coalescence of oil droplets (Athas et al., 2014; Brochu et al., 1986). However, research efforts on examining the effectiveness of a biosurfactant product as the sole surface active component of an oil spill dispersant are still at the preliminary stage (Cai et al., 2016; Cao, 2015; Freitas et al., 2016; Lv et al., 2016; Marti et al., 2014; Moshtagh and Hawboldt, 2014).

1.5 Objectives and Structure of the Thesis

This thesis aims to face identified research gaps and tackles the advancement of biosurfactant production and applications for oil contaminate control. It entails 1) the development of novel and robust biosurfactant producing microorganisms and their hypo-production mutants; 2) the examinination of the functionality of produced biosurfactants; and 3) the investigation of the potential for using these produced biosurfactants in diverse applications concerning oily wastewater treatment and oil pollution control. The structure

and main contents of the thesis (Chapters 2-7) is summarized in Table 1.3. Chapter 2 serves as the foundation of the remaining chapters as it showcases the screening and characterization of biosurfactant-producing bacteria from offshore oil and gas platforms in North Atlantic Canada. Among the 114 isolated biosurfactant producers, there are a newly discovered *Alcanivorax atlantics* species (stated in chapter 3), first *Exiguobacterium* strain with emulsification ability (chapter 4), novel marine strains demuslfies O/W emulsions (chapter 5), and *Rhodococcus* strains with the oil dispersion potential (lead to chapters 6 and 7)

Chapter 3 presents the results of in-depth characterization of the newly discovered *Alcanivorax atlantics* species and the proposed type strain *Alcanivorax atlantics* N3-7A^{T}. According to the unique genomic and phenotypic features of this strain, a novel bacterial species, *Alcanivorax atlantics* is proposed and named. The type strain of the new species is an oil-degrading/biosurfactant producing bacterium, which is stored in microbial curators in Germany and Belgium. It also reports the complete genome sequence and curated annotation of *Alcanivorax atlantics* N3-7A^{T}.

Table 1.3 Structure and main contents of the thesis

		Chapter 2	Chapter 3	Chapter 4	Chapter 5	Chapter 6	Chapter 7
Biosurfactant production	Novel producing microorganisms	Screened 114 biosurfactant producers	Newly discovered species*	Novel bioemulsifiers	Novel biodemulsifier producers*		
	Hyper-production mutants					√	
	Harvested product characterization			√		√	4 types of products
Biosurfactant function examination	Surface/interfacial tension reduction	√	√	√	√		√
	Emulsification	√	√	√*	√		√
	Micelle formation	√			√	√	√
	Cell hydrophobicity				√		
	Demulsification				√*		
	Oil affinity						√
	Toxicity						√
	Biodegradability						√
Biosurfactant aided oil contaminate control	Oily wastewater treatment				√		
	Oil spill dispersion					√	√*
	Soil washing			√			
	Enhance bioremediation			√			

*Contains in-depth characterization and analysis

Chapter 4 reports an *Exiguobacterium* strain N4-1P as a bioemulsifier producer for the first time. It contains detailed characterization of the bacterial strain, the composition analysis of the produced bioemulsifier, and the performance and stability of the emulsifier (exmulsins) under diverse conditions with multiple hydrocarbons. It also discusses the application potential of exmulsins as an agent for enhancing soil washing and bioremediation. The complete genome sequence and curated annotation of *Exiguobacterium* strain N4-1P are discussed.

Chapter 5 focuses on the development of a biological demulsification technique for the treatment of oily wastewater. It reports thirty-seven novel marine O/W emulsion demulsifying bacterial strains belong to 5 genera and 15 species, among which *Halomonas* venusta N3-2A is the first *Halomonas* strain reported with demulsifying ability. It also includes detailed genotype and phenotype characterization of *Halomonas* venusta N3-2A and its performance and mechanisms of O/W emulsion breaking. It includes the complete genome sequence and curated annotation of *Halomonas* venusta N3-2A. This chapter also recommends optimized screening strategy for O/W emulsion breaking bacteria based on statistically supported results.

Chapter 6 starts to focus on oil spill dispersion. It illustrates the development of hyper-production *Rhodococcus erythropolis* mutant M47 using UV mutagenesis. It also demonstrates the promising performance of the crude biosurfactant generated by M47 as the key component of an oil spill bio-dispersant.

Chapter 7 further explores biosurfactant applications in oil spill dispersion. It includes not only the trehalose lipids produced from the 3rd generation mutant M36 based on Chapter 6, but also three other crude oil biosurfactants generated in our lab (i.e., surfactins, rhamnolipids and exmulsins). It demonstrates the performance of these 4 types of crude oil biosurfactants when using them at different dispersant : oil ratios to treat different crude oils. It explores the synergic effects between these biosurfactants and speculates the governing factors affecting dispersion effectiveness. Oil affinity, fatty acid composition, toxicity and biodegradability of these biosurfactants are also included. The chapter shows selected biosurfactants can be equally competent when compared with Corexit 9500A with the advantages of high oil affinity, low toxicity and high biodegradability.

Chapter 8 concludes the thesis with summarized findings, contributions and recommendations for future research activities.

CHAPTER 2 SCREENING OF BIOSURFACTANT-PRODUCING BACTERIA FROM OFFSHORE OIL AND GAS PLATFORMS IN NORTH ATLANTIC CANADA

This chapter is based on and expanded from the following papers:

Cai, Q., Zhang, B., Chen, B., Song, X., Zhu, Z., Cao, T. (2015) Screening of biosurfactant-producing bacteria from offshore oil and gas platforms in North Atlantic Canada. *Environmental Monitoring and Assessment*, 187(5):4490

Roles: Cai designed and conducted the study under the guidance of Dr. Baiyu Zhang and Dr. Bing Chen and acted as the first author of the manuscript. Xing Song, Zhiwen Zhu and Tong Cao participated in the experiments. Most contents of this paper were written by Cai and further polished by the other co-authors.

Cai, Q., Zhang, B., Chen, B., Zhu, Z., Lin W., Cao, T. (2014) Screening of biosurfactant producers from petroleum hydrocarbon contaminated sources in cold marine environments. *Marine Pollution Bulletin*, 86:402-410

Roles: Cai designed and conducted the study under the guidance of Dr. Baiyu Zhang and Dr. Bing Chen and acted as the first author of the manuscript. Weiyun Lin, Zhiwen Zhu and Tong Cao participated in the experiments. Most contents of this paper were written by Cai and further polished by the other co-authors

2.1 Introduction

Biosurfactants are surface-active amphiphilic molecules produced by microorganisms (Banat et al., 2010). The global biosurfactants market was estimated to be $1,735.5 million in 2011 and is expected to reach $2,210.5 million in 2018 with a compound annual growth rate of 3.5%. This rate is significantly higher than the overall surfactant market (2%) (Transparency Market Research, 2011). Oilfield and environmental applications are among the primary market sectors due to the environmental friendly nature of bisourfactants (Mulligan, 2009; Pacwa-Płociniczak et al., 2011). Although biosurfactants possess advantages such as less toxic, biodegradable and remaining active under extreme conditions (Mulligan, 2009; Pacwa-Płociniczak et al., 2011), their applications are still limited due to the high production cost (Banat et al., 2010; Mukherjee et al., 2006). One promising strategy to overcome the economic obstacle is to develop new biosurfactant producers that have potential to produce effective and versatile biosurfactants with high yields (Han and Parekh, 2005; Mukherjee et al., 2006; Mulligan, 2009).

The screening and isolation of new biosurfactant producers have been primarily conducted with terrestrial sources (Das et al., 2010). In recent years, diverse habitats with extreme salinity, temperature and pressure were starting to be explored as sources for biosurfactant producers (Bull et al., 2000; Joshi et al., 2008; Satpute et al., 2010a; Yakimov et al., 1995). The ocean harbors microorganisms with extraordinary metabolic and physiological features that are different from their terrestrial counterparts (Satpute et al.,

2010a). These microorganisms are underexplored with limited published reports (Das et al., 2010; Satpute et al., 2010a).

In addition, oil reservoirs could provide a hydrocarbon rich environment for the enrichment of diverse biosurfactant producers (Christofi and Ivshina, 2002). The extreme conditions (e.g., high temperature, high pressure, and low oxygen concentration) in oil reservoirs would formulate a microbial community that distinguished from others (Grabowski et al., 2005). Several researchers have isolated biosurfactant producers from oil reservoirs in China (She et al., 2011; Wang et al., 2011; Xia et al., 2013), Brazil (Gudiña et al., 2012), India (Pruthi and Cameotra, 2003), Iran (Lotfabad et al., 2009) and Germany (Yakimov et al., 1995), most of which are from inland reservoirs. The reported biosurfactant producers in reservoirs included *Bacillus licheniformis* strains (She et al., 2011; Yakimov et al., 1995), *Bacillus cereus* strain (She et al., 2011), *Bacillus subtilis* strains (Gudiña et al., 2012; Wang et al., 2011), *Pseudomonas aeruginosa* strains (Gudiña et al., 2012; Lotfabad et al., 2009; Xia et al., 2013), *Pseudomonas putida* strain (Pruthi and Cameotra, 2003). Those biosurfactant producers identified from oil field samples are limited to the genera of *Bacillus* and *Pseudomonas*.

Till now, very limited work has been conducted to isolate marine biosurfactant producers from petroleum hydrocarbon contaminated samples from offshore oil and gas fields. Hardly any reports discussed and compared the culturable biosurfactant producers from different components of offshore oil and gas production processes such as extraction (crude oil and formation water), mud circulation (drilling mud), water/oil separation

(produced water) and produced water discharge (seawater in the vicinity of the platform). Therefore, the current study attempted to fill the knowledge gap by isolating biosurfactant producers from crude oil, formation water, drilling mud and treated produced water samples from offshore oil and gas platforms. The genotype and phylogenetic relation of these isolates were investigated. The diversity of the biosurfactant producer communities in different platform samples were analyzed and compared. Subsequent characterization of the isolates provided information on the properties and associated possible blends/applications of the produced biosurfactants.

2.2 Materials and Methods

2.2.1 Samples for the isolation

In total, 10 samples were taken from the offshore oil and gas platforms areas. They are two formation water samples, two drilling mud samples and two crude oil samples from two different oil and gas platforms as well as two seawater samples in the vicinity of these two platforms. In addition, oily contaminated water samples from two heavy traffic harbours and sediment samples from coastline near a refinery were collected for screening as well. The denotations of sample IDs were summarized in Table 1. Samples were shipped to the lab with ice bags and stored in amber bottles at 4^{o}C.

Table 2.1 Samples for screening biosurfactant producers

Sample source	Sample ID
Formation water	RA and RB
Crude oil	COA and COB
Drilling mud	DMA and DMB
Treated produced water	PWA, PWB and P1-P10
Seawater near platforms	SA and SB
Water samples from two heavy traffic harbours	SJ and CBS
Sediment samples near a refinery company	N1-N4

2.2.2 Screening and isolation

Each collected sample was first enriched with two different recipes of medium in 125 mL conical flasks. The first one composed of $(NH_4)_2SO_4$, 10 g; NaCl, 15 g; $FeSO_4 \cdot 7H_2O$, 2.8×10^{-4} g; KH_2PO_4, 3.4 g;$K_2HPO_4 \cdot 3H_2O$, 4.4 g; $MgSO_4 \cdot 7H_2O$, 1.02 g; yeast extract, 0.5 g; trace element solution, 0.5 mL in 1 L of distilled water, with 2% (v/v) n-hexadecane as the sole carbon source and salinity adjusted to 3.5%, which was adopted and modified from Peng et al. (Peng et al., 2007a). The trace element solution contained $ZnSO_4$, 0.29 g; $CaCl_2$, 0.24 g; $CuSO_4$, 0.25 g; $MnSO_4$, 0.17 g L^{-1} and was sterilized separately. The second recipe is a modified Atlas oil agar medium (Atlas, 2004) composed of $MgSO_4$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 0.05 g; KH_2PO_4, 3.4 g; $K_2HPO_4 \cdot 3H_2O$, 4.4 g; $(NH_4)_2NO_3$, 1 g; $FeCl_3$, 0.05 g; NaCl, 26 g in 1 L of distilled water, with 1% (v/v) clear diesel fuel as the sole carbon source. The chemicals used were analytical grade, unless otherwise specified. Enrichment was conducted at 30°C, 200 rpm for 3-5 days until observable turbidity occurred.

After enrichment, the consortia were serial diluted to 10^8 times and spread on agar plates. The resulting plates were incubated at 30°C for 3-5 days until slow growers formed clear colonies. Morphologically different colonies were picked for screening. Subsequently, a drop collapsing test modified from Bodour and Miller-Maier (1998) was used to screen biosurfactant producers (Cai et al., 2014). Briefly, 2 µL of mineral oil was added to each well of a 96-well microtiter plate lid, and the lid was equilibrated at room temperature. Five µl of distilled water was added to the surface of oil, after which a colony was picked up by

a loop and added in that 5 µl of distilled water. After 1 min, the flatness of the water droplet was observed and recorded as "+". Those cultures that form the same droplet as the control were scored as "-". The cultures gave positive responses were subjected to purification with the streak plate technique for 3 times.

2.2.3 Identification and phylogenetic analysis of isolates

The purified isolates were subjected to 16S ribosomal DNA (rDNA) sequencing using universal bacterial primers F27 and R1493 (position in *Escherichia Coli* 8-27 and 1512-1493, respectively). An aliquot of each culture was used as a DNA template in a polymerase chain reaction (PCR) using the primer pair. After gel electrophoresis confirmation of a successful PCR reaction, PCR products were subjected to a clean-up process and measured by a NanoDrop spectrophotometer to determine the concentrations. Lastly, sequencing reactions with the last PCR products were conducted and measured with Applied Biosystems 3130 and/or 3730 systems in Core Research and Instrument Training Network at the Memorial University of Newfoundland. The obtained DNA sequence was matched with Basic Local Alignment Search Tool database.

Phylogenetic trees and distances were calculated using the software package MEGA (Molecular Evolutionary Genetics Analysis) version 5.1, after alignment of sequences with ClustalX. Distances were calculated using the Kimura two-parameter model (Kumar et al., 2007). Trees were reconstructed using the neighbour-joining method (Saitou and Nei, 1987) with the use of 'default settings' and the bootstrap values were

calculated based on 1000 replications, including all available species under the selected genera with validly published names, as well as the type strains.

2.2.4 Performance of the isolated biosurfactant producers

A production medium (PM) composed of $MgSO_4$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 0.05 g; KH_2PO_4, 3.4 g; $K_2HPO_4 \cdot 3H_2O$, 4.4 g; $(NH4)_2NO_3$, 1 g; $FeCl_3$, 0.05 g; Glucose, 1g; NaCl, 26 g in 1L of distilled water, with 3% (v/v) n-hexadecane were selected to investigate the performance of biosurfactant production. A loopful of colony was inoculated to each flask containing 25 mL of the PM and incubated for 4 days at 30ºC while shaking at 150 rpm. Three parameters were applied to evaluate the efficacy of the produced biosurfactants. Relevant analytical methods of the parameters are indicated below.

Surface tension measurement: Culture samples were centrifuged at 3,000 rpm for 25 min to remove the cells and the supernatant was submitted to surface activity measurements. The surface tension (ST) was determined in triplicate with a surface tensiometer (DuNouyTensiometer, Interfacial, CSC Scientific) at room temperature. The surface tension determination was triplicated.

Emulsification activity assay: The emulsification index (E24) of culture samples was determined by adding 5 mL of light mineral oil to the same amount of culture, mixing with a vortex for 2 min, and leaving to stand for 24 h. The emulsification activity was evaluated by E24 using Equation (1):

$$E24 = H_{EL}/H_S \times 100\% \quad (1)$$

where $H_{EL}$is the height of the emulsion layer and H_S is the height of the total solution.

Critical micelle dilution (CMD): CMD can reflect the concentration of produced biosurfactants. It was defined as the dilution of the culture broth upon reaching the critical micelle concentration (Shavandi et al., 2011). After centrifuging at 10,000 rpm for 10 min and discarding the pellet, the cell free broths were diluted with distilled water, while the surface tension of each dilution was measured. The CMD was determined as the highest dilution with which the surface tension did not significantly increase. As the broth consists of both aqueous and oil phase, each dilution was conducted after sonication to ensure homogeneity. Before each measurement, the sonicated solution was allowed to stand for 15-20 min to achieve equilibrium.

2.2.5 Diversity analysis

The diversity and relationships between species isolated in this study were evaluated by several species-diversity indices (Atlas and Bartha, 1997). The following parameters were used: the number of species (S); Simpson's index (λ); Simpson's index of diversity (1- λ); Simpson's reciprocal index (1/ λ); the Shannon-Weaver index (H); and the evenness index (J).

2.3 Results and Discussion

2.3.1 Phylogenetic analysis of isolates

As shown in Table 2.2, in total, 114 biosurfactant producers and possible petroleum hydrocarbon degraders were isolated and identified according to their 16S rDNA

sequencing results. The isolated strains belonged to 8 different genera, i.e. *Bacillus, Rhodococcus, Halomonas, Alcanivorax, Exiguobacterium, Halomonas, Pseudomonas and Streptomyces*. Among them, three were recently established, i.e. *Alcanivorax*, *Exiguobacterium* and *Halomonas*. The genus *Alcanivorax* is particularly young and was established in 1998 (Yakimov et al., 1998), with only 11 species identified so far. All of them were isolated from marine environments and were found to be important alkane degrading bacteria (Lai et al., 2013b). The genus of *Exiguobacterium* comprises species that can grow at a wide temperatures span of -6^{o}C to 55^{o}C and are considered extremophiles (Vishnivetskaya et al., 2009). Several *Exiguobacterium* strains have been found with unique properties such as production of diverse enzymes that are stable at a broad range of temperature, ability to neutralize alkaline wastewater, ability to remove pesticide and reduce heavy metals (Vishnivetskaya et al., 2009), whereas no *Exiguobacterium* strain has been reported in the literature as a biosurfactant producer. The genus *Halomonas* comprises halophilic strains capable of growing at salinity between 5 and 10% (w/v) (Sánchez-Porro et al., 2010). Few studies discussed the biosurfactants produced by *Halomonas* strains (Gutiérrez et al., 2007b; Pepi et al., 2005), but the efforts were limited compared with other well-studied biosurfactant producing genus.

The further analysis of these isolates will be limited within the isolates from Samples RA, RB, COA, COB, DMA, DMB, PWA, PWB, SA and SB (total 59 isolates), as the rest were described previously (Cai, 2014).

Most of the 59 strains belonged to genera of *Bacillus* (35 strains) (Table 2.3). The representative strains of each clustered groups were then selected to generate the complete phylogenetic trees with close related type strains of the genera (Figure 2.1). The *Bacillus* isolates were closely related to *B. cereus, B. thuringiensis, B. subtilis, and B. licheniformis*. They have been commonly isolated from marine environments (Ivanova et al., 2010). The *Rhodococcus* isolates were closely related to *R. erythropolis* and *R. phenolicus*. Both of them are well known degraders of diverse persistent organic pollutions (Shinoda and Kunieda, 1983; Yakimov et al., 1997). The *Halomonas* isolates were closely related to *H. glaciei*, *H. neptunia* and *H. boliviensis.* These halophilic bacteria have been found at harboring locations with extreme conditions such as Antarctica, deep-sea hydrothermal-vent, and hypersaline lake (Kaster et al., 2012; Lazar et al., 2007; Lotfabad et al., 2009). The *Pseudomonas* isolates were closely related to *P. fluorescens* which was reported producing rhamolipid-type biosurfactants that are effective under extreme temperature, salinity and pH (Yang et al., 2006).

As shown in Figure 2.2, biosurfactant producers have been found in both *Eubacteria* and *Archaea* in the division of *Proteobacteria*, *Firmicutes, Actinobacteria, Cytophage-Flexibacter-Bacteroides, Crenachaeota* and *Euryarchaeota*. This Figure is an update of a previous work (Bodour et al., 2003) by clustering 16S rDNA sequence of the recent studies and representative strains found in this study. The strains isolated in this study were distributed between *Proteobacteria, Actinobacteria* and *Firmicutes* divisions.

The biosurfactant producers and the corresponding biosurfactants published after 2003 were summarized in Table 2.4.

Table 2.2 Identification of the isolated biosurfactant producers

Sample	No. of isolates	Isolate ID	Species name with highest match	Max identity
RA	11	RA-1A	*Rhodococcus erythropolis*	100
		RA-2A	*Halomonas glaciei*	99
		RA-3A	*Halomonas glaciei*	99
		RA-4A	*Bacillus cereus*	99
		RA-5A	*Bacillus subtilis*	99
		RA-6A	*Halomonas. Neptunia*	99
		RA-7A	*Halomonas neptunia*	99
		RA-8A	*Bacillus subtilis*	99
		RA-9A	*Halomonas boliviensis*	99
		RA-2P	*Bacillus subtilis*	100
		RA-3P	*Bacillus licheniformis*	99
RB	5	RB-1A	*Rhodococcus erythropolis*	100
		RB-2A	*Rhodococcus erythropolis*	100
		RB-3A	*Bacillus licheniformis*	99
		RB-4A	Bacillus licheniformis	99
		RB-2P	*Bacillus clausii*	100
SA	5	SA-1A	*Bacillus cereus*	99
		SA-2A	*Bacillus licheniformis*	98
		SA-1P	*Bacillus subtilis*	99
		SA-2P	*Halomonas boliviensis*	99
		SA-3P	*Rhodococcus erythropolis*	99
SB	4	SB-1A	*Rhodococcus erythropolis*	99
		SB-2A	*Halomonas boliviensis*	99
		SB-1P	*Rhodococcus phenolicus*	99
		SB-2P	*Bacillus subtilis*	100
PWA	4	PWA-1P	*Bacillus subtilis*	99
		PWA-1A	*Rhodococcus erythropolis*	100
		PWA-2P	*Bacillus subtilis*	98
		PWA-3P	*Bacillus subtilis*	99
PWB	3	PWB-1P	*Bacillus subtilis*	100
		PWB-2P	*Bacillus subtilis*	100
		PWB-1A	*Rhodococcus phenolicus*	99
DMA	8	DMA-1P	*Rhodococcus erythropolis*	99
		DMA-2P	*Bacillus subtilis*	99

		DMA-3P	*Bacillus thuringiensis*	99
		DMA-4P	*Pseudomonas fluorescens*	100
		DMA-5P	*Bacillus cereus*	100
		DMA-6P	*Rhodococcus erythropolis*	99
		DMA-7P	*Rhodococcus erythropolis*	100
		DMA-8P	*Bacillus cereus*	100
DMB	3	DMB-1P	*Bacillus cereus*	100
		DMB-3P	*Bacillus cereus*	99
		DMB-5P	*Bacillus cereus*	99
COA	5	COA-1P1	*Bacillus cereus*	99
		COA-1P2	*Rhodococcus erythropolis*	99
		COA-2P	*Bacillus subtilis*	100
		COA-3P	*Bacillus cereus*	99
		COA-1A	*Bacillus subtilis*	99
COB	11	COB-1P	*Bacillus cereus*	100
		COB-1A	*Bacillus subtilis*	99
		COB-2P	*Pseudomonas fluorescens*	99
		COB-2P2	*Pseudomonas fluorescens*	100
		COB-2A	*Bacillus subtilis*	99
		COB-3P	*Pseudomonas fluorescens*	99
		COB-4P	*Rhodococcus erythropolis*	100
		COB-5P	*Bacillus subtilis*	99
		COB-6P	*Bacillus cereus*	100
		COB-7P	*Bacillus cereus*	100
		COB-3A	*Bacillus pumilus*	99
CBS	1	CBS-1P	*Bacillus Thuringiensis*	100
SJ	1	SJ-1P	*Rhodococcus yunanensis*	100
N1	2	N1-1P	*Rhodococcus phenolicus*	99
		N1-2P	*Bacillus subtilis*	100
N2	7	N2-1P	*Rhodococcus zopfii*	99
		N2-3P	*Bacillus subtilis*	100
		N2-4P	*Rhodococcus erythropolis*	100
		N2-5P	*Rhodococcus erythropolis*	100
		N2-6P	*Bacillus subtilis*	99
		N2-7P	*Bacillus subtilis*	98
		N2-2A	*Rhodococcus erythropolis*	100
N3	17	N3-1P	*Bacillussubtilis*	100
		N3-2P	*Rhodococcus zopfii*	98

		N3-3P	*Rhodococcus wratislaviensis*	98
		N3-4P	*Bacillus licheniformis*	100
		N3-5P	*Rhodococcus phenolicus*	98
		N3-6P	*Pseudomonas peli*	99
		N3-7P	*Bacillus flexus*	99
		N3-8P	*Bacillus mycoides*	100
		N3-9P	*Bacillus subtilis*	100
		N3-11P	*Bacillus subtilis*	96
		N3-2A	*Halomonas venusta*	99
		N3-3A	*Rhodococcus fascians*	99
		N3-4A	*Rhodococcus cercidiphyllis*	99
		N3-5A	*Rhodococcus fascians*	99
		N3-6A	*Streptomyces venezuelae*	98
		N3-7A	*Alcanivorax venustensis*	96
		N3-8A	*Halomonas variabilis*	99
N4	4	N4-1P	*Exiguobacterium oxidotolerans*	99
		N4-2P	*Bacillus subtilis*	100
		N4-3P	*Rhodococcus yunnanensis*	100
		N4-1A	*Rhodococcus erythropolis*	99
P1	7	P1-1P	*Rhodococcus opacus*	98
		P1-2P	*Bacillus subtilis*	99
		P1-3P	*Bacillus subtilis*	98
		P1-4P	*Rhodococcus opacus*	98
		P1-5P	*Rhodococcus wratislaviensis*	98
		P1-6P	*Bacillus subtilis*	96
		P1-1A	*Acinetobacter calcoaceticus*	99
P2	1	P2-2P	*Rhodococcus erythropolis*	100
P3	1	P3-1P	*Bacillus subtilis*	99
P4	2	P4-1P	*Rhodococcus opacus*	99
		P4-2P	*Rhodococcus erythropolis*	99
P5	2	P5-1P	*Rhodococcus erythropolis*	99
		P5-1A	*Acinetobacter oleivorans*	100
P6	7	P6-2P	*Bacillus subtilis*	100
		P6-3P	*Bacillus subtilis*	98
		P6-4P	*Rhodococcus erythropolis*	99
		P6-5P	*Rhodococcus erythropolis*	100
		P6-6P	*Rhodococcus erythropolis*	100
		P6-7P	*Bacillus thuringiensis*	100

		P6-9P	*Rhodococcus erythropolis*	99
P7	1	P7-1A	*Acinetobacter oleivorans*	100
P9	1	P9-1A	*Acinetobacter calcoaceticus*	99
P10	1	P10-1P	*Bacillus subtilis*	99

Note: Annotation of Isolate ID is sample ID-No. agar type (P = Peng's agar, A = Atlas oil agar).

Table 2.3 Distribution of the isolates

Genus	No of isolates	Distribution	
		Atlas oil agar (no. of strains)	Peng's agar (no. of strains)
Bacillus	35	COA, COB, RA, RB, SA (11)	COA, COB, DMA, DMB, RA, RB, PWA, PWB, SA, SB (24)
Halomonas	7	RA, SB (6)	SA (1)
Pseudomonas	4	COB, DMA (4)	-
Rhodococcus	13	RA, RB, PWA, PWB, SB (6)	COA, COB, DMA, SA, SB (7)

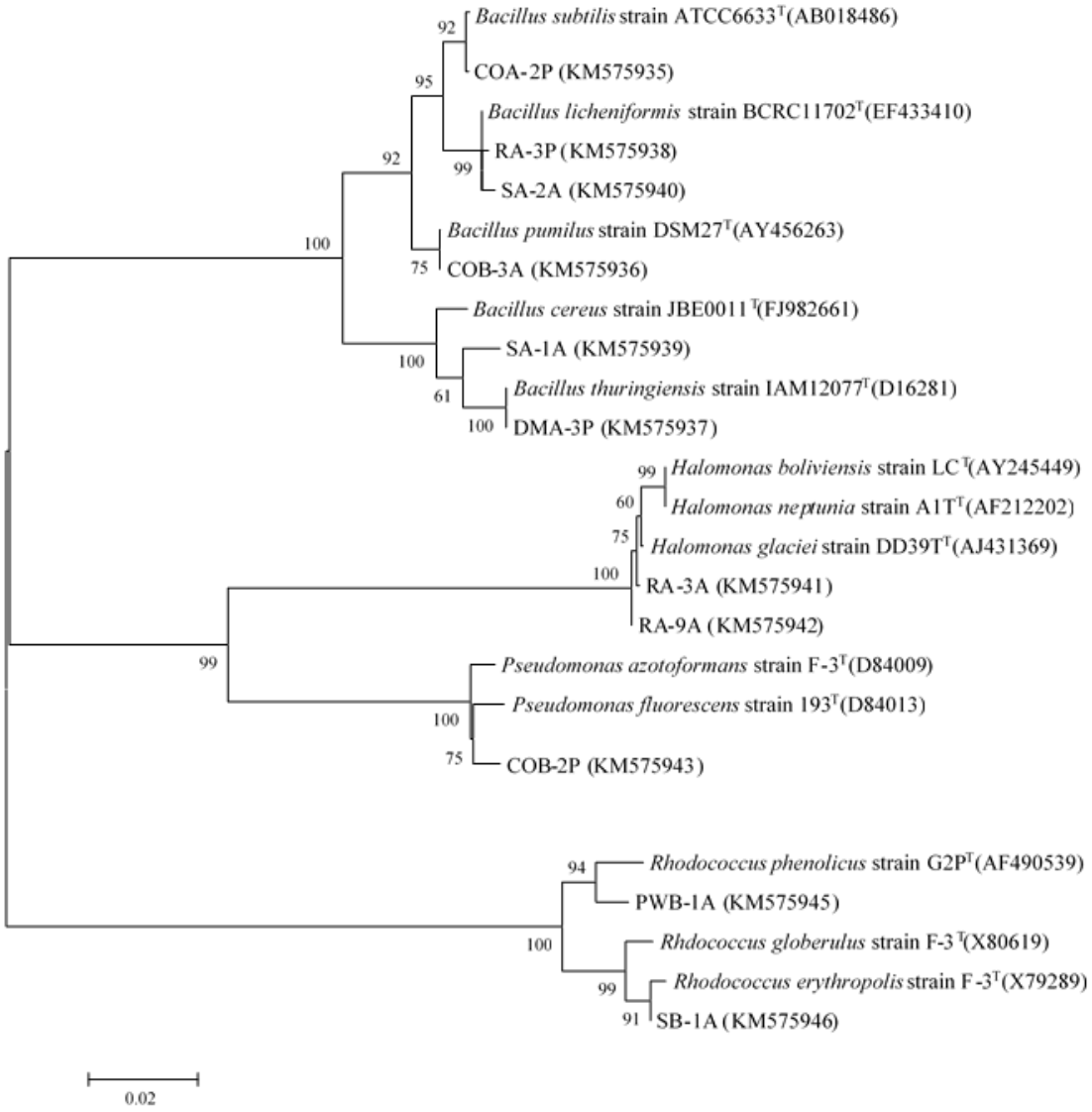


Figure 2.1 Phylogenetic tree based on 16S rRNA gene sequences. (Note: The tree was constructed using the NJ method. The numbers at nodes represent bootstrap values (based on a resampling of 1000). Bootstrap values >70 % are indicated. The GenBank accession numbers for the 16S rRNA gene sequences of all the strains are listed in parentheses. Bar, 0.01 nucleotide substitutions per site.

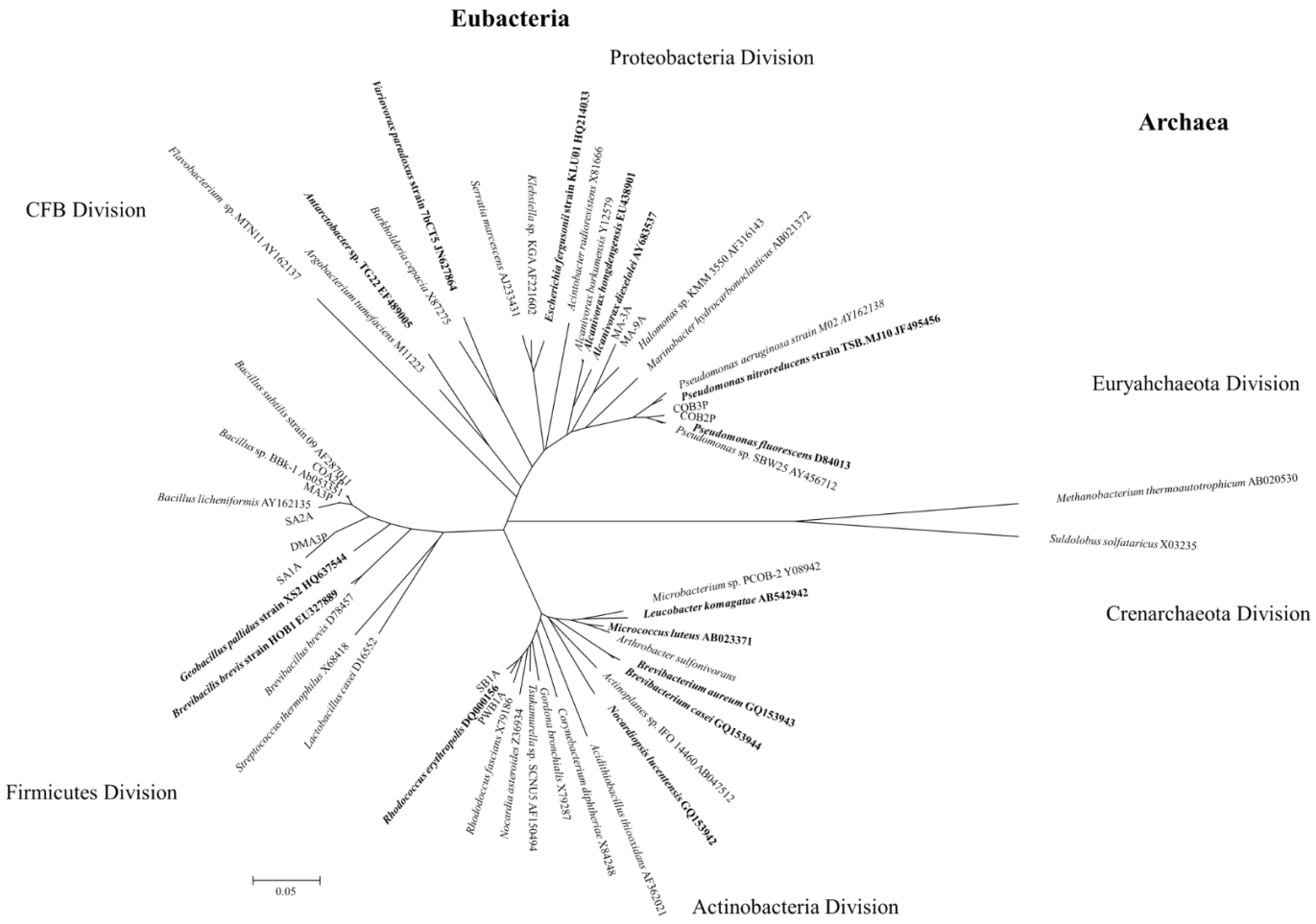
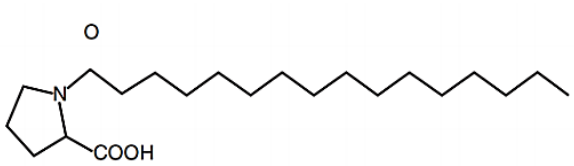


Figure 2.2 Phylogenetic tree based on 16S rDNA sequence from representative biosurfactant producers isolated in this study and those published in the literature. CFB=Cytophage-Flexibacter-Bacteroides.

Table 2.4 Biosurfactant producers and the corresponding biosurfactants published after 2003

Biosurfactant producers	Produced biosurfactants	Ref.
Lipopeptide-type biosurfactant producers		
Alcanivorax dieselolei strain B-5		Qiao and Shao (2010)
Alcanivorax hongdengensis strain A-11-3	Lipopeptides with fatty acid moiety of C15:0 (46.3%) and C17:0 (40.2%), while the amino acid moiety consists of two amino acids; one of which was detected as tyrosine.	Wu et al. (2009)
Brevibacilis brevis strain HOB1	Surfactin isoform	Haddad et al. (2008)
Brevibacterium aureum strain MSA13	A lipopeptide with a hydrophobic moiety of octadecanoic acid methyl ester and a peptide part of four amino acid including proline-leucine-glycine-glycine	Kiran et al. (2010a)
Escherichia fergusonii strain KLU01	Lipopeptides showed 2 compounds in HPLC chromatogram	Sriram et al. (2011)
Leucobacter komagatae strain 183	A Lipopeptide similar with surfactin and lichenysin	Saimmai et al. (2012)
Pseudomonas nitroreducens strain TSB.MJ10	A lipopeptides containing lysine, serine and iso-leucine	de Sousa and Bhosle (2012)
Glycolipid-type biosurfactant producers		
Brevibacterium casei strain MSA19	A glycolipid with a hydrophobic nonpolar hydrocarbon chain (9,12,15-octadecatrienoic acid, 2-[(trimethylsilyl)oxy]-1-[[(trimethylsilyl) oxy]methyl] ethyl ester, (Z,Z,Z)) and the hydrophilic sugar part as 1H-cyclopenta[c]furan-3(3aH)-one, 6,6a-dihydro-1-(1,3-dioxolan-2-yl)-, (3aR,1-trans,6a-cis)	Kiran et al. (2010a)
Micrococcus luteus strain BN56	Two trehalose tetraesters with molecular mass of 876 and 848 g/mol	Tuleva et al. (2009)

Nocardiopsis lucentensis strain MSA04	A glycolipid with a hydrophobic non-polar hydrocarbon chain and hydrophilic sugar, 3-acetyl 2,5 dimethyl furan	Kiran et al. (2010b)
Rhodococcus erythropolis strain 3C-9	A glucolipid, a trehalose lipid and free fatty acids	Peng et al. (2007b)
High-molecular-weight biosurfactant producers		
Antarctobacter sp. TG22	A glycoprotein (>2,000 kDa) with high uronic acids content	Gutiérrez et al. (2007a)
Geobacillus pallidus strains XS2	A polusaccharide based biosurfactant (271,785 Da) consisted of carbohydrates (68.6%), lipids (22.7%) and proteins (11.3%)	Zheng et al. (2011b)
Variovorax paradoxus strain 7bCT5	A polysaccharide based biosurfactant (165-186 kDa) consisted of carbohydrates (95%), lipids (4%) and proteins (1%)	Franzetti et al. (2012)

2.3.2 Distribution and diversity of the isolates

The distribution of the 4 genera in the platform samples was summarized in Table 2.3. The isolated strains were location and/or medium specific. *Bacillus* were widely found in platform samples including raw and treated produced water, crude oil, drilling mud and nearby seawater samples. More specifically, when using Peng's agar, all the platform samples contained *Bacillus*, whereas Atlas oil agar only lead to isolation of *Bacillus* from crude oil, raw produced water and seawater samples. For *Halomonas*, they were only found in raw produced water samples and sea water samples and Atlas oil agar had better chance to isolate *Halomonas. Pseudomonas* strains were all isolated with Atlas oil agar and they only presented in crude oil samples and drilling mud samples. *Rhodococcus* were almost equally isolated from both medium. However, they were primarily isolated from raw and treated produced water with Atlas oil agar, whereas when using Peng's agar, they were mainly isolated from crude oil samples and drilling mud samples. Both Peng's agar and Atlas oil agar have been intensively used for isolation of oil-degrading/biosurfactant producing bacteria. While Peng's agar provided comprehensive nutrients and non-toxic carbon source, Atlas oil agar is more nutrient limited with moderately toxic carbon source (diesel). Therefore, Atlas oil agar tends to enrich bacteria sustainable with limited nutrients and oil contaminated environments.

The diversity of biosurfactant producers in the 10 platform samples were summarized in Table 2.5. Several diversity indices were calculated based on the isolates we obtained using the isolation protocols tailored to biosurfactant producing microrganims.

The purpose is to provide a relative comparison among the platform samples and provide guidance for future screening studies with similar interests. The number of species associates with community richness. It is a simple yet important way to analyze diversity. Besides, the lower the Simpson's index, the higher the diversity of the community, whereas higher Gini-Simpson's index and Simpson's reciprocal index are associated with higher diversity. Meanwile, Shannon-Weaver index is positively correlated with diversity. The last but not the least, the high Evenness index indicates the abundance of each species is relatively equal and considered to havce higher diversity than the situations with low Evenness index (community dominated by limited species) (Atlas and Bartha, 1997). Among the 10 different samples, raw produced water sample A (RA) had the highest number of species, Gini-Simpson's index, Simpson's reciprocal index and Shannon-Weaver index. It also had a high Evenness index of 0.9. RA harbored a biosurfactant producer community of high diversity. In contrast, drilling mud samples B had the lowest values for all the parameters indicating the biosurfactant producer community had the poor diversity. In sum, the diversity of the 10 platform samples following the order of RA > SA > SB > DMA > COB > RB > COA> PWB > PWA > DMB. Raw produced water samples harbored biosurfactant producers with higher diversity than treated produced water samples. Besides, seawater samples in the vicinity of platforms were found as a good source of isolating diverse biosurfactant producers.

Table 2.5. Diversity parameters of different samples

Sample ID	Number of species	Simpson's index	Gini-Simpson's index	Simpson's reciprocal index	Shannon-Weaver index	Evenness index
	S	$\lambda = \sum_{n}^{i} P_i^2$	1- λ	1/ λ	$H = \sum_{n}^{i} P_i lnP_i$	J=H/Hmax
COA	3	0.56	0.44	1.79	0.99	0.62
COB	5	0.26	0.74	3.85	1.49	0.61
DMA	5	0.25	0.75	4.00	1.49	0.72
DMB	1	1	0	1.00	0	0
RA	7	0.17	0.83	5.88	2.15	0.9
RB	3	0.38	0.62	2.63	1.04	0.75
PWA	2	0.62	0.38	1.61	0.56	0.40
PWB	2	0.56	0.44	1.79	0.64	0.58
SA	5	0.20	0.80	5.00	1.61	1.00
SB	4	0.25	0.75	4.00	1.38	1

Note: Pi is the fraction of the entire population made up of species i; Hmax equals to lnS (S=number of species encountered).

2.3.3 Performance evaluation of all isolated biosurfactant producers

The biosurfactant production of the 59 isolated biosurfactant producers were analyzed with the parameters of surface tension, E24 and CMD of cultures (Figure 2.3). The surface tension of water is around 72 dynes/cm, when biosurfactants were produced in the water, the surface tension decreased with increasing concentration of biosurfactants until it reaches the critical micelle concentration (CMC) (Mulligan, 2005). The lowest surface tension the solution can reach is mainly determined by the molecular structure and composition of the biosurfactants (Cai et al., 2014). It has been found that Rhamnolipids produced by *Pseudomonas* strains reduced the surface tension of water to lower than 30 dynes/cm (Gutiérrez et al., 2007a). Trehaloselipids produced by *Rhodococcus* strains were found to reduce the surface tension of water to 25-30 dynes/cm (Franzetti et al., 2010). Surfactin produced by *Bacillus* strains was found to reduce the surface tension of water to around 27 dynes/cm (Nitschke and Pastore, 2006). In this study, diverse strains in genera of *Bacillus*, *Pseudomonas*, *Rhodococcus* reduced the surface tension to less than 30 dynes/cm. *Halomonas* strains were generally found with lower ability of reducing surface tension which agreed with previous findings that high-molecular-weight biosurfactants had less tendency to reduce surface tension (Martínez-Checa et al., 2007).

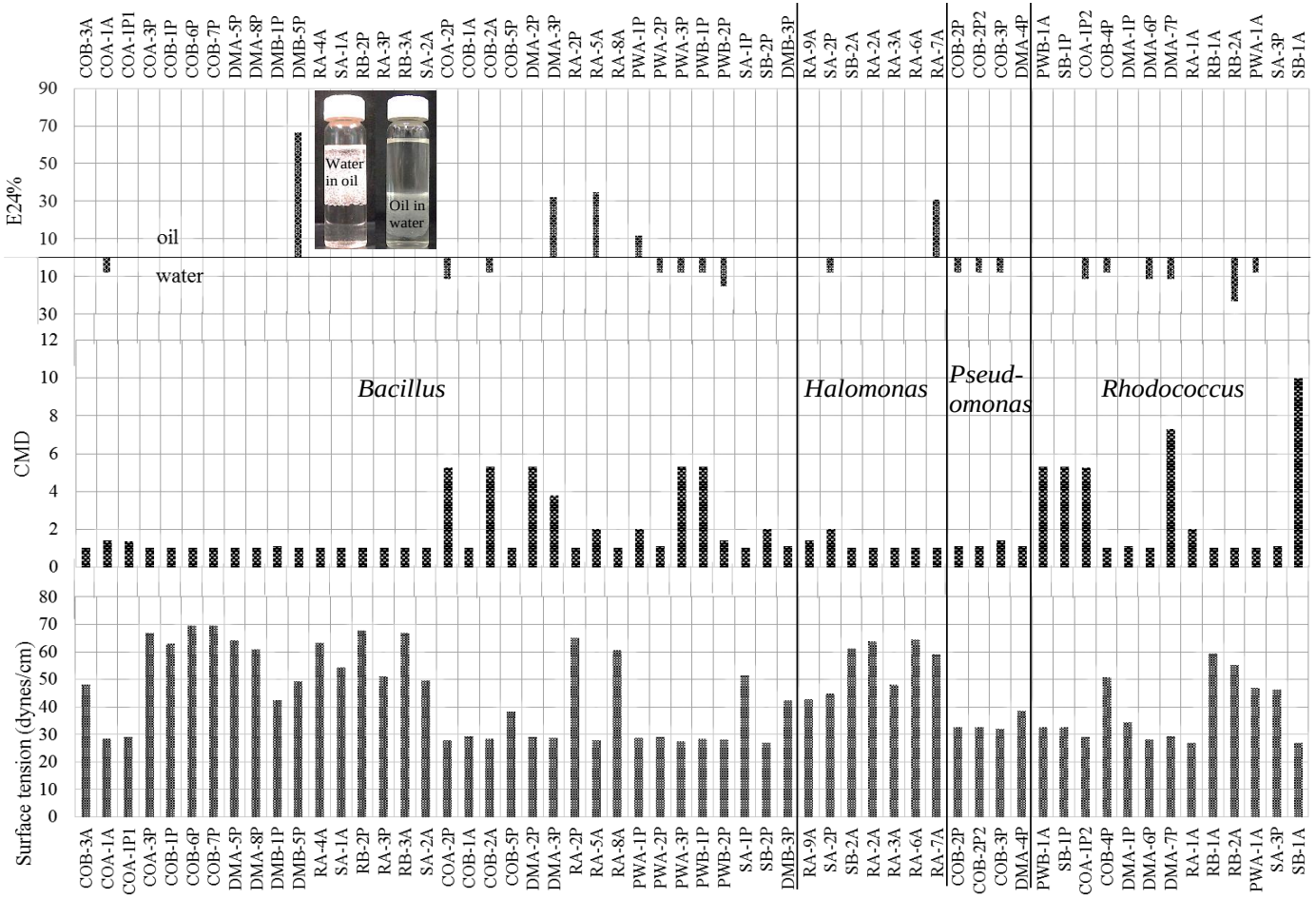


Figure 2.3 Surface tension, CMD and E24 of the 59 isolates (Note: for E24, the location of emulsion layer in either water or oil phase was identified with bars upon or below the solid line indicating the oil/water interface. Insert: a photo demonstrating the oil-in water emulsion vs. oil in water emulsion)

CMD has been used as an approximate measure of the concentration of biosurfactants (Shavandi et al., 2011). Some isolates belong to genera of *Rhodococcus* and *Bacillus* with high CMD after 4 days of incubation showed high potential of producing biosurfactants at high rate and yields. They are *Bacillus* sp. COA-2P, COB-2A, DMA-2P, PWA-3P, PWB-1P and *Rhodococcus* sp. PWB-1A, SB-1P, COA-1P2, DMA-7P, SB-1A and they have CMD>5. *Rhodococcus* sp. SB-1A had the highest CMD (=10) and highest production rate and yield. The E24 were used to evaluate the emulsification ability. When conducting the test, it was observed that emulsion layers appeared in water or oil phase reflecting their ability to form oil-in-water emulsions or water-in-oil emulsions.

Surfactants with relatively high hydrophilic-lipophilic balance (HLB) values tend to stabilize oil-in-water emulsions whereas surfactants with low HLB tend to stabilize water-in-oil emulsions (Pacwa-Płociniczak et al., 2011). Thus, the observations from the E24 tests indicate that the HLB values for the biosurfactants produced in this study span a wide range. *Bacillus* sp. DMB-5P, DMA-3P and RA-5A and *Halomonas* sp. RA-7A had high emulsification ability and formed emulsions in the oil phase. *Rhodococcus* RB-2A had the highest emulsification ability to form emulsion in the water phase. It is worth noting that some isolates (i.e. *Bacillus* sp. DMA-3P and RA-5A) could effectively reduce surface tension and stabilize emulsions. This results agreed with properties of surfactants which have previously been produced by *Bacillus subtilis* (Abdel-Mawgoud et al., 2008).

Most studies in the literature have presented a single isolate with in-depth characterization (Banat et al., 2010; Das et al., 2010; Satpute et al., 2010a). However, prior

research has found that multiple biosurfactant-related mechanisms worked towards a single outcome which could be effective dispersion, microbial enhanced oil recovery or soil washing, etc. (Pacwa-Płociniczak et al., 2011). For example, effective dispersion of weathered crude oil required a HLB of 10-12 (Becker et al., 1997; Board, 1989; Fingas, 2011). A blend of one surfactant with higher HLB and one with lower HLB to give an average HLB of 10-12 performed even better as both molecular penetrated further into water or oil phase (Board, 1989). Based on prior E24 observations, *Bacillus* sp. DMB-5P was found as a potential producer of biosurfactants with a relatively low HLB value (7-11) (Shinoda and Kunieda, 1983). Prior work found that *Rhodococcus* sp. RB-2A formed effective oil-in-water emulsions with biosurfactants that had a relatively high HLB value (12-16) (Shinoda and Kunieda, 1983). Therefore, the blend of the biosurfactants produced by these two strains may lead to synergic dispersion effects.

Another potential application of biosurfactants is enhanced oil recovery. The primary mechanisms behind microbial enhanced oil recovery are interfacial tension reduction and wettability alteration (Kowalewski et al., 2006). It is not likely for a single biosurfactant to exert both properties (Karimi et al., 2012; Kowalewski et al., 2006; Zargari et al., 2010). When bacterial cultures were applied in field trials to enhance oil recovery, mixed cultures were selected based on bench scale experiments and adapted to the reservoir environments were used in most cases (Lazar et al., 2007). *Rhodococcus* SB-1A reduced the surface tension of the medium to less than 30 dynes/cm. Besides, it had the highest CMD value indicating it produced the highest concentration of biosurfactants within the 4

days' timeframe. This strain is a good example of producer with potential to effectively reduce the interfacial tension. In addition, some *Pseudomonas fluorescens* strains (Kaster et al., 2012) and *Bacillus licheniformis* strains (Yakimov et al., 1997) were found with the ability to alter the wettability of the rock formation. It could be interesting to investigate the combined effects of *Rhodococcus* sp. SB-1A and *Bacillus lichniformis* strain RA-3P and/or *Pseudomonas fluorescens* strains COB-2P, COB-2P2, COB-3P and DMA-4P on enhanced oil recovery.

The mechanisms of biosurfactant aided soil washing, such as mobilization, solubilization and emulsification, also rely on different properties of biosurfactants (Pacwa-Płociniczak et al., 2011; Urum and Pekdemir, 2004). A biosurfactant with a high CMD value would be effective in the mobilization and solubization of oil whereas biosurfactants with high E24 (oil in water emulsion) value would be better at emulsification (Pacwa-Płociniczak et al., 2011). Mixed surfactant systems have been found to synergistically enhance soil washing efficiency (Yang et al., 2006; Zhao et al., 2005). The blend of biosurfactants produced by *Bacillus* sp. SB-1A and *Bacillus* sp. RB-2A would be capable of utilizing the three mechanisms spontaneously to achieve the synergistic removal efficiency. For the abovementioned applications, several biosurfactants with different properties blended as a mixture may generate outstanding efficiency. It is thus of great significance to screen and isolate various versatile biosurfactant producers from a promising source such as samples from offshore oil and gas platforms.

2.4 Summary

Crude oil, formation water, drilling mud, treated produced water and seawater samples were collected in offshore oil and gas platforms in North Atlantic Canada. Using n-hexadecane or diesel as the sole carbon source, 114 biosurfactant producers were identified and characterized. They belong to 8 different genera, i.e. *Bacillus*, *Rhodococcus*, *Halomonas, Alcanivorax, Exiguobacterium, Halomonas*, *Pseudomonas and Streptomyces*. The genetically closely related species of the isolates were clustered and summarized. Moreover, the 16S rDNA sequences of biosurfactant producers in the literature were combined with those of the representative strains isolated in this study to construct a comprehensive phylogenetic tree. The isolates were found to fall in the division of *Proteobacteria, Actinobactera* and *Firmicutes.* The distributions of the isolates were site and medium specific. The diversity of biosurfactant producer communities in the platform samples followed the sequence of RA > SA > SB > DMA > COB > RB > COA > PWB > PWA > DMB. Diverse isolates were found with properties such as reduction of surface tension, production of biosurfactants at high rate and stabilization of water-in-oil or oil-in-water emulsion. These isolates were found in habitats potentially rich in petroleum hydrocarbon produced biosurfactants while using petroleum hydrocarbon as the sole carbon source. The producers and their corresponding biosurfactants thus could facilitate bio-transformation of petroleum hydrocarbons, leading to promising potential in offshore oil spill control, enhancing oil recovery for crude oil production and soil washing treatment of petroleum hydrocarbon contaminated sites. The possible blends of isolates or their

biosurfactants and their capacities for applications were discussed. These blends will be tested at bench/batch scales to compare their performance in future studies.

CHAPTER 3 *ALCANIVORAX ATLATICUS* SP. NOV., A BIOSURFACTANT PRODUCING AND ALKANE DEGRADING BACTERIUM ISOLATED FROM PETROLEUM HYDROCARBON CONTAMINATED COASTAL SEDIMENT

This chapter is based on and expanded from the following papers:

Cai, Q., Zhang, B., Chen, B., Zhu, Z., Lv, Z (2018) *Alcanivorax atlaticus* sp. nov., a biosurfactant producing and alkane degrading bacterium isolated from petroleum hydrocarbon contaminated coastal sediment. *International Journal of Systematic and Evolutionary Microbiology* (ready for submission)

Roles: Cai designed and conducted the study under the guidance of Dr. Baiyu Zhang and Dr. Bing Chen and acted as the first author of the manuscript. Zhiwen Zhu and Ze Lv participated in the experiments. Most contents of this paper were written by Cai and further polished by the other co-authors.

Cai, Q., Ye, X., Chen, B., Zhang, B. (2018) Complete genome sequence of *of Alcanivorax altacticus strain N3-2A*, an oil degrading bacterium and biosurfactant producer isolated from cold marine environment in North Atlantic Canada. *Marine genomics, (ready for submission)*

Roles: Cai designed and conducted the study under the guidance of Dr. Baiyu Zhang and Dr. Bing Chen and acted as the first author of the manuscript. Xudong Ye participated in the data analysis. Most contents of this paper were written by Cai and further polished by the other co-authors.

3.1 Introduction

The genus *Alcanivorax*, which includes the most important alkane degrading bacteria in the marine environment (Lai et al., 2016), was proposed by Yakimov et al. (1998) and it currently comprises eleven recognized species, *A. borkumensis* (Yakimov et al., 1998), *A. jadensis* (Bruns and Berthe-Corti, 1999), *A. venustensis* (Fernindez-Martinez et al., 2003), *A. dieselolei* (Liu and Shao, 2005), *A. balearicus* (Rivas et al., 2007), *A. hongdengensis* (Wu et al., 2009), *A. pacificus* (Lai et al., 2011), *A. marinus* (Lai et al., 2013a), *A. xenomutans* (Rahul et al., 2014), *A. gelatiniphagus* (Kwon et al., 2015) and *A. nanhaiticus* (Lai et al., 2016). All of them were isolated from marine environments, except for *A. xenomutans*, which was isolated from a shrimp cultivation pond in India. The strains belonging to genus *Alcanivorax* are Gram-reaction-negative, aerobic, straight rods, non-motile or motile by polar flagella, halophilic, and can use aliphatic hydrocarbons as the sole source of carbon and energy (Yakimov et al., 1998). Seven out of the eleven species were isolated from the South/East China sea and/or the associated coastal environments, one was isolated from the Northern Ocean in Germany, one was isolated from the Yellow sea in China, one was isolated from shrimp cultivation pond in southern India, and one was from the Indian Ocean.

In this study, we described a novel strain N3-7A isolated from the coastal sediment near a refinery factory wastewater outlet during screening of oil-degrading/biosurfactant producing bacteria, which expands this intriguing genus. Comparative 16S rRNA gene sequence analysis indicated that strain N3-7A belonged to genus *Alcanivorax* and differed significantly from the eleven existing *Alcanivorax* species. Characterization and

classification of strain N3-7A were carried out using a polyphasic approach. Consequently, a novel species, *Alcanivorax atlaticus* sp. nov. represented by strain N3-7A^T (International microorganism curator ID =LMG 30071^T =DSM 105152^T) was proposed.

3.2 Methodology

3.2.1 Isolation of the bacterial strain

Oily contaminated coastal sediment samples in the vicinity of a refinery were collected for the isolation. Before the collection of sediment samples, temperature, salinity, conductivity and total dissolved solids (TDS) were measured on site with a conductivity meter (Thermo Scientific Orion Star A222). The temperature, salinity, conductivity and TDS of the sediment sample were 22.2ºC, 20.85‰, 33.4 ms/cm and 16.4 ppt, respectively. The samples were shipped with ice bags and stored in amber bottles at 4ºC. The sample site located at site N3 (E54º1', N48º48') in the southeast coast of Newfoundland (Placentia Bay), Canada. Each collected sample was enriched with a modified Atlas oil agar (MAOA) medium composed of $MgSO_4$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 0.05 g; KH_2PO_4, 3.4 g; $K_2HPO_4 \cdot 3H_2O$, 4.4 g; NH_4NO_3, 1 g; $FeCl_3$, 0.05 g; NaCl, 26 g L^{-1} of distilled water, with 1% (v/v) clear diesel fuel as the sole carbon source before isolation. The isolated strain was maintained on DSMZ® Medium 514 which composed of Bacto peptone 5 g, Bacto yeast extract 1 g, Fe(III) citrate 0.1 g, NaCl 19.45 g, $MgCl_2$ anhydrous 5.9 g, Na_2SO_4 3.24 g, $CaCl_2$ 1.8 g, KCl 0.55 g, $NaHCO_3$ 0.16 g, KBr 0.08 g, $SrCl_2$ 34 mg, H_3BO_3 22 mg, Na-silicate 4 mg, NaF 2.4 mg, NH_4NO_3 1.6 mg, Na_2HPO_4 8 mg L^{-1} with 1% sodium pyruvate at 28ºC.

3.2.2 16S rDNA sequencing and phylogenetic analysis

The purified isolates were subjected to 16S ribosomal DNA (rDNA) sequencing using universal bacterial primers F27 and R1493 (position in *Escherichia Coli* 8-27 and 1512-1493, respectively). An aliquot of each culture was used as DNA template in a polymerase chain reaction (PCR) using the primer pair. After gel electrophoresis confirmation of a successful PCR reaction, PCR products were subjected to a clean-up process and measured by a NanoDrop spectrophotometer to determine the concentrations. Lastly, sequencing reactions with the last PCR products were conducted and measured with Applied Biosystems 3130 and/or 3730 systems in CREAIT of MUN. Sequence of related taxa were obtained from the GenBank database. The 16S rRNA gene sequence similarity was determined using the Align sequence tool of the Basic Local Alignment Search Tool (BLAST). Phylogenetic trees and distances were calculated using the software package MEGA (Molecular Evolutionary Genetics Analysis) version 5.1, after alignment of sequences with CLUSTALX. Distances were calculated using the Kimura two-parameter model (Kimura, 1980). Trees were reconstructed using the neighbour-joining method (Saitou and Nei, 1987) with the use of ‘default settings’ and the bootstrap values were calculated based on 1000 replications.

3.2.3 Cell morphology analysis

General cell morphology was studied using scanning electron microscopy (SEM) (FEI MLA 650F) system as well as through visual observation of colonies on agar plates.

Cells grown on marine agar plates for 24 h were harvested twice with 2 mm loop from the third streak of the quadrant streaked plates, and then were transferred to 15 mL centrifugal tubes. The bacteria were fixed, washed and dehydrated following the methods used by de Sousa and Bhosle (2012). Briefly, bacterial cells were fixed overnight in 2% glutaraldehyde prepared in 50 mM potassium phosphate buffer. The fixed cells were washed three times with phosphate buffer and dehydrated using an increasing gradient of acetone in distilled water (30%, 50%, 70%, 80% and 90%, respectively) for 10 min each and finally in 100% acetone for 30 min and air dried. The specimens were sputter coated with gold using an auto fine coater and visualized using SEM. The coating process and the SEM visualization were conducted in the Micro Analysis Facility (MAF IIC) at the CREAIT of MUN.

3.2.4 Bacterial growth conditions and bacterial motility

The growth temperature was determined over the range of 4-45^{o}C on DSMZ® medium 514. Tolerance of NaCl was tested by using DSMZ® medium 514 supplemented with NaCl concentration of 0, 0.5, 1, 3, 5, 7, 10, 12, 15, 17 and 20 % (w/v), respectively. The range of growth temperature and salinity were determined based on the observation of colonies after 7 days of incubation.

Bacterial motility were measured using semi-solid agar (Tittsler and Sandholzer, 1936). Briefly, agar slants made of DSMZ medium 514 with 0.5% agar were prepared. Inoculations were made by the stab method with a straight needle. The source cultures were

on agar plate. After 6 days of incubation at 30ºC, the motility was manifested macroscopically by a diffuse zone of growth spreading form the line of inoculation.

3.2.5 Biochemical tests

The BIOLOG® microbial identification system was used to test the utilization of the 95 sole carbon sources simultaneously by strain N3-7A. The strain was grown on DSMZ medium 514 agar at 30ºC for 24h. The cells were then harvested with loop and transferred to 0.4 M aqueous NaCl solution. After thoroughly mixed with the NaCl solution, the cell density OD_{590} was determined by measuring absorbance at 590 nm with a spectrophotometer and was adjusted to 0.3 ± 0.05 with the NaCl solution. Subsequently, BIOLOG GN2 plate was inoculated with 150 µL of the cell suspension per well. The inoculated plates were incubated at 30ºC. The results were read visually after incubation for 1 day (Ivanova et al., 1998). The test was duplicated.

3.2.6 Membrane-based phospholipid-derived fatty acid (PLFA) analysis

Fatty acids of cells grown aerobically in DSMZ medium 514 agar at 30 °C for 72 h were harvested twice with a 2-mm loop from the third streak of the quadrant-streaked plates. The cells were then transferred in duplicate to reaction tubes with 1 ml of Reagent 1 (150 g NaOH in 1 L of 50% aqueous methanol) for saponification. Samples within the tubes were incubated for 30 min at 100°C in water bath. To methylate liberated fatty acids, 2 mL of Reagent 2 (6 N HCl in aqueous methanol) was added to each tube. Samples within the tubes were incubated again for 10 min at 80°C in water bath. Fatty acid methyl esters (FAME) were extracted from the aqueous phase by the addition of 1.15 mL of Reagent 3

(hexane/methyl *tert*-butyl ether, 1:1, v/v) to each tube. Then samples were rotated end-over-end for 10 min. After removing the aqueous (lower) phase, 3 mL of aqueous 1.2% NaOH (Reagent 4) was added and the tubes were again rotated for 5 min (Sasser, 1990). Finally, the organic (upper) phase containing FAMEs was transferred to a gas chromatography (GC) vial. The FAMEs were quantified by a GC/ mass spectrometry (GC/MS) system (Agilent Technologies 7890A GC connected to an Agilent technologies 5975C inert mass spectroscopy detector) in the Northern Region Persistent Organic Pollution Control (NRPOP) laboratory. Separation of FAMEs was conducted using an Agilent BD-5MS fused-silica capillary column. The column temperature was programmed from 50^{o}C to 120^{o}C at a rate of 10^{o}C/min, and then to 280^{o}C at a rate of 3^{o}C/min. Individual compounds were identified from their mass spectra and from comparing their retention times with the standard spectra. The concentration of each individual compound was determined based on the GC/MS response relative to that of the known quantitative standards. The test was conducted in duplicate.

3.2.7 Complete genome sequencing

Whole-genome shotgun sequencing of strain N3-7A was performed at the Donnelly Sequencing Center at University of Toronto (Toronto, Canada) using Illumina MiSeq 2500 using 300 cycle Miseq kit V2. This generated a total of 6,020,451 filtered paired-end reads, providing 383-fold coverage of the genome. Quality control was conducted through FastQC (Andrews, 2010). To assemble the data, SPAdes genome assembler was used (Bankevich et al., 2012). The plasmidSPAdes was used to assemble plasmids from whole genome sequencing data (Antipov et al., 2016). Subsequently, the assembled contigs was

re-ordered by Mauve multiple genome alignment using Alcanivorax dieselolei strain B5 as the reference genome (Rissman et al., 2009). Gene annotation was performed by using the NCBI prokaryotic Genome Annotation Pipeline (http://www.ncbi.nlm.nih.gov/genome/annotationprok/) tRNA and rRNA sequences were identified using tRNAscan-SE and RNAmmer, respectively (Lagesen et al., 2007; Lowe and Eddy, 1997).

3.2.8 Biosurfactant production

The biosurfactant producing abilities of the strain N3-7A^T were investigated with a MAOA medium with different carbon sources consisting of a non-hydrocarbon part and a hydrocarbon part. The non-hydrocarbon part of the carbon sources was standardized as 0.05% starch, 0.02% glucose, 0.02% sucrose and 0.1% citrate salt according to the recipe used by Wu et al. (2009). For the hydrocarbon part, four conditions, namely, 3% hexadecane, 3% diesel, 0.25% benzoate and zero hydrocarbon, were tested respectively. After 7 days of incubation, the culture broths were centrifuged at 12,000 rpm for 5 min to remove cells and precipitants. Subsequently, the broths were subjected to the surface tension determination and E24 test following protocols used by Cai et al. (2014). The clear broths were concentrated with lyophilisation and then mixed with three volumes of cold acetone. Subsequently, the solution was stored at 4^{o}C for 3 days to fully precipitatet the crude biosurfactants products. The supernatant was removed by centrifugation and the pellet was washed twice with acetone to remove the hydrocarbon residues. Finally, the pellet was grinded and dried first in fume hood then in dissectors. When constant weight was obtained for each sample, the yield was then determined (Cai et al., 2017a).

3.3 Results and Discussion

3.3.1 Phylogenetic analysis

The 16S rRNA gene was sequenced in five replicates and each sequence agreed well with each other. Subsequently, a nearly full-length 16S rRNA gene sequence (1498 nt) of strain N3-7A^T was obtained (Gene bank ccession number: KX714224). Phylogenetic analysis of the strain N3-7A^T indicated that it belonged to the class *gammaproteobacteria*, forming a robust clade within the genus *Alcanivorax* (shown in Figure 3.1). Using an improved Myers-Miller global alignment algorithm (Huang and Miller, 1991), the closest related species was calculated as *A. gelatiniphagus* MEBiC08158^T and *A. marinus* R8-12^T (96.8%), followed by *A. venustensis* DSM 13974^T (96.0%), *A. xenomutans* JC109^T (95.8%), *A. dieselolei* B-5^T (95.7%), *A. pacificus* W11-5^T (95.0%), *A. balearicus* MACL04^T (94.9%), *A. nanhaiticus* 19-m-6^T (94.1%), *A. hongdengensis* A-11-3^T (93.1%), *A. borkumensis* SK2^T (93.0%), and *A. jadensis* T9^T (92.7 %). As strain N3-7A^T has the highest match in the gene bank with <97% similarity, the strain could be a newly discovered bacterium without necessity of conduct DNA-DNA hybridization analysis (Tindall et al., 2010).

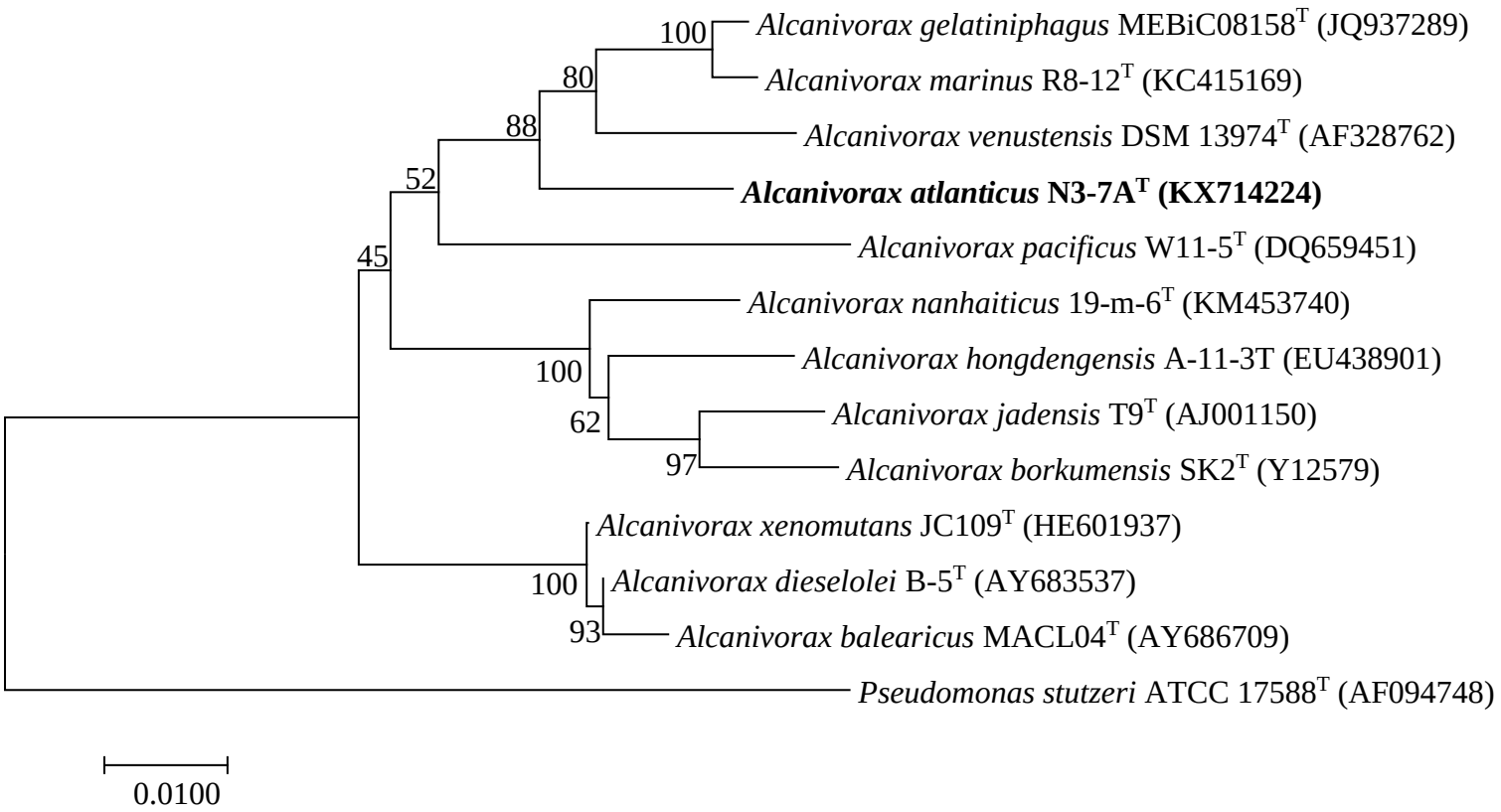


Figure 3.1 Neighbour-joining tree showing the phylogenetic positions of strain N3-7A^T and the type strains of all species of the genus *Alcanivorax*, based on 16S rRNA gene sequences. Bootstrap values (expressed as percentages of 1000 replications) are shown at branch points. Bar, 0.01 nucleotide substitution rate (Knuc) units. *Pseudomonas stutzeri* ATCC 17588T (AF094748) was used as an outgroup.

3.3.2 Cell morphology

The SEM image of strain N3-7A^T is shown in Figure 3.2. The strain is rod shaped bacteria with a length of 1-2 µm and diameter of around 0.5 µm. The general morphology of the strain is similar to the other member of the genus. When growing on the MAOA medium, the colonies are irregular, undulate, flat and transparent. When growing on the DSMZ medium 514, the colonies are circular, entire, convex and transparent.

3.3.3 Bacterial properties

The optimal growth temperature was determined over the temperature range of 4-45^{o}C on DSMZ medium 514. Tolerance of NaCl was tested by using DSMZ medium 514 supplemented with NaCl concentration of 0-20 % (w/v). The biochemical tests were carried out by using Biolog GN2 MicroPlate Panel, according to the manufacturer's instructions, with the adjustment of NaCl concentration to 3.5%. Bacterial motility was measured using semi-solid agar. The obtained results are compiled in Table 3.1 with the obtained results from the closely related type strains of other *Alcanivorax* species. The DNA G+C contents of the new isolate N3-7A was 63.02 mol% according to draft genome sequence (accession number CP022307-CP022309) and it was the closest to *A. xenomutans* KCTC 23751^T (62.1 mol%) and *A. gelatiniphagus* KCTC 23751^T (65.2%). It was also within the range (48.4-66.4 mol%) of genus *Alcanivorax*.

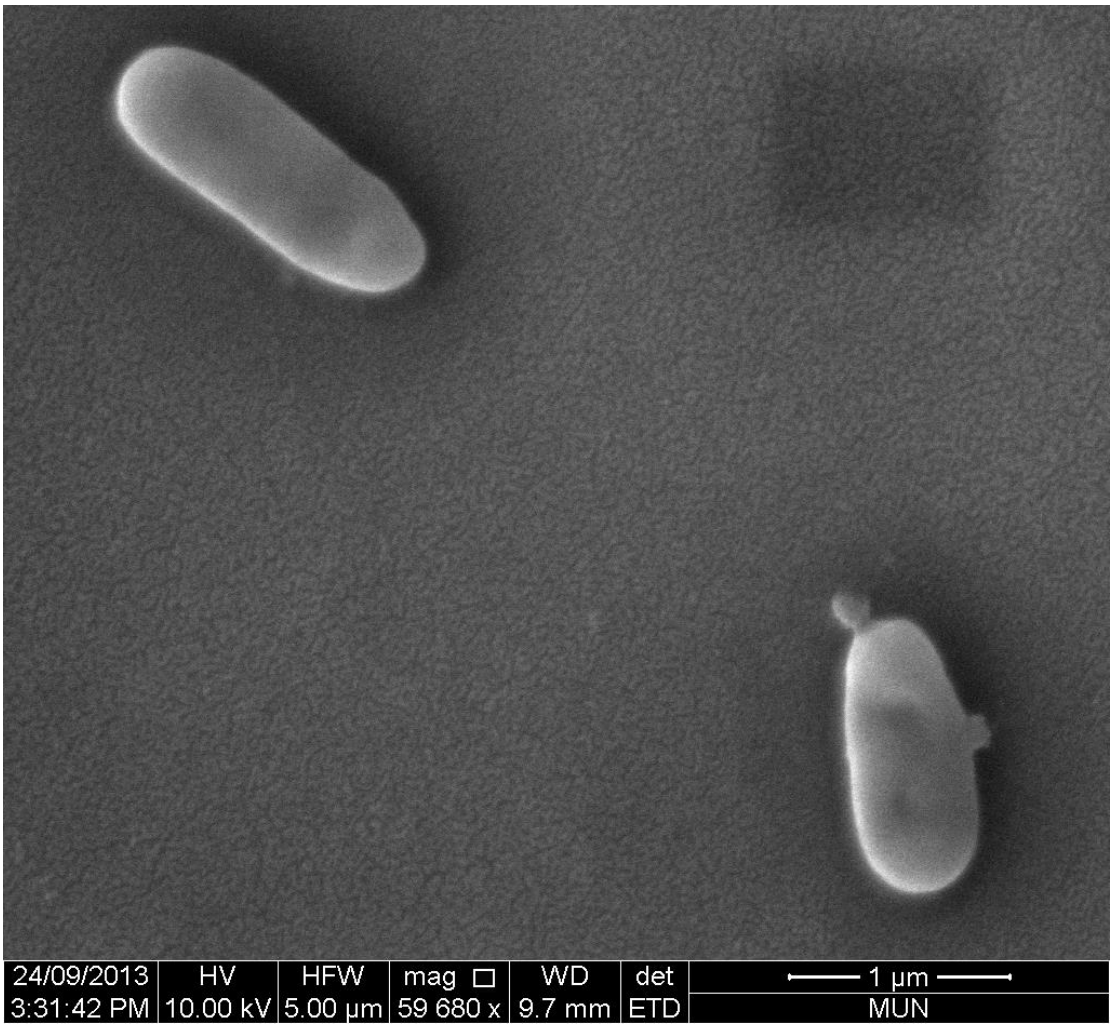


Figure 3.2 SEM visualization results of Alcanivorax sp. N3-7A^{T}

Table 3.1 Differential characteristics between strain N3-7A^T and closely related species in the genus *Alcanivorax*

Characteristic	**1**	**2**	**3**	**4**	**5**	**6**
Motility	-	+	+	+	+	+
Temperature range (^{o}C)	4-37	15-43	10-42	4-40	25-40	15-45
NaCl range (%, w/v)	1-15	0.5-16	0.5-15	1-15	0.5-20	1-15
Utilization of coumpounds as sole source of carbon and energy						
Formate	-	+	+	-	-	-
Phenylacetate	-	+	+	-	+	+
β-/γ Hydroxybutyric Acid	+	-	+	+	+	+
Bromosuccinic acid	+	-	-	-	-	+
a-Ketobutyric acid	-	+	-	-	-	-
a-Hydroxybutyric acid	-	+	-	-	-	-
Arabinose	+	-	-	-	+	-
Arabitol	+	-	-	-	-	-
Cellobiose	+	-	-	-	-	-
Dextrin	+	-	-	-	-	-
Galactose	+	-	-	-	-	-
Glutamic Acid	+	-	-	-	-	-
D,L-Alanine	+	-	-	-	-	-
D,L-Glucose	+	-	-	-	-	-
L-Asparagine	+	-	-	-	-	-

L-Proline	+	-	-	-	-	-
Sucrose	-	-	-	-	+	-
Mannitol	-	-	-	-	+	-
2,3-Butanediol	-	-	-	-	+	+
citric acid	-	-	-	-	+	+
DNA G+C content (mol%)	63.02	65.2	66.1	66.4	54.5	62.1

Strains: 1, N3-7A^T (data were obtained in the present study); 2, *A. gelatiniphagus* JCM 18425^T (data from this study and from Kwon et al., 2015); 3, *A. marinus* LMG 24621^T (data from this study and from Lai et al., 2013); 4, *A. venustensis* DSM 13974^T (data from this study and from (Fernindez-Martinez et al., 2003); 5, *A. xenomutans* KCTC 23751^T (data from this study and from Rahul et al., 2014); 6. *A. dieselolei* DSM 16502^T (data from this study and from Liu & Shao, 2005). +, positive reaction, -, negative reaction. All strains are Gram-reaction-negative and capable of using acetate, D, L-lactate, propionate, methyl pyruvate, methyl succinate, succinate, sebaccinate, Tween 40 and 80.

3.3.4 Complete genome sequence

A chromosome type replicon (4,305,015 bp), with G+C content of 63.02%, made of 210 contigs/scaffolds was obtained for *Alcanivorax atlantica* strain N3-7A^{T}, which harbors 4,162 coding sequences, including 3,309 proteins with identified functions. Three rRNA operons and 47 tRNAs were also annotated. The plasmids, containing a total of 407 coding sequences and sizes of 427,202 bp and 15,963 bp, respectively, were obtained. The G+C contents of the plasmids are 63.43% and 55.11%. As expected, diverse oxygenases, dehydrogenases and cytochromes involved in aerobic hydrocarbon biodegradation pathway are found in the genome, both in the chromosome and the plasmids, explaining its ability to degrade hydrocarbons. Several lipopolysaccharide and polysaccharide biosynthesis genes are presented in the chromosome linking to its ability to produce biosurfactants that are capable of reducing surface tension and stablizing water/oil emulsions. This is the 4th reported draft genome for the genus *Alcanivorax* but the only one isolated from petroleum hydrocarbon contaminated marine sediment samples in North Atlantic Ocean. It will provide a reference for many further phylogenetic, comparative genomic, metagenomic, and functional studies of this hydrocarbonoclastic genus.

The genome sequence has been deposited in DDBJ/EMBL/GenBank under accession number CP022307-CP022309. The version described in this paper is the first version, CP022307.1- CP022309.1.

3.3.5 Membrane PLFA profile

As shown in Table 3.2, the predominant fatty acids of the closely related type strains were C16:0, cy19, and C18:1, which accounted for 44.8~68.1 % of the total fatty acids. The predominant fatty acids of N3-7A^T were C16:0 (19.6 %), C18:1 (19.4 %), C16:1 (12.0 %), 3-OH C12:0 (11.7%) and cy19 (5.8). The chemotaxonomically characteristic fatty acid C19:0 cyclo was detected. In addition, strain N3-7A^T can significantly differ from its closest type strains *A. gelatiniphagus* JCM 18425^T and *A. marinus* LMG 24621^T based on PLFA analysis.

3.3.6 Biosurfactant production

The strain N3-7A^T was found with the ability to produce biosurfactants under all 4 conditions. The addition of hydrocarbons stimulated the production yields of the biosurfactants. Hexadecane and diesel lead to the production of biosurfactants that can significantly reduce surface tension, while the aromatic hydrocarbon (i.e., benzoate) stimulated the formation of biosurfactants that stabilized oil/water emulsions. Without hydrocarbons, the produced biosurfactants can effectively stabilize oil/water emulsions (Table 3.3).

Table 3.2 Fatty acid composition (%) of strain N3-7A^T and the type strains of closely related species of the genus *Alcanivorax*

Fatty acid	**1**	**2**	**3**	**4**	**5**	**6**
Saturated						
$C_{10:0}$	1.6	3.0	3.1	2.5	5.9	2.6
$C_{12:0}$	1.8	9.5	7.8	3.9	10.2	8.9
$C_{14:0}$	3.0	TR	TR	1.2	1.0	1.0
$C_{16:0}$	19.6	28.5	30.6	36.4	31.0	32.1
$C_{17:0}$	3.3	TR	1.3	TR	-	-
$C_{18:0}$	4.1	3.0	1.3	4.4	TR	0.9
Unsaturated						
$C_{14:1}$	2.8	-	-	-	-	-
$C_{16:1}$	12.0	5.0	6.9	6.7	8.6	11.3
$C_{18:1}$	19.4	5.6	20.0	18.1	7.8	22.4
Hydroxy						
$C_{12:0}$ 2-OH	-	-	-	-	2.1	0.9
$C_{12:0}$ 3-OH	11.7	8.8	9.3	7.6	7.3	2.9
Cyclopropane						
cy17	4.7	1.8	TR	TR	4.8	1.1
cy19	5.8	32.5	13.9	13.6	18.0	14.3

Strains: 1, N3-7A^T (data were obtained in the present study); 2, *A. gelatiniphagus* JCM 18425^T (data from this study and from Kwon et al., 2015); 3, *A. marinus* LMG 24621^T

(data from this study and from Lai et al., 2013); 4, *A. venustensis* DSM 13974^{T} (data from this study and from (Fernindez-Martinez et al., 2003); 5, *A. xenomutans* KCTC 23751^{T} (data from this study and from Rahul et al., 2014); 6. *A. dieselolei* DSM 16502^{T} (data from this study and from Liu & Shao, 2005). -, not detected; TR, trace amount <1%.

Table 3.3 Surface activities of the cultivating broth and the yields of biosurfactants of strain N3-7A^T when growing with different carbon sources.

Carbon source	Surface tension (dynes/cm)	E24 (%)	Yield (g/L)
Non-hydrocarbon carbon sources (NHC)	56.2	76.2	4.6
Hexadecane + NHC	37.0	0	13.1
Diesel +NHC	40.1	0	11.8
Benzonate +NHC	59.6	52.4	12.6

3.4 Summary

On the basis of morphological, physiological, and chemotaxonomic characteristics, as well as the phylogenetic analysis based on the 16S rDNA sequences described above, strain N3-7A^T should be placed into a new species of the genus *Alcanivorax*. As shown in Figure 1 and Tables 1-2, there are some significant characteristics distinguish strain N3-7A^T from the closely related species. The name *Alcanivorax atlanticus* sp. nov. is proposed. Its description is as following: *Alcanivorax atlanticus* (at.'lan. tic.us. L. masc. adj. atlanticus, pertaining to the Atlantic Ocean).

Its cells are Gram-reaction-negative, short non-motile rods, 1–2 mm * 0.5 mm. When growing on the DSMZ medium 514, the colonies are circular, entire, convex and transparent. They are moderately halophilic and can grow in 1–15% (w/v) NaCl and at 4–37 ^{o}C. The principal fatty acids are C16:0 (19.6%), C18:1 (19.4%), C16:1 (12.0%) and 3-OH C12:0 (11.7%). Among the 95 carbon sources in the Biolog system (GN2 plate), tests are positive for utilization of arabinose, arabitol, cellobiose, dextrin, galactose, glutamic acid, D,L-alanine, D,L-glucose, L-asparagine, L-proline, acetate, D, L-lactate, propionate, methyl pyruvate, methyl succinate, succinate, sebaccinate, γ hydroxybutyric acid, bromosuccinic acid, Tween 40 and 80. They grow well in Atlas oil agar with n-alkane or benzoate as carbon source. The produced biosurfactants are capable of reducing surface tension or stabilize oil/water emulsions while utilizing different substrates. The type strain, N3-7A^T, is isolated from coastal sediment of the Atlantic Ocean. The DNA G+C content of the type strain is 63.02 mol%.

CHAPTER 4 A NOVEL BIOEMULSIFIER PRODUCED BY *EXIGUOBACTERIUM* SP. STRAIN N4-1P ISOLATED FROM PETROLEUM HYDROCARBON CONTAMINATED COASTAL SEDIMENT

This chapter is based on and expanded from the following papers:

Cai, Q., Zhang, B., Chen, B., Zhu, Z., Zhao, Y.M. (2017) A novel bioemulsifier produced by Exiguobacterium sp. strain N4-1P isolated from petroleum hydrocarbon contaminated coastal sediment. *RSC Advances*, 2017(7):42699-42708

Roles: Cai designed and conducted the study under the guidance of Dr. Baiyu Zhang and Dr. Bing Chen and acted as the first author of the manuscript. Dr. Yumin Zhang provided guidance for the product structural analysis. Zhiwen Zhu participated in the experiments. Most contents of this paper were written by Cai and further polished by the other co-authors.

Cai, Q., Ye, X., Chen, B., Zhang, B. (2017) Complete genome sequence of *Exiguobacterium* sp. Strain N4-1P, a psychrophilic bioemulsifier producer isolated from cold marine environment in North Atlantic Canada. *Microbiology Resource Announcements*, 5(44):e01248-17

Roles: Cai designed and conducted the study under the guidance of Dr. Baiyu Zhang and Dr. Bing Chen and acted as the first author of the manuscript. Xudong Ye participated in the data analysis. Most contents of this paper were written by Cai and further polished by the other co-authors.

4.1 Introduction

Emulsifiers are compounds which assemble at the interfaces helping in the dispersion of droplets of one immiscible liquid within another, and preventing them from coalescing (Mittal and Shah, 2013). Their desirable characteristics such as solubility enhancement, detergency power and emulsion stabilization have enabled their applications in various industries, such as household cleaning, food processing, pharmaceutical, petroleum, agriculture, and textile (Calvo et al., 2009; Shete et al., 2006). They have been found with promising applications in the environmental engineering field, e.g. soil washing and bioremediation enhancement agents (Cai et al., 2015a; Pacwa-Płociniczak et al., 2011; Ron and Rosenberg, 2014). However, chemically synthesized emulsifiers suffer from concerns about toxicity and environmental impacts (Shete et al., 2006). Thus, identifying alternatives that may reduce these concerns, such as bioemulsifiers, could be important. Bioemulsifiers are surface-active molecules produced by microorganisms. They are high molecular weight polymers or lipopeptides (Satpute et al., 2010b). They have the advantages of lower or no toxicity, high biodegradability, and high stability at extreme salinity, pH and temperature when compared to their chemically synthetic counterparts (Muthusamy et al., 2008). Moreover, they can be produced from diverse substrates including waste streams (Makkar et al., 2011; Mukherjee et al., 2006). Despite all these advantages, the applications of bioemulsifiers have been hindered by low yields, as well as high recovery and purification costs (Banat et al., 2010). To overcome such obstacles, research efforts on developing novel bioemulsifier producers are highly important.

Diverse microorganisms including algae, bacteria and fungi have been found as bioemulsifier producers. A summary of such findings is shown in Table 1, along with their identified bioemulsifier compositions. Bacterial trains belonging to genera including *Acinetobacter* (Dams-Kozlowska et al., 2008), *Aeribacillus* (Zheng et al., 2011a), *Alcaligenes* (Toledo et al., 2008), *Amycolatopsis* (Colin et al., 2013a), *Azotobacter* (Thavasi et al., 2009), *Bacillus* (Liu et al., 2010b), *Beijerinckia* (Paul et al., 1986), *Corynebacterium* (Thavasi et al., 2007), *Enterobacter* (Hua et al., 2010), *Geobacillus* (Zheng et al., 2011a), *Halomonas* (Calvo et al., 1998), *Klebsiella* (Lee et al., 2008), Myroides (Maneerat et al., 2006), *Pedobacter* (Beltrani et al., 2015), *Propionibacterium* (Hajfarajollah et al., 2014), *Pseudomonas* (Husain et al., 1997), *Solibacillus* (Markande et al., 2013), *Streptomyces* (Colin et al., 2013b), *Varovorax* (Franzetti et al., 2012), have been reported to be bioemulsifier producers (Table 4.1). To date, however, no strains belong to the genus of *Exiguobacterium* have been reported as bioemulsifier producers.

Table 4.1 Summary of bioemulsifier producers and their produced bioemulsifiers

Phylum	Genus	Species	Bioemulsifiers composition/structure	Reference
Alga	*Dunalialla*	*D. salina*	Polysaccharides	Mishra et al. (2011)
	Porphiridium	*P. cruentum*	Polysaccharides	de Jesús Paniagua-Michel et al. (2014)
Archaea	*Methanobacterium*	*M. thermoautotrophicum*	Cell-associated protein with a molecular weight greater than 5000 Da	Trebbau de Acevedo and McInerney (1996)
	Halovivax	*H. sp.* A21	Sugar, protein and lipid	Kebbouche-Gana et al. (2009)
	Haloarcula	*H. sp.* D21	Glycoproteins	
Eubacteria	*Acinetobacter*	*A. calcoaceticus*	Emulsan: lipopolysaccharide and polysaccharide	Dams-Kozlowska et al. (2008)
		A. radioresistens	Alasan: protein polysaccharide (1 mDa)	Navon-Venezia et al. (1995)
	Aeribacillus	*A. pallidus* YM-1	A complex of carbohydrates (41·1%), lipids (47·6%) and proteins (11·3%)	Zheng et al. (2012)
	Alcaligenes	*A. faecalis*	Carbohydrate:protein (50.93:22.05)	Toledo et al. (2008)

Amycolatopsis	*A. tucumanensis* DSM 45259	Lipopolysaccharides or polysaccharides based on carbon source	Colin et al. (2013a)
Azotobacter	*A. chroococcum*	Lipid and protein (31.3:68.7)	Thavasi et al. (2009)
Bacillus	*B. licheniformis*	Lipopeptide: Lichenysin (1006-1034 Da)	Yakimov et al. (1995)
	B. subtilis	Lipopeptide: Surfactin (1036 Da)	Peypoux et al. (1999)
	B. velezensis	Lipopeptide: nC14-surfactin and anteisoC15-surfactin	Liu et al. (2010b)
Beijerinckia	*B. indica ss lacticogenes*	Polysaccharide PS-7	Paul et al. (1986)
Corynebacterium	*C. kutscheri*	Carbohydrate:lipid:protein (40:27:29)	Thavasi et al. (2007)
Enterobacter	*E. aerogenes*	Carbohydrate:protein (41.79:24.59)	Toledo et al. (2008)
	E. cloacae TU	Polysaccharide was found to be composed of glucose and galactose with molecular weight of 12.4 ± 0.4 kDa	Hua et al. (2010)
Geobacillus	*G. Pallidus*	271,785 Da (carbohydrates (68.6%), lipids (22.7%) and proteins (8.7%);	Zheng et al. (2011a)

		526,369 Da (carbohydrates (41.1%), lipids (47.6%) and proteins (11.3%).	
Halomonas	*H. eurihalina*	Sulfated heteropolysaccharide	Calvo et al. (1998)
Klebsiella	*K. sp.* Y6-1	Lipopeptide (1000-1500 Da); polysaccharide (3.5% protein)	Lee et al. (2008); Shepherd et al. (1995)
	K. oxytoca BSF-1	Lipopolysaccharide (1,700-2000kDa); polysaccharide fraction contained l-rhamnose,d-galactose,d-glucose, andd-glucuronic acid at a molar ratio of 3∶1∶1∶1; fatty acid content consisted of C16∶0, 3-OH C12∶0 and C12∶0.	Kim and Kim (2005)
Myroides	*M. sp.* SM1	mixture of l-ornithine lipids	Maneerat et al. (2006)
Pedobacter	*Pedobacter sp.* MCC-Z	67% of carbohydrates; 30% of lipids; 3% of proteins	Beltrani et al. (2015)
Propionibacterium	*P. acidipropionici*	Polysaccharides	Gorret et al. (2001)
	P. freundenreichii ss shermanii	Lipopeptide	Hajfarajollah et al. (2014)
	P. jensenii	Polysaccharides	Shepherd et al. (1995)
	P. thoenii	Polysaccharides	Shepherd et al. (1995)

	Pseudomonas	*P. nautica*	Proteins, carbohydrates and lipids (35:63:2)	Husain et al. (1997)
		P. fluorescens	Trehaloselipid-o-dialkyl monoglycerides-protein	Desai et al. (1988)
	Solibacillus	*S. silvestris* AM1	MW of 200 kDa and containing 30 kDa monomeric subunits flagellin-like protein	Markande et al. (2013)
	Streptomyces	*Streptomyces sp.* S1	Proteins and carbohydrates (82:18)	Kokare et al. (2007)
		Streptomyces sp. MC1	Glycoprotein	Colin et al. (2013b)
	Varovorax	*V. Paradoxus*	Polysaccharides	Franzetti et al. (2012)
Fungi	*Aspergillus*	*A. Niger* MYA 135	Glycolipid	Colin et al. (2010)
	Candida	*C. lipolytica*	Liposan (83% carbohydrate and 17% protein) glycoprotein	Cirigliano and Carman (1985)
		C. tropicalis	Polysaccharide	Shepherd et al. (1995)
		C. utilis	Carbohydrate (79~98%)	Shepherd et al. (1995)
	Geotrichum	*G. sp.* CLOA40		Monteiro et al. (2010)
	Lipomyces	*L. starkeyi*	Polysaccharides	Zhang and Greasham (1999)
	Pichia	*P. anomala*	Polysaccharides	Shepherd et al. (1995)

Rhodospiridium	*R. diobovatum*	Polysaccharides	Shepherd et al. (1995)
Rhodotorula	*R. glutinis*	Carbohydrate-protein complex	Oloke and Glick (2005)
	R. graminis	Polysaccharides	Shepherd et al. (1995)
	R. rubra	Polysaccharides	Shepherd et al. (1995)
Saccharomyces	*S. cerevisiae*	Mannoprotein (90% mannose 5-10% protein)	Cameron et al. (1988)
Trichosporon	*T. mycotoxinivorans* CLA2		de Souza Monteiro et al. (2012)
	T. loubieri CLV20		Monteiro et al. (2010)
	T. montevideense CLOA70		Monteiro et al. (2010)
Yarrowia	*Y. lipolytica*	Yansan: glycoprotain complex	Trindade et al. (2008)

In this study, a bioemulsifier producing bacterium, *Exiguobacterium sp.* strain N4-1P, was isolated from petroleum hydrocarbon contaminated coastal sediment in North Atlantic Canada is reported for the first time. Its 16S rDNA sequence and complete genome sequence was reported. Its phenotypic properties were characterized, and the composition and structure of the produced bioemulsifier was investigated. The produced bioemulsifier was also tested in terms of its emulsification ability with different hydrocarbons and the stability of the formed emulsions under different pH, temperature and salinity conditions.

4.2 Materials and Methods

4.2.1 Screening and isolation

Exiguobacterium sp. strain N4-1P was isolated previously from a coastal sediment sample in the vicinity of a refinery company in Northern Atlantic Canada (Cai et al., 2014). Briefly, approximately 1 g of each sediment sample was first enriched with 50 mL of medium in 125 mL conical flasks. The medium composed of $(NH_4)_2SO_4$, 10 g; NaCl, 15 g; $FeSO_4 \cdot 7H_2O$, 2.8×10^{-4} g; KH_2PO_4, 3.4 g; $K_2HPO_4 \cdot 3H_2O$, 4.4 g; $MgSO_4 \cdot 7H_2O$, 1.02 g; yeast extract, 0.5 g and trace element solution, 0.5 mL L^{-1} of distilled water, with 2% (v/v) *n*-hexadecane as the sole carbon source. The trace element solution contained $ZnSO_4$, 0.29 g; $CaCl_2$, 0.24 g; $CuSO_4$, 0.25 g; $MnSO_4$, 0.17 g L^{-1} and was sterilized separately. The enrichment was shaken at 200 rpm at 30^{o}C for 3-5 days until observable turbidity occurred. Subsequently, the consortia were serial diluted up to10^8 times, spread on agar plates with the enrichment medium and incubated at 30^{o}C for 3-5 days. The emerged colonies were tested with a modified drop collapsing test to screen bacterial colonies with surface

activities (Cai et al., 2014). As a result, the *Exiguobacterium sp.* strain N4-1P was isolated along with 54 other isolates.

4.2.2 Ribosomal DNA sequencing and phylogenetic analysis

The purified isolate was then subjected to 16S ribosomal DNA (rDNA) sequencing using universal bacterial primers F27 and R926 (position in *Escherichia Coli* 8-27 and 926-907, respectively). An aliquot of each culture was used as DNA template in a polymerase chain reaction (PCR) using the primer pair. After gel electrophoresis confirmation of successful PCR reaction, the PCR products were subjected to a clean-up process and were measured by a NanoDrop spectrophotometer to determine the concentrations. Lastly, sequencing reactions with the last PCR products were conducted and measured with Applied Biosystems 3130 and/or 3730 systems in Core Research and Instrument Training Network (CREAIT) at Memorial University. The obtained DNA sequence was matched with Basic Local Alignment Search Tool (BLAST) database. Phylogenetic trees and distances were calculated using the software package MEGA (Molecular Evolutionary Genetics Analysis) version 5.1, after alignment of sequences with CLUSTALX. Distances were calculated using the Kimura two-parameter model (Kimura, 1980). Trees were reconstructed using the neighbor-joining method (Saitou and Nei, 1987) with the use of 'default settings' and the bootstrap values were calculated based on 1000 replications.

4.2.3 Complete genome sequence

Whole-genome shotgun sequencing of *Exiguobacterium* sp. strain N4-1P was performed at the Donnelly Sequencing Center at University of Toronto (Toronto, Canada) using Illumina MiSeq 2500 using 300 cycle Miseq kit V2. This generated a total of 2,260,831 filtered paired-end reads, providing 233-fold coverage of the genome. Quality control was conducted through FastQC (Andrews, 2010). To assemble the data, SPAdes genome assembler was used (Bankevich et al., 2012). plasmidSPAdes was used to assemble plasmids from whole genome sequencing data (Antipov et al., 2016). Subsequently, the assembled contigs was re-ordered by Mauve multiple genome alignment using *Exiguobacterium Antarctica* B7 as the reference genome (Rissman et al., 2009). Gene annotation was performed by using the NCBI prokaryotic Genome Annotation Pipeline (http://www.ncbi.nlm.nih.gov/genome/annotation_prok/) tRNA and rRNA sequences were identified using tRNAscan-SE and RNAmmer, respectively (Lagesen et al., 2007; Lowe and Eddy, 1997).

4.2.4 Bacterial phenotype characterization

BIOLOG® microbial identification and characterization

The BIOLOG® microbial identification system was used to test the utilization of the 95 sole carbon sources simultaneously by *Exiguobacterium* sp. strain N4-1P. The strain was grown on marine agar plates at 30ºC for 24h. The cells were then harvested with loop and transferred to 0.4 M aqueous NaCl solution. After thoroughly mixed with the NaCl solution, the cell density OD_{590} was determined by measuring absorbance at 590 nm with a spectrophotometer and was adjusted to 0.3 ± 0.05 with the NaCl solution. Subsequently, BIOLOG GP2 plate was inoculated with 150 µL of the cell suspension per well. The

inoculated plates were incubated at 30ºC. The results were read visually after incubation for 1 day (Ivanova et al., 1998). The test was duplicated.

Membrane-based phospholipid-derived fatty acid (PLFA) analysis

Cells grown on marine agar plates for 24 h were harvested twice with a 2-mm loop from the third streak of the quadrant-streaked plates. The cells were then transferred in duplicate to reaction tubes with 1 ml of Reagent 1 (150 g NaOH in 1 L of 50% aqueous methanol) for saponification. Samples within the tubes were incubated for 30 min at 100°C in water bath. To methylate liberated fatty acids, 2 mL of Reagent 2 (6 N HCl in aqueous methanol) was added to each tube. Samples within the tubes were incubated again for 10 min at 80°C in water bath. Fatty acid methyl esters (FAME) were extracted from the aqueous phase by the addition of 1.15 mL of Reagent 3 (hexane/methyl *tert*-butyl ether, 1:1, v/v) to each tube. Then samples were rotated end-over-end for 10 min. After removing the aqueous (lower) phase, 3 mL of aqueous 1.2% NaOH (Reagent 4) was added and the tubes were again rotated for 5 min (Sasser, 1990). Finally, the organic (upper) phase containing FAMEs was transferred to a gas chromatography (GC) vial. The FAMEs were quantified by a GC/ mass spectrometry (GC/MS) system (Agilent Technologies 7890A GC connected to an Agilent technologies 5975C inert mass spectroscopy detector) in the NRPOP laboratory. Separation of FAMEs was conducted using an Agilent BD-5MS fused-silica capillary column. The column temperature was programmed from 50ºC to 120ºC at a rate of 10ºC/min, and then to 280ºC at a rate of 3ºC/min. Individual compounds were identified from their mass spectra and from comparing their retention times with the standard spectra. The concentration of each individual compound was determined based

on the GC/MS response relative to that of the known quantitative standards. The test was conducted in duplicate.

Scanning electron microscope (SEM) analysis

Cells grown on marine agar plates for 24 h were harvested twice with a 2-mm loop from the third streak of the quadrant streaked plates, and then were transferred to 15 mL centrifugal tubes. The bacteria were fixed, washed and dehydrated following the methods used by de Sousa et al. (2012). Briefly, bacterial cells were fixed overnight in 2% glutaraldehyde prepared in 50 mM potassium phosphate buffer. The fixed cells were washed three times with phosphate buffer and dehydrated using an increasing gradient of acetone in distilled water (30%, 50%, 70%, 80% and 90%, respectively) for 10 min each and finally in 100% acetone for 30 min and air dried. The specimens were sputter-coated with gold using an auto fine coater and visualized using SEM (FEI MLA 650F). The coating process and the SEM visualization were conducted in the Micro Analysis Facility (MAF IIC) at CREAIT.

4.2.5 Bioemulsifier production with different carbon sources

A serial of production media composed of essentially the enrichment medium and four different carbon sources were tested, respectively, and compared. Three different hydrocarbon sources were applied, namely, hexadecane, diesel and benzoate. The fourth carbon source was a non-hydrocarbon one which was a mixture of 0.05% starch, 0.02% glucose, 0.02% sucrose and 0.1% citrate salt. After 7 days of incubation, the culture broths were centrifuged at 12,000 rpm for 5 min to remove cells and precipitates. The surface

tension and emulsification index (E24) of the culture broth were tested (Cai et al., 2015a). The clear broths were concentrated to approximately 1/5 of the volume with lyophilisation, and then were mixed with three volumes of cold acetone. Subsequently, the solution was stored at 4^{o}C for 3 days to fully precipitate the crude bioemulsifier products. The supernatant was removed by centrifugation, and the pellet was washed twice with acetone to remove the hydrocarbon residues. Finally, the pellet was finely ground and dried first in a fume hood, and then in dissectors. When constant weight was obtained for each sample, the yield was then determined. The medium with the highest yield, E24 and lowest surface tension in the broth was selected for further characterization.

4.2.6 Composition analysis of the bioemulsifier

The crude bioemulsifier was subjected to a dialysis process with 1kDa MW cut dialysis tubes to remove salts and small compounds. The crude bioemulsifiers before and after dialysis were subjected to protein content determination (Bradford, 1976), total lipid content determination (Pande et al., 1963) and total carbohydrate analysis (Dubois et al., 1956).

Further analysis of the fatty acid composition of the lipid content was conducted. The lipid content was extracted according to Parrish (1999). The extracted samples were homogenized in a 2:1 mixture of ice-cold chloroform: methanol with a Polytron PCU-2-110 homogenizer (Brinkmann Instruments, Rexdale, Ontario, Canada). The chloroform extracted aqueous layer was added to the sample to bring the ratio of cholorform:methanol:water to 8:4:3. The sample was sonicated for 4 to 10 minutes in an ice bath and centrifuged at 5000 rpm for two minutes. The bottom organic layer was

removed. Chloroform was then added back to the extraction test tube and the entire procedure was repeated 3 more times. All liquids located in the organic layers were pooled into a lipid-free vial. The sample was concentrated using a flash-evaporator (Buchler Instruments, Fort Lee, N.J.). The lipid extract was trans-esterified using methanol/sulfuric acid for 1 hour at 100^{o}C. The FAMEs were analyzed on a HP 6890 GC/Flame Ionized Detector (GC/FID) equipped with a 7683 Autosampler. The column temperature began at 65^{o}C and was held at this temperature for 0.5 minutes. The temperature was then ramped to 195^{o}C at a rate of 40^{o}C/min, held for 15 minutes, and finally ramped to a temperature of 220^{o}C at a rate of 2^{o}C/min. This final temperature was held for 0.75 minutes. Peaks were identified using retention times from standards purchased from Supelco, namely 37 component FAME mix, Bacterial acid methyl ester mix, polyunsaturated fatty acids (PUFA) 1 and PUFA 3. The tests were conducted in triplicate.

Fourier transform infrared (FT-IR) spectroscopy analysis of the bioemulsifier was conducted on a Bruker Alpha with KBr disc. A ninhydrin-based assay was applied to quantify amino acids and peptides using tryptophan as the standard (Starcher, 2001).

4.2.7 Emulsification ability of the bioemulsifier

The emulsification index (E24) was used for performance evaluation. The bioemulsifier solutions were mixed with equal amount of diesel and vortexed for 2 min. After settling for 24 hours, E24 was determined by the fraction height of the emulsion layer out of the total height of the mixture (Cai et al., 2015). The emulsification abilities of the bioemulsifier solution, against different hydrocarbons including hexadecane, mineral oil, crude oil, diesel, xylene, toluene, dodecane, and iso-octane were tested. The effects of

concentrations of bioemulsifiers on the emulsification ability were also measured. The emulsification stability under different salinity (levels of 0, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20 and 25%), temperature (-20, 0, 4, 30, 50, 70 and 100 ^{o}C), and pH conditions (2, 4, 6, 8, 10 and 12) were tested. The tests were duplicated.

4.3 Results and Discussion

4.3.1 Ribosomal DNA sequencing and phylogenetic analysis

The PCR reaction obtained almost full length (1,500 bp) 16S rDNA fragments for the isolate. The five replicates of the sequencing results agreed well with one another. *Exiguobacterium sp.* strain N4-1P (GenBank Accession number: KX714225) was closely related to *E. oxidotolerans* strain T-2-2^{T} with 99.47% similarity; to *E. antarcticum* B7 ^{T} with 98.70% similarity; and to *E. sibiricum* 255-15^{T} with 98.63% similarity. The phylogenetic tree of *Exiguobacterium sp.* strain N4-1P and the closely-related type strains are shown in Figure 4.1. The genus *Exiguobacterium* consists of gram positive, facultative anaerobes with low G+C (Vishnivetskaya et al., 2009). *Exiguobacterium* species isolated from diverse habitats over a wide temperature range (-12 to 55°C), such as glacial ice, hot springs, Siberian permafrost, and tropical soils, (Carneiro et al., 2012). *Exiguobacterium* strains possess interesting properties such as temperature acclimation proteins and enzymes which have potential applications in food industry, environmental remediation, and in the pharmaceutical industry (Carneiro et al., 2012; Vishnivetskaya et al., 2009). However, genomic investigations of *Exiguobacterium* sp. have been limited (Vishnivetskaya et al., 2009).

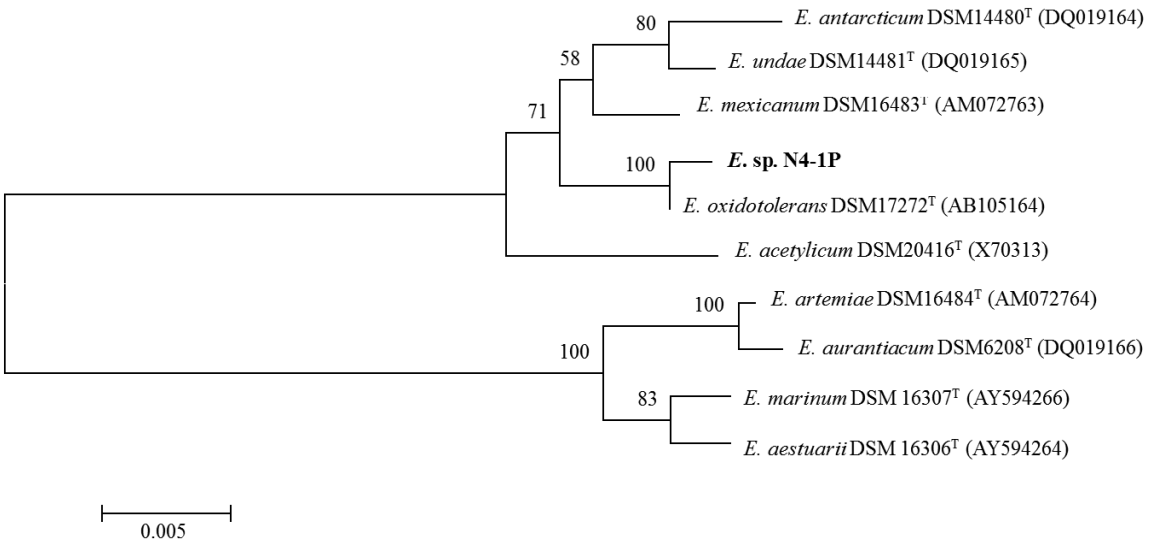


Figure 4.1 Phylogenetic tree based on 16S rRNA gene sequences. (Note: The tree was constructed using the NJ method. The numbers at nodes represent bootstrap values (based on a resampling of 1000). Bootstrap values >70 % are indicated. The GenBank accession numbers for the 16S rRNA gene sequences of all the strains are listed in parentheses. Bar, 0.01 nucleotide substitutions per site

As shown in Figure 4.2, bioemulsifier producers have been found in both *Eubacteria* and *Archaea* in the division of *Proteobacteria*, *Firmicutes, Actinobacteria, Cytophage-Flexibacter-Bacteroides*, and *Euryarchaeota*. The 16S rDNA sequences of *Exiguobacterium sp.* strain N4-1P and the published producers with the nearly full length 16S rDNA genebank deposits were clustered and aligned. *Exiguobacterium sp.* strain N4-1P isolated in this study belongs to the *Firmicutes* divisions. Bioemulsifiers is a unique group of biosurfactants. Most well-identified biosurfactants were recognized based on surface/interfacial tension reduction while bioemulsifiers are exceptional due to their strong emulsion stabilizing abilities. Relatively, very little research has been reported on the discovery and characterization of new biodemulsifiers when compared with that of surface/interfacial tension reducing biosurfactants (Uzoigwe et al., 2015).

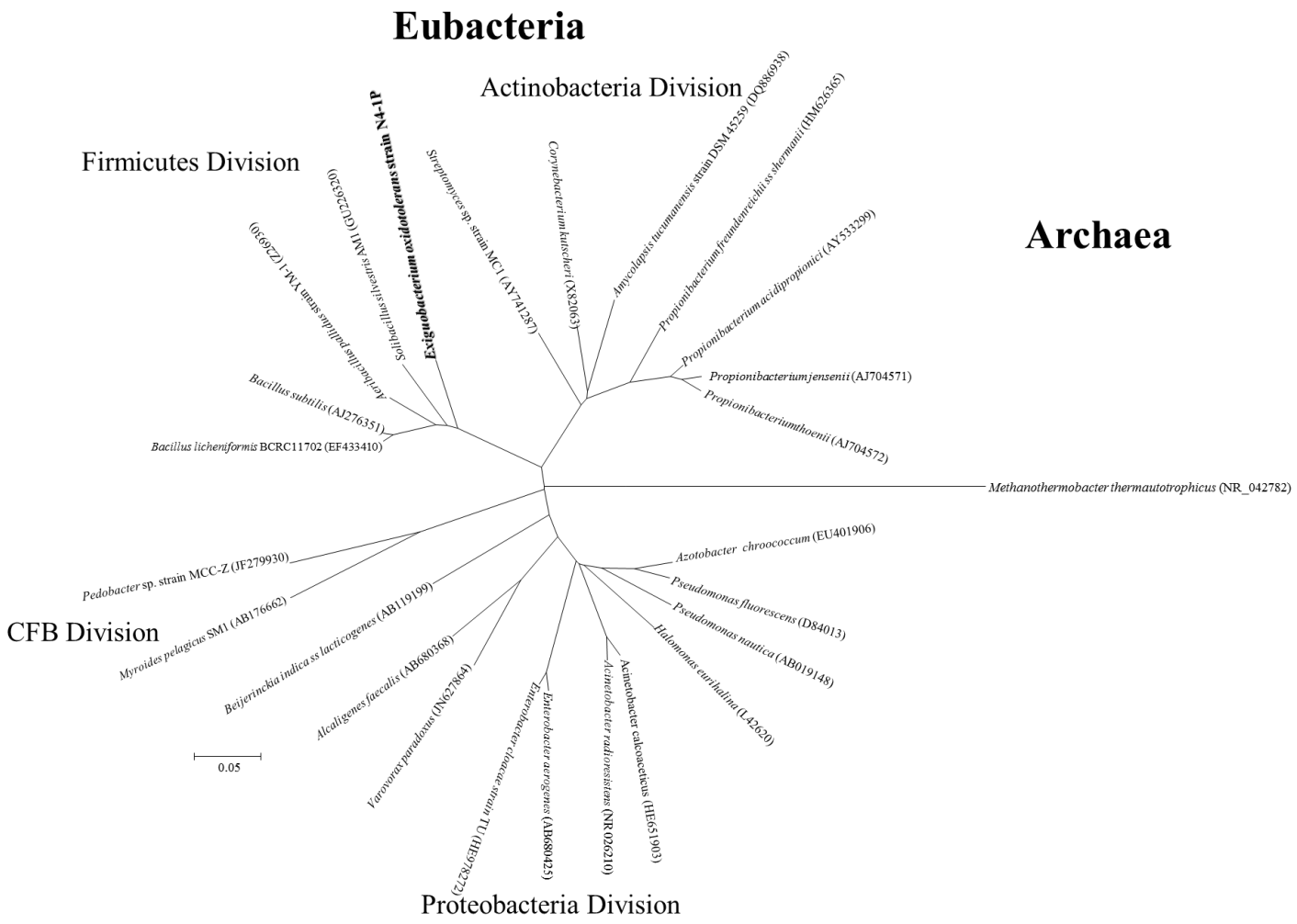


Figure 4.2 Phylogenetic tree based on 16S rDNA sequence from representative biosurfactant producers isolated in this study and those published in literature. CFB=Cytophage-Flexibacter-Bacteroides

4.3.2 Complete genome sequence

A chromosome type replicon (3,032,448 bp), with G+C content of 46.8%, made of 54 contigs/scaffolds was obtained for *Exiguobacterium* sp. strain N4-1P, which harbors 3,112 coding sequences, including 2,989 proteins with identified functions. Eleven rRNA operons and 67 tRNAs were also annotated. The plasmids, containing a total of 702 coding sequences and sizes of 130,902 bp, 406,323 bp, 64,131 bp, 5,498 bp and 4,905 bp, respectively, were obtained. The G+C contents of the plasmids range from 36.6% to 37.6%. As expected, several cold shock proteins (Csp) are found in both chromosome and the second plasmids, explaining the cold adaptability of the strain. Diverse mono- or di-oxygenases, dehydrogenases and cytochromes involved in aerobic hydrocarbon biodegradation pathway are found in the genome, explaining its ability to degrade hydrocarbons. Several lipoproteins, lipopolysaccharide, polysaccharide biosynthesis genes are presented in the genome linking to its ability to produce bioemulsifiers.

This is the 9th draft genome for the genus *Exiguobacterium* but the only one isolated from petroleum hydrocarbon contaminated marine sediment samples. It will provide a reference for many further phylogenetic, comparative genomic, metagenomic, and functional studies of this extremophilic genus. This whole-genome shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession no. CP022236-CP022241.

4.3.3 Phenotypic properties

According to BIOLOG® metabolic fingerprinting results, *Exiguobacterium* sp. N4-1P could use 32 different carbon sources out of 95 on the GP2 plates. These include are 14 out of the 36 carbohydrates (cellobiose, fructose, glucose, sucrose, maltose, mannitol,

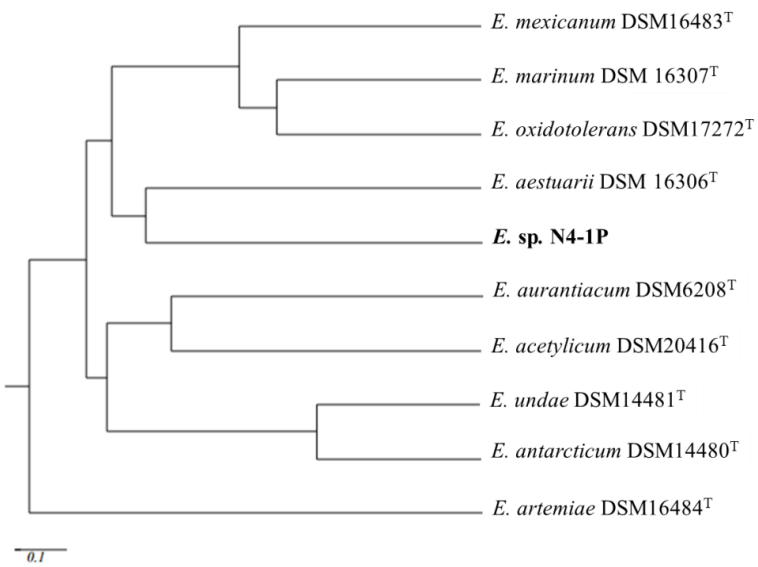
mannose, methyl-D-glucoside, trehalose, dextrin, maltotriose, palatinose, d-ribose and pslcose); 3 out of the 16 carboxylic acids (acetic acid, d-gluconic acid and α-ketovaleric acid); 2 amides among the 4 amides(*N*-acety-D- galactosamine and *N*-acetyl mannosamine); 2 polymers (Tween 40 and 80); 2 out of the 3 esters (methylpyruvate and methylsuccinate); 2 among the 7 alcohols (d-Sorbitol and glycerol); 6 out of 10 aromatic chemicals could be used:(arbutin, amygdalin, adenine, 2'-deoxy adenosine inosine, thymidine and uridine); and 1 out of the 9 amino acids (l-serine). No amines, phosphorylated chemicals, brominated chemicals included in the GP2 plates could be used as sole carbon sources for this strain. The strain is able to utilize the 10 substrates that other types strains in this genus can use, which defined the *Exiguobacterium* species (López-Cortés et al., 2006). The carbon source utilization differentiation within the genus is shown in Table 4.2. The dissimilarities among the *Exiguobacterium* strains are illustrated in Figure 4.3. The N4-1P strain was found to be closely related to *E. aestuarii* DSM 16306^T and *E. oxidotolerans* DSM17272^T with Pearson coefficients of 0.309 and 0.260, respectively, in carbon utilization.

Table 4.2 BIOLOG® metabolic profile

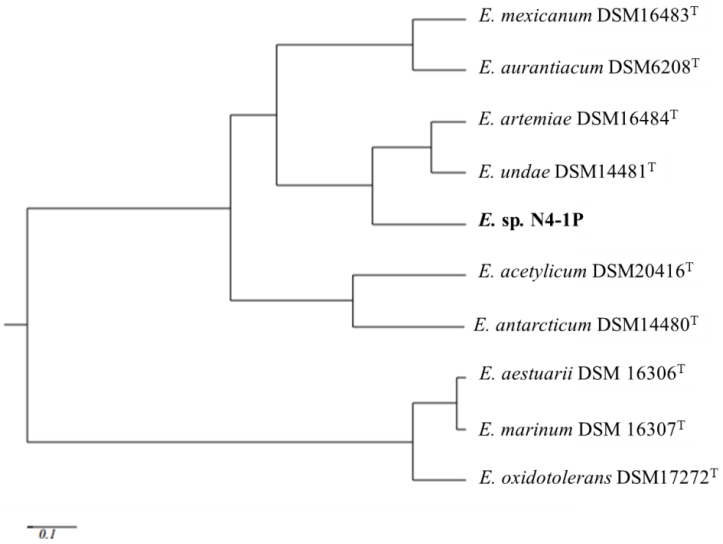
Characteristics	*E. mexicanum* DSM 16483^T	*E. aurantiacum* DSM 6208^T	*E. aestuari* DSM 16306^T	*E. marinum* DSM 16484^T	*E. artemiae* DSM 16484^T	*E. acetylicum* DSM 20146^T	*E. undae* DSM 14481^T	*E. antarcticum* DSM 14480^T	*E. oxidotolerans* DSM 17272^T	*E.* sp. N4-1P
α-Cyclodextrin	+	-	+	+	+	-	-	-	+	-
β-Cyclodextrin	+	-	+	+	+	-	-	-	+	-
Glycogen	+	-	+	+	+	+	+	+	+	-
Mannan	-	+	-	+	-	+	w	w	+	-
N-*Acetyl* mannosamine	+	-	+	w	-	-	w	w	-	w
Cellobiose	-	-	-	w	+	+	+	+	+	+
d-Galactose	-	-	-	-	-	-	+	+	+	-
d-Gluconic acid	-	-	-	w	+	-	-	-	-	w
d-Mannitol	-	-	+	+	+	+	+	-	+	+
3-Methyl glucose	-	+	+	+	+	+	+	+	w	-
d-Raffinose	-	-	+	-	-	-	+	+	+	-
d-Ribose	+	-	+	+	+	-	+	+	+	w
Salicin	-	-	+	+	+	-	-	-	-	-
d-Sorbitol	+	-	+	+	+	+	-	-	+	w
Turanose	+	-	-	-	+	-	-	-	-	-
d-Xylose	+	-	-	-	-	-	-	-	-	-
Acetic acid	+	-	+	+	+	w	+	+	+	w

γ-Hydroxy-butiric acid	-	-	+	+	-	-	w	w	-	-
α-ketovaleric acid	+	+	+	+	-	+	+	+	+	+
d-Lactic acid methyl ester	-	-	-	-	+	-	-	-	-	-
l-Lactic acid	+	-	-	-	-	-	-	-	-	-
Methylpyruvate	+	+	+	-	-	-	-	-	+	w
Methylsuccinate	-	-	-	-	-	-	w	w	-	w
Propionic acid	+	-	+	+	-	-	-	w	+	-
d-Alanine	-	-	-	-	+	-	-	-	-	-
l-Alanine	-	-	-	-	+	+	w	w	-	-
l-Alanyl glycine	-	-	-	-	-	w	-	+	+	-
l-Glycyl glutamic acid	-	-	-	-	-	-	-	+	-	-
l-Serine	-	-	-	w	-	w	-	w	-	w
2,3-Butanediol	-	-	+	+	-	-	w	+	-	-
Thymidine	+	+	+	+	-	+	+	+	+	+
Adenosine 5′-monophosphate	-	-	-	+	-	-	-	+	-	-
Thymidine 5′-monophosphate	-	-	-	+	+	-	w	+	-	-
Uridine 5′-monophosphate	-	-	-	+	-	-	-	+	-	-

Fructose 6-phosphate		-	-	-	w	-	+	-	-	-	-
Glucose 1-phosphate		-	-	-	-	-	+	-	-	-	-
Glucose 6-phosphate		-	-	-	-	-	+	-	-	-	-
dl-α-Glycerol Phosphate		-	-	-	-	-	+	-	-	-	-
Tween 40		-	-	-	+	-	-	-	-	-	+
Tween 80		-	-	-	+	-	-	-	-	-	+
Reference		López-Cortés et al. (2006)	Frühling et al. (2002)	Kim et al. (2005)	Kim et al. (2005)	López-Cortés et al. (2006)	López-Cortés et al. (2006)	Frühling et al. (2002)	Frühling et al. (2002)	Yumoto et al. (2004)	This study



(A)



(B)

Figure 4.3 Phenogram obtained from the carbon source utilization and membrane PLFA composition of the ten *Exiguobacterium* strains based on the UPGMA method (unweighted pair-group method using arithmetic averages) after measuring similarity/dissimilarity among the strains using the coefficient of simple matching, which considers the same weight positive and negative similarities

The membrane PLFA composition is shown in Tables 4.3. The composition was found to be corresponded well with other members of the *Exiguobacterium* genus. Terminally branched-chain fatty acids are biomarkers of gram positive bacteria (White et al., 1996). For *Exiguobacterium* sp. N4-1P, the primary PLFAs were iso-C15:0 (15.52%), iso-C17:0 (13.64%), C16:0 (12.51%), anteiso-C13:0 (11.83%), C16:1 (11.4%) and iso-C13:0 (8.92%). This pattern is very close to *E. artemiae* DSM16484^T (Vishnivetskaya et al., 2009) and *E. undae* DSM14461^T (Frühling et al., 2002), which further demonstrated the assignment of the N4-1P stain as an *Exiguobacterium* (Figure 4.3B). The SEM visualization results are shown in Figure 4.4. The *Exiguobacterium* sp. N4-1P was rod-shaped with a length of 2-3 μm and diameter of around 0.5 μm after 48 hours of incubation. Growing on the marine agar, the colonies were circular, entire, convex and had orange/yellow pigment. These morphological properties are similar to other members of the genus (López-Cortés et al., 2006; Vishnivetskaya et al., 2009).

Table 4.3 PLFA composition of *Exiguobacterium* sp. N4-1P and relevant strains (Unit: %, n=3)

Fatty acids	*E. mexicanum* DSM 16483^T	*E. aurantiacum* DSM 6208^T	*E. aestuari* DSM 16306^T	*E. marinum* DSM 16484^T	*E. artemiae* DSM 16484^T	*E. acetylicum* DSM 20146^T	*E. undae* DSM 14481^T	*E. antarcticum* DSM 14480^T	*E. oxidotolerans* DSM 17272^T	*E.* sp. N4-1P
iC11:0	1.5	2	0	0	0	0	0	0	0	0
iC12:0	2.1	3	1.7	2.6	1.6	0	2	3	1.4	2.7
C12:0	8.3	2	0	0	0	1	0	1	0	0
iC13:0	**11.2**	**18**	**11.5**	**11.5**	**13.2**	5	9	**12**	8.5	8.9
aC13:0	8.9	**12**	**15.6**	**18.1**	**12**	6	9	**11**	9	**11.8**
iC14:0	0	0	1.3	0	1.2	1	2	1	2.7	2.3
C14:0	6.1	3	0	0	1.3	**13**	3	2	0	2.3
C14:1 ω5c	0	0	0	0	0	2	0	0	0	0
iC15:0	1.7	4	**13.1**	**10.4**	**11.8**	8	**10**	**11**	**20.7**	**15.5**
aiC15:0	0	0	3.2	2.6	2.9	1	3	2	4.2	5.1
iC16:0	0	0	7.1	5	1.4	0	2	0	7.1	0
C16:1	16.8	10	0	0	4.5	41	8	21	0	**11.4**
C16:0	**32.8**	**27**	5.3	4.3	**22.9**	**10**	**17**	**13**	2.9	**12.5**
iC17:0	0	0	**27.2**	**34.4**	**12.2**	1	7	5	**23.3**	**13.6**
aiC17:0	0	6	8.2	7.1	2.1	1	2	0	6.1	5.6
C18:1	0	2	0	0	1.1	7	6	6	0	**0**
C18:0	7	5	1.7	0	7.7	1	6	5	0	4.82

Reference	López-Cortés et al. (2006)	Frühling et al. (2002)	López-Cortés et al. (2006)	Kim et al. (2005)	Kim et al. (2005)	López-Cortés et al. (2006)	Frühling et al. (2002)	Frühling et al. (2002)	Yumoto et al. (2004)	This study

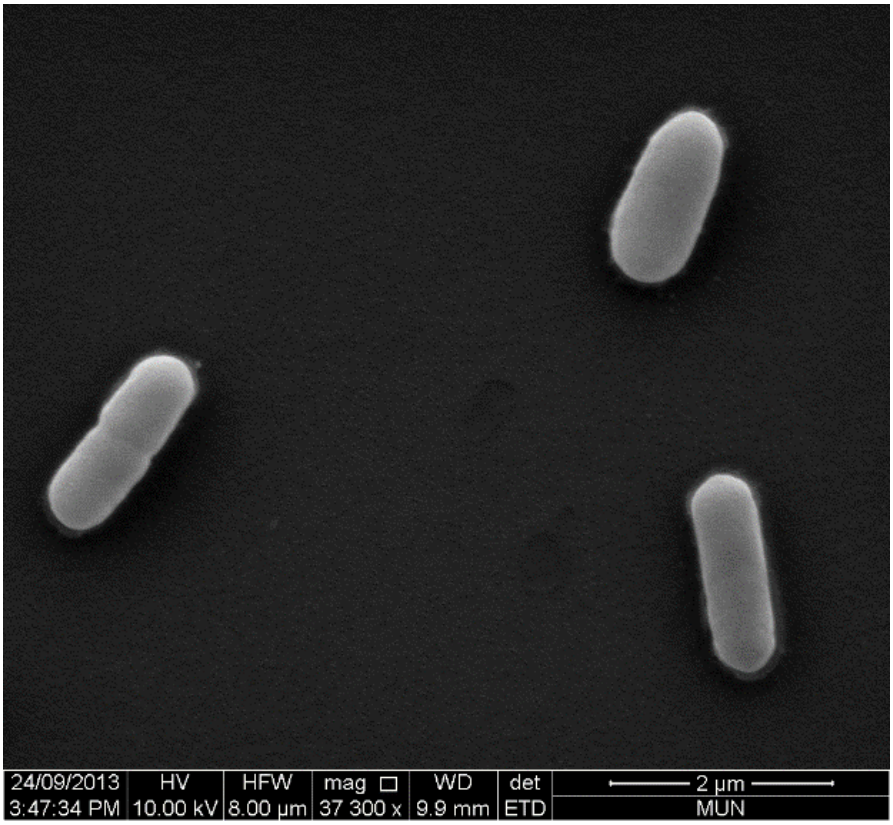


Figure 4.4 SEM visualization results of *Exiguobacterium* sp. N4-1P

4.3.4 Bioemulsifier production with different carbon sources

The general properties of the produced bacterial broth are summarized in Figure 4.5. Hexadecane is a low toxicity water immiscible hydrocarbon. Diesel is composed of diverse hydrocarbons (mostly water immiscible) with moderate toxicity. It is an economic production substrate when compared with hexadecane and benzoate (Makkar and Cameotra, 2002). However, its toxicity could hinder the production to some extent (Sadouk et al., 2008). Benzoate is a water-soluble hydrocarbon with moderate toxicity. It was found to stimulate surface-active compound production for some other biosurfactant producers (de Sousa and Bhosle, 2012), while eliminating the concerns of immiscible mixture during the post-processing and testing stages. The addition of all three types of hydrocarbons promoted the production of the emulsion-stabling bioemulsifer. The emulsification ability of the broths followed a sequence of diesel > hexadecane > benzoate. In terms of the yield, addition of hydrocarbon significantly increased the yield for the strain, and diesel addition generated the highest yield of the crude bioemulsifier. Interestingly, the addition of diesel produced bacterial broth can reduce surface tension significantly, indicating the simultaneous production of lower-molecular-weight biosurfactants capable of reducing surface tension (Cai et al., 2015a). The obtained results indicated that the strain thrived with the moderate toxicity inherent with diesel. As a relative economic substrate (~$0.9/L), diesel has the potential for pilot to large scale production. Further testing on waste based substrates will be included in future studies to achieve economic production.

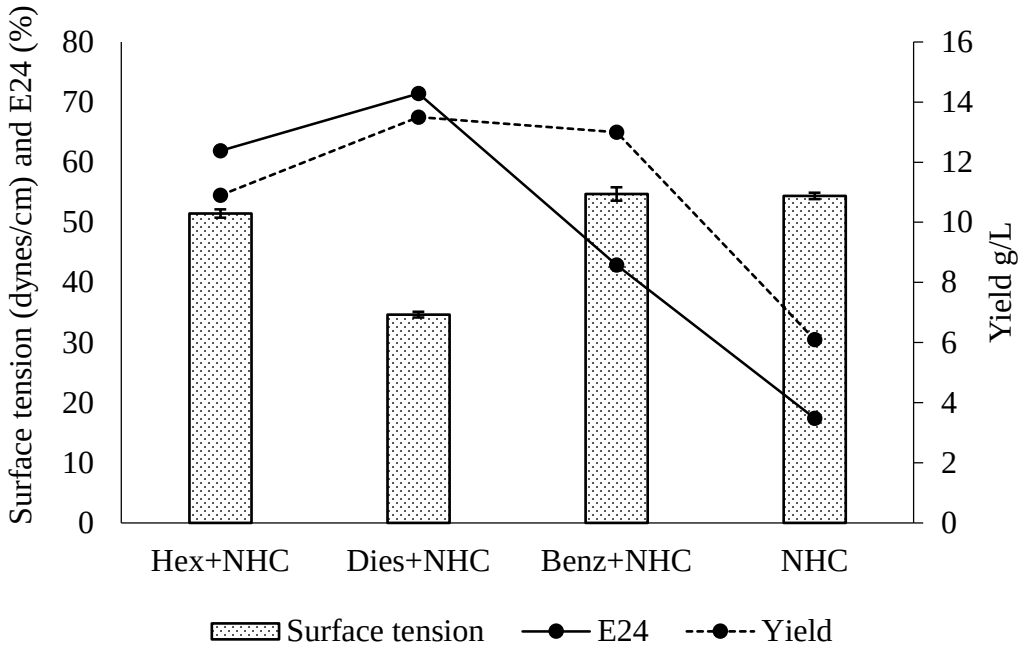


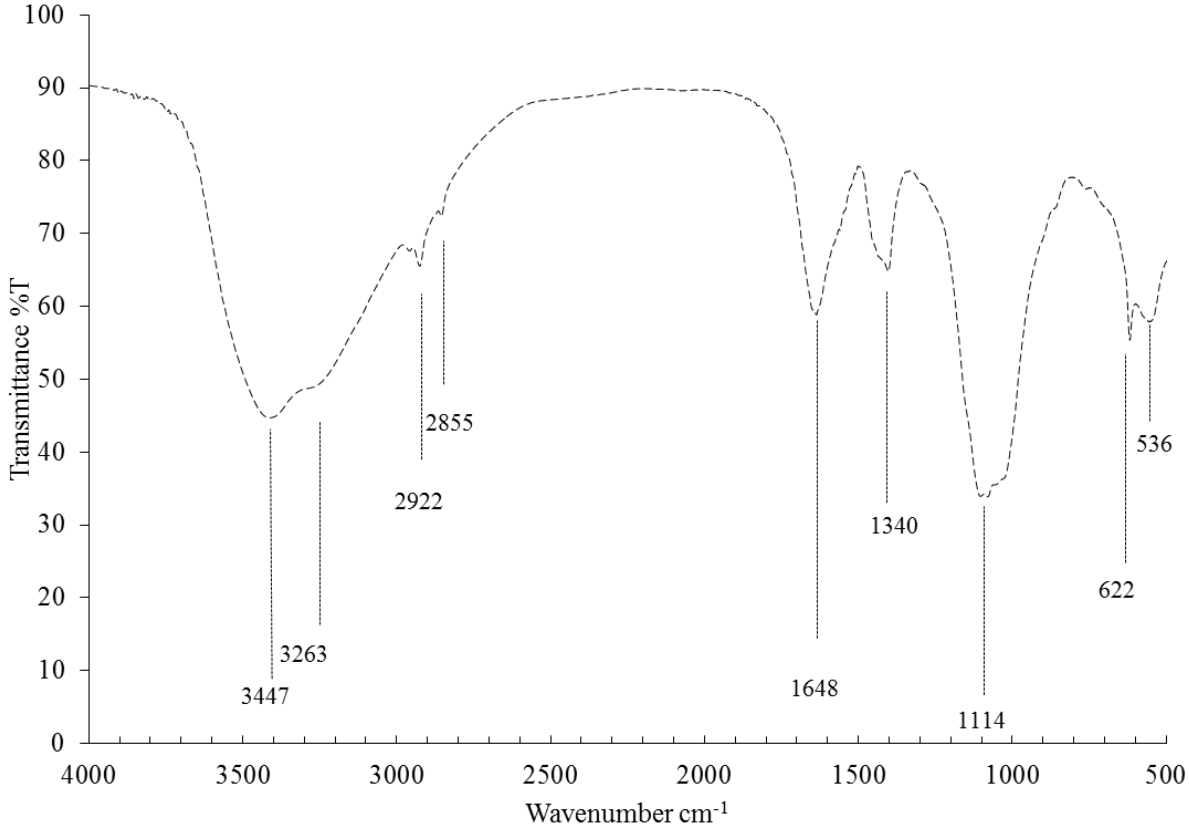
Figure 4.5 General properties of the clear broths obtained with different carbon sources (Hex=hexadecane, Dies=Diesel, Benz=Benzoate, NHC=non-hydrocarbon carbon sources; n=3)

4.3.5 Composition of the crude bioemulsifier

The bioemulsifier before dialysis consist of 14.64% lipid, 0.14% carbohydrate and 0.37% protein per the results of the colorimetric tests. After dialysis, the composition changed to 45.32% lipid, 5.1% carbohydrate and 0.93% protein. Eighteen fatty acids (FA) out of the 70 tested ones were detectable (Table 4.4). Results showed that the hydrophobic moiety of the bioemulsifiers was primarily saturated FA (77.14%). Among which, C16:0 (32.18%) and C18:0 (40.99%) were the most abundant FA in the hydrophobic moiety. Unsaturated FA were mainly composed of monounsaturated FA (14.88) while polyunsaturated fatty acids only constituted 0.5% of total fatty acids. The hydrophobic moiety dominated by long chain FA (C16 and C18) in the bioemulsifer molecule as shown in this study is quite unique and seldom reported. The long chain FA (C16 and C18) might represent a significant improvement of emulsification activity Johri et al. (2002). The FT-IR spectrum indicates the presence of carboxylic groups (3600-2600 cm^{-1}, carboxyl -OH stretch; 1648 cm^{-1}, carboxyl -C=O stretch; 1114 cm^{-1}, carboxyl -C-O stretch) and alkyl groups (2922 cm^{-1} and 2855 cm^{-1}, alkyl C-H stretch; 1340 cm^{-1}, alkyl -C-H bending) (Figure 4.6). The ninhydrin-based total amino acids/peptides test showed that the amino acids/peptides accounted for 13.11% before dialysis and 50.82% after dialysis (weight calibrated as tryptophan). When compared with the Bradford test results, the ninhydrin-based tests gave much higher readings. The Bradford test only targets proteins but not smaller peptides. The results indicate the obtained bioemulsifier is mainly a complex of lipopeptides with small fraction of glycolipids.

Table 4.4 Composition of the bioemulsifiers produced by *Exiguobacterium* sp. N4-1P (n=3)

Composition	%	Hydrophobic moiety fatty acids	%
Protein	0.93	C14:0	2.58
Amino acids/peptides	50.82	C14:1	3.73
Carbohydrate	5.1	iC15:0	2.38
Lipid	45.32	aiC15:0	1.07
		C15:0	0.27
		C15:1	3.05
		iC16:0	0.63
		C16:0	32.18
		C16:1w9	0.46
		C16:1w7	0.30
		iC17:0	2.00
		aiC17:0	1.40
		C17:0	1.13
		C18:0	40.99
		C18:1w9	4.45
		C18:1w7	1.42
		C18:3w3	0.50
		C22:1w9	1.48
		∑Saturated	84.63
		∑Monounsaturated	14.88
		∑polyunsaturated	0.50
		∑Branched	7.64



Wavenumber cm^{-1}	Functional group and type of vibration
3600-2600	Alcohol O-H stretch and amine N-H stretch
2922 and 2855	Alkyl C-H stretch
1648	Carboxyl C=O stretch
1340	Alkyl -C-H bending
1114	Carboxyl C-O stretch

Figure 4.6 FT-IR spectrum of the bioemulsifier

4.3.6 Performance of the bioemulsifier

Emulsification ability of the bioemulsifier with different hydrocarbons is shown in Figure 4.7. The experiment was conducted in parallel with a chemically-synthesized emulsifier, sodium dodecyl sulfate (SDS). The E24 was above 60% when using the single component aliphatic and aromatic hydrocarbons. With aromatic hydrocarbons, the bioemulsifier generally has slightly higher values of emulsification efficacy. With mixed hydrocarbons (mineral oil, crude oil and diesel), the E24 varied. High E24 values were observed with mineral oil and diesel. With crude oil, the E24 values were slightly lower than those of other hydrocarbons. The emulsification ability of the bioemulsifer is generally comparable with that of SDS. Since the bioemulsifier was capable of effectively emulsifying both aromatic and aliphatic hydrocarbons, it could be used for hydrocarbon remediation and oil recovery (Ilori et al., 2005). The bioemulsifier produced by *Exiguobacterium* sp. N4-1P showed strikingly no-foaming characteristics even at high concentration (10%). This property would eliminate the cost of chemical defoament, sterilized pulse addition, and foam level detection during fermentation which is inevitable with common emulsifiers. In addition, the no-foaming surface-active agents were also found to have wider applications as wetting agents, rinse agents, and soil washing solutions. (Hirata et al., 2009). This is because the above processes rely largely on the scrubbing effect of vigorous jets or sprays of liquid which would be rendered relatively ineffective if cushioned by the action of large amounts of foam. Moreover, the foam formation will largely reduce the amount of effective liquids during applications (Hirata et al., 2009).

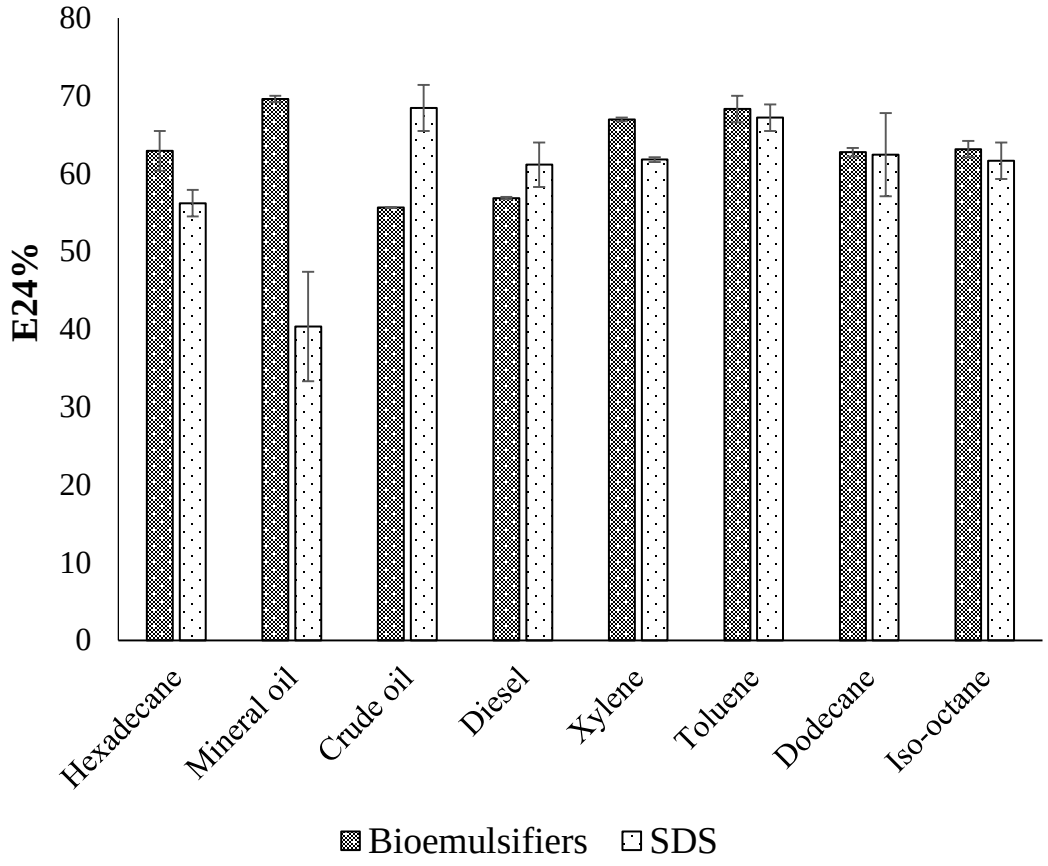


Figure 4.7 Emulsification ability of *Exiguobacterium* sp. N4-1P with different hydrocarbons (n=2)

As shown in Figure 4.8, the E24 of the bioemulsifiers stabilized water-diesel emulsion increased when the concentrations of the bioemulsifiers increased till they reached the plateau. Before dialysis, the concentration required to reach the plateau was 15 g/L. After the dialysis, the concentration required was reduced to 3 g/L. Dialysis substantially improved the unit effectiveness of the bioemulsifier product. The formed oil-in-water emulsion under microscope displayed as Oil Red O-dyed oil droplets surrounded by continuous water phase with size ranging from a few microns to around 100 microns (Fig. 7). The bioemulsifier was also found able to reduce surface tension of water from 71 dynes/cm to 44.9 ± 0.26 dynes/cm. The abilities of the produced bioemulsifier to stabilize oil-in-water emulsion and reduce surface tension enables its application as a soil washing agent to remove subsurface hydrocarbon plumes (Cai et al., 2015a; Pacwa-Płociniczak et al., 2011). Moreover, such abilities also lead to improved bioavailability of the hydrocarbon containments, thus enhance the bioremediation process (Ron and Rosenberg, 2014).

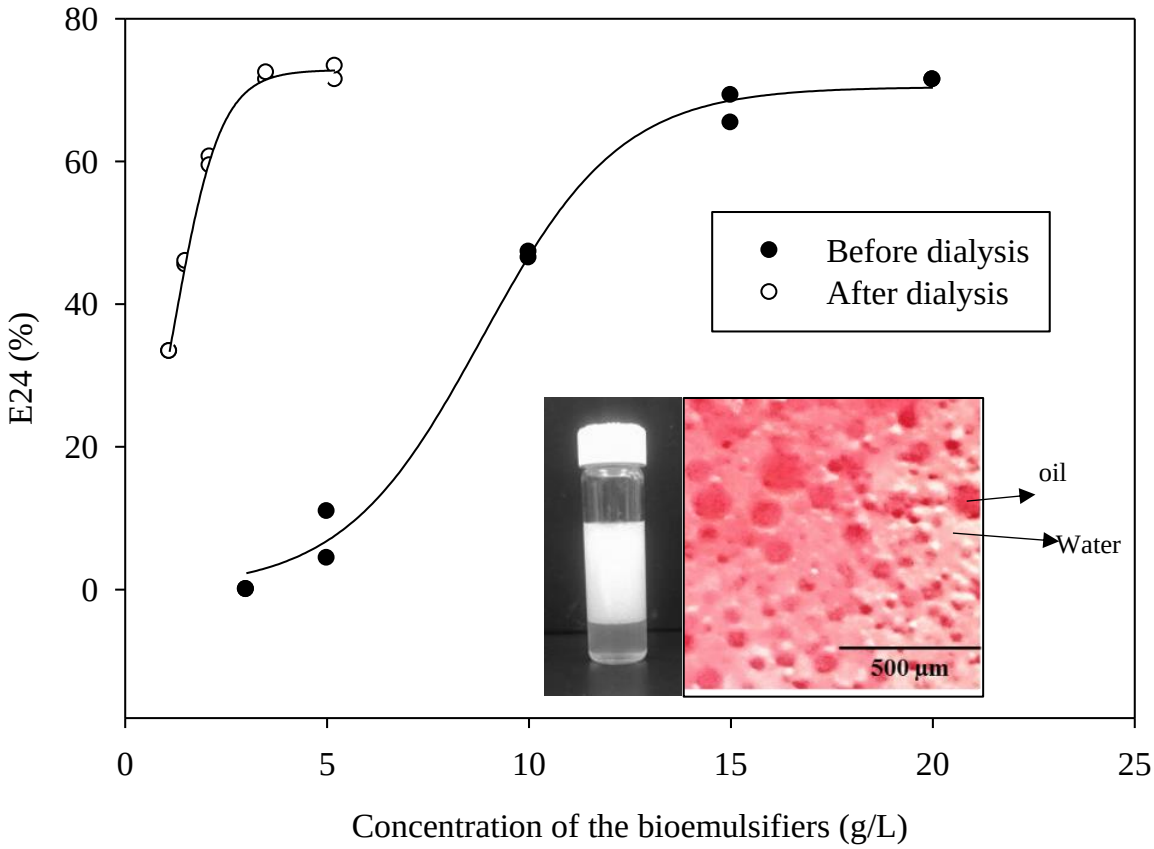


Figure 4.8 Effectiveness of the emulsifier at different concentrations (n=2; insert: optical microscopy of cream phase of emulsions after 24 h, oil phase was dyed with Oil Red O)

As shown in Figure 4.9, the water-diesel emulsion formed with the bioemulsifier was very stable with salinity from 0 to 25%. Under acidic conditions, higher E24 values suggesting the bioemulsifiers are more effective. The temperature had significant effects on destabilization of the emulsion. When temperature was increased up to 50^{o}C, the emulsion started to collapse. At 100^{o}C, only around 20% emulsion remained. However, the emulsion was very stable and the E24 readings were the highest at or below 10^{o}C (>70%). *Exiguobacterium* sp. N4-1P is a cold-adapted bacterial strain isolated from a petroleum hydrocarbon contaminated sample in North Atlantic Canada. It, therefore, easily adapts to and thrives in the cold environment with abundant hydrocarbons. The produced bioemulsifier could perform excellent under low temperature as a soil washing agent and/or a bioremediation enhancement agent. The crude bioemulsifier when stored in an air-tight container under room temperature remained the same level of effectiveness after more than a year of storage (data not shown).

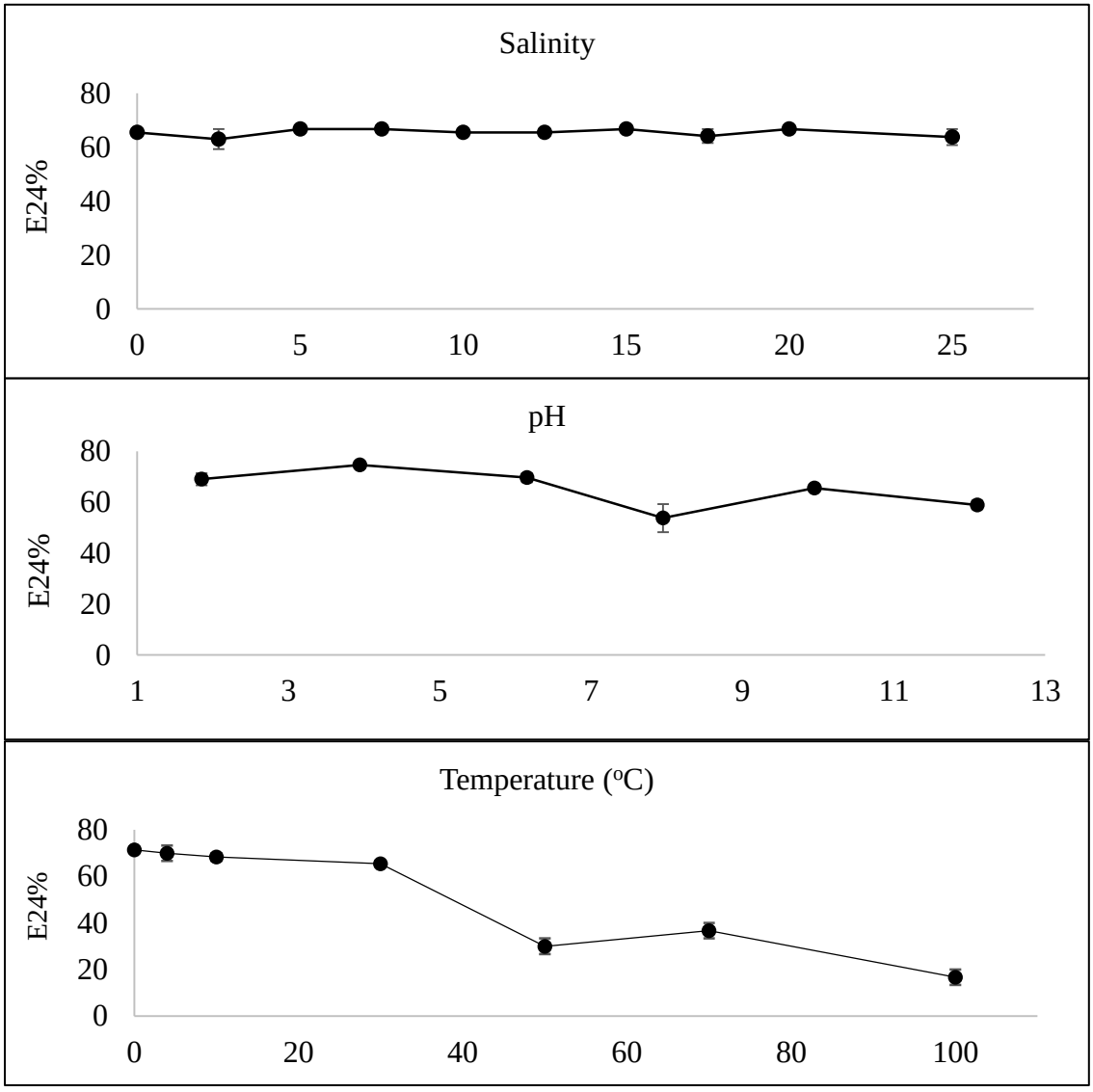


Figure 4.9 Stability of the emulsion under different salinity, pH and temperature conditions (n=2)

4.4 Summary

Exiguobacterium N4-1P is reported herein as a bioemulsifier producer for the first time. The genotypic and phenotypic properties of the strain were determined. Different carbon sources were used for bioemulsifier production and diesel stimulated the yield. The bioemulsifier is a complex mainly consist of lipopeptides with C16:0 (32.18%) and C18:0 (40.99%) as the primary FA. The produced bioemulsifier could form emulsions effectively with diverse hydrocarbons without foam generation, which facilitates the potential commercialization. The formed oil-in-water emulsions were stable upon a wide span of salinity (5-25%), pH (2-12), and temperature (below 50^{o}C). *Exiguobacterium* N4-1P and the produced bioemulsifier have promising application potential in environmental engineering, especially for soil washing and bioremediation targeting subsurface hydrocarbon pollution.

CHAPTER 5 NOVEL OIL-IN-WATER EMULSION BREAKING MARINE BACTERIA FOR DEMULSIFYING OILY WASTEWATER

This chapter is based on and expanded from the following paper (s)/manuscript (s):

Cai, Q., Zhu, Z., Chen, B., Zhang, B. (2018) Novel oil-in-water emulsion breaking marine bacteria for demulsifying oily wastewater. *Water Research (revised version submitted)*

Roles: Cai designed and conducted the study under the guidance of Dr. Baiyu Zhang and Dr. Bing Chen and acted as the first author of the manuscript. Zhiwen Zhu participated in the experiments. Most contents of this paper were written by Cai and further polished by the other co-authors.

Cai, Q., Ye, X., Chen, B., Zhang, B. (2018) Complete genome sequence of an oil degrading, oil-in-water emulsion breaking bacterium *Halomonas venusta* strain N3-2A. *Marine genomics (ready for submission)*

Roles: Cai designed and conducted the study under the guidance of Dr. Baiyu Zhang and Dr. Bing Chen and acted as the first author of the manuscript. Xudong Ye participated in the data analysis. Most contents of this paper were written by Cai and further polished by the other co-authors.

5.1 Introduction

Cost effective and rapid treatment of oily wastewater generated by oil and gas platforms, petroleum refineries, and chemical processing and manufacturing plants (Mysore et al., 2005; Scholz and Fuchs, 2000; Yang et al., 2012) is an increasing need. Discharge of poorly treated or untreated oily wastewater to the environment can create ecological problems (Han et al., 2015; Jing et al., 2015). The challenge of oily wastewater treatment lies in the presence of stable oil-in-water (O/W) emulsions, which renders traditional treatment methods, such as gravity separation and coagulation/flocculation less effective, and demulsification is required (Al-Anzi and Siang, 2017; Yang et al., 2016; Zhang et al., 2014). Demulsification techniques are generally classified as chemical, physical and biological approaches (Jamaly et al., 2015; Zolfaghari et al., 2016). Traditional chemical demulsification uses synthesized demulsifiers to assist in O/W separation (Zolfaghari et al., 2016). Biological demulsification, is similar in that it uses demulsifiers (biosurfactants produced by bacteria), but also the microbial cell surface to achieve effective emulsion breaking (Hou et al., 2014; Huang et al., 2014). The bacterial cell surface, particularly the cell surface-associated proteins or lipids has been found to play important roles in the demulsification process (Huang et al., 2014). The produced biosurfactants have also been found with demulsifying power (Amirabadi et al., 2013; Li et al., 2012) and generally have the advantages of lower toxicity and higher biodegradability and effectiveness over a wider span of pH, salinity and temperature conditions when compared with synthetic surfactants (Muthusamy et al., 2008; Shekhar et al., 2015). When demulsifying bacteria are applied to the emulsions, they may produce

biosurfactants with demulsifying ability *in situ* and the cells can be re-used (Das, 2001). In view of these intriguing properties, biological demulsifiers have been proposed as promising alternatives to chemically synthesized demulsifiers (Liu et al., 2010a).

Microorganisms have many roles when interacting with oil in water, such as direct biodegradation and the formation of O/W emulsion to enhance biodegradation, which have been reported extensively (Calvo et al., 2002; Cappello et al., 2016). Here, we focus on another way of utilizing bacteria which has attracted increased research interests in recent years, that is to destabilize emulsions for the subsequent separation of oil and water phases achieved using processes such as gravity separation, and coagulation/flocculation filtration (Amirabadi et al., 2013; Hou et al., 2014; Huang et al., 2010; Huang et al., 2014).

Research on biological demulsification has primarily focused on water-in-oil emulsions, which are commonly present in waste oil sludge (Zolfaghari et al., 2016). Biological demulsification of oil-in-water emulsions is still at a preliminary stage with few bacteria strains reported (Coutinho et al., 2013; Das, 2001; Li et al., 2012; Park et al., 2000). The ones that have been studied belong to the genera of *Micrococcus*, *Pseudomonas*, *Streptomyces* and *Bacillus*. These demulsifying bacteria were isolated from soil sampled from a gas station in India (Das, 2001); contaminated soil of Daqing Oil Field in China (Li et al., 2012); soil contaminated with refined oil products in Brazil (Coutinho et al., 2013), and sediment samples from South Korea and Antarctica (Park et al., 2000). They are mostly of terrestrial origin. Marine microorganisms have extraordinary metabolic and physiological capabilities that are rarely found in their terrestrial counterparts (Cai et al.,

2014; Satpute et al., 2010a). Thus, further exploration of novel demulsiftying bacteria for O/W emulsion, especially those of marine origins, is warranted.

An effective and high-throughput screening approach to quantify the performance of a large number of microbes, both natural and metabolically engineered, is critical for rapid development of biotechnology (Dietrich et al., 2010). Currently, no such screening approach has been identified to specifically target O/W demulsifying microbes. Huang et al. (2009) screened water in-oil-emulsion (W/O) demulsifying bacteria using measurements of surface tension, oil spreading, and blood-plate hemolysis. Whether the results can be extended to O/W emulsion is unknown. Moreover, the tested methods may overlook the mechanism of cell surface interactions during the demulsification (Huang et al., 2014).

In this study, thirty-seven novel marine bacteria isolated from offshore platforms and coastal sediment in North Atlantic Canada were studied to determine their O/W demulsification ability and define species/genus specific patterns. In addition, an oil drop collapse test (ODC), an oil spreading test (OS), surface tension measurements (ST), critical micelle dilution measurements (CMD), the emulsification index (EI), and cell hydrophobicity (CH) were evaluated as potential screening methods for demulsification ability.

5.2 Materials and Methods

5.2.1 Demulsifying bacterial strains

The demulsifying bacterial strains used for this study were from marine biosurfactant producing/surface-active bacteria previously isolated from an offshore

platform and coastal sediment in North Atlantic Canada (Cai et al., 2015a; Cai et al., 2014). The bacteria were cultivated in the Production Medium (PM) composed of $MgSO_4$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 0.05 g; KH_2PO_4, 3.4 g; $K_2HPO_4 \cdot 3H_2O$, 4.4 g; $(NH_4)_2NO_3$, 1 g; $FeCl_3$, 0.05 g; Glucose, 1g; NaCl, 26 g in 1L of distilled water, with 3% (v/v) diesel (Cai et al., 2014). Aliquots of bacterial broths were used for the emulsion breaking tests, ODC and OS tests. The remaining broth were subjected to centrifuge at 8,720 x g for 10 min. The supernatants were collected for the measurement of ST, EI and CMD, while the cell pellets were collected for the CH test.

5.2.2 Characterization of the demulsifying bacteria targeting oily wastewater with O/W emulsion

The purified isolates were subjected to 16S ribosomal DNA (rDNA) sequencing using universal bacterial primers 27F and 1493R (position in *Escherichia Coli* 8-27 and 1512-1493, respectively) following the protocol used previously (Cai et al., 2017). The phylogenetic distances were calculated using the software package MEGA (Molecular Evolutionary Genetics Analysis) version 5.1 (Tamura et al., 2011), after alignment of sequences with software package ClustalX (Multiple alignment of nucleic acid and protein sequences). Distances were calculated using the Kimura two-parameter model (Kimura, 1980). Trees were reconstructed using the neighbour-joining method (Saitou and Nei, 1987) with the use of 'default settings' and the bootstrap values were calculated based on 1000 replications.

The BIOLOG® microbial identification system was used to test the utilization of the 95 sole carbon sources simultaneously by the selected strains and reveal their metabolic

fingerprints (Ivanova et al., 1998). The strain was grown on Difco Marine Agar 2216 at 30^{o}C for 24h. The cells were then harvested with a loop and transferred to 0.4 M aqueous NaCl solution. After thoroughly mixing with the NaCl solution, the cell density OD_{590} was determined using a Thermo Scientific^{TM} Genesys^{TM} 10S UV/Vis spectrophotometer and adjusted to 0.3 ± 0.05 by adding more NaCl solution. Subsequently, BIOLOG GN2 plate was inoculated with 150 µL of the cell suspension per well. The inoculated plates were incubated at 30^{o}C. The results were read visually after incubation for 1 day.

Membrane-based phospholipid-derived fatty acid (PLFA) analysis was conducted to reveal strain specific patterns. Briefly, cells of the selected strain were harvested and treated with saponification reagent with heat to liberate fatty acids. The liberated fatty acids were methylated and the extracted for quantification using gas chromatography/ mass spectrometry (GC/MS) system. The detailed protocol can be founding our previous study (Cai et al., 2017b). The test was conducted in duplicate.

Scanning electron microscope (SEM) analysis was conducted to reveal the morphology of the selected isolates following the method used by de Sousa and Bhosle (2012). Briefly, bacterial cells were fixed overnight in 2% glutaraldehyde prepared in 50 mM potassium phosphate buffer. The fixed cells were washed three times with phosphate buffer and dehydrated using an increasing gradient of acetone in distilled water (30%, 50%, 70%, 80% and 90%, respectively) for 10 min each and finally in 100% acetone for 30 min and air dried. The specimens were sputter-coated with gold using an auto fine coater and visualized using SEM (FEI MLA 650F).

5.2.3 Complete genome sequence of the selected demulsifying strain

Whole-genome shotgun sequencing of *Halomonas venusta* strain N3-2A was performed at the Donnelly Sequencing Center at University of Toronto (Toronto, Canada) using Illumina MiSeq 2500 using 300 cycle Miseq kit V2. This generated a total of 2,187,466 filtered paired-end reads, providing 139-fold coverage of the genome. Quality control was conducted through FastQC (Andrews, 2010). To assemble the data, SPAdes genome assembler was used (Bankevich et al., 2012). plasmidSPAdes was used to assemble plasmids from whole genome sequencing data (Antipov et al., 2016). Subsequently, the assembled contigs was re-ordered by Mauve multiple genome alignment using *Exiguobacterium Antarctica* B7 as the reference genome (Rissman et al., 2009). Gene annotation was performed by using the NCBI prokaryotic Genome Annotation Pipeline (http://www.ncbi.nlm.nih.gov/genome/annotation_prok/) tRNA and rRNA sequences were identified using tRNAscan-SE and RNAmmer, respectively (Lagesen et al., 2007; Lowe and Eddy, 1997).

5.2.4 Screening tests for demulsifying bacteria targeting oily wastewater with O/W emulsion

The results of **Oil drop collapse test (ODC)** have been found to be positively correlated with the concentrations of the surface tension reduction materials (Bodour and Miller-Maier 1998). The test was conducted following Bodour and Miller-Maier (1998). Briefly, 2 µL of mineral oil was added to each well of a 96-well microtiter plate lid. A 5 µL aliquot of bacterial broth was delivered into the center of the well. After 1 min, the flatness of the water droplet was observed and recorded. The droplets had 100% collapsing,

50%~100% collapsing and 10~50% collapsing were marked as +++, ++, and +, respectively.

Oil spreading test (OS) was conducted following Morikawa et al. (2000). Ten µL of crude oil was gently added to the surface of 40 mL of distilled water in a petri dish (Ø = 150 mm) to form a thin oil membrane. Ten µL of bacterial broth was gently added to the center of the oil membrane. A clear zone was formed due to the activity of the surfactants. The area of the clear zone was used to reflect the concentration of the produced surfactants. The results were illustrated as the diameter of the clear zone.

Surface tension measurement (ST) uses du Nouy ring method to measures surface/interfacial tension (Harkins and Alexander, 1959). It was determined in triplicate with a surface tensiometer (DuNouy Tensiometer, Interfacial, CSC Scientific) at 20±3^{o}C following the manufacturer's instruction.

Emulsification index (EI) reflects the ability of the biosurfactant to form and stabilize emulsions (Cai et al. 2017). EI of the cell free supernatant was determined by adding 5 mL of light mineral oil to the same amount of culture, mixing with a vortex for 2 min, and letting stand for 24 h. The emulsifying activity (E24) was calculated using Equation (1):

$$E24=H_{EL}/H_S\times100\% \quad (1)$$

where H_{EL} is the height of the emulsion layer and H_S is the height of the total solution.

Critical micelle dilution measurement (CMD) of the cell free supernatant was determined following Cai et al. (2015a). CMD can be used to reflect the concentrations of

(bio)surfactant that reduce surface/interfacial tension (Shavandi et al. 2011). After centrifuging at 10000 rpm for 10 min and discarding the pellet, the cell free broth samples were diluted with distilled water, while the surface tension of each dilution was measured. The CMD was determined as the highest dilution with which the surface tension did not significantly increase. As the broth consists of both an aqueous and an oil phase, each dilution was conducted with sonication to ensure homogeneity. Before each measurement, the sonicated solution was allowed to stand for 15-20 min to achieve equilibrium.

Cell hydrophobicity (CH) was evaluated by the method of microbial adhesion to the hydrocarbon (MATH) (Park et al., 2000). The cell pellets obtained after centrifuge separation of the whole culture were rinsed with 50 mM phosphorus buffer (PBS, pH = 7.0) twice and then diluted to an initial OD_{580} value of around 0.8–1.0. Then 5 mL of this cell suspension was mixed with 1 mL of kerosene in a test tube on a vortex mixer for 2 min. The mixture was left undisturbed for 20 min, and then the final OD_{580} of the aqueous phase was measured again. MATH was calculated as follows (2):

$$\text{MATH} = \left(1 - \frac{OD_{580}(final)}{OD_{580}(initial)}\right) \times 100\% \quad (2)$$

High MATH values indicate high affinity of the cells for oils.

5.2.5 Oil-in-water emulsion breaking test

Span–Tween–Kerosene O/W emulsion prepared following Coutinho et al. (2013) was used for this test. The emulsion type was confirmed using microscopic visualization after dyeing the oil phase using hydrophobic dye Oil Red O (Lee and Lee, 2000). The fresh emulsion had an emulsion breaking ratio of <15% at 35^{o}C within 48 h. For screening test,

4-day culture of strains were used. For the detailed characterization of the featured isolate, *Halomonas* sp. N3-2A, a 7-day culture was used in which the growth of bacteria in terms of accumulated biomass reached plateau. Three types of samples were subjected to demulsification assays, i.e. whole culture broth, cell free supernatant obtained from centrifuging, and cell suspension obtained by dissolving PBS washed cell pellets in the fresh medium. The demulsification assay was conducted following Wen et al. (2010). Briefly, 1 mL sample or blank were added into a 15 ml vial containing 9 mL of emulsion and sealed with silicone rubber. The test tube was agitated in a vortex for 30 s. The tube was kept undisturbed in an upright position in an incubator at 35°C. The changes in the volume of the oil phase (top), water phase (bottom), and emulsion phase (in-between) were recorded at certain time intervals for up to 48 hours. The tests were duplicated. Demulsification performance was evaluated by the emulsion breaking ratio according to the following equations:

$$\text{Emulsion breaking ratio (EBR)} = \left(1 - \frac{remaining\ emulsion\ volume}{original\ emulsion\ volume + added\ volume}\right) \times 100\% \quad (3)$$

Each EBR was normalized as EBR_n using the following equation:

$$\text{EBR}_n = \left(\frac{EBR_{sample} - EBR_{control}}{1 - EBR_{control}}\right) \times 100\% \quad (4)$$

5.2.6 Statistical analysis and quality control

Analysis of variance (ANOVA) for multiple linear regression was employed to fit a regression line for a response variable (EBRn 24h) using explanatory variables (ODC,

OS, CMD, ST, EI and CH values). The model was reduced using stepwise regression with α to enter = 0.05 and α to exit = 0.05. Principal component analysis (PCA) of the data matrix were conducted with corrplot package in the R project for statistical computing (R Core Team, 2013). The data were first log transferred, and then centered and scaled to a mean of 0 and standard deviation of 1 prior to the PCA analysis (Venables and Ripley, 2013). The graphic display of the dominant patterns in the matrix was made using ggplot 2 (Wickham, 2016). It colors each point according to the bacterial species/genera and draws a normal contour line with ellipse covering probability (default to 68%) for each group. The Mahalanobis distances, i.e. a measure for the separation of the groups taking into account the spreading of the samples within one group, were calculated in the overlap data after PCA analysis (Mahalanobis, 1930) using R project.

Randomly selected strains (20%) were subjected to duplicate runs of all the 7 tests. The relative standard error (RSE) in the duplication has been less than ±20%, demonstrating the accuracy and repeatability of the measurements for strains reported in this study.

5.3 Results and Discussion

5.3.1 Emulsion breaking performance of the isolates

The O/W emulsion breaking performance of the isolates is summarized in Figure 5.1. After 24 h, the EBR values were found to be in a range of 19.41% to 97.60% for the tested strains. EBR_n 12h and EBR_n 24h are comparable, except for few strains (e.g. N3-8A and N2-3P) which exhibited a lag in demulsification. Figure 5.1 also shows the result trends of the 6 screening tests sorted by a descending EBR_n 24h.

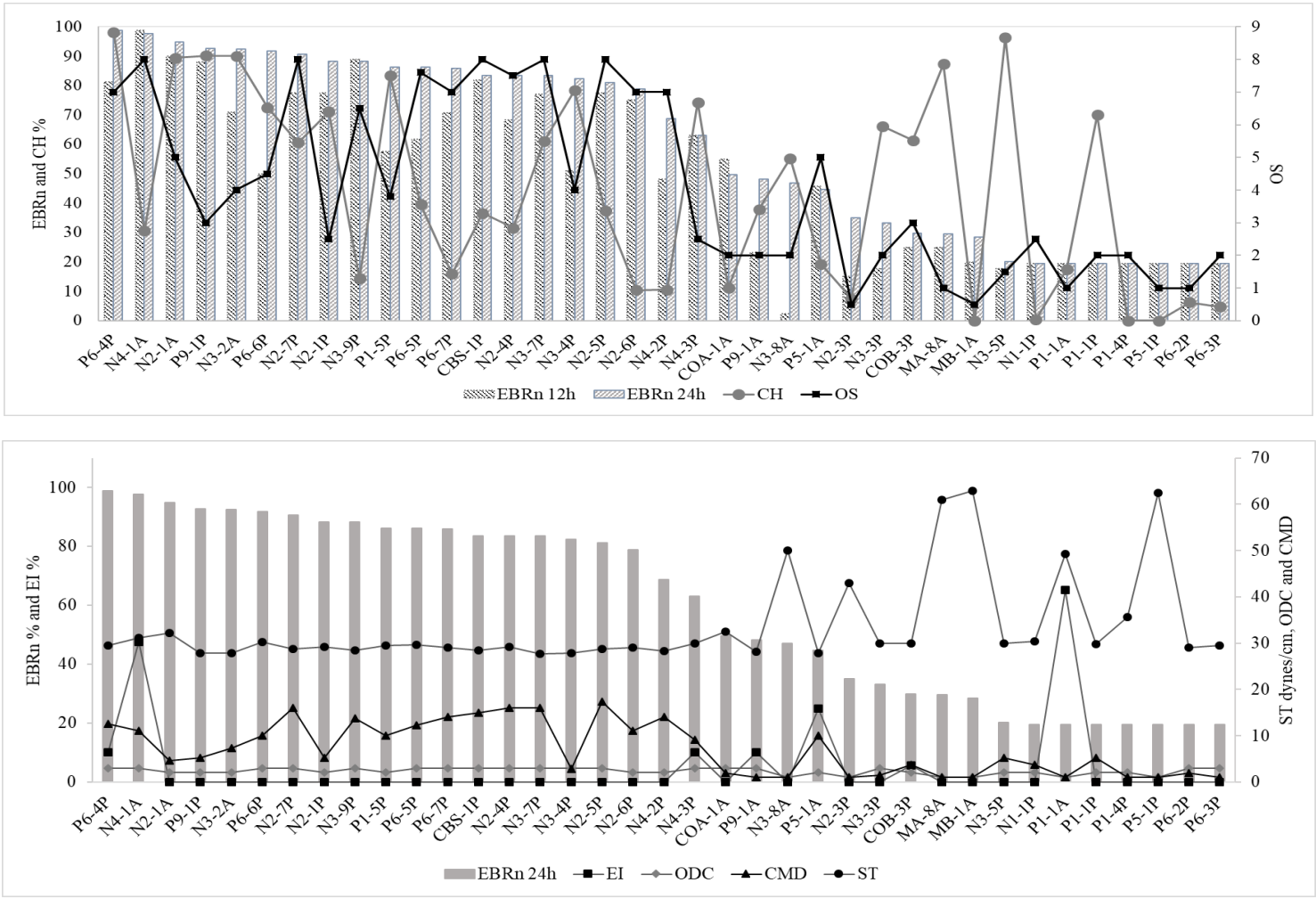


Figure 5.1 Emulsion breaking performance of the isolates and the trend of screening tests sorted by descending EBR_n 24h values (A: Oil spreading test (OS) and Cell hydrophobicity (CH); B: Emulsification index (EI), Oil drop collapse test (ODC), critical micelle dilution (CMD) and surface tension measurement (ST))

OS showed similar descending trend with that of EBR_n 24h. Interestingly, CH served a supplementary role to OS (Figure 5.1A). For the strains with high EBR_n 24h, their OS were generally high, or when OS was low, it was accompanied by a high CH to achieve a high EBR_n.

OS is an assay based on change of the contact angle at the oil-water interface with the quantity of the added (bio)surfactants (Morikawa et al., 1993). CH demonstrates the affinity of microbial cells to the oil phase. The native cell hydrophobicity has been attributed to certain proteins and lipids present in the cell wall (Huang et al., 2014; Kaczorek et al., 2008). The generation of surface active compounds also likely lead to "cell-bound" components that affect the CH readings (Kuyukina et al., 2001). Therefore, OS and CH correlate to the concentrations of surface/interfacial active compounds and the affinity of the microbial cells to the oil phase, respectively. Studies on the biological demulsification of water-in-oil emulsions have found that microorganisms modify the emulsion properties by employing either their hydrophobic cell surfaces or the extracellular amphiphilic biosurfactants to replace the emulsifier molecules (Zolfaghari et al., 2016). As a result, interfacial tension gradient is reduced, which leads to the thinning of the interfacial film and eventually phase separation (Wen et al., 2010). In this study, both OS and CH readings were found to be positively and significantly influencing the EBR_n readings. This suggests that similar mechanisms are behind the demulsification of O/W emulsion with wide variety of bacteria examined in this study. The results obtained agreed with the findings of Park et al. (2000) and Coutinho et al. (2013) that strains with high demulsification efficiency tend to have high cell hydrophobicity and interfacial activity.

Detailed investigation on how cell surface compositions of the diverse bacterial strains isolated in this study affect the cell hydrophobicity and interfacial activity, and thus the demulsification of O/W emulsions will be included in future studies.

5.3.2 Identification of the demulsifying bacteria targeting oily wastewater with O/W emulsion

Table 5.1 provides a summary of the results of bacterial identification, emulsion breaking test and the 6 screening tests. Thirty-seven demulsifying bacteria belong to 5 genera, namely *Acinetobacter*, *Bacillus*, *Halomonas*, *Pseudomonas* and *Rhodococcus* were isolated (Figure 5.2). The sources of these bacteria were seawater from a ship harbor (sample ID: CBS), offshore crude oil samples sample IDs: COA and COB), raw produced water from offshore oil reservoirs (sample IDs: MA and MB), four coastal sediment samples collected near a refinery wastewater outlet (sample IDs: N1, N2, N3, and N4) and four treated produced water samples collected from offshore oil platforms (sample IDs: P1, P5, P6 and P9). The isolated bacteria belong to 15 species as illustrated in the phylogenetic tree (Figure 1). The tree also shows three O/W emulsion breaking bacteria described in the literature, i.e. *Pseudomonas aeruginosa* MSJ (Coutinho et al., 2013), *Bacillus mojavensis* XH1 (Li et al., 2012), and Streptomyces sp. AA8321 (Park et al., 2000) (Figure 5.2). These species are in the divisions of Firmicutes, Actinobacteria and Proteobacteria (Figure 5.2). The 15 new bacteria reported here significantly expands the knowledge of O/W emulsion breaking bacteria.

Table 5.1 Strains and the test results

Strain ID	Species name with highest match	Max identity %	ODC	OS cm	CMD	CH %	EI %	ST dynes /cm	EBRn 12h %	EBRn 24h %
CBS-1P	*Bacillus thuringiensis*	100	+++	8	15	36.57	0	28.48	81.89	83.49
COA-1A	*B. subtilis*	99	+++	2	1.98	11.16	0	32.47	55.00	49.59
COB-3P	*Pseudomonas fluorescens*	99	++	3	3.7	61.39	5.56	29.87	25.00	29.80
MA-8A	*B. subtilis*	99	+	1	1	87.37	0	60.87	25.00	29.58
MB-1A	*Rhodococcus erythropolis*	100	+	0.5	1	0	0	62.87	20.00	28.37
N1-1P	*R. phenolicus*	99	++	2.5	3.7	0.49	0	30.33	19.41	19.41
N2-1A	*R. zopfii*	99	++	5	4.56	89.39	0	32.2	90.00	94.80
N2-1P	*R. zopfii*	99	++	2.5	5.26	71.16	0	29.13	77.44	88.33
N2-3P	*B. subtilis*	100	+	0.5	1	7.53	0	42.97	15.00	35.00
N2-4P	*R. erythropolis*	100	+++	7.5	16	31.56	0	29.23	68.41	83.49
N2-5P	*R. erythropolis*	100	+++	8	17.3	37.39	0	28.67	77.43	81.13

N2-6P	*B. subtilis*	99	++	7	11.1	10.46	0	29.1	75.18	78.77
N2-7P	*B. subtilis*	98	+++	8	16	60.61	0	28.77	77.43	90.56
N3-2A	*Halomonas venusta*	99	++	4	7.3	90.09	0	27.83	70.85	87.03
N3-3P	*R. wratislaviensis*	98	+++	2	1.5	66.17	0	29.9	17.74	33.23
N3-4P	*B. licheniformis*	100	+++	4	2.83	78.35	0	27.77	50.95	82.43
N3-5P	*R. phenolicus*	98	++	1.5	5.26	96.4	0	29.87	17.74	20.09
N3-7P	*B. flexus*	99	+++	8	16	60.99	0	27.73	76.97	83.49
N3-8A	*H. variabilis*	99	+	2	1	55.18	0	50.03	2.50	46.94
N3-9P	*B. subtilis*	100	+++	6.5	13.7	14.37	0	28.47	88.89	88.20
N4-1A	*R. erythropolis*	99	+++	8	11.1	30.56	47.62	31.13	96.80	97.60
N4-2P	*B. subtilis*	100	++	7	14	10.53	0	28.3	48.10	68.71
N4-3P	*R. yunnanensis*	100	+++	2.5	9.1	74.25	10	29.9	63.16	63.16
P1-1A	*R. opacus*	98	+	1	1	17.5	65.22	49.2	19.41	19.41
P1-1P	*R. opacus*	99	++	2	5.26	69.96	0	29.8	19.41	19.41
P1-4P	*R. opacus*	98	++	2	1	0	0	35.6	19.41	19.41
P1-5P	*R. wratislaviensis*	98	++	3.8	10	83.52	0	29.5	57.69	86.19
P5-1A	*Acinobacter oleivorans*	100	++	6.5	10	19.35	25	27.9	45.84	44.64
P5-1P	*R. erythropolis*	99	+	1	1	0	0	62.37	19.41	19.41

P6-2P	*B. subtilis*	100	+++	1	1.98	6.29	0	29.07	19.41	19.41
P6-3P	*B. subtilis*	98	+++	2	1	4.74	0	29.5	19.41	19.41
P6-4P	*R. erythropolis*	99	+++	7	12.5	98.02	10	29.47	81.20	98.85
P6-5P	*R. erythropolis*	100	+++	7.6	12.3	39.57	0	29.67	61.64	86.19
P6-6P	*R. erythropolis*	100	+++	4.5	10	72.52	0	30.3	50.00	91.70
P6-7P	*B. thuringiensis*	100	+++	7	14	15.89	0	29.03	70.66	85.85
P9-1A	*A. calcoaceticus*	99	+++	2	1	37.88	10	28.17	23.28	48.10
P9-1P	*A. calcoaceticus*	99	++	3	5.26	90.18	0	27.83	87.99	92.63

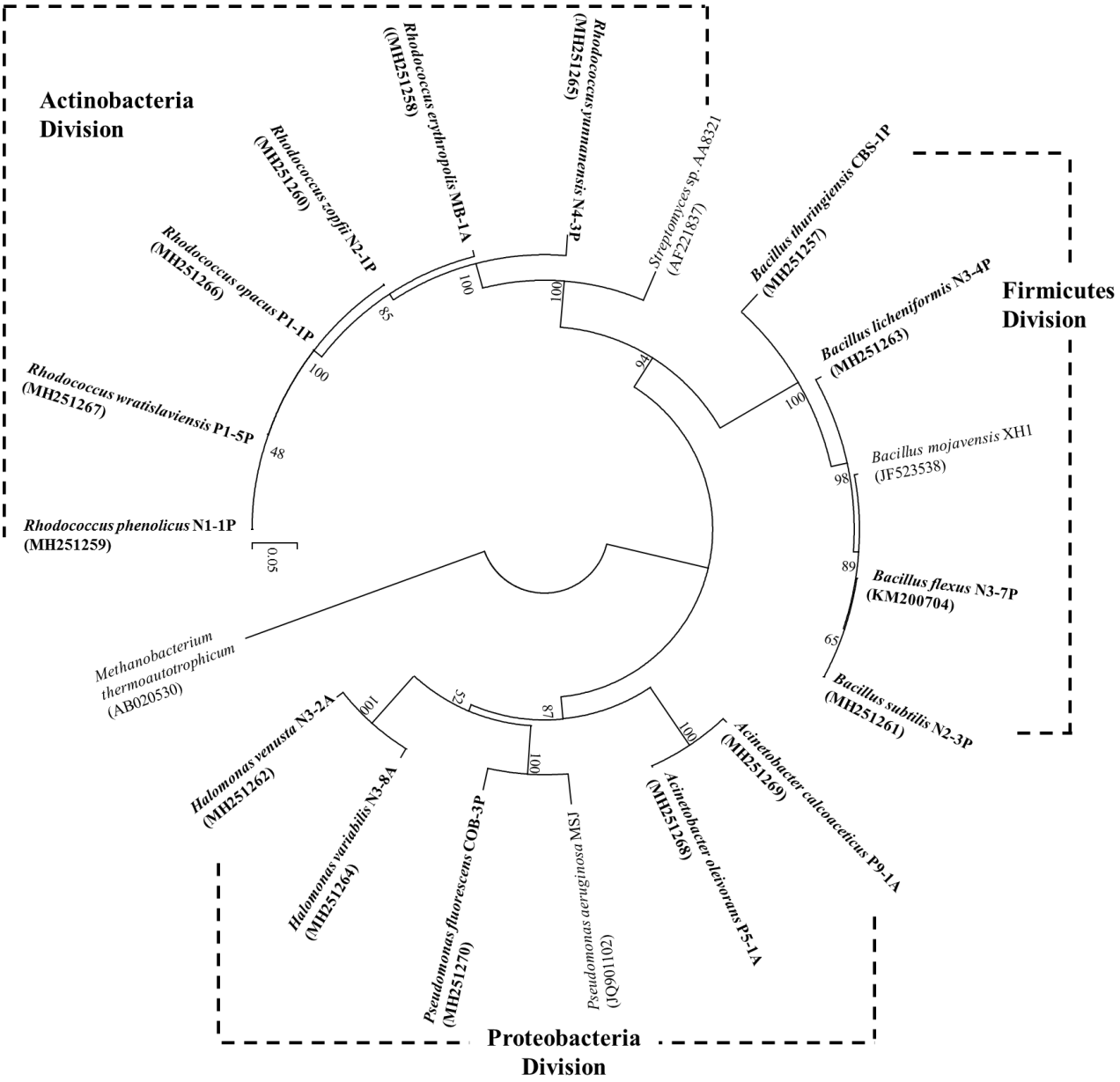


Figure 5.2 Phylogenetic tree based on 16S rDNA sequence from representative O/W emulsion breaking bacteria of the 15 species that was isolated in this study (bold) and those published in the literature (GenBank accession numbers in parentheses). *Methanobacterium thermoautotrophicum* is served as the out group. Percentages of 1000 bootstrap are shown at the nodes of the phylogenetic tree

5.3.3 Demulsification kinetics and mechanisms of *Halomonas venusta* strain N3-2A

When the whole broth of *Halomonas venusta* strain N3-2A was used to treat the model emulsion, the EBR_n was low in the first 4 hours and jumped at the 5^{th} hour. Subsequently, the EBR_n was increasing with a steady rate until it reached around 92.5% at 24 hours. When only supernatant was used, no emulsion breaking was observed within the first 5 hours, and then the EBR_n steadily increased to 77%. When only cells were used, no emulsion breaking was observed within the first 9 hours, and then EBR_n slowing increased with a rate that much lower than with whole broth and supernatant till it reached 25% (Figure 5.3A). This indicates that both cellular (cell-bound) and extracellular bio-demulsifiers contributed to the demulsification. The combined EBR_n of supernatant and cells were significantly lower than the whole broth within the first 12 hours (Figure 5.3B). This significantly more rapid increase in EBR of the whole broth could be important. It indicates that there may be a loss of certain cell soluble components that contributed to the demulsification due to the pellet washing using PBS or a synergetic effect between bacterial cells and extracellular bio-demulsifiers. Similar phenomenon was also observed by Nadarajah et al. (2002) when using a mixed bacterial culture. Extracellular bio-demulsifiers (biosurfactants) were found with stronger impact on emulsion breaking. *Halomonas* strains have been found with the ability to produce glycoprotein type (Gutierrez et al., 2007) or glycolipid type (Dhasayan et al., 2014; Pepi et al., 2005) of biosurfactants. Further characterization of the extracellular bio-demulsifiers (biosurfactants) produced by *Halomonas venusta* strain N3-2A will be conducted in future research.

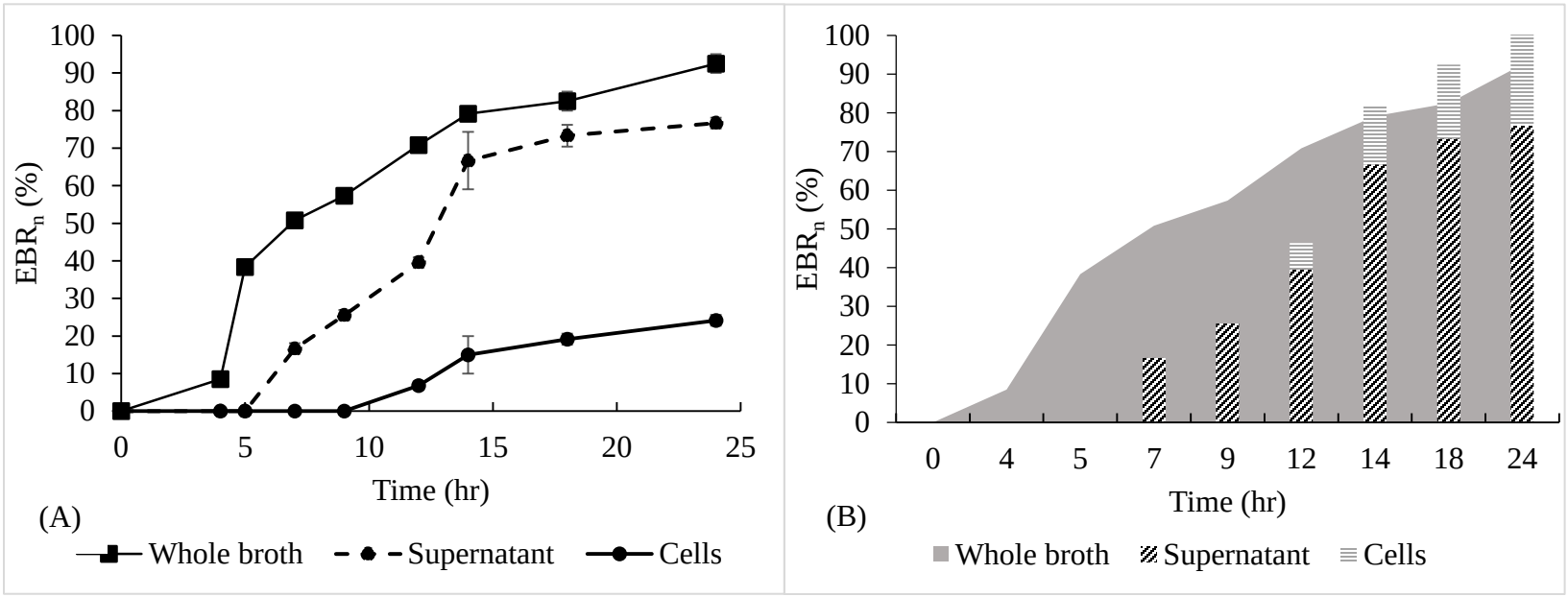


Figure 5.3 Emulsion breaking ability of whole broth, cell-free supernatant and cells of Halomonas sp. N3-2A (A: dot and line graph; B: supernatant and cells as staked columns)

Oily wastewater oil can contain oil that is floating, dispersed, emulsified and dissolved based on the size of the oil droplets (Coca et al. 2011). Stable emulsified oil poses a major challenge for O/W separation during wastewater treatment. Stable emulsified oil has droplet diameters < 2 μm (Coca et al. 2011). They are immune from some primary treatments such as gravity separation owing to the small droplet size (Stewart and Arnold 2008). The presence of emulsifiers often renders some secondary treatments such as a coagulation/flocculation based on traditional coagulants (e.g. $AlCl_3$ and $FeCl_3$) ineffective (Ibrahim et al. 2009). Microfiltration and ultrafiltration as secondary wastewater treatment techniques can effectively remove O/W emulsion. However, membrane fouling due to surfactant or oil adhere on the pore walls can significantly lower the membrane life and increase the treatment costs (Ahmad et al. 2005). The addition of effective demulsifiers to coalesce the droplets into significantly larger sizes or even separated phases prior to gravity separation and coagulation/flocculation is thus a promising approach to treat oily wastewater with emulsified oil (Yang et al. 2016, Coca et al. 2011). Today, the dominate players in the O/W demulsifier market are chemically synthesized polyelectrolytes, surfactants and polymers (Coca et al. 2011, Talingting-Pabalan et al. 2010). Bacterial demulsifiers, although they may have great potential, lag far behind in terms of the maturity of research and development. This study assists in their development. The results demonstrated the high emulsion breaking efficiency of *Halomonas venusta* strain N3-2A in a batch-scale test that are typically used to screen potential demulsifiers (Nishimaki et al. 1999). The emulsion breaking efficiency is comparable with recently reported O/W demulsifying bacteria, such as *Pseudomonas aeruginosa* MSJ (Coutinho et al. 2013) and

Bacillus mojavensis XH1 (Li et al. 2012). The feasibility of the reported biodemulsifier will be further examined in multiple-scale treatment tests in future studies.

5.3.4 Characterization of a novel demulsifying bacterium *Halomonas venusta* strain N3-2A

The polymerase chain reaction (PCR) reaction obtained almost full length (1,500 bp) 16S rDNA fragments for the selected isolates. The five replicates agreed well with each other (genebank accession no.: MH251262). The novel demulsifying bacterium N3-2A was found to be closely related to *H. venusta* DSM 4743^T, *H. hydrothermalis* ATCC BAA-800^T and *H. axialensis* ATCC BAA-802^T with similarities of 99.5%, 99.3% and 97.9%, respectively. The phylogenetic tree based on 16S rDNA sequences of *Halomonas* sp. N3-2A and 6 close related type strains is constructed (Figure 5.4A). *Halomonas* belong to the order of Oceanospirillales. Most species of this order are halophilic and halotolerant (Garrity et al., 2005). Hitherto, none of the strains belonging to this genus have been reported with demulsifying ability. The similarity/dissimilarity of the N3-2A and the 6 closely related type strains according to their metabolic profile and PLFA compositions are illustrated in Figures 5.4B and 5.4C. Metabolic traits of N3-2A were similar with *H. venusta* DSM 4743^T, *H. meridiana* DSM 5425^T, and *H. variabilis* DSM 3051^T. The membrane PLFA profiles of *H. venusta* DSM 4743^T, *H. hydrothermalis* ATCC BAA-800^T, *H. axialensis* ATCC BAA-802^T, *H. meridiana* DSM 5425^T, and *H. sulfidaeris* ATCC BAA-803^T were closely resemble each other. N3-2A is slightly different from the above 5 strains. N3-2A was rod shaped bacterium with a length of 0.5-1 μm and diameter of around 0.5 μm (Figure 5.4D) and was significantly smaller than the 6 closely related strains.

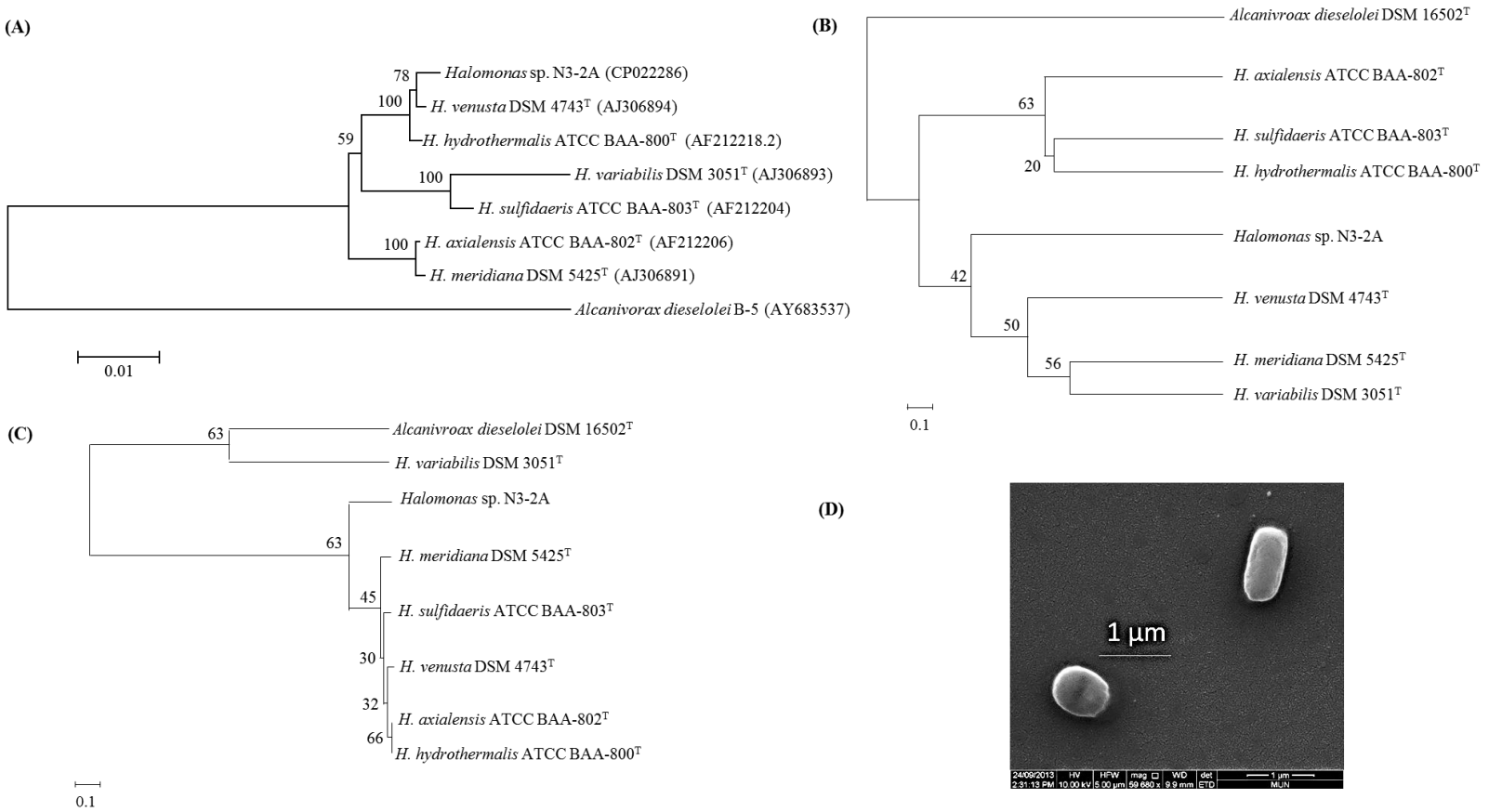


Figure 5.4 Characterization of *Halomonas* sp. N3-2A. (A) Phylogenetic tree based on 16S rRNA gene sequences. (Note: The tree was constructed using the NJ method, the GenBank accession numbers for the 16S rRNA gene sequences of each reference species are listed in parentheses); (B), (C) Phenograms using unweighted pair group method with arithmetic mean (UPGMA) based on metabolic and PLFA profile, respectively; (D) SEM image of the bacterium (Percentages of bootstrap are shown on the nodes; *Alcanivorax diselolei* DSM 16502^T is used as the out group)

5.3.5 Complete genome sequence of *Halomonas venusta* strain N3-2A

A chromosome type replicon (4,723,828 bp), with G+C content of 52.91%, made of 116 contigs/scaffolds was obtained for *Halomonas venusta* strain N3-2A, which harbors 4,193 coding sequences, including 3,253 proteins with identified functions. Eight rRNA operons and 58 tRNAs were also annotated. No plasmids were identified by plasmidSPAdes. As expected, diverse di-oxygenases, dehydrogenases and cytochromes involved in aerobic hydrocarbon biodegradation pathway are found in the genome, explaining its ability to degrade hydrocarbons. Several lipopolysaccharide and polysaccharide biosynthesis genes are presented in the genome linking to its ability to produce biosurfactants that are capable of reducing surface tension and breaking oil-in-water emulsions.

The genome sequence has been deposited in DDBJ/EMBL/GenBank under accession number CP022286. The version described in this paper is the first version, CP022286.1.

5.3.6 Evaluation of the screening methods for identifying demulsifying bacteria targeting oily wastewater with O/W emulsion

In this study, in order to evaluate the efficacy of the above tests in relation to emulsion breaking ratio, multiple linear regression model and non-linear models including quadratic and linear with interaction models were employed. They were used to fit the experimental data and shed light on the relationship between the results of the six screening methods (explanatory variable) and EBR (the response variable). During pre-screening, the

quadratic model and the two-factor interaction model both had sequential p-values larger than 0.05 (data not shown) and were excluded. The linear models (i.e. a full model and a reduced model) were applied to fit the experimental data. When all six screening parameters were used to fit the linear model, the model *F*-value of 10.18 implied the model was significant (Table 5.2: left side). The explanatory variables, OS and CH were significant with a *p*-value (prob>F) less than 0.05. The explanatory variables that weren't significant, i.e. ODC, CMD, EI and ST are supposed to be removed to improve the model. The model R^2 value was at 0.6706, indicating 67.06% of variability in the response was explained by the explanatory variables. The predicted R^2 of 0.5273 was in reasonable agreement with the adjusted R^2 of 0.6047 (difference <0.2). Adequate precision is a measure of the signal to noise ratio and the value of 10.43 (> the desirable value 4) indicated an adequate signal. Coefficient of Variation (C.V. %) is the error expressed as a percentage of the mean. PRESS is a measure of how well a particular model fits each point in the design. Both C.V.% and PRESS can be used to evaluate the performances of varies models. The lower values indicate better structure of the candidate models. After the model terms that were insignificant were removed, the model was reduced (Table 5.2: right side). The new model R^2 value was very close to the full model, indicating the majority of variability in the response was explained by the two significant explanatory variables, i.e. OS and CH. The reduced model had improved adjusted R^2, predicted R^2 and adequate precision, indicating better model explainability, predictability and signal to noise ratio. The reduced model also had lower C.V.% and PRESS indicating it has lower error and better model structure. After the reduction, the regression model was as follows:

Table 5.2 ANOVA for multiple linear regression (Left: complete model; right: reduced model)

	Sum of Squares	df	Mean Square	F value	p-value Prob>F
Model	21953.32	6	3658.89	10.18	< 0.0001*
A-ODC	205.83	1	205.83	0.57	0.4551
B-OS	2638.44	1	2638.44	7.34	0.0110*
C-CMD	9.86	1	9.86	0.03	0.8695
D-CH	3264.74	1	3264.74	9.08	0.0052*
E-EI	13.45	1	13.45	0.04	0.7582
F-ST	34.71	1	34.71	0.10	
Residual	10784.91	30	359.50		
Corrected Total SS	32738.23	36			
R^2	0.6706		Predicted R^2		0.5273
Adjusted R^2	0.6047		Adequate precision		10.43
C.V.%	31.63		PRESS		15474.50

Factor	Coefficient Estimate (coded)	Std. Error	95% CI Low	95% CI High	Coefficient Estimate (actual)
Intercept	64.07	6.38	51.05	77.10	-2.98
A-ODC	5.12	6.77	-8.71	18.95	5.12
B-OS	32.49	11.99	8.00	56.99	8.66

	Sum of Squares	df	Mean Square	F value	p-value Prob>F
Model	21682.92	2	10841.46	33.34	< 0.0001*
B-OS	17860.74	1	17860.74	54.93	< 0.0001*
D-CH	3799.09	1	3799.09	11.68	0.0017*
Residual	11055.31	34	325.16		
Corrected Total SS	32738.23	36			
R^2	0.6623		Predicted R^2		0.6013
Adjusted R^2	0.6424		Adequate Precision		16.17
C.V.%	30.08		PRESS		13053.61

Factor	Coefficient Estimate (coded)	Std. Error	95% CI Low	95% CI High	Coefficient Estimate (actual)
Intercept	64.30	3.04	58.12	70.48	12.83
B-OS	32.18	4.34	23.36	41.01	8.58
D-CH	14.99	4.39	6.08	23.90	0.30

C-CMD	-2.01	12.11	-26.73	22.72	-0.25
D-CH	15.45	5.13	4.98	25.92	0.32
E-EI	-2.33	12.02	-26.88	22.23	-0.04
F-ST	2.77	8.92	-15.44	20.98	0.16

$$EBR_n 24h = 12.83 + 8.58 \cdot OS + 0.30 \cdot CH \quad (5)$$

According to the results, OS and CH are significant explanatory factors that are both positively contribute to the emulsion breaking. This finding validated the observation in Section 3.1. Results of OS and CH can be used to predict the emulsion breaking using the obtained regression model. OS coupled with CH are recommended as the strategy for the screening of demulsifying microorganisms. Both tests are fast, require no specialized equipment and have potential to be automated to further increase throughputs.

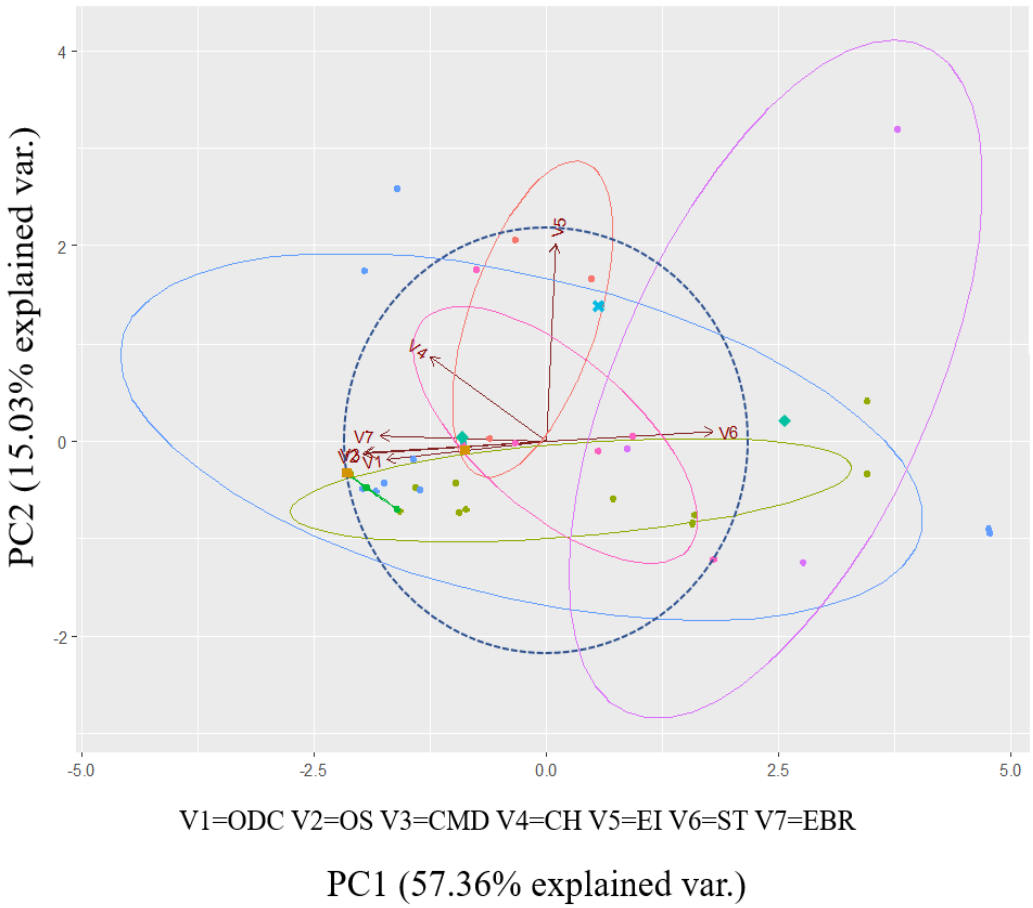
5.3.7 Species/genus specific patterns of demulsifying bacteria targeting oily wastewater with O/W emulsion

Principal component analysis (PCA) was applied to investigate the structure of the data and infer species/genus specific patterns of the demulsifying bacteria in response to the tests. Varimax rotated principal component matrix of the 7 factors is summarized in Table 5.3. The components 1 and 2 accounting for 72.01% of total variance in the dataset. The dominant variables contributing to variance along component 1 were ODC (-0.40), OS (-0.46), CMD (-0.45), ST (0.42) and EBR (-0.42). ST and EI showed positive loadings while the others show negative loadings, indicating an inverse association between these variables. Variance along component 2 was accounted for primarily by EI (0.91).

The circle of correlations and plot of loadings along components 1 and 2 are shown in Figure 5.5 as vectors V1 to V7. The grouping of bacterial species/genera based on their PCA scores is illustrated in Figure 5.5 to reflect how strains perform differently in the 7 tests. Certain degree of overlapping between groups was observed. Thus, the Mahalanobis distances between groups are calculated (Figure 5.5, insert).

Table 5.3 Varimax rotated principal component matrix of the 7 factors. The higher the loading value, the higher the contribution of that variable along the component. Significant contributions (loadings above 0.4) are marked with an asterisk

Variable	Component						
	1	2	3	4	5	6	7
ODC	-0.3988	-0.0843	0.5246*	-0.2545	0.4485*	-0.5080*	-0.1861
OS	-0.4577*	-0.0579	-0.0190	0.3936	-0.1220	-0.2307	0.7509*
CMD	-0.4491*	-0.0543	-0.0797	0.3623	-0.5254*	-0.1463	-0.6002*
CH	-0.2892	0.3898	-0.5220*	-0.6344*	-0.1694	-0.2353	0.0733
EI	0.0227	0.9122*	0.2977	0.2710	0.0446	0.0517	-0.0271
ST	0.4160*	0.0438	-0.4137*	0.3496	0.2509	-0.6767*	-0.1039
EBR	-0.4154*	0.0258	-0.4310*	0.2256	0.6436*	0.3890	-0.1563
Eigenvalue	3.988	1.053	0.811	0.669	0.244	0.155	0.080
Percent of variance	56.97	15.04	11.59	9.56	3.48	2.21	1.15
Cumulative percent of variance	56.97	72.01	83.60	93.16	96.64	98.85	100



Acinetobacter
Bacillus others
Bacillus subtilis
Bacillus thuringiensis
Halomonas
Pseudomonas
Rhodococcus erythropolis
Rhodococcus opacus
Rhodococcus others

		4.61	12.55	10.80	2.80	1.20	9.68	1.72
	4.61		3.36	7.63	5.33	0.43	11.36	5.28
	12.55	3.36		1.76	3.72	0.42	1.82	0.93
	10.80	7.63	1.76		28.45	1.05	19.38	10.95
	2.80	5.33	3.72	28.45		0.20	0.79	0.40
	1.20	0.43	0.42	1.05	0.20		1.47	0.04
	9.68	11.36	1.82	19.38	0.79	1.47		4.40
	1.72	5.28	0.93	10.95	0.40	0.04	4.40	

Figure 5.5 Circle of correlations (blue dotted cycle) and plot of the loadings along components 1 and 2, overplayed with grouped score plot, showing individual strain data points plotted in coordinated space along component 1 and 2 (insert: the Mahalanobis distance matrix between groups)

These results illustrated that when evaluating the isolated bacteria using the seven tested parameters, some species/genera indeed showed distinct patterns after incubated under the same conditions. Strains belongs to *B. thuringiensis* were the most distinct group which had significant distances (Mahalanobis distances >5) from *Acinetobacter*, the *Bacillus* others (*B. flexus* and *B. licheniformis*), *Halomonas*, *R. opacus* and *Rhodococcus* others (*R. phenolicus, R. wratislaviensis*, *R. zopfii*, and *R. yunnanensis*). *B. thuringiensis* strains had PCA scores that grouped tightly to the high ODC, OS, CMD and EBR side and low ST side, indicating they produced high level of surface tension reducing biosurfactant, effectively broke O/W emulsion. *R. opacus* strains were significantly distant from *Acinetobacter*, *Bacillus* others and *B. thuringiensis.* PCA scores of *R. opacus* strains were grouped at the side of vector V6 and opposite side of vectors V1, V2, V3 and V7, indicating that they had lower efficiency in producing biosurfactants and breaking O/W emulsion. *Acinetobacter* strains were significantly distant from strains belong to *B. subtilis*, *B. thuringiensis*, and *R. opacus*, and they mostly had high EI readings (Positive V5) with strong ability to form/stabilize emulsions. *B. subtilis* strains only had significant distance from *Acinetobacter* strains. Strains closely related to *R. erythropolis* overlapped with most of the other groups and showed low Mahalanobis distances. The understanding of the performance patterns of the species/genera fills the knowledge gap and provides guidance on the selection of strains for certain biotechnological applications. For example, *Acinetobacter* strains are suitable for emulsification applications which is evident in many studies (Navon-Venezia et al., 1995; Zosim et al., 1982) with a commercialized product, i.e. Emulsan.

5.4 Summary

Thirty-seven marine demulsifying bacteria belonging to 5 genera, namely *Acinetobacter*, *Bacillus*, *Halomonas*, *Pseudomonas* and *Rhodococcus* were reported. OS and CH were found to best explain the effectiveness of O/W emulsion breaking. Their readings were complementary and both positively correlated to the emulsion breaking ratio, which implied high cell hydrophobicity and interfacial activity were driving the emulsion breaking. *Halomonas venusta* strain N3-2A was reported as a new demulsifying bacterium which achieved 92.5% EBR for O/W emulsion within 24 h with combined forces of its extracellular biodemulsifiers (biosurfactants) and bacterial cell surfaces. Its 16S rRNA and phenotypes were characterized in detail and compared to the closely related type strains in the *Halomonas* genus. The 37 demulsifying bacteria showed species/genus specific patterns in their abilities to reduce surface/interfacial tension, produce biosurfactant, stabilize/destabilize emulsion, and adhere to hydrocarbons under the same incubation conditions. The reported marine demulsifying bacteria and the screening strategy contribute to the development of biological demulsification of oily wastewater stream as a highly effective yet environmental friendly option.

CHAPTER 6 BIOSURFACTANT PRODUCED BY A *RHODOCOCCUS ERYTHROPOLIS* MUTANT AS AN OIL SPILL RESPONSE AGENT

Cai, Q., Zhang, B., Chen, B., Cao, T., Lv, Z. (2016) Biosurfactant produced by a Rhodococcus erythropolis mutant as an oil spill response agent. Water Quality Research Journal of Canada. 51(2): 97-108.

Roles: Cai designed and conducted the study under the guidance of Dr. Baiyu Zhang and Dr. Bing Chen and acted as the first author of the manuscript. Tong Cao and Ze Lv assist in the experiments. Most contents of this paper were written by Cai and further polished by the other co-authors.

6.1 Introduction

Offshore oil spills are of tremendous concern due to the enormous economic loss and the harm to ecological systems that they may cause. Among diverse oil spill response technologies such as *in situ* burning, booming and skimming, absorption and solidification, dispersion has high feasibility and effectiveness in open water (Fingas, 2016). It is not as restricted by the limitation factors such as accessibility, weather conditions, sea states, and oil thickness as other countermeasures (Walker et al., 2000). Dispersants induce oil dispersion and they consist of surfactants and solvents. Solvents help transfer surfactants into the oil phase where they can rapidly move to oil-water interfaces that form when an oil slick encounters mixing energy, e.g., from waves, and thus beak down oil slicks into small droplets (Fingas, 2016). They are used to reduce the impact of oil on the shorelines, birds and mammals living on the water surface as well as to promote the biodegradation of oil (National Research Council, 2005). Dispersants were used as the primary combating agent for the Deepwater Horizon oil spill (Lehr et al., 2010). In total, 4.05 million liters of Corexit dispersants (Corexit 9500 and Corexit 9527) were applied on the surface, while 2.9 million liters were applied to the subsea discharge point (National Commission on the BP Deepwater Horizon Spill, 2011).

The USEPA has provided concentration benchmarks based on available ecological data to aid in the assessment of potential risk associated with some common dispersant components. The methods and the corresponding benchmarks are presented in Table 6.1. Among these dispersant chemicals, the surfactant, dioctylsulfosuccinate, sodium salt (DOSS) has the lowest benchmark value and is of the highest toxicity.

Table 6.1 Summary of USEPA analytical methods and screening levels of dispersant chemicals in water samples (Zukunft, 2010)

Compound	CAS number	EPA method ID	Technology	Reporting limits	EPA aquatic life benchmark
Propylene Glycol	57-55-6	EPA SW 846 Modified 8270	Direct inject GC/MS	500 µg/L	500 mg/L
2-Butoxyethanol	111-76-2	EPA R5/6 LC	Direct inject LC/MS/MS	125 µg/L	165 µg/L
Di(propylene Glycol) Buty Ether (DPNB)	29911-28-2	EPA R5/6 LC	Direct inject LC/MS/MS	1 µg/L	1 mg/L (chronic)
DOSS	577-11-7	EPA RAM-DOSS	LC/MS/MS	20 µg/L	40 µg/L (chronic)

The toxicity data used as the reference for the benchmark were basically LC_{50} (median lethal concentration) of constant exposures which was regarded as problematic by toxicologists (Schmidt, 2010). Moreover, this benchmark used a concentration that is highly possible to be toxic to species at certain stages, especially larvae. Kujawinski et al. (2011) published their method and results of monitoring the DOSS associated with Deepwater Horizon oil spill. The fate analysis of DOSS showed the presence of DOSS even after six months of the spill, which indicated their recalcitrant nature in the ecosystem (Zukunft, 2010).

Due to these concerns with DOSS, it is timely and important to develop better alternatives. Biosurfactant-based dispersants, with the proven features of high effectiveness, lower toxicity and persistency, can be a promising option. However, the current bottleneck of biosurfactant application is the high production cost. It was estimated that biosurfactants would cost 3–10 times of synthetic surfactants (Mulligan, 1993). The development of hyper producing mutants was proposed as one of the strategies to eliminate economic constraints (Mukherjee et al., 2006). The hyper producing mutants in the literature only belonged to a limited genus of *Pseudomonas*, *Bacillus* and *Acinetobacter* which are the producers of rhamnolipid, surfactin and emulsan, respectively (Mukherjee et al., 2006). Previously, no studies have attempted to genetically modify *Rhodococcus* strains for hyper production of biosurfactant. In addition, the dispersing abilities of the above mentioned hyper production mutants have not been investigated. The dispersant capability of a biosurfactant is highly dependant on the hydrophilic-lipophilic balance (HLB) value. When the HLB is between 10 and 12, the biosurfactant should stabilize oil-in-water

emulsions to facilitate break down of oil slicks and dispersion into the ocean (Pacwa-Płociniczak et al., 2011). A *Rhodococcus erythropolis* strain SB-1A was isolated from oily contaminated seawater in Newfoundland (Cai et al., 2015b) with proven potential to disperse crude oil in our preliminary test. Therefore, a further investigation of the oil dispersion efficacy of biosurfactants produced by the hyper production *Rhodococcus* mutant is highly desired.

The objective of this study is to investigate the biosurfactant production by a *Rhodococcus erythropolis* mutant as an oil spill response agent. The hyper producing mutant of *Rhodococcus erythropolis* strain SB-1A with oil spreading technique was screened. The resulting mutant was characterized and its feasibility as an oil spill response agent was tested. This is the first study that investigated the oil dispersion efficacy of biosurfactants produced by a hyper production mutant.

6.2 Materials and Methods

6.2.1 Bacterial strains and growth condition

Rhodococcus erythropolis strain SB-1A was isolated from seawater samples in the vicinity of offshore platforms (Cai *et al.* 2014). The strain was cultured with the production medium (PM) composed of $MgSO_4$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 0.05 g; KH_2PO_4, 3.4 g; $K_2HPO_4 \cdot 3H_2O$, 4.4 g; $(NH4)_2NO_3$, 1 g; $FeCl_3$, 0.05 g; Glucose, 1 g; nutrient broth 0.1 g; NaCl, 26 g in 1 L of distilled water, with 3% (v/v) n-hexadecane. Incubation was maintained at 30 ºC while shaking at 200 rpm (Cai et al., 2014).

6.2.2 Ultraviolet mutagenesis

The *R. erythropolis* strain SB-1A was grown to logarithmic phase and then approximately 3000 cells were plated on PM agar plates. The cells were UV radiated for 45 s with a Thermo scientific 1300 Series Class II, Type A2 Biological Safety Cabinet. This dosage of UV radiation gave around 10–20% survival of the colonies. The UV irradiated cells were then incubated on the agar plates at 30 ºC in the dark until colonies were visible (Mulligan et al., 1989).

6.2.3 Screening technique for hyper producing mutants

The UV irradiated colonies were inoculated to 2 mL Eppendorf tubes with 1 mL PM and incubated at 30 ºC while shaking at 200 rpm for 48 h. Subsequently, an oil spreading technique was used to screen hyper producing mutants (Morikawa et al., 2000). Ten µL of crude oil was gently added to the surface of 40 mL of distilled water in a petri dish (D.I. 150 mm) to form a thin oil membrane. Ten µL of bacterial culture was gently added to the center of the oil membrane. A clear zone was formed due to the activity of the surfactants. The area of the clear zone was used to reflect the concentration of the produced surfactants.

6.2.4 Determination of critical micelle dilution (CMD)

The selected hyper producing mutant was incubated in 1 L PM medium for 4 days at 30 ºC while shaking at 200 rpm. The resulting culture was used for the determination of CMD. CMD can reflect the concentration of produced biosurfactants. It was defined as the dilution of the culture broth upon reaching the critical micelle concentration (Shavandi et

al., 2011). After centrifuge at 10,000 rpm for 10 min and discarding the pellet, the 10 mL of cell free broth was diluted with distilled water, while the surface tension of each dilution was measured. The surface tension data were plotted against concentrations of the broth. The CMD values were then found as intersection points of tangential lines (Sheppard and Mulligan, 1987). As the broth consists of both aqueous and oil phase, each dilution was conducted with sonication to ensure homogeneity. Before each measurement, the sonicated solution was allowed to stand for 15–20 min to achieve equilibrium.

6.2.5 Production and recovery of crude biosurfactants

The remaining cell free broth was extracted by shaking with methyl tert-butyl ether (MTBE) of the same volume for 24 h. The upper phase was collected and concentrated with rotary evaporation. The concentrated solution was washed with petroleum ether to remove the remaining hexadecane in the solution (Kuyukina et al., 2001). After washing, the crude biosurfactant products were collected and stored at –20 ºC before analysis and testing.

6.2.6 Thin layer chromatography (TLC) analysis

After MTBE extraction and removal of upper phase, the remaining aqueous phase was again extracted with chloroform and methanol 2:1. Ten µL aliquots of the MTBE extracted solution, chloroform/methanol extracted solution and the remaining aqueous phase were added on the TLC plates. Subsequently, ninhydrin n-butanol-acetic acid and phenol-sulfuric acid were sprayed on the TLC plates respectively. The plates were then heated at 110 ºC for 10 min for color development, in order to illustrate the presence of

amino acids and carbohydrates in different extracts and the remaining aqueous phase. The recipe of ninhydrin n-butanol-acetic acid: 100 mL n-butanol dissolves 0.3 g ninhydrin followed by the addition of 3 mL acetic acid. The target compounds were amino acids, which showed red or purple. The recipe of phenol-sulfuric acid: 3 g phenol and 5 mL concentrated H_2SO_4 were added in 95 mL ethanol (Touchstone, 1992). The target compounds were carbohydrates, which showed red or brown.

The concentrated MTBE extracts were dried and re-dissolved in chloroform and subjected to TLC analysis on silica gel F_{254} with the following solvent system: chloroform/methanol/water (85:15:2, v/v/v) for lipopeptides. To detect functional groups, ninhydrin n-butanol-acetate acid stain was used.

6.2.7 Baffled flask test (BFT) for evaluation of dispersant effectiveness

The baffled flask test was conducted following the protocol proposed by Sorial et al. (2004). Briefly, artificial sea water was prepared as 3.5% sea salt solution. One hundred and twenty mL of the artificial sea water equilibrated at the desired temperature was added to the baffle flasks. One hundred µL of crude oil (from a Newfoundland offshore platform) was added on the surface of the synthetic sea water. Then, crude biosurfactants were added to the center of the oil slick. The flasks were placed on an orbital shaker and mixed for 10 min at 200 rpm. After 10 min of settling, the first 2 mL of the sample was drained from the stopcock and discarded, then 30 mL of the sample was collected in a separatory funnel and extracted three times with 5 mL dichloromethane (DCM). The extracts were then diluted to a final volume of 20 mL and subjected to spectroscopy analysis at the

wavelengths of 340, 370 and 400 nm respectively with DCM as blank. The calculation of dispersion efficiency followed the procedure below. The dispersion efficiency of the biosurfactants produced by the mutated strains was compared with Corexit dispersants and biosurfactants produced by wild type strain as references, each treatment had an oil free control

The area under the absorbance vs wavelength curve between 340 and 400 nm was calculated by using the trapezoidal rule according to Equation (1):

$$\text{Area} = \frac{(Abs_{340}+Abs_{370})\times 30}{2} + \frac{(Abs_{370}+Abs_{400})\times 30}{2} \quad (1)$$

$$\text{Concentration of the dispersed oil (g/L)} = \left(\frac{Area\ as\ determined\ by\ Equation\ (1)}{Slope\ of\ the\ crude\ oil\ calibration\ curve}\right) \quad (2)$$

$$\text{Total oil dispersed (g)} = \text{concentration of the dispersed oil} \times 20\ \text{mL DCM} \times \frac{120\ mL}{30\ mL} \quad (3)$$

$$\text{Dispersion efficiency, \%} = \frac{Total\ dispersed\ oil}{Mass\ of\ oil\ added} \times 100 \quad (4)$$

The calibration standards were prepared with crude oil-DCM stock solution which was made by adding 2 mL crude oil to 18 mL DCM. Specific volumes of 20, 50, 100, 150, 200, and 300 µL of crude oil-DCM stock were added to 30 mL of synthetic seawater in separatory funnels and extracted three times with DCM. The final DCM volume for each standard solution was adjusted to 20 mL and subjected to spectroscopy analysis with DCM as the blank at 340, 370 and 400 nm. The area of each standard was calculated according to Equation (1). The slope of the calibration curve was thus determined by plotting the area

against the concentration of the crude oil in the standards. The concentrations of biosurfactants and Corexit used were based at the same times of their CMCs. The experiments were conducted under room temperature (i.e. around 25 ºC).

6.2.8 Data analysis

All of the tests were conducted in duplicate and the typical error in the measurement was less than ±5%. The statistical analyses agreed to within 95% confidence demonstrating the accuracy of measurements reported in this study.

6.3 Results and Discussion

6.3.1 Screening of hyper producing mutant

In total, 71 UV irradiated mutants of *Rhodococcus ethroypolis* SB-1A were collected for the screening of hyper producing mutants using an oil spreading technique. In most relevant prior? studies, the high throughput method used to screen the hyper producing mutants was the hemolytic activity test (HA) (Iqbal et al., 1995; Mulligan et al., 1989) and blue agar plate test (BAP) (Lin et al., 1998; Tahzibi et al., 2004). However, Youssef et al. (2004) demonstrated that 38% biosurfactant producing strains showed no response in the HA tests. In addition, HA tests showed low correlation with surface tension indicating that HA is not a reliable method to detect biosurfactant. Meanwhile, Satpute et al. (2008) used 45 marine biosurfactant producing strains to evaluate the performance of different screening methods and discovered that the HA test was not totally reliable. In their study, only one strain showed a positive response to the BAP test. In both studies, the oil spreading technique was found as a reliable technique and was recommended by both authors. Good

correlation (r^2=0.997) was found between the concentration of biosurfactant and the diameter of the clear zone (Youssef et al., 2004). The oil spreading technique was found to have high sensitivity even when the concentration of biosurfactant was low and water-insoluble (Morikawa et al., 2000).

In Figure 7.1A, plate #1 show the testing result of the wild type strain. The remaining 71 plates show the results of the mutants. It can be seen from Figure 7.1A that Mutant 47 had the largest clear zone with a diameter of around 2.5 cm while the wild type strain had a clear zone with a diameter of around 1.5 cm **(**Figure 7.1B**)**.

6.3.2 Biosurfactant production

The CMD has been used as a measure of biosurfactant concentration (Shavandi et al., 2011). When incubated with the same medium under the same condition. Mutant #47 had a CMD value of 62.5 while the wild type strain only has a CMD value of 15.4 (Figure 7.2). The biosurfactant concentration produced by mutant #47 was 4.07 times of the wild type. The CMD test was conducted after 4 days of incubation, while the oil spreading test was conducted after 2 days of incubation. The diameter of mutant #47 was 1.7 times of the wild type. Based on the estimation of both methods, mutant #47 produced biosurfactants with higher rates after the first 48 h. Other studies found the CMD of culture broth of wide type *Rhodococcus* strains were higher than the present study. Philp et al. (2002) found that the culture of a *Rhodococcus ruber* strain had a CMD of around 90 after 4 days of incubation. Shavandi et al. (2011) found the culture broth of a *Rhococococcus* sp. strain TA6 had a CMD of around 35 under diverse conditions.

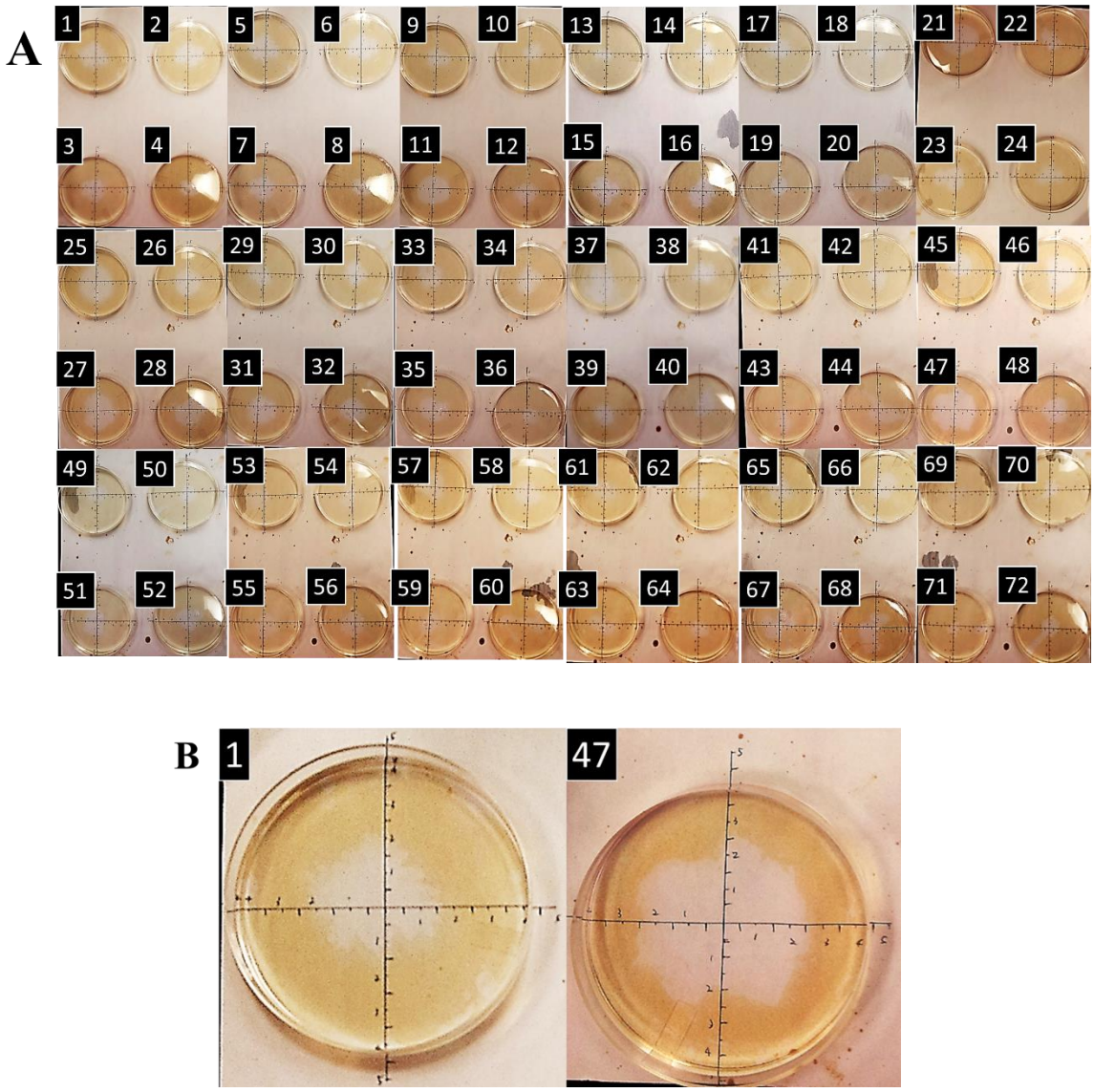


Figure 6.1 Results of oil spreading test. A) Wild strain and the 71 mutants; B) wild type and mutant #47).

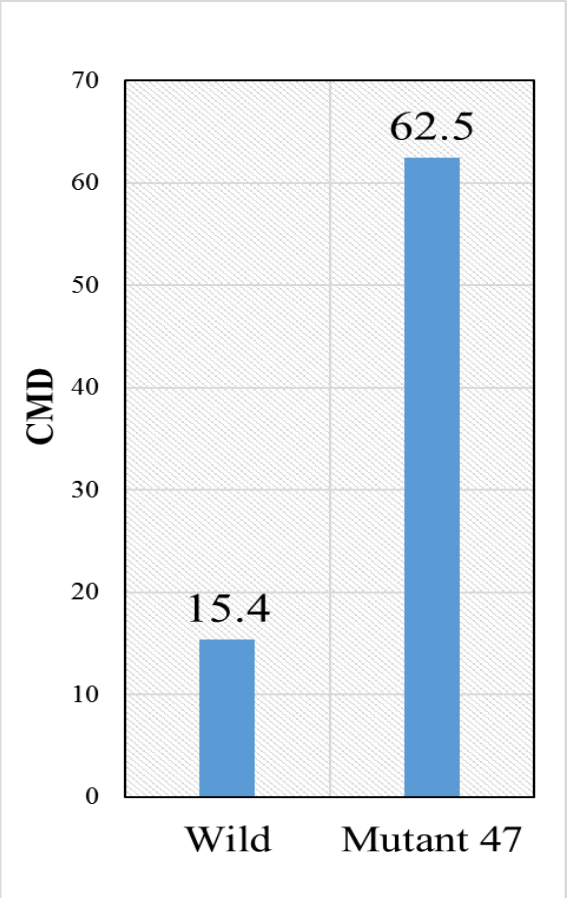


Figure 6.2 CMD improvement of mutant #47

We surmise the possible reason for the higher CMD was that both studies did not apply sonication while diluting the culture broth. In the present study, sonication was applied to ensure each dilution was homogeneous. The produced biosurfactants mainly present at the interface between aqueous and water-insoluble carbon source phases, especially for biosurfactants produced by *Rhodococcus* strains (Franzetti *et al.* 2010). The dilution procedure accompanied by sonification would be more efficient than direct dilution of two-phased culture broth. However, sonication before dilution would also lead to lower dilution factors.

6.3.3 Characterization of the produced biosurfactants

Methanol (2:1 v:v) have been commonly used for the extraction of biosurfactants from bacterial culture broth (Franzetti et al., 2010). Methyl tert-butyl ether (MTBE) was later found as a comparable alternative of the chloroform:methanol solvent system and was recommended due to its low toxicity and flammability for large scale application (Kuyukina et al., 2001). The biosurfactants are mainly composed of fatty acid moiety and either carbohydrate moiety (glycolipid) or amino acid moiety (lipopeptide) (Soberón-Chávez and Maier, 2011). As shown in Figure 7.3, MTBE extracts contained similar amount of lipopeptides with chloroform:methanol extracts. However, the MTBE extracts were concentrated for 10 times while the chloroform:methanol extracts were not concentrated. Besides, MTBE was used for the first round extraction while chloroform:methanol was used for the second round. Therefore, MTBE showed poor recovery efficiency for lipopeptides when compared with the chloroform:methanol solvent.

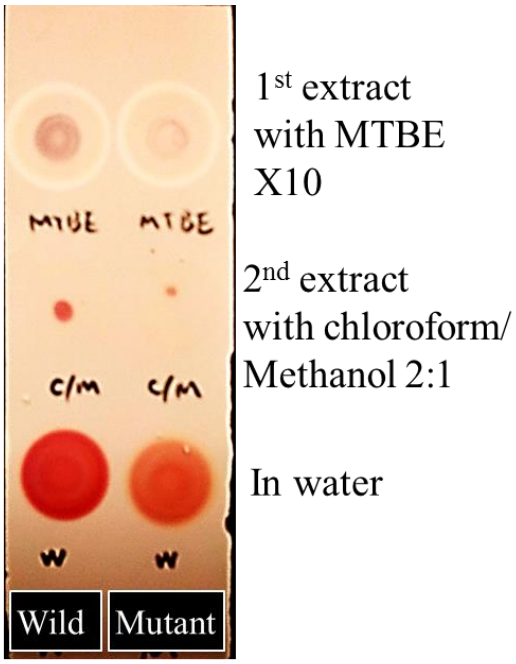


Figure 6.3 Ninhydrin stain for amino acid moiety.

In contrast, as shown in Figure 7.4, MTBE extracts contained many more glycolipids than chloroform:methanol extracts. MTBE showed good efficiency for extracting glycolipids. Moreover, the remaining culture broth after extraction still had a large amount of amino acids and limited amount of carbohydrate. This might be explained by the following: the original medium contained 1 g glucose (carbohydrates) and 0.1 g nutrient broth (amino acids); after 4 days of microbial transfer, most glucose was consumed while nutrient broth was barely used. It is surprising that the culture broth of wild type had more glycolipids and lipopeptides than mutant #47 while the biosurfactant concentration in the culture broth of mutant #47 was around 3 times higher. We surmise that mutant #47 produced certain biosurfactants that cannot be effectively extracted by both MTBE and chloroform:methanol solvent. According to the results in Figures 3 and 4, both glycolipids and lipopeptides were presented in the culture broth of the wild type strain and mutant #47.

Moreover, on the TLC plate developed with chloroform:methanol:water = 65:25:4 and stained with ninhydrin agent, the spot of lipopeptides of both wild type strain and mutant #47 appeared with the same response factor (Rf). The result indicated that the culture broth of the wild type strain and mutant #47 contained one type of lipopeptide and the lipopeptide was the same in both culture broths.

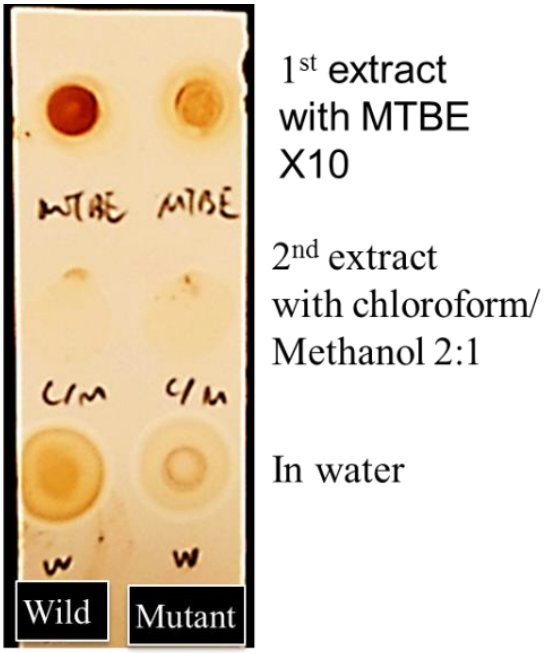


Figure 6.4 Phenol-sulfuric acid stain for sugar moiety

6.3.4 Dispersion efficiency of the *Rhodococcus Erythropolis* mutant

The dispersion efficiency of crude biosurfactants from *Rhodococcus erythropolis* SB-1A wild type strain and mutant #47 are summarized in Table 7.2**.** The dispersion efficiency of crude biosurfactants produced by mutant #47 was 1.35 times of the biosurfactants produced by the wild type strain. When compared with the Corexit dispersants, both biosurfactants had better performance than the Corexit 9527, while they were not as good as Corexit 9500. The result agreed with the conclusion of Blondina and Sowby (1997) that Corexit 9500 was generally more effective than 9527. They also found that Corexit 9500 was less affected by the variations in water salinity conditions (Blondina and Sowby, 1997). The biosurfactant from mutant #47 is close to Corexit 9500 for dispersing crude oil produced from Newfoundland offshore. The toxicity and persistency of the produced biosurfactant will be determined in future studies. The productivity could be further improved with technologies such as genome shuffling (Zhao et al., 2012) using some superior mutants screened in this study as the parent strains. Moreover, to further reduce the cost of production, the incubation media/conditions and the post-processing process can be further optimized to achieve economic production that may compete with DOSS (Mukherjee et al., 2006).

Table 6.2 Absorbance of BFT effluent and dispersion efficiency

	Mutant #47	**Wild strain**
Absorbance (340 nm)	0.329	0.25
Absorbance (370 nm)	0.176	0.13
Absorbance (400 nm)	0.114	0.8
Dispersion efficiency (% Corexit 9500)	77.26	57.34
Dispersion efficiency (% Corexit 9527)	221.45	164.34

6.4 Summary

A *Rhodococcus erythropolis* SB-1A strain isolated from oily wastewater from Newfoundland offshore was used as the parent strain to develop hyper producing mutants that produced biosurfactants as oil dispersion agents. Genetically improved biosurfactant production was studied for the first time using a *Rhodococcus* strain while such biosurfactant was barely reported as an oil spill response agent. The parent strain was previously found to produce biosurfactants with an HLB shown to disperse crude oil in our lab. UV induced mutagenesis was conducted to generate possible mutants, and subsequently, an oil spreading technique was applied as the high throughput method to screen hyper producing mutants. The oil spreading technique was found as a reliable and semi-quantitative approach to effectively screen biosurfactant hyper producing strains. Subsequently, mutant #47 was found as the superior mutant and was subjected to further analysis. The culture broth of both the wild type strain and mutant #47 contained lipopeptides and glycolipid. The lipopeptides in both cultures were the same and of a single component. The dispersion efficiency determined by BFT showed that mutant #47 was 1.35 times of the biosurfactants produced by the wild type strain. The dispersion efficiency of mutant #47 is comparable to the Corexit 9500, while it is better than Corexit 9527 when dispersing the Newfoundland offshore crude oil.

The detailed composition and structure of the produced biosurfactants will be analyzed in future studies. Several mutants developed in this study will be used as the parent strains for genome shuffling to further improve the productivity and the corresponding dispersion efficiency.

CHAPTER 7 BIOSURFACTANTS PRODUCED BY *RHODOCOCCUS ERYTHROPOLIS* MUTANT M36, *BACILLUS SUBTILIS* N3-1P, *EXIGUOBACTERIUM* SP. N4-1P, AND *PSEODOMONAS AERUGINOSA* AS MARINE OIL SPILL DISPERSANTS

Cai, Q., Zhu, Z., Chen, B., Zhang, B. (2018) Biosurfactants produced by *Rhodococcus erythropolis* mutant M36, *Bacillus subtilis* N3-1P, *Exiguobacterium* sp. N4-1P, and *Pseodomonas aeruginosa* as marine oil spill dispersants. Journal of Hazoudous Materials *(Ready for submission)*

Roles: Cai designed and conducted the study under the guidance of Dr. Baiyu Zhang and Dr. Bing Chen and acted as the first author of the manuscript. Zhiwen Zhu assist in the experiments. Most contents of this paper were written by Cai and further polished by the other co-authors.

7.1 Introduction

Marine oil spills are of great concerns due to the long-term significant impacts on ecological integrity and enormous economic loss that may cause. For instance, the catastrophic Deepwater Horizon oil spill in 2010 has caused deaths of millions of seabirds and billions of fish eggs (Corn, 2010) and threatened over $5.5 billion of fishing, tourism, industrial economic entities and more than 200,000 employment opportunities (Hagerty and Ramseur, 2010). To combat marine oil spills, the application of dispersants is considered as an important response option that can yield net environmental benefits in many instances; particularly, for large offshore spills (Lee et al., 2015; Li et al., 2016) and spills in harsh environment conditions (Li et al., 2016; Mullin, 2014).

Traditional dispersants are chemically synthesized and can be defined as a blend of surfactants, solvents, and additives (Kujawinski et al., 2011). They can reduce oil-water interfacial tension, and with sufficient mixing energy, break an oil slick into small droplets and diffuse them into the ocean column (Li et al., 2016). After Deepwater Horizon oil spill, increasing research efforts have been put to formulate new dispersants in forms of particles, polymer/gel and bio-based materials (Nyankson et al., 2016). Examples of newly developed particle-based dispersants include functionalized carbon black (Powell and Chauhan, 2014), surfactant-loaded halloysite nanotubes (Owoseni et al., 2014), and modified natural minerals (Dong et al., 2014). Polymer/gel based dispersants such as dendritic polymers (Geitner et al., 2012) and gel-like dispersants produced by ExxonMobil (Nedwed, 2010; Nedwed et al., 2011) have been reported recently. New bio-based dispersants include plant-based lecithin (Nyankson et al., 2015) and cactus mucilage

(Alcantar et al., 2015), animal-based diethylolamide and monoethylolamide fats (Asadov et al., 2012), and biosurfactant (Cai et al., 2016; Freitas et al., 2016; Lv et al., 2016; Marti et al., 2014; Moshtagh and Hawboldt, 2014) based dispersants.

In view of the perceived public health and environmental concerns of synthesized dispersants, the development of environmentally benign and bio-based dispersants has been highly motivated (Doshi et al., 2018; Nyankson et al., 2016). As one of such bio-based dispersants, biosurfactant based products have myriads of advantages such as low toxicity, short half-lives, and specific effectiveness under extreme conditions of temperature, salinity and pH, as well as usage of recycling wastes as production substrates (Shekhar et al., 2015). However, research efforts on examining the effectiveness of biosurfactants as the sole surface active component of oil spill dispersants have been very limited. Cai et al. (2016), Lv et al. (2016), and Cao (2015) studied biosurfactant-based dispersants produced from *Rhodococcus* strains, while Marti et al. (2014) and Freitas et al. (2016) investigated those produced from *Bacillus* strain and yeast *Candida bombicola*, respectively. Some of these studies used results from emulsification assay, surface tension reduction measurement, oil displacement test and dispersant-to-hexane ratio test as indications of oil dispersion efficiency (Freitas et al., 2016; Moshtagh and Hawboldt, 2014). However, they did not use established testing systems such as Baffled Flask Test (BFT) and Swirling Flask Test (SFT) to confirm the effectiveness of the produced biosurfactant based dispersants.

To fill the knowledge gaps, four types of biosurfactant-based dispersants from strains belonging to the genus of *Rhodococcus*, *Bacillus*, *Pseudomonas*, and *Exiguobacterium*

were produced. Their dispersion effectiveness against a light crude oil, Arabic light crude oil (ALC), and a medium crude oil, the weathered Alaska North Slope crude oil (WANS), under different dispersant: oil ratio (DOR) was evaluated using BFT. The diverse properties of these biosurfactants were characterized to facilitate the understanding of the governing factors of their dispersion efficiencies and possible synergic effects. Lastly, we touched on the eco-toxicity and biodegradation aspects of these biosurfactant-based dispersants.

7.2 Materials and Methods

7.2.1 Bacteria and biosurfactants

Rhodococcus erythropolis M36 was a 3rd generation mutant obtained using runs of UV random mutagenesis and screening based on previous works in our lab (Cai et al., 2016; Lv et al., 2016). The crude biosurfactant product was generated following the pre-developed protocol (Cai et al., 2016; Kuyukina et al., 2001). The main biosurfactant ingredients were identified as glycolipids (trehalose lipids) (Franzetti et al., 2010). *Bacillus subtilis* N3-1P was isolated from petroleum hydrocarbon contaminated coastal sediment from North Atlantic Canada (Cai et al., 2014). The crude biosurfactant product was obtained using a protocol described previously (Zhu et al., 2016). The primary active ingredients were identified as lipopeptides (surfactins) (Zhu et al. 2018). *Exiguobacterium* sp. N4-1P was isolated from petroleum hydrocarbon contaminated coastal sediment from North Atlantic Canada (Cai et al., 2014). The crude biosurfactant product was obtained following the protocol of Cai et al. (2017a) and the effective ingredient was identified as a complex mainly composed of lipopeptides with C16: 0 (32.18%) and C18: 0 (40.99%) as

the primary fatty acids (Cai et al., 2017a). We here name such a complex "Exmulsins". Crude Rhamnolipid product was obtained from a commercial source produced by *Pseudomonas aeruginosa* mutants. All the biosurfactants used in this study were crude products. For the ease of discussion, the key effective ingredients will be used to refer each crude biosurfactant product as trehalose lipids, sufactins, exmulsins and rhamnolipids, respectively.

7.2.2 Crude oils and Corexit 9500A

Crude oil samples, i.e. ALC and WANS were provided by the Environment and Climate Change Canada. The Corexit 9500A used in this study was provided by Nalco Environmental Solutions LLC (Taxes, USA).

7.2.3 Surface tension reduction and critical micelle concentration

The surface tension (ST) of each crude biosurfactant solutions or the Corexit 9500A solution was determined in triplicate with a surface tensiometer (DuNouyTensiometer, Interfacial, CSC Scientific) at room temperature (Cai et al., 2015b).

The ST of each solution decreases with the increased amount of a surfactant until it reaches a plateau once a biosurfactant product is presented in micelles form. The concentration corresponding to this turning point is defined as critical micelle concentration (CMC) as shown in Figure 7.1(A) (Rosen and Kunjappu, 2012). However, due to the presence of impurities, sometimes the abruptness of the curve cannot be observed. Instead a relatively flat curve is plotted as exemplified in Figure 7.1(B) (Sheppard and Mulligan, 1987). In the latter case, the transition point on the curve is determined as point

D in Figure 1(B) which is drawn from the intersection point (B) of two linear fitting lines to intersect the tangent E-F at 90 degrees (Sheppard and Mulligan, 1987). In this study, data points in each surface tension vs. concentration plot were fitted with both two linear lines and a non-linear curve with the highest fitting R^2. When the data fitted better with the non-linear curve, the method shown in Figure 1B is used; otherwise, the method shown in Figure 1A is adopted.

CMC is a direct indication of the surface activity of a surfactant product. The lower the CMC, the smaller quantity of the surfactant is needed to reach the micellar stage, thus the greater the associated surface activity (Rosen and Kunjappu, 2012). CMC is, therefore, a more important measurement than the actual quantity of a surfactant used, especially when applied with a biosurfactant product, as its surface activity may vary with cultural conditions and between batches of production. In this study, the concentration of a biosurfactant product used in studies is expressed as times of CMC.

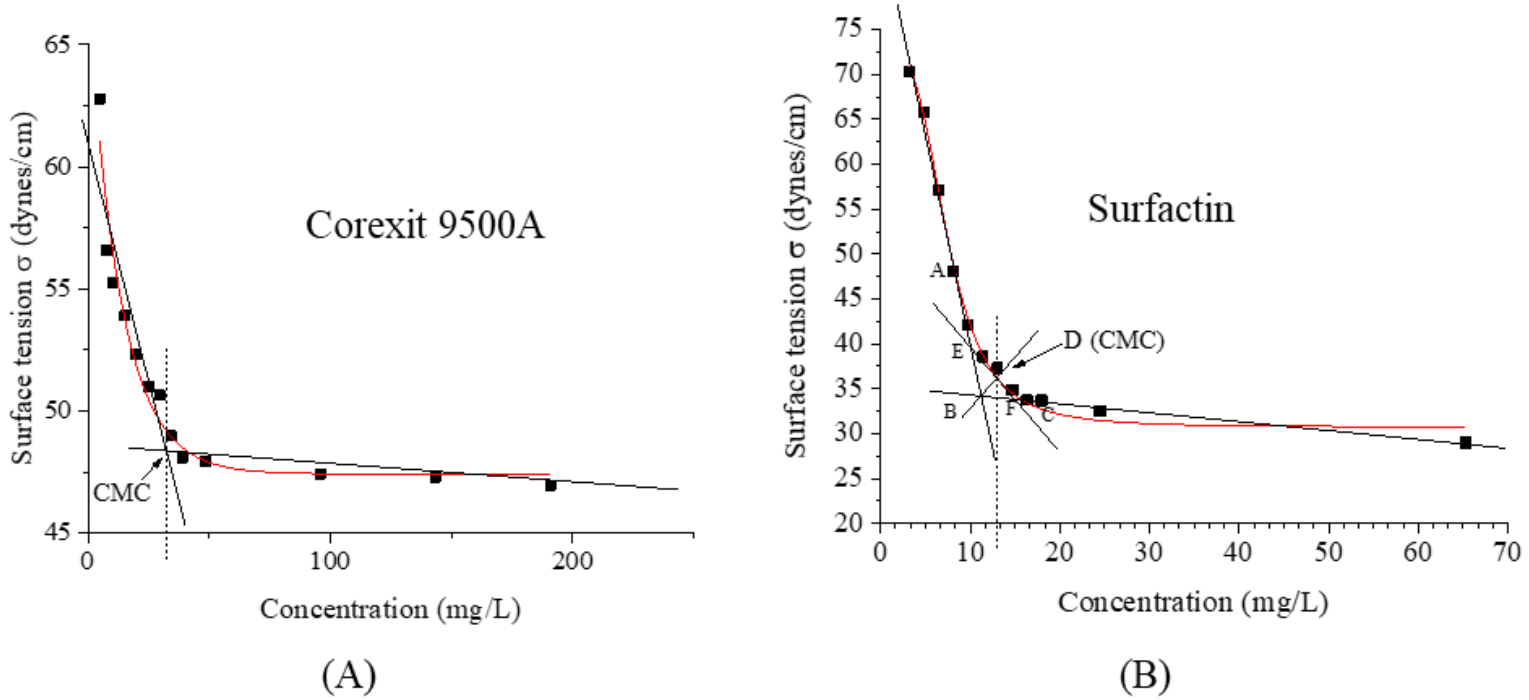


Figure 7.1 Determination of CMCs of dispersants using concentration vs surface tension plots. (A) Corexit 9500A with abrupt transition; (B) Surfactins with flat transition (data points are the average of triplicated measurements)

7.2.4 Emulsification index and critical emulsion concentration

The emulsification index (E24) of a biosurfactant solution or Corexit 9500A solution was determined by adding 1 mL of diesel to the same volume of a surfactant solution, mixing the liquid with a vortex for 2 min, and leaving it to stand for 24 h. The emulsification activity was evaluated by E24 using Equation (1):

$$E24=H_{EL}/H_S\times100\% \quad (1)$$

where H_{EL} is the height of the emulsion layer and H_S is the height of the total solution (Cai et al., 2017a).

It was observed that E24% of a surfactant product follows a similar trend as surface tension, in that with increasing concentration of a surfactant, the E24% value increases until it reaches a plateau (Figure 7.2). Beyond which, the percentage of the emulsion formed in the system will not be changed even with additional surfactant. We term this transition point as critical emulsion concentration (CEC). Like CMC plots, the transition can be abrupt or flat depending on the level of impurities. Therefore, similar principle is adopted to determine the CECs as shown in Figure 7.2A and 7.2B, in which CEC is used as a direct indication of the emulsification ability of a surfactant. The lower the CEC, the smaller quantity of a surfactant is needed to reach the maximum emulsion level, thus the more effective the product serves as an emulsifier. It is a more reasonable parameter than the quantity of a biosurfactant as it rules out the activity fluctuations in different production conditions. In this study, concentrations of emulsifiers used are calculated as times of CEC to reflect their capacity for stabilizing oil/water emulsion.

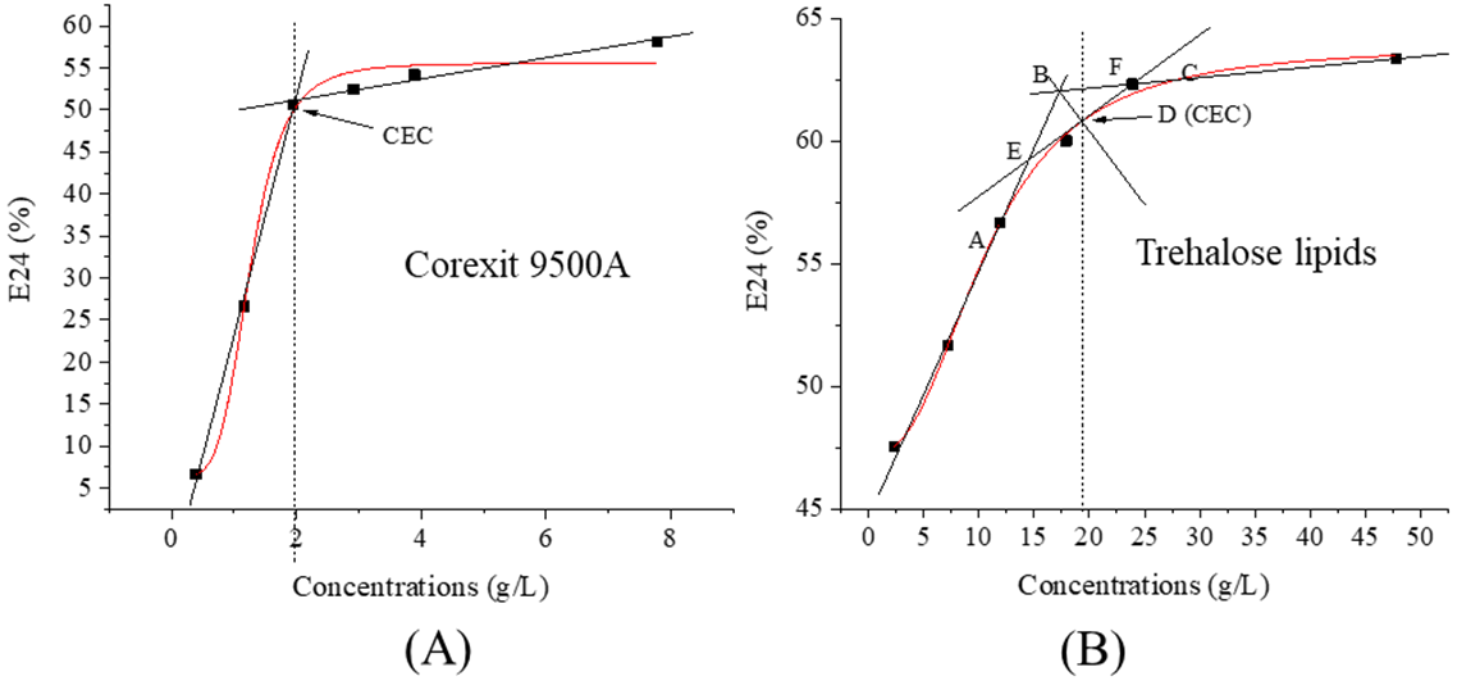


Figure 7.2 Determination of CECs of dispersants using concentration vs E24 plots. (A) Corexit 9500A with abrupt transition; (B) Trehalose lipids with flat transition (data points are the average of duplicated measurements)

7.2.5 Baffled Flask Test (BFT)

The BFT was conducted following the protocol proposed by Sorial et al. (2004). Each biosurfactant product was used as a biodispersant without the addition of solvents and/or additives for the testing. Briefly, the artificial sea water was prepared as 3.5% sea salt solution. One hundred and twenty mL of the artificial sea water equilibrated at the desired temperature was added to the baffle flasks. One hundred µL of a crude oil (ALC or WANS) was added on the surface of the synthetic sea water. Each biosurfactant product or Corexit 9500A was then added to the center of the oil slick. The flasks were placed on an orbital shaker and mixed for 10 min at 200 rpm. After 10 min of settling, the first 2 mL of the sample was drained from the stopcock and discarded, then 30 mL of the sample was collected in a separatory funnel and extracted three times with 5 mL dichloromethane (DCM). The extracts were further diluted to a final volume of 20 mL and subjected to spectroscopy analysis at the wavelengths of 340, 370 and 400 nm, respectively, with DCM as blank. Each testing run was triplicated. The calculation of oil removal percentage (ORP%) followed the procedure stated below (Lee et al., 2012; Wang et al., 2013).

The area under the absorbance vs wavelength curve between 340 and 400 nm was calculated by using the trapezoidal rule according to Equation (2):

$$\text{Area} = \frac{(\text{Abs}_{340}+\text{Abs}_{370})\times 30}{2} + \frac{(\text{Abs}_{370}+\text{Abs}_{400})\times 30}{2} \quad (2)$$

$$\text{Concentration of the dispersed oil (g/L)} = \left(\frac{\text{Area as determined by Equation (2)}}{\text{Slope of the crude oil calibration curve}}\right) \quad (3)$$

$$\text{Total oil dispersed (g)} = \text{concentration of the dispersed oil} \times 20\ \text{mL DCM} \times \frac{120\ \text{mL}}{30\ \text{mL}}$$

Oil removal percentage (ORP) is a measurement of the fraction of oil removed from the surface slick layer, and it is calculated as:

$$\text{Oil removal percentage (ORP)} = \frac{\text{Total dispersed oil}}{\text{Mass of oil added}} \times 100\% \quad (5)$$

The calibration standards were prepared with a crude oil-DCM stock solution which was made by adding 2 mL crude oil (ALC and WANS) to 18 mL DCM. Specific volume of 20, 50, 100, 150, 200, and 300 µL of crude oil-DCM stock was added to 30 mL of synthetic seawater respectively in separatory funnels and extracted three times with DCM. The final DCM volume for each standard solution was adjusted to 20 mL and subjected to spectroscopy analysis with DCM as the blank at 340, 370 and 400 nm. The area of each standard was calculated according to Equation (2). The slope of the calibration curve was thus determined by plotting the area against the concentration of the crude oil in the standards.

The concentration of each biosurfactant solution tested was determined as the CMC (mg/L) or CEC (g/L) equivalent of Corexit 9500A at various DORs. When the CMC was applied, the biosurfacant concentration under a DOR of 1:50, 1:25 or 1:10 equaled to 0.5 (level 0.5), 1 (level 1) and 2.5 (level 2.5) CMCs, respectively, the same as that of Corexit 9500A. Once applying CEC, at DOR of 1:50, 1:25 and 1:10, the concentration of Corexit 9500A or each biosurfactant solution was $8.2*10^{-3}$ (level 0.5), $1.6*10^{-2}$ (level 1) and $4.1*10^{-2}$ (level 2.5) CECs, respectively. All BFT tests were conducted in triplicates and under room temperature (i.e., 20 ºC). Permutational analysis of variance (PERMANOVA)

was conducted to examine the effect of treatments, using PERMANOVA in vegan-package under R-project for statistical computing (R Core Team, 2013).

7.2.6 Investigation of synergetic effects using response surface methodology

Preliminary results indicated possible synergetic effects between rhamnolipids and exmulsins, which primarily functions as a surface tension reducing agent and an oil-water emulsion stabilizer, respectively. In order to simulate the interplay of these two forces in the dispersion of a crude oil, a response surface methodology (RSM) namely central composite design (CCD) was used. In addition, the optimized combination of these two biosurfactant products was determined using the desirability function (DF) as maximize criterion of the response (Ng et al., 2010). The actual levels of the independent variables were coded as (-α,-1,0,+1 and +α) according to Equation (6)

$$Z_i = \frac{x_i - x_0}{\Delta x_i} \quad (6)$$

Where Z_i is the dimensionless value of an independent variable, x_i represents the real value of the independent variable, x_0 is the real value of the independent variable at the center point, and Δx_i is the step change. The number of experimental runs is equal to Equation (7)

$$N = 2^k + 2k + n_c \quad (7)$$

Where k and n_c are the number of factors and center point runs, respectively.

A general model is presented as equation (8) for simulation and prediction:

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \sum_{j=1}^{k} \beta_{ij} x_i x_j + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \varepsilon \quad (8)$$

where y is the response, $\beta_0, \beta_i, \beta_{ij}$ and β_{ii} are the regression coefficients of variable for intercept, linear, interaction and quadratic terms, respectively. x_i and x_j are the independent variables, k is the number of factors and ε is the residual term. Experimental data were fitted to a second-order polynomial equation, and regression coefficients were obtained. The analysis of variance (ANOVA) was performed to evaluate the statistical significance and adequacy of the developed models. Design-Expert v.8.0.6 (Stat-Ease, Inc., Minneapolis, USA) were used for the design and analysis of the experiment.

The layout of the design is shown in Table 7.1 with concentrations of rhamnolipids (CMC) and exmulsins (CEC*10^{-3}) as the independent variables and ORP% as response. In total, 5 levels of the factors were examined, leading to 12 runs with 4 replicates. Identical design was used for both ALC and WANS.

Table 7.1 Setup of the CCD

Factors	Units	Levels				
		-α (-1.414)	-1	0	+1	+α (+1.414)
A.Rhamnolipids	CMC	0.086	0.5	1.5	2.5	2.914
B. Exmulsins	CEC*10^{-3}	1.006	2	4.4	6.8	7.794
Response	Unit					
ORP	%					
Center point=4		Total runs =12				
Experiment layout as coded factors						
Standard order	Run order	A. Rhamnolipids (CMC)		B. Exmulsins (CEC*10^{-3})		
1	9	-1		-1		
2	10	1		-1		
3	4	-1		1		
4	1	1		1		
5	8	-1.414		0		
6	6	1.414		0		
7	3	0		-1.414		
8	12	0		1.414		
9	7	0		0		
10	5	0		0		
11	2	0		0		
12	11	0		0		

7.2.7 Partitioning coefficients in water/n-octane system

The partitioning coefficients K_p in the water/n-octane system was measured using the equilibrium surface tension method developed by Catanoiu et al. (2011) to indirectly determine the surfactant concentration in the water and octane phases. Aqueous surfactant solutions with an initial surfactant concentration ($C_{water,0}$) of lower than 1 CMC (~0.7 CMC) were prepared, and their equilibrium surface tension were measured using DuNouyTensiometer. A hundred mL of each surfactant solution was then placed in a separating funnel and 10 mL n-octane was carefully placed on top of the aqueous surfactant solution without causing turbulence to avoid the formation of emulsion. The separating funnel was caped, and the two phases were kept in contact to allow the surfactant to diffuse from the water phase to the oil phase until the partitioning equilibrium condition was reached. Subsequently, the two phases were carefully separated by draining the aqueous phase from the bottom. During the process, the surfactant concentration in the water phase was reduced and the surface tension of the solution was thus increased as illustrated in Figure 7.3. Such an increase will be detected with the tensiometer and K_p can be determined using Equation (9)

$$K_p = \frac{C_{oil}}{C_{water}} = \frac{V_{water}}{V_{oil}} \left(\frac{C_{water,0} - C_{water}}{C_{water}} \right) \quad (9)$$

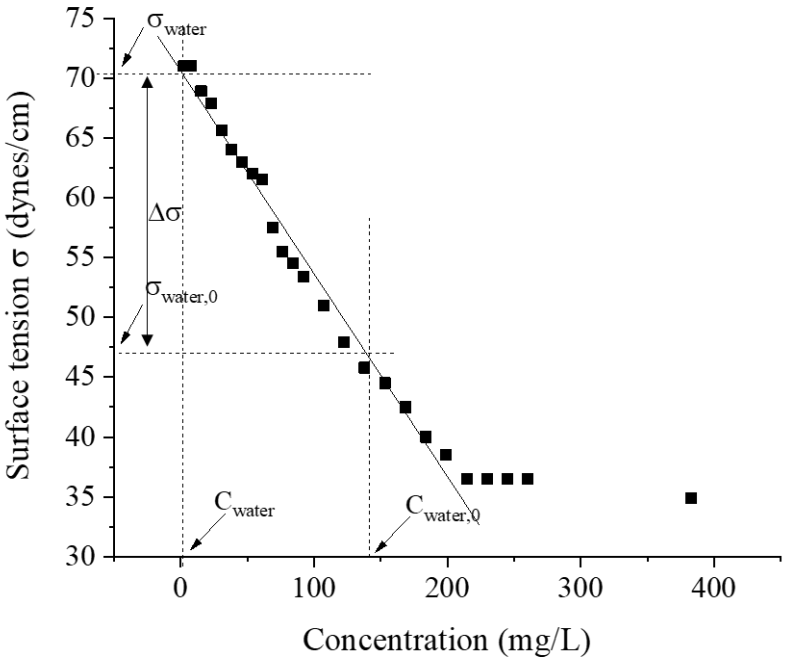


Figure 7.3 Determination of K_p using the relationship between surface tension of the solution and the concentration of the surfactants

7.2.8 Biosurfactant fatty acid composition analysis

The lipid content was extracted from each crude biosurfactant product according to Parrish (1999). Each extracted sample was homogenized in a 2:1 mixture of ice-cold chloroform: methanol with a Polytron PCU-2-110 homogenizer (Brinkmann Instruments, Rexdale, Ontario, Canada). The chloroform extracted aqueous layer was added to the sample to bring the ratio of cholorform:methanol:water to 8:4:3. The sample was sonicated for 4 to 10 minutes in an ice bath and centrifuged at 5000 rpm for two minutes. The bottom organic layer was removed. Chloroform was then added back to the extraction test tube and the entire procedure was repeated 3 more times. All liquids located in the organic layers were pooled into a lipid-free vial. The mixture was concentrated using a flash-evaporator (Buchler Instruments, Fort Lee, N.J.). Afterwards, the lipid extract was trans-esterified using methanol/sulfuric acid for 1 hour at 100^{o}C. The fatty acid methyl ethers (FAMEs) in each extract were analyzed on a HP 6890 GC/Flame Ionized Dectector (GC/FID) equipped with a 7683 Autosampler. The column temperature began at 65^{o}C and was held at this temperature for 0.5 minutes. The temperature was then ramped to 195^{o}C at a rate of 40^{o}C/min, held for 15 minutes, and finally ramped to a temperature of 220^{o}C at a rate of 2^{o}C/min. This final temperature was held for 0.75 minutes. Peaks were identified using retention times from standards purchased from Supelco, namely 37 component FAME mix, bacterial acid methyl ester mix, polyunsaturated fatty acids (PUFA) 1 and PUFA 3. Each test was conducted in triplicate.

7.2.9 Microtox® toxicity test

The acute general or systemic toxicity was measured using the Microtox toxicity assay according to standard method (ISO, 2007). The method is based on measured light emission in the auto luminescent bacterium *Vibrio fischeri*. Its light intensity relates to the amount of toxic stress. The tests were carried out using a Microtox M500 analyzer (Osprey Scientific, Edmonton, Canada) according to the Microtox Manual (1992: standard procedure). The half effective concentrations (EC50) of biosurfactants after 5 min and 15 min of exposure time were reported.

7.2.10 Total C mineralization of biosurfactant-based dispersants

AER-800 Research Respirometer (Challenge technology, Arkansas, USA) with flasks contained their own oxygen supply system and oxygen-uptake monitoring system was used to track the mineralization process of the surfactins, trehaloselipids and Crexit 9500A. Seawater samples from Newfoundland offshore were collected to prepare 50 ppm dispersant solutions (500 mL). Nutrient solutions including phosphor buffer, magnesium sulfate solution, calcium chloride solution and ferric chloride solutions were prepared according to USEPA standard method 5210B. One ml of each nutrient solution was added to the dispersant/seawater solution. All treatments were continuously mixed (500 rpm) using Teflon coated stir-bars. Respirometer experiments were carried out following the manufacturer protocol in duplicate at room temperature (20^{o}C). The readings are data of oxygen uptake in mg for treatments and controls and are recorded every minute during the time course of the experiments. Negative controls for the respirometry experiments contained seawater and nutrient solutions only. The respiration measured in the negative

controls (2 replicates) was subtracted from the respirometer treatments. Mineralization was estimated as a fraction of theoretical chemical oxygen demand (ThOD). An empirical conversion factor from ThOD to total organic carbon (TOC) equals 3.3 was adopted (Hodzic, 2011). TOC of each dispersant solution was measured in triplicate using TOC-L analyzer (Shimadzu, Kyoto, Japan), following the standard manufacturer protocol.

7.3 Results and Discussion

7.3.1 Surface activity and emulsification ability

The ability of the 4 types of crude biosurfactant products and Corexit 9500A to reduce surface tension of seawater? with increased concentrations is shown in Figure 7.4. Their emulsion forming ability (illustrated as E24%) with increasing concentrations is shown in Figure 7.5. Crude rhamnolipids did not form emulsion even when used without any dilution. The density of the rhamnolipids was found as 1.016 g/ml and CEC was represented as >1016 g/L. The surface activity and emulsification ability of all products are summarized in Table 7.2.

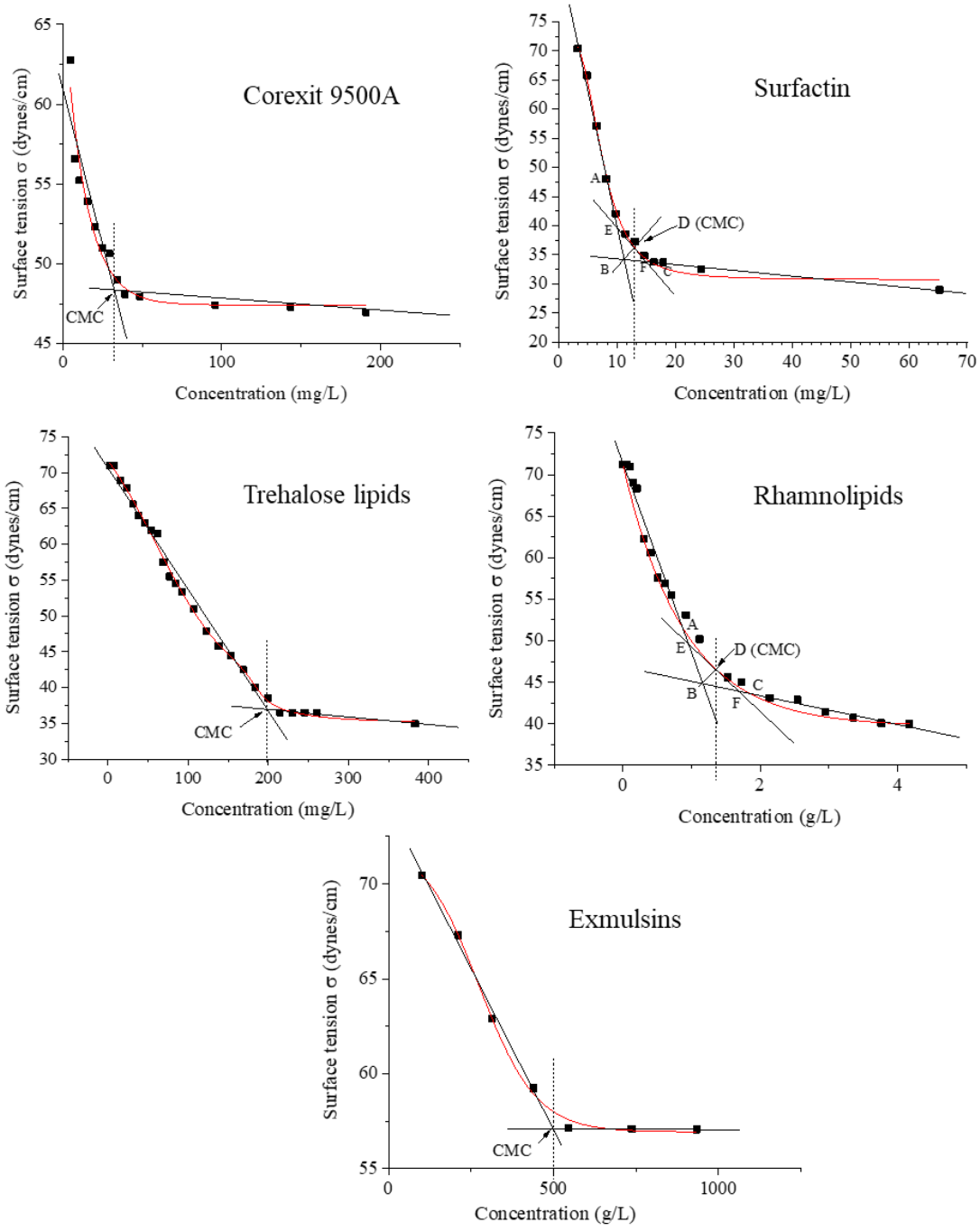


Figure 7.4 The surface tension of the surfactant solution vs the concentration of the surfactants and the determination of CMCs (n=3)

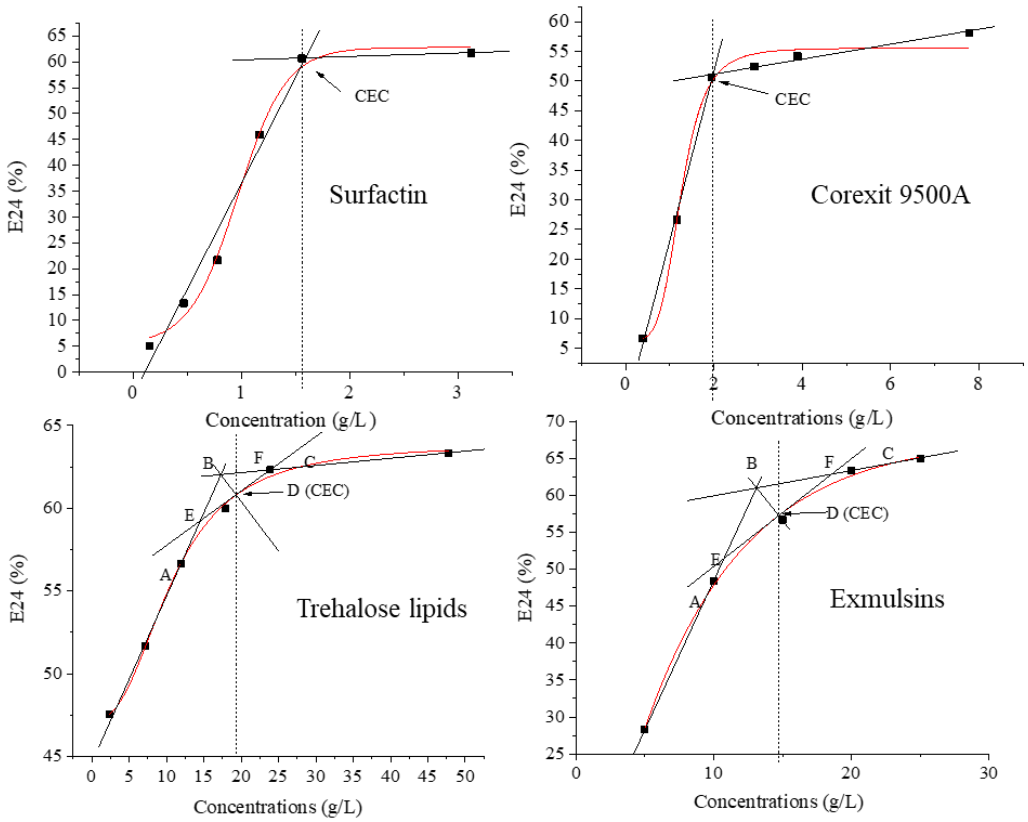


Figure 7.5 The E24% of the surfactant solution vs the concentration of the surfactants and the determination of CECs (n=2)

Table 7.2 Surface activity and emulsification ability of the biosurfactants and Corexit 9500A

Surface-active parameters	Units	Surfactins	Rhamno-lipids	Trehalose lipids	Exmulsins	Corexit 9500A
CMC	(mg/L)	13.01	1355.71	199.16	500512.65	32.41
Minimum surface tension	(dynes/ cm)	28.98	39.97	34.90	57.06	39.96
CEC	(g/L)	1.56	>1016	19.37	14.73	1.98
Maximum E24	(%)	60.00	0.00	63.33	65.00	56.25
CMC/1000CEC	1	0.0083	<0.0013	0.010	33.98	0.016

Surfactins was found to reduce the surface tension of water from 71.2 dynes/cm to as low as 28.98 dynes/cm. Trehalose lipids was the second best at reducing the surface tension and would reach 34.9 dynes/cm. Both rhamnolipids and Corexit 9500A could lower the surface tension to around 40 dynes/cm. Exmulsins was found with the least ability to reduce surface tension to only 57.06 dynes/cm. According to the CMCs, the crude surfactins had the strongest surface activity and could reach micellar stage with the concentration as low as 13.01 mg/L, which is about a 2.5-fold effectiveness of Corexit 9500A. Crude trehalose lipids came the second with CMC of 199.16 mg/L, followed by Crude rhamnolipids which had a CMC of 1355.71 mg/L. Exmulsins was found with limited ability to reduce surface tension even at a high concentration, which implied the ingredient that could reduce surface tension in exmulsins might be a minor component.

In terms of emulsification ability, surfactins, trehalose lipids and exmulsins all showed excellent emulsification ability with maximum E24 above 60%, especially exmulsins which had maximum E24 of 65%. These E24 readings were slightly higher than those of Corexit 9500A, which had the maximum E24 of 56.25%. Surfactins was found with the lowest CEC of 1.56 g/L indicating its superior ability to form emulsion even with a small quantity. Corexit 9500A also had a low CEC of 1.98 g/L, while trehaloselipids and exmulsins had their CECs at 19.37 and 14.73 g/L, respectively. In our study, the crude rhamnolipids had a relatively low level of purity and effective concentration resulting in a high CMC and undetectable CEC.

CMC/1000CEC is a unitless term calculated according to Equation (10) to reflect the relative ability of surface/interfacial tension reduction vs. emulsification.

$$CMC/1000CEC = \frac{CMC\ mg/L}{CEC\ g/L} \times \frac{1}{1000} \times \frac{g}{mg} \quad (10)$$

The higher value of $CMC/1000CEC$ suggests that the emulsification ability of the associated product is stronger than the surface/interfacial tension reducing ability, and vice versa. Emulsins was found with the highest CMC/1000CEC of 33.98. Its surface tension ability was negligible when compared with its emulsification ability. The primary function of emulsins was serving as a bioemulsifier. In the contrast, rhamnolipids had a relatively weak emulsification ability and will be primarily considered as a surface tension reducing agent. Other than these two extremes, surfactins and trehalose lipids both showed duel abilities of surface tension reduction and emulsion stabilizing in a balanced way. Their CMC/1000CEC values were comparable to Corexit 9500A albeit a little smaller.

7.3.2 Oil dispersion effectiveness

US EPA recommended a DOR of 1:50 to 1:10 for Corexit 9500A application (USEPA, 1995), and DOR of 1:25 has been also frequently used as the target DOR for oil dispersion (Techtmann et al., 2017). It was also found in our study that when applied at DOR 1:25, Corexit 9500A concentration in the BFT system is around its CMC level. Therefore, these three levels, 1:50 (level 0.5), 1:25 (level 1) and 1:10 (level 2.5), were selected to evaluate the oil dispersion effectiveness of biosurfactants. The concentrations of biosurfactants used in this study were designed based on the CMC (for surfactins, trehaloselipids and rhamnolipids) or CEC (for exmulsins) equivalents of Corexit 9500A at

these three levels. The performances of the selected biosurfactants when treating ALC and WANS in artificial seawater are shown in Figure 5 and Figure 6, respectively.

As shown in Figure 7.6, the ORP% for Corexit 9500A when treating ALC at all three concentrations were around 80% without significant difference. Surfactins and trehalose lipids exerted the same level of performances when used at level 1 and level 2.5. However, when used at level 0.5, their ORP% decreased dramatically. Rhamnolipids showed moderate ORP% at level 1 and level 2.5 (~50%) and when used at level 0.5, it achieved ORP% <20%. Exmulsins was found with negligible oil dispersion ability at all three levels. Similar trends were observed when using WANS (Figure 7.7). Corexit 9500A dispersed around 60% of WANS crude oil under all three DORs. Surfactins and trehalose lipids could also reach the same degree of effectiveness when using at level 1 and level 2.5. Rhamnolipids showed significantly lower ORP%. Emulsins was again found without oil dispersion ability.

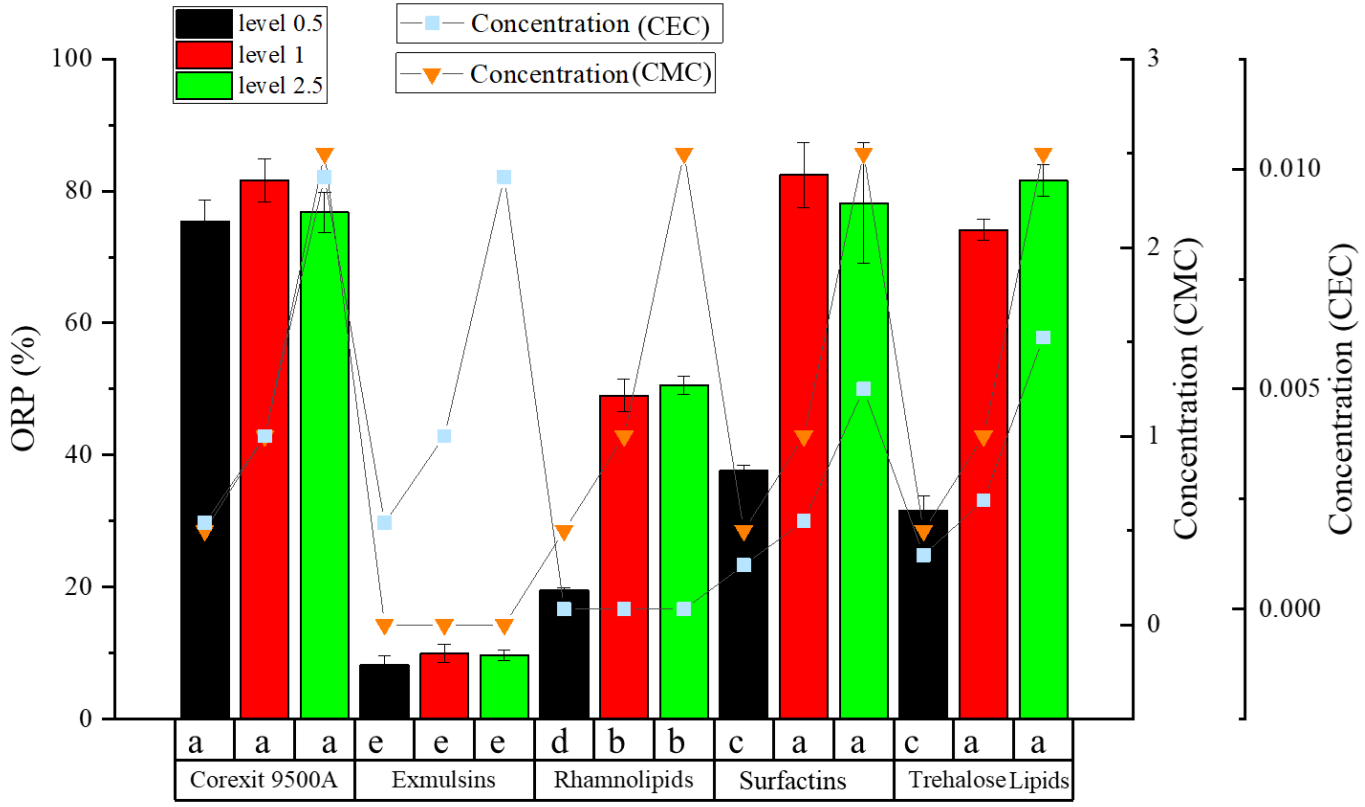


Figure 7.6 Oil dispersion effectiveness of diverse dispersants when treating ALC. Bar chart represents the ORP% at three different levels. Line and scatter overlays show the concentrations of dispersants in terms of unit CMC or unit CEC. The same letters underneath the bar chart indicate no significant difference according to PERMANOVA ($p > 0.05$)

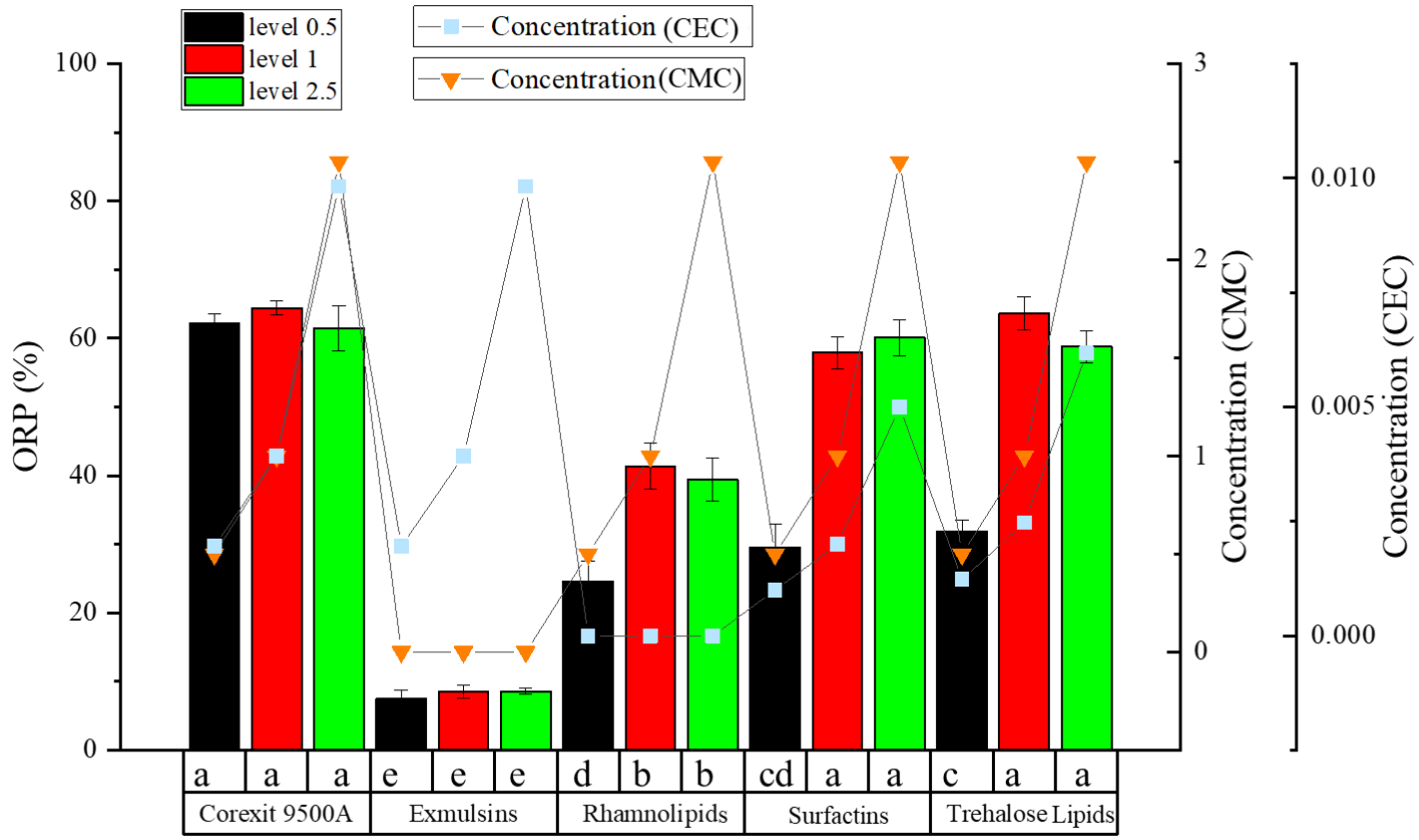


Figure 7.7 Oil dispersion effectiveness of diverse dispersants when treating WANS. Bar chart represents the ORP% at three different levels. Line and scatter overlays show the concentrations of dispersants in terms of unit CMC or unit CEC. The same letters underneath the bars indicate no significant difference according to PERMANOVA ($p > 0.05$)

Taking a closer look at the surface tension reduction and emulsification ability of these biosurfactants (illustrated as concentrations in unit CMC and CEC in Figures 7.5 and 7.6), it can be found that Corexit 9500A, surfactins and trehalose lipids shared a common trait of having a balanced surface/interfacial tension reduction and emulsification ability. Under the same CMC concentration, Corexit 9500A had a slightly higher CEC value than surfactins and trehalose lipids. At level 0.5, Corexit 9500A had the emulsification ability similar to that of trehalose lipids and surfactins when using at level 1, which might explain the superior performance of Corexit 9500A at level 0.5 to trehalose lipids and surfactins. In the contrast, crude rhamnolipids and exmulsins in this study were found with either surface reduction ability or emulsification ability, and their ORP% values were significantly lower than the other three types of dispersants with balanced abilities.

Studies concerning the effectiveness of dispersants may shed light on the mechanisms of biosurfactants-based dispersants. On one hand, oil dispersants are expected to lower the oil/water interfacial tension so as to entrain small oil droplets into the water column even at relatively lower energy inputs, as the mixing energy required to entrain oil droplets is proportional to oil-water interfacial tension as shown in Equation (11) (NRC, 2005).

$$W_k = \gamma_{o/w} A_{o/w} \tag{11}$$

Where W_k is the mixing energy (g-cm^2-s^{-2}), $\gamma_{o/w}$ is the oil-water interfacial tension (dynes-cm^{-1}), and $A_{o/w}$ is the oil-water interfacial area (cm^2). In the oil spill models, the role of dispersants has also been linked to their impacts on oil-water interfacial tension

(Johansen et al., 2015; Li et al., 2017; Zhu et al., 2016). On the other hand, several studies considered oil dispersant process essentially an emulsification process, and the most efficient dispersants blends have the structural compatibility of surfactants that offers a stable arrangement at the interface (Athas et al., 2014; Brochu et al., 1986). Moreover, such understanding also leads to the development of particle-based dispersants forming "Pickering emulsion" by particle arrangement at the oil droplet-water interface (Nyankson et al., 2016; Pickering, 1907). It was also found that a combination of surfactants and particles can provide the optimized emulsion in which surfactants lower the interfacial tension to facilitate drop formation, while particles stabilize the formed droplets (Kraft et al., 2010). Our study echoed that the biosurfactants with both abilities to reduce surface/interfacial tension and stabilize oil-water emulsion delivered the best oil dispersion effectiveness. To further examine this hypothesis, we studied the synergetic effects of rhamnolipids and exmulsins.

7.3.3 Synergetic effects

The response surface plots of independent variables (the concentration of rhamnolipids in unit CMC and the concentration of exmulsins in unit CEC*10^{-3}) and the response (ORP%) are illustrated in Figure 7.7. Generally, a higher concentration of rhamnolipids led to a higher level of ORP. The response surface plots for both ALC and WANS demonstrated that the combination of rhamnolipids and exmulsins at varies levels led to a much higher value of ORP% than when they were used alone.

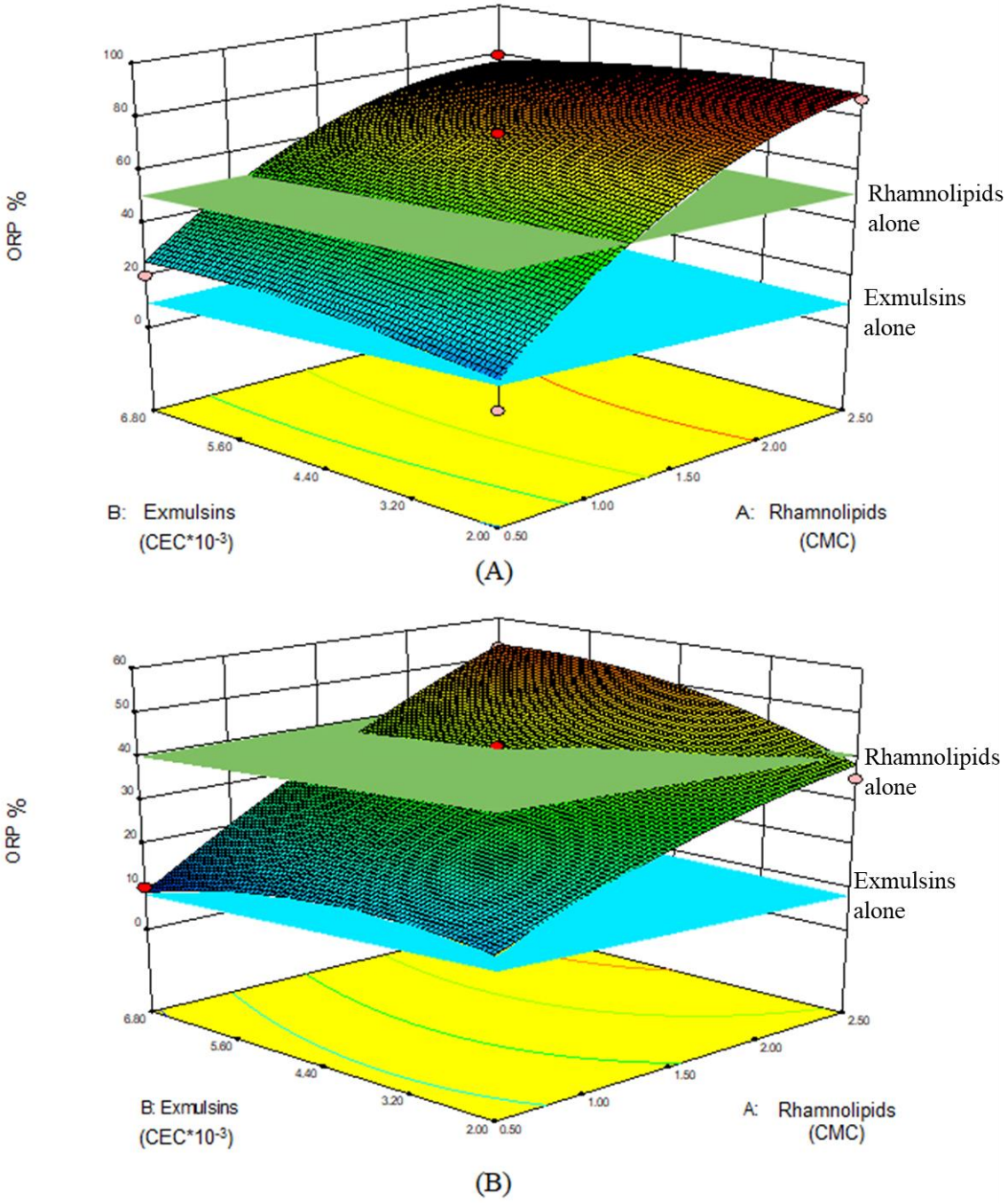


Figure 7.8 Response surface plots depicting the interactions of independent variables and their response (A) when dispersing ALC (B) when dispersing WANS. Green and blue planes represent the ORP% levels of rhamnolipids and exmulsins when used alone.

Results of the ANOVA test, the simulated model, as well as the optimized conditions for synergetic dispersions are shown in Tables 7.3 and 7.4 for ALC and WANS, respectively. Both models were significant with insignificant Lack of Fit. The model R^2 values of the two models were 0.96 and 0.95, respectively, indicating the response can be well demonstrated by the explanatory variables. The two predicted R^2 values were in reasonable agreement with both adjusted R^2 values (difference <0.2). Adequate precision is a measure of the signal to noise ratio. The value of 15.33 and 14.90, respectively, (> the desirable value 4) indicated adequate signals. Coefficient of Variation (C.V. %) is the error expressed as a percentage of the mean. PRESS is a measure of how well a particular model fits each point in the design. Both C.V.% and PRESS values indicated model adequacy. In addition, DF was used to find the maximum ORP% when the variables are in the range of the CCD design (Derringer and Suich, 1980). When treating ALC, the optimized condition was achieved at a concentration of 2.33 CMC for rhamnolipids and of 3.01 CEC*10^{-3} for exmulsins. The optimized ORP% led to the highest effectiveness and reached as high as 87.22% which was found to be comparable to Corexit 9500A, surfactins and trehalose lipids. When treating WANS, the optimized condition was found with 2.5 CMC of rhamnolipids and 6.33 CEC*10^{-3} of exmulsins. The required concentration of exmulsins was significantly higher when treating the heavier oil WANS. The optimized ORP% of the WANS oil is 53.56%, which is slightly lower than that of Corexit 9500A, surfactins and trehalose lipids. When treating both the oils, the optimized ORP% values were significantly higher than those of the treatments when using rhamnolipids and exmulsins alone.

Table 7.3 Results of ANOVA test of fitted model, the simulated model and the optimized conditions for synergetic dispersion of ALC

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	8572.83	5.00	1714.57	25.60	0.0006	significant
A-Rhamnolipid (CMC)	7307.59	1.00	7307.59	109.11	< 0.0001	
B-Exmulsins (CEC*10^{-3})	12.51	1.00	12.51	0.19	0.6807	
AB	85.41	1.00	85.41	1.28	0.3019	
A^2	1161.76	1.00	1161.76	17.35	0.0059	
B^2	83.30	1.00	83.30	1.24	0.3074	
Residual	401.86	6.00	66.98			
Lack of Fit	302.88	3.00	100.96	3.06	0.1914	not significant
Pure Error	98.98	3.00	32.99			
Cor Total	8974.69	11.00				

Std. Dev.	8.18	R^2	0.96
Mean	58.34	Adj R^2	0.92
C.V. %	14.03	Pred R^2	0.74
PRESS	2329.77	Adeq Precision	15.33

Simulation model:

$$ORP\% = 69.73 + 30.22 \times A - 1.25 \times B - 4.62 \times A \times B - 13.47 \times A^2 - 3.61 \times B^2$$

Optimized conditions and the solution:	
Rhamnolipid (CMC)	2.33
Exmulsins (CEC*10-3)	3.01
ORP%	87.22

Table 7.4 Results of ANOVA test of fitted model, the simulated model and the optimized conditions for synergetic dispersion of WANS

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	2581.17	5.00	516.23	25.21	0.0006	significant
A-Rhamnolipid (CMC)	2273.38	1.00	2273.38	111.02	< 0.0001	
B-Exmulsins (CEC*10^{-3})	31.36	1.00	31.36	1.53	0.2621	
AB	117.46	1.00	117.46	5.74	0.0537	
A^2	49.22	1.00	49.22	2.40	0.172	
B^2	136.13	1.00	136.13	6.65	0.0419	
Residual	122.86	6.00	20.48			
Lack of Fit	30.01	3.00	10.00	0.32	0.8108	not significant
Pure Error	92.86	3.00	30.95			
Cor Total	2704.04	11.00				

Std. Dev.	4.53	R^2	0.95
Mean	31.58	Adj R^2	0.92
C.V. %	14.33	Pred R^2	0.86
PRESS	378.47	Adeq Precision	14.90

Simulation model:

$$ORP\% = 36.5 + 16.86 \times A + 5.42 \times B + 5.42 \times A \times B - 2.77 \times A^2 - 4.61 \times B^2$$

Optimized conditions and the solution:	
Rhamnolipid (CMC)	2.50
Exmulsins (CEC*10-3)	6.33
ORP%	53.56

The results supported the hypothesis that when combing the surface/interfacial reduction force and emulsification stabilizing force of a biosurfactant product in a balanced manner, its oil dispersion effectiveness can be significantly improved. This finding is valuable for the formulation of biosurfactant-based oil dispersants.

7.3.4 Partitioning coefficients of biodispersants in water/n-octane system

According to the definition, the partitioning coefficients Kp in water/n-octane system reflect the affinity of the biosurfactant towards the oil phase vs. aqueous phase. They can also illustrate the tendency of the surfactant molecular migrating from one phase to another phase (Catanoiu et al., 2011). As shown in Table 7.5, the affinities of the crude biosurfactants and Corexit 9500A towards oil phase are following the order of trehalose lipid>rhamnolipid>Corexit 9500A>surfactins. When surfactants present in a dynamic equilibrium between the continuous phase (water) and the disperse (oil) phase at interface, the surfactants with relatively high affinities towards aqueous phase can desorb from a droplet and diffuse through the aqueous phase, especially in marine oil spill scenarios when water is typically more abundant (Athas et al., 2014; Riehm and McCormick, 2014). This may leave the droplet interfaces relatively vulnerable for coalescence (Morrison and Ross, 2002). Therefore, surfactants with higher affinity towards the oil phase may have a slight upper-hand in this regard, such as lechitin (Athas et al., 2014). In our case, trehalose lipids might sustain the dispersion without diffusing into the aqueous phase when compared with rhamnolipids and surfactins. Future studies will further explore this aspect.

Table 7.5 Partitioning coefficients Kp of biodispersants in water/n-octane system

Sufactant	Δσ	Kp	log Kp
Corexit	0.033	0.052	-1.29
Trehalose Lipids	22.68	449.29	2.65
Rhamnolipid	7.55	4.85	0.69
Surfactins	0.017	0.0043	-2.37

Δσ is the difference in surface tension of aqueous phase before the test and when the system achieves equibilium. It is calculated using the following equation: $\Delta\sigma = \sigma_{water,0} - \sigma_{water}$

7.3.5 Fatty acid composition of biosurfactant-based dispersants

The fatty acid composition of the hydrophobic moiety help explains some properties of the selected biosurfactants, especially their oil affinity (Table7.6). The hydrophobic tails of trehalose lipids primarily consisted of fatty acid chains of C16:0, C16:2ω4 and C18:1ω9. In the case of rhamnolipids, C18 chains, especially the unsaturated ones dominated the product. Surfactins had fatty acids in a shorter range with anteiso C15:0, C16:0 and anteiso C17:0 as the primary fatty acids. Exmulsins had C16:0 and C18:0 as the primary fatty acids. Biosurfactants have been produced by microorganisms as complex mixtures of up to 40 congeners which varied intensely at the hydrophobic moieties (Monteiro et al., 2007). This is quite a unique feature of biosurfactants when compared with chemically synthesized surfactants. The variation extended the structural diversity and accorded biosurfactants remarkably intriguing behaviors at interfaces (Soberón-Chávez and Maier, 2011). The variance in the congeners can fine-tune their emulsification properties (Das et al., 2014) as well as the shape and formation of the self-assembly (Dhasaiyan et al., 2017). Such feature if better understood and employed effectively may serve as remarkable leverage for biosurfactant-based dispersants.

During the commercialization of biosurfactants, 70%-80% of the overall cost has been spent on the downstream processing which removes impurities and precisely harvests the target components (Santos et al., 2016). When biosurfactants are directly applied to the environment as treating agents, their crude forms may have higher cost-to-benefit ratios as some expensive purification steps can be eliminated (Freitas et al., 2016).

Table 7.6 Fatty acid composition of the hydrophobic moieties of the selected biosurfactants (n=3; the primary fatty acids are in bold, fatty acids constitute <1% are omitted)

Fatty acids	% of total fatty acids			
	Trehalose lipids	Rhamnolipids	Surfactins	Exmulsins
14:0	N.D.	0.03	0.63	2.58
14:1	3.02	N.D.	N.D.	3.73
i15:0	3.53	N.D.	6.28	2.38
ai15:0	4.23	1.62	**37.63**	1.07
15:1	2.28	N.D.	N.D.	3.05
i16:0	3.42	N.D.	**23.03**	0.63
ai16:0	2.56	N.D.	0.38	0.98
16:0	**15.95**	3.80	7.38	**32.18**
16:1ω11	2.70	N.D.	0.15	N.D.
16:1ω9	1.56	0.05	N.D.	0.46
16:1ω7	2.43	0.10	N.D.	0.30
16:1ω5	6.45	N.D.	N.D.	N.D.
i17:0	N.D.	N.D.	6.00	2.00
ai17:0	5.24	0.54	**12.25**	1.40
16:2ω4	**13.66**	0.21	0.23	N.D.
17:0	3.16	N.D.	0.19	1.13
17:1	2.87	N.D.	N.D.	N.D.
18:0	2.33	**4.56**	2.25	**40.99**
18:1ω9	**15.95**	**78.88**	0.61	4.45
18:1ω6	2.82	N.D.	N.D.	N.D.
18:1ω7	N.D.	0.77	N.D.	1.42
18:2ω6	N.D.	**4.26**	0.41	N.D.
20:4ω3	2.08	N.D.	N.D.	N.D.
22:0	N.D.	2.53	N.D.	N.D.
22:1ω9	N.D.	N.D.	N.D.	1.48

N.D.: not detectable

In the case of biosurfactant-based dispersants, it will be a tradeoff between the purification cost and the quantities of biodispersants required. Surfactins had a significantly lower CMC than Corexit9500A and is already a competitive candidate with an effective DOR of 1:53.3 (w/w). Further purification procedure will be developed for trehalose lipids, exmulsins and rhamnolipids to improve the overall competitiveness in potential biodispersants market.

7.3.6 Microtox® toxicity of biosurfactant-based dispersants

Microtox® toxicity testing results of the 4 types of biosurfactant-based dispersants are listed in Table 7.7. For trehalose lipids or exmulsins, the toxicity was not detectable even when the concentrations were way above its saturation levels. Rhamonlipids was found with EC50 at 0.968 g/L after 5 min of exposure and 0.977 g/L after 15 min of exposure. Surfactins was found with the highest toxicity effects and had EC50 at 0.243 g/L after 5 min of exposure and 0.255 g/L after 15 min of exposure, which was still slightly less toxic than Corexit 9500, which was found with an EC50 of 0.17 g/L (Fuller et al., 2004).

The toxicity observed here may reflect the antimicrobial properties of the biosurfactants. Surfactins and rhamnolipids have been known for their antimicrobial properties and have been applied as therapeutic agents (Rodrigues et al., 2006). Their mode of action may be elicited by their surface/membrane active properties, such as anti-adhesive activity (Cameotra and Makkar, 2004). The effects of biosurfactant-based dispersants at

the oil-water-microbe interfaces, and the subsequent impacts on biotransformation of hydrocarbon will be an interesting topic, which needs further exploration.

Table 7.7 Microtox® toxicity test results for biosurfactant-based dispersants

Biosurfactants	EC50 (g/L)			
	5 min exposure	95% confidence range	15 min exposure	95% confidence range
Surfactins	0.243	0.122-0.486	0.255	NA[a]
Trehalose	>6.150	NA[b]	>6.150	NA[b]
Rhamnolipid	0.968	0.730-1.285	0.977	0.614-1.556
Exmulsins	>5.005	NA[b]	>5.005	NA[b]

[a]Regression confidence couldn't be calculated as 2 out of 4 levels gave 0 readings after exposure

[b]Regression confidence couldn't be calculated as all 4 levels gave readings with insignificant differences after exposure

7.3.7 Total C mineralization of bio-dispersants

The total C mineralization of trehalose lipids, surfactins and Corexit 9500A are illustrated in Figure 8.8. During the 30-day treatment, trehalose lipids reached total mineralization around 45%, which is higher than surfactins (~30%). The biodegradation of surfactins had a lag phase of about 1 day, while trehalose lipids experienced a 3-day lag phase (Figure 7.9). The longer lag phase for trehalose lipids may be introduced by lower water affinity. Trehalose lipids and surfactins showed significantly higher biodegradation rates than Corexit 9500A (Figure 7.9B). The curves of oxygen uptakes of Corexit 9500A and negative control entangled during the measurement, resulting in a fluctuating mineralization% curve. The final mineralization% of Corexit 9500A was in a range of 10%-20%. The concentration of bio-dispersants and Corexit 9500A used in the tests was set at 50 ppm, which is around the upper range limit of studies that worked on the biodegradation of Corexit 9500A (Brakstad et al., 2018; McFarlin et al., 2014). We selected a relatively high concentration in this study as the bio-dispersants (e.g., crude trehalose lipids) may have higher effective DORs (i.e. higher expected concentrations) in the field than Corexit 9500A. The results here thus illustrated the relative biodegradation rates and their persistency in the environments.

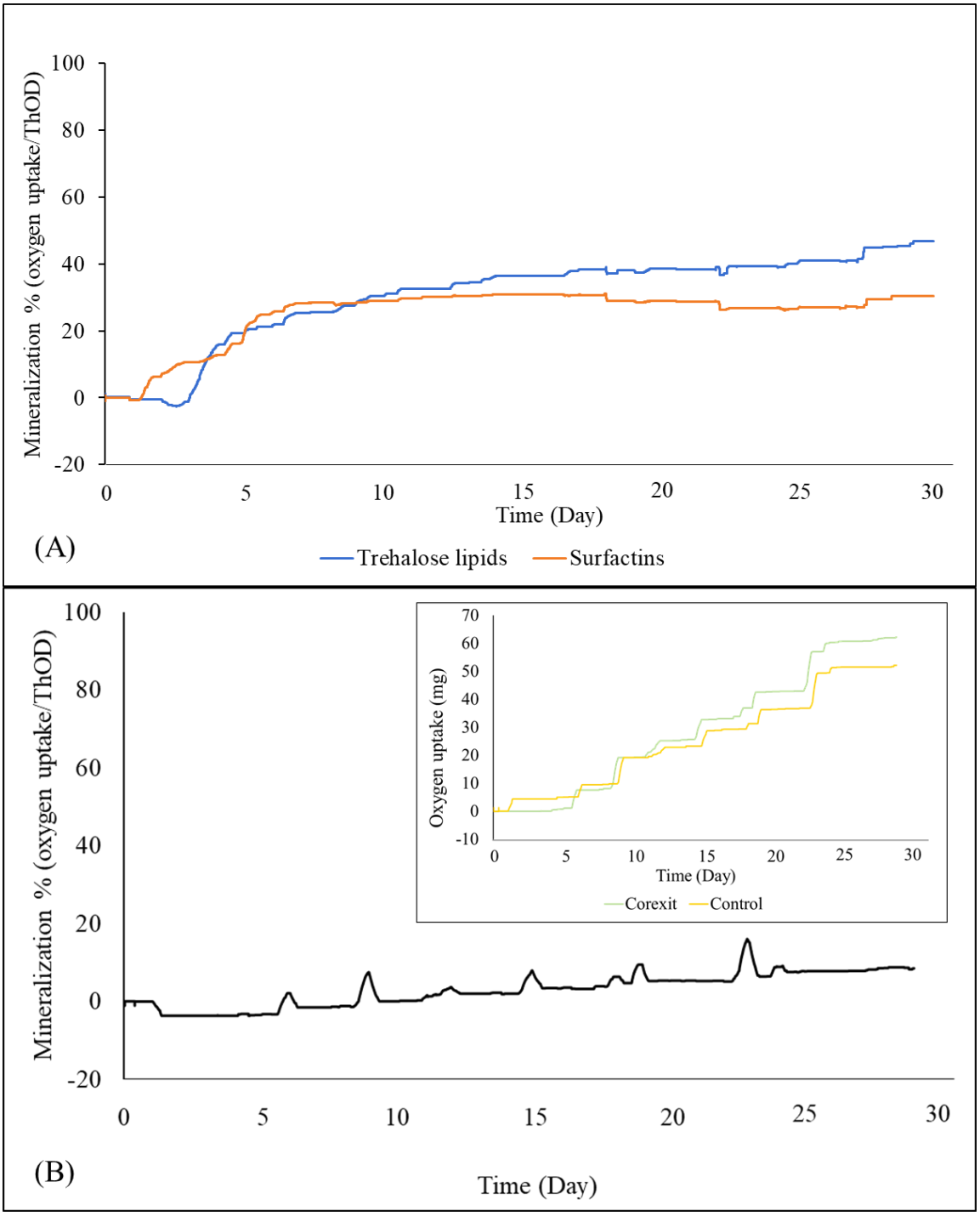


Figure 7.9 Total C mineralization of dispersants during 30 days in seawater. (A) Trehalose lipids and surfactins; (B) Corexit 9500A (insert: oxygen uptake of Corexit 9500A and negative control). Data are the average of duplicates.

7.4 Summary

Trehalose lipids and surfactins, when applied at concentrations above 1 CMC exerted comparable dispersion effectiveness to Corexit 9500A for treating both ALC and WANS. Rhamnolipids (primarily as a surface/interfacial tension reducing agent) and exmulsins (primarily as emulsifier) when used alone showed significantly lower dispersion effectiveness at all test levels, and when used together exhibited synergetic effects that led to similar performances with trehalose lipids and surfactins. Our study demonstrated that when combining the abilities of surface/interfacial tension reduction and emulsion stabilization in a balanced manner, the optimum oil dispersion effectiveness was achieved. Such findings facilitated the understanding of the mechanisms behind biosurfactant-based oil dispersion and support the development of new formulas. The affinities of 4 types of dispersants towards oil phase followed the order of trehalose lipids>rhamnolipids>Corexit 9500A>surfactins. Surfactins as dispersant achieved effective DOR as low as 1:53.3 (w/w). Trehalose lipids showed low toxicity and high biodegradation rate when compared with other biodispersants, but need further purification to reduce the effective DOR. Both surfactins and trehalose lipids have potential to be competitive candidates for oil spill dispersion.

CHAPTER 8 CONCLUSIONS AND RECOMMENDATIONS

8.1 Conclusions

Surfactants are amphiphilic compounds that can affect interfacial properties. Biosurfactants are surfactants produced by microorganism during their growth. They have many advantages over the traditional chemically synthesized surfactants, such as environmentally friendly, renewable, enormous structural diversity, ability to function in wide ranges of pH, temperature and salinity as well as greater selectivity, lower critical micellar concentrations (CMCs), and the feasibility to use renewable and waste streams/by-products as the source of production. They are considered as multifunctional biomolecules of the 21st century with a thriving global market share. They have been primarily applied as environmental remediation agents and oilfield chemicals owing to their environmental friendly virtues. The bottleneck of biosurfactants applications lays in their production costs and low yields. Among the key components involved in a biosurfactant production process, the inoculum/microorganism is the engine of the process, which determines the maximum yield potential and the functionality of biosurfactants. The thesis aims at developing novel and robust biosurfactant producing microorganisms and their hyper-production mutants and to examine the functionality of the produced biosurfactants and their potential applications in oil contaminate control.

From offshore oil and gas platforms in North Atlantic Canada, crude oil, formation water, drilling mud, treated produced water and seawater samples were collected for screening potential biosurfactant producers. In total, 114 biosurfactant producers belong to 8 different genera, i.e. *Bacillus, Rhodococcus, Halomonas, Alcanivorax, Exiguobacterium,*

Halomonas, *Pseudomonas and Streptomyces* were identified and characterized. Phytogenetic trees based on 16S ribosomal Deoxyribonucleic acid (16S rDNA) were constructed with isolated strains plus their closely related strains and isolated strains with biosurfactant producers in the literature, respectively. The distributions of the isolates were site and medium specific. The richness, diversity and evenness of biosurfactant producer communities in oil and gas platform samples have been analyzed. Diverse isolates were found with featured properties such as effective reduction of surface tension, producing biosurfactants at high rate and stabilization of water-in-oil or oil-in-water emulsion. The producers and their corresponding biosurfactants had promising potential in applications such as offshore oil spill control, enhancing oil recovery and soil washing treatment of petroleum hydrocarbon contaminated sites.

Alcanivorax atlanticus N3-2A^{T} was found as a novel species with intriguing surface activity. Cells are Gram-reaction-negative, short non-motile rods, 1–2 mm * 0.5 mm. When growing on the DSMZ medium 514, the colonies are circular, entire, convex and transparent. Moderately halophilic. Grows in 1–15% (w/v) NaCl (optimum 3–5 %) and at 4–37 ^{o}C (optimum 25–30 ^{o}C). The principal fatty acids were C16:0 (19.6%), C18:1 (19.4%), C16:1 (12.0%) and 3-OH C12:0 (11.7%). Among the 95 carbon sources in the Biolog system (GN2 plate), tests are positive for utilization of arabinose, arabitol, cellobiose, dextrin, galactose, glutamic acid, D,L-alanine, D,L-glucose, L-asparagine, L-proline, acetate, D, L-lactate, propionate, methyl pyruvate, methyl succinate, succinate, sebaccinate, γ hydroxybutyric Acid, bromosuccinic acid, Tween 40 and 80. Grows well in Atlas oil agar with n-alkane or benzoate as carbon source. Produces biosurfactants capable

of reducing surface tension or stabilize oil/water emulsions while utilizing different substrates. The type strain, N3-2A^T, was isolated from coastal sediment of the Atlantic Ocean. The DNA G+C content of the type strain is 63.1 mol%.

Exiguobacterium N4-1P was reported herein as a bioemulsifier producer for the first time. The strain was found closely related to *E. oxidotolerans* strain T-2-2^T, *E. antarcticum* B7^T, and *E. antarcticum* B7^T with similarities of 99.47, 98.70 and 98.63%, respectively. Its phenotypic properties such as metabolic fingerprints, membrane composition, and cell morphology were determined. Different carbon sources were used for bioemulsifier production and diesel was confirmed to stimulate the yield effectively. The produced bioemulsifier is a complex mainly composed of lipopeptides with C16:0 (32.18%) and C18:0 (40.99%) as the primary fatty acids. The produced bioemulsifier could form emulsions effectively with diverse hydrocarbons. No foams were formed during the production and applications, which would facilitate the commercialization. The bioemulsifier was stable over a wide range of salinity (0-25%), pH (2-12), and temperature (below 50^{o}C). *Exiguobacterium* N4-1P and the produced bioemulsifier fills knowledge gaps and have promising application potential in diverse fields, especially for soil washing and bioremediation targeting subsurface hydrocarbon pollution.

Oily wastewater is a large waste stream produced by a number of industries. This wastewater often forms stable oil-in-water (O/W) emulsion. These emulsions require demulsification in order to effectively treat the water prior to release. Although biological demulsification of O/W emulsion has advantages over traditional approaches, its development is at a preliminary stage with few demulsifying bacteria reported and a need

for effective screening methods for such bacteria. In this study, thirty-seven novel marine O/W emulsion demulsifying bacterial strains belonging to 5 genera and 15 species were reported. Cell hydrophobicity and interfacial activity played key roles in the emulsion breaking. One of the highly effective demulsifying bacteria, *Halomonas venusta* strain N3-2A was identified and characterized. Both its extracellular biosurfactant and cell surface contributed to demulsification resulting in breaking of 92.5% of the emulsion within 24 hr. A high throughput and effective screening strategy targeting O/W emulsion breaking bacteria using oil spreading test coupled with cell hydrophobicity test was proposed. In addition, the 37 demulsifying bacteria showed a certain degree of species/genus specific patterns of surface activity and cell hydrophobicity. The novel bacteria and the screening strategy have promising potential for the biological demulsification of O/W emulsions and oily wastewater treatment.

A *Rhodococcus erythropolis* SB-1A strain isolated from oily wastewater from Newfoundland offshore was used as the parent strain to develop hyper producing mutants that produced biosurfactants as oil dispersion agents. Genetically improved biosurfactant production was studied first time based on a *Rhodococcus* strain while such biosurfactant was barely reported as an oil spill response agent. The parent strain was previously found with proper HLB to disperse crude oil in our lab. UV induced mutagenesis was conducted to generate possible mutants, and subsequently, an oil spreading technique was applied as the high put through method to screen hyper producing mutants. The oil spreading technique was found as a reliable and semi-quantitative approach to effectively screen biosurfactant hyper producing strains. Subsequently, mutant #47 was found as the superior

mutant and was subjected to further analysis. The culture broth of both the wild type strain and mutant #47 contained lipopeptides and glycolipid. The lipopeptides in both cultures were the same and of a single component. The dispersion efficiency determined by BFT showed that mutant #47 was 1.35 times of the biosurfactants produced by the wild type strain. The dispersion efficiency of mutant #47 is comparable to the Corexit 9500, while it is better than Corexit 9527 when dispersing the Newfoundland offshore crude oil.

Dispersants are crucial marine oil spill treating agents. With varied structures and functions, certain biosurfactants may serve as the core ingredients of dispersants. Their virtues of low toxicity and persistency in the ecosystem make them competent alternatives of existing dispersants which receives mixed public perceptions. Four types of crude biosurfactants, i.e. lipopeptides (surfactins) produced by *Bacillus subtilis* N3-1P, glycolipids (trehalose lipids) produced by *Rhodococcus erythropolis* mutant M36, lipopeptides (exmulsins) produced by *Exiguobacterium* sp. N4-1P and commercial crude rhamnolipids were tested as oil spill dispersants. Trehalose lipids and surfactins exerted comparable dispersion effectiveness to Corexit 9500A. Rhamnolipids (primarily as a surface/interfacial tension reducing agent) and exmulsins (primarily as emulsifier) when used alone showed significantly lower dispersion effectiveness. Together they exhibited synergetic effects that lead to similar performances with trehalose lipids and surfactins indicating the balanced surface/interfacial tension reduction and emulsion stabilization abilities is the key for effective oil dispersion. Surfactins achieved effective DOR as low as 1:53.3 (w/w). Trehalose lipids showed advantages of high oil affinity, low toxicity, and

high biodegradation rate, but needs further purification to reduce the effective DOR. Both surfactins and trehalose lipids can be competitive candidates for oil spill dispersion.

8.2 Research Contributions

This research can be summarized and highlighted by the following contributions:

1) It is the first study on marine biosurfactant producers from petroleum hydrocarbon contaminated sites in North Atlantic Canada. It is also the first study isolating biosurfactant producers from samples from offshore oil and gas platforms in North Atlantic Canada (crude oil, formation water, drilling mud, treated produced water and seawater samples).
2) Among these isolates, *Alcanivorax atlaticus* N3-2A has <98% match with the 16S rDNA database and many unique phenotypic features indicating it is a newly discovered bacterial species. Other *Alcanivorax* published in the literature were only found to produce biosurfactant capable of reducing surface tension. However, *Alcanivorax atlaticus* N3-2A could produce biosurfactants that could stabilize emulsion or reduce surface tension depending on the culturing substrates. The complete genome of *Alcanivorax atlaticus* N3-2A was reported for the first time.
3) Among these isolates, *Exiguobacterium* sp. N4-1P was demonstrated as a biosurfactant producer belonging to the genus *Exiguobacterium* for the first time. The biosurfactants it produced, and the relevant producing process was characterized. They possess interesting features lead to the development of novel

and superior bioemulsifiers, which are especially potent in cold conditions. Its complete genome was reported for the first time.

4) Among these isolates, thirty-seven marine bacteria destabilizing O/W emulsion were reported. It was the first report and characterization of a Halomonas demulsifying strain. *Halomonas venusta* N3-2A achieved 92.5% EBR of O/W emulsion within 24 h. Bacterial species/genus specific patterns in surface activities were illustrated. Oil spreading test coupled with cell hydrophobicity test was recommended as a high throughput screening strategy for O/W demulsifying bacteria. The complete genome of *Halomonas venusta* N3-2A was reported for the first time.
5) It is the first study attempted to genetically modify *Rhodococcus* strains for hyper production of biosurfactant and to investigate the dispersing abilities of the above-mentioned hyper production mutants and the produced biosurfactants. The strains, the products and the bioprocess can be of great value to both scientific understanding and the environmental applications.
6) It is the first study investigating 4 types of biosurfactants produced by *Rhodococcus erythropolis* mutant M36, *Bacillus subtilis* N3-1P, *Exiguobacterium* sp. N4-1P, and *Pseodomonas aeruginosa* as the core ingredients for oil spill dispersion using BFT. The results demonstrated that a balanced surface/interfacial tension reduction and emulsion stabilization abilities is the key for effective oil dispersion. Surfactins achieved effective DOR as low as 1:53.3 (w/w). Trehalose lipids showed advantages of high oil affinity, low

toxicity, and high biodegradation rate. Both surfactins and trehalose lipids were found as competitive candidates for oil spill dispersion.

8.3 Publications

Paper under preparation

1) **Cai, Q.**, Zhu, Z., Chen, B., and Zhang, B (2018) Biosurfactants produced by *Rhodococcus erythropolis* mutant M36, *Bacillus subtilis* N3-1P, *Exiguobacterium* sp. N4-1P, and *Pseodomonas aeruginosa* as marine oil spill dispersants. Journal of hazardous materials, to be submitted.
2) **Cai, Q.**, Ye, X., Chen, B., Zhang, B (2018) Complete genome sequence of an oil degrading, oil-in-water emulsion breaking bacterium Halomonas venusta strain N3-2A. Marine Genomics, to be submitted.
3) **Cai, Q.**, Zhang, B., Chen, B., Zhu, Z., and Lv, Z (2018) *Alcanivorax atlaticus* sp. nov., a biosurfactant producing and alkane degrading bacterium isolated from petroleum hydrocarbon contaminated coastal sediment. International Journal of Systematic and Evolutionary Microbiology, to be submitted.
4) **Cai, Q.**, Ye, X., Chen, B., and Zhang, B (2018) Complete genome sequence of of *Alcanivorax altacticus* strain N3-7A, an oil degrading bacterium and biosurfactant producer isolated from cold marine environment in North Atlantic Canada. Marine Genomics, to be submitted.

Paper under review

1) **Cai, Q.**, Zhu, Z., Chen, B., and Zhang, B. (2018) Novel marine bacteria for demulsifying oily wastewater with oil-in-water emulsion. Water Research (WR45038, revised version submitted)

Referred Journal papers

1) **Cai, Q.**, Ye, X., Chen, B., and Zhang, B. (2017) Complete genome sequence of *Exiguobacterium* sp. strain N4-1P, a psychrophilic bioemulsifier producer isolated from a cold marine environment in North Atlantic Canada. Genome Announcements. 5(44): e01248-17.

2) **Cai, Q.**, Zhang, B., Chen, B., Zhu, Z. and Zhao, Y. (2017) A novel bioemulsifier produced by *Exiguobacterium* sp. strain N4-1P isolated from petroleum hydrocarbon contaminated coastal sediment. RSC Advances. 7(68): 42699-42708.

3) Lin, W., Jing, L., Zhu, Z., **Cai, Q.**, and Zhang, B. (2017) Removal of heavy metals from mining wastewater by micellar-enhanced ultrafiltration (MEUF): experimental investigation and Monte Carlo-based Artificial Neural Network modeling. Water, Air, & Soil Pollution. 228(6): 206-216.

4) Pi. Y., Chen, B., Bao, M., Fan, F., **Cai, Q.**, Lv, Z., and Zhang, B. (2017) Microbial degradation of four crude oil by biosurfactant producing strain *Rhodococcus* sp. Bioresource Technology. 232: 263-269.

5) Kazemi, K., Zhang, B., Lye, L., **Cai, Q.**, and Cao, T. (2016) Design of Experiment (DOE) based screening of factors affecting municipal solid waste (MSW) composting. Waste Management. 58: 107-117.

6) Li, P., **Cai, Q.**, Lin, W., Chen, B., and Zhang, B. (2016) Offshore oil spill response practices and emerging challenges. Marine Pollution Bulletin. 110(1):6-27.

7) Song, X., Zhang, B., Chen, B., and **Cai, Q.** (2016) Use of sesquiterpanes, steranes and terpanes for forensic fingerprinting of chemically dispersed oil. Water, Air, & Soil Pollution. 227: 281-295.

8) **Cai, Q.**, Zhang, B., Chen, B., Cao, T., and Lv, Z. (2016) Biosurfactant produced by a Rhodococcus erythropolis mutant as an oil spill response agent. Water Quality Research Journal of Canada. 51(2): 97-108.

9) Zhu, Z., Zhang, B., Chen, B., **Cai, Q.**, and Lin, W. (2016) Biosurfactant production by marine-originated bacteria *Bacillus subtilis* and its application for crude oil removal. Water, Air, &Soil Pollution. 227(9): 1-14.

10) **Cai, Q.**, Zhang, B., Chen, B., Song, X., and Zhu, Z. (2015) Screening of biosurfactant producing bacteria from offshore oil and gas platforms in North Atlantic Canada. Environmental Monitoring and Assessment. 187(5): 184-192.

11) **Cai, Q.**, Zhang, B., Chen, B., Zhu, Z., and Lin, W. (2014) Screening of biosurfactant producers from petroleum hydrocarbon contaminated sources in cold

marine environments in Atlantic Canada. Marine Pollution Bulletin. 86(1-2): 402-410.

Other Referred Publications

(1) Lv, Z.*, **Cai, Q.*,** Zhang, B., and Chen, B. (2016) A new high-yielding bio-dispersant producer mutated from Rhodococcus erythropolis strain P6-4P. Proceedings in the 2016 CSCE conference, June 1-4, London, Canada. (*Equally contribution) (oral presentation and paper; National conference)

(2) **Cai, Q.**, Zhang, B., Chen, B., Cao, T., and Lv, Z. (2014) Biodispersants produced by a Rhodococcus erythropolis mutant as an oil spill response agent. Proceedings in the International Conference on Marine and Freshwater Environments (iMFE 2014) conferences, August 6-8, St. John's, Canada. (oral presentation and paper; international conference)

(3) **Cai, Q.**, Zhang, B., Chen, B., Li, P., Song, X., and Zhu, Z. (2014) Behavior of Corexit dispersants in the Gulf of Mexico after the Deepwater Horizon oil spill. Proceedings of the 2014 International Conference on Marine and Freshwater Environments (iMFE 2014), August 6-8, St. John's, Canada. (oral presentation and paper; international conference)

(4) Cao, T., Zhang, B., Chen, B., **Cai, Q.**, Zhang, H., Zhu, Z., Zhang K. (2014) Biosurfactant production by Rhodococcus erythropolis sp. SB-1A isolated from North Atlantic Ocean: study on the influence of environmental conditions.

Proceedings in the iMFE 2014 conferences, August 6-8, St. John's, Canada. (oral presentation and paper; international conference)

8.4 Recommendations for Future Research

1) In-situ burning is an oil mitigation option particularly suited to remote, ice-covered seawater in the north. The key to effective in-situ burning is thick oil slicks. In drift ice conditions and open water, spilled oil can rapidly spread to become too thin to ignite. Oil-herding surfactants (herders) can thicken slicks even in light ice conditions. Available herders are chemical synthesized blends grouped into hydrocarbon based, silicone based, and fluoro-surfactants. They contain ingredients with significant persistency in the ecosystem. Some of the screened isolates are able to produce biosurfactants that can dramatically reduce surface tension of water from 70 dynes/cm to as low as 25 dynes/cm. Such ability warrants a strong spreading pressure of monolayer on water, making them excellent environmentally friendly candidates for oil herding application. Further research exploring their oil herding performances and examine the possibilities to enhance their production yield and reduce the production cost through metabolic engineering will be of great interests.
2) Genome shuffling has been suggested as a novel whole-genome engineering approach for rapid improvement of complex traits of an organism using recursive protoplast fusion strategy. This approach was found to be more effective than the traditional mutagenesis. It has been successfully used to yield bio-products, but not much for biosurfactant production. Furthermore, optimization of fermentation conditions can also help to improve the yield of the bio-products. The addition of a

small quantity of solid porous carriers (e.g., activated carbon, expanded clay, and fly ash) into fermentation broth could increase the biosurfactant production by providing a large surface area for microorganisms to adhere.

3) Crude biosurfactants produced in this study can be further purified to harvest the most effective components and eliminate unwanted impurities. The bioemulsifier, biodemulsifier, and biodispersants described in this study will all benefit significantly from an optimized purification strategy tailored to the specific strain. The end results will be significantly lowered effective concentrations to meet specific goals in oil pollution control applications of soil washing, enhanced bioremediation, oily wastewater treatment and oil dispersion, and so on.
4) When applied as biodispersants, solvents are normally required to add to biosurfactants to help delivering the effective ingredients into the water/oil interfaces. Research efforts are suggested to place on the selection of proper solvent system for each type of biodispersants based on their specific solubility properties. Hansen solubility parameters (dispersion, polarity and hydrogen-bonding) can be used to quantify the solubility relationships between biosurfactants and solvents. Moreover, the selected biosurfactants/solvents systems with different affinities with crude oils can be used to examine the hypothesis that dispersant mixture with stronger crude oil affinity tend to mobile effortlessly with oil slick and maintain the effective concentrations of biosurfactants at the interfaces without desorbing into seawater which is vastly more abundant than oil in this case.

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