Screening and Characterization of Biosurfactant Producers from Petroleum Hydrocarbon Contaminated Marine Sources in North Atlantic Canada for Oil Spill Responses

By © Qinhong Cai A Thesis submitted

to the School of Graduate Studies in partial fulfillment of the

requirements for the degree of

Master in Engineering

Memorial University of Newfoundland

May 2014

St John's   Newfoundland

ABSTRACT

As one of the oil spill responses, oil dispersion was found effective in open sea and under harsh conditions. However, currently used chemical surfactant-based dispersants may harm the environment due to the toxicity and persistency. Thus novel, environmentally friendly biosurfactant-based dispersants are desired.

Biosurfactants are less toxic, biodegradable, and can be biologically produced. Their establishment is impeded by a lack of economic and versatile products. Discovery of new biosurfactant producers is the key to overcome the obstacles. This dissertation will thus fill the research gap through screening and characterization of biosurfactant producing microorganisms from petroleum hydrocarbon contaminated marine sources in the North Atlantic Canada. Fifty-five biosurfactant producers belong to 8 genera were isolated. Some of the isolated strains were found with properties such as greatly reducing surface tension, stabilizing emulsion and producing flocculant. Three strains with interesting characteristics and limited relevant publications were selected for genetype and phenotype characterization. The strains, the products and the bioprocess can be of great value to both scientific understanding and the environmental applications in offshore oil spill responses.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS AND SYMBOLS

ANN   artificial neural network

B/W   Buoyance to weight ratio

BEFs   extracellular biopolymeric flocculants

BFT   baffled flask test

BLAST   Basic Local Alignment Search Tool

CMC   critical micelle concentration

CMD   critical micelle dilution

CMT   critical micelle temperature

CREAIT   Core Research and Instrument Training Network

CROSERF   chemical response to an oil spills: ecological research forum

DNA   deoxyribonucleic acid

DOSS   dioctylsulfosuccinate, sodium salt

DPNB   di(propylene glycol) buty ether

E24   emulsification index

EC_{50} median effective concentration

EPS   exopolysaccharides

FAME	fatty acid methyl esters
GA	genetic algorithm
GC/MS	gas chromatography coupled with mass spectroscopy
HLB	hydrophilic-lipophilic balance
LC/MS/MS	ultra-high resolution mass spectrometry and liquid chromatography with tandem mass spectrometry
LC_{50}	median lethal concentration
MAF IIC	Micro Analysis Facility
MEGA	molecular evolutionary genetics analysis
MSD	mass spectroscopy detector
MUN	Memorial University of Newfoundland
NCP	National Oil and Hazardous Substances Pollution Contingency Plan
NRPOP	Northern Region Persistent Organic Pollution Control
OD	optical density
OSCs	on-scene-coordinators
PAHs	polycyclic aromatic hydrocarbons
PCR	polymerase chain reaction

PLFA	phospholipid-derived fatty acid
PM	production medium
R&D	research and develop
rDNA	16S ribosomal deoxyribonucleic acid
RSM	response surface methodology
SEM	scanning electron microscope
SERVS	The ship escort response vessel system
SFT	swirling flask test
SMART	Special monitoring of applied response technologies
TDS	total dissolved solid
USEPA	US Environmental Protection Agency
UV	ultraviolet
WAC	wood-based activated carbon
WSL	Warren Springs Laboratory

CHAPTER 1

INTRODUCTION

Offshore oil spills are of tremendous concerns due to the enormous economic loss and the harm to ecological systems they may cause. The catastrophic Deepwater Horizon oil spill occurred on April 20, 2010 threatened over $5.5 billion fishing and tourism industrial economic entities and more than 200,000 employment opportunities (Hagerty and Ramseur, 2010). Millions of seabirds and billions of fish eggs (Corn and Copeland, 2010) were killed and a dramatic population decline and shift of microorganisms, phytoplankton and other flora were observed (Widger et al., 2011). Although intensive efforts have been made to prevent offshore oil spills, accidental spills are inevitable. Furthermore, increasing risk of spills in the Northern Atlantic and Arctic regions has been widely recognized due to the expansion of oil and gas industry and the openings of new transportation routes in the north as a result of global warming. With the estimated undiscovered potential of 6 billion barrels of oil, Newfoundland and Labrador accounts for about 40% of Canada's conventional light crude output with promising prospects (DBNL, 2012). The thriving oil industry needs to be introduced to improved technologies to alleviate the potential impacts of accidental spills. Some traditional mechanical countermeasures, such as controlled burning, skimming and vacuum/centrifuge have disadvantages such as narrow operational time window, fire retarded boom requirements, interferences caused by currency and wind, and insufficiency in separation (USEPA, 2011). These disadvantages limit their applications in open sea spills with unfriendly weather conditions. Dispersants have been widely used as a key measure to prevent landfall of oil by breaking the oil slick into droplets (micelles) which can be easily dispersed by turbulence and diluted downwards in the water column (CRRC, 2010). During Deepwater Horizon oil spill, about 1.84 million gallons of Corexit 9500 and 9527

dispersants were applied on the surface and the subsea discharge point to disperse the oil (National Commission Final Report, 2011) The dispersed droplets can be then degraded by indigenous microbes (Hazen, 2010). Various gaps in the knowledge concerning dispersants operation have been addressed while a general concern about the toxicity effect and efficacy of currently used surfactants, which is the primary ingredient of dispersants, has been widely recognized (Fingas, 2010d). Therefore, the development of superior surfactants with low toxicity and higher effectiveness and biodegradability is of great significance. The promising development of surfactants focuses on biosurfactants which are surfactants produced as metabolic by-products from microorganisms. Biosurfactants were found to be more effective and stable, less toxic and better at enhancing biodegradation than chemically synthetic surfactants (Muthusamy et al., 2008). Furthermore, they could be produced from various sources (e.g., industrial wastes) with facile procedures (Muthusamy et al., 2008).

Nevertheless, the development of biosurfactants is still at a preliminary stage that requires enormous efforts before field applications. The primary challenge is the lack of availability of economic and versatile biosurfactants (Mukherjee, 2006). Production of biosurfactants has suffered from low yields and high cost hitherto (Mukherjee, 2006). It was estimated that biosurfactants would cost 3-10 times of chemically synthetic surfactants in current stage (Muthusamy et al., 2008). Besides, with the harsh environment in the North Atlantic Canada, the efficacy of surfactants should be reassessed as they would remain crystalline if the ambient temperature was lower than their critical micelle temperature (CMT) (Gu and Sjöblom, 1992). Biosurfactants with low CMT are thus highly desired for application in Atlantic Canada. Moreover, Marine

environments harbor microbes with unique metabolic and physiological capabilities. These microorganisms can thrive in extreme conditions and have high potential to produce novel metabolites. Till now, marine originated biosurfactant producing microorganisms are extremely underexplored with only around 57 related study reported, and these studies only focusing on tropical regions (Satpute et al., 2010a). In order to address these challenges, this research tackles the screening and characterization of biosurfactant producing microorganisms from petroleum hydrocarbon contaminated marine sources in North Atlantic Canada for oil spill responses. The biosurfactant producers are expected to cost-effectively yield biosurfactans with the high efficacy, low CMT and good capacity in enhancing biodegradation. It is the first study on marine biosurfactant producers from petroleum hydrocarbon contaminated sites in North Atlantic Canada. The strains, the products and the bioprocess can be of great value to both scientific understanding and the environmental applications in offshore oil spill responses.

This thesis consists of five chapters. Chapter 2 presents a comprehensive review on oil spill responses, dispersants and biosurfactants. Chapter 3 is a study of screening of biosurfactant producers from petroleum hydrocarbon contaminated sources in cold marine environments in Atlantic Canada. The chapter describes the unique characteristics of the sampling sites, the isolating procedures, and a series of research findings associate with some promising producers isolated in this study. The characterization of these isolates and their corresponding biosurfactant products is illustrated in details in Chapter 4. Finally, the conclusions are drawn up with the research achievements and the recommendations for future work in Chapter 5.

CHAPTER 2

LITERATURE REVIEW

2.1 Offshore Oil Spills

2.1.1. Background

The sources of oil spills can originate from various sectors of the industry, namely, exploration/production, transportation (tank ships/pipelines), refinery, transportation of refined products due to structural failures, operational errors, weather and Earth crust related events and even vandalism (Fingas, 2010b). According to the size class of U.S. marine oil spills, most occasions of spills (71.9%) were below 0.003 tones while the disaster spills (>30 tonnes) accounted for approximately 60% of the total amount of spilled oil (Etkin, 2001) Some of the large-scale spills were summarized in **Table 2.1**. The spills were of various types of oil ranging from diverse crude oil to a large group of refined products which included heavy persistent compounds and lighter, less persistent, but more toxic ones (Boström et al., 2002; Fingas, 2010b).

2.1.2. Impacts

Offshore oil spills are of tremendous concern due to the enormous economic loss and the harm to ecological systems, public health, society and community they may cause. During the long run of oil and gas exploitation, the adverse impacts of oil spills have been documented in various aspects including economy, ecology and environment, public health and society/community.

Table 2.1 Major oil spills in history worldwide (Camilli et al., 2011; Fingas, 2010b)

Time	Name of spill souces	Location	Spill amount (Tonnes)
10-Mar-1991	700 oil wells	Kuwait	71,428,571
20-Jan-1991	Min al Ahmadi Terminalyz	Kuwait	857,143
3-Aug-2000	Oil wells	Russia	700,000
24-May-2010	Deep Horizon	U.S.A	559,000
3-Jun-1979	Ixtoc I well	Mexico	476,190
1-Feb-1991	Bahra oil fieldsy	Iraq	377,537
2-Mar-1990	Oil well	Uzbekistan	299,320
19-Jul-1979	Tank vessel Atlantic Empressx	Trinidad/ Tobago	286,354
25-Oct-1994	Kharyaga-Usinsk Pipeline	Russia	285,714
4-Feb-1983	No. 3 Well (Nowruz)	Iran	272,109
6-Aug-1983	Tank vessel Castillo de Bellver	South Africa	267,007
16-Mar-1978	Tank vessel Amoco Cadiz	France	233,565
10-Nov-1988	Tank vessel Odyssey	Canada	146,599
11-Apr-1991	Tank vessel Haven	Italy	144,000
1-Aug-1980	D-103 concession well	Libya	142,857
6-Jan-2001	Pipeline	Nigeria	142,857
19-Jan-1991	Tank vessel Al Qadasiyahyz	Kuwait	139,690
19-Jan-1991	Tank vessel Hileeny	Kuwait	139,690
18-Mar-1967	Tank vessel Torrey Canyon	United Kingdom	129,857
19-Dec-1972	Tank vessel Sea Star	Oman	128,891
23-Feb-1980	Tank vessel Irenes Serenade	Greece	124,490
19-Jan-1991	Tank vessel Al-Mulanabbiyz	Kuwait	117,239
7-Dec-1971	Tank vessel Texaco Denmark	Belgium	107,143
19-Jan-1991	Tank vessel Tariq Ibn Ziyadyz	Kuwait	106,325
20-Aug-1981	Storage tanks	Kuwait	106,003
26-Jan-1991	Min al Bakar Terminalyz	Kuwait	100,000

For offshore oil spills and the ship-sourced oil pollution, the prominent affected industries have been the commercial and recreational fisheries and coastal tourism. The catastrophic Deepwater Horizon oil spill which occurred on April 20, 2010 threatened over $5.5 billion fishing and tourism industrial economic entities and more than 200,000 employment opportunities (Hagerty, 2010). In the Gulf of Mexico, the commercial fisheries industry was estimated with a dockside value of $659 million in 2008 (NMFS, 2008). This industry as well as the related processor, wholesale and retailed businesses supported over 200,000 jobs with related income impacts of $5.5 billion (NMFS, 2008). In terms of recreational fisheries, recreational anglers contributed $12 billion on equipment and trips in 2008 and supported businesses including charters, bait and tackle shops, restaurants and hotels (NMFS, 2008). On the other hand, the tourist industry accommodated 21.9 million visitors in 2009 and contributed to 620,000 jobs and corresponding income impact of $6 billion (Ache, 2008). In terms of oil spill cleaning cost, the cleanup methods were estimated with the sequence of natural attenuation ($1,286/tonne) < in-situ burning ($3,128/tonne) < dispersants ($5,634) < mechanical ($9,612/tonne) < manual (23,403/tonne) (Etkin, 1998).

About 250,000 seabirds were killed by the Exxon Valdez oil spill (Piatt and Ford, 1996). Significantly higher pink salmon egg mortality was found in oiled than in unoiled streams in 1989-1991 (Bue et al., 1993). Other studies also showed that significantly higher Dolly Varden fish (Hepler et al., 1993; Marty et al., 1997), Herring (Biggs and Baker, 1993; Funk, 1994), shrimp (Armstrong et al., 1995; Donaldson and Ackley, 1990) and snail (Highsmith et al., 1995; Houghton et al., 1993) mortality in oiled than unoiled

stream. During Deepwater Horizon oil spill, the estimated deaths of seabirds was 23,400 (Corn, 2010). The benthos has experienced a broad range of effects from the Deepwater Horizon oil spill (Corn, 2010). The coastal baseline community metrics were completed and will be compared with the post-spill community structure and composition to determine the effect of BP oil spill (Demopoulos and storm, 2012). During the Deepwater Horizon oil spill, approximately 692 km of marsh shorelines were oiled, among which 41% (283 km) were either heavily or moderately oiled (Zengel and Michel, 2011). Although few quantitative data were yet available on the extent of vegetation impacts, recent findings for the salt marshes in the Bay Jimmy area of northern Barataria Bay, Louisiana documented variable impacts depending on oiling intensity (Lin and Mendelssohn, 2012). Since the spill, some recovery has been noted for oiled marshes (Mendelssohn et al., 2011).

In regards with transference to the food chain, studies have demonstrated that oil-contaminated food can cause genotoxic damage in consumers (Chaty et al., 2008; Lemiere et al., 2005). Chaty et al. (2008) showed evidence for the bioaccumulation of oil compounds and their transference to the food chain in oil-contaminated marine food, which was agreed with Bro-Rasmussen (1996) that persistent chemicals might create a human hazard after bioconcentration when climbing the food chain. The study also demonstrated the induction of deoxyribonucleic acid (DNA) damage by the metabolic transferred products which might be more toxic than their parent compounds (Chaty et al., 2008). After the Prestige oil, significantly higher DNA damage occurred, but not cytogenetic damage (Laffon et al., 2006; Pérez-Cadahú et al., 2006 and 2007), and also alterations in the endocrine status in relation to the exposure (Pérez-Cadahú et al., 2008a).

They also found general decrease in the proliferation index in the individuals with longer time of exposure (Pérez-Cadahía et al., 2008b). It was found that the cultural and ethnical differences were important in the perception of the Exxon Valdez spill (Palinkas et al., 1992). Gill and Picou (1998) conducted a 4-year (1989–1992) study on the impact of the Exxon Valdez spill, and revealed the chronic nature of stress, such as increased out-migration expectations and desires, social disruption, high levels of event-related psychological stress between 1989 and 1991. After the Erika oil spill, Schvoerer et al. (2000) identified the duration of the cleaning work as a risk factor. As a result of the disaster of the tanker Prestige, Suarez et al. (2005) found a significant correlation between proper health-protection briefing and use of protective devices and lower frequency of health problems. It has been found that approximately 1.5 years after the accident, health-related quality of life and mental health levels were not deteriorated (Carrasco et al., 2007; Sabucedo et al., 2009). According to the survey of Columbia University's National Center for Disaster Preparedness, 40% of the Gulf coastal residents reported that they had experienced physical health problem related to the spill (CUMSPH, 2010).

The sociocultural and psychological impacts of the Exxon Valdez oil spill were examined by Palinkas et al., (1993) in a population-based study one year after the spill occurred. Their results suggested that the impact on the psychosocial environment was significant. According to the survey of Columbia University's National Center for Disaster Preparedness, 20% of Gulf Coast residents reported a drop in income, and 25% thought they might have to move away from the Gulf (CUMSPH, 2010).

2.1.3 Oil Spill Responses Technologies

The primary objective of a spill response operation is to minimize the size of the affected area and damage to vulnerable resources (Owens, 2010). At different stages of response strategies, there are some common treatment options available and proven by authorities. These options could be grouped as physical/mechanical removal technologies (e.g., Booming, skimming, manual recovery, and sorption), biological removal technologies (e.g., bioremediation and natural attenuation) and chemical removal technologies (e.g., dispersion, emulsification, in situ burning, and solidification) (Fingas, 2010b).

Booms are used to retaining oil so that it can be recovered or treated (Separovich 1991). A boom commonly consists of a means of flotation, a freeboard member and a skirt to avoid oil being swept over and underneath the boom, and the tension members to support the entire boom (Fingas, 2001). The performance of a boom and its ability to contain oil were affected by hydrodynamic conditions such as water currents, waves, and winds (Delgado et al., 2004; Fang and Johnston 2001a, 2001b, 2001c; Violeau et al., 2007), as well as boom parameters such as buoyance to weight ratio (B/W), initial draft, heave response and roll response (Castro et al., 2010; Muttin, 2008). Their interactions in different scenario have also been studied (Amini et al., 2008; Badesha et al., 1993; Wong and Barin, 2003).

Skimmers are mechanical devices designed to remove oil from the water surface. They vary greatly in size, application, and capacity, as well as in recovery efficiency (Potter, 2008; Schulze, 1998). A skimmer's performance was affected by a number of factors including the oil thickness, the viscosity of the oil, the presence of debris, and

wind/current conditions at the time of recovery operations (Meyer et al., 2009; Potter, 2008; Schwartz, 1979; Ventikos et al., 2004).

Sorbents were used mainly to clean up the final traces of oil spills on water or land. Besides, they were also used as an enhancement to the boom. When working offshore, they were only suitable for small spills (Fingas, 2010a).

As the oldest countermeasures to oil spills, **in-situ burning** has not been explored in scientific depth until recently (Fingas, 2010c). Oil slick generally burns at a rate of about 0.5 to 4 mm per minute (Crawley et al., 1982; Evans et al., 1991). The amount of vapors produced which was critical to achieve steady-state burning was dependent on the amount of heat radiated back to the oil (Buist et al., 1994; Nakakuki, 2002). Most oils ignite under similar conditions if no stable emulsion formation was formed (Buist and Morrison, 2005; McCourt et al., 2000). For the stable emulsion with water content higher than 25%, de-emulsifiers might be required (Mullin and Champ, 2003).

Dispersants consist of surfactants and solvents. Solvents help distribute surfactants into the oil/water interface whereafter surfactants changes the interfacial properties thus beak down oil slicks into small droplets. They were 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. However, several concerns such as effectiveness, toxicity, the effect of dispersants on biodegradation, and long-term considerations withdrew its applications (NAS, 2006). Several researchers and organizations have conducted comprehensive reviews and written books and reports concerning these issues in the decade (Fingas and Ka'aihue, 2005; Michel et al., 2005; Fingas, 2008a; SL Ross, 2010; Fingas, 2010d). Some general conclusion of these reviews were as follows: 1) Oil

composition is the most important factor influencing dispersion effectiveness, followed by sea energy and the dispersant dosage; (2) half-lives of the oil dispersions were between 4-24 hours; (3) toxicity of dispersants vary in various species, and dispersants were less toxic than the dispersed oil; (4) approximately half of the studies found inhibition of biodegradation by dispersants. Franklin and Warner (2011) reviewed the role and regulation of dispersants during its history in America. It seems that the regulation of dispersants has been towards a more complete level with new bills mandate the more extensive safety, health and environmental testing and set more stringent standards for US Environmental Protection Agency (USEPA) to add a product to the National Oil and Hazardous Substances Pollution Contingency Plan (NCP) list. Michel et al. (2005) also reviewed the decision-making process of dispersants application in detail.

Solidifiers are materials react with oil and change it to solid (Rosales et al., 2010). It is used to constraint oil quickly, to recover thin sheens, and it is commonly used with small spills (Fingas, 2008b). The main concern with solidifier application has been their effectiveness which generally is a function of oil types and composition (Fingas et al., 1996). Besides, the dosage of solidifier when treating oil spills was relatively high and varied with 16-200% by weight (Fingas et al., 1991), and it was not compatible with other oil spill countermeasures (Fingas, 2008b).

If natural processes are good enough to limit the migration of significant levels of an oil spill, a monitoring only plan might be sufficient for the site (McAllister and Chiang, 2007). Several studies were conducted to understand **natural attenuation** and determine its potential. Cozzarelli et al. (2001) found the extent of contaminant migration and compound-specific behavior were affected by iron redox. Alonso-Gutiérrez et al. (2009)

and Acosta-González et al. (2013) conducted studies to analyze bacterial communities, and their results proved the indigenous microbes have natural attenuation potential in terms of their responds against contamination.

Bioremediation was used extensively in the Exxon Valdez spill with the addition of fertilizers to enhance oil biodegradation (Bragg et al., 1994). Following the field tests, fertilizers with 107,000 lbs of nitrogen were introduced to the affected area between 1989 and 1991 (Atlas and Hazen, 2011). Subsequently, about 25-30% of total hydrocarbon was degraded within the first week. In the meantime, the population of oil-degrading bacteria grew dramatically from 1-10% of the total heterophrophic bacteria population to 40% which returned to background levels in around one and a half year (Bragg et al., 1992; Bragg et al., 1994; Pritchard and Costa, 1991). The follow-up monitoring showed the oil declination rate was 22% per year between 1991 to 2001 and slowed down to 4% after 2001 (Atlas and Bragg, 2009; Boufadel et al., 2010; Short et al., 2004, 2007).

Comparison of the existing technologies

The above discussed countermeasures all have their own advantages and disadvantages. The overall comparison of the above discussed methods is listed in **Table 2.2.** It can be seen from the table that oil dispersion has the advantages of high feasibility and effectiveness in open water. In the meantime, it is not restricted by the limitation factors such as accessibility, weather conditions, sea states, and oil thickness that other countermeasures require. It was used as the primary countermeasures for the Deepwater Horizon oil spill (The Federal Interagency Solutions Group, 2010).

Table 2.2 Overview of oil spill response technologies

Response technology/agents	Mechanisms of action	When to use	Target Areas	Characteristics of products	Limiting factors	Waste generation	Oil types	Impacts to sensitive resources	References
Booms, skimmers	Mechanical containment and removal of oil from the water surface	Typically first line of defense during a response	Varies	Contains, removes spilled product	Weather conditions; Site accessibility	Varies by method	Varies	Response personnel interruption; may be invasive/destructive to land habitats	Fingas 2010a; NOAA, 2010; Scientific and Environmental Associates, Inc., 2003
Sorbents	Absorption (uptake into the sorbent material) and adsorption (coating of the sorbent surface)	To treat spill on land or hard surface; to create a physical barrier; to immobilize small spill	Shorelines; Hard surfaces with recoverable oil	Low application rate; easy to recover	Access to deploy and retrieve products	Concern if only lightly oiled; may be burned or recycled	Light to heavy oils; not effective on viscous oils	May cause smothering of benthic/attached wildlife if not recovered; May be ingested by wildlife if not recovered	Scientific and Environmental Associates, Inc., 2003; Rosales, 2010; Fingas 2008b;
In situ **Burning**	Removal of free oil or oily debris from the water surface or land surface from burning oil in place	To quickly remove oil to prevent its spread to sensitive areas; to reduce waste; limited access and recovery	Remote areas wth land or water where oil is thick enough for an effective burn	Removal of free oil from the water or land surface; require certain oil thickness	Heavy, weathered or emulsified oils may not ignite; wind can affect smoke; need air quality monitoring	Residue may sink, a semi-solid, tar-like layer may need to be recovered	Oil thickness required increases with oil weathering, & heavy-component content	Consult with Resource Trustees on environmental issues.	Evans et al., 2001; Scientific and Environmental Associates, Inc., 2003;
Dispersants	Break of oil into small droplets that mix into the water and do not reflect	When dispersion has less impact than slicks that strand onshore or affect surface water resources	Open water	Products have to pass a dispersant effectiveness test to be listed	Low effectiveness with heavy, weathered, or emulsified oils	Can significantly reduce the volume of oil wastes, if effective	Any oil with a viscosity less than 20,000-40,000 cP	Consult with Resource Trustees on environmental issues.	Michel et al., 2005; Scientific and Environmental Associates, Inc., 2003;
Solidifiers	Physically or chemically bond of solidifers (most of which are polymers) with the oil, turning it into a coherent mass	To immobilize oil, preventing further spread; to apply to edge to form a temporary barrier; to reduce vapors		Low application rate (10-25% by weight); cure time of a few hours; forms a cohesive mass; easily applied	Not effective with viscous oils; must be able to recover the solidified oil;	Minimal increase in volume; mostly not reversible; must be disposed of or burned	Light to heavy oils; not effective on viscous oils	Consult with Resource Trustees on environmental issues.	Rosales et al., 2010; Scientific and Environmental Associates, Inc., 2003;

Bioremediation Agents	Acceleration of degradation rate by adding nutrients, microbes, and/or surfactants;	After removal of gross contamination; when further oil removal will be destructive, or ineffective; when nutrients are limiting natural degradation rates	Where other cleanup methods would be destructive or ineffective; as a polishing tool for any size spill	Treated samples show oil degradation greater than control samples in lab tests; key factors are site-specific	Nutrient availability; temperature (>60ºF); pH 7-8.5; moisture requirements; surface area of oil	Can significantly reduce the volume of oily wastes, if effective	Less effective on heavy refined products and evaporative fraction;	Unionized ammonia can be toxic to aquatic life in low concentrations; dissolved O2 levels may be affected	Correa et al., 1997; Rӧling et al., 2002; Scientific and Environmental Associates, Inc., 2003
Natural Attenuation	Oil left in-situ with no treatment and recovery technology applied	Access to spill site is limited or other methods will not provide value	Where other response strategies result in more harm than value	-	slow process requires monitoring	Not applicable	Varies	No additional impacts other than the effect of the oil alone	McAllister and Chiang, 2007; Scientific and Environmental Associates, Inc., 2003;

Oil spill response in harsh environments

Booms and skimmers are designed for different wave environments and had to meet standards (e.g. ASTM-F1523) to work with wave of 0.4 m/s (¾ knot) /2 m height or less (Grenier, 2010; Pinks, 2010). In-situ burning is not feasible and safe in high winds and currents (Nuka Research and Planning Group, 2007). Visibility was also crucial to offshore spill-response operations relying on visual tracking, planning and surveillance that an effective spill-response was not practical when the visibility is < 1 km and at night (Brandvik et al., 2006; Nuka Research and Planning Group, 2007). The presence of ice may affect a spill-response operation in terms of impeding access to the spill, skimmer and boom operation and dispersion mixing (Angus and Mitchell, 2010; Nuka Research and Planning Group, 2007). The low temperature condition would have a negative impact on personal safety, skimmers function, ignition, dispersion efficiency as viscosity might increase and bioremediation effectiveness might decrease (Nuka Research and Planning Group, 2007).

In North Atlantic Canada, the water temperatures are 7-10°C lower than at corresponding latitudes on the west coasts of North America and Europe due to the presence of the Labrador Current (Macpherson, 1997). The strong wind also frequently affects the offshore oil platforms in this region (Turner, 2010). The mean surface water temperature are lower than 4°C with more than 50% of ice coverage from January to May in the sampling area (Whitford, 2003), while the daylight hour are less than 10 h from November to early February (Turner, 2010). The cold and harsh environments in North Atlantic Canada limited the applications of other countermeasures require relatively calm sea and acceptable accessibility. Under such circumstances, dispersants are expected to be the most feasible combat agents for oil spill management.

2.2 Dispersants

As reviewed in the previous session, dispersants have been used to reduce the impact on sensitive shoreline resources, birds and mammals living on the water surface, as well as to promote the biodegradation of oil (NAS, 2006). One of the first attempts of using dispersants to combat oil spill occurred in 1967 after the Torrey Canyon Tanker spill off the English Coast (Skinner and Reilly, 1989). The results were found to be catastrophic as the dispersants in use was similar to industrial degreasing agents which greatly enhanced the adverse effects (Skinner and Reilly, 1989). After this accident, USEPA started working with states to develop the first NCP which was issued in 1975. Nowadays, the review and listing process of dispersants remain similar to the original process established in the first NCP (Franklin and Warner, 2011). During the Exxon Valdez oil spill, dispersants were applied in little extent as the dispersants and the operational equipment are not readily available; beside, the wave energy at that time was not sufficient for an effective dispersion treatment (USEPA, 2013). Within the first week of the Deepwater Horizon oil spill, the use of dispersants was approved by the first-acting federal on-scene-coordinators (OSCs) in order to fight this spill as far away from the coastline as possible. In total, 1.07 million gallons of Corexit dispersants (Corexit 9500 and Corexit 9527) were applied on the surface, while 771,000 gallons were applied to the subsea discharge point (National Commission Final Report, 2011). Dispersants have been considered as critical agents to combat oil spill for decades. They have been applied to more than 60 documented spills world-wide and 25 spills in U.S. waters (Franklin and Warner, 2011). Nevertheless, the application of dispersants as an oil spill response agents

has never been without controversy due to the issues of the effectiveness, toxicity and persistency (Fingas, 2010b).

2.2.1 Effectiveness

Several types of procedures and apparatus for testing the effectiveness of dispersants at batch/bench scale have been described in the literature. Among them, the Warren Springs Laboratory (WSL; Labofina) test, the swirling flask test (SFT), and the baffled flask test (BFT) were commonly used (NAS, 2006). Fingas (2000) conducted a comparison among these tests and found the ranking of effectiveness determined in each test was consistent. Besides, as all the bench/batch scale tests had higher effectiveness data than field values due to the higher energy levels in the laboratory tests (Lunel et al, 1995). It was difficult to scale the results of these tests to predict field performance due to the factors of energy regimes, advection induced dilution and turbulent diffusion (Fingas, 2000). Wave tanks have also been used in laboratory to provide effectiveness data on a much more realistic scale under controlled conditions (Fingas and Ka'aihue, 2004a). However, as spills in test tanks were still confined by walls and dispersant was applied in controlled, optimized ways, the effectiveness measured should be considered as upper limits to real-world conditions (NAS, 2006). One hundred and thirty seven times of field tests of dispersants effectiveness in open sea have been conducted primarily in North Sea, Mediterranean and near shore of USA and Canada (Fingas, 2010d). However, the effectiveness determined during these trial tests varied significantly. The analytical method used like colorimetric and fluorometric methods were questionable (Fingas and Ka'aihue, 2004a). Besides, the approaches mainly relied on establishing a mass balance which was very difficult to achieve in a large scale natural system (Fingas and Ka'aihue, 2004a). In order to monitor the effectiveness in the field, some protocols such as SMART

(special monitoring of applied response technologies) and SERVS (the ship escort response vessel system) were proposed and recommended (Fingas, 2010d). In Deep Horizon oil spill, the estimation of the effectiveness of these operations was found to be the most challenging work due to the knowledge gaps and immature state of the technologies mentioned above (The Federal Interagency Solutions Group, 2010). They eventually made the estimation that 16% of the total oil was dispersed with much larger uncertainties than other human intervention categories as shown in **Figure 2.1**.

2.2.2 Fate and Transport

The USEPA has provided benchmarks based on available ecological data to aid in the assessment of potential risk associated with dispersant chemicals, namely propylene glycol, 2-butoxyethanol, di(propylene glycol) buty ether (DPNB), dioctylsulfosuccinate, sodium salt (DOSS) (OSAT, 2010). Water and sediment samples associated with the Deepwater horizon oil spill were analyzed to investigate the fate and transport of the dispersants. The chemical analytical methods were either modified from the existing method to enhance its performance to a specific compound, or newly developed (OSAT, 2010). The used methods and the corresponding benchmarks were presented in **Table 2.3**.
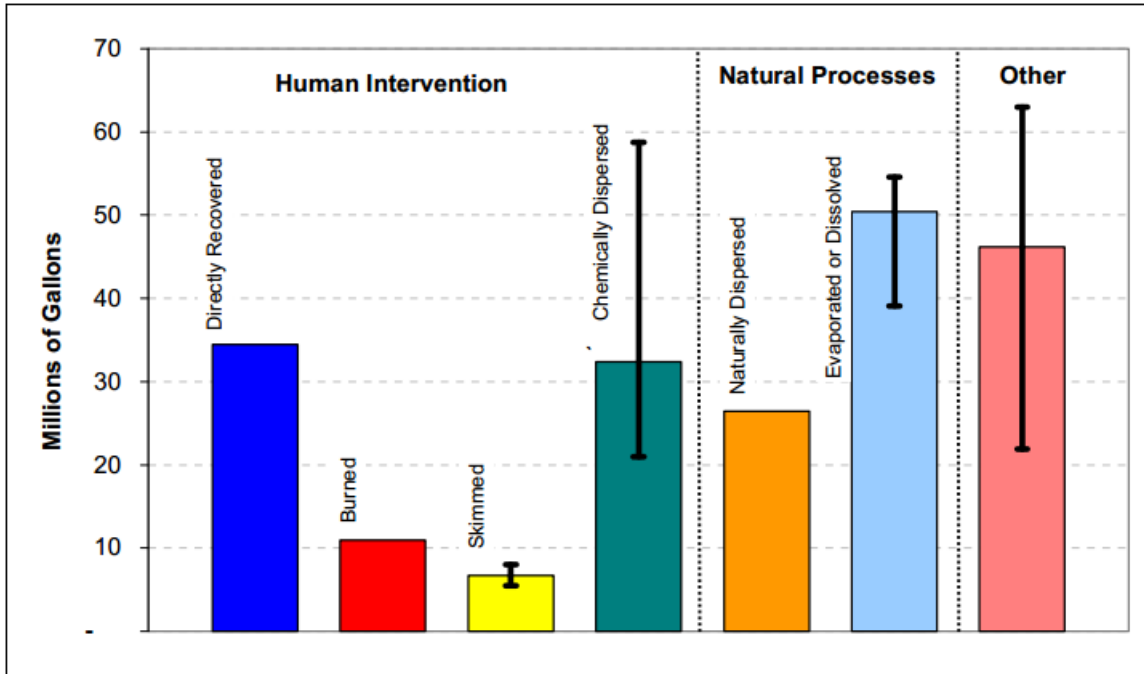
Figure 2.1 Oil budget estimates and ranges of uncertainty Deep Horizon oil spill

(excerpt from Ramseur (2010).

Note: Error bars indicate the ranges of uncertainty.

Table 2.3 Summary of USEPA analytical methods and screening levels of dispersant chemicals in water samples (excerpted from OSAT, 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	500000 μg/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)
Dioctylsulfosuccinate, sodlium salt	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). Furthermore, as the formulas of Corexit dispersants were confidential, the exact ratio of the tested ingredients in the original load was unknown. It was hard to make a comparison between benchmark of each ingredient to the combined toxicity of Corexit dispersants. Nevertheless, this benchmark used a concentration that is highly possible to be toxic to species at certain stages, especially larvae. Moreover, USEPA had not benchmarks for dispersants-related chemicals in sediment samples (OSAT, 2010).

The methods applied to monitoring the fates of the ingredients had not yet been finalized and were being reviewed for posting (USEPA, 2010). However, Kujawinski et al., (2010) published their method and results of monitoring the DOSS associated with Deepwater Horizon oil spill. The method was adapted by USEPA. They collected water samples from wide locations and depths of the accident. After liquid-liquid extraction or solid phase extraction, they injected extracts into gas chromatography coupled with mass spectroscopy (GC/MS) and ultra-high resolution mass spectrometry and liquid chromatography with tandem mass spectrometry (LC/MS/MS). The fates of dispersants in water samples even after 6 months of the spill showed that the presence of dispersants chemicals even after 6 months of the spill which indicated their recalcitrant nature in the ecosystem (OSAT, 2010).

2.2.3 Toxicity

Hundreds of studies regarding the toxicity of the dispersants have been published and have been reviewed by Fingas (2008a, 2010d) and Lewis and Pryor (2013). The following general conclusions were drawn: 1) Toxicities of dispersants were species specific and were generally lower than the toxicity of dispersed oil (Fingas, 2008a); 2) Newer test set departing from only traditional lethal aquatic toxicity assay which focus on other aspects, such as genotoxicity, endocrine distruption, and long term effects was in demand (Fingas, 2010d); 3) "A proactive, multispecies and experimentally-consistent approach was recommended to provide threshold toxic effect concentrations for sensitive life stages of aquatic plants inhabiting diverse ecosystems" (Lewis, 2013).

Toxicity effects in terms of LC50 or EC50 (median effective concentration) varied in 6 orders of magnitude because of the diversity of measuring methods (Lewis, 2013). In order to standardize test methods and reduce interlaboratory variability, chemical response to an oil spills: ecological research forum (CROSERF) was proposed (Aurand and Coelho, 2005). Besides, several studies noted that when exposed to ultraviolet (UV) light or UV fraction of sunlight, the dispersed oil had greater toxicity as oil contaminates such as polycyclic aromatic hydrocarbons (PAHs) generated photolysis intermediates with higher toxicity (Barron et al., 2003; Kirby et al., 2007). This photoenhanced toxicity raised the concern about the validity of laboratory tests without the UV fraction of light (Fingas, 2010d).

2.3 Biosurfactants

As reviewed in Section 2.2, currently used dispersants (surfactants mixed with solvents) had the concerns of insufficient effectiveness, persistency in the ecosystem and toxicity to some sensitive life stages of organisms. 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.

2.3.1 Background

Surfactants are key ingredients of dispersants (Fingas, 2010d). They are amphiphilic molecules containing both hydrophilic and hydrophobic moieties that allow them to array at the interface of polar and nonpolar media (Satpute et al., 2010b). The abilities to increase the solubility of hydrophobic materials, to form water-oil emulsions, and to reduce surface and interfacial tension determined the physiochemical characteristics of a surfactant (Desai and Banat, 1997). When surfactants were added into a solution, the surface tension would decrease until the surfactant concentration reached the critical micelle concentration (CMC), at which point surfactant molecules started to form aggregates like micelles with hydrophobic tails directed to the oil phase and hydrophobic heads facing water phase (Mulligan, 2005). These aggregates were structured with various weak chemical forces such as hydrophobic, Van der Waals and hydrogen bonding (Soberón-Chváez and Maier, 2011). When the surfactant had a relatively high hydrophilic-lipophilic balance (HLB) value, the micelles would stabilize the oil-in-water emulsions thus breaking down the oil slick and dispersing the oil into the ocean column through the mixing of currents (Pacwa-Płociniczak et al., 2011). The CMC and HLB values were determined by the molecular structure of surfactants and can be

affected by pH, ionic strength and temperature of the solution (Mulligan, 2005; Soberón-Chváez and Maier, 2011).
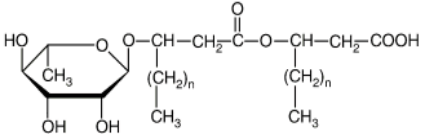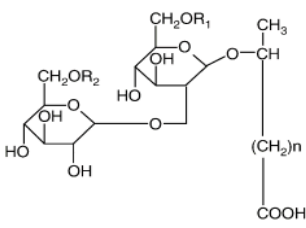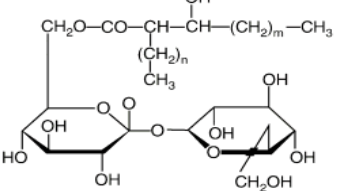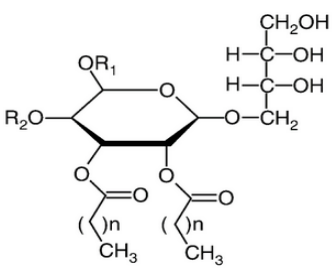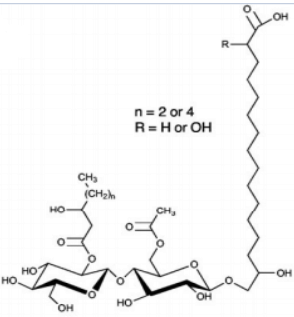
Biosurfactants are surfactants produced extracellularly by microorganisms (Mulligan, 2005). They could be roughly classified as two groups: Low-molecular-weight compounds such as lipopeptides, glycolipids and proteins; high-molecular-weight compounds such as polysaccharides, lipopolysaccharides proteins or lipoproteins (Neu, 1996). Generally, the former group effectively reduced the surface/interfacial tension, while the latter group tended to stabilize oil-in-water emulsions but did not reduce much surface tension (Rosenberg and Ron, 1999; Smyth et al., 2010). Some important biosurfactants and their corresponding producers were summarized in **Table 2.4**. 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).
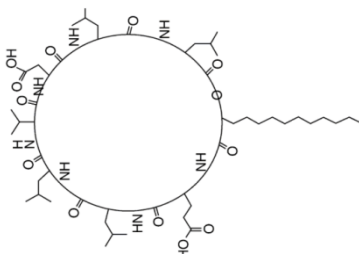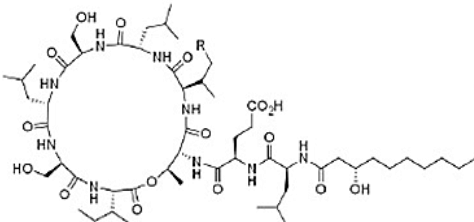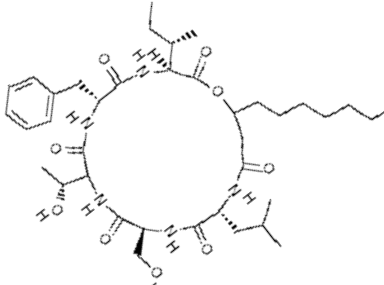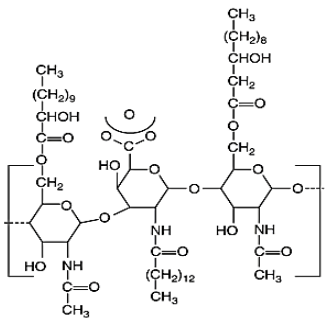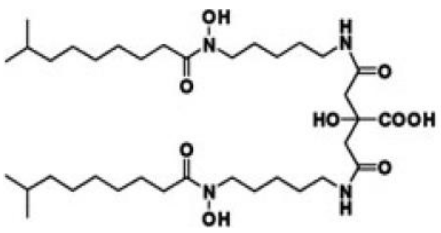
2.3.2 Superiority over the Chemically Synthetic Counterparts

As discussed above, current surfactants have significant toxic concerns and they remain persistent in the environment for an undesirably long period. Therefore, development of better alternatives of surfactants is needed. Biosurfactants which are produced by microorganisms during their growth have been suggested as a promising and renewable source.

Biosurfactants are found to be less toxic, more effective and stable at extreme pH, temperature and salinity, and better at enhancing biodegradation (Muthusamy et al., 2008). Poremba et al., (1991) compared the toxicity of 8 synthetic and 9 biosurfactants

Table 2.4 Examples of some common biosurfactants and their origins.

Head group	Biosurfactant	Molecular structure	Microorganism	Ref.
Glycolipids	Rhamolipids	Monorhamnolipids	*Pseudomonas aerihompsa*	Reiling et al., 1986
	Sophorolipids	Acidic sophorolipid	*Corynebacterium batistae, Thiobacillus bombicalo*	Van Bogaert et al., 2007
	Trehaloselipids	m+n = 27 TO 31 Trehalose monomycolates	*Rhodococcus* sp., *Arthrobacter* sp., *Nocardia erythropolis*	Lang and Philip, 1998
	Mannosyleryth ritol lipids		*Pseudozyma* sp., *Candida antartica, Ustilago maydis*	Kitamoto et al., 2002
	Cellobiose lipids	n = 2 or 4 R = H or OH	*Ustilago zeae, Ustilago maydis*	Hewald et al., 2005

Lipopeptides	Surfactin		*Bacillus subtilis, Bacillus pumilus A*	Seydlova and Svobodova, 2008
	Viscosin		*Pseudomonas fluorescens, P. libanensis*	Laycock et al., 1991
	Serrawettin		*Serratia marcescens*	Matsuyama et al., 1992
Polymeric	Emulsan		*Acinetobacter calcoaceticus*	Rosenberg and Ron, 1999
Others	Flavolipids		*Flavobacterium*	Bodour et al., 2004

that have potential applications in oil spill remediation with mixed and pure cultures of marine bacteria, microalgae, and protozoa. The rankings of the toxicity results showed that most biosurfactants were less toxic than synthetic surfactants. The *Menidia beryllina* 96h LC50 data of 13 chemically and 4 biosurfactants were summarized from the literature and listed in **Table 2.5**. This toxicity test is authorized by USEPA for the toxicity evaluation of oil dispersants. It was found that the mean *Menidia beryllina* 96h LC50 of the listed biosurfactants was about twice of synthetic surfactants indicating the toxicity of biosurfactants were generally lower than the chemically synthetic surfactants.

Some of biosurfactants had much lower CMC so that less biosurfactants was necessary to get a maximum decrease in surface tension (Soberón-Chváez and Maier, 2011). The CMC values of some chemically synthetic surfactants and biosurfactants were listed in **Table 2.6**. The mean CMC of the listed biosurfactants was about one thirds of their chemically synthetic counterparts indicating much smaller dosage of biosurfactants was needed compared to chemically synthetic surfactants. As shown in **Table 2.4**, biosurfactants had amazingly diverse chemical structures. Biosurfactants were produced by microorganisms as complex mixtures of up to 40 congeners which varied intensely at the hydrophobic moieties (Monteiro et al., 2007). The variation further extended the structural diversity and accorded biosurfactants remarkably different properties and behaviors at interfaces (Soberón-Chváez and Maier, 2011). Biosurfactants also had shorter alkyl chains than typical synthetic surfactants which made them easily dissolved in water (Soberón-Chváez and Maier, 2011). These structural properties were intriguing and exhibited surprisingly high surface activities (Soberón-Chváez and Maier, 2011).

Table 2.5 *Menidia beryllina* **96 h LC50 values of some chemically synthetic surfactants and biosurfactants**

Chemically synthetic surfactants	*M. beryllina* 96h LC50 (mg/L)	Ref.	Biosurfactants	*M. beryllina* 96h LC50 (mg/L)	Ref.
BIODISPERS	13.5	Berninger et al., 2011	JE1058BS	91.7	Saeki et al., 2009
COREXIT®EC9500A	25.2		BIOEM	14.7	Edward et al., 2003
COREXIT®EC9527A	14.6		EMULSAN	345.3	
DISPERSIT SPC 1000 ™	3.5		PES-51	21.7	
FINASOL® OSR 52	11.7		Mean	118.4	
JD-109	1.9		S.D.	155.2	
JD-2000 ™	407				
NEOS AB3000	91				
NOKOMIS 3-AA	34.2				
NOKOMIS 3-F4	29.8				
SAF-RON GOLD	29.4				
SEA BRAT #4	30				
ZI-400	31.8				
Mean	55.7				
S.D.	107.9				

Table 2.6 CMC values of some chemically synthetic surfactants and biosurfactants

Chemically synthetic surfactants	CMC (µM)	Ref.	Biosurfactans	CMC (µM)	Ref.
Triton X-100	240	Nantes et al., 2011	Rhamnolipids	5	Desai and Banat, 1997
Brij 35	60		Trehalolipids	12	
Tween 80	12		Peptide-lipid	16	
Arachidonic acid	60		Surfactin	91.5	
DTAO	32		Carbohydrate-protein-lipid	10	
Mean	80.8		Mean	26.9	
S.D.	91.3		S.D.	36.3	

For example, muticomponet mono-rhamolipid had CMC values ranging from <1 μM to 10 μM (Lebron-Paler, 2008). In contrast, the chemically synthesized alkyl glucosides with similar structure had CMC values higher that 10 mM (Soderman and Johansson, 2000). Moreover, when applied to enhance bioremediation, some chemically synthetic surfactants were found to inhibit the PAH biodegradation through toxic interactions, sequestration of PAHs into micelles, or promoting surfactant degraders instead of PAHs degraders. In contrast, biosurfactants were found with no such inhibition (Makkar and Rockne, 2003). Besides, the half-lives of biosurfactants were shorter than that of their chemically synthetic couterparts and the biodegradation of biosurfactants were found to be complete (Finnerty, 1994). Furthermore, Biosurfactants have enormous diversity, and could be produced from various sources, such as industrial wastes with simple and inexpensive procedures (Desai and Banat, 1997; Muthusamy et al., 2008).

Other than the surface activities, biosurfactants have also been found to protect the producing microorganism from toxic heavy metals through various mechanisms (Sandrin et al., 2000), and other microorganisms by acting as antibiotics (Peypoux et al, 1999). They also regulate the attachment and detachment of microorganisms to and from surfaces (Neu, 1996).

2.3.3 High Cost Issue and Possible Overcoming Strategies

Despite of the above advantages, the application of biosurfactants has been significantly restricted due to their low yields and high production cost as well as the lack of desired producing microorganisms (producers) (Mukherjee et al., 2006). It was estimated that biosurfactants would cost 3-10 times of synthetic surfactants (Mulligan and Gibbs, 1993).

Three strategies have been proposed to cope with the economic constraints associated with biosurfactat production: i) the use of waste streams or cheap substrates to reduce the initial raw material costs; ii) the improvement of efficiency of the production process (e.g., optimization of cultural condition and cost-effective separation process); iii) the development and use of superior biosurfactant producing microogranisms (Mukherjee et al., 2006).

Following the first strategy, several studies have been conducted using inexpensive raw materials. Some of these studies were summarized in **Table 2.7.** For the second strategy, recent studies used statistical methods based on response surface methodology (RSM) (Rodrigues et al., 2006b; Sen and Swaminathan, 2005) and modeling strategy based on artificial neural network (ANN) plus a genetic algorithm (GA) were conducted (Sivapathasekaran et al., 2010). Some studies applied both methods to optimize other fermentation processes and compare the effectiveness of these two strategies (Lu et al., 2011).

Another path for the second strategy is to optimize the downstream process to achieve fast, efficient and cheap product recovery. It was estimated that the downstream processing (recovery of biosurfactant from the culture broth) costs accounted for around 60% of the total production cost (Mukherjee et al., 2006). Conventional recovery process such as acid precipitation, solvent extraction, crystallization, ammonium sulfate precipitation were not feasible for the large-scale continuous mode (Mukherjee et al., 2006). Recently, some novel methods such as foam fractionation (Davis et al., 2001), ultrafiltration (Sen and Swaminatham, 2005), adsorption-desorption on resins and wood-based activated carbon (WAC) (Dubey et al., 2005) have been studied. These methods

Table 2.7 Summary of inexpensive raw materials for biosurfactant production

(Mukherjee et al., 2006)

Raw material	Biosurfactant type	Producer microbial genus	Maximum yields (g/L)
Rapeseed oil	Rhamnolipids	Pseudomonas	45
Babassu oil	Sophorolipids	Candida	-
Turkish corn oil	Sophorolipids	Candida	400
Sunflower and soybean oil	Rhamnolipid	Pseudomonas	4.31
Sunflower oil	Lipopeptide	Serratia	-
Soybean oil	Mannosylerythritol lipid	Candida	95
Waste frying oils	Rhamnolipid	Pseudomonas	2.7
Soybean soapstock waste	Rhamnolipid	Pseudomonas	11.72
Sunflower oil soapstock waste	Rhamnolipid	Pseudomonas	16
Oil refinery wastes	Glycolipids	Candida	10.5
Soybean oil refinery wastes	Rhamnolipids	Pseudomonas	9.5
Curd whey and distillery wastes	Rhamnolipid	Pseudomonas	0.92
Potato process effluents	Lipopeptide	Bacillus	-
Cassava flour wastewater	Lipopeptide	Bacillus	2.2-3.0

were designed for large-scale continuous recovery of biosurfactants from culture broth which may further reduce the production cost.

In order to develop novel and superior biosurfactant producing microogranisms. Several commonly used screening methods such as drop collapse, oil spreading, blood agar lysis, hemlytic assay, tilted glass slide, blue agar plate, hydrocarbon overlaid agar plate, emulsification index, emulsification assay and surface tension measurement were used (Youssef et al., 2004; Satpute et al., 2008; Yilmaz et al., 2009). Several studies were conducted to assess different screening methods for selecting biosurfactant producing bacteria. Youssef et al., (2004) compared drop collapse, oil spreading, blood agar lysis methods, and surface tension measurements with 205 environmental strains. The results suggested the use of the drop collapse method as a primary methods followed by the oil spreading tests would be quick and reliable. Satpute et al., (2008) compared hemlytic assay, drop collapse method, tilted glass slide, blue agar plate, hydrocarbon overlaid agar plate, emulsification index, emulsification assay and concluded that singe method was not sufficient to identify different types of biosurfactant producers. They recommended the use of drop collapse, tilted glass slide test, oil spread method followed by emulsification assay as the primary screening protocols.

While extensive research efforts have been put on the first two strategies, the research efforts on developing microorganism that can produce effective biosurfactants with high product yields are limited. As shown in **Table 2.4**, biosurfactant production was still limited to several categories and compounds so far in the literature. Thus, more efforts in this direction should be made to address these challenges (Mukherjee et al., 2006).

2.3.4 Sources for Screening Superior Biosurfactant Producers

The biosurfactant producers are commonly obtained from diverse terrestrial sources (Das et al., 2010). In recent decades, the searching for novel biosurfactant producers have been extended to diverse habitats with extreme salinity, temperature and pressure, such as oceans, hot springs, and oil wells (Joshi et al., 2008; Satpute et al., 2010a; Yakimov et al., 1995). The marine environment consititutes nearly 75% of the earth's surface and is a robust reservoir of diverse microflora including biosurfactant producers (Das et al., 2010). Marine microorganisms have extraordinary metabolic and physiological capabilities that are rarely found in their terrestal counterparts (Satpute et al., 2010a). Moreover, the exploration of marine biosurfactant producers are relatively rare (Das et al., 2010; Satpute et al., 2010a). A comprehensive literature review has been conducted in this study and 57 publications focusing on isolation of marine biosurfactant producers were identified (**Table 2.8** and **Figure 2.3**). These studies mainly concentrated on low latitude areas and none of them from the North Atlantic Canada. Only one study was reported in the 8 Arctic states hitherto (Gerard et al., 1997), despite the fact that the northen region with cold and harsh environments has been attracting growing attention and considered as one of the hotest spots for novel organism discovery (Bull et al., 2000).

Moreover, when applied in a cold environment, biosurfactants with low CMT are desired, as when the CMT was higher than the applied sites biosurfactants would remain crystaline and have no surface-active functions (Gu and Sjöblom, 1992). The cold adapted microorganisms would be capable of producing biosurfactants with low CMT and thus having potential of applications under diverse conditions including the cold and harsh environments.

Table 2.8 Summary of the isolated biosurfactant producers from marine environments

Location	No.	Sampling details	Isolates	References
Canada (Masset Inlet, BC)	1	Leafy red algae and tube worm	1 *Pseudomonas*	Gerard et al., 1997
Germany (North sea, Borkum and some northern areas)	4	2 from seawater/sediment samples, 1 from deep oil reservoirs, 1 unknown	2 *Alcanivorax*, 1 *Alcaligenes*, 1 *Arthrobacter*, 1 *Bacillus*	Passeri et al., 1992; Schulz et al., 1991; Yakimov et al., 1998; Yakimov et al., 1995
UK (Menai straits and some unknown regions)	3	1 from seawater, 2 unknown	1 *Pseudomonas*, 1 *Antarctobacter*, 1 *Halomonas*	Fletcher and Floodgate, 1973; Gutiérrez et al., 2007a; Gutiérrez et al., 2007b
France (Gulf of Fos and Gulf of Marseille)	3	2 from Refinery areas, 1 from hydrocarbon polluted sea surface water	1 *Marinobacter*, 1 *pseudomonas*, 1 *Alcaligenes*	Al-Mallah et al., 1990; Bonin et al., 1987; Goutx et al., 1987
Georgia	1	unknown	1 *Acinetobacter*	Taylor and Juni, 1961
Japan (Sagami Bay and some unknown regions)	3	1 from deep ocean clam, 2 unknown	1 *Dietzia,* 1 *Bacillus,* 1 *Pseudozyma*	Arima et al., 1968; Konishi et al., 2010; Nakano et al., 2011
Israel	3	unknown	3 *Acinetobacter*	Navon-Venezia et al., 1995; Reisfeld et al., 1972; Rosenberg et al., 1988
Iran (Khark, Siri, Hormoz, Bushehr, Bandar-Abbas and Qeshm islands and some unknown regions)	3	1 from oil contaminated sediment and seawater, 1 from toluene contaminated seawater, 1 from seawater	1 *Acinetobacter,* 1 *Rhodococcus,* 2 *Halomonas,* 1 *Alcanivorax,* 1 m*arinobacter,* 1 *Microbacterium,* 1 *Sporosarcina,* 1 *Bacillus* and 1 *Bacterium EX-DG74*	Abari et al., 2012; Hassanshahian et al., 2012
China (Xiamen)	1	Seaside soil	*Rhodococcus*	Peng et al., 2007
Nigeria (Lagos lagoon)	1	Water sample	*Aeromonas*	Ilori et al., 2005
Singapore (straits of Malacca and Singapore)	1	Surface seawater	*Alcanivorax hongdengensis*	Wu et al., 2009

Country (regions)	No.	Source	Genera	References
India (Alang coast, Gujarat, Mumbai, Alibag, Janjira, Goa coastal, Andhra Pradesh, Andaman and Nicobar Islands, Tamil Nadu,Tuticorin harbour, and southwest, west, south coast and some other unidentified regions)	22	1 from crude oil contaminated seawater, 1 from refinery sludge, 3 from marine sediment, 8 from seawater, 5 from marine sponge, 1 from seawater, sediment, sand, mussels and shells, 1 from petroleum contaminated mangrove sediment, 2 unknown	1 *Nocardia*, 1 *Planococcus*, 1 *Acinetobacter*, 3 *Nocardiopsis*, 1 *Yarrowia*, 3 *Streptomyces*,4 *Pseudomonas*, 1 *Aspergillus*,3 *Bacillus*, 2 *Brevibacterium*, 1 *Azobobacter*, 1 *Corynebacterium*	Coelho et al., 2003; Das et al., 2008; de Sousa and Bhosle, 2012; Desai et al., 1988; Gandhimathi et al., 2009; Gnanamani et al., 2010; Hanson et al., 1994; Khopade et al., 2012a; Khopade et al., 2012b; Kiran et al., 2009; Kiran et al., 2010a; Kiran et al., 2010b; Kiran et al., 2010c; Kokare et al., 2007; Kumar et al., 2007; Shubhrasekhar et al., 2013; Thavasi et al., 2007, 2008; Thavasi et al., 2011; Thavasi et al., 2009; Vyas and Dave, 2011; Zinjarde and Pant, 2002
Thailand (Songkhla, Songkhla lagoon, Gulf of Thailand and southern regions)	5	3 from oil contaminated seawater, 1 from mangrove sediment, 1 from seawater	*1 Pseudomonas, 1 Leucobacter, 1 Ochrobactrum, 1 Myroides, 2 Vibro, 2 Bacillus, 2 Micrococcus, 2 Acinetobacter*	Gerard et al., 1997; Maneerat et al., 2005; Maneerat and Phetrong, 2007; Phetrong et al., 2008; Saimmai et al., 2012; Thaniyavarn et al., 2006
Indonesia (Cilacap coast)	1	Petroleum hydrocarbon contaminated water and sediment from mangrove system	3 *Bacillus,* 1 *Flexibacteraceae,* 1 H*alobacillus,*1 *Rhodobacteraceae*	Syakti et al., 2013
Papua New Guinea (Loloata island)	1	Tissue of tube worm	*Brevibacillus*	Desjardine et al., 2007
Brazil (Rio de Janeiro)	1	N/A	*Yarrowia*	Amaral et al., 2006
Australia (Hautman Reef)	1	Marine sponge Ircinia sp	*Bacillus*	Kalinovskaya et al., 1995
Uruguay (Montevideo bay)	1	Hydrocarbon polluted sediment	*Pseudomonas*	Bonilla et al., 2005
Antarctica	1	Superficial seawater	*Halomonas*	Pepi et al., 2005

Figure 2.2 World distribution of studies regarding isolation of marine biosurfactant producers

On the other hand, production of biosurfactants occurs predominently on hydrophobic substrates such as petroleum hydrocarbons (Ward, 2010). Petroleum contaminated environments have high potential of providing favorable conditions for biosurfactants producers, and the associated bioremediation potentials have been preliminarily examined (Abari et al., 2012; de Sousa and Bhosle, 2012; Syakti et al., 2013). As shown in **Figure 2.4A**, there were 15 studies using the petroleum hydrocarbon contaminated sources to isolate the biosurfactant producers and the bioremediation potential of the isolates was reported in 10 of them. Therefore, it is highly desired to further explore marine biosurfactant producers from petroleum hydrocarbon contaminated sources in high-latitude seas where the cold weather and harsh environments prevail.

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(A)

(B)
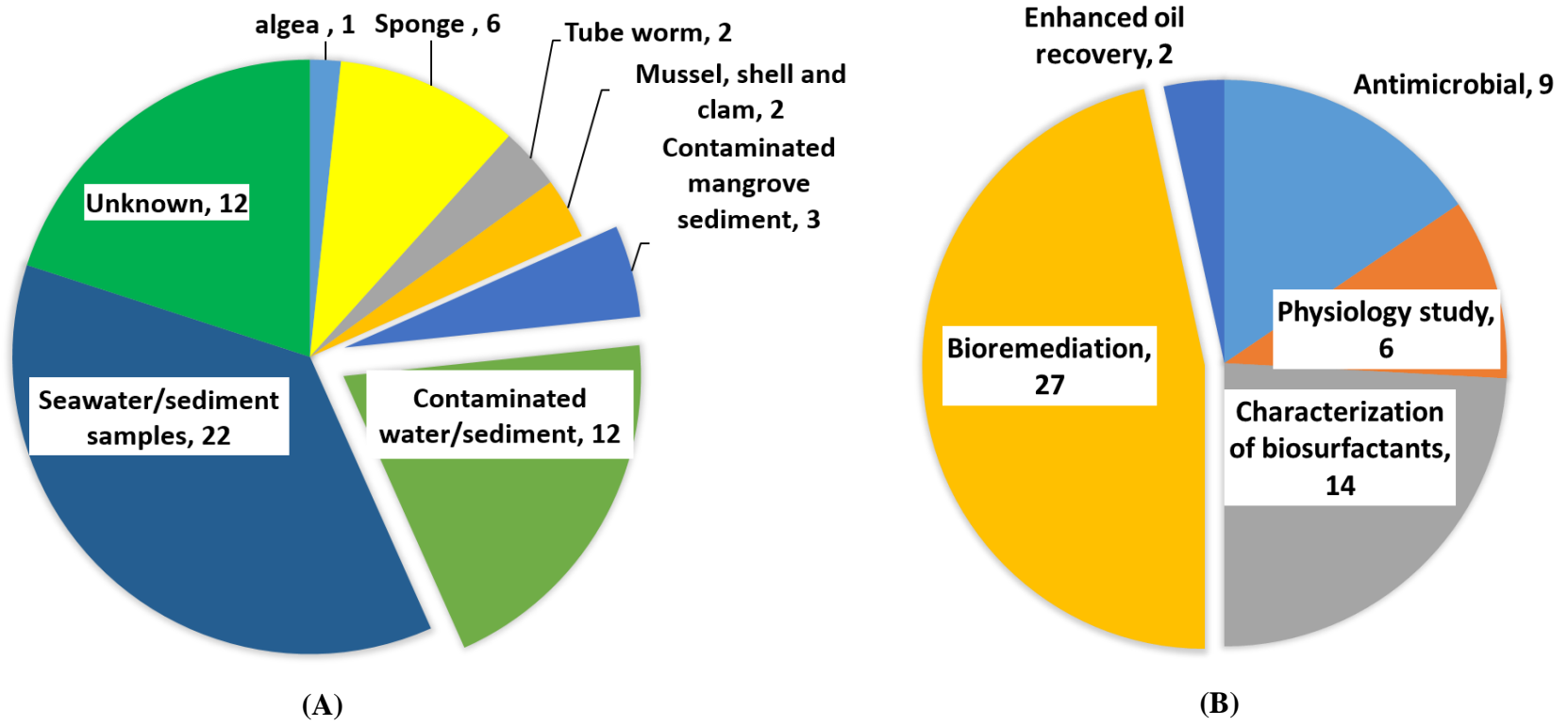
Figure 2.3 The isolation sources (A) and suggested application fields (B) of the 57 reviewed studies (Exploded slices represents those studies from petroleum hydrocarbon contaminated sites aiming at bioremediation purposes)

2.4 Summary

This chapter started with literature review of oil spills. Some basic physiochemical properties of oil spills have been reviewed, followed by the history of oil spills. Subsequently, the review extended to the impacts of oil spills and the relevant response technologies. The review from this part concluded that dispersants can be an effective combat agent for offshore oil spill even in cold and harsh environment, and it has indeed played a very important role during the response of Deepwater Horizon oil spill. The following section reviewed dispersants including their effectiveness, fate and transport, and toxicity concerns. These three aspects of dispersants all revealed some of the disadvantages and research and develop (R&D) direction of dispersants. Currently used chemically synthetic dispersants were found to be not very effective, especially when dealing with weathered or emulsified oil; their toxicity concerns were prevailing among public; they were found to be persistent in the ecosystem. Under such circumstances, biosurfactants-based dispersants were proposed as possible alternatives. In the third section of this chapter, several aspects of biosurfactants were reviewed. Firstly, some fundmental concepts of biosurfactants were discussed. Then, the superiorities over chemical surfactants such as lower toxicity and CMC, less persistent and better at enhancing biodegradation.Then, their high cost issue and possible overcoming strategies were discussed. Finnaly, sources for screening superior biosurfactant producers were reviewed and it was found marine sources contaminated with petroleum hydrocarbon were underexplored and of great potential. The screening of such marine biosurfactant producers have not been done in North Atlantic Canada.

CHAPTER 3

SCREENING OF BIOSURFACTANT PRODUCERS FROM PETROLEUM HYDROCARBON CONTAMINATED SOURCES IN MARINE ENVIRONMENTS IN ATLANTIC CANADA

3.1. Background

As reviewed in Section 2.3.4, marine sources contaminated with petroleum hydrocarbon had potential to be the effective sources for isolating superior biosurfactant producers. Besides, such producers have never been published in the region of North Atlantic Canada. The aim of this study is thus to fill the knowledge gaps by screening biosurfactant producers from petroleum hydrocarbon contaminated sources in cold marine environments in Atlantic Canada, characterizing isolated producers and examining their diversity and regional distributions, and testing the efficacy of produced biosurfactant.

3.2 Methdology

3.2.1 Sampling

Oily contaminated water samples from two heavy traffic harbors and sediment samples from coastal zone in the vicinity of a refinery company were collected for screening novel biosurfactant producers. Before the collection of sediment samples, temperature, salinity, conductivity and total dissolved solid (TDS) were measured on site with a conductivity meter (Thermo Scientific Orion Star A222). Samples were shipped with ice bags and stored in amber bottles at 4°C.

The information of the sampling sites and physiochemical properties of the samples were summarized in **Table 3.1**, while the photos of sampling were shown in **Figure 3.1**. All samples were taken within N43.9^{o} to N47.8^{o} and the locations were shown in **Figure 3.2**. The cold and harsh environments prevail in the offshore sampling areas.

Table 3.1 Physiochemical properties of the marine and coastal samples

Sample ID	Conductivity (ms/cm)	TDS (ppt)	Salinity (ppt)	Temperature (°C)
P1	47.9	>200	29.76	N/A
P2	74.2	>200	46.43	N/A
P3	232.4	>200	>80	N/A
P4	75.8	>200	47.37	≈60
P5	78.5	>200	48.98	N/A
P6	22.68	11.11	12.57	N/A
P7	75.5	>200	47.33	≈60
P8	74.8	>200	47.36	≈60
P9	75.4	>200	47.16	≈60
P10	74.8	>200	46.82	≈60
SJ	44.5	>200	26.76	N/A
CBS	49.9	>200	30.46	N/A
N1	37.6	18.4	23.63	19
N2	95.6	5.13	5.984	25.3
N3	33.4	16.4	20.85	22.2
N4	588	>200	0.331	11.8

Note: the temperature data for P4, P7-P10 were measured in situ and provided by the sample providers
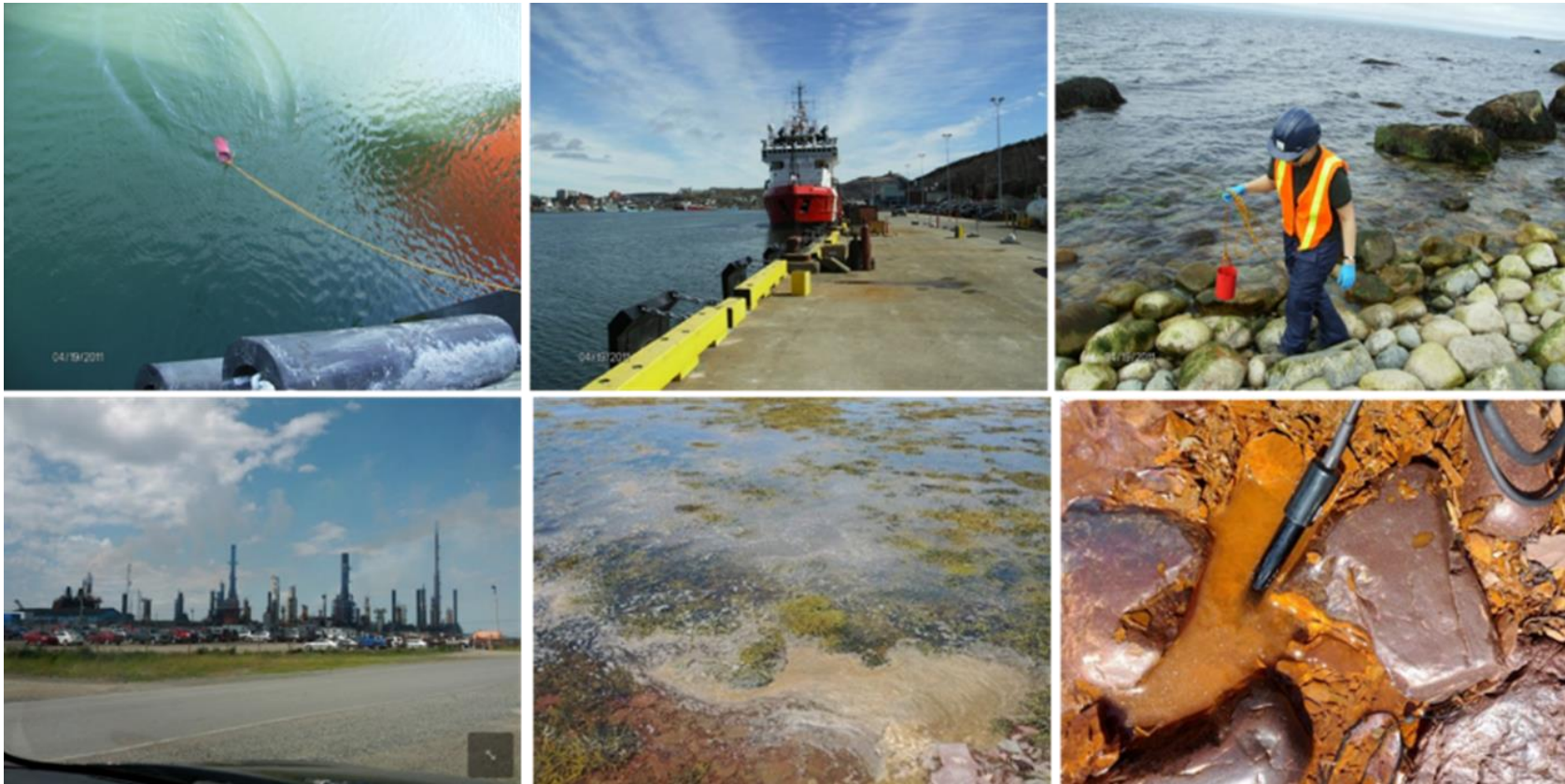
Figure 3.1 Water and sediment sampling (detailed information of the photos were emitted due to the confidential consideration)
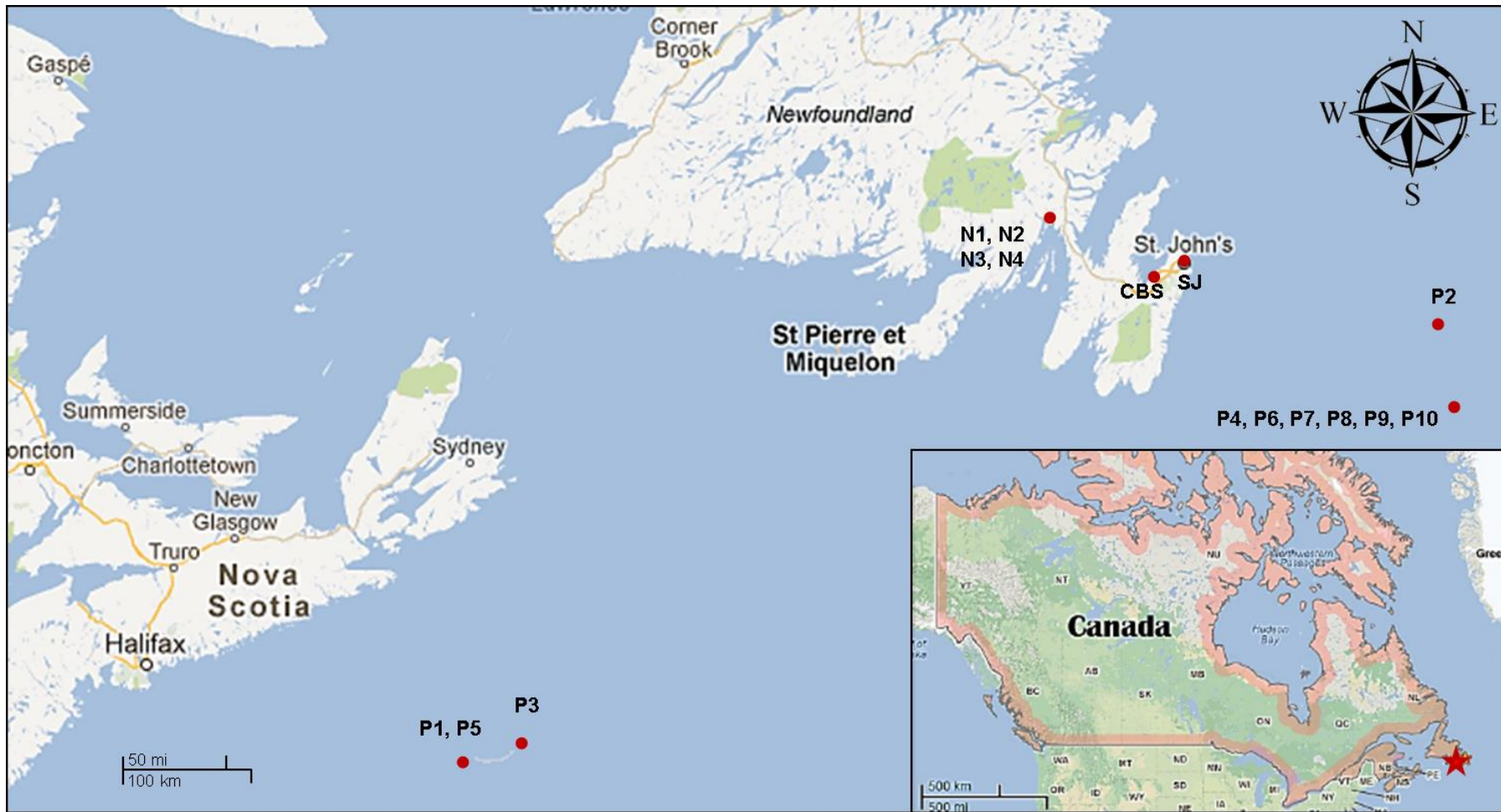
Figure 3.2 Sampling locations (Note: Dots indicate the sampling sites and annotation indicates the corresponding sample ID; offshore samples were provided by other research entity)

3.2.2 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 and trace element solution, 0.5 mL L^{-1} of distilled water, with 2% (v/v) n-hexadecaneas as the sole carbon source, which was adopted and modified from Peng et al. (2007) with salinity adjusted to 3.5%. 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 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 L^{-1} 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 $30^{o}C$, 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 plate with the same composition as the enrichment medium. The resulting plates were incubated at $30^{o}C$ for 3-5 days until slow growers form clear colonies. Subsequently, a drop collapsing test modified from Bodour and Miller-Maier (1998) was used to screen biosurfactant producers. The traditional method operated with liquid cultures which excluded sessile bacteria. Besides, the liquid media cultivation might increase the risk of contamination and was time consuming. The modified method overcame these problems with direct colonies addition. 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.

3.2.3 Identification and Morphological Characterization of Isolates

The purified isolates were 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 the 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, 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 (CREAIT) of MUN. 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 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. The morphology of isolates on the agar plates used during isolation and in liquid media were observed and recorded.

3.2.4 Performance of the Isolated Biosurfactant Producers

(1) *Performance evaluation of all isolated biosurfactant producers*

Three parameters were applied to evaluate the efficacy of the newly generated biosurfactants. Relevant analytical methods of the parameters are indicated below.

Biomass determination: Each isolate was incubated using the same conditions as isolation procedure for 7 days. Subsequently, 10 mL culture sample was filtered through a pre-weighted 0.2 μm filter membrane and washed two times with 10 mL distilled water. The filter membrane was then dried in oven (70°C) for 24 h and cooled down in desiccators before reading the final weight. The biomass was determined as cell dry weight (g/L).

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 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 (3-1):

$$E24 = H_{EL}/H_S \times 100\% \tag{3-1}$$

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

Performance demonstration of two selected isolates

One isolate that most effectively reduced surface tension and emulsified n-hexadecane/water and one isolate that significantly reduced surface tension and had high flocculating activity were selected to further demonstrate the performance of biosurfactant production. 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; $(NH_4)_2NO_3$, 1 g; $FeCl_3$, 0.05 g; Glucose, 1g; NaCl, 26 g L^{-1} of distilled water, with 3% (v/v) n-hexadecane was selected based on screening of incubation conditions. The PM was then used to investigate the performance of biosurfactant production. The incubation was conducted at 30^oC while shaking at 150 rpm. The two selected isolates were first incubated using the above conditions for 48 hrs. A 200 μL aliquot of the resulting culture was inoculated to each flask containing 20 mL of the PM. The overall 22 flasks were used and incubated under the same conditions. The following two tests at time intervals of 12, 24, 30, 36, 42, 48, 54, 60, 66, 72 and 96 hours, respectively were conducted in duplication:

1) Surface tension measurement and emulsification activity assay as mentioned in section 3.2.4.

2) Critical micelle dilution (CMD): 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 discard the pellet, the cell free broth 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 with sonication to ensure homogeneity. Before each measurement, the sonicated solution was allowed to stand for 15-20 min to achieve equilibrium.

In addition, for the selected isolates formed flocculants, the flocculating activity of culture medium after 4 days incubation was tested according to a slight modified previous method (Shih et al., 2001), in which bacterial cells of the selected isolates was chosen as the suspended solid. Briefly, freshly prepared PM was added to culture broths with various volumes to make each 2 mL after mixing in a test tube, followed by the addition of 2 ml cell suspension in each mixture. The mixture was shaking at 150 rpm overnight and kept standing for 5 min. The formation of flocs floating at the surface was observed. The optical density (OD) of the lower phase was measured at 550 nm with a spectrophotometer. A control experiment with only 2 mL of freshly prepared PM was also included. All the measurements were duplicated. Flocculating activity was calculated according to equation (3-2):

Flocculating activity$=1/OD_{550} - 1/(OD_{550})_c$, $\qquad\qquad\qquad$ (3-2)

in which

OD_{550} is the optical density of the sample at 550 nm,

$(OD_{550})_c$ is the optical density of control at 550 nm

3.3 Results and Discussion

3.3.1 Phylogenetic Analysis of Isolates

As shown in **Table 3.2**, in total 55 biosurfactant producers and possible petroleum hydrocarbon degraders were isolated and identified according to their 16S rDNA sequencing results. The isolated strains belonged to eight different genera among which three were recently established, i.e. *Alcanivorax*, *Exiguobacterium* and *Halomonas*. The genus *Alcanivorax* currently includes 8 species; all of them were isolated from marine environments and were found to be important alkane degrading bacteria (Lai et al., 2013). The genus of *Exiguobacterium* comprises of species that can grow at a wide temperatures span of -6ºC to 55ºC and is considered as extremophiles (Vishnivetskaya et al., 2009). Several *Exiguobacterium* strains were found with unique properties such as production of diverse enzymes that were stable at a broad range of temperature, neutralizing alkaline wastewater, removal of pesticide and reducing heavy metals (Vishnivetskaya et al., 2009), whereas no *Exiguobacterium* strains has been reported 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 that some *Halomonas* strains produced (Gutiérrez et al., 2007b; Pepi et al., 2005), but the efforts were limited compared with other well-studied biosurfactant producing genus. These three interesting isolates and their corresponding biosurfactants will be further characterized.

In addition, most of the 55 strains belonged to genera of *Rhodococcus* (24 strains) and *Bacillus* (20 strains) (**Table 3.2**). The *Rhodococcus* and *Bacillus* strains were first clustered separately to construct two phylogenetic trees. Both genera had 4 phyletic groups and one representing strain was selected to generate the complete phylogenetic trees with the other type strains of the genus. Under the genus of *Bacillus*, N3-4P, N3-7P,

Table 3.2 Identification of the isolated biosurfactant producers

Isolate ID	Species name with highest match	Max identity%	Liquid medium morphology	Agar plate morphology (shape/margin/elevation/color)
CBS1	*Bacillus Thuringiensis*	100	Pellicle	Circular/curled/umbonate/light orange
SJ-1A	*Rhodococcusyunanensis*	100	Flocculent	Circular/entire/convex/orange
N1-1P	*R. phenolicus*	99	Turbidity	Circular/curled/raised/orange
N1-2P	*B. subtilis*	100	Pellicle	Irregular/undulate/flat/light brown
N2-1P	*R. zopfii*	99	Turbidity	Circular/entire/convex/orange
N2-3P	*B. subtilis*	100	Turbidity	Irregular/undulate/flat/light brown
N2-4P	*R. erythropolis*	100	Pellicle	Irregular/undulate/umbonate/beige
N2-5P	*R. erythropolis*	100	Flocculent	Irregular/undulate/umbonate/beige
N2-6P	*B. subtilis*	99	Pellicle	Circular/undulate/umbonate/beige
N2-7P	*B. subtilis*	98	Pellicle	Circular/undulate/umbonate/beige
N2-2A	*R. erythropolis*	100	Flocculent	Circular/undulate/umbonate/beige
N3-1P	*B. subtilis*	100	Flocculent	Irregular/lobate/flat/bown
N3-2P	*R. zopfii*	98	Turbidity	Circular/entire/convex/orange
N3-3P	*R. wratislaviensis*	98	Turbidity	Circular/entire/convex/orange
N3-4P	*B. licheniformis*	100	Turbidity	Circular/entire/convex/orange
N3-5P	*R. phenolicus*	98	Pellicle	Circular/entire/convex/orange
N3-6P	*Pseudomonas peli*	99	Pellicle	Circular/undulate/umbonate/beige
N3-7P	*B. flexus*	99	Pellicle	Circular/undulate/umbonate/beige
N3-8P	*B. mycoides*	100	Turbidity	Irregular/undulate/flat/light brown
N3-9P	*B. subtilis*	100	Pellicle	Circular/undulate/umbonate/beige
N3-11P	*B. subtilis*	96	Turbidity	Circular/undulate/convex/orange
N3-2A	*Halomonas venusta*	99	Flocculent	Circular/entire/convex/beige
N3-3A	*R. fascians*	99	Pellicle	Circular/undulate/convex/yellow
N3-4A	*R. cercidiphyllis*	99	Pellicle	Circular/undulate/convex/yellow
N3-5A	*R. fascians*	99	Pellicle	Circular/undulate/convex/yellow
N3-6A	*Streptomyces venezuelae*	98	Granular	Filamentous/filamentous/flat/grey
N3-7A	*Alcanivorax venustensis*	96	Sediment	Irregular/undulate/flat/transparent
N3-8A	*H. variabilis*	99	Granular	Circular/undulate/convex/yellow
N4-1P	*Exiguobacterium antarcticum*	99	Turbidity	Irregular/lobate/flat/orange
N4-2P	*B. subtilis*	100	Pellicle	Circular/undulate/flat/brown
N4-3P	*R. yunnanensis*	100	Turbidity	Circular/undulate/umbonate/yellow
N4-1A	*R. erythropolis*	99	Flocculant	Circular/undulate/convex/orange
P1-1P	*R. opacus*	98	Turbidity	Irregular/undulate/convex/orange
P1-2P	*B. subtilis*	99	Pellicle	Circular/undulate/convex/beige

P1-3P	*B. subtilis*	98	Turbidity	Irregular/undulate/flat/light brown
P1-4P	*R. opacus*	98	Turbidity	Circular/entire/convex/orange
P1-5P	*R. wratislaviensis*	98	Flocculent	Circular/entire/convex/light orange
P1-6P	*B. subtilis*	96	Pellicle	Circular/undulate/umbonate/orange
P1-1A	*Acinetobactercalc oaceticus*	99	granular	Circular/entire/convex/beige
P2-2P	*R. erythropolis*	100	Pellicle	Circular/curled/umbonate/beige
P3-1P	*B. subtilis*	99	Flocculent	Circular/lobate/flat/light brown
P4-1P	*R. opacus*	99	Turbidity	Circular/entire/convex/orange
P4-2P	*R. erythropolis*	99	Flocculent	Circular/curled/umbonate/beige
P5-1P	*R. erythropolis*	99	Flocculent	Circular/entire/convex/yellow
P5-1A	*A. oleivorans*	100	Turbidity	Circular/entire/convex/beige
P6-2P	*B. subtilis*	100	Turbidity	Irregular/undulate/flat/brown
P6-3P	*B. subtilis*	98	Turbidity	Irregular/lobate/flat/brown
P6-4P	*R. erythropolis*	99	Pellicle	Circular/curled/umbonate/beige
P6-5P	*R. erythropolis*	100	Pellicle	Circular/curled/umbonate/beige
P6-6P	*R. erythropolis*	100	Pellicle	Circular/curled/umbonate/beige
P6-7P	*B. thuringiensis*	100	Turbidity	Irregular/lobate/flat/brown
P6-9P	*R. erythropolis*	99	Pellicle	Circular/undulate/umbonate/light orange
P7-1A	*A. oleivorans*	100	Turbidity	Circular/entire/convex/beige
P9-1A	*A. calcoaceticus*	99	Pellicle	Circular/entire/convex/beige
P10-1P	*B. subtilis*	99	Turbidity	Irregular/lobate/flat/brown

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

oil agar)

N3-8P, and P6-7P were selected to construct the phylogenetic tree as shown in **Figure 3.3A.** Under the genus of *Rhodococcus*, N3-2P, P2-2P, SH-1A and N3-4A were selected and the phylogenetic tree was shown in **Figure 3.3B**. The selected *Bacillus* strains formed a stable phyletic group within a heterogeneous cluster of *B. thuringiensis, B. mycoides, B. licheniformis* and *B. subtilis* which were commonly isolated from marine environments (Ivanova et al., 1992; Ivanova et al., 2010). In the contrast, *Rhodococcus* strains were scattered and their closely related species were ubiquitous.

The distribution of the 4 genera in North Atlantic Canada is summarized in **Table 3.3**. Sampling site N3 showed the highest diversity of biosurfactant producers which may be due to the moderate temperature and salinity conditions as shown in **Table 3.1**. The isolated genera were location and/or medium specific. *Bacillus* strains were isolated with Peng's agar while *Acinetobater* were isolated with Atlas oil agar from offshore samples. When using Atlas oil agar, *Rhodococcus* were only isolated from coastal samples.

3.3.2 Morphological Characters of Isolated Culture

The morphological characters of isolates are summarized in Table 2. The morphology of isolates on agar plates varied with different isolates. In liquid medium, some isolates, most notably, P1-5P and N4-1A formed flocculants during their growth (**Figure 3.4**). Flocculation in microbial systems has been investigated extensively and a correlation has been established between cell aggregation and secretion of extracellular biopolymeric flocculants (EBFs) (Salehizadeh and Shojaosadati, 2001). The EBFs have been reported to be polysaccharides, proteins, lipids, glycolipids and glycoproteins (Zheng et al., 2008). Some of the biosurfactants shared the same structure and composition with EBFs. As discussed in the introduction, the primary bottleneck of large-

(A)

Bacillus vireti LMG 21834^T (AJ542509)
Bacillus novalis LMG21837^T (AJ542512)
Bacillus soli LMG21838^T (AJ542513)
Bacillus drentensis LMG 21838^T (AJ542506)
Bacillus bataviensis LMG 21838^T (AJ542508)
Bacillus niacin IFO 15566^T (AB021194)
Bacillus fumarioli LMG 17489^T (AJ250056)
Bacillus pocheonensis Gsoil 420^T (AB245377)
Bacillus foraminis CV53^T (AJ717382)
Bacillus subterraneus DSM 13966^T (FR733689)
Bacillus boroniphilus T-15Z^T (AB198719)
Bacillus jeotgali YKJ-10^T (AF221061)
Bacillus selenatarsenatis SF-1^T (AB262082)
Bacillus thioparans BMP-1^T (DQ371431)
Bacillus persicus B48^T (HQ433471)
Bacillus purgationiresistens DS22^T (FR666703)
Bacillus horneckiae DSM 23495^T (FR749913)
Bacillus oceanisediminis H2^T GQ292772)
Bacillus firmus NCIMB 9366^T (X60616)
Bacillus asahii MA001^T (AB109209)
Bacillus psychrosaccharolyticus ATCC 23296^T (AB021195)
Bacillus butanolivorans K9^T (EF206294)
Bacillus simplex NBRC 15720^T (AB363738)
Bacillus muralis LMG 20238^T (AJ628748)
Bacillus flexus DSM 1320^T (AB021185)
N3-8P
P6-7P
Bacillus thuringiensis GTC 02829^T (AB592540)
Bacillus mycoides DSM 2048^T (AB021192)
N3-4P
Bacillus licheniformis BCRC 11702^T (EF433410)
N3-7P
Bacillus subtilis subsp. Subtilis DSM 10^T (AJ276351)

0.01

56

(B)

- *Rhodococcus imtechensis* RKJ300[T] (AY525785) — 99
- *Rhodococcus wratislaviensis* NCIMB 13082[T] (Z37138)
- *Rhodococcus opacus* NCIMB 13082[T] (X80630)
- *Rhodococcus percolatus* MBS 1[T] (X92114)
- *Rhodococcus koreensis* DNP 505[T] (AF124342)
- *Rhodococcus marinonascens* DSM 43752[T] (X80617)
- *Rhodococcus jostii* IFO 16295[T] (AB046357)
- *Rhodococcus maanshanensis* M712[T] (AF416566) — 97
- *Rhodococcus tukisamuensis* Mb8[T] (AB067734)
- SH-1A
- *Rhodococcus kyotonensis* DS472[T] (AB269261) — 81
- *Rhodococcus yunnanensis* YIM 70056[T] (AY602219) — 100
- *Rhodococcus fascians* DSM 20669[T] (X79186)
- N3-4A — 79, 91
- *Rhodococcus cerastii* C5[T] (FR714842)
- *Rhodococcus triatomae* IMMIB RIV-085[T] (AJ854055) — 86
- *Rhodococcus rhodnii* DSM 43336[T] (X80621)
- *Rhodococcus erythropolis* DSM 43066[T] (X79289) — 100
- P2-2P
- *Rhodococcus globerulus* DSM 4954[T] (X80619) — 87, 88
- *Rhodococcus jialingiae* djl-6-2[T] (DQ185587)
- *Rhodococcus baikonurensis* GTC 1041[T] (AB071951) — 82
- *Rhodococcus qingshengii* djl-6[T] (DQ090961)
- *Rhodococcus kunmingensis* YIM 45607[T] (DQ997045)
- *Rhodococcus equi* DSM 20307[T] (X80614) — 96
- *Rhodococcus trifolii* T8[T] (FR714843)
- *Rhodococcus kroppenstedtii* K07-23[T] (AY726605) — 99
- *Rhodococcus corynebacterioides* DSM 20151[T] (AF430066) — 92
- N3-2P
- *Rhodococcus phenolicus* G2P[T] (AY533293) — 85
- *Rhodococcus zopfii* ATCC 51349[T] (AF191343) — 77
- *Rhodococcus coprophilus* DSM 43347[T] (X80626)
- *Rhodococcus aetherivorans* 10bc312[T] (AF447391) — 96
- *Rhodococcus ruber* DSM 43338[T] (X80625)
- *Rhodococcus rhodochrous* DSM 43241[T] (X79288) — 75
- *Rhodococcus gordoniae* W 4937[T] (AY233201) — 56
- *Rhodococcus pyridinivorans* PDB9[T] (AF173005) — 57

82, 96

0.005

**Figure 3.3 Phylogenetic tree based on 16S rDNA 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 rDNA
gene sequences of each reference species are listed in parentheses. Bar, 0.01
nucleotide substitutions per site. A. *Bacillus* strains; B. *Rhodococcus* strains).**

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Table 3.3 Distribution of isolates and the media effects

Genus	Established (author, year)	No.	Peng's agar	Atlas oil agar
Rhodococcus	Zopf, 1891	25	N1,N2,N3,N4,P1,P2,P4,P5,P6	SH,N1,N2,N3,N4
Bacillus	Cohn, 1872	20	CBS,N1,N2,N3,N4,P1,P3,P5,P6,P10	-
Acinetobacter	Beijerinck, 1954	4	-	P1,P5,P7,P9
Halomonas	Vreeland, 1980	2	-	N3
Pseudomonas	Migula, 1894	1	N3	-
Alcanivorax	Yakimov, 1998	1	-	N3
Stretomyces	Waksman and Henrici, 1943	1	-	N3
Exiguobacterium	Collins, 1983	1	N4	-
Total no.		55		
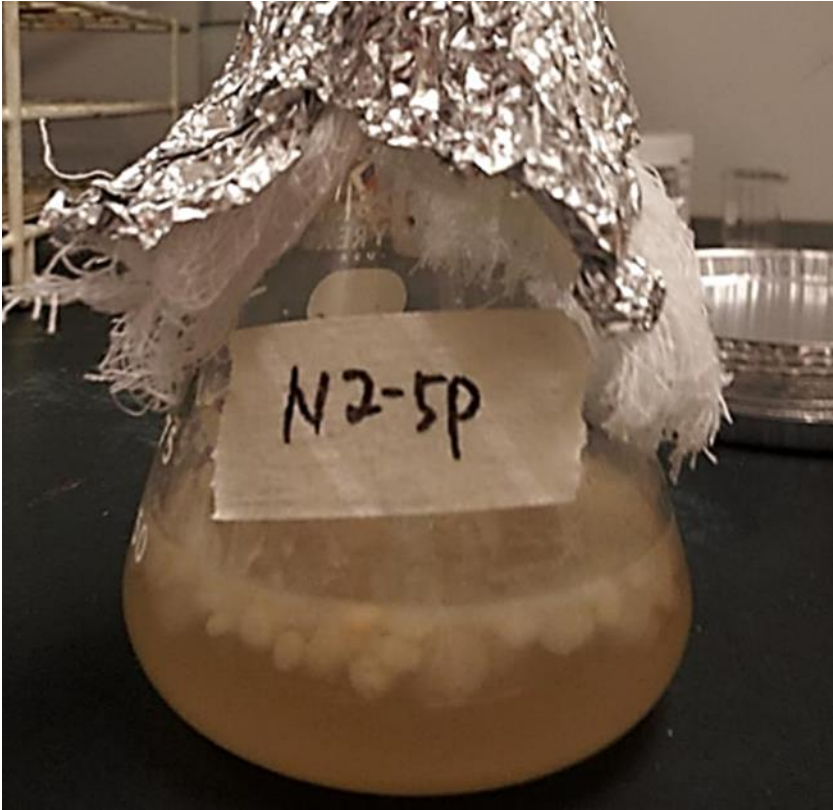
Figure 3.4 Photo of a flocculent culture

scale applications of biosurfactants is the high production cost. Generally, the downstream processing (mainly recovery and purification of the products) of a biotechnological product account for about 60% of the total production costs (Mukherjee et al., 2006). The recovery of biosurfactants commonly starts with removal of bacteria cells and cell debris by filtration and centrifugation. The flocculant formation significantly facilitated the separation by reducing the processing time and allowing use of smaller equipment (Salehizadeh and Shojaosadati, 2001). Besides, flocculated cells were also find to be superior with continuous fermentation system as cells could be easily retaining in the reactor while producing a clean broth for downstream processing (Andrietta et al., 2008). The flocculant forming isolates will be selected in future study of optimization of production processes and the EBFs responsible for the flotation will be analyzed and determined.

3.3.3 Performance of the Isolated Biosurfactant Producers

Performance evaluation of all isolated biosurfactant producers

The biomass accumulated after 7 days of incubation, the surface tension and E24 of the cell free culture of the 55 isolates were determined (**Figure 3.5**). Among them, thirteen isolates could reduce the surface tension of water to less than 40 dynes/cm, which indicated the capability of *Bacillus* and *Rhodococcus* strains isolated from sites of N1-N4 and P6 in reducing surface tension. For example, the cell free culture of the selected 5 isolates, i.e. N2-2A, N3-1P, N3-3A, N3-5A and P6-4P, had the surface tension of 31.9, 31.6, 33.5, 35.6 and 31.3 dynes/cm respectively. These findings can also be

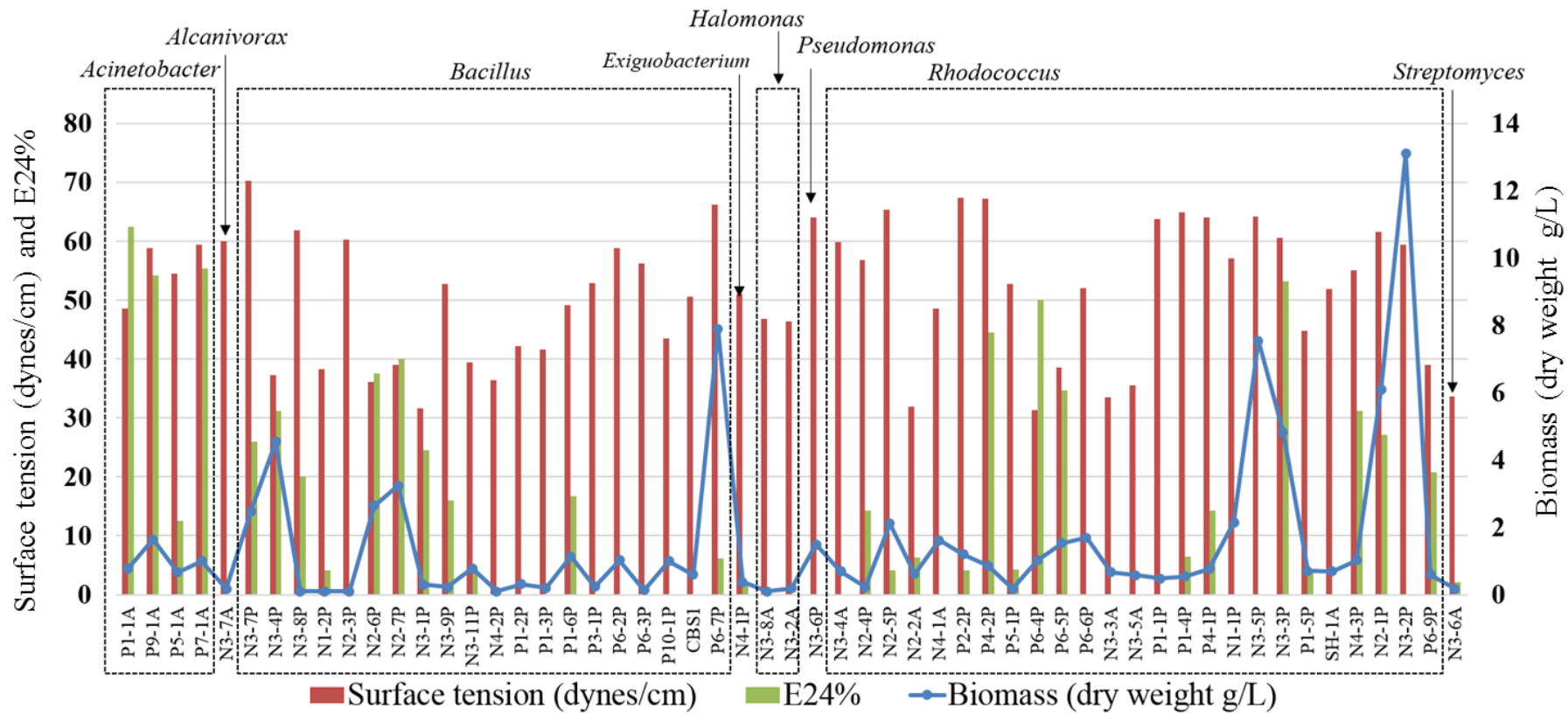**Figure 3.5 Surface tension, E24 and biomass of the 55 selected bacterial cultures**

observed using the spreading of their liquid culture on parafilm as shown **Figure 3.6**. The most effective isolates were P6-4P (*Rhodococcus* sp.) and N3-1P (*Bacillus* sp.). The results are comparable with literature. The biosurfactants produced by *Rhodococcus* sp. were found to reduce the surface tension of water to 25-30 dynes/cm (Franzetti et al., 2010). The biosurfactants produced by Bacillus sp. were also found to reduce the surface tension of water to around 27 dynes/cm (Nitschke and Pastore, 2006).

It was observed that three *Acinetobacter* (P1-1A, P7-1A and P9-1A) and two *Rhodococcus* (P5-1A and P6-4P) strains could significantly emulsify n-hexadecane (E24 > 50%). The P1-1A presented the highest emulsification ability (62.5%). The results showed that the surface tension reduction and emulsification were not necessarily correlated. Biosurfactants are surface active molecules, they can form micelles at the interface of immiscible liquids by either reduction of surface and interfacial tension or form stable emulsions between immiscible liquids, the latter are more commonly referred as bioemulsiers (de Sousa and Bhosle, 2012). It is interesting to notice that *Acinetobacter* cultures tend to have high emulsifying ability. High E24 values (> 50%) were observed in three out of four *Acinetobacter* strains, while the P5-1A culture showed lower E24 (12.5%). The biomass of P5-1A culture was 0.67 g/L, indicating that the cultivation conditions were suboptimal.

The *Acinetobacter* spp. have been well known for producing polymeric bioemulsifiers (Phetrong et al., 2008; Reisfeld et al., 1972; Rosenberg et al., 1988). The well-studied polymeric biosurfactant, emulsan, was also produced by *Acinetobacter calcoaceticus* RAG-1 (Reisfeld et al., 1972). The results also supported that *Acinetobacter* spp. had the tendency to produce bioemulsifiers. The isolates generating
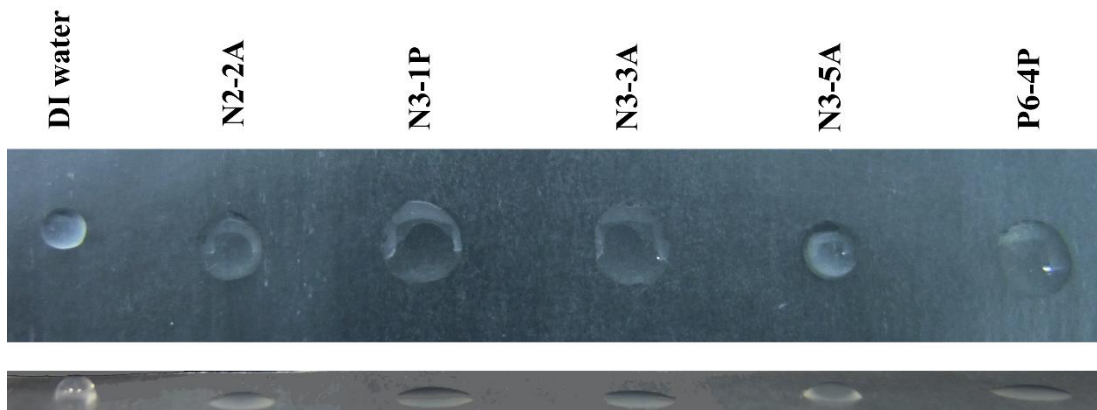
Figure 3.6 Surface tension of the culture supernatant reduced by secretion of amphipathic biosurfactants as indicated by the flatten of droplet on parafilm

cell free cultures of lower surface tension and higher E24 will be studied in terms of the optimization of production and application in the environmental fields.

The biomass production of the selected isolates was also summarized in **Figure 3.5**. Several *Bacillus* strains (i.e., N3-4P and P6-7P) and *Rhodococcus* strains (i.e., N3-5P and N3-2P) produced biomass exceeding 5 g dry weight/L under the experimental conditions. The biomass data of most Bacillus subtilis strains (N1-2P, N2-3P, N2-6P, N2-7P, N3-1P, N3-9P, N3-11P, N4-2P, P1-2P, P1-3P, P1-6P, P3-1P P6-2P and P6-3P) showed a similar trend with the corresponding data of the emulsification index. Some isolates, such as N1-2P (0.09 g/L), N3-8P (0.09 g/L) and N4-2P (0.10 g/L), developed significantly low biomass when growing in the liquid medium although they formed moderate colonies on agar plates. This indicated that they might be sessile bacteria. Many marine bacteria are sessile, growing exclusively or preferentially attached to a solid surface (Zobell, 1943). Their growth and biosurfactant production could be optimized through introducing a solid surface in the medium or even using solid state fermentation instead (Seghal Kiran et al., 2010). However, when using traditional drop collapsing test to do the screening, these isolates would be eliminated from the pool as they barely grew in liquid. Therefore, the colony based oil drop collapsing test as described previously was used in this study for the comprehensive biosurfactant screening. The modified method can eliminate the step of culturing isolates in liquid medium, thus saving time and reducing risk of contamination. No visual precipitants were observed in the cell free culture of the 55 isolates after equilibrium at 4°C, indicating that the generated biosurfactants had CMT lower than 4°C. The CMT temperature of each biosurfactant could be further determined after the separation and purification of each surface-active components.

These isolates originally inhabited in the petroleum hydrocarbon contaminated sites and were producing biosurfactants while using petroleum hydrocarbon as the sole carbon source. Given that all the isolates adapted to the cold marine environments of northern Atlantic Canada and their cell free culture remained clear at 4°C, the biosurfactants they produced could thus be effective under low temperature. The produced biosurfactants thus could facilitate bio-transfer of petroleum hydrocarbons, leading to promising potential in enhancing bioremediation of petroleum hydrocarbon contaminated sites and offshore oil spill responses.

Performance demonstration of two selected isolates

Two isolates were selected in this section. P6-4P was selected as its culture broth has the lowest surface tension and high E24. P1-5P was selected as its culture broth has low surface tension and prominent flocculating ability (**Figure 3.5**). As shown in **Figure 3.7**, the surface tension of the culture broth was gradually decreased to a plateau of around 28 dynes/cm, while the CMD and E24 were gradually increased to a plateau.

The change of emulsion layer with time is illustrated in **Figure 3.8**. It was observed that the height of emulsion layer was increased along with time as the produced biosurfactant accumulated. Generally speaking, low-molecular-weight biosurfactants lower surface and interfacial tensions, whereas the high-molecular-weight ones were effective at stabilizing oil-in-water emulsions (Rosenberg and Ron, 1999). The biosurfactants produced by P6-4P may be a mixture of both low and high molecular weight surface-active agents. Good correlation (correlation coefficient=0.867) could be found between CMD and E24 (**Figure 3.9**), indicating these two types of biosurfactants were accumulated proportional in the culture broth. When applying as oil clean-up agents, the biosufactants produced by P6-4 can work under the three proposed mechanisms of
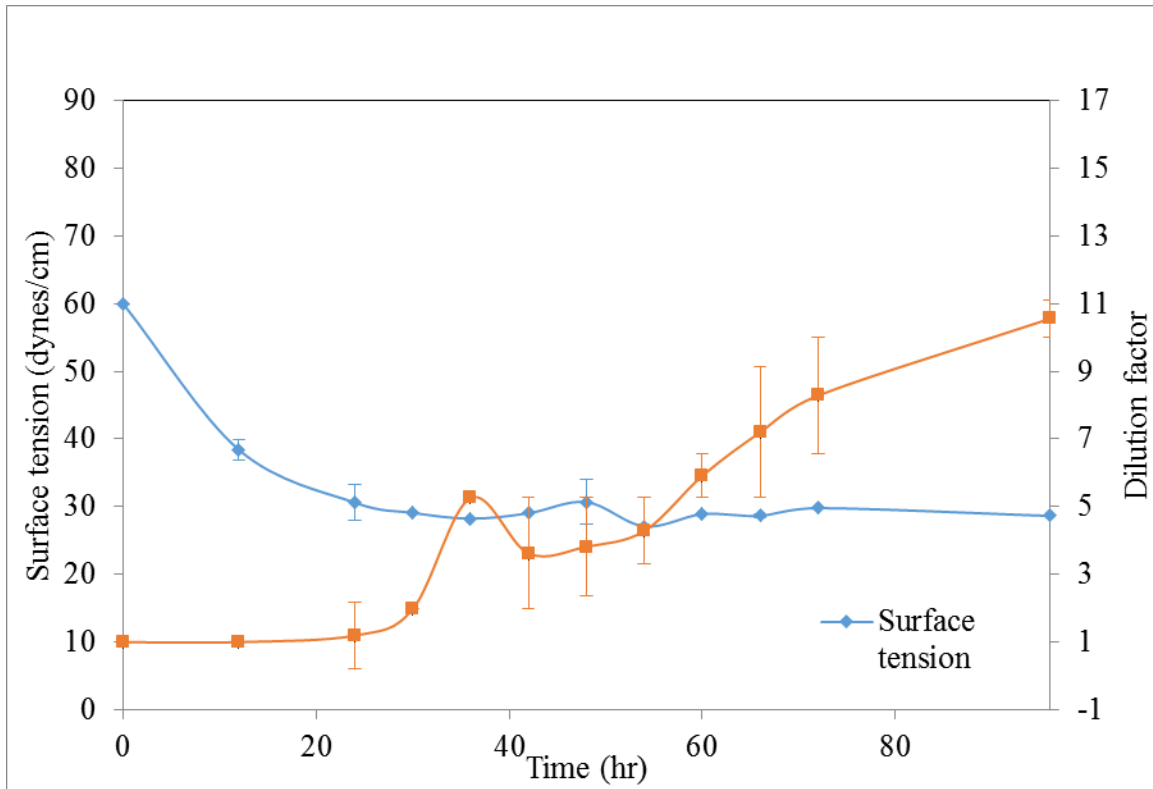
Figure 3.7 Changes of surface tension, CMD and E24 of the P6-4P culture along with the incubation time
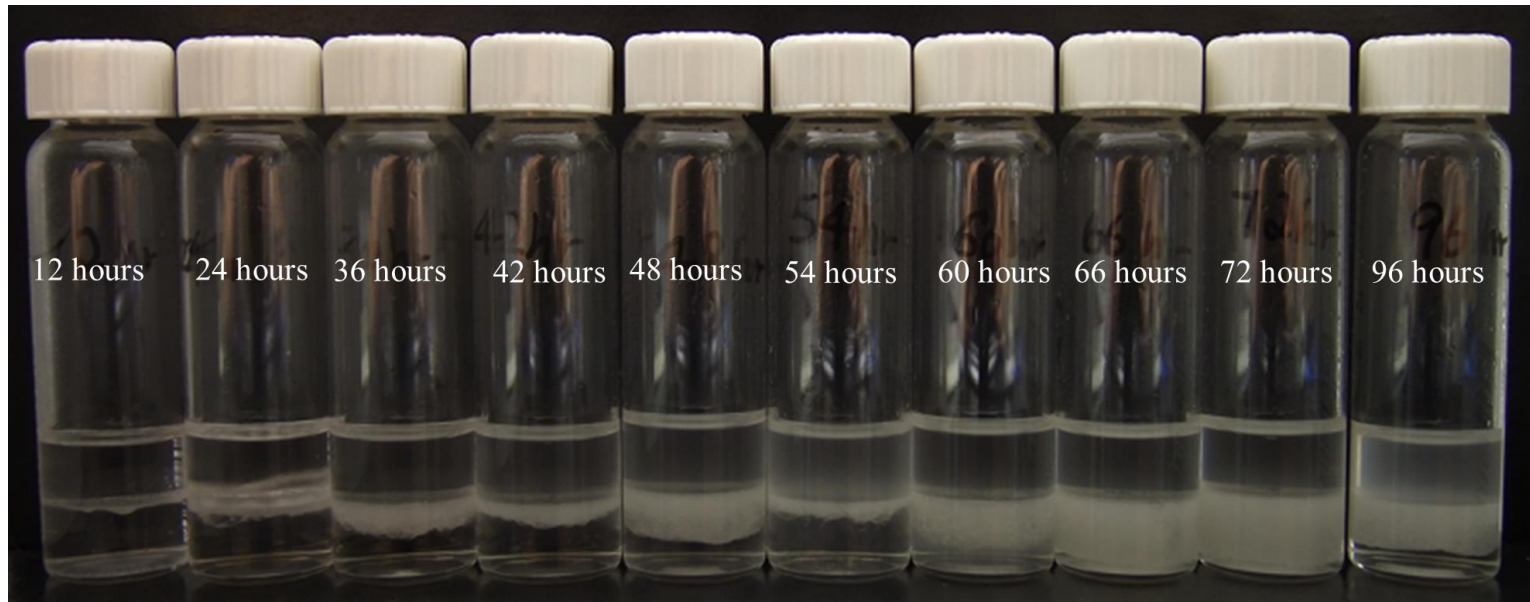
Figure 3.8 Changes of emulsion layer of the P6-4P culture with the incubation time

$$y = 4.89x + 11.401$$
$$R^2 = 0.7474$$
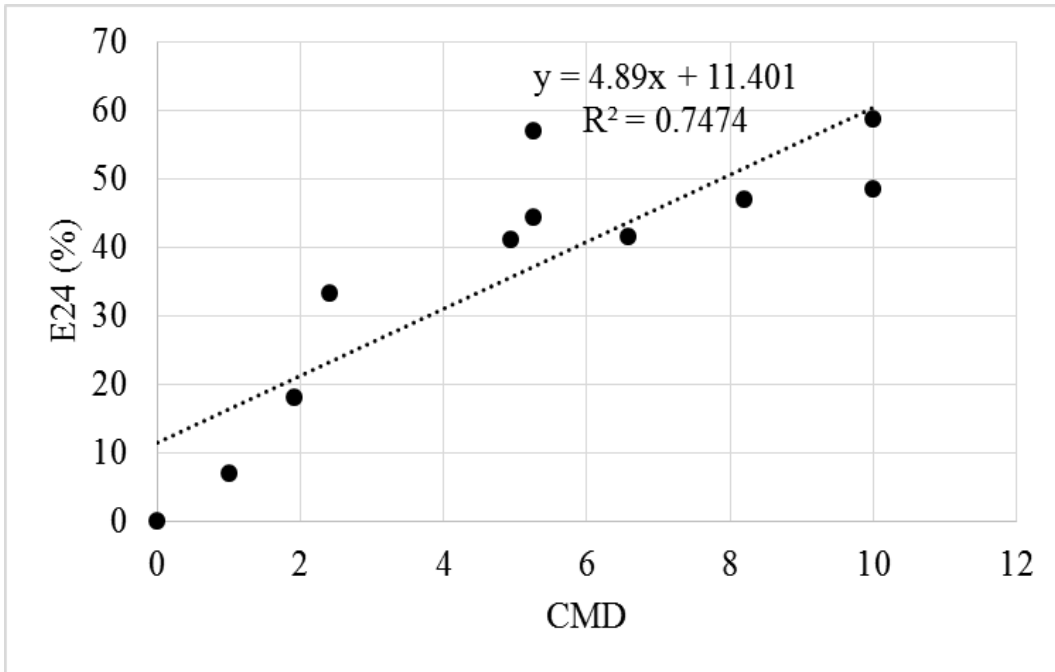
Figure 3.9 correlation regression between CMD and E24

mobilization, solubilisation and emulsification to effectively remove oil from soil/rock and keep them in the liquid phase (Pacwa-Płociniczak et al., 2011). Similar trends of surface tension (can reach as low as 28 dynes/cm) and CMD were obtained for P1-5P whereas it has no emulsification ability (**Figure 3.10**). On the other hand, the increasing dosage of flocculant (culture broth of P1-5P) had higher flocculating ability to suspend and aggregate the bacterial cells. As discussed in section 3.2, this ability would facilitate the post-processing of biosurfactant production, thus reducing the production cost. Isolates P6-4P and P1-5P as well as their biosurfactants will be further characterized in future studies.

3.4 Summary

In this chapter, an overview of the literature on isolating marine biosurfactant producers was conducted and vacancy of work in North Atlantic Canada was identified. Water and sediment samples were collected in petroleum hydrocarbon contaminated sources. A modified colony-based drop collapsing method was used to cover sessile bacteria, showing the advantages of saving time and reducing contamination risks. By using n-hexadecane or diesel as the sole carbon source, 55 biosurfactant producers were identified and characterized. They belong to 8 genera, namely, *Alcanivorax*, *Exiguobacterium*, *Halomonas*, *Rhodococcus*, *Bacillus*, *Acinetobacter*, *Pseudomonas*, and *Streptomyces*. The first three genera have not been long established with limited relevant publications. They were generally found to be alkane degrading, extremophile and halophile, respectively. The distributions of the 55 isolates were site and medium specific. Diverse isolates were found with featured properties such as flocculant production,
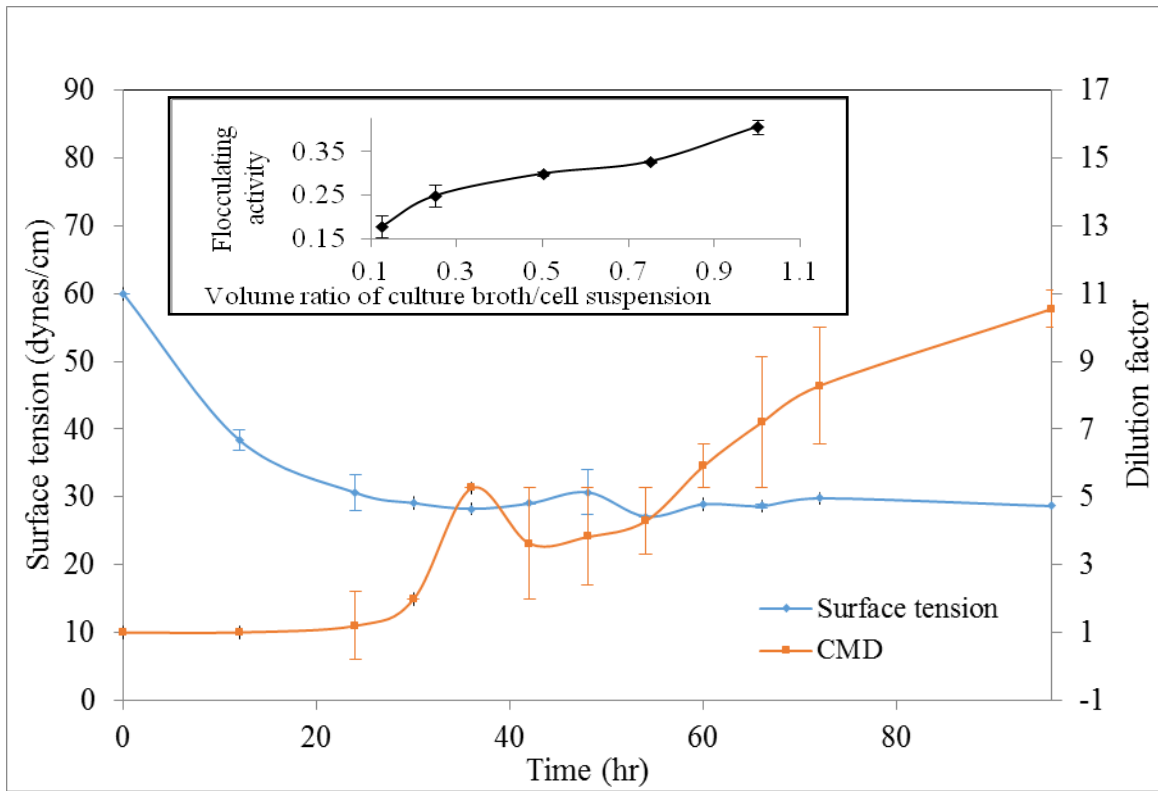
Figure 3.10 Changes in surface tension and CMD of the P1-5P culture along with the incubation time (insert: effect of volume ratio of culture broth/cell suspension on the flocculating activity)

significant reduction of surface tension and stabilization of water-n-hexadecane emulsion. Isolates P6-4P and P1-5P were selected to demonstrate the performance of biosurfactant production. They reduced the surface tension to as low as 28 dynes/cm and have the ability to stabilize emulsions or facilitate biosurfactant recovery through cell flocculation.

These isolates originally inhabited in the petroleum hydrocarbon contaminated sites and were producing biosurfactants while using petroleum hydrocarbon as the sole carbon source. Given that all the isolates adapted to the cold marine environments of northern Atlantic Canada and their cell free culture remained clear at 4ºC, the biosurfactants they produced could thus be effective under low temperature. The produced biosurfactants thus could facilitate bio-transfer of petroleum hydrocarbon, leading to promising potential in offshore oil spill responses even under harsh conditions. \

CHAPTER 4

CHARACTERIZATION OF SELECTED ISOLATES: HALOMONAS SP. N3-2A, ALCANIVORAX SP. N3-7A AND EXIGUOBACTERIUM SP. N4-1P

4.1 Background

As mentioned in Section 3.3.1, three isolates, i.e. *Halomonas* sp. N3-2A, Alcanivorax sp. N3-7A and *Exiguobacterium* sp. N4-1P, belong to relative younger genus with a limited number of members at species level. Thus, the phenotype and genotype of these isolates were not well investigated and the relevant information was hard to obtain from the literature. Besides, limited efforts were made on their properties as biosurfactant producing bacteria, particularly true for genus *Exiguobacterium,* which was never been published for biosurfactant production. Three strains belonging to the genus *Alcanivorax* have been published to produce biosurfactants. Passeri et al. (1992) found the first species belongs to this genus, *Alcanivorax borkumensis,* was a hydrocarbon-degrading and surfactant-producing marine bacterium. The structure of the produced lipid was deducted as shown in **Figure 4.1A**. Qiao and Shao (2010) found that when incubated with n-hexadecane as the sole carbon source, *Alcanivorax dieselolei* can reduce the surface tension to 29.8-32.8 mN m^{-1}. They also analyzed the structure and composition of the biosurfactant which was confirmed to be a lipopeptide as shown in **Figure 4.1B**. Wu et al. (2009) found *Alcanivorax hongdengensis* produced 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.
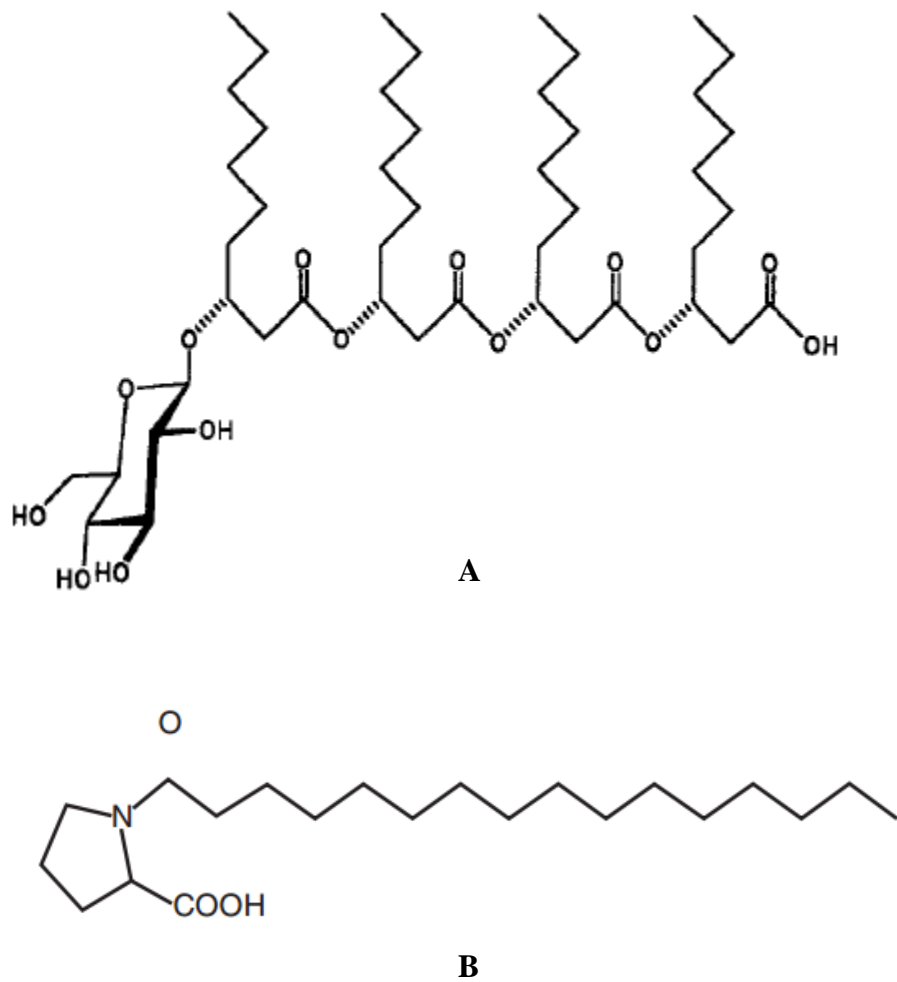
Figure 4.1 Molecular structure of (A) the glycolipid produced by *Alcanivorax borkumensis* (Passeri et al. 1992); and (B) lipopeptide produced by *Alcanivorax dieselolei* (Qiao and Shao, 2009)

Instead of producers like *Alcanivorax* who could generate low-molecular-weight glycolipids and lipopeptides, *Halomonas* were generally found to produce high-molecular-weight polymers. Several *Halomonas eurihalina* strains were found to produce large amount of exopolysaccharides (EPS) with higher emulsification ability than commercial surfactants. The chemical composition of the EPS was found to be primarily carbohydrates and sulfates followed by proteins, uronic acids and acetyls (Calvo et al., 2002; Martínez-Checa et al., 2002; Martínez-Checa et al. 2007). Gutiérrez et al. (2007b) found *Halomonas* sp. Strain TG39 produced a heterogeneous polymer with a polydispersity index of 1.8, proof anionic nature and protein content of EPS which affected the emulsifying qualities. The chemical composition of bioemulsifier varies according to the substrate. EPS produced on hydrocarbons have less carbohydrate and protein components but are richer in uronic acids than EPS produced on glucose. Uronic acids in particular may be involved in the detoxification of hydrocarbons thus representing a form of adaptation. One interesting aspect of *Halomonas* bioemulsifiers is that, like other biosurfactants, they are able to stimulate the growth of other hydrocarbon-degrading bacteria such as *Bacillus*, *Pseudomonas*, *Micrococcus* and *Arthrobacter* (Calvo et al., 2002; Martínez -Checa et al., 2002).

The main objectives of this study are to characterize the genotype (the genetic makeup of an organism) and phenotype (observable characteristics of an organism) properties of these three newly generated isolates and disclose their properties of the biosurfactant products. According to the recommendation of Tindall et al. (2010) the characterization of prokaryote strains would better include the results from 16S rDNA sequencing, metabolic trait analysis, cell membrane composition, morphology of the cell

through electronic microscopy and their interesting bioproducts. The results will fill the knowledge gaps and provide the researchers worldwide with the basic information of these intriguing microorganisms. Morevoer, the information on their bioproducts will lead to future applications of these microorganisms as the producers of the bio-dispersants.

4.2 Methodology

4.2.1 Ribosomal DNA Sequencing and Phylogenetic Analysis

These three isolates were subjected to 16S ribosomal DNA sequencing using universal bacterial primers F27 and R1493 (position in *Escherichia Coli* 8-27 and 1493-1512, respectively) to obtain full length ribosomal DNA. Other protocols were the same with Section 3.2.3, except when using Applied Biosystems, the standard mode were used instead of the fast mode. The sequencing for each isolates was replicated 5 times to ensure the accuracy. The obtained DNA sequence was matched with the BLAST database to check if the isolates belong to the genus that determined in Section 3.3.1. Phylogenetic trees were constructed by the method described in Section 3.2.3. The phylogenetic tree involved the three selected strains, several type strains in their genus, and some other selected representative strains from the 55 isolated strains which included the four representative strains from *Bacillus* and *Rhodococcus*, respectively; one representative strains of *Acinetobacter* and *Pseudomonas,* respectively.

4.2.2 BIOLOG® Microbial Identification and Characterization

The BIOLOG® microbial identification system was used to test the utilization of the 95 sole carbon sources simultaneously. Strains were grown on marine agar plates at

30°C for 24h. The the cells were then harvested with loop and transferred to 0.4 M 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 (for *Alcanivorax* sp. and *Halomonas* sp.) or GP2 plates (for *Exiguobacterium* sp.) were inoculated with 150 μL of the cell suspension per well. The inoculated plates were incubated at 30°C. The results were read visually after incubation for 1 day (Ivanova et al., 1998).

4.2.3 Phospholipid-Derived Fatty Acid (PLFA) Analysis

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. The cells were then transferred in duplicate to reaction tubes with 1 ml of reagent 1 (150 g NaOH in 1 L of 50% 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 aqueous (lower) phase, 3 mL of 1.2% NaOH in H_2O (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 GC vial. The FAMEs were quantified by a GC/MS system (Agilent technologies 7890A GC system connected to an Agilent technologies 5975C inert mass spectroscopy detector (MSD) 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°C to 120°C at a rate of 10°C/min, and then to 280°C at a rate of 3°C/min. Individual compounds were identified from their mass spectra and from comparing the retention time 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.

4.2.4 Scanning Electron Microscope (SEM) Analysis

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 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) of CREAIT of MUN.

4.2.5 Production and Recovery of Crude Biosurfactants

The first recipe in Section 3.2.2 was used as the basic incubation medium to produce biosurfactants from the three selected strains. However, the carbon source of this

recipe has been changed to diverse forms to test how this factor affected the biosurfactant production. More specifically, the carbon source combinations consist of non-hydrocarbon part and hydrocarbon part. The non-hydrocarbon part 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 as introduced in session 3.2.4. The clear broths were concentrated with lyophilisation and then mixed with three volumes of cold acetone. Subsequently, the solution was stored at 4°C for 3 days to fully precipitant 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.

4.2.6 Properties Analysis of the Crude Biosurfactants

The abilities of the crude biosurfactants to lower the surface tension and stabilize emulsions were determined. For the surface tension measurement, 50 mg of each crude biosurfactant was dissolved in 10 mL water by sonification. The solution was measured in triplicate a surface tensiometer (DuNouyTensiometer, Interfacial, CSC Scientific) at room temperature. For the E24 determination, 50 mg of each crude biosurfactant was dissolved in 2 mL of water by sonification. Then 2 mL of n-hexadecane was added. The

two phases were vortexed vigorously for 2 min. The E24 was determined following the description of session 3.2.4.

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 three selected isolates. The five replicates of each strain agreed well with each other. The isolates *Halomonas* sp. N3-2A was found to be closely related to *H. venusta* strain DSM 4743^T with 99.53% similarity. *Exiguobacterium* sp. N4-1P was closely related to *E. oxidotolerans* strain T-2-2 with 99.47% similarity; to *E. antarcticum* B7^T with 98.70% similarity; and to *E. sibiricum* 255-15^T 98.63% similarity. *Alcanivorax* sp. N3-7A was closely related to *A. dieselolei* B-5^T with 95.67% similarity. As *Alcanivorax* sp. N3-7A has the highest match in the gene bank with <97% similarity, the strain could be a newly discovered bacterium (Tindall et al., 2010) and its characterizations will be detailed in later sessions. As shown in **Figure 4.2**, isolates that belong to genus *Alcanivorax* is closely related to isolates belongs lwyto genus *Pseudomonas*, *Halomonas* and *Acinetobacter*. They are gram negative bacteria and all belong to the phylum of γ-Proteobacteria. On the other hand, isolates classified as *Rhodococcus* and *Steptomyces* (phylum of Actinobacteria) are more closely related to isolates that belong to genus *Bacillus* and *Exiguobacterium* (phylum of Firmicutes). They are all gram positive bacteria.

The Actinobacteria have high G+C content and play important roles in organic matter turnover and the carbon cycle (Dworkin et al., 2006). They are also well known as
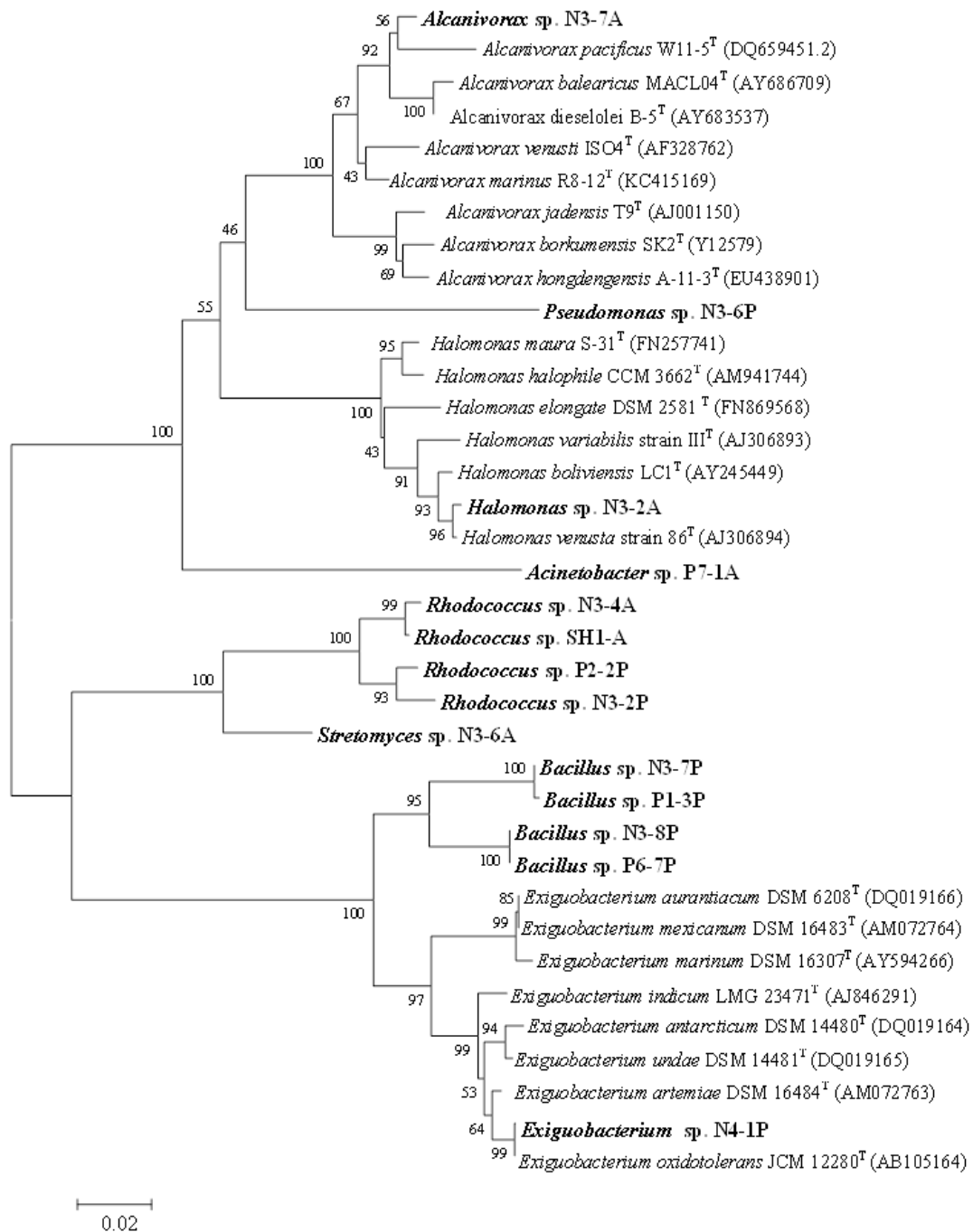
Figure 4.2 Phylogenetic tree based on 16S rDNA gene sequences
(Note: The tree was constructed using the NJ method. The numbers at nodes represent bootstrap values (based on a resampling of 1000). The GenBank accession numbers for the 16S rDNA gene sequences of each reference species are listed in parentheses. Bar, 0.02 nucleotide substitutions per site.

a secondary metabolite producing bacteria, and have attracted wide attention in terms of pharmacological and commercial research and development (Dworkin et al., 2006). The Firmicutes have low G+C content, and are well known to produce endospores and thus can survive under extreme conditions (Dworkin et al., 2006)

4.3.2 Metabolic Properties

The metabolic profiles of the three selected bacteria are illustrated in **Figures 4.3-4.5**. According to these results, it can be found that *Halomonas* sp. N3-2A can digest the highest diversity of the 95 carbon sources, following by *Exiguobacterium* sp. N4-1P and *Alcanivorax* sp. N3-7A. The testing results for *Halomonas* sp. N3-2A and *Exiguobacterium* sp. N4-1P correlated well with the studies of Mata et al. (2002) and Rodrigues et al. (2006a), respectively. For *Alcanivorax* Sp. N3-7A, there were limited comprehensive phenotypic data existed in the literature. Some of their metabolic properties were published when they were characterized as a novel species, during which only features different from the existed relevant species were listed. In order to compare the metabolic fingerprinting in between relevant species within genus of *Exiguobacterium* and *Alcanivorax*, type strains of each species may need to be purchased to conduct parallel experiments, which is beyond the scope of this study but recommended for future research.

A1 Water	A2 α-cyclodextrin	A3 Dextrin	A4 Glycogen	A5 Tween 40	A6 Tween 80	A7 N-Acetyl-D-Galactosamine	A8 N-Acetyl-D-Galactosamine	A9 Adonitol	A10 L-Arabinose	A11 D-Arabitol	A12 D-cellobiose
B1 i-Erythritol	B2 D-Fructose	B3 L-Fucose	B4 D-Galactose	B5 Gentiobiose	B6 α-D-Glucose	B7 m-inositol	B8 α-D-lactose	B9 Lactulose	B10 Maltose	B11 D-mannitol	B12 D-mannose
C1 D-melibiose	C2 ρ-methyl-D-Glucoside	C3 D-Psicose	C4 D-Raffinose	C5 L-Rhamnose	C6 D-Sorbitol	C7 Sucrose	C8 D-Trehalose	C9 Turanose	C10 Xylitol	C11 Pyruvic Acid Methyl Ester	C12 Succinic Acid Mono-Methyl-Ester
D1 Acetic Acid	D2 Cis-Aconitic Acid	D3 Citric Acid	D4 Formic Acid	D5 D-galactonic Acid Lactone	D6 D-Galacturonic Acid	D7 D-Gluconic Acid	D8 D-Glucosaminic Acid	D9 D-Glucuronic Acid	D10 α-Hydroxybutyric Acid	D11 β-Hydroxybutyric Acid	D12 γ-Hydroxybutyric Acid
E1 p-Hydroxy Phenylacetic Acid	E2 Itaconic Acid	E3 α-Keto Butyric Acid	E4 α-Keto Glutaric Acid	E5 α-Keto Valeric Acid	E6 D,L-Lactic Acid	E7 Malonic Acid	E8 Propionic Acid	E9 Quinic Acid	E10 D-Saccharic Acid	E11 Sebacic Acid	E12 Succinic Acid
F1 Bromosuccinic Acid	F2 Succinamic Acid	F3 Glucuronamide	F4 L-Alaninamide	F5 D-Alanine	F6 L-Alanine	F7 L-Alanyl-Glycine	F8 L-Asparagine	F9 L-Aspartic Acid	F10 L-Glutamic Acid	F11 Glycyl-L-Aspartic Acid	F12 glycyl-L-Glutamic Acid
G1 L-Histidine	G2 Hydroxy-L-Proline	G3 L-Leucine	G4 L-Omithine	G5 L-Phenylalanine	G6 L-Proline	G7 L-Pyroglutamic Acid	G8 D-Serine	G9 L-Serine	G10 L-Threonine	G11 D,L-Camitine	G12 γ-Amino Butyric Acid
H1 Urocanic Acid	H2 Inosine	H3 Uridine	H4 Thymidine	H5 Phenyethyl-amine	H6 Putrescine	H7 2-Aminoethanol	H8 2,3-Butanediol	H9 Glycerol	H10 D,L-α-Glycerol Phosphate	H11 α-D-Glucose-1-Phosphate	H12 D-Glucose-6-Phosphate

Figure 4.3 Metabolic fingerprints of *Alcanivorax* sp. N3-7A

A1 Water	A2 α-cyclodextrin	A3 β-cyclodextrin	A4 Dextrin	A5 Glycogen	A6 Inulin	A7 Mannan	A8 Tween 40	A9 Tween 80	A10 N-Acetyl-D-Galactosamine	A11 N-Acetyl-D-Mannosamine	A12 amygdalin
B1 L-Arabinose	B2 D-Arbutin	B3 Arbutin	B4 D-Cellobiose	B5 D-Fructose	B6 L-Frucose	B7 D-Galactose	B8 D-Galacturonic acid	B9 Gentiobiose	B10 D-Gluconic Acid	B11 α-D-Glucose	B12 m-insitol
C1 α-D-Lactose	C2 Lactulose	C3 Maltose	C4 Maltotriose	C5 D-Mannitol	C6 D-Mannose	C7 D-Melezltose	C8 D-Melbiose	C9 α-methyl-D-Galactoside	C10 β-methyl-D-Galactoside	C11 3-Methyl Glucose	C12 α-methyl-D-Glucoside
D1 β-methyl-D-Glucoside	D2 α-methyl-D-Mannoside	D3 Palatinose	D4 D-Pslcose	D5 D-Raffinose	D6 D-Rhamnose	D7 D-Ribose	D8 Salicin	D9 Sedoheptulosan	D10-Sorbitol	D11 Stachyose	D12 Sucrose
E1 D-Tagatose	E2 D-Trehalose	E3 Turanose	E4 Xylltol	E5 D-xylose	E6 Acetic Acid	E7 α-Hydroxy butyric acid	E8 β-Hydroxy butyric acid	E9 γ-Hydroxy butyric acid	E10 ρ-Hydroxy butyric acid	E11 α-Keto Glutaric Acid	E12 α-Keto Valeric Acid
F1 lactamide	F2 D-lactic Acid Methyl Ester	F3 L-Lactic Acid	F4 D-Malic Acid	F5 L-Malic Acid	F6 Methyl Pyruvate	F7 Mono-methyl succinate	F8 Propionic Acid	F9 Pyruvic Acid	F10 Succinamic Acid	F11 Succinic Acid	F12 N-Acetyl-L-Glutamic Acid
G1 L-Alaninamide	G2 D-Alanine	G3 L-Alanine	G4 L-Alanyl-Glycine	G5 L-Asparagine	G6 L-Glutamic Acid	G7 Glycyl-L-Glutamic Acid	G8 L-Pyroglutamic Acid	G9 L-Serine	G10 Putrescine	G11 2,3-Butanediol	G12 Glycerol
H1 Adenaine	H2 2'-Deoxy Adenosine	H3 Inosine	H4 Thymidine	H5 Uridine	H6 Adenosine-5'-Monophosphate	H7 Thymidine-5'-Monophosphate	H8 Uridine-5'-Monophosphate	H9 Fructose-6-phosphate	H10 glucose-1-Phosphate	H11 Glucose-6-phosphate	H12 D-L-α-Glycerol-Phosphate

Figure 4.4 Metabolic fingerprints of *Exiguobacterium* sp. N4-1P

A1 Water	A2 α–cyclodextrin	A3 Dextrin	A4 Glycogen	A5 Tween 40	A6 Tween 80	A7 N-Acetyl-D-Galactosamine	A8 N-Acetyl-D-Galactosamine	A9 Adonitol	A10 L-Arabinose	A11 D-Arabitol	A12 D-cellobiose
B1 i-Erythritol	B2 D-Fructose	B3 L-Fucose	B4 D-Galactose	B5 Gentiobiose	B6 α-D-Glucose	B7 m-inositol	B8 α-D-lactose	B9 Lactulose	B10 Maltose	B11 D-mannitol	B12 D-mannose
C1 D-melibiose	C2 ρ–methyl–D-Glucoside	C3 D-Psicose	C4 D-Raffinose	C5 L-Rhamnose	C6 D-Sorbitol	C7 Sucrose	C8 D-Trehalose	C9 Turanose	C10 Xylitol	C11 Pyruvic Acid Methyl Ester	C12 Succinic Acid Mono-Methyl-Ester
D1 Acetic Acid	D2 Cis-Aconitic Acid	D3 Citric Acid	D4 Formic Acid	D5 D-galactonic Acid Lactone	D6 D-Galacturonic Acid	D7 D-Gluconic Acid	D8 D-Glucosaminic Acid	D9 D-Glucuronic Acid	D10 α-Hydroxybutyric Acid	D11 β–Hydroxybutyric Acid	D12 γ–Hydroxybutyric Acid
E1 p-Hydroxy Phenylacetic Acid	E2 Itaconic Acid	E3 α-Keto Butyric Acid	E4 α-Keto Glutaric Acid	E5 α-Keto Valeric Acid	E6 D,L-Lactic Acid	E7 Malonic Acid	E8 Propionic Acid	E9 Quinic Acid	E10 D-Saccharic Acid	E11 Sebacic Acid	E12 Succinic Acid
F1 Bromosuccinic Acid	F2 Succinamic Acid	F3 Glucuronamide	F4 L-Alaninamide	F5 D-Alanine	F6 L-Alanine	F7 L-Alanyl-Glycine	F8 L-Asparagine	F9 L-Aspartic Acid	F10 L-Glutamic Acid	F11 Glycyl-L-Aspartic Acid	F12 glycyl-L-Glutamic Acid
G1 L-Histidine	G2 Hydroxy-L-Proline	G3 L-Leucine	G4 L-Omithine	G5 L-Phenylalanine	G6 L-Proline	G7 L-Pyroglutamic Acid	G8 D-Serine	G9 L-Serine	G10 L-Threonine	G11 D,L-Camitine	G12 γ–Amino Butyric Acid
H1 Urocanic Acid	H2 Inosine	H3 Uridine	H4 Thymidine	H5 Phenyethyl-amine	H6 Putrescine	H7 2-Aminoethanol	H8 2,3-Butanediol	H9 Glycerol	H10 D,L-α–Glycerol Phosphate	H11 α–D–Glucose-1–Phosphate	H12 D-Glucose-6-Phosphate

Figure 4.5 Metabolic fingerprints of *Halomonas* sp. N3-2A

4.3.3 PLFA Composition

The PLFA composition of the three selected isolates were listed in **Tables 4.1-4.3**. Generally speaking, the composition was similar to, and corresponded well with those members of their corresponding genera.

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%), which agreed well with previous results. The primary PLFAs of *Alcanivorax* sp. N3-7A were C16:0 (19.65%), C18:1 (19.39%), C16:1 (11.95%) and 3-OH C12:0 (11.74%). For *Halomonas* sp. N3-2A, the primary PLFAs were C18:1 (43.16%), C16:1 (23.37%), C16:0 (11.04%), C12:0 (9.34%). Both *Alcanivorax* sp. N3-7A and *Halomonas* sp. N3-2A had a relatively similar PLFA profile, except *Halomonas* Sp. N3-2A had no presence of hydroxyl fatty acids. Both *Halomonas* and *Alcanivorax* belong to the order of Oceanospirillales. Most species of this order are halophilic and halotolerant (Garrity et al., 2005), which were also found as important members involved in the biodegradation processes of dispersed oil plume after the Deepwater Horizon oil spill (Hazen et al., 2010).

Table 4.1 PLFA composition of *Exiguobacterium* sp. N4-1P and relevant strains

(Unit: %)

Fatty acid	*Exiguobacterium* sp. N4-1P	*E. oxidotolerans* T-2-2[T a]	*E. sibiricum* 7-3[T b]	*E. antarcticum* DSM 14480[T b]
iC12:0	2.70±0.08	1.4	2	3
C12	-	-	1	1
iC13:0	8.92±0.02	8.5	9	12
aC13:0	11.83±0.12	9	11	11
iC14:0	2.30±0.02	2.7	1	1
C14:0	2.33±0.02		3	2
iC15:0	15.52±0.01	20.7	13	11
aC15:0	5.10±0.40	4.2	3	2
C16:1	11.4±0.15	3	9	21
C16:0	12.51±0.02	2.9	17	13
C17:1	-	-	2	3
iC17:0	13.64±0.10	6.2	9	5
aC17:0	5.61±0.07	-	3	-
C17:0	3.28±0.28	1.2	-	
C18:1	-	-	4	-
C18:0	4.82±0.02	1.6	4	5

Note: [a] data were excerpted from Yumoto et al., 2004

[b] data were excerpted from Rodrigues et al., 2006a

Table 4.2 PLFA composition of *Alcanivorax* sp. N3-7A and relevant strains (Unit: %)

Fatty acid	*Alcanivorax* sp. N3-7A	*A. Dieselolei* B-5[T a]	*A. pacificus* W11-5[T b]	*A. balearicus* MACL04[T c]
C10:0	1.60±0.02	2.55	1.2	2.4
C12:0	1.82±0.01	8.89	2.9	2.7
2-OH C12:0	-	0.92	4	3.1
3-OH C12:0	11.74±0.21	2.91	8	4.8
C14:0	3.04±0.01	0.58	2.5	0.6
C14:1	2.83±0.06	-	-	-
C16:1	11.95±0.03	11.32	-	-
C16:0	19.65±0.10	32.12	29.1	29.9
cy17	4.67±0.02	1.11	-	2
C17:0	3.32±0.06	0.25	0.8	-
C18:1	19.39±0.30	22.41	29.1	9.7
C18:0	4.09±0.01	0.64	3.5	10.01
cy19	5.85±0.09	14.27	1.5	22.4

Note: [a] data were excerpted from Shao et al., 2005

[b] data were excerpted from Lai et al., 2011

[c] data were excerpted from Liu and Rivas et al., 2007

Table 4.3 PLFA composition of *Halomonas* sp. N3-2A and relevant strains (Unit: %)

Fatty acid	*Halomonas* sp. N3-2A	*H. Shengliensis* SL014B-85[T a]	*H. smyrnensis* AAD6[T b]	*H. lutea* YIM91125[T c]
C10:0	3.77±0.02	1.73	-	6
C12:0 2-OH	1.54±0.02	2.7	1.3	7.9
C12:0 3-OH	-	-	-	-
C12:0	9.34±0.27	8.41	10.7	10.7
C14:0	3.13±0.03	-	-	-
C14:1	2.11±0.03	-	-	-
C16:1	23.37±0.06	6.98	21.4	-
C16:0	11.04±0.06	24.81	35	17
cy17	-	0.48	-	4.6
C17:0	-	0.54	-	-
C18:1	43.16±0.26	43.05	22.6	25.1
C18:0	2.52±0.04	0.57	-	-
cy19	-	10.14	5.6	13.6

Note: [a] data were excerpted from Wang et al., 2007

[b] data were excerpted from Poli et al., 2013

[c] data were excerpted from Wang et al., 2008

4.3.4 Micro-structure

The SEM visualization results were shown in **Figures 4.6-4.8**. The *Exiguobacterium* sp. N4-1P were rod shaped bacteria with a length of 2-3 μm and diameter of around 0.5 μm. After 24 h of incubation, a large portion of the population was undergoing a fission process. The *Alcanivorax* sp. N3-7A were also rod shaped bacteria with a length of 1-2 μm and diameter of around 0.5 μm. *Halomonas* sp. N3-2A were prominently shorter than the other two strains. They were rod shaped bacteria with a length of 0.5-1 μm and diameter of around 0.5 μm. The similar results were also reported by Rodrigues et al. (2006a), Liu and Shao (2005) and Wang et al. (2008), respectively.
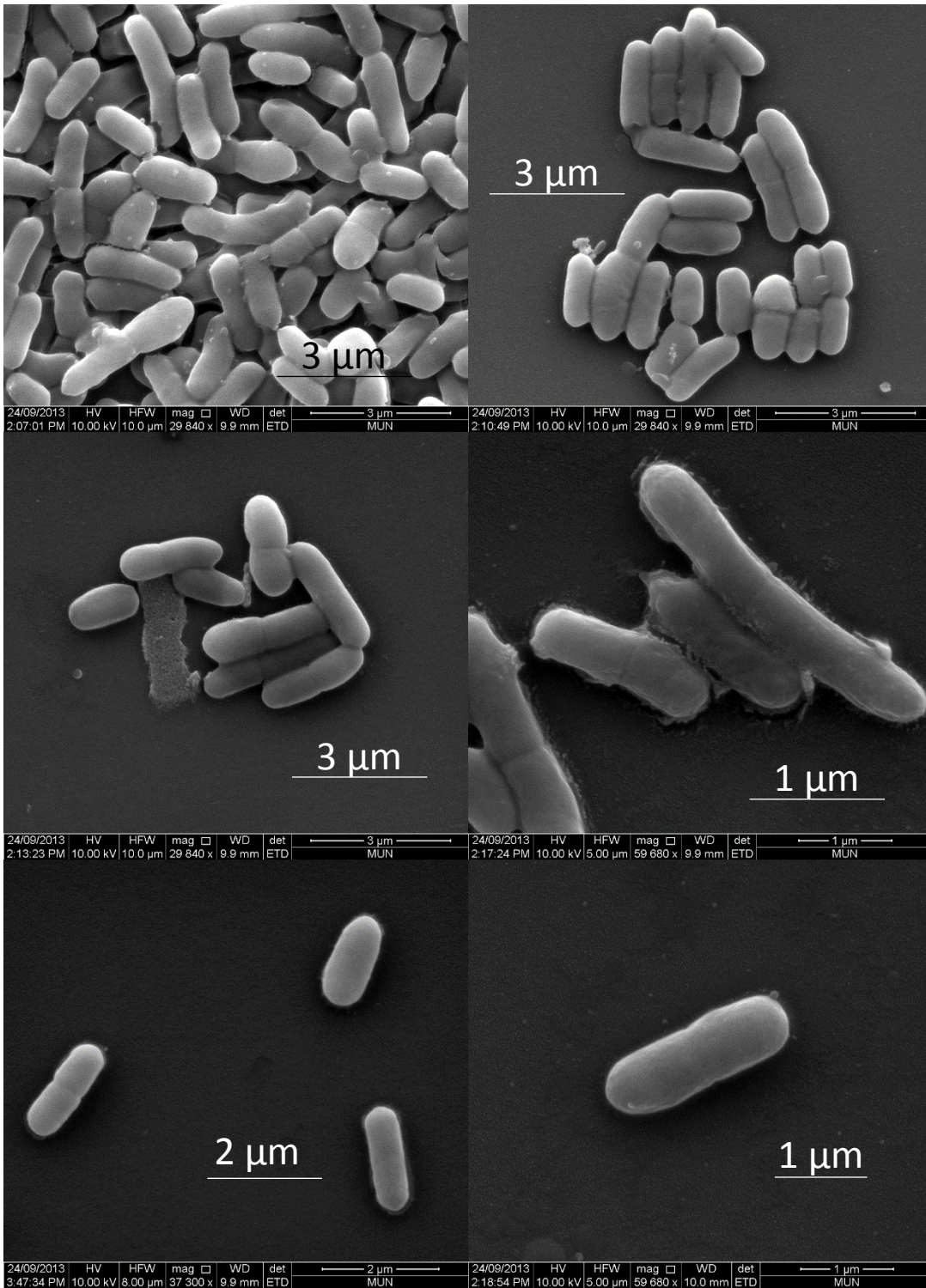
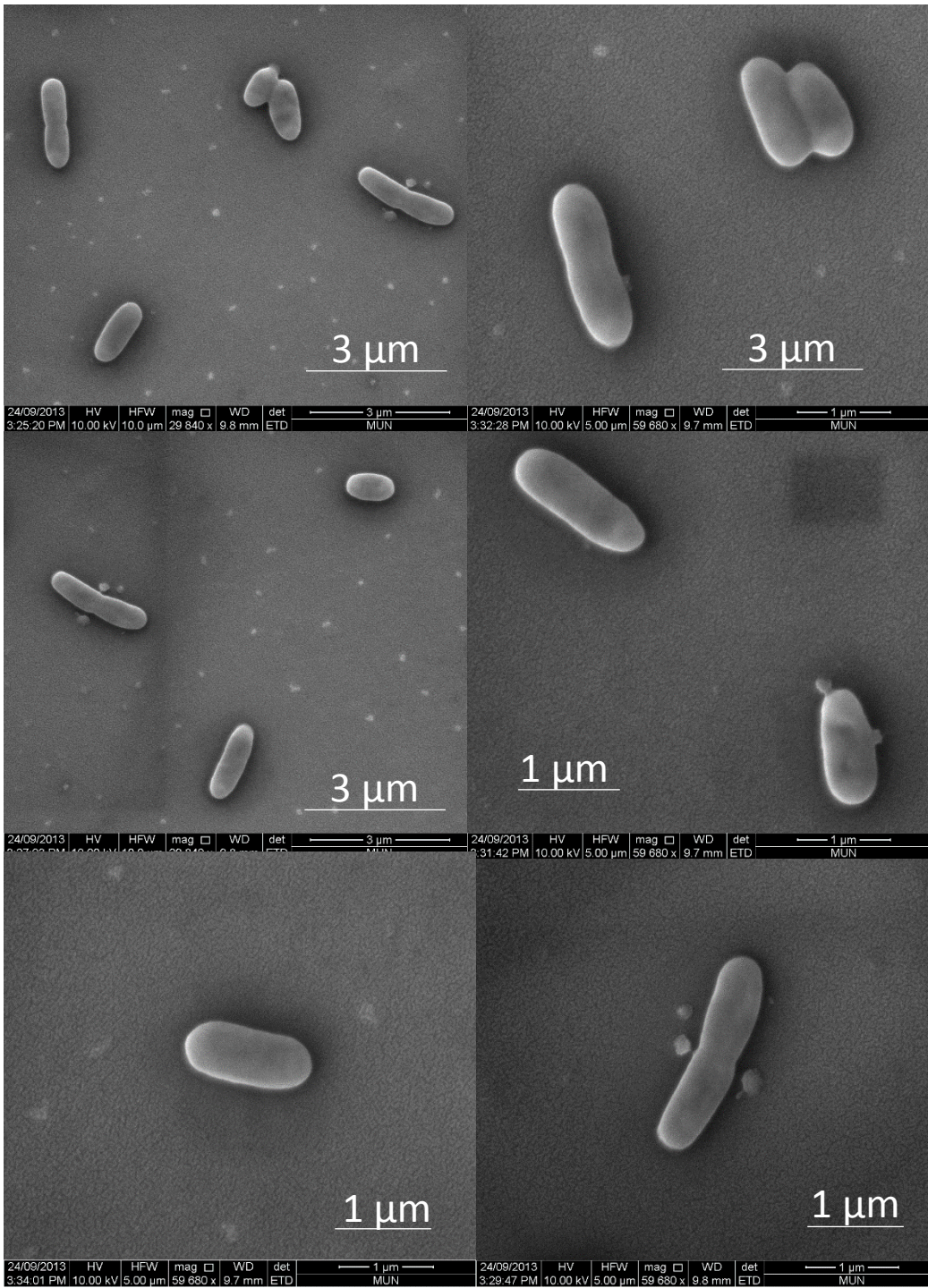**Figure 4.6 SEM visualization results of *Exiguobacterium* sp. N4-1P**

Figure 4.7 SEM visualization results of *Alcanivorax* sp. N3-7A
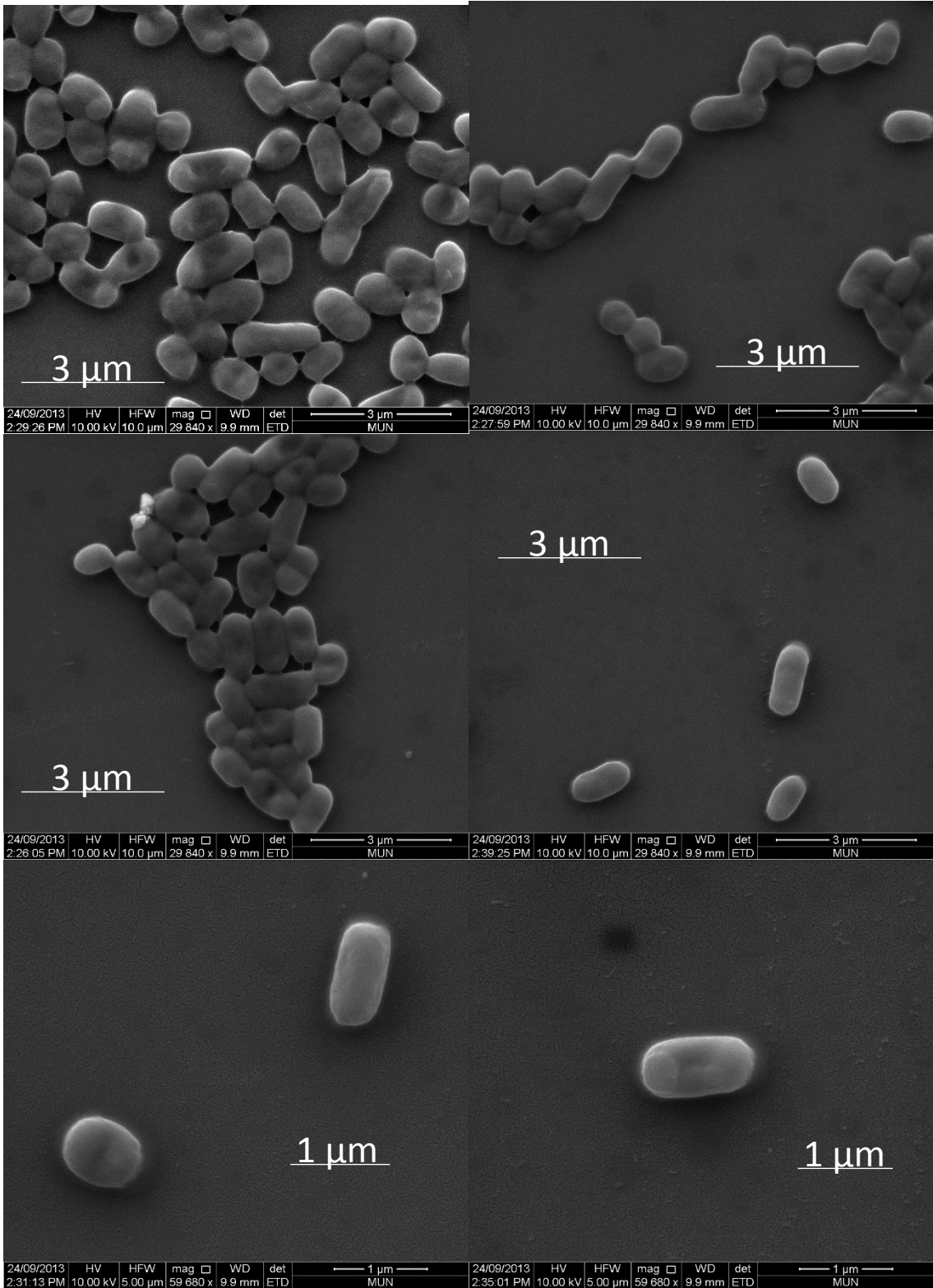
Figure 4.8 SEM visualization results of *Halomonas* sp. N3-2A

4.3.5 Production and Recovery of Crude Biosurfactants

      The general properties of the 16 clear broth are summarized in **Table 4.4**. For *Alcanivorax* sp. N3-2A and *Halomonas* sp. N3-7A, the addition of hexadecane and diesel stimulated the reduction of surface tension. Generally, benzoate had no effect on reducing the surface tension and the broths with non-hydrocarbon only all have high surface tensions. For *Alcanivorax* sp. N3-2A, addition of hydrocarbons suppresses its ability to produce biosurfactants that can stabilize emulsions, especially hexadecane and diesel. In contrast, for *Exiguobacterium* sp. N4-1P, the addition of hydrocarbon promoted the production of high-molecular-weight biosurfactants, following the sequence of diesel>hexadecane>benzoate. For *Halomonas* sp. N3-7A, only hexadecane significantly promoted generation of emulsion stabilizing biosurfactants. In terms of yields, addition of hydrocarbon significantly increased the yield for all three strains. *Halomonas* sp. N3-7A had slightly higher overall production. For *Alcanivorax* sp. N3-2A, hexadecane addition generated the highest yield; while for *Exiguobacterium* sp. N4-1P diesel addition generated the highest yield.

4.3.6 Properties of the Crude Biosurfactants

      The general properties of the biosurfactants from the 16 clear broth are summarized in **Table 4.5**. For surface tension, the trend observed in the broths was not that clear for the crude biosurfactant products. One possible explanation to this was the concentration of crude biosurfactants (5g/L) used in this test was lower than the concentration in the broths. The CMC was not yet achieved. For E24, the trend was

Table 4.4 General properties of the clear broths obtained with different strains and carbon sources.

Strain	Carbon source	Surface tension (dynes/cm)	E24 (%)	Yield (g/L)
Alcanivorax sp. N3-2A	Hex+NHC	37.3, 37.2, 36.6	0	13.1
	Dies+NHC	40.8, 39.7,39.8	0	11.8
	Benz+NHC	60.0, 59.8, 58.9	52.4	12.6
	NHC	56.5, 56, 56	76.2	4.6
Exiguobacterium sp. N4-1P	Hex+NHC	51.7, 52, 50.7	61.9	10.9
	Dies+NHC	34.1, 35.0, 34.8	71.4	13.5
	Benz+NHC	56.0, 54.0, 54.1	42.9	13.0
	NHC	55.0, 54.0, 54.2	17.4	6.1
Halomonas sp. N3-7A	Hex+NHC	45.3, 45.5, 45.6	66.7	14.1
	Dies+NHC	44.4, 45.0, 44.8	28.6	13.6
	Benz+NHC	55.8, 55.0, 55.2	33.3	12.9
	NHC	59.5, 59, 58.9	28.6	6.7

Note: Hex=hexadecane, Dies=Diesel, Benz=Benzoate, NHC=non-hydrocarbon carbon sources.

Table 4.5 General properties of the crude biosurfactants

Source strain	Carbon source	Surface tension (dynes/cm)	E24 (%)
Alcanivorax sp. N3-2A	Hex+NHC	43.5, 43.2, 43	15.00
	Dies+NHC	53, 52.7, 52.5	30.00
	Benz+NHC	57.7, 58.1, 58	70.00
	NHC	44.1, 44, 43.9	71.43
Exiguobacterium sp. N4-1P	Hex+NHC	52, 51.8, 51.7	47.62
	Dies+NHC	54, 54.3, 54.2	55.00
	Benz+NHC	58.7, 59.2, 59.4	66.67
	NHC	56.7, 56.8, 57.2	57.14
Halomonas sp. N3-7A	Hex+NHC	55.0, 54.9, 54.8	66.67
	Dies+NHC	54.5, 54.0, 54.4	28.57
	Benz+NHC	54.0, 54.3, 54.2	33.33
	NHC	44.1, 44.0, 43.9	66.67

Note: Hex=hexadecane, Dies=Diesel, Benz=Benzoate, NHC=non-hydrocarbon carbon sources.

similar with the broths, except crude biosurfactants generated with non-hydrocarbon carbon source tended to have higher E24 than those in broths.

4.4 Summary

In this Chapter, three featured bacteria were selected based on the results of previous chapter were characterized. The Chapter starts with a literature review of these three bacteria as biosurfactant producers. Then the phylogenetic relationship between them, other isolated bacteria in this study and their closely related species were investigated based on 16S rRNA analysis. Subsequently, their metabolic fingerprints were determined using BIOLOG ® GN2 and GP2 plates. The PLFA composition of their membrane were also revealed and agreed well with literature which reassured the taxonomical conclusions. These results contribute to the understanding of these selected isolates from both aspects of genotype and phenotype. The bacterial cells were also visualized using SEM. The cell shape and size were recorded and compared with the literature. The results correlated well with the literature. Lastly, biosurfactants produced by these bacteria were recovered from the culture broth and characterized.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Summary

This dissertation research has focused on screening of novel and superior biosurfactant producing bacteria from petroleum hydrocarbon contaminated marine environment and characterizing the featured isolates and their produced biosurfactants.

Firstly, some basic physiochemical properties, history, impact and response technologies of oil spills have been reviewed. The review from this part concluded that dispersants can be an effective combat agent for offshore oil spill even in cold and harsh environment. The following section reviewed dispersants including their effectiveness, fate and transport, and toxicity concerns. Currently used chemically synthetic dispersants were found to be not very effective, especially when dealing with weathered or emulsified oil. Their toxicity concerns were prevailing among public and they were found to be persistent in the ecosystem. Under such circumstances, biosurfactants-based dispersants were proposed as possible alternatives. Some fundmental concepts of biosurfactants were discussed. Then, the superiorities over chemical surfactants such as lower toxicity and CMC, less persistent and better at enhancing biodegradation were reviewed. Then, their high cost issue and possible overcoming strategies were discussed. Finally, the sources for screening superior biosurfactant producers were reviewed and it was found that marine sources contaminated with petroleum hydrocarbon were of great potential. The screening of such marine biosurfactant producers have not been done in North Atlantic Canada.

Subsequently, water and sediment samples were collected in petroleum hydrocarbon contaminated sources. A modified colony-based drop collapsing method was used to cover sessile bacteria, showing the advantages of saving time and reducing contamination

risks. Through using n-hexadecane or diesel as the sole carbon source, 55 biosurfactant producers were identified and characterized. They belonged to 8 genera, namely, *Alcanivorax*, *Exiguobacterium*, *Halomonas*, *Rhodococcus*, *Bacillus*, *Acinetobacter*, *Pseudomonas*, and *Streptomyces*. The first three genera had not been long established with limited relevant publications. They were generally found to be alkane degrading, extremophile and halophile, respectively. The distributions of the 55 isolates were site and medium specific. Diverse isolates were found with featured properties such as flocculant production, significant reduction of surface tension and stabilization of water-n-hexadecane emulsion. Isolates P6-4P and P1-5P were selected to demonstrate the performance of biosurfactant production. They reduced the surface tension to as low as 28 dynes/cm and had the ability to stabilize emulsions (E24>50%) or facilitate biosurfactant recovery through cell flocculation.

These isolates originally lived in petroleum hydrocarbon contaminated sites and were able to produce biosurfactants while using petroleum hydrocarbon as the sole carbon source. Given that all the isolates adapted to the cold marine environments of northern Atlantic Canada and their cell free culture remained clear at 4°C, the biosurfactants they produced could thus be effective under low temperature. The produced biosurfactants thus could facilitate bio-transfer of petroleum hydrocarbon, leading to promising potential in offshore oil spill responses even under harsh conditions.

Subsequently, three featured bacteria namely *Halomonas* sp. N3-2A, *Alcanivorax* sp. N3-7A and *Exiguobacterium* sp. N4-1P were selected as their corresponding genus were relatively new with significant knowledge gaps. The phylogenetic tree was constructed to reveal the relationships between the representative bacteria in this study and their closely

related species based on 16S rRNA analysis. Subsequently, their metabolic fingerprints have been determined using BIOLOG® GN2 and GP2 plates which showed which carbon sources out of the 95 set on the plates can be digested by the selected strains. The PLFA composition of their membrane were also revealed and agreed well with literature values. The bacterial cells were visualized using SEM. The cell shape and size were recorded and compared with the literature. With medium recipes of different combination of the carbon source, biosurfactants were produced with the selected three isolates. The yield, surface tension and E24 of the obtained biosurfactant products were determined.

5.2 Research Achievements

It is the first study on marine biosurfactant producers from petroleum hydrocarbon contaminated sites in North Atlantic Canada. A colony based oil drop collapsing test was modified for the screening process to cover sessile bacteria. Fifty five cold-adapted and hydrocarbon degrading biosurfactant producers have been isolated. The isolated biosurfactant producers belong to 8 genera. Some isolates formed flocculants which facilitate economic production, while some isolates effectively reduced surface tension and/or stabilized emulsion. *Halomonas* sp. N3-7A had slightly higher overall production.

From these isolates, *Exiguobacterium* sp. N4-1P has never been demonstrated as a biosurfactant producer in the literature. The biosurfactants it produced and the relevant producing process are unknown. They have great potential to possess interesting features leads to the development of novel and superior bio-dispersants. *Alcanivorax* sp. N3-2A has only 94% match with the 16S rDNA database indicating it is a potentially newly discovered bacteria. Other *Alcanivorax* published in the literature were only found to produce small molecular biosurfactant. However, *Alcanivorax* sp. N3-2A could produce

biosurfactants with high E24 indicating the products were high-molecular biosurfactants. These findings are novel and will also contribute to the development of new and superior bio-dispersants. *Halomonas* sp. N3-7A was demonstrated as an effective producer of bio-emulsifier with high E24.

The strains, the products and the bioprocess can be of great value to both scientific understanding and the environmental applications in offshore oil spill responses.

The contents in the dissertation research have also led to the following publications/presentations or potential publications:

1. Li, P., Cai, Q.H., Lin, W.Y., Chen, B., and Zhang B.Y. 2013. From challenges to opportunities: Towards future strategies and a decision support framework for oil spill preparedness and response in harsh environment. Environ. Rev. (drafted).

Roles: co-writer of the manuscript

2. Cai, Q.H., Zhang B.Y., Chen, B., Zhu, Z.W., Lin, W.Y., and Cao, T. 2013. Screening of biosurfactant producers from petroleum hydrocarbon contaminated sources in cold marine environments. Mar. Pollut. Bull. (under review).

Roles: Group leader of the team, editorial writer of the manuscript

3. Cai, Q.H., Zhang, B.Y., Chen, B., Lin, W.Y., Zhu, Z.W. 2013. Isolation of biosurfactant producers from marine sources for offshore oil spill responses in harsh environments. Poster presentation in BIT's 3rd Annual World Congress of Marine Biotechnology, Hangzhou, China

Roles: Group leader of the team, editorial writer of the poster

4. Cai, Q.H., Zhang, B.Y., Chen, B., Zhu, Z.W., Lin, W.Y. 2013. Screening of biosurfactant producers from petroleum hydrocarbon contaminated sources in cold marine environments in Atlantic Canada. Orally presented at the 36th AMOP Technical Seminar, June 4-6, Halifax, Canada.

Roles: Group leader of the team, presentor of this study

5. Cai, Q.H., Zhang B.Y., Chen, B., Zhu, Z.W., Lin, W.Y., and Cao, T. 2013. Isolation and characterization of a lipopolysaccharide type biosurfactant produced by an *Exiguobacterium oxidotolerans* strain isolated from petroleum hydrocarbon contaminated sea sediment. Bioresource Tech. (under preparation).

Roles: Group leader of the team, editorial writer of the manuscript

6. Cai, Q.H., Zhang B.Y., Chen, B., Zhu, Z.W., Lin, W.Y., and Cao, T. 2013. *Alcanivorax xxxxx* sp. nov. a novel alkane degrading and biosurfactant producing bacterium isolated from petroleum hydrocarbon contaminated sea sediment. Int. J. Sys. Evol. Micr. (under preparation).

Roles: Group leader of the team, editorial writer of the manuscript

7. Cai, Q.H., Zhang B.Y., Chen, B., Zhu, Z.W., Lin, W.Y., and Cao, T. 2013. Isolation and characterization of an alkane degrader and biosurfactant producer - *Alcanivorax xxxx* - and its potential application to oil dispersion and enhanced bioremediation. J. hazar. Mater. (under preparation).

Roles: Group leader of the team, editorial writer of the manuscript

8. Cai, Q.H., Zhang B.Y., Chen, B., Zhu, Z.W., Lin, W.Y., and Cao, T. 2013. Isolation and characterization of a lipopolysaccharide type biosurfactant produced by *Halomonas venusta* isolated from petroleum hydrocarbon contaminated sea sediment. Bioresource Tech. (under preparation).

Roles: Group leader of the team, editorial writer of the manuscript

5.3 Recommendations for Future Research

The study can be further extended as follows:

(1) A full genome sequence should be conducted to reveal the possible coding site for biosurfactant production. This information will be used to identify the DNA fragments. Through overexpressing of the target DNA fragments, the production yield could be greatly enhanced.

(2) The fermentation processes should be further investigated with the one-factor-at-a-time (OFAT) approach to study the effects of diverse medium compositions and environmental conditions on biosurfactant production. These factors should also be investigated with fermentation kinetic models and degradation competition models. Subsequently, as OFAT results may establish the proper levels of factors, a response

surface methodology (RSM) and artificial neural network (ANN) based optimization approach can be used to obtain the optimum conditions.

(3) The production should be eventually scaled-up and even changed to continuous mode. Several newly developed downstream processing techniques can be tested and further optimized accordingly to achieve the final goal of economic biosurfactant production.

(4) The produced biosurfactants can be used to develop advanced biodispersants after formulating with suitable solvents. The effectiveness of the biodispersants can be then tested in both laboratory scale reactors and wave tanks.

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