GENERATION OF BIODISPERSANTS FOR OFFSHORE OIL SPILL RESPONSE

By © Tong Cao

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ABSTRACT

Biodispersants are detergent-like products made by dissolving biosurfactants in solvents. Biosurfactants, comparing with their chemical counterparts, have advantages of lower eco-toxicity, as well as higher biodegradability and stability. Biodispersants have the great potential to be applied as the reagent for offshore oil spill response. However, relevant topics were rarely reported in literature due to the extremely limited biodispersants available and the high cost of biosurfactant production.

This study thus tried to fill the research gap through enhancing the producting of biosurfactants so as to decrease the cost and generating biodispersant products for oil spill response. The biosurfactants were produced by *Rhodococcus erythropolis* sp. SB-1A, a strain isolated from the North Atlantic Ocean. Effects of culturing conditions including the carbon source, the nitrogen source, pH and salinity were investigated through the One-factor-at-a-time (OFAT) experiments. Surface tension and the reciprocal of critical micelle concentration (CMC^{-1}) of the cell-free culture were monitored periodically. A kinetics model was established to represent the time course of biosurfactant production. Under the determined culturing conditions (3.5 v/v% N-hexadecane, 0.7 g/L NH_4NO_3, pH 7 and 26 g/L NaCl), the surface tension of culture mediums was reduced by 40 dynes/cm with a CMC^{-1} of 11.9 after 40 hours of cultivation. The produced biosurfactants were further characterized. Results indicated that the total carbohydrate content in 1 g of biosurfactants was 8.4 mg in term of D-glucose, and the total lipid content in 1 g of biosurfactants was 11.6 mg in term of Palmitic acid.

Multiple solvents were selected to mixed with the biosurfactants, respectively, to obtain the biodispersants. The solvents were screened based on their toxicity and the effectiveness of relevant dispersants generated. The final formula of the biodispersant was determined as 16.7%/ 83.3% (biosurfactants/ PEG 400). The biodispersant-based dispersion was further examined using a motor oil sample and a crude oil sample. The performance was compared with the commercial chemical dispersant Corexit 9527. Results showed that compared with Corexit 9527, the biodispersant could achieve a compatible dispersant effectiveness (DE) when treating the motor oil and a higher DE for treating the crude oil. Through the biodegradation test, 45% of the biodispersed crude oil was biodegraded by the biodispersant assisted dispersion after 28 days of dispersion treatment without spiking any oil-degrading bacteria. The toxicity of the biodispersed oil was reduced by 50% after 28 days. The research outputs provided an evidence for applying biodispersants as a promising alternative reagent for offshore oil spill response.

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TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

BFT	baffled flask test
C9500	Corexit 9500
C9527	Corexit 9527
CLP	cyclic lipopeptides
CMC	critical micelle concentration
CMC^{-1}	the reciprocal of critical micelle concentration
CMD	critical micelle dilution
CMT	critical micelle temperature
DCM	Dichloromethane
DE	dispersion effectiveness
DER	dispersion effectiveness ratio
DMSO	Dimethyl sulfoxide
DOR	dispersant to oil ratio
DWAF	dispersed oil water-accommodated fraction
EC_{50}%	median effective concentration
EI 24	emulsification index 24
GC/MS	gas chromatography coupled with mass spectroscopy

HLB	hydrophilic-lipophilic balance
HPLC-MS	High pressure liquid chromatography and mass spectroscopy
IFP	Institut Français du Petrole
IR	Infrared
MNS	Mackay-Nadeau-Steel-man
MTBE	methyl tertiary-butyl ether
ND	natural dispersion
NMR	Nuclear magnetic resonance
OFAT	one-factor-at-a-time
PAHs	polycyclic aromatic hydrocarbons
PEG 400	Polyethylene glycol 400
QA/QC	Quality Assurance and Quality Control
SFT	swirling flask test
Solketal	DL-12-isopropylidene glycerol
TLC	Thin layer chromatography
TPH	total petroleum hydrocarbons
WAF	water-accommodated fraction
W/O	water-in-oil
WSL	Warren Spring Laboratory

CHAPTER 1

INTRODUCTION

1.1 Background

Oil spills have gain the global concern due to their impacts on marine environment along with the large economic loss. After the Exxon Valdez oil spill in 1989, more than 36,000 seabirds died immediately and most of the rescued 1,800 living oiled seabirds eventually died after they were brought to rehabilitation centers (Piatt and Ford, 1996). The negative effects of oil spills on benthic organisms were demonstrated based on the research of the multiple ecological processes and ecosystem functions that these organisms support (Mendelssohn et al., 2012). Human health can also be affected by the consumption of oiled seafood. It was revealed that marine food such as mussels contaminated with polycyclic aromatic hydrocarbons (PAHs) coming from oil spills can cause genotoxic damage in consumers (Lemiere et al., 2005). The total economic loss due to an oil spill event can be broken down into the socioeconomic loss, cleanup cost, environmental damage, research cost, and other costs (Liu and Wirtz, 2006).

The offshore oil and gas industry in Newfoundland and Labrador (NL) is booming. NL produces more than 300,000 barrels of crude oil per day, representing about 12 percent of Canada's total crude oil production. Consequently, promising oil spill alternatives are required. Several response techniques including physical/mechanical and chemical counter-measurements have been developed to help the offshore oil and gas industry reduce the impacts on marine systems, local communities and fisheries, and human health. Booms are mechanical devices used to contain an oil spill and prevent it from spreading to a particular area, to divert it to another area where it can be recovered or treated, or to concentrate the oil so that it can be recovered, burned, or

otherwise treated (Fingas, 2010). Sometimes solidifiers were used in combination with the booms to recover oil from smaller areas on a rapid basis to prevent the spread of slicks, to recover thin sheens, and to protect areas and wildlife (Dahl et al., 1996). Skimmers are mechanical devices composed by disks, belts, drums, and brushes designed to remove oil from the water surface in conjunction with booms without changing its properties so it can be reprocessed and reused (Hammoud, 2006; Schulze, 1998; Schwartz, 1979). In situ burning is a thermal mean of oil spill remediation that can be proceeded with minimal specialized equipment with higher rates of oil removal efficiency (Dave and Ghaly, 2011). Nevertheless, the applicability and effectiveness of current mechanical countermeasures can be affected by strong currents and wind in regions such as the North Atlantic Oceans (Chen et al., 2011; Dave and Ghaly, 2011; Jing et al., 2012).

The global awareness of dispersants was raised after the Deepwater Horizon oil spill as being a promising alternative response technique regardless of severe weather (Walker et al., 2003). They are usually applied by spraying the water or by underwater injection (Sittig, 1974). An oil slick can be broken down into smaller droplets and transferred into the water column where it undergoes rapid dilution and can be easily degraded (Lessard and DeMarco, 2000). They also allow for rapid treatment, slow down the formation of oil-water emulsions, and make the oil less likely to stick to surfaces (including animals) (Nomack, 2010). Nevertheless, there are rising concerns about environmental harm due to the toxicity and non-biodegradability of some chemical dispersants and dispersed oil. Hence, novel, environmental friendly and cost-effective biosurfactants and associated biodispersants are being considered as the alternatives to adequately address the safety and environmental concerns (Muthusamy et al., 2008). Despite the various advantages and diverse potential applications of

biosurfactants that have been reported, difficulties exist in their applications on a commercial level due to the low yields and thus the high cost in the production process (Mukherjee et al., 2008). Moreover, biosurfactant-based oil dispersion has rarely been studied.

1.2 Objective

The core value of this research was to fill the gap through enhancing the production of biosurfactants so as to decrease the cost and generating biodispersant products for oil spill response. The produced biodispersants should have favorable dispersion effectiveness (DE) and evironmental friendly characteristics.

It entails the following tasks:

(1) To study important factors in biosurfactant production by a biosurfactant producer isolated from North Atlantic and to enhance the productivity based on selected conditions;

(2) To generate biosurfactant products and isolate them from the culturing media;

(3) To study the surface activity and structural characteristics of the biosurfactants;

(4) To investigate the formulation of the biodispersants by testing candidate solvents and selecting the favorable biosurfactants/solvent ratio;

(5) To evaluate the generated biodispersant product by testing its DE using a motor oil sample and a light crude oil sample, and compared it with commercial chemical dispersants; and

(6) To assess the effect of the biodispersant product on crude oil biodegradation and the toxicity of the water-accommodated fraction of biodispersed crude oil.

1.3 Thesis structure

The thesis consists of five chapters and a list of references. Chapter 2 describes the background of oil spills, as well as chemical dispersants and biodispersants including their physicochemical properties, mechanisms of action, and their applications. It also presents reviews of previous research findings in the potential of biosurfactants for oil spill response including effective biosufactant producers, characterization, and applications. Chapter 3 describes the cost-effective production of biosurfactants by *Rhodococcus erythropolis* sp. SB-1A isolated from the North Atlantic ocean and relevant kinetics study. Chapter 4 indicates the generation of biodispersant and the evaluation of its performance as the oil spill response alternative. Chapter 5 summarizes the finds and significance of this study, and presents recommendations for future work.

CHAPTER 2

LITERATURE REVIEW

2.1 Dispersants

Dispersants are detergent-like products made of surfactants dissolved in one or more solvents. As important oil spill response reagents, they have been used to reduce the impact of oil spills on shorelines and habitats. An ideal dispersion is generally designed with a chemical affinity for both oil and water. By spraying onto oil slicks or by underwater injecting, the surfactants diffuse to the oil/water interface, and the interfacial tension can be reduced, which leads to oil dispersion into the water column at very low concentrations (Lessard and DeMarco, 2000). The oil dispersion can enhance the degradation of the oil by microorganisms in natural waters (Swannell and Daniel, 1999).

2.1.1 Chemical surfactants

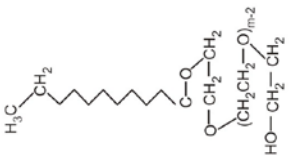
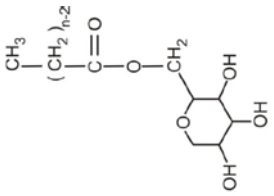
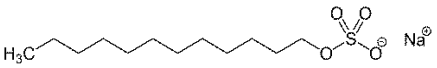
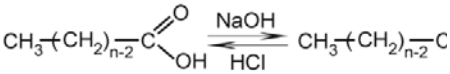
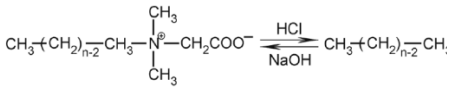
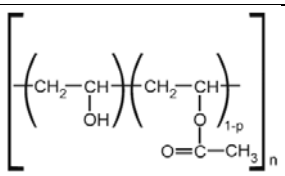
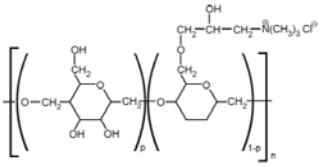
Surfactants are a unique class of chemical compounds with both hydrophobic groups (or tails) and hydrophilic groups (or heads) (Schramm et al., 2003). As surface-active compounds, effective surfactants can reduce the surface tension between the water and air from 72 to 30 mN/m and the interfacial tension between the water and n-hexadecane from 40 to 1 mN/m (Schramm, 2000). Some surfactants are named micelle-forming molecules as the surface or interfacial tension will stop decreasing when the concentration of surfactants reaches the critical micelle concentration (CMC) (Schramm, 2000).

The applications of surfactants are diversified due to their remarkable ability to influence the properties of surfaces and interfaces (Schramm et al., 2003). Surfactants are usually applied in the detergent industry based on their surface wettability. Surfactants that can rapidly diffuse and adsorb at appropriate interfaces are considered

as good wetting agents. For example, the surface active fatty acid salt is usually used in the soap production (Schramm, 2001). In the cosmetics industry, surfactants such as alkyl polyglyosides were used for improving the stability of micro-emulsions by increasing the temperature ranges to reduce skin irritation and to create the formulation of O/W (Schueller and Romanowski, 1998). In the food production and processing industry, surfactants can be added as food coating modifiers. For instance, surfactants such as sorbitan monostearate and polysorbate 60 were used to stabilize and blend the fat and the cocoa butter in chocolate coating (Dziezak, 1988). Surfactants such as xanthan and carboxymethyl cellulose were good for enhancing smoothness, reducing ice, lactose crystal and melting in the processing of popsicle (Goff, 1997). Surfactants also have a big application value in the petroleum industry from oil-in-water emulsification, differential sticking prevention, shale-swelling inhibitors, to foaming/ defoaming addition (Quintero, 2002). Surfactants are potentially useful surface-active agents for fuel additives and lubricants, pharmaceuticals, adhesives, paints, agrochemicals, and environmental remediation techniques (Schramm et al., 2003).

According to the chemical structure of surfactants, chemical surfactants can be classified as the low molecular mass surfactants and polymeric surfactants. The classification has been indicated in Table 1 (Denkov et al., 2009).

Table 1. Classification of chemical surfactants

Types	Example compounds	Molecular formula
	Low molecular mass surfactants	
Nonionic	Alkylpolyoxyethylenes	
	Spans	
Ionic	Sodium dodecyl sulfate, SDS	
	Cetylpyridinium chloride, CPC	
Amphoteric	alkylcarboxylates, Lipids	
	Betaines	
	Polymeric surfactants	
Synthetic	Polyvinyl alcohol, PV	
	Modified polysacharides	

2.1.2 Chemical dispersants

(1) Advantages of Chemical Dispersants

Compared with other oil spill response alternatives, dispersants have unique advantages. Dispersants can be applied in harsh weather conditions (e.g., rough seas, strong winds and currents) where the use of mechanical containment and recovery techniques such as booms and skimmers is limited. Dispersant treatment can be rapidly applied to large oil spills such as the Deepwater Horizon oil spill. This can be accomplished by large aircraft. Dispersants application also might enhance the natural biodegradation process by increasing the surface area of oil available to bacteria. Dispersed oil is unlikely to stick to sediment, wildlife, shorelines, and vessels due to the presence of the surfactants on the surface of the droplets (Lessard and DeMarco, 2000).

(2) Structural Properties

One critical parameter that affects the performance of the chemical dispersants is the hydrophile-lipophile balance (HLB), which can be used to characterize the tendency of the surfactant to preferentially dissolve in either the oil phase (low HLB) or the aqueous phase (high HLB). HLB can be calculated based on theoretical equations measuring the balance between the length of the water-soluble portion of the surfactant and the oil-soluble portion of the surfactant. Generally, water-in-oil (W/O) emulsions can be created under an HLB between 1 and 8, and oil-in-water (O/W) emulsions exist under an HLB between 12 and 20 (Fingas, 2010; Porter and Porter, 1991). This is due to the fact that the dominant group of the surfactant molecules will tend to orient in the outer phase to form a droplet of either oil or water. (Porter and Porter, 1991). Consequently, the components of commercial chemical dispersants are

usually comprised of two or more surfactants with different HLBs with an overall HLB in the range of 9 to 11 (Clayton et al., 1993). Due to such HLB property, dispersants may avoid the formation of emulsions (mousse) and enhance the time window for response (Lessard and DeMarco, 2000).

Another important parameter for the dispersion is CMC. Above this threshold level, micelles can be created as surfactant molecules aggregate because of the chemical interactions between the polar head groups and the non-polar tail groups including hydrophobic, Van der Waals' force, and hydrogen bonding (Soberón-Chávez and Maier, 2011).

CMC varies with the structure of surfactants, pH, ionic strength, temperature, and the polarity of the solvent. CMC values of some chemically synthetic surfactants and biosurfactants are listed in Table 2 (Desai and Banat, 1997; Nantes et al., 2011).

Table 2. CMC values of example 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	

(3) Factors Affecting the Effectiveness of Chemical Dispersants

As a prior consideration for selecting a dispersant, the effectiveness is influenced by many factors including oil composition, sea energy, temperature, salinity of the water, oil weathering, type of dispersant, and the applied amount (Fingas, 2010). It is widely recognized that oil composition affects the dispersant effectiveness and the time window for response. Heavy oils are more resistant to be dispersed since their high viscosity keeps themselves from being penetrated, which is a necessary condition to produce dispersed oil droplets (Kaku et al., 2006). Heavy oils are also more likely to form emulsion (mousse). Wax and asphaltenes have been found to be stabilizing agents for water-in-crude oil emulsions. La Rosa crude oil was found to be more

likely to form a more stable, higher water content emulsion than other oils such as Murban crude oil because of wax and asphaltenes (Bridie et al., 1980). However, if the oil is too light, the formed oil droplets have to be very small to overcome buoyancy. This means that the dispersion of oil by a dispersant is dependent on the type of dispersant/oil pair (Kaku et al., 2006).

Dispersion of oil droplets can be increased by turbulence due to the mixing energy from waves, especially breaking waves (Delvigne, 1993). In a calm sea stage, the dispersant cannot penetrate the oil and gathers in small pools within the slick. During the comparison study of two common dispersion effectiveness tests in the laboratory, the Swirling Flask test (SFT) and the Baffled Flask test (BFT), BFT was found to have smaller oil droplets than SFT. It was noted that BFT have more uniformly distributed mixing and energy dissipation rates as measured by a hot wire anemometer. Thus, BFT is more preferable for a dispersant test in the laboratory due to its superior turbulence in the flask (Kaku et al., 2006).

The salinity can also impact the dispersant effectiveness, which have been shown in some studies. An overall increase in dispersion with increasing salinity was observed in the Labofina-rotating flask test to determine the effect of salinity on dispersant effectiveness under low temperatures and high-energy conditions (Byford et al., 1983). By using the swirling flask test, three types of crude oil were shown to be increasingly dispersed with an increase in salinity from 0 to 45 psu (Clayton et al., 1993; Fingas, 1991). From experimental studies, it was demonstrated that higher salinity may prevent the migration of surfactants into the water phase due to the salting-out effect of surfactants from the saline medium, which can decrease the solubility of

dispersants in water and generate more available surfactants to interact and mix with the oil (Mackay et al., 1984).

There are regional concerns that dispersants may not be effective on oil spills in cold water. It is widely recognized that low temperatures can decrease the dispersion effectiveness because the viscosity of the spilled oil and dispersant is increased inhibiting the dispersion, which has been proven in several studies (Belore et al., 2009). Cold dispersant ineffective in cold oil-water system was used in warm oil-water systems and the results were better than that of warm dispersant (Cox and Schultz, 1981). The interfacial tensions in dispersant-oil-sea water systems were higher than that in cold-water, which indicated that dispersants might be less effective in cold conditions by Mackay. However, he also noted that the influence of temperature on dispersion effectiveness might be very complex due to dispersant-oil mixing and interfacial tension modification processes as well as oil viscosity issues (Mackay and Hossain, 1982). Furthermore, higher oil viscosities in cold waters may prevent the re-coalescence of dispersed oil droplets and increase the density, which may facilitate dispersion (Byford et al., 1983).

The weathering of crude oil is a complicated process affected by other factors such as oil composition, natural dispersion, emulsification, photo-oxidation and evaporation. Many experiments have tried to simulate the real process of oil weathering and to acquire valuable information on the time- window of the usage of dispersants from small-scale to pilot-scale levels. However, it is difficult to consider all the factors in one experiment simultaneously. The weathering processes was simulated in small-scale study by evaporation and water-in-oil (W/O) emulsification after three days. By using Institut Français du Petrole (IFP), Mackay-Nadeau-Steel-man (MNS) and

Warren Spring Laboratory (WSL) tests, it was concluded that emulsion breaking played an important role in chemical dispersion (Lewis et al., 1995). Two dispersants, Corexit 9500 and Dasic Slickgone NS, were tested in field studies on the weathered crude oils and heavy fuel oils to investigate to what extent the dispersants can break and disperse the high viscosity emulsions formed by oil that has weathered on the sea surface for several days and to what extent heavy fuel oils can be chemically dispersed (Lewis et al., 1998). It was concluded that that the window of opportunity for dispersant use is wider than previously considered, but emulsions with lower water contents are more resistant to the effect of dispersants by surface sampling, sub-surface oil concentration monitoring and airborne remote sensing (Lewis et al., 1998).

2.1.3 Toxicity of chemical dispersants and/or dispersed oil

The negative effects of chemical dispersants on marine life and the environment raised increasing concern among scientists and environmentalists, especially after the Deepwater Horizon oil spill. The toxicity of chemical dispersants and chemically dispersed oil on different marine species in various ecosystems has been studied based on impact of both acute and chronic toxicity (Gulec and Holdway, 1997; Fucik et al., 1994; Burridge and Shir, 1995; Baca et al., 1996; Kirby et al., 2007).

The toxicity of oil and the dispersant Corexit 9527 was studied using the amphipod, Allorchestes compressa. The acute 96-hour LC50 for A. compressa exposed to Corexit 9527, dispersed crude oil and the water-accommodated fraction of Bass Strait crude oil was 3 mg/L, 16.2 mg/L and 311,000 mg/L respectively. The EC50 for sublethal effects after exposure for 30 minutes was 50.2 mg/L, 65.4 mg/L and 190,000 mg/L (Gulec and Holdway, 1997). The toxicity of Corexit 9527 and dispersed oil to

some species from the Gulf of Mexico, including shrimp (both *Penaeus aztecus* and *Penaeus setiferus*), the blue crab (*Callinectes sapidus*), eastern oyster (*Crassostrea virginica*), inland silverside larvae (*Menidia beryllina*) , silverside embryos (*Menidia beryllina*), Atlantic menhaden (*Brevoortia tyrannus*), the Spot (*Leiostomus xanthurus*), and red drum (*Sciaenops ocellatus*). The results indicated that dispersant plus oil had a higher LC50 than dispersant or oil alone for most species, while such dispersed oil also displayed a lower LC50 for the blue crab (*Callinectes sapidus*) and Atlantic menhaden (*Brevoortia tyrannus*) (Fucik et al., 1994). The toxicity of different Corexit products including Corexit 7664, Corexit 8667, Corexit 9500, and Corexit 9527 were compared based on the 48-hour EC50 for marine algae. It was noted that the most significant effect on the germination was the dispersant/oil combinations, of which the EC50 was 4,000 mL/L for Corexit 7664, 2,500 mL/L for Corexit 8667, 20 mL/L for Corexit 9500, and 200 mL/L for Corexit 9527 (Burridge and Shir, 1995). The chronic toxicity of oil and dispersed oil on three tropical ecosystems (i.e., sea grass beds, mangrove forests, and coral reefs) was studied individually on the Caribbean coast of Panama. The experiment demonstrated that the influence of dispersed oil on the abundance, and growth of the dominant flora and fauna in each habitat was obvious with two years but disappeared over ten years. By contrast, the single oil had severer effects especially on the survival of mangroves and associated fauna even after ten years (Baca et al., 1996). It was noteworthy that the toxicity may be enhanced by UV illumination. Chemically dispersed Kuwait crude oil showed a toxicity effect at 25% and 5% dilutions under the room and UV conditions, respectively. A comparison study of the non-observed effect concentrations demonstrated that UV illumination reduced the concentration of the toxicity of dispersed Kuwait crude by approximately 10 fold. Such study demonstrated that the use of chemical dispersants on oil increased

the toxicity of the water-accommodated fraction (WAF) and augmented the magnitude of the UV-mediated toxicity (Kirby et al., 2007).

2.1.4 Biodegradation of chemically dispersed oil

It is important to expand the scientific understanding of the fate of dispersed oil along with chemical dispersants in combination with the study of long-term environmental impacts. Biodegradation is the crucial process that consumes oil and dispersants by microbial degradation. Many studies were conducted to investigate if the chemical dispersants have potential to enhance bioavailability, and hence, the biodegradation of oil.

Nevertheless, the results indicated that biodegradation is a very complex process to be simulated. Inhibition of dispersion was pointed out by some scholars yet some observed no effects with the addition of chemical dispersants (Fingas, 2010). The effects of the initial oil concentration and the Corexit 9500 dispersant on the bioremediation of petroleum hydrocarbons were investigated with a series of ex-situ seawater samples. The results showed that the presence of dispersant enhanced crude oil biodegradation and bioremediation were not effective when oil concentrations were higher than 2,000 mg/L (Zahed et al., 2010). The rate of biodegradation may be influenced by temperature. The biodegradation of oil after BFT dispersion by Corexit 9500 with 3.5% artificial seawater was tested at 20 °C and 5 °C. In the study, oil compositional analysis was performed by gas chromatography/mass spectrometry (GC/MS) to evaluate the biodegradability. The results indicated that dispersed oil was biodegraded more rapidly at 20 °C than the counterpart result at 5 °C, which was in line with the hypothesis that the ultimate fate of dispersed oil in the sea is rapid loss

by biodegradation (Venosa and Holder, 2007). However, most studies only demonstrated the effective biodegradation of chain hydrocarbons, while successful biodegradation of PAHs (Polycyclic Aromatic Hydrocarbons) was rarely reported based on previous studies. It was found that microbial mineralization favored particular components of crude oil in the order of 2-methyl-naphthalene > dodecane > phenanthrene > hexadecane > pyrene, but the rate was not affected by the addition of nutrients or sediment. When provided as carbon sources, the gross mineralization favored Corexit 9500, followed by fresh oil, weathered oil, and dispersed oil. Adding the dispersant inhibited the mineralization of hexadecane and phenanthrene but did not affect dodecane and 2-methyl-naphthalene mineralization (Lindstrom and Braddock, 2002). Furthermore, some studies also noted that observed results may have been confounded by the biodegradation of the readily biodegradable dispersants (Fingas, 2010).

2.2 Biosurfactant-based biodispersants

2.2.1 Biosurfactants

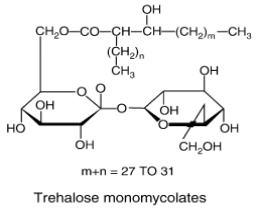
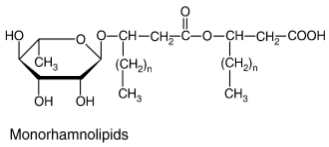
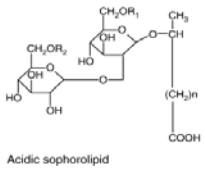
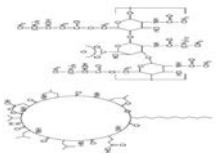
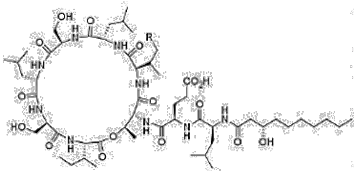
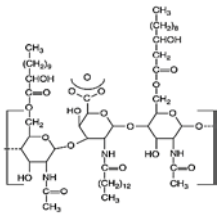
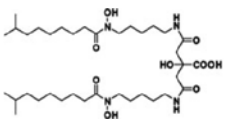
Biosurfactants are being considered as possible replacements of chemical surfactants as a result of current demand for industries (Banat et al., 2010). Such surface-active biomolecules produced by microorganisms, are superior alternatives for chemical surfactants due to their unique properties (Geys et al., 2014; Mukherjee et al., 2006). Biosurfactants are environmentally friendly since they can also be readily biodegraded and less damaging to the environment than the more recalcitrant chemical surfactants. Their excellent toleration under extreme conditions such as high

temperatures and high salt concentrations makes them attractive components for many industrial products (Banat et al., 2010).

Biosurfactants can be produced by bio-producers such as bacteria, yeast and fungi, and the products have a wide range of structures. Some example biosurfactants are listed and classified in Table 3. These compounds have similar suface properties displayed by chemically synthesized surfactants (Desai and Banat, 1997).

Biosurfactants also displayed unique biological functions such as antibiotic, antifungal, insecticidal, antiviral, immunomodulator, and anti-tumoral activities. They had shown a potential of special applications including the biological control of pests in medicine and pharmaceutics, cancer treatment (Saini et al., 2008), and wound healing (Piljac et al., 2007; Stipcevic et al., 2006).

Table 3. Classification of example biosurfactants

Head group	Biosurfactants	Molecular formula	Microorganism	Reference
Glycolipids	Trehalolipids	 m+n = 27 TO 31 Trehalose monomycolates	*Rhodococcus* sp., *Arthrobacter* sp., *R. erythropolis*, *N. erythropolis*	(Lang and Philp, 1998)
	Rhamnolipids	 Monorhamnolipids	*Pseudomonas* sp., *P. aeruginosa*	(Reiling et al., 1986)
	Sophorolipids	 Acidic sophorolipid	*C. batistae, T. bombicola, C. lypolytica,*	(Van Bogaert et al., 2007)
Lipopeptides	Surfactin		*Bacillus subtilis, Bacillus pumilus A*	(Seydlová and Svobodová, 2008)
	Viscosin		*Pseudomonas fluorescens, P. libanensis*	(Laycock et al., 1991)
Polymeric	Emulsan		*Acinetobacter calcoaceticus*	(Rosenberg and Ron, 1999)
Siderophore	Flavolipids		*Flavobacterium*	(Bodour et al., 2004)

2.2.2 Biodispersants

Despite the large amount of studies on chemical dispersants, there is very little research focusing on the use of biosurfactants in dispersant tests, given the fact that they have the great potential, particularly for enhancing oil biodegradation and solubilisation (Mulligan, 2005). The bottleneck exists that operating biosurfactant production is an expensive matter. Some experiments regarding biodispersant test were conducted in the laboratory using only commercial purified Rhamnolipids. The feasibility of Rhamnolipids for dispersing oil slicks was tested at 25 °C and a salinity of 35 ppt (Holakoo, 2001). The enhancement of DE by adding solvents such as ethanol and octanol was demonstrated in a comparison study. It was found that dispersion efficiency decreased at lower temperatures and lower salinity but altering the formulation could increase efficiencies (Holakoo, 2001). A low concentration of Rhamnolipids (1 g/L) was able to convert an emulsified oil back into an O/W solution then disperses the oil to undetectable levels (Nakata and Ishigami, 1999). The dispersion ability of a spray-dried sterilized culture broth of *Gordonia* sp. strain JE-1058 was tested as a dispersant without any solvent in the BFT. It showed a strong potential to be applied as an oil spill dispersant and stimulated the degradation of weathered crude oil (ANS 521) by the activity of the indigenous marine bacteria at sea or even contaminated sea sand (Saeki et al., 2009).

2.2.3 Toxicity of biodispersants and/or dispersed oil

Environmental toxicity events due to biosurfactants have been rarely reported, particularly when based on the results of standard toxicity tests and chronic effects on marine species. More research is needed to investigate whether biodispersants have

less environmental effects than that of chemical dispersants by studying the responses of various marine species. Acute and chronic toxicities of three hemical dispersants and surfactants (TRITON-X100, COREXIT 9500 and PES61) and three biosurfactants (BIOEM, PES51 and EMULSAN) were investigated for the estuarine epibenthic invertebrate, *Mysidopsis bahia* and the inland silverside, *Menidia beryllina*. After the standard 4–7 day static and static-renewal tests, the three biosurfactants and PES61 generally had higher LC50 values than TRITON-X100 and COREXIT 9500 based on the survival, growth and fecundity of M. bahia and M. beryllina (Edwards et al., 2003). It was found that Rhamnolipids had a lower toxicity than the counterpart chemical surfactants for marine flagellates microalgae by IC50 test (Elucidation et al., 1987). Higher initial EC50 values for the Glycolipids produced by *Rhodococcus* species H13-A indicated that they exhibited lesser aqueous toxicity as compared to Tween-80. When evaluating the toxicity per mass of PAH basis, the result showed that the Tween-80 system was approximately 50% more toxic than the biosurfactant system (Kanga et al., 1997).

2.2.4 Biodegradation of dispersed oil

It was previously concluded that the rate of hydrocarbon biodegradation can be promoted by biosurfactants in the ways of either increasing solubilisation and dispersion of the hydrocarbons or changing the affinity between microbial cells and hydrocarbons by inducing increases in cell surface hydrophobicity (Zhang and Miller, 1992, 1994). The biodegradability of a Brent crude oil with Corexit 9500 was compared to that with a commercial biosurfactant product JBR 425. Such biosurfactant product is Rhamnolipid-generated as a metabolic by-product of *Pseuomonas aeruginosa*. The GC/MS total petroleum hydrocarbons (TPHs) and microbial counts after the 35-day experiment in 250-mL flasks showed that the biodispersant and biological agent mixed was the most bio-available followed by JBR 425, oil only, and finally Corexit 9500 only. The results indicated that the use of the Rhamnolipid biosurfactants promoted biodegradation whereas Corexit 9500 suppressed biodegradation (Dagnew, 2004). In a biosurfactant-based remediation agent test, the spray-dried sterilized culture broth of *Gordonia* sp. strain JE-1058 displayed a strong potential to be applied as an oil spill dispersant and stimulated the degradation of weathered crude oil (ANS 521) by the activity of the indigenous marine bacteria at sea or even contaminated sea sand (Saeki et al., 2009).

Although effective PAH biodegradation enhanced by biosurfactants was not reported in literature, the PAH solubility can be significantly enhanced using biosurfactant aqueous solutions. The enhancement of the solubility of naphthalene and its methyl-substituted derivatives by Glycolipids produced by *Rhodococcus* species H13-A and Tween-80 (polyoxyethylene sorbitan monooleate) was studied (Kanga et al., 1997). The two-ring aromatics showed a substantial increase in their apparent solubilities in

the presence of both surfactants, and Glycolipids showed significantly greater enhancement than Tween-80. Highly substituted derivatives had greater solubility than lesser substituted compounds (Kanga et al., 1997).

2.3 Production of biodispersants

2.3.1 Biosurfactant producers

Biosurfactant producers were isolated from a variety of environments including sea water, soil, marine sediments, oil fields (Yakimov et al., 1998) and some were from harsh environment (Cameotra and Makkar, 1998). Some genera such as *Pseudomonas* have multiple species which can generate different kinds of surfactants. For instance, *P. aeruginosa* produces Rhamnolipids, but *P. fluorescens* produces cyclic lipopeptides (CLP), which are similar to surfactin and other CLPs can also be produced by *Bacillus* (Raaijmakers et al., 2006). Major groups of biosurfactants discovered by far including the rhamnolipids, surfactin and trehalose lipids are presented in this section.

(1) Rhamnolipids:

Rhamnolipids are glycosides which consist of a glycon part and an aglycon part linked to each other via an O-glycosidic structure. Based on the glycon part, rhamnolipids can be divided into one or two rhamnose moieties (Edwards and Hayashi, 1965). Despite in some rare homologs, rhamnolipids can be acylated with a long chain alkenoic acid, the 2-hydroxyl structure of the distal rhamnose group remains generally free (Yamaguchi et al., 1976). Rhamnolipids are mainly generated by *Pseudomonas* species, among which the primary producing species is *P.*

aeruginosa. Nevertheless, many other *Pseudomonas* species have been reported to be the producers of rhamnolipids (Gunther IV et al., 2006; Gunther et al., 2005; Onbasli and Aslim, 2009). Some rhamnolipids producers such as *Acinetobacter calcoaceticus* are not in the family of *Pseudomonadacae* (Rooney et al., 2009). Other producers such as *Pseudoxanthomonas* sp are not even in the same order as *P. aeruginosa* (Vasileva-Tonkova et al., 2006).

(2) Surfactin:

Products in the family of surfactin mainly consist of about 20 different lipopeptides (Jacques, 2011) except for esperin. These lipopeptides share some common structural properties (Thomas and Ito, 1969). The first surfactin was found as an exocellular compound with an exceptional biosurfactant activity isolated from the supernatant of a *Bacillus subtilis* culture in 1968 (Kakinuma et al., 1968). A surfactin-like compound was isolated from *Bacillus pumilus* cultural supernatants and was called pumilacidin (Morikawa et al., 1992). Ever since iturins was discovered, the great potential of lipopeptide biosynthesis in this genus was recognized and more lipopeptides produced by different strains of *Bacillus* spp. have been found (Jacques, 2011). Nowadays, the great potential benefits of surfactin have been realized, especially in healthcare areas such as being the inhibitor of fibrin clot formation, antibacterial agents, anti-tumour agents and hypocholesterolemic agents.

(3) Trehalolipid:

Trehalolipid is a non-reducing disaccharide in which the two glucose units are linked in an a,a-1,1-glycosidic structure, which is the basic component of the cell wall Glycolipids in *Mycobacteria* and *Corynebacteria* (Franzetti et al., 2010). Different

types of trehalose containing Glycolipids are known to be produced by several microorganisms belonging to the mycolates group, such as *Mycobacterium, Rhodococcus, Arthrobacter, Nocardia* and *Gordonia*, among which different structures have been elucidated particularly in *Rhodococcus* genus. For instance, the characterisation of the organic extract of *Rhodococcus erythropolis* DSM43215 revealed the production of trehalose-6-monocorynomycolates, trehalose- 6,60-diacylates (e.g. 3-oxo-2-alkyl alkanoic acid), and trehalose-6-acylates (e.g. 3-oxo-2-alkyl alkanoic acid) (Kretschmer et al., 1982). Within *Rhodococcus*, trehalose lipids were subsequently isolated from *R. erythropolis* by Ristau and Wagner (Ristau and Wagner, 1983). The Glycolipid synthesised by *Rhodococcus* strain H13-A is a non-ionic trehalose lipid, consisting of one major and ten minor components (Singer and Finnerty, 1990). Flocculating properties were found caused by Glycolipids of *R. erythropolis* S-1, and the carbohydrate is acylated with C10–C22 saturated and unsaturated fatty acids, C35–C40 mycolic acids, hexanedioic, dodecanedioic acids, 10-methyl hexadecanoic, and 10-methyl octadecanoic acids (Kurane et al., 1995).

2.3.2 Factors affecting biosurfactant production

Although the biosurfactant products have displayed valuable potential, given the fact that such biotechnology fermentation is a complex, expensive and low-yield process, different factors that may enhance the production are still being increasingly studied. To conquer the bottleneck associated with biosurfactant production, two basic strategies were developed to make the process more cost-effective: the use of inexpensive waste substrates in the formulation of fermentation media to reduce the initial raw material costs involved in the process, and the development of successfully optimized bioprocess, including the optimization of the culture conditions, cost-effective purification and recovery methods (Saharan et al., 2011). To enhance the biosurfactant production, the important factors affecting the process were summarized in below.

(1) The carbon source

Biosurfactant producers can utilize a variety of soluble and insoluble organic compounds as the source of carbon and energy for their growth. Among the soluble organic carbon sources, glucose was the mostly used substrate followed by saccharose and fructose. Efficient insoluble organic carbon sources include glucose, glycerol and hexadecane (Akpa et al., 2001). In rhamnolipids production by *Pseudomonas aeruginosa*, water soluble carbon sources including glycerol, glucose mannitol and ethanol were widely used (Robert et al., 1989). The 6% glucose was used as the sole carbon source and 1400-1500 mg/L Rhamnolipids were produced. It was noted that the inhibition effect of the carbon source was observed when glycerol was used as a carbon source as the Rhamnolipids level decreased sharply when glycerol

concentration was over 2%, and Rhamnolipids production approximated to 0 when glycerol concentration was about 6-7% (Monteiro et al., 2007). The 3.9 g/L Rhamnolipids product was obtained during the cultivation of *P. aeruginosa* DAPUPE614 on glycerol and ammonium nitrate within 216 hours (Gunther et al., 2005).

Compared with soluble organic carbon sources, insoluble organic hydrocarbons were deemed as more favorable biosurfactant inducers. Different types of vegetable oil have been reported to produce Rhamnolipids (Desai and Banat, 1997). The Rhamnolipids production with a range of 3.0 g/L was achieved using 10% olive oil. When using at 6% sunflower and grape seed oil, 2 g/L Rhamnolipids were produced. Different types of fuel were also proven to be excellent carbon sources. The 1.3 and 2.1 g/L Rhamnolipids were produced using 6% and 5% diesel and kerosene oil as carbon sources, respectively (Desai and Banat, 1997). Alkanes is another type of popular carbon sources in previous studies (Vasileva-Tonkova et al., 2006). Many types of hydrocarbons including n-Hexane, n-Heptane, n-Hexadecane, Kerosene, Benzene, Toluene, Xylene, n-Paraffins, and Mineral oils were investigated to produce biosurfactants with excellent surface activity and emulsifying activity (Vasileva-Tonkova et al., 2006).

(2) Nitrogen source

During the fermentation process, nitrogen sources are required for most microorganisms to synthesize proteins, nucleic acids and other cellular components. Nevertheless, it is crucial to acquire the appropriate dose of nitrogen source as inhibition effect was observed when the nitrogen was overdosed in many

biosurfactant studies. The production of ustilipids and ustilagic acid using *U. maydis* was more effective under conditions of nitrogen starvation and could reach a rate of up to 23 g/L (Hewald et al., 2005). The occurrence of inhibition may also be due to the types of nitrogen sources. The production of Rhamnolipids was inhibited by the presence of $NH4^+$, glutamine, asparagine, and arginine as nitrogen sources, but was promoted by NO_3, glutamate, and aspartate (Köhler et al., 2000; Ramana and Karanth, 1989; Van Alst et al., 2007). Several reports have displayed that NO_3 may be the best nitrogen source for Rhamnolipids production (Arino et al., 1996; Manresa et al., 1991). High levels of $NH4^+$ or glutamine reduced the production of Rhamnolipids which was correlated with a lower glutamine synthase activity (Mulligan and Gibbs, 1989). The mechanisms for the preference of different nitrogen sources by microorganisms and the inhibition biosurfactant production processes still remained unknown.

(3) Salinity and pH

Salinity and pH are two important interactive environmental factors, and their effects on biosurfactant production were usually studied together. They were found not only to affect the structure but also the property and production rate of biosurfactants.

Although there are many exceptions, the optimum pH for the growth of most producers ranges from 5 to 8 (Munro, 1970). The morphology of biosurfactants has been proven to be significantly affected by changes in pH, which in turn could affect the properties of biosurfactants such as the degree of solubility enhancement. It was demonstrated that the effect of a Rhamnolipids biosurfactant on the surface tension and dispersion of phenanthrene was a function of pH (Shin et al., 2004). Similar

results were found in a study in association with naphthalene (Vipulanandan and Ren, 2000). Studies also demonstrated that the morphology of Rhamnolipids biosurfactants was a function of pH (Champion et al., 1995; Ishigami et al., 1987). With the increasing pH the morphology of Rhamnolipids biosurfactants changed from lamellar to vesicular, ultimately micellar (Shin et al., 2008). Metabolism is pH sensitive because pH is the important factor that affects the chemical reactions of the living cells. It was observed that there was a maximum production of biosurfactants at pH range from 6 to 6.8 and the production rate was decreased sharply when pH increased above 7 (Guerra-Santos et al., 1986).

Similar as pH, the ionic strength or salinity of the medium could also influence the property of biosurfactants. It has been found that the presence of electrolytes could render a decrease in the CMC and therefore increase the solubility of hydrocarbons by Rhamnolipids (Wang et al., 2007). The micelle formation could be affected by the formation of complex compounds between ions and biosurfactants (Ochoa-Loza et al., 2001). Consequently, it is important to adjust the pH and the salinity to improve the performances of biosurfactant systems.

2.3.3 Kinetics of biosurfactant production

Kinetic data are needed to develop basic understanding of fermentation processes by microorganism and to obtain a rational design of continuous and efficient fermentation processes (Luedeking and Piret, 1959). Studies on the biosurfactant production kinetics and the conditional requirements of *rhodococci* acquired worthy information on microbial metabolism that allowed the conditional parameters to be adjusted to meet the production target of biotechnology (Pacheco et al., 2010). The

kinetics of surfactin production by *Bacillus subtilis* LAMI005 revealed that the best medium contained clarified cashew apple juice and distilled water and 1.0 g/L of (NH_4), which decreased the surface tension of water to 30 dyne/cm with a critical micelle concentration (CMC) of 63.0 mg/L and a kerosene emulsification index 24 (EI_{24}) of 67% (Freitas de Oliveira et al., 2013). Rhamnolipids production by *Pseudomonas aeruginosa* O-2-2 was drastically enhanced from 28.8 g/L to 70.56 g/L in a pH stage-controlled fed-batch fermentation at 500 rpm and 30 °C based on a kinetics model through studying the cell growth, product synthesis, and substrate consumption (Zhu et al., 2012).

2.3.4 Characterization of Biosurfactants

Given the fact the biosurfactant products from the fermentation process are generally complex bio-polymeric compounds. A combination of analytical methods were essential to isolate, purify, and characterize various structures of biosurfactants (Banat, 1993). The analytical methods are generally classified into two groups to identify both the biosurfactant surface activity and structure properties.

(1) Characterization of biosurfactant surface active properties

Surface tension: As a physical property of any liquid, the surface tension of a cell free culture can be reduced due to the existence of surfactants by adsorbing at the liquid-gas interface. The surface tension can be determined with a surface tensiometer (e.g., DuNouy Tensiometer, Interfacial, CSC Scientific).

The reciprocal of critical micelle concentration (CMC^{-1}): The CMC^{-1} is defined as the dilution factor of the cell free culture upon reaching the critical micelle concentration (CMC) (Sheppard and Mulligan, 1987). The CMC is the point at which the surface tension abruptly increases and can be determined by measuring the surface tension of a cell free culture at various dilutions. It is considered as an indirect measurement of surfactant concentration (Mulligan et al., 2001).

(2) Characterization of biosurfactant structure properties

Protein content: The protein content can be quantified by a colorimetric method using the protein reagent including Coomassie Brilliant Blue G, ethanol , and phosphoric acid. Color intensity could be measured at 595 nm by a spectrophotometer based on

the developed calibration curve with different concentrations of a mix of Bovine serum albumin stock solutions and protein reagents. Protein content in a biosurfactant can be quantified using this assay (Bradford, 1976).

Total lipids: The total lipid can be quantified by a colorimetric method by applying a semi-micro method (Pande et al., 1963). A solution with 2.0% potassium dichromate (w/v) in 98% (w/v) sulfuric acid (Lipid reagent) was prepared and 2 g potassium dichromate was dissolved in 100 ml 98% sulfuric acid at room temperature. Color intensity was measured as absorbance at 595 nm by a spectrophotometer against a calibration curve with different concentrations of the mix of Palmitic acid stock solutions and petroleum ether. The test tube was heated in a boiling water bath for 15 minutes. The test tube was then cooled in running water and added with 4.5 ml water.

Absorbance at 595 was measured with the solution generated in column 1 (reagent blank) as blank. The 0.1 g biosurfactants was weighted and dissolved in 1 ml water. Then 0.1 ml the above solution was transferred into the test tube and mixed with 3 ml 2% potassium dichromate in 98% sulfuric acid. The test tube was heated in a boiling water bath for 15 minutes and cooled in running water. Then 4.5 ml water was added followed by mixing and re-cooling. The absorbance at 590 nm was measured with the solution generated in column 1 (reagent blank) as blank (Pande et al., 1963).

Total carbohydrate: The total carbohydrate can be quantified by a colorimetric method using the phenol solution in the presence of concentrated sulfuric acid (Dubois et al., 1956). The 80% phenol solution was firstly prepared by adding 2 g of water to 8 g of phenol. Standard curve was generated by mixing different amounts of glucose, stock solutions, water, phenol, and concentrated sulfuric acid. After mixing, all the tubes

were settled for 10 minutes, and shaked for 15 minutes at 30ºC. Tthe absorbance at 490 was measured with the solution generated in column 1 (reagent blank) as blank. The sample could be analyzed by the following steps: the 0.01 g biosurfactants was dissolved into 100 ml water. 2 ml the above solution was taken into a test tube, and mixed with 50 µl 80% phenol. 5 ml concentrated sulfuric acid was added and settled for 10 minutes. Then the tube was shaked for 15 minutes at 30ºC. The absorbance was measured at 490nm with the solution generated in column 1 (reagent blank) as blank (Dubois et al., 1956).

Thin layer chromatography (TLC) analysis: TLC is one of the most commonly used technique to characterize biosurfactants. The principle lies in that the solutes compete with the solvent for the surface sites of the adsorbent. Different compounds were distributed on the surface of the adsorbents and the distribution coefficient was used to quantify the process. Carrying over or cross contamination of samples and sorbent regeneration procedures could be avoided by the separation of each sample on fresh layers. A solvent system can be selected based on different types of biosurfactants. Generally single solvent systems could meet the requirement for mobilization of different functional groups which could be sequentially identified with different developing reagents (Makkar and Cameotra, 1997).

High pressure liquid chromatography and mass spectroscopy (HPLC-MS) analysis: HPLC-MS could be used generally for the separation of biosurfactants and the analysis of the molecular mass of each fraction. Biosurfactants were treated with trifluoroacetic acid and further centrifuged for the removal of solid particles. The sample solution was carried by the mobile phase and migrated over the solid stationary phase. Components were migrated at different speeds due to noncovalent

interactions of the compounds with the column and were then separated. The detector emited a response due to the elution of the sample and subsequently signaled a peak on the chromatogram (Aguilar, 2004). The separated products were detected and the fractions collected for individual peaks were used to analyze the structure of each moiety (Siegmund and Wagner, 1991).

Gas chromatography mass spectroscopy (GC-MS) analysis: GC-MS is the most sensitive method for the identification and quantification of Glycolipids biosurfactants. The compounds require a complex pre-treatment before GC-MS analysis. Firstly, they needs a hydrolytic cleavage between the carbohydrate or peptide/protein part of the biosurfactants and the lipid portions. Secondly, the fatty acid chains are derived to fatty acid methyl esters or to trimethylsilyl derivatives (Yakimov et al., 1995). The esterification step by diazomethane is important for the detection of compounds using GC-MS (Peng et al., 2007). FA methyl esters can be recovered with Hexane, and then concentrated under nitrogen blowing for GC-MS analysis.

Infrared (IR) spectroscopy analysis: IR is being increasingly used to analyze the functional groups and structural elucidation of biosurfactants. Surfactin, Lichenysin and Rhamnolipids have been characterized by the IR technique (Das et al., 2008). Alkyl, carbonyl, ester compounds of biosurfactants could be detected clearly when 100 scans were used in 0.23 mm KBr liquid cell (Tuleva et al., 2002). Translucent pellets was obtained from 10 mg freeze-dried crude biosurfactants by adding potassium bromide (100 mg) and pressure with 7500 kg for 30 seconds in association with IR spectrum (Thavasi et al., 2007). The principle lies in the measurement of the absorption of different IR frequencies of a sample positioned in the path of an IR beam.

Nuclear magnetic resonance (NMR) analysis: The exact location of functional groups, the position of linkages within the carbohydrate, and lipid molecules and structural isomers can be obtained by NMR analysis. The biosurfactants should be hydrolysed (HCl), and then FA extraction is carried out with solvents such as acetic acid, acetone, benzene, chloroform, dimethyl sulfoxide, methanol pyridine, and water. The principle of NMR is based on transitions in atoms with a magnetic moment when an external magnetic field is applied by energy dependent on the magnetic-field strength and magnetogyric ratio. The response is the absorbance of radio frequency radiation by a nucleus in a strong magnetic field. The nuclear spin could realign or flip in the higher-energy direction under the radiation absorption (Satpute et al., 2010).

2.3.5 Generation of biodispersants

Despite the large amount of research on chemical dispersants, the generation of biosurfactants based biodispersants and their applications have rarely been reported. Biodispersants have the potential to be used as the oil spill response alternatives since they are less toxic and more persistent than chemically synthetic ones, particularly for enhancing oil biodegradation and solubilization. The extremely limited commercial biodispersant products have increased the difficulty of the applications.

Some experiments were conducted to investigate the dispersion ability of biosurfactants (Saeki et al., 2009; Chakrabarty, 1985; Shafeeq et al., 1989; Chhatre et al., 1996; Holakoo, 2001; Lang et al., 1987; Song et al., 2013). Solvents were added into biosurfactants in some studies. JE1058BS displayed a strong potential to be applied as an oil spill dispersant even in the absence of a solvent for the bioremediation of oil spills at sea or on shorelines (Saeki et al., 2009). Oil dispersion

and biodegradation were highly enhanced by the addition of an emulsifier produced by *Pseudomonas aeruginosa* SB30 (Chakrabarty, 1985). 70% of the Gulf and Bombay High Crude oil was successfully degraded by the biosurfactants produced by *P. aeruginosa* S8 during the biodegradation of a hydrocarbon mixture (Shafeeq et al., 1989). Rhamnolipids biosurfactants produced by four bacterial isolates enhanced biodegradation through the emulsification of the crude oil (Chhatre et al., 1996). The feasibility of biosurfactants for dispersing oil slicks at 25°C and a salinity of 35 ppt was verified by using a solution of 2% Rhamnolipids diluted in saline water at a dispersant to oil ratio (DOR) of 1:2, and immediately dispersed 65% of a crude oil. Meanwhile, the effectiveness was promoted to 82% by the addition of 60% ethanol and 32% octanol with 8% Rhamnolipids applied at a DOR of 1:8 improved dispersion (Holakoo, 2001). Comparison of the dispersion behaviour to the control revealed that the Rhamnolipids when mixting with solvents had excellent potential as non-toxic oil dispersing agents (Lang et al., 1987). A type of mousse oil was successfully de-emulsified by a low concentrations of Rhamnolipids (1 g/L) and bio-remediated to undetectable levels (Nakata and Ishigami, 1999). One of the recent studies tried to develop more efficient and less toxic biodispersants by using Rhamnolipids and sophorolipid biosurfactants, and a low toxic solvent (Ethylene glycol butyl ether). Via a series of optimization experiments for dispersant generation, the two dispersants were obtained and showed a high dispersion effectiveness for treating heavy crude oil at the dispersant-to-oil ratio below 1:25 and the temperature above 5˚C in a wide range of salinity and pH values with low effects on two kinds of fish (*Danio rerio* and *Microgobius gulosus*) (Song et al., 2013).

2.4 Applications of dispersants in offshore oil spills

2.4.1 Worldwide

Given the increasing concern about various negative effects of chemical dispersants on marine environments, the permission of dispersant applications around the world has been declining steadily. By far the dispersants are permitted in Canada, United States, Great Britain, France, Norway, Italy, Spain, South Africa, Nigeria, Singapore, Malaysia, Indonesia, Japan, countries in Arabian Gulf, India, and Australia (Chapman et al., 2007; Fingas, 2010). In Canada and the United States, any commercial dispersant has to pass the standard procedures for testing toxicity and effectiveness before they get the permission. Current approved commercial chemical dispersants in Canada and United States are listed in Table 4.

The largest dispersant usage in recorded oil spill history is the application of Coerxit 9500 and 9527 in the Deepwater Horizon oil spill in 2010, the largest marine oil spill in Gulf of Mexico. Around 1.8 million gallons of dispersants was used in the Gulf, among which approximately 42% of this dispersant was applied at the point where oil was escaping the wellhead (Ramseur, 2010).

Table 4. List of approved chemical dispersants in Canada and United States (Fingas, 2010).

Product	Manufacturer	Canada	United States
Corexit 9500	Exxon, Houston	✓	✓
Corexit 9527	Exxon, Houston	✓	✓
Enersperse xx	BP, Britain (old stocks)	✓	
Biodispers	USA, Newport, NH		✓
Dispersit SPC1000	Polychem, Chestnut Ridge, NY		✓
Finasol OSR 62	Total Fluides, France		✓
JD (109, 2000)	Globemark, Houston, TX		✓
Mare Clean (20, 200, 505)	Taiho, Japan		✓
NEOS AB-300	Neos, Japan		✓
Nokomis (3-AA, 3-F4)	Mar-Len, Hayward, CA		✓
Saf-Ron Gold	Sus. Env. Tech., Mesa, AZ		✓
Sea Brat #4	Alabaster, Pasadena, TX		✓
ZI-400	Studio City, CA		✓

2.4.2 Northern regions

In northern regions such as North Atlantic, oil spill response is facing special challenges due to the prevailing harsh environments (e.g., low temperature, strong winds, rough seas, low visibility, and sea-ice (Chen et al., 2011). Containment and recovery response techniques have severe limitations in its applicability, particularly during winter months. Yet the use of dispersants shows a strong potential as an alternative response technique in northern regions where the weather may not limit the effectiveness and the sea-state may enhance the oil dispersion (Chen et al., 2011). Therefore, novel, environmental friendly and cost-effective dispersants and technologies are much desired to adequately address the associated safety and environmental concerns.

According to the record, some dispersant usage cases occurred in northern regions. In Canada, there was no limitation in dispersant usage in the late 1970s and early 1980s, but nearly all stockpiles and equipment have now been sold (Fingas, 2010). It was recorded that dispersants were last used in Canada in about 1984 (Etkin, 1998) and that 12 ton Corexit was used to disperse 5000 ton Bunker C in 1970 while the dispersion was ineffective (Fingas, 1989). In 1979 in Denmark, 400 ton heavy fuel oil was reported to be dispersed to some extent by using an unknown dispersant. In 1979 in Ireland, Saudi Arabian crude was successfully dispersed by BP 1100 WD (Fingas, 2010).

2.5 Summary

This chapter started with a literature review on chemical surfactants. Some basic physiochemical properties including surface-active reduction, emulsification, and CMC were introduced. The wide applications of chemical surfactants to food industry, as well as oil recovery and environmental remediation were stated. They can be classified based on their structure properties. Subsequently, the review extended to the chemical dispersants and their working principle. Their potential was concluded based on the statement of various advantages compared with other oil spill response techniques. Different factors affecting dispersant effectiveness were also demonstrated. Facing the controversial usage of chemical dispersants, section 2.2.3 concluded their disadvantages and a research and development (R&D) direction of dispersants based on a review of their toxicity and biodegradation efficiency.

Section 2.2.4 began with a background on biosurfactants including their physiochemical properties, classification, and demonstrated their special advantages compared with chemical dispersants. The feasibility of biosurfactants for oil spill response was supported by a review of current experimental studies using biosurfactants such as Rhamnolipids. The last part concluded that biosurfactants had less negative effects on environments than that of chemical surfactants by studying the responses of various marine species. In the section 2.3, the production of biosurfactant based biodispersants was stated. The section started with the introduction of some biosurfactant producers including Rhamnolipids, surfactin and trehalose lipids. The significant production factors including the carbon source, the nitrogen source, pH and salinity were then discussed to investigate the necessity of production optimization. A review of kinetics studies were followed to develop a

basic understanding of fermentation processes followed by the introduction of common characterization methods including colorimetric chemical analysis, as well as the TLC, HPLC-MS,GCMS, IR, and NMR analysis. The section ended with the potential and the knowledge gap in the development of biodispersants. Finally, the chapter reviewed the current applications of dispersants around the world especially in the northern regions, and discussed the challenge and need of novel biodispersants.

CHAPTER 3

BIOSURFACTANT PRODUCTION BY RHODOCOCCUS ERYTHROPOLIS SP. SB-1A ISOLATED FROM THE NORTH ATLANTIC OCEAN

3.1 Background

Biosurfactants, surface-active biomolecules produced by microorganisms, are a superior alternative over chemical surfactants due to their unique properties such as lower eco-toxicity, higher biodegradability, and greater stability (Geys et al., 2014; Mukherjee et al., 2006). In the past few decades, biosurfactants have shown great potential in environmental bioremediation, specifically, in the desorption, solubilisation, and biodegradation of hydrophobic organic contaminants in the environments (Ivshina et al., 1998; Kanga et al.,1997; Kuyukina et al., 2005).

Many members of the genus *Rhodococcus* are known to be effective biosurfactant producers. *Rhodococcus* species can naturally persist and grow in various temperate or extreme environments especially in hydrocarbon-contaminated soils and waters (Kuyukina and Ivshina, 2010). Biosurfactants are considered as a by-product promoting the biodegradation of hydrocarbons by enhancing the adherence of genus *Rhodococcus* to hydrophobic phases (Neu, 1996), by providing easy access to enter microbial cells, by reducing the interfacial tension between the phases (Fiechter, 1992), and by increasing the microbial attack based on hydrocarbon dispersion(Finnerty, 1994).

Different trehalose containing Glycolipids are known to be produced throughout *Rhodococcus* genus including *R. erythropolis*, "*R. longus*," *R. opacus*, and *R. ruber* (Franzetti et al., 2010). Among *Rhodococci R. erythropolis* was reported to have unique bioconversion and biodegradation abilities due to its diversified enzyme system (De Carvalho and Da Fonseca, 2005). *R. erythropolis* can also generate Mycolate-containing Glycolipids in term of bioflocculants working on a variety of

suspended solids (Kurane et al., 1994; Kurane et al.,1995; Kurane and Tomizuka, 1992).

It has been intensively investigated that important culturing conditions affecting the biosurfactant production rate include the carbon source, the nitrogen source, pH, and salinity (Bicca et al., 1999). However, few studies have performed a systematic analysis of all these parameters in biosufactant production by *Rhodococcus*. Thus, it is desired to acquire worthy information on the conditional requirements of *rhodococci* to meet the target of biotechnology (Pacheco et al., 2010). In addition, only limited studies have been performed so far on the kinetic study of biosurfactant production by *Rhodococcus*. This chapter tried to fill the research gap through producing biosurfactant by *Rhodococcus* and investigate the appropriate culturing conditions and relevant kinetic of the process. The effects of the carbon source, the nitrogen source, pH, and salinity on the biosurfactant production by a *Rhodococcus erythropolis* strain isolated from the North Atlantic Ocean were explored. A kinetic model was then used to analyze the data. The biosurfactants were finally produced under the selected culturing conditions. The research outputs could help to increase the production rate and thus reduce the cost for biosurfactant production.

3.2 Methodology

3.2.1 Biosurfactant producer and culture nutrients

R. erythropolis sp. SB-1A isolated from a water sample collected from the North Atlantic Ocean was selected during the study (Cai et al., 2014). The bacterium was maintained in NBS mineral salt medium plates. The composition per litre of the NBS

plate contained: Nutrient Broth Broth, 25 g; Agar, 15 g; and NaCl, 22 g (Zhou et al., 2005).

The bacterium was cultivated in a revised Atlas oil agar medium (Atlas, 2004). The mineral composition consisted of per litre: $MgSO_4.7H_2O$: 0.2 g; $FeCl_3$: 0.05 g ; and $CaCl_2.2H_2O$: 0.05 g. Glucose was added in a concentration of 1g/L as an organic carbon source to stimulate the cell growth in the early phase. All chemicals were analytical grade reagents unless specified.

3.2.2 Investigation of factors affecting biosurfactant production

In order to evaluate effects of the organic carbon source, salinity, pH, and the nitrogen source on the biosurfactant production, one-factor-at-a-time (OFAT) experiments were performed as shown in Table 5. The range of each factor was determined in preliminary experiments using revised Atlas oil agar medium. The 15 ml sterile cultures were inoculated with 1.5% volume aliquot of a preculture grown for 48 hours, and incubated at 30°C, 200 rpm in a rotary shaker for 96 hours in 125 ml flasks.

Table 5. Experimental design of four factors studied in biosurfactant production.

No.	Factors	Levels			
1	n-Hexadecane (v/v%) (carbon source)	0.5	2.0	3.5	5.0
2	Salinity (g/L)	13	26	39	52
3	NH_4NO_3 (g/L) (nitrogen source)	0.4	0.7	1.0	1.3
4	pH (KH_2PO_4/ K_2HPO_4)	6	7	8	9

3.2.3 Isolation of biosurfactants

The cell free broth supernatant was extracted using isometric methyl tertiary-butyl ether (MTBE) at 25 °C on a rotary shaker at 250 rpm for 24 hours (Kuyukina et al., 2001). The upper organic phase was separated from the aqueous phase. Solvent was then removed by rotary evaporation at 45 °C under reduced pressure. The crude biosurfactants were stored at 4 °C in the fridge.

3.2.4 Characterization of biosurfactants

To measure the surface tension reduction, flasks were sacrificed periodically to collect culture samples followed by the 10,000 rpm centrifugation for 15 minutes to remove the cells. A 10 ml of the cell free broth supernatant was used for surface tension measurements. The surface tension was determined by a surface tensiometer (DuNouyTensiometer, Interfacial, CSC Scientific) at 25 °C.

For evaluation of biosurfactant content in the cell free broth, the CMC^{-1} was measured. The CMC was determined by measuring the surface tension of the supernatant at various dilutions (Mulligan et al., 2001). As the broth consists of both aqueous and organic phases, each dilution was conducted with sonification to ensure homogeneity. Before each measurement, the treated solution was allowed to stand for 10 minutes to achieve equilibrium.

3.2.5 Kinetics study of biosurfactant production

Experimental data of biosurfactant production were fitted into the modeling software by OriginLab 9.0 (OriginLab Corporation, Northampton, MA, USA), by nonlinear regression using the least-squares method. The model was ever evaluated for the analysis of lactic acid production (Mercier et al., 1992) and subsequently used for the fermentation study of *Lactobacillus* strains (Rodrigues et al., 2006). The relationship between relative biosurfactant concentration and time can be stated using Eq. (1).

$$\frac{dP}{dt} = P_r P\left(1 - \frac{P}{P_{max}}\right) \quad (1)$$

where t is time (h), P is relative biosurfactant concentration in term of CMC^{-1}, P_{max} is the maximum relative concentration of biosurfactants in term of CMC^{-1}, and P_r is the ratio between the initial volumetric rate of product formation r_p and the initial relative product concentration P_0 in term of CMC^{-1}. The Eq. (1) can be further used to deduce the Eq. (2).

$$P = \frac{P_0 P_{max} e^{P_r t}}{P_{max} - P_0 + P_0 e^{P_r t}} \quad (2)$$

The model parameters P_0 , P_{max} and P_r can be calculated based on the series of experimental data.

3.2.6 Quality Assurance and Quality Control (QA/QC)

The *Rhodococcus* strain was cultured on a NBS mineral salt medium plate regularly and incubated for 48 hours for purity check. Surface tension was measured in triplicates during the determination of surface tension reduction and CMC^{-1}. When testing CMC^{-1}, each dilution was conducted with sonification to ensure homogeneity as the culture consists of both aqueous and organic layers, and each treated solution was allowed to stand for 10 minutes to achieve equilibrium before measurement.

3.3 Results and discussion

To enhance the biosurfactant production rate, factors including carbon source, salinity, pH and nitrogen source were analyzed based on the results of both surface tension reduction and the kinetic modeling of biosurfactant production.

3.3.1 Effects of the carbon source

The use of n-hexadecane as the carbon source in biosurfactant production has been widely recognized as an effective biosurfactant inducer. In the pre-testing of this study, n-hexadecane was used as the sole carbon source, and the cell growth was found to be very slow. Glucose was then added as a supplemental carbon source and significantly stimulated the cell growth in the early phase. Therefore, glucose (1g/L) was used along with n-hexadecane. It has been found that due to the production and accumulation of biosurfactants during the growth of biosurfactant producers, the surface tension decreased between the logarithm phase and stationary phases (Toledo et al., 2008). In Fig. 1, surface tension declined after 12 hours in all the four levels of n-hexadecane and remained stable after 60 hours. The surface tension in the system using lower levels (0.5% and 2%) of n-hexadecane was reduced by 40 dynes/cm, while that of using higher levels (3.5% and 5%) of n-hexadecane was reduced by 35 dynes/cm. This indicated that redundant n-hexadecane can inhibit the biosurfactant production by reducing the interaction between air and the culture.

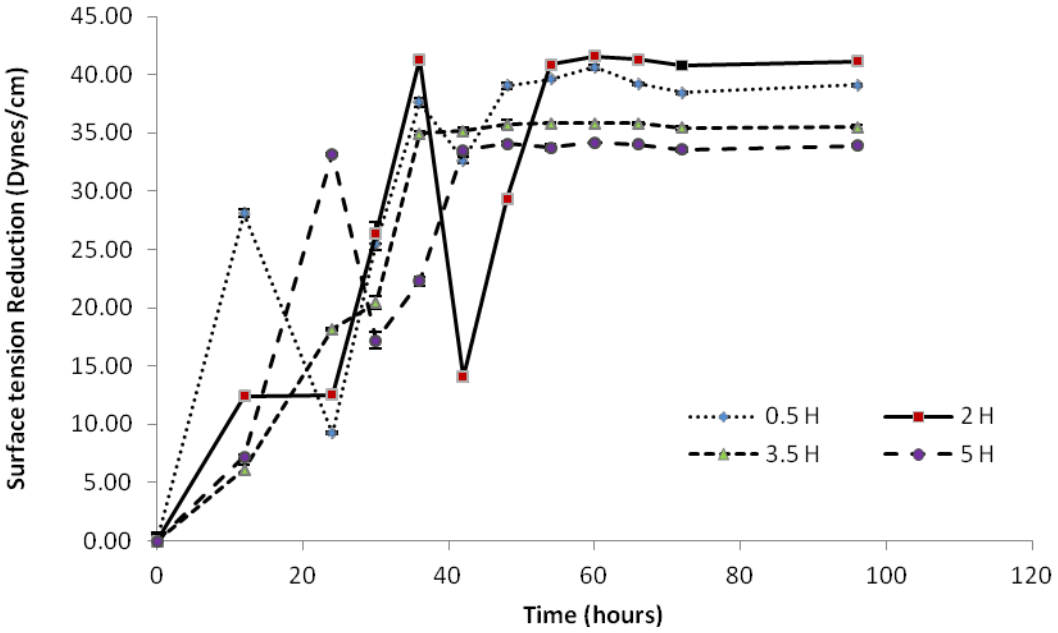


Figure 1. Effects of the carbon source on surface tension reduction. Medium (g/L): KH_2PO_4, 3.4; K_2HPO_4, 4.4; $MgSO_4 \cdot 7H_2O$, 0.2; $FeCl_3$, 0.05; $CaCl_2.2H_2O$, 0.05; glucose, 1; NaCl, 26; NH_4NO_3, 1; pH= 6.5.

3.3.2 Effects of salinity

Salinity is one of the critical factors for controlling the production of biosurfactants especially for those producers isolated from salty environments. Biosurfactants produced by *Bacillus mycoides* isolated from an Iranian oil field was enhanced with a high salinity, while low salinity had negative effect on biosurfactant production and cell growth (Najafi et al., 2010). Till now, effects of salinity on the biosurfactant production by *Rhodococcus* have been seldom studied. In this study NaCl was used and its concentration was set from 13 to 52 g/L given that the biosurfactant producer was from a marine environment. The surface tension was reduced by 35 dynes/cm rapidly in 40 hours in a system with lower salinity (13g/L and 26 g/L); By contrast,

the surface tension was ultimately reduced by 30 dynes/cm in a system with a relative high salinity (39 g/L) after 70 hours of production. The biosurfactant production was significantly inhibited in a system with the salinity of 52 g/L, where the surface tension was reduced by 30 dynes/cm after 100 hours, and the stable phase was not observed. The study indicated that the biosurfactant production by this *Rhodococcus* isolate achieved the best performance in the system with a salinity of 26 g/L (NaCl), which is equivalent to the 35 ppt seawater salinity.

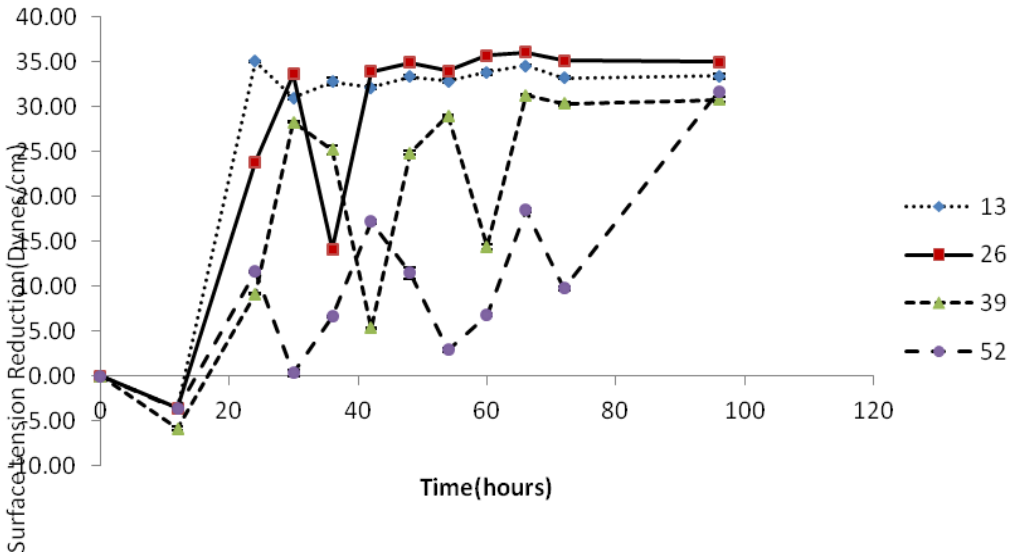


Figure 2. Effects of salinity on surface tension reduction. Medium (g/L):KH_2PO_4, 3.4; K_2HPO_4, 4.4; $MgSO_4•7H_2O$, 0.2; $FeCl_3$, 0.05; $CaCl_2.2H_2O$, 0.05; glucose, 1; n-Hexadecane, 3.5 v/v%; NH_4NO_3, 1; pH= 6.5.

3.3.3 Effects of pH

pH is an important parameter that may need periodical monitoring and adjustion in the biosurfactant production process. The effect of pH on biosurfactant production varied among different biosurfactant producers but only limited studies investigated the effect of pH using *Rhodococcus* species. The surface tension of the biosurfactants produced by *Pseudomonas aeruginosa* from an oil-contaminated soil was stable at a large range of pH between 2 and 10 (Saikia et al., 2012). In this study, pH was controlled by a Potassium Phosphate buffer along with (10%) NaOH or (10%) HCl solutions. The results indicated that there were no significant when between pH changed from 6 to 8. Systems with all levels of pH had shown the reduction of surface tension. The highest surface tension reduction was achieved when pH was 7. The bad performance on surface tension reduction in the system with pH 5 indicated that the production favored a non-acid condition. Similar findings were reported in Rhamnolipids production by *Pseudomonas aeruginosa* (Abdel-Mawgoud et al., 2009).

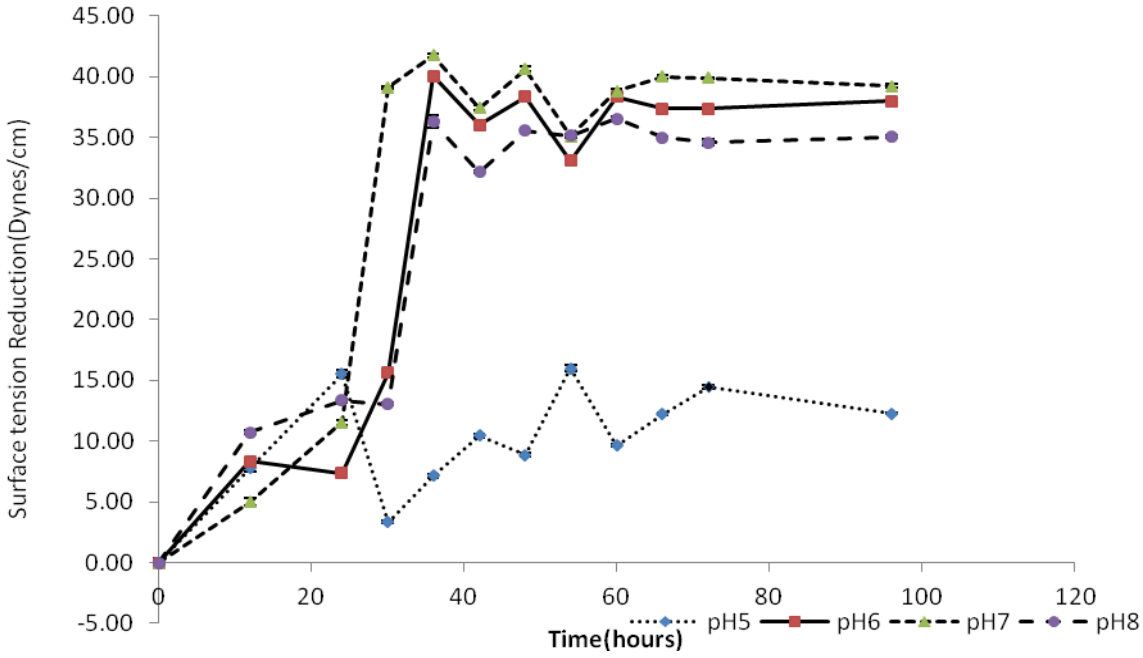


Figure 3. Effects of pH on surface tension reduction. Medium (g/L):$MgSO_4$•$7H_2O$, 0.2; $FeCl_3$, 0.05; $CaCl_2.2H_2O$, 0.05; glucose, 1; n-Hexadecane, 3.5 v/v%; NH_4NO_3, 1; NaCl, 26.

3.3.4 Effects of the nitrogen source

NH_4NO_3 has been recognized as an effective inorganic nitrogen source for biosurfactant production by *Pseudomonas aeruginosa* (Cha et al., 2008) and *Rhodococcus erythropolis* (Gogotov and Khodakov, 2008). The production of biosurfactants by some biosurfactant producers has been reported to yield high product only under limited concentrations of the nitrogen source (Chayabutra et al., 2001; Patel and Desai, 1997). In this study, the effect of NH_4NO_3 at low concentrations on surface tension reduction was investigated, and the results were shown in Fig. 4. The surface tension declined quickly under the concentration of 0.7 g/L and 1.0 g/L NH_4NO_3. Nonetheless, no significant differences were observed using NH_4NO_3 with the concentration between 0.4 and 1.3 g/L.

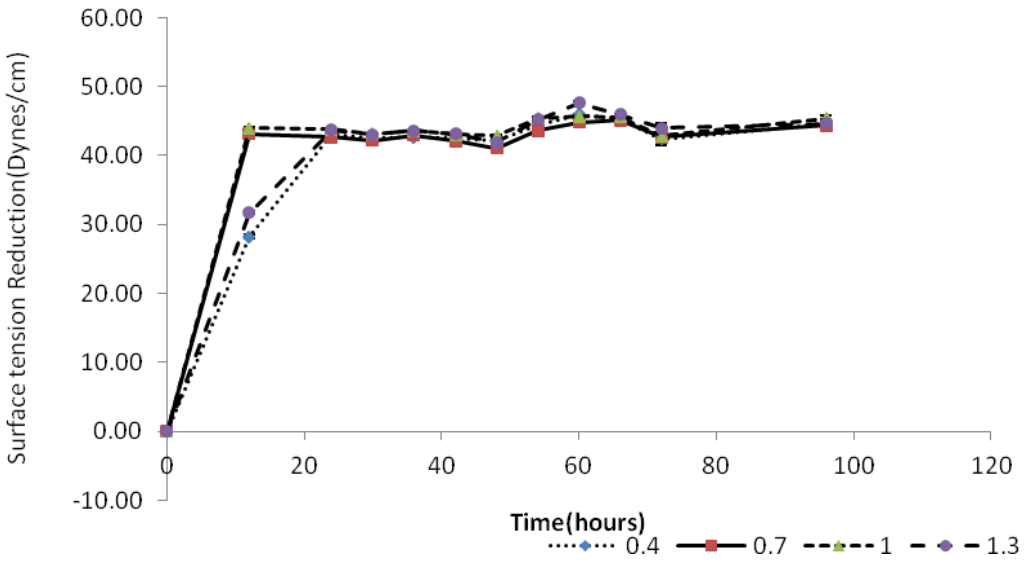


Figure 4. Effects of the nitrogen source on surface tension reduction. Medium (g/L): $MgSO_4 \cdot 7H_2O$, 0.2; $FeCl_3$, 0.05; $CaCl_2.2H_2O$, 0.05; glucose, 1; n-Hexadecane, 3.5 v/v%; NaCl, 26; pH= 7.

3.3.5. Kinetics of biosurfactant production

In this study, CMC^{-1} was used and determined by measuring the surface tension at varying dilutions of the cell free culture. The dilution at which the surface tension abruptly increased was the factor by which the biosurfactant concentration exceeded the CMC. Fig. 5 indicated that when CMC was reached, the surface tension sharply increased from 30 to 45 dynes/cm with the further decrease of biosurfactant concentration in the solution. Consequently, the CMC^{-1} was defined as the dilution factor by which surface tension was higher than 30 dynes/cm.

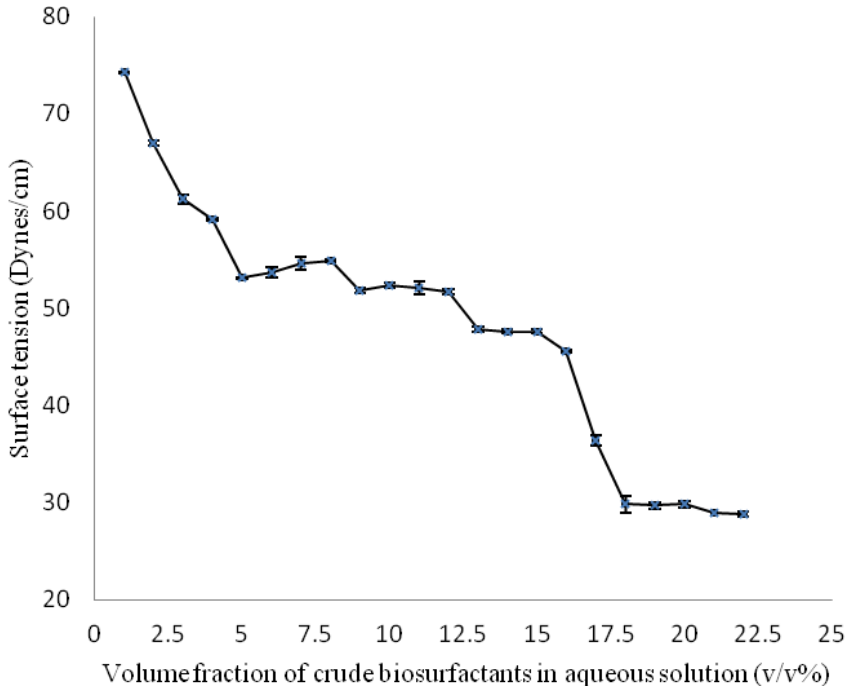


Figure 5. CMC-1 of the biosurfactants.

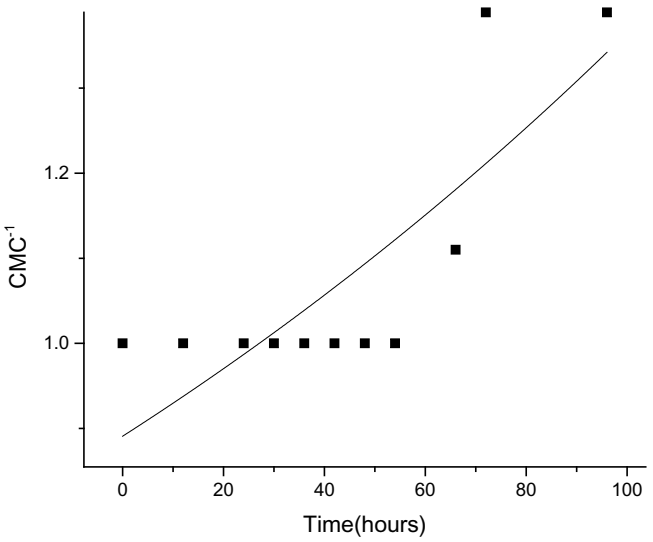
Once the CMC^{-1} values were obtained, the effects of the carbon source on time course of CMC^{-1} was investigated and presented in Fig. 6. The biosurfactant concentration increased with time, and finally reached the stationary phase.

The influence of the different factors on biosurfactant production was represented by the experimental data and used for the kinetic study. Through using the Eq. (2), the P_{max} of each production scenario was calculated and presented in Table 6. It was found that the rate of biosurfactants produced under higher concentrations (3.5 or 5 v/v%) of n-hexadecane was significantly higher with a

Table 6. Results obtained by regression of biosurfactant production for *Rhodococcus erythropolis* under four levels of the carbon source, salinity, pH and the nitrogen source.

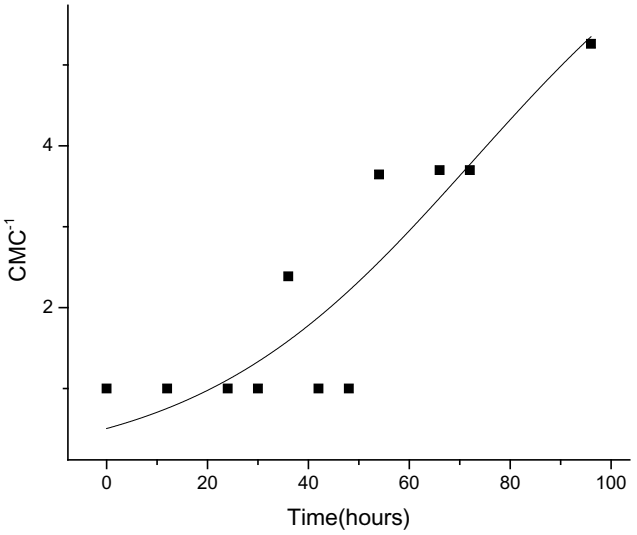
	n-Hexadecane (v/v%)				NaCl (g/L)				pH				NH_4NO_3 (g/L)			
	0.5	2	3.5	5	13	26	39	52	5	6	7	8	0.4	0.7	1.0	1.3
P_0	0.89	0.51	0.04	0.24	0.11	0.63	0.27	0.77	-	0.21	0.02	0.19	0.01	0.01	0.37	0.02
P_{max}	7.33	7.64	11.51	11.81	4.80	16.24	7.32	2.33	-	11.01	11.27	10.11	11.91	11.27	11.60	11.56
$P_r\,(10^{-2})$	0.42	3.64	9.58	6.17	15.34	3.78	4.48	0.71	-	7.70	13.10	9.01	40.40	32.24	16.60	33.66
R^2	0.65	0.81	0.77	0.85	0.59	0.88	0.69	0.44	-	0.90	0.88	0.80	0.89	0.92	0.81	0.91
F-value	411.54	43.76	25.11	45.57	27.50	58.38	22.51	88.54	-	87.62	68.27	39.33	144.11	251.57	72.62	133.41

P_{max} equal to 11.51 or 11.81 than that under lower concentrations with P_{max} 7.33 or 7.64. Similar production rate has been achieved when n-hexadecane concentration increased from3.5 v/v% to 5 v/v% (P_{max} changed from 11.51 to 11.81). To decrease the usage of raw material thus decrease the production cost, and to maintain a relative high production rate, n-hexadecane concentration of 3.5 v/v% was finally selected.



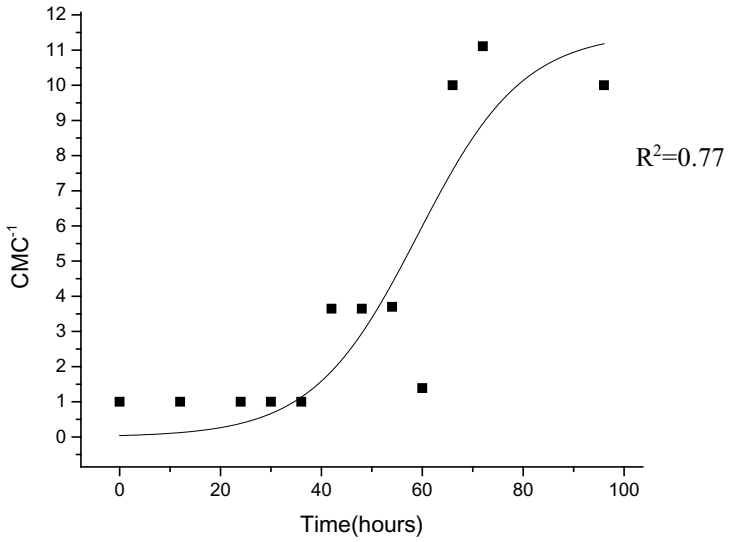
R^2=0.65

(a)



R^2=0.81

(b)



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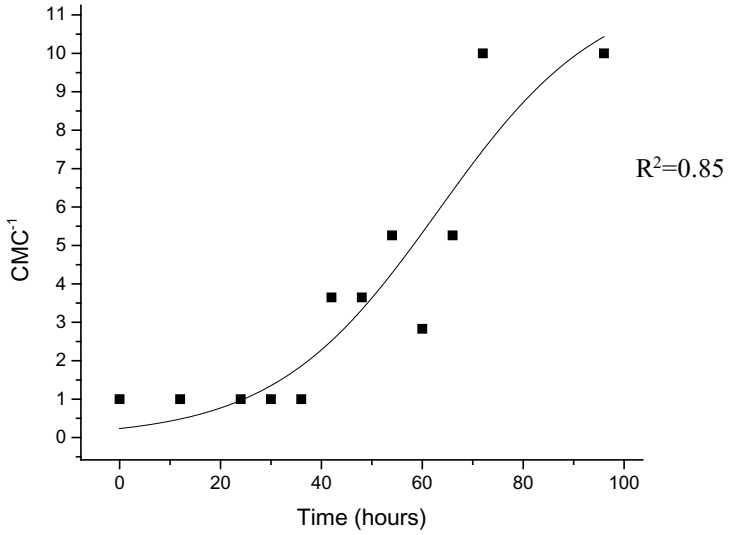
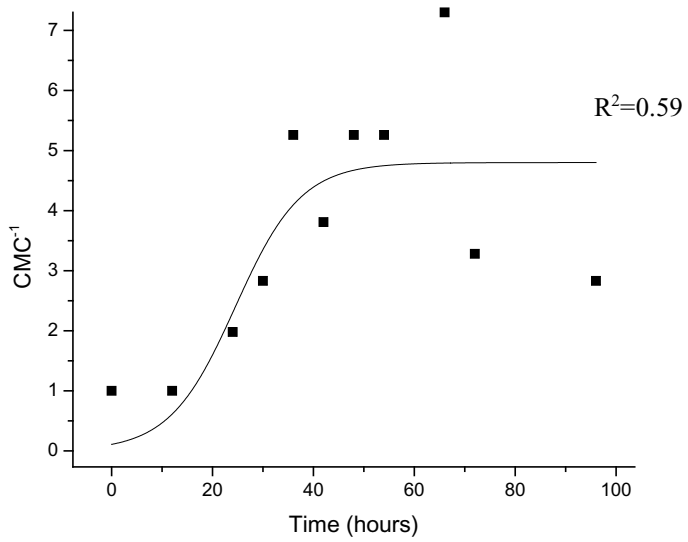


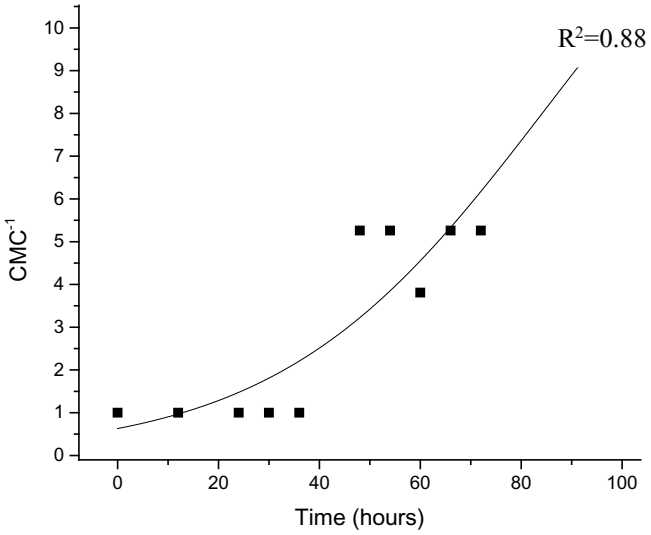
(d)

Figure 6. Effects of the carbon source on time course of CMC^{-1} using (a) 0.5 v/v% n-Hexadecane; (b) 2 v/v% n-Hexadecane; (c) 3.5 v/v% n-Hexadecane; (d) 5 v/v% n-Hexadecane. (pH 6.5, NaCl 26g/L, NH_4NO_3 1g/L, $MgSO_4.7H_2O$ 0.2 g/L, $FeCl_3$ 0.05 g/L, $CaCl_2.2H_2O$ 0.05 g/L and glucose 1g/L)

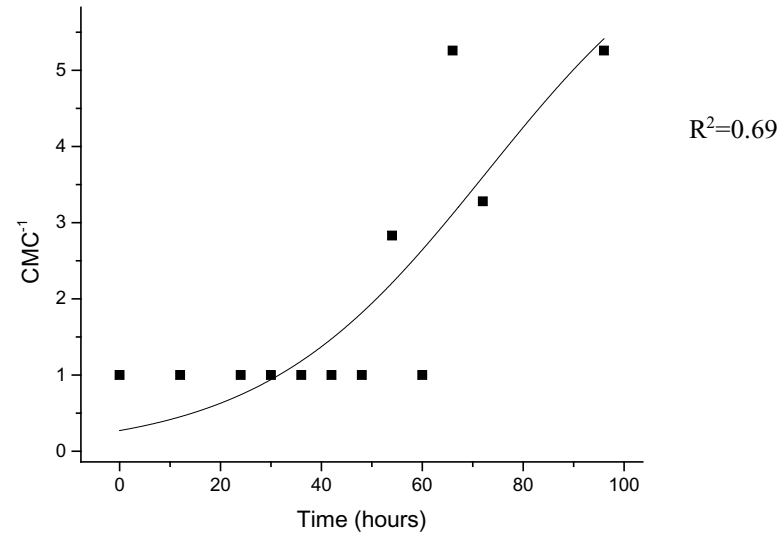
Effect of salinity on biosurfactant production has been further investigated using the kinetic model and the results were presented in Fig.7. CMC^{-1} increased with time for all scenarios. Inhibition of higher salinity displayed that it may affect microbial processes and reduce n-hexadecane biodegradation activity. The highest production rate was achieved when NaCl concentration was 26g/L (P_{max}=16.24). Meanwhile, the fitting of data for the fermentation at 26g/L NaCl achieved a favorable R^2 of 0.88. Therefore, 26g/L NaCl was selected for further biosurfactant production.


R2=0.59
CMC-1
Time (hours)
0
1
2
3
4
5
6
7
0
20
40
60
80
100

(a)


R2=0.88
CMC-1
Time (hours)
0
1
2
3
4
5
6
7
8
9
10
0
20
40
60
80
100

(b)



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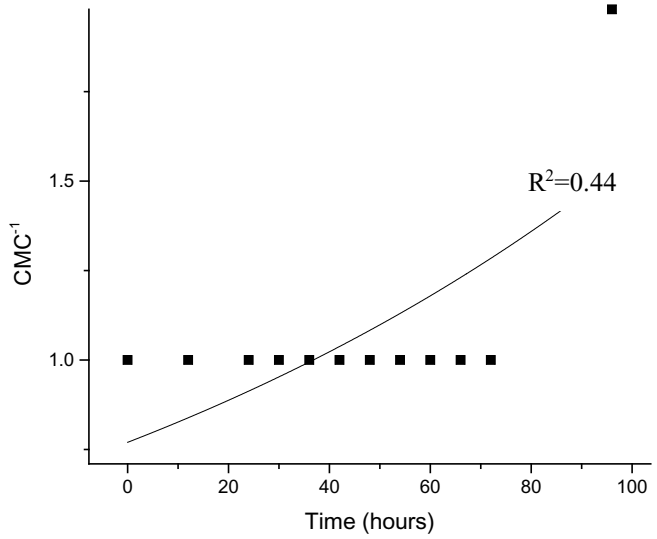
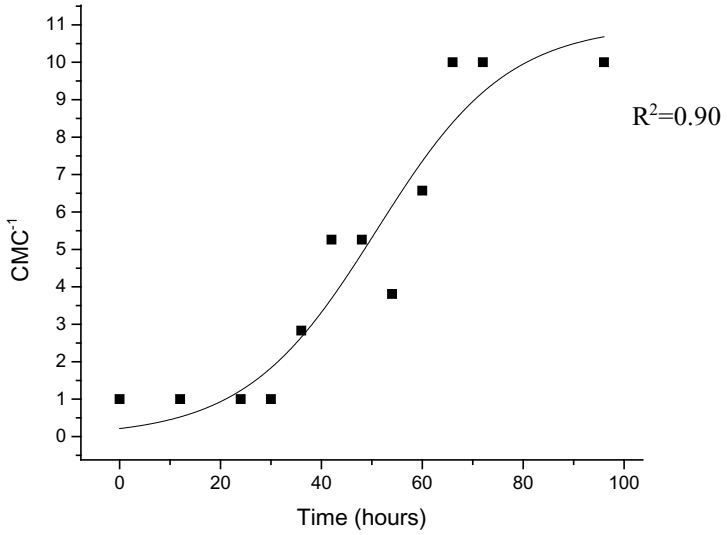


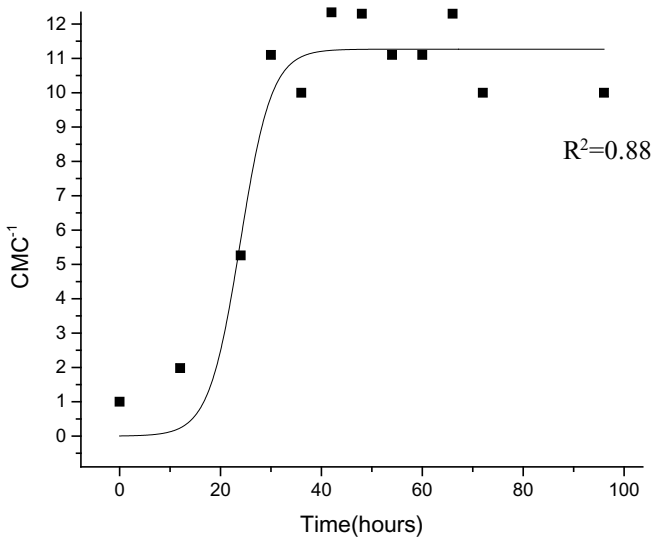
(d)

Figure 7. Effects of salinity on time course of CMC-1 using (a) 13g/L NaCl; (b) 26g/L NaCl; (c) 39g/L NaCl; (d) 52g/L NaCl. (pH 6.5, n-hexadecane 3.5 v/v%, NH_4NO_3 1g/L, $MgSO_4.7H_2O$ 0.2 g/L, $FeCl_3$ 0.05 g/L, $CaCl_2.2H_2O$ 0.05 g/L and glucose 1g/L)

Effect of pH on CMC-1 was presented in Fig. 8. No CMC^{-1} was observed during the fermentation at pH 5. However, similar production rate was observed at pH 6, 7 and 8 with a P_{max} of 11.01, 9.67 and 10.11 respectively. It was noted that the data fitting for all three pH levels was reasonable (R^2 higher than 0.8). Considering both the production rate and surface tension reduction in Fig. 3, pH 7 was selected for further biosurfactant production. When pH is 7, the exponential phase and stationary phase of the predicted curve was obvious with a turning points (Fig. 7 (b)). Furthermore, the duration of each phase was shortened as an exponential phase started between 20 and 40 hours, and a stationary phase was observed after 60 hours.



(a)



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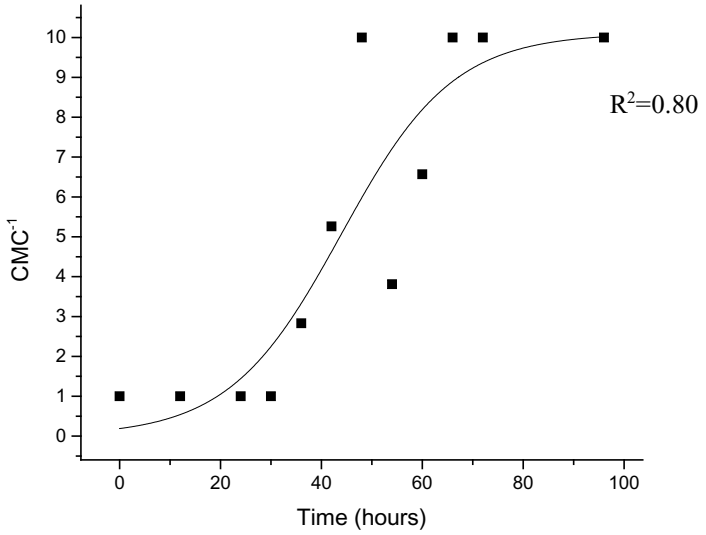
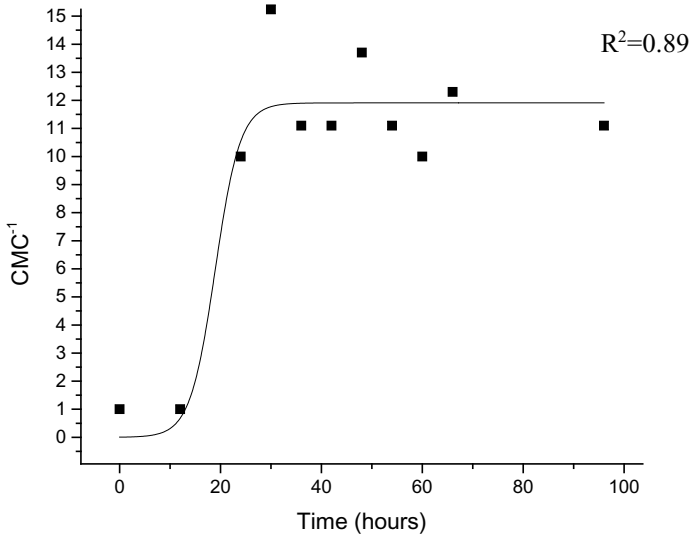


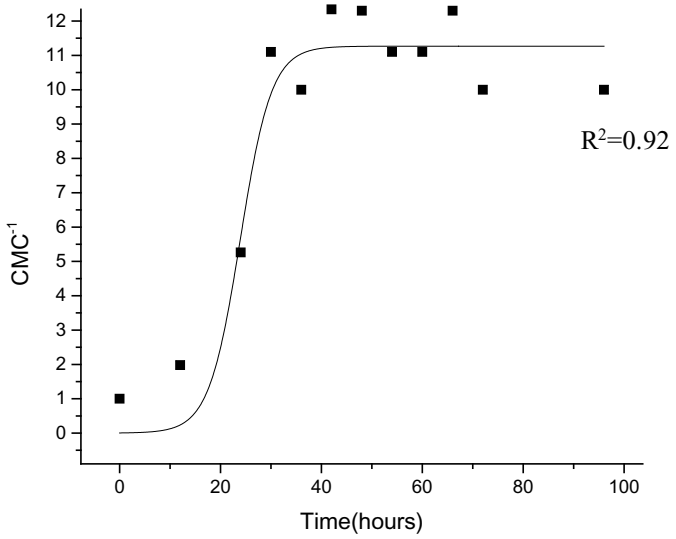
(c)

Figure 8. Effects of pH on time course of CMC-1 using (a) pH6; (b) pH7; (c) pH8 (n-hexadecane 3.5 v/v%, NaCl 26g/L, NH_4NO_3 1g/L, $MgSO_4.7H_2O$ 0.2 g/L, $FeCl_3$ 0.05 g/L, $CaCl_2.2H_2O$ 0.05 g/L and glucose 1g/L)

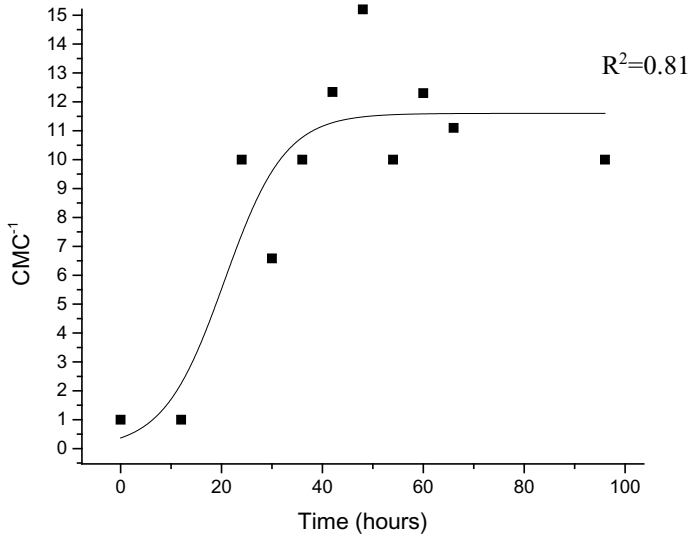
The effect of NH_4NO_3 on the CMC-1 was presented in Fig. 9. The limiting effect of nitrogen was not obvious within the NH_4NO_3 concentration range between 0.4 and 1.3 g/L. Similar predicted curve was achieved for each level with a P_{max} between 11 and 12. What was remarkable was that the P_r was much higher than previous runs. The exponential phase started within 20 hours, and the stationary phase was achieved before 30 hours. Considering the production rate and surface tension reduction (Fig. 4), NH_4NO_3 with a concentration of 0.7g/L was selected for further biosurfactant production. Final cultural conditions were 3.5 v/v% n-hexadecane, 0.7g/L NH_4NO_3, pH 7 and 26g/L NaCl.



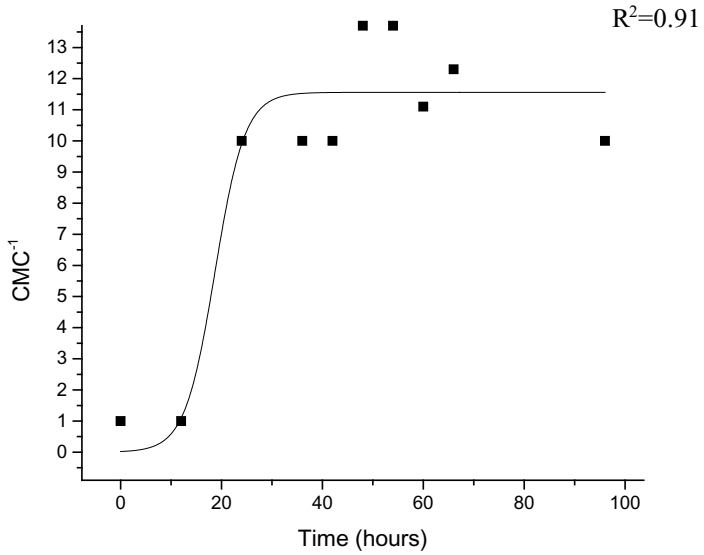
(a)



(b)



(c)



(d)

Figure 9. Effects of the nitrogen source on time course of CMC-1 using (a) 0.4g/L NH_4NO_3; (b) 0.7g/L NH_4NO_3; (c) 1g/L NH_4NO_3; (d) 1.3g/L NH_4NO_3 (pH 6.5, n-hexadecane 3.5 v/v%, NaCl 26g/L, $MgSO_4.7H_2O$ 0.2 g/L, $FeCl_3$ 0.05 g/L, $CaCl_2.2H_2O$ 0.05 g/L and glucose 1g/L

3.4 Summary

The dynamics of biosurfactant production by marine biosurfactant producers isolated from the North Atlantic Ocean was studied for the first time and presented in this chapter. A non-growth associated kinetics model was applied for tracking the biosurfactant production process with fermentation time. This study used both the biosurfactant capability of surface tension reduction and the production rate of biosurfactants by CMC^{-1} to determine the appropriate culturing conditions. Based on the experimental results, the carbon source (3.5 v/v%), salinity (26g/L NaCl), pH (7), and the nitrogen source (0.7g/L NH_4NO_3) were selected as the culturing conditons for future biosurfactant production. The surface tension of the culture was reduced by 40 dynes/cm with a CMC^{-1} of 11.9. Based on the experimentally optimized levels of, the surface tension of the culture can be reduced. The duration before reaching stationary phase was shortened to 30 hours.

CHAPTER 4

BIODISPERSANT GENERATION AND PERFORMANCE EVALUATION

4.1 Background

The escalating offshore oil spills are serious accidents caused by vessel collisions, exploration of offshore oil and gas development, and operational discharges of vessels (Doerffer, 1992). They have long-term negative impacts on the environment, ecology, communities, and socio-economic activities in offshore regions. Oil spill response techniques are being developed according to the stricter environmental laws to alleviate their impacts on the marine environment (Etkin, 2001). However, the applicability and effectiveness of mechanical countermeasures (e.g., controlled burning, skimming, and vacuum/centrifuge) are limited by variable and cold weather conditions in harsh environment such as the North Atlantic Ocean (Chen et al., 2011; Dave and Ghaly, 2011; Jing et al., 2012).

Chemical dispersants are less costly than the physical methods. Furthermore, they can be used on rough seas for rapid treatments where there are high winds (Holakoo, 2001). Nevertheless, there are raising concerns about the toxicity and non-biodegradability of chemical dispersants and dispersed oil, especially after the Deepwater Horizon oil spill (Walker et al., 2003).

Biosurfactant based biodispersants are considered as an environmental friendly alternative to chemical ones as some studies have demonstrated the potential of biosurfactants, (Mulligan, 2005). However, studies on the application of biosurfactants in oil spill response were rarely reported and one crucial bottleneck is that there are extremely limited biodispersant products available in the market (Mukherjee et al., 2008).

In this study, biodispersant products were generated by using selected low-toxic solvents and biosurfactants produced using the *Rhodococcus* strain isolated from the North Atlantic Ocean. The formulation of biodispersants was determined by evaluating the dispersion effectiveness (DE) of a light crude oil sample using the BFT. The DE of this light crude oil sample and another motor oil sample (Pennzoil® 10W-30) was further evaluated and compared with the commercial chemical dispersant Corexit 9527. The acute toxicity of the water-accommodated fraction (WAF) of the biodispersed oils and their biodegradation were tested to assess the environmental impact of the selected biodispersant product.

4.2 Methodology

4.2.1 Biosurfactant production, isolation and purification

The biosurfactant-producer *R. erythropolis* sp. SB-1A isolated from in the North Atlantic Ocean was cultivated in revised Atlas oil agar medium based on previous study in chapter 3 (Atlas, 2004). The selected medium composition consisted of per litre: glucose: 1 g; $MgSO_4.7H_2O$: 0.2 g; $FeCl_3$: 0.05 g; $CaCl_2.2H_2O$: 0.05 g; n-Hexadecane: 3.5%, v/v; NaCl: 26 g; NH_4NO_3: 0.7 g and pH 7.0. All chemicals used were analytical grade reagents unless specified. The strain was streaked on NBS mineral salt medium plates regularly (with the composition per litre as following : Nutrient Broth Broth, 25 g; Agar, 15 g; NaCl, 22 g) and incubated for 48 hours for purity check and storage (Zhou et al., 2005).

The biosurfactant production by *R. erythropolis* sp. SB-1A was carried out in shake flasks holding 700 ml medium per flask. Sterile cultures were inoculated with 1.5% volume aliquot of the inoculums, and incubated for 120 hours at 30°C, 200 rpm in a rotary shaker.

After incubation, the whole culture was extracted using MTBE at 25 °C by magnetic stirring for 24 hours (Kuyukina et al., 2001). After settling for 10 minutes, the upper organic layer and the middle emulsion layer were extracted. MTBE was then removed by rotary evaporation at 45 °C. The remnant n-Hexadecane in the extracted solution was rinsed out using petroleum ether in a separating funnel. The extracted biosurfactants were stored at 4 °C in the fridge.

4.2.2 Characterization of structural properties of the biosurfactants

The concentration of Glycolipid in the biosurfactant product was estimated by a phenol sulfuric acid method to quantify the total carbohydrate (Dubois et al., 1956). The calibration standard curve was firstly generated by mixing 50 μL 80% phenol solution and 5 mL concentrated sulfuric acid with 2 mL D-glucose stock at various concentrations in 20 mL tubes. After settling for 10 minutes, all the tubes were shaked for 15 min at 30°C. A 0.01 g biosurfactant solution was added into 100 ml water. A 2 mL of dilution was operated using the same procedure as that used for the calibration standard. The absorbances of the calibration standard points and the sample was measured at 490 nm by a spectrophotometer. The total carbohydrate in the sample solution was expressed in terms of D-glucose (g/ 100mL).

The total lipids in the biosurfactant product was quantified by a colorimetric method using Palmitic acid as the calibration standard (Pande et al., 1963). The 2.0% potassium dichromate (w/v) in sulfuric acid (Lipid reagent) was prepared. The 0.1 g Palmitic acid was dissolved in 10 mL petroleum ether. To prepare the calibration curve, petroleum ether in Palmitic acid stock solutions of different concentrations was firstly dried by air-blowing in 20 mL tubes, and then 3 mL of 2.0% potassium dichromate solution was added to each tube. After heating in a water bath for 15 minutes, the tubes were cooled using running water. Another 4.5 mL water was then added to each tube. Color intensity was measured at 595 nm by a spectrophotometer once the calibration curve was developed successfully. 0.1 g biosurfactant solution was dried through air-blowing in a tube and operated using the same procedure as that was applied to treat the calibration standard. The total lipids in the sample solution was expressed in terms of Palmitic acid (g/ 100mL).

The CMC of the biosurfactant product was determined. The surface tension of 10 mL diluted biosurfactant solution at various concentrations was determined in triplicate with a surface tensiometer (DuNouyTensiometer, Interfacial, CSC Scientific) at 25°C. The CMC was determined by plotting the surface tension versus the concentration of biosurfactants in the solution. The CMC point was defined as the intersection of the two best-fit lines for ≤1,000mg/L and ≥5,000 mg/L of biosurfactants (Carpena et al., 2002).

4.2.3 Screening of solvents

Dimethyl sulfoxide (DMSO), 2-(2-Butoxyethoxy) ethanol, Polyethylene glycol 400 (PEG 400), Propylene glycol, Ethyl lactate, and DL-12-isopropylidene glycerol (Solketal) were considered as candidate solvents due to their favorable characteristics (e.g., low toxicity, high water-solubility, high biodegradability, high stability, and low volatility) based on literature review. The biosurfactant product was mixed with each solvent at the ratio of 50% to generate series biodispersant products. BFT was used to determine the DE of each biodispersant when treating a crude oil sample at 200rpm and 25 ºC. Two candidate solvents were obtained by considering both toxicity (ORL-RAT LD50 (mg/kg)) and the DE of biodispersants.

4.2.4 Determination of biodispersant formulation

The biosurfactant solution was mixed with each of the two candidate solvents at seven different concentrations: 3.3%, 10%, 16.7%, 33.3%, 50%, 66.7%, and 83.3%, respectively. For each concentration level, the performance of generated bidispersant was evaluated by its DE when treating crude oil sample. BFT was used to determine the effectiveness of biodispersants in triplicate (Chandrasekar et al., 2003; Venosa et al., 2002).

4.2.5 BFT

To evaluate the DE, BFT was conducted using a crude oil sample and a motor oil sample (Pennzoil® 10W-30). The physicochemical characteristics of these two types of oil are listed in Table 7.

Table 7. Physicochemical characteristics of oil samples

Type	Supplier	Specific density@ 25°C	API gravity@ 60ºF	kinematic viscosity @20 °C
Pennzoil® 10W-30	Pennzoil-Quaker State Canada.Inc	0.85g/mL	33.21°	17.0 mm^2/s
Crude oil	Canada	0.86g/mL	31.29°	208.3 mm^2/s

Hexane was used instead of DCM during the BFT test due to the fact that DCM can dissolve biodispersants. Standard calibration curves were generated using a spectrophotometer at 340, 370 and 400 nm. Four types of samples were used, control sample with no dispersant, oil treated by biodispersants, and oil treated by Corexit 9500 and 9527 for method calibration. In the BFT procedure, 100 ml 25°C synthesized seawater at a concentration (salinity) of 35 ppt created by mixing distilled water with Instant Ocean® synthetic sea salt was added into a modified 150 mL glass baffled trypsinizing flask with a screw cap at the top and a teflon stopcock near the bottom (Song et al., 2013). The 100 μL crude oil/motor oil was then spiked onto the surface of the synthetic seawater. Finally, 3 mL of each crude biodispersant (approximate 8.4 mg total carbohydrate content in 1 g crude biosurfactant product) or 4 μL Corexit 9527/9500 was added onto the oil layer. The flask was shaked for 10 minutes at 200 rpm and allowed to remain stationary for another 10 minutes. After discarding the first 2 mL sample from the stopcock, 30 mL sample was collected and extracted with 20 mL Hexane. The absorbance of the sample extracts and the standard solutions were measured at 340, 370 and 400 nm by a spectrophotometer. To calculate the DE, the area under the absorbance curve for the standards and experimental samples between 340 and 400 nm was calculated using Eq. (3):

$$\text{Area} = \frac{30(\text{Abs}_{340} + \text{Abs}_{370})}{2} + \frac{30(\text{Abs}_{370} + \text{Abs}_{400})}{2} \quad (3)$$

In order to compare the DEs of different dispersants, the final results were displayed in term of the dispersion effectiveness ratio (DER) between chemical dispersants and biodispersants:

$$DER = \frac{\text{Total oil dispersed (dispersant A)}}{\text{Total oil dispersed (dispersant B)}} \times 100\% \quad (4)$$

where the total oil dispersed is the mass of oil×100 ml/30 ml;

the mass of oil (g) is the concentration of oil×V_{Hexane};

V_{Hexane} is the volume of Hexane-extract of the water sample (0.020 L);

the concentration of oil (g/L) is the area determined by Eq.(3) divided by the slope of the calibration curve (Chandrasekar et al., 2006).

4.2.6 Biodegradation test

The biodegradation test was investigated in flasks under simulated marine conditions in dark condition. Synthesized seawater was created by mixting distilled water with Instant Ocean® Synthetic Sea Salt to reach a concentration (salinity) of 35 ppt. No microbial inoculum was added to the seawater prior to testing. The flasks filled with 40 mL seawater and 100 µL crude oil were used as blank samples. The control flasks with biodispersant inside were loaded with 0.5 mL biodispersants (biosurfactants/solvent ratio of 5:1) to generate the biodispersed oil samples. All the flasks were shaken in an orbital shaker at 100 rpm and 25°C. After 0, 7, 14, and 28 days of incubation, flasks were sacrificed and Total Petroleum Hydrocarbons (TPH) analysis was conducted by a spectrophotometer (Tellez et al., 2005). Microbial growth in each flask was determined by a spectrophotometer at 600 nm. Cultures with an OD (600 nm) greater than 0.2 were deemed as the positive growth (Bej et al., 2000; Margesin et al., 2003).

4.2.7 Toxicity test

Blank and biodispersed oil samples were prepared using the same procedure as that stated in 4.2.6. The solution of oil WAF or dispersed oil water-accommodated fraction (DWAF) in flasks were prepared through magnetic stirring (Couillard et al., 2005). The speed of the magnetic stirrer was adjusted to form a vortex of 20–25% of the water depth. For biodispersed samples, the vortex was kept for 18 hours followed by a 6-hour settling period. For blank samples without biodispersants, a 24-hour mixing period was used without settling period. The WAF/DWAF in each flask was collected for toxicity test using a Microtox® Model 500 (M500) analyzer according to the manufacturer's standard protocols (Yassine et al., 2012). The pH of each sample was adjusted between 6 and 8 with (10%) NaOH or (10%) HCl. The data were analyzed by MicrotoxOmni® 4.1 software. Color correction was conducted if there was red/brown color in the samples by reading the absorbance of the samples at 490 nm and the data were re-analyzed by MicrotoxOmni®4.1. The 5- and 15- min EC50s were obtained.

4.2.8 Quality Assurance and Quality Control (QA/QC)

The correlation coefficient of each standard calibration curve for total lipid and total carbohydrate analysis and BFT was greater than 0.99 and the repeatability of these calibration curves was confirmed. During the characterization of biosurfactants, the biosurfactant product was rinsed before each test to eliminate the noice from the medium. Each treatment of BFT was performed in triplicates. In toxicity and biodegradation tests, 50% of the samples were analyzed in duplicates. The relative percent difference values should be within 20% and re-evaluated immediately if a

value was larger than 20%. The pre-treatment of WAF/DWAF samples included color correction and pH adjustion if necessary. The bacterial reagent was sealed and stored frozen at –20°C before use. Micortox test was performed following the protocol, and the data were analyzed using MicrotoxOmni® 4.1 software.

4.3 Results and discussion

4.3.1 Isolation and purification of biosurfactant

In previous studies, it was demonstrated that *Rhodococci* produced both cell-bound and extracellular trehalose containing Glycolipids (Ciapina et al., 2006; Lang and Philp, 1998). Based on the results, in this study the culturing media was extracted using MTBE without eliminating bacterial cells by centrifugation. When extraction was completed, a stable emulsion layer existed between the top solvent layer and lower water layer. This may be due to the fact that the cell-bound Glycolipids cannot be entirely extracted by MTBE (Kuyukina et al., 2001). Both emulsion layer and solvent layer were collected and concentrated by rotary evaporation. The biosurfactant was produced at a rate of 57.2 ml per litre of culture on average. The morphology of the crude product was shown in Fig. 10.

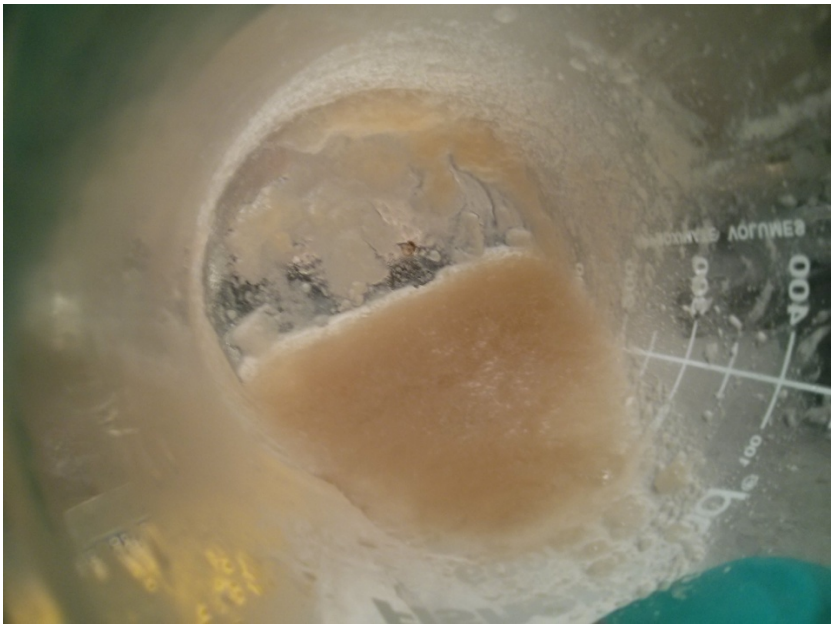


Figure 10. Biosurfactant generated by *R. erythropolis* sp. SB-1A

4.3.2 Biosurfactant characterization

Biochemical composition of the biosurfactant revealed that the total carbohydrate content was 8.4 mg in term of D-glucose and the total lipid content was 11.6 mg in term of Palmitic acid in 1g product.

The surface activity can be represented by CMC, surface tension reduction, and CMC^{-1}. It can demonstrate surfactant functions including emulsification, solubilization, and foaming (Yin et al., 2009). In this study it the surface activity of the biosurfactant was evaluated. Its CMC was approximately 1,500 mg/L as determined in Fig. 11.

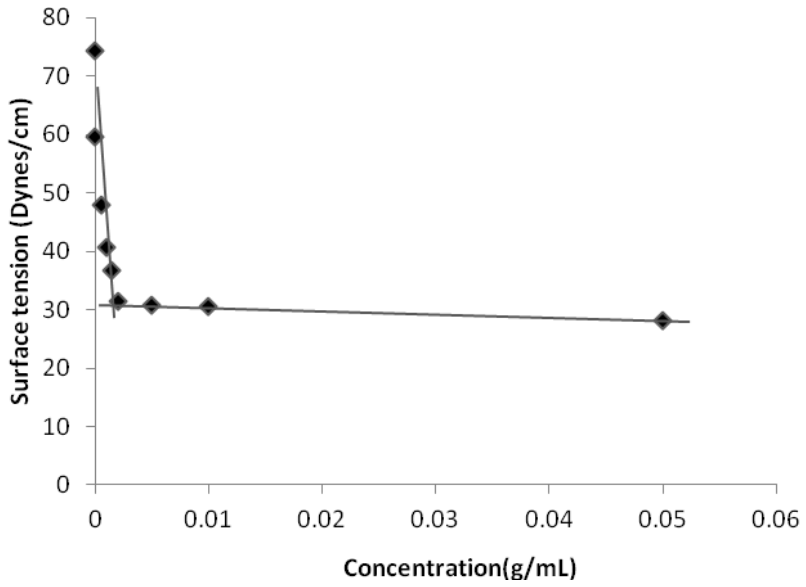


Figure 11. CMC determination of the biosurfactants

4.3.3 Screening of solvents

Dispersants consist of surfactants and solvents. The surfactants can reduce the interfacial tension and enhance the dispersion of oil droplets. The solvents are applied to carry surfactant and to promote the solubility of oil. Consequently, the solvents for dispersant should be miscible with both water and oil. In this study, six types of solvents were considered and evaluated for biodispersant generation based on the DER, toxicity, volatility and viscosity. As shown in Table 8, the DER of 2-(2-Butoxyethoxy) ethanol-based biodispersant was 2.39 fold greater than that of the Solketal-based biodispersant. Nonetheless, it was the most toxic solvent with a LD50 of 5660 (mg/kg). PEG 400-based biodispersant had a DER 1.83 times that of the Solketal-based biodispersant and the lowest toxicity of 30200 (mg/kg). No significant difference was observed among DMSO, Ethyl lactate, and Propylene glycol on their DERs but Propylene glycol was significantly less toxic than the other two with a

LD50 of 20000 (mg/kg). Consequently, PEG 400 and Propylene glycol were selected as candidate solvents for generating biodispersants and performance evaluation tests.

Table 8. Characteristics and DERs of solvents

Type	DER*	Toxicity	Volatility	Kinematic viscosity
		ORL-RAT LD50 (mg/kg)	(boiling point °C)	(mm^2/s @ 20 °C)
Dimethyl sulfoxide (DMSO)	1.30	14500	189	1.80
2-(2-Butoxyethoxy) ethanol	2.39	5660	231	6.28
Polyethylene glycol 400 (PEG 400)	1.83	30200	200	106.48
Propylene glycol	1.16	20000	188	38.85
Ethyl lactate	1.25	8200	155	2.50
DL-12-isopropylidene glycerol (Solketal)	1.00	7000	188	10.35

* DER indicates the ratio between the DE of each solvent and the DE of Solketal.

4.3.4 Determination of biodispersant formulation

In this study, the crude biosurfactant product was mixed with the two selected solvents with multiple ratios to generate biodispersants. The BFT was selected to evaluate the DE of each type of biodispersant to determine the final biodispersant formula. As a bench-scale dispersion test, BFT has been proven to provide more energy to allow the dispersant to react adequately than Swirling Flask Test (SFT). Therefore, dispersed oil droplets in BFT can better reach into the deeper water column by the energy of wave (Kaku et al., 2005). Based on the CMC test, the minimum effective concentration of biosurfactants in biodispersants was approximately 1,500 mg/L. Correspondingly the minimal biosurfactant concentration in biodispersants was 3.3%, and seven concentrations (3.3%, 10%, 16.7%, 33.3%, 50%, 66.7%, 83.3%) were selected for screening.

Figure 12. Baffled Flask Test Apparatus

The dispersability of crude oil through using natural dispersion, Corexit dispersants (i.e., 9500 and 9527) and lab-generated biodispersants at 200rpm, 25 ºC was tested. The results were presented in Fig. 13 and 14. The DER of natural dispersion on crude oil was not obvious. Corexit 9500 reached 76% DER of Corexit 9527. When using PEG 400 as the solvent to generate biodispersants, the highest DER of 1.48 was reached with the biosurfactant concentration of 16.7% in the biodispersant (Fig.13), and no further obvious increase was observed with concentrations higher than 16.7%. When using Propylene glycol as the solvent (Fig.14) the DER of Propylene glycol biodispersant remained stable between 0.64 and 0.83 as the concentrations increased. The reproducibility of Corexit dispersion was better than that of Biodispersants with smaller error bars as shown in Fig. 13 and 14. The main reason is that unpurified crude biosurfactants were applied, leading to heterogeneous in the biodispersant

products. Overall, PEG 400 obtained better performance than Propylene glycol. Therefore, PEG 400 was selected for biodispersant generation.

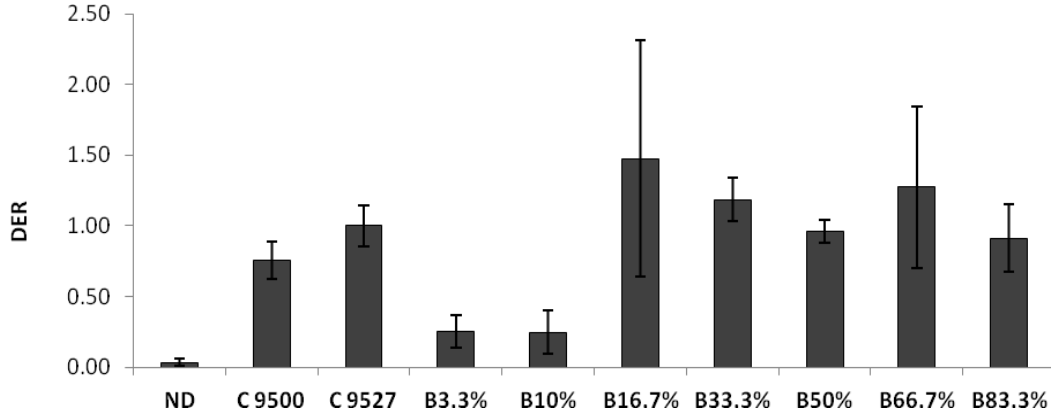


Figure 13. Comparison of the dispersability of light crude oil by natural dispersion (ND), Corexit 9500 (C 9500), Corexit 9527 (C 9527) and PEG 400 biodispersants (B). The error bars represent ±1 standard deviation unit. DER denominator stands for DE of C 9527.

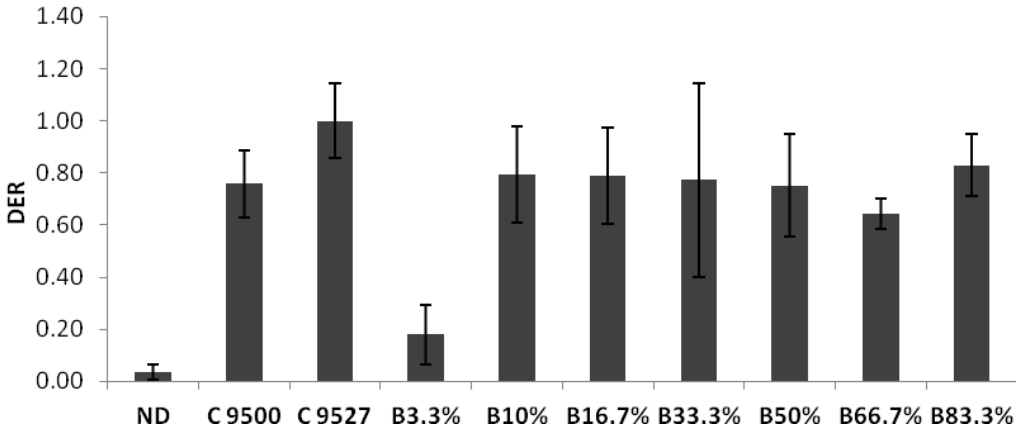


Figure 14. Comparison of the dispersability of light crude oil by natural dispersion (ND), Corexit9500 (C9500), Corexit 9527 (C9527) and Propylene glycol biodispersants (B). The error bars represent ±1 standard deviation unit. DER denominator stands for DE of C9527.

4.3.5 Comparison of the DER between Corexit and biodispersant using crude oil and motor oil

After the formulation of biodispersant, 16.7% crude biosurfactant was mixed with 83.3% PEG400 to generate biodispersant. By using two types of oil, the dispersion performance of both Corexit 9257 and biodispersant was evaluated shown in Fig.15. The results indicated that motor oil with a DER of 0.28 was more easily dispersed than crude oil under natural dispersion. The DER of the biodispersant was close to that of Corexit 9527 when using motor oil. A DER of 1.48 was achieved when using the crude oil. The two kinds of oil have similar dispersibility regardless of their viscosity difference. The results illustrated the possibility of applying this lab-generated crude biodispersant for real oil spill response.

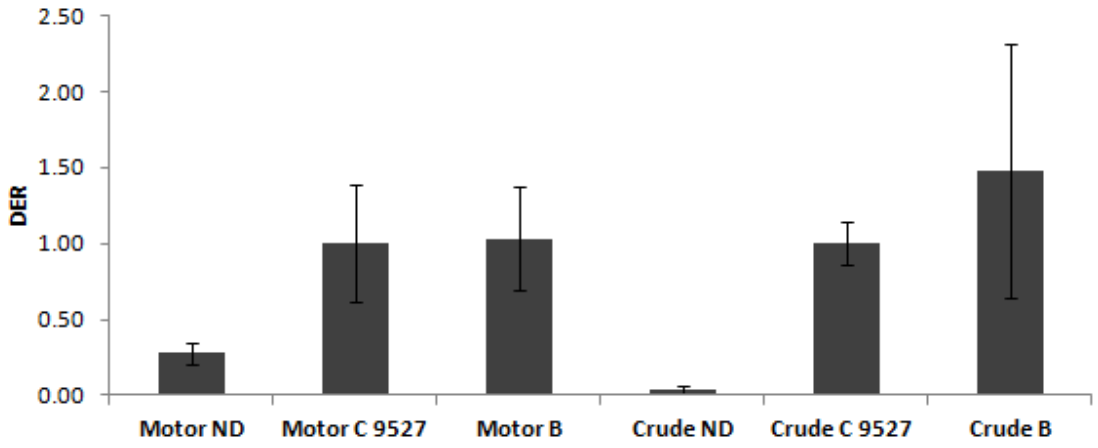


Figure 15. Comparison of the dispersability of light crude oil (crude) and motor oil (motor) by natural dispersion (ND), Corexit 9527 (C 9527) and biodispersants (B). The error bars represent ±1 standard deviation unit. DER denominator stands for DE of C 9527.

4.3.6 Biodegradation test

Biodegradation is a crucial process that consumes oil by microbial degradation but it is a very complex process to be simulated. Past chemical dispersant studies have displayed enhanced biodegradation with the addition of chemical dispersants yet some observed the inhibition of dispersion (Fingas, 2010; Zahed et al., 2010). For the biodegradation of biodispersed oil, the studies have been rarely reported. In this study, biodegradation test was conducted to evaluate the performance of the lab-generated biodispersant product using crude oil. The biodegradation (TPH) and microbial growth (OD 600nm) results of both biodispersed oil samples and naturally dispersed oil samples were presented in Fig.16. For the biodegradation in flasks with biodispersant, the biodegradation started significantly after 7 days, and the OD 600 nm increased simultaneously. Approximate 45% of the crude oil was biodegraded by biodispersant -assisted dispersion after 28 days with the same tendency as OD 600 nm. The lack of final stable phase in the results revealed the limitation of flask study without spiked oil-degrading bacteria and suggested a extended period longer than 28 days in further studies. Overall, the biodispersed oil can be obviously biodegraded compared with naturally dispersed oil.

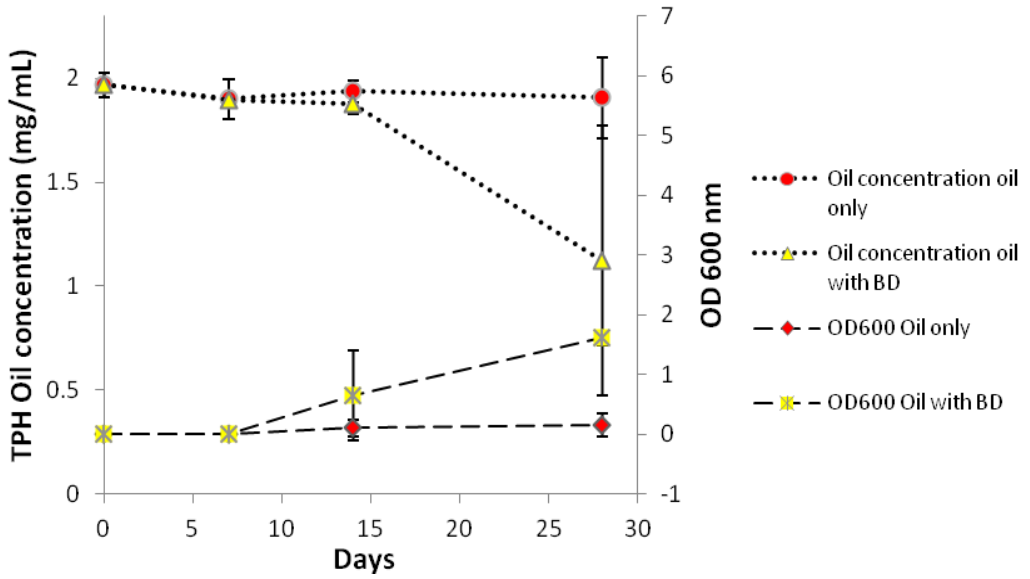


Figure 16. Comparison of the biodegradation of natural dispersed oil, biodispersant dispersed oil. The error bars represent ±1 standard deviation unit.

4.3.7 Toxicity test

The toxicity of Corexit was not allowed to be studied as required by the agreement with the supplier. The acute toxicity of the PEG 400 biodispersant was evaluated by Microtox® and compared with that of Corexit 9527 in literature. The 15min EC50 concentration was 43.60 g/L, which was significantly less toxic than that of Corexit 9527 with a 15min EC50 concentration of 4.9-12.8 mg/L according to literature (George-Ares and Clark, 2000; George-Ares et al., 1999). It was revealed that DWAF was more toxic than that of WAF or dispersants themselves (Cohen and Nugegoda, 2000; Gulec and Holdway, 2000). DWAF contained higher concentration of water soluble components (mainly Low-molecular-weight hydrocarbons) from light crude oil. In Table 9, both 5-min EC50 (%) and 15-min EC50 (%) of ND-WAF increased after 7 days and remained stable in the following days. The concentration of dispersed oil in those flasks estimated by BFT test remained 0.01 mg/mL. For biodispersant dispersion (BD) DWAF, the increasing tendency was observed in both 5-min EC50 (%) and 15-min EC50 (%) through 28 days which reached approximately half of initial EC50% after 28 days. Consequently, the toxicity of biodispersed oil samples was obviously reduced compared with that of naturally dispersed oil samples.

Table 9.* Comparison of 5-min and 15-min EC 50(%) of ND-WAF and BD-DWAF by Microtox®

Days	ND		Dispersed oil (mg/mL)	BD		Dispersed oil (mg/mL)
	EC50-5 min (%)	EC50-15 min (%)		EC50-5 min (%)	EC50-15 min (%)	
0	21.40	26.58	0.01	8.20	11.94	0.60
7	24.13	30.40	0.01	12.44	16.78	0.57
14	26.59	32.05	0.01	11.46	16.90	0.56
28	25.09	31.54	0.01	14.06	22.11	0.34

* The Microtox® results didn't stand for the toxicity of the whole experimental system and indicated that of undersurface environment.

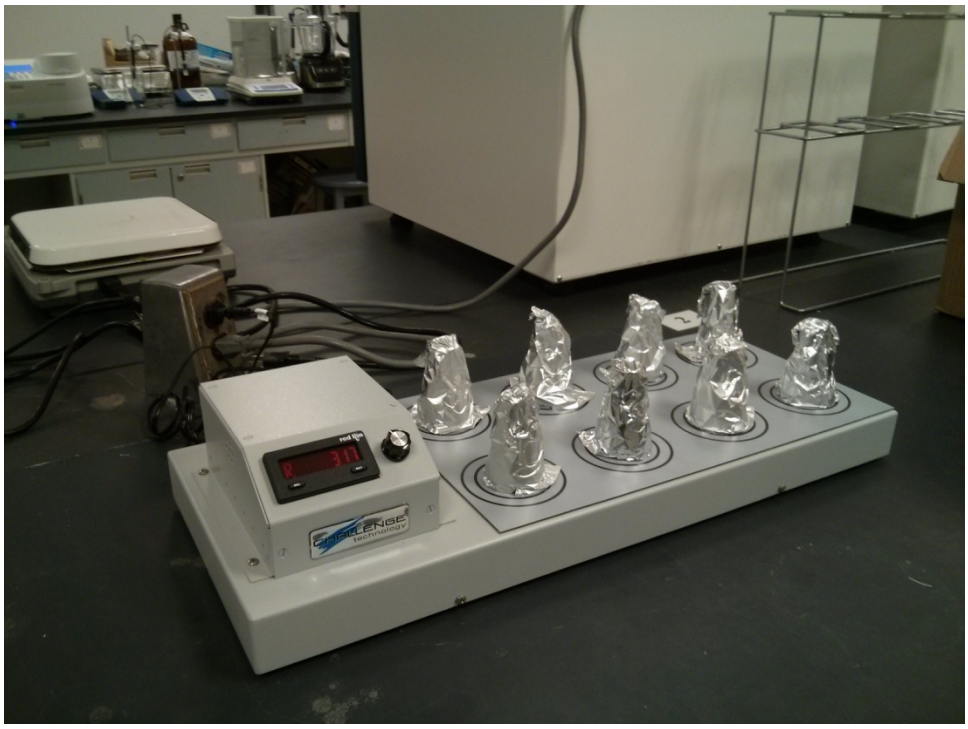


Figure 17. Preparation of WAF and DWAF

4.4 Summary

In this chapter, a biosurfactant-producer *R. erythropolis* sp. SB-1A isolated in the North Atlantic Ocean was cultivated to produce a biosurfactant. The biochemical composition of the crude biosurfactant product was determined. The product was then mixed with solvents to generate biodispersants. Six types of solvents were considered and two of them were finally selected based on the DER, toxicity, volatility and viscosity. The formulated biodispersant displayed compatible DE on a stubborn light crude oil sample by mixing solvents in the laboratory compared with Corexit dispersants. After the determination of the biodispersant formulation, it can effectively disperse another motor oil sample under cold/weathering conditions. During the biodegradation test, 45% of the crude oil was biodegraded by biodispersant assisted dispersion after 28 days without spiking oil-degrading bacteria. The toxicity was found to be reduced by half after 28 days using Microtox®.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

This research tackled the development of biosurfactants produced by *Rhodococcus erythropolis,* a strain isolated from the North Atlantic Ocean, as well as the generation and performance evaluation of relevant biosurfactant-based biodispersants.

The effects of the carbon source, the nitrogen source, pH and salinity on biosurfactant production were investigated. A non-growth associated kinetic model was applied and validated for biosurfactant production with a favorable significance level and R^2 of 0.92 under experimentally defined conditions. By periodically monitoring the surface tension and CMC^{-1} of the culture during the production and integrating the results with the outputs of the kinetic model, the appropriate values of the carbon source (3.5 v/v%), salinity (26g/L NaCl), pH (7) and the nitrogen source (0.7g/L NH_4NO_3) for further production were obtained. The surface tension of the culture can be reduced by 40 dynes/cm with a CMC^{-1} of 11.9. Results also indicated that the exponential phase started within 20 hours, and the stationary phase could be achieved before 30 hours as displayed in the kinetic curve.

The biochemical compositions of the crude biosurfactant were detected. The total carbohydrate content in 1 g of the biosurfactant was 8.4 mg in term of D-glucose, and the total lipid content in 1 g of the biosurfactant was 11.6 mg in term of Palmitic acid.

Biodispersants were generated subsequently through mixing the crude biosurfactant and solvents. Dimethyl sulfoxide (DMSO), 2-(2-Butoxyethoxy) ethanol, PEG 400, Propylene glycol, Ethyl lactate, and Solketal were considered as solvents and screened

based on their toxicity and the effectiveness of relevant dispersants generated. The final formula of the biodispersant was determined as 16.7%/ 83.3% (biosurfactants/ PEG 400).

The biodispersant-based dispersion was examined using a motor oil and a crude oil. Its performance was compared with the commercial chemical dispersant Corexit 9527. Results showed that compared with Corexit 9527, the biodispersant could achieve a compatible dispersant effectiveness (DE) when treating two types of oil.

Finally, the environmental impact of the biodispersant product was assessed by analyzing oil biodegradation using a spectrophotometer and by analyzing the acute toxicity of the WAF of oil dosing solutions with/without biodispersants using Microtox®. During the biodegradation test, 45% of the crude oil was biodegraded by biodispersant assisted dispersion after 28 days without spiking oil-degrading bacteria. The toxicity was found to be reduced by half after 28 days.

5.2 Scientific achievements

(1) This study, for the first time, combined the kinetic study with system optimization for biosurfactant production by *Rhodococcus*. Multiple variables including the carbon source, the nitrogen source, pH and salinity were considered in kinetic analysis during biosurfactant production.

(2) This study, for the first time, used a biosurfactant producer isolated from the North Atlantic Ocean to generate biosurfactant-based biodispersants. The products were proven to be capable of dispersing light crude oil and motor oil with a compatible and performance than the Corexit dispersants.

(3) This study, for the first time, demonstrated the effectiveness of the crude (un-purified) biodispersants on oil spill dispersion, which would greatly decrease the cost in large scale field applications.

5.3 Recommendations for future work

(1) Design of experiments (DOE) can be applied to investigate the biosurfactant production process based on the results of OFAT experiments in this study to further improve the system performance and the production efficiency. Biomass and substrate consumption should also be monitored in a kinetic study to evaluate the effects of microorganisms and other nutrients on biosurfactant production.

(2) Purification procedures (e.g., freeze-drying and cell isolation) should be conducted following current isolation operations to further purify the crude biosurfactant product and improve the dispersion performance of related biodispersant products.

(3) Biosurfactant structures should be further identified with advanced characterization methods including TLC, HPLC-MS, GCMS, IR and NMR to analyze how the functional groups of biosurfactants could affect the effectiveness of oil dispersion.

(4) Pilot-scale (e.g., wave tank) examination should be conducted to evaluate the effectiveness of biodispersants. The performance evaluation should also be extended to the examination in harsh environments with complicated conditions (e.g., using weathered oils, with rough waves, under low temperatures).

(5) Oil biodegradation could be monitored to track the compositional change of both saturated and aromatic hydrocarbons.

(6) The toxicity of dispersed oils can be further analyzed using different marine species to obtain information about their effects on the environments at a short- and long-terms.

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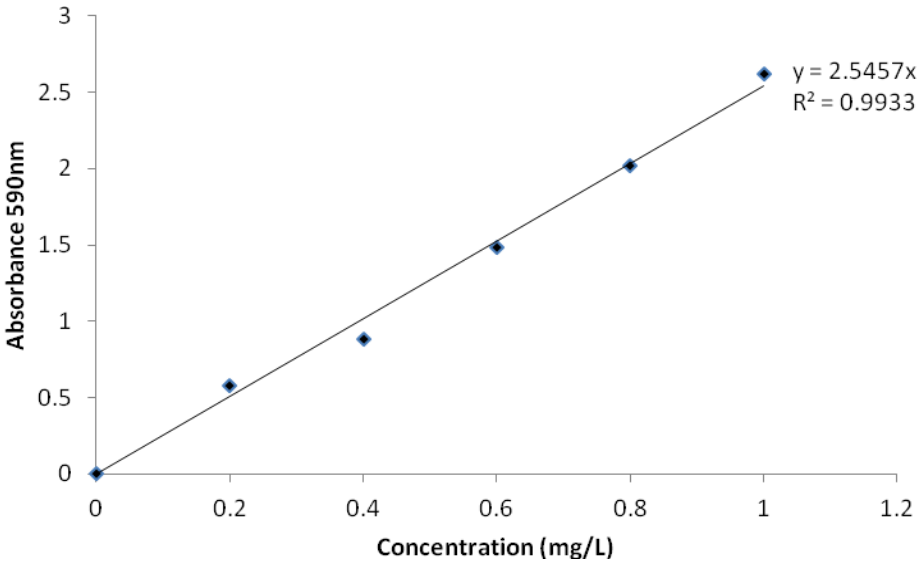
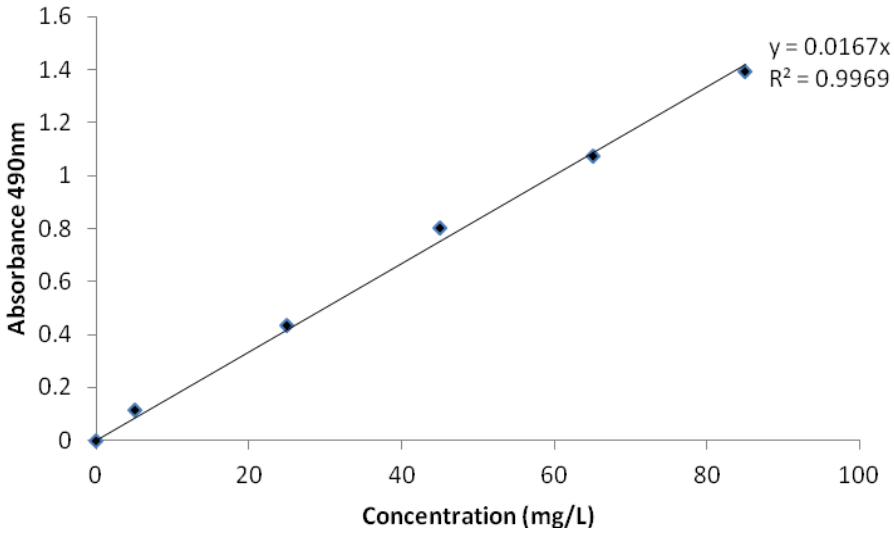
APPENDIX



Total lipid calibration curve



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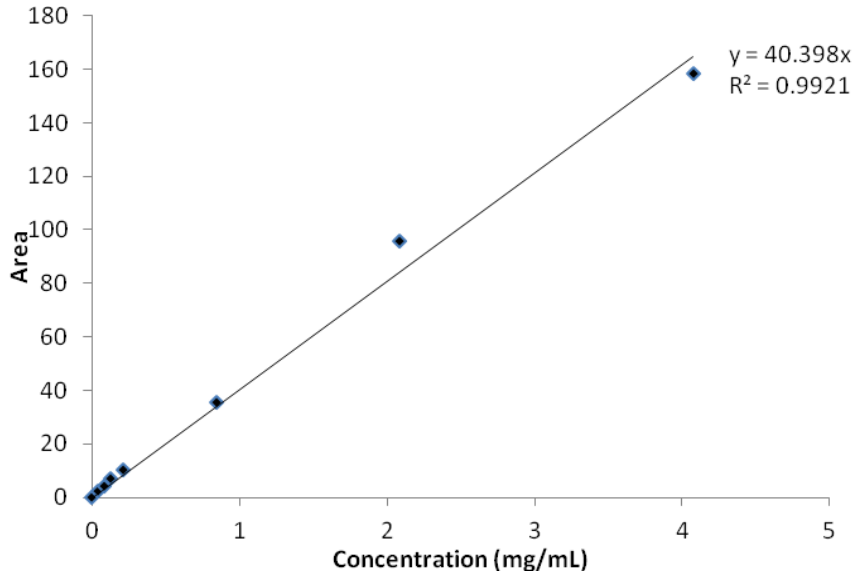
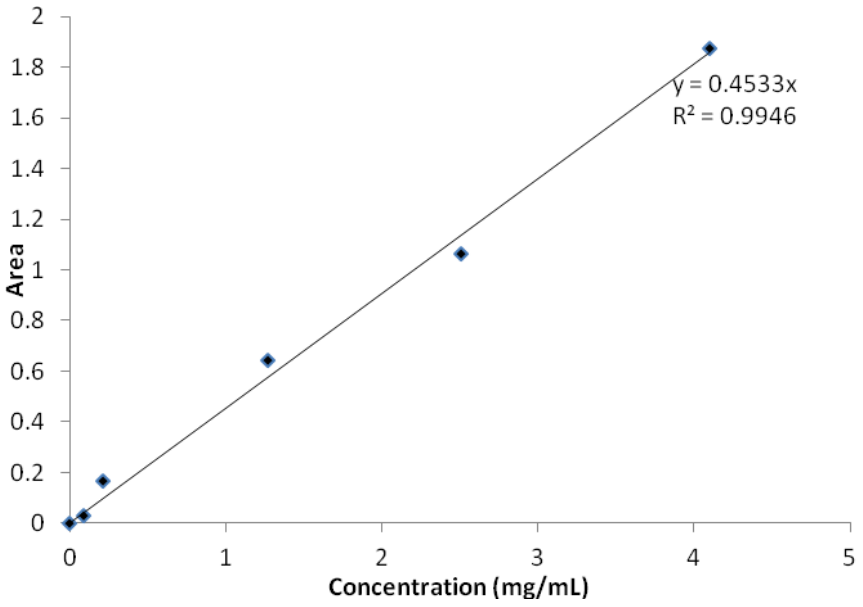


BFT calibration curve for biodispersants and crude oil/crude oil only control



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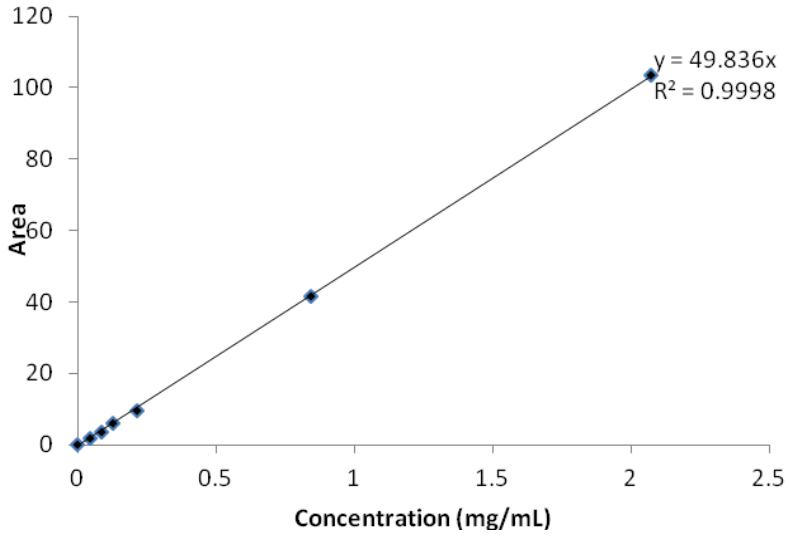
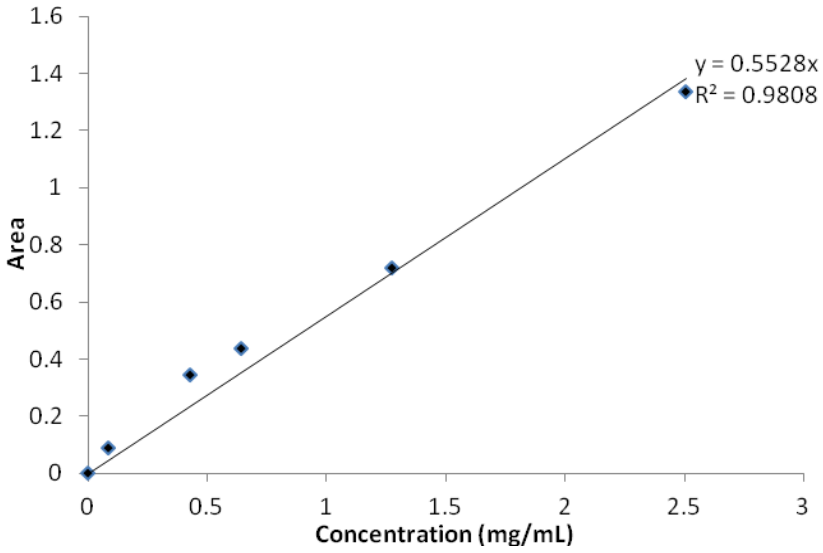


BFT calibration curve for Corexit 9527 and crude oil control



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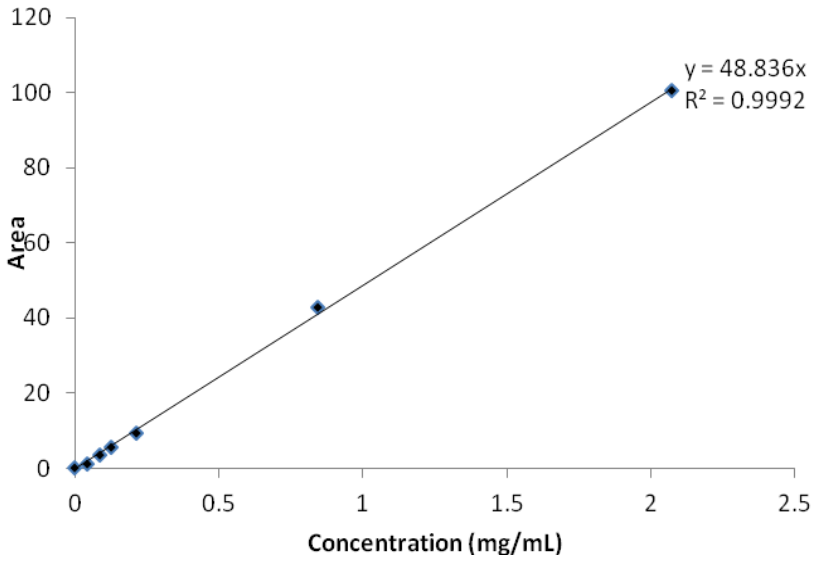

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