

Bio-dispersant produced by a *Rhodococcus erythropolis* mutant as an oil spill response agent

Qinhong Cai¹, Baiyu Zhang^{1,*}, Bing Chen¹, Tong Cao¹ and Ze Lv¹

¹The Northern Region Persistent Organic Pollution Control (NRPOP) Laboratory, Faculty of Engineering and Applied Science, Memorial University of Newfoundland, St. John's, Newfoundland, Canada A1B 3X5

*Corresponding Author's Email address; bzhang@mun.ca

Abstract

Bio-dispersants have been considered as superior alternatives of currently used dispersants as they are generally more biodegradable, less toxic, and better at enhancing biodegradation. However, the application of bio-dispersants is limited by the availability of economic products and the corresponding producers that can work effectively. Hyperproducers generated by metabolic engineering of biosurfactant producers are highly desired to overcome this obstacle. A *Rhodococcus erythropolis* SB-1A strain was isolated from Newfoundland offshore oily water samples. One of its mutant derived by random mutagenesis with ultraviolet radiation, producing high levels of biosurfactants was selected by the oil spreading technique. The mutant produces biosurfactants with critical micelle dilutions (CMD) approximately 4 times of the parent strain. The results obtained with thin layer chromatography (TLC) indicated the produced biosurfactant remained unchanged between the mutant and the parent strain. In addition, the produced biosurfactants were recovered with solvent extraction method and applied as the oil spill response agents to effectively disperse oil slick. Based on the baffled flask test (BFT) results, the dispersion efficiency of the biosurfactants produced by the mutant is higher than that induced by the parent strain and Corexit 9527 while comparable to the Corexit 9500.

Keywords: biosurfactant, hyper producing mutants, oil spill response agent, *Rhodococcus erythropolis*

Introduction

Offshore oil spills are of tremendous concerns due to the enormous economic loss and the harm to ecological systems they may cause. Among diverse oil spill response technologies such as *in situ* burning, booming and skimming, absorption and solidification, dispersion has high feasibility and effectiveness in open water. In the meantime, it is not restricted by the limitation factors such as accessibility, weather conditions, sea states, and oil thickness that other countermeasures require Scientific and Environmental Associates (2003). Dispersants induce oil dispersion and they consist of surfactants and solvents. Solvents help distribute surfactants into the oil/water interface whereafter surfactants changes the interfacial properties thus break down oil slicks into small droplets. They were used to reduce the impact of oil on the shorelines, birds and mammals living on the water surface as well as to promote the biodegradation of oil (Board of Ocean Studies, 2005). Dispersants were used as the primary combating agent for the Deepwater Horizon oil spill (The Federal Interagency Solutions Group, 2010). In total, 1.07

million gallons of Corexit dispersants (Corexit 9500 and Corexit 9527) were applied on the surface, while 771,000 gallons were applied to the subsea discharge point (National Commission Final Report, 2011).

The USEPA has provided benchmarks based on available ecological data to aid in the assessment of potential risk associated with dispersant chemicals. The used methods and the corresponding benchmarks were presented in **Table 1**. Among these dispersant chemicals, the surfactant, Dioctylsulfosuccinate, sodium salt (DOSS), has the lowest benchmark value and is of the highest toxicity.

Table 1. Summary of USEPA analytical methods and screening levels of dispersant chemicals in water samples (excerpted from OSAT, 2010)

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

The toxicity data used as the reference for the benchmark were basically LC₅₀ (median lethal concentration) of constant exposures which was regarded as problematic by toxicologists (Schmidt, 2010). Moreover, this benchmark used a concentration that is highly possible to be toxic to species at certain stages, especially larvae. Kujawinski et al. (2011) published their method and results of monitoring the DOSS associated with Deepwater Horizon oil spill. The fates analysis of DOSS showed that the presence of DOSS even after 6 months of the spill which indicated their recalcitrant nature in the ecosystem (OSAT, 2010).

Due to the above mentioned concerns of DOSS, it is timely and important to develop better alternatives. Biosurfactant-based dispersants, with the proven features of high effectiveness, lower toxicity and persistency, can be a promising option. However, the current bottleneck of biosurfactant application is the high production cost. It was estimated that biosurfactants would cost 3-10 times of synthetic surfactants (Mulligan and Gibbs, 1993). The development of hyper producing mutant was proposed as one of the strategies to cope with the economic constrains (Mukherjee et al., 2006). The hyper producing mutants in the literature mainly belonged to the genera of *Pseudomonas*, *Bacillus* and *Acinetobacter* which are the producers of rhamnolipid, surfactin and emulsan, respectively. A *Rhodococcus erythropolis* strain SB-1A was isolated from oily contaminated seawater (Cai et al., 2014). In the preliminary test, it produced biosurfactants with proper hydrophilic-lipophilic balance (HLB) to disperse crude oil. No studies genetically modified *Rhodococcus* strains for hyper production of

biosurfactant while such biosurfactant was barely used as oil spill response agents in the literature.

In the present study, hyper producing mutant of *Rhodococcus erythropolis* strain SB-1A was screened with oil spreading technique. The resulting mutant was characterized and their feasibility as oil spill response agent was tested.

Materials and Methods

Bacterial Strains and Growth Condition

Rhodococcus erythropolis strain SB-1A was isolated from seawater samples in the vicinity of offshore platforms (Cai et al., 2014). The strain was cultured with the production medium (PM) composed of MgSO₄, 0.2 g; CaCl₂·2H₂O, 0.05 g; KH₂PO₄, 3.4 g; K₂HPO₄·3H₂O, 4.4 g; (NH₄)₂NO₃, 1 g; FeCl₃, 0.05 g; Glucose, 1g; nutrient broth 0.1g; NaCl, 26 g in 1L of distilled water, with 3% (v/v) n-hexadecane. The incubation maintained at 30°C while shaking at 200 rpm (Cai et al., 2014).

Ultraviolet Mutagenesis

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

Screening Technique for Hyper Producing Mutants

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

Determination of Critical Micelle Dilution (CMD)

The selected hyper producing mutant was incubated in 1 L PM medium for 4 days at 30°C while shaking at 200 rpm. The resulting culture was used for the determination of CMD. CMD can reflect the concentration of produced biosurfactants. It was defined as the dilution of the culture broth upon reaching the critical micelle concentration (Shavandi et al., 2011). After centrifuge at 10,000 rpm for 10 min and discard the pellet, the 10 mL of cell free broth were diluted with distilled water, while the surface tension of each dilution was measured. The CMD was determined as the highest dilution with which the surface tension did not significantly increase. As the broth consists of both aqueous and oil phase, each dilution was conducted with sonication to ensure homogeneity. Before each measurement, the sonicated solution was allowed to stand for 15-20 min to achieve equilibrium.

Production and Recovery of Crude Biosurfactants

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

Thin Layer Chromatography (TLC) Analysis

After the MTBE extraction and removal of upper phase, the remaining aqueous phase was again extracted with chloroform and methanol 2:1. Ten µL aliquots of The MTBE extracted solution, chloroform/methanol extracted solution and the remaining aqueous phase were added on the TLC plates. Subsequently, ninhydrin n-butanol-acetic acid and phenol-sulfuric acid were sprayed on the TLC plates respectively. The plates were then heated at 110°C for 10 min for color development, in order to illustrate the presence of amino acids and carbohydrates in different extracts and the remaining aqueous phase. The recipe of ninhydrin n-butanol-acetic acid: 100mL n-butanol dissolves 0.3 g ninhydrin followed by the addition of 3 mL acetic acid. The target compounds were amino acids, which showed red or purple. The recipe of phenol-sulfuric acid: 3 g phenol and 5 ml concentrated H₂SO₄ were added in 95 mL ethanol (Touchstone, 1992). The target compounds were carbohydrates, which showed red or brown.

The concentrated MTBE extracts were dried and re-dissolved in chloroform and subject to TOC analysis on silica gel F₂₅₄ with the following solvent system: chloroform/methanol/water (85:15:2, v/v/v) for lipopeptides. To detect functional groups, ninhydrin n-butanol-acetate acid stain was used.

Baffle Flask Test (BFT) for Dispersant Effectiveness

The baffle flask test was conducted following the protocol proposed by Sorial et al. (2004). Briefly, artificial sea water was prepared as 3.5% sea salt solution. One hundred twenty mL of the artificial sea water equilibrated at the desired temperature was added to the baffle flasks. One hundred µL of crude oil (from Newfoundland offshore platform) was added on the surface of the synthetic sea water. Crude biosurfactants were then mixed with solvent and added to the center of the oil slick with Corexit dispersants and biosurfactants produced by wild type strain as references, each treatment had an oil free control. The flasks were placed on an orbital shaker and mixed for 10 min at 200 rpm. After the 10 min of settling, the first 2 mL of sample was drained form the stopcock and discarded, then, 30 mL of the sample was collected in a separatory funnel and extracted three times with 5 mL dichloromethane (DCM). The extracts was then diluted to a final volume of 20 mL and subjected to spectroscopy analysis at the wavelength of 340 nm, 370 nm and 400 nm respectively with DCM as blank. The calculation of dispersion efficiency followed the below procedure:

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

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

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

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

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

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

Results and discussion

Screening of hyper producing mutant

In total 71 UV irradiated mutants of *Rhodococcus ethroypolis* SB-1A were collected for the screening of hyper producing mutants using oil spreading technique. In most relevant studies, the high put through method used to screen the hyper producing mutant were hemolytic activity test (HA) (Iqbal et al., 1995; Mulligan et al., 1989) and blue agar plate test (BAP) (Lin et al., 1998; Tahzibi et al., 2004). However, Youssef et al. (2004) demonstrated that 38% biosurfactant producing strains showed no response in the HA tests. In addition, HA tests showed low correlation with surface tension indicating that HA is not a reliable method to detect biosurfactant. Meanwhile, Satpute et al. (2008) used 45 marine biosurfactant producing strains to evaluate the performance of different screening methods and found out HA test was not totally reliable. In their study, only one strain showed positive response to BAP test. In both studies, oil spreading technique was found as a reliable technique and was recommended by both authors. Good correlation ($r^2=0.997$) was found between concentration of biosurfactant and the diameter of the clear zone (Youssef et al., 2004). The oil spreading technique was found with high sensitivity even when the concentration of biosurfactant was low and water-insoluble (Morikawa et al., 2000).

In this study oil spreading technique was applied first time as the high putthrough screening method for the hyperproducers. In **Figure 1A**, #1 plate showed the testing result of the wild type strain. The rest 71 plates showed in results of the mutants. It can be seen from **Figure 1A** that Mutant 47 had the largest clear zone with diameter around 2.5 cm while the wild type strain had a clear zone with the diameter around 1.5 cm (**Figure 1B**).

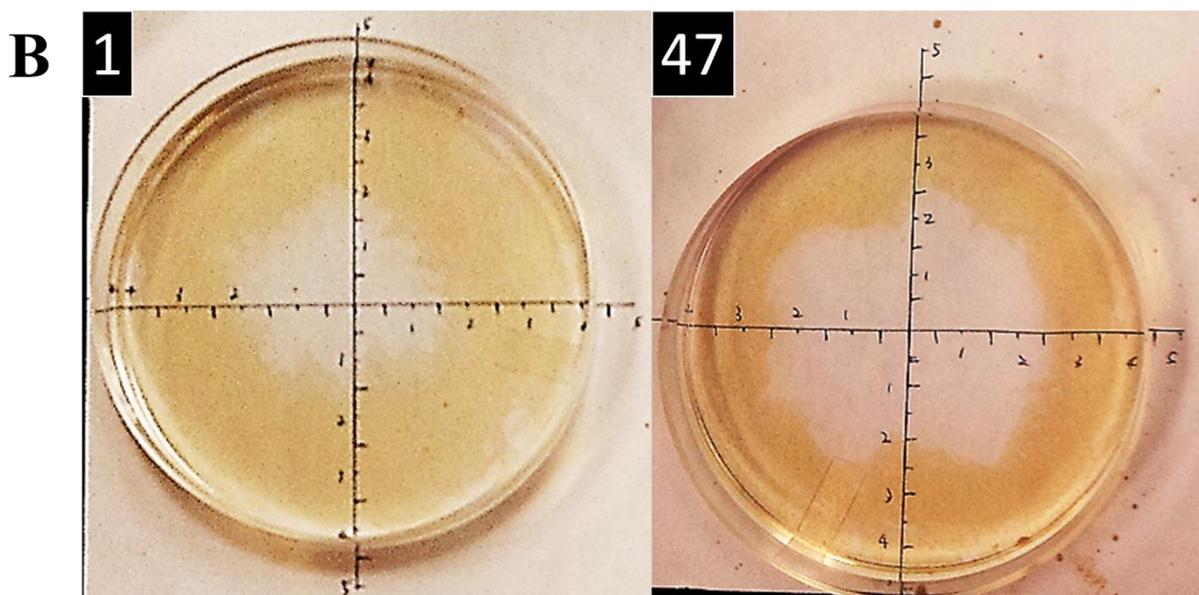
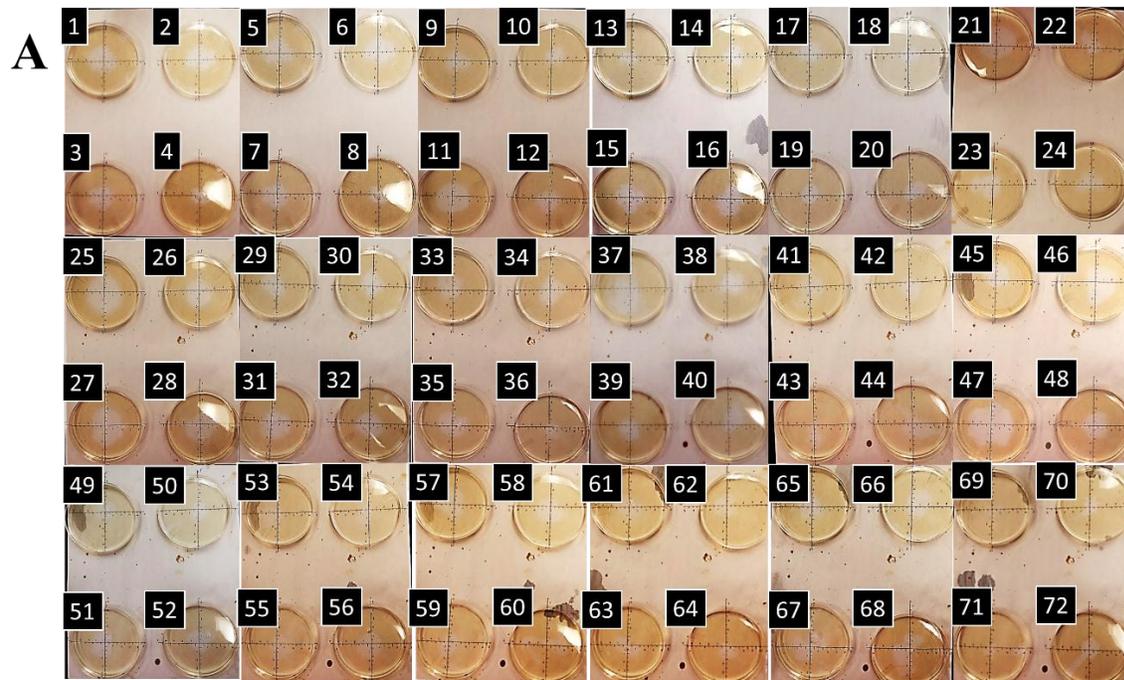


Figure 1. Results of oil spreading test (A. wild strain and the 71 mutants; B. wild type and mutant #47)

Biosurfactant production

The CMD have been used as a measure of biosurfactant concentration (Shavandi et al., 2011). When incubated with the same medium under the same condition. Mutant #47 had a CMD value of 62.5 while the wild type strain only have a CMD value of 15.4 (Figure 2). The biosurfactant concentration produced by the mutant #47 was 4.07 times of the wild type. The CMD test was conducted after 4 days of incubation, while the oil spreading test was conducted after 2 days of incubation. The diameter of the mutant #47 was 1.7 times of the wild type. Based

on the estimation of both methods, the mutant #47 produced biosurfactants with higher rate after the first 48 h. Other studies found the CMD of culture broth of wide type *Rhodococcus* strains were higher than the present study. Philp et al. (2002) found culture of a *Rhodococcus ruber* strain had the CMD of around 90 after 4 days of incubation. Shavandi et al. (2011) found the culture broth of a *Rhodococcus* sp. strain TA6 had the CMD around 35 under diverse conditions. We surmise the possible reason for the higher CMD was that both studies did not apply sonication while diluting the culture broth. In the present study, sonication was applied to ensure each dilution was homogeneous. The produced biosurfactants mainly present at the interface between aqueous and water-insoluble carbon source phases, especially for biosurfactants produced by *Rhodococcus* strains (Franzetti et al., 2010). The dilution procedure accompanied by sonification would be more efficient than direct dilution of two-phased culture broth leading to lower dilution factors.

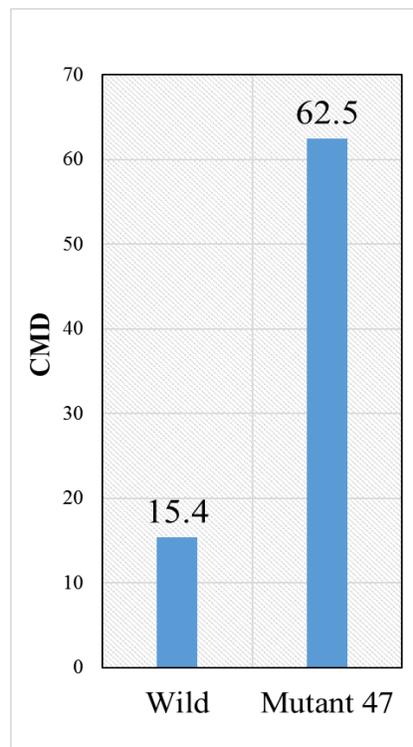


Figure 2. CMD improvement of the Mutant #47

Characterization of the produced biosurfactants

Solvent system of chloroform: methanol (2:1 v:v) have been commonly used for extraction of biosurfactants from the bacterial culture broth (Franzetti et al., 2010). MTBE was later found as a comparable alternative of the chloroform: methanol solvent system and was recommended due to its low toxicity and flammability for large scale application (Kuyukina et al., 2001). The biosurfactants mainly composed of fatty acid moiety and either carbohydrate moiety (glycolipid) or amino acid moiety (lipopeptide) (Soberón-Chávez and Maier, 2011). As shown in **Figure 3**, MTBE extracts contained similar amount of lipopeptides with chloroform: methanol

extracts. The MTBE extracts were concentrated for 10 times while the chloroform: methanol extracts was not concentrated. Besides, MTBE was used for the first round extraction while chloroform: methanol was used for the second round. MTBE showed poor recovery efficiency for lipopeptides when comparing with chloroform: methanol solvent. In the contrast, as shown in **Figure 4**, MTBE extracts contained much more glycolipids than chloroform: methanol extracts. MTBE showed good efficiency for extracting glycolipids. Moreover, the remaining culture broths after extraction still have large amount of amino acids and limited amount of carbohydrate. The original medium contained 1 g glucose (carbohydrates) and 0.1 g nutrient broth (amino acids). After 4 days of microbial transfer, most glucose was consumed while nutrient broth was barely used. It is surprising that culture broth of wild type had more glycolipids and lipopeptides than the mutant #47 while the biosurfactant concentration in the culture broth of mutant #47 was around 3 times higher. We surmise that the mutant #47 produced certain biosurfactants that cannot be effectively extracted by both MTBE and chloroform: methanol solvent. According to the results in **Figure 3-4**, both glycolipids and lipopeptides were presented in the culture broth of the wild type strain and the mutant #47.

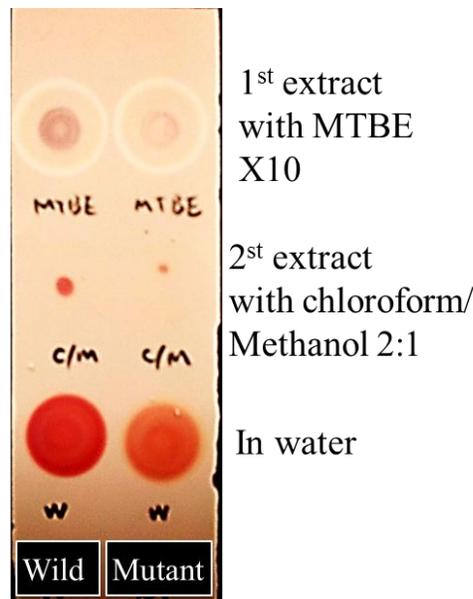


Figure 3. Ninhydrin stain for amino acid moiety

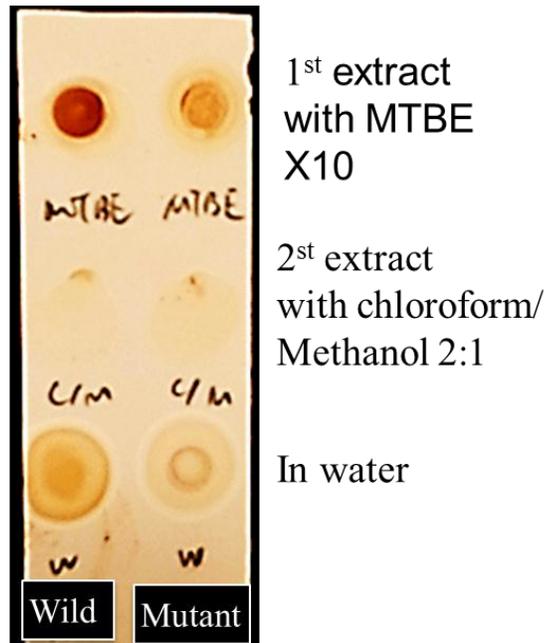


Figure 4. Phenol-sulfuric acid stain for sugar moiety

Moreover, the TLC plate developed with chloroform: methanol: water = 65:25:4 and stained with ninhydrin agent was shown in **Figure 5**. The spot of lipopeptides of both wild type strain and the mutant #47 appeared with the same response factor (Rf). The result indicated that the culture broth of the wild type strain and the mutant #47 contained one type of lipopeptide and the lipopeptide was the same in both culture broths.



Figure 5. TLC of wild type strain and mutant #47 (developed with Chloroform:methanol:water=65:25:4; stained with ninhydrin agent)

Dispersion efficiency of the *Rhodococcus erythropolis* mutant

The dispersion efficiency of crude biosurfactants from *Rhodococcus erythropolis* SB-1A wild type strain and mutant #47 was summarized in **Table 2**. The dispersion efficiency of crude biosurfactants produced by the mutant #47 was 1.35 times of the biosurfactants produced by the wild type strain. When compared with the Corexit dispersants, both biosurfactants had better performance than the Corexit 9527 while not as good as Corexit 9500. The result agreed with the conclusion of Blondina and Sowby (1997) that Corexit 9500 was generally more effective than 9527. They also found that Corexit 9500 was less affected by the variations in water salinity conditions (Blondina and Sowby, 1997). The biosurfactant from the mutant #47 is comparable with the Corexit 9500 demonstrated its potential to replace DOSS as a more environmental friendly alternative. The productivity could be further improved with technologies such as genome shuffling (Zhao et al., 2012) using some superior mutants screened in this study as the parent strains. Moreover, to further reduce the cost of production, the incubation media/conditions and the post-processing process can be further optimized to achieve economic production that may compete with DOSS (Mukherjee et al., 2006).

Table 2. Absorbance of BFT effluent and dispersion efficiency

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

Conclusions

A *Rhodococcus erythropolis* SB-1A strain isolated from oily wastewater from Newfoundland offshore was used as the parent strain to develop hyper producing mutants that produced biosurfactants as oil dispersion agents. Genetically improved biosurfactant production was studied first time based a *Rhodococcus* strain while such biosurfactant was barely reported as an oil spill response agent. After the UV induced mutagenesis, oil spreading technique was applied as the high put through method to screen hyper producing mutants. Subsequently, mutant #47 was found as the superior mutant and was subjected to further analysis. The culture broth of both the wild type strain and the mutant #47 contained lipopeptides and glycolipid. The dispersion efficiency determined by BFT showed that the mutant #47 was 1.35 times of the

Joint Conferences:

The 2014 Annual Conference of the International Society for Environmental Information Sciences (ISEIS)

The 2014 Atlantic Symposium of the Canadian Association on Water Quality (CAWQ)

The 2014 Annual General Meeting and 30th Anniversary Celebration of the Canadian Society for Civil Engineering Newfoundland and Labrador Section (CSCE-NL)

The 2nd International Conference of Coastal Biotechnology (ICCB) of the Chinese Society of Marine Biotechnology and Chinese Academy of Sciences (CAS)

biosurfactants produced by the wild type strain. The dispersion efficiency of the mutant #47 is comparable to the Corexit 9500 while better than Corexit 9527 when dispersing the Newfoundland offshore crude oil.

Acknowledgements

The authors would like to express the gratitude to Petroleum Research Newfoundland and Labrador (PRNL) and Research & Development Corporation (RDC) of Newfoundland and Labrador for their support.

References

- Blondina G. and Sowby M. (1997). Comparative efficacy of two Corexit R dispersants as measured using California's modified swirling flask test.
- Board of Ocean Studies (2005). Oil Spill Dispersants:: Efficacy and Effects, National Academies Press.
- Cai Q., Zhang b., Chen B., Song X. and Cao T. (2014). Screening of biosurfactant producing bacteria from offshore oil and gas platforms in North Atlantic Canada. Journal of Industrial Microbiology and Biotechnology (submitted).
- Franzetti A., Gandolfi I., Bestetti G., Smyth T.J. and Banat I.M. (2010). Production and applications of trehalose lipid biosurfactants. European journal of lipid science and technology 112(6), 617-627.
- Iqbal S., Khalid Z. and Malik K. (1995). Enhanced biodegradation and emulsification of crude oil and hyperproduction of biosurfactants by a gamma ray - induced mutant of *Pseudomonas aeruginosa*. Letters in Applied Microbiology 21(3), 176-179.
- Kujawinski E.B., Kido Soule M.C., Valentine D.L., Boysen A.K., Longnecker K. and Redmond M.C. (2011). Fate of dispersants associated with the Deepwater Horizon oil spill. Environmental science & technology 45(4), 1298-1306.
- Kuyukina M.S., Ivshina I.B., Philp J.C., Christofi N., Dunbar S.A. and Ritchkova M.I. (2001). Recovery of *Rhodococcus* biosurfactants using methyl tertiary-butyl ether extraction. Journal of Microbiological Methods 46(2), 149-156.
- Lin S.C., Lin K.G., Lo C.C. and Lin Y.M. (1998). Enhanced biosurfactant production by a *Bacillus licheniformis* mutant. Enzyme and microbial Technology 23(3), 267-273.
- Morikawa M., Hirata Y. and Imanaka T. (2000). A study on the structure–function relationship of lipopeptide biosurfactants. Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids 1488(3), 211-218.
- Mukherjee S., Das P. and Sen R. (2006). Towards commercial production of microbial surfactants. Trends in biotechnology 24(11), 509-515.
- Mulligan C.N., Chow T.Y.-K. and Gibbs B.F. (1989). Enhanced biosurfactant production by a mutant *Bacillus subtilis* strain. Applied microbiology and biotechnology 31(5-6), 486-489.
- Mulligan C.N. and Gibbs B.F. (1993). Factors influencing the economics of biosurfactants. SURFACTANT SCIENCE SERIES, 329-329.
- National Commission Final Report (2011). Deep Water, The Gulf Oil Disaster and the Future of Offshore Drilling (Commission Report)
- OSAT (2010). Summary report for subsea and subsurface oil and dispersant detection: Sampling and monitoring, Operational Science Advisory Team, Darby, USA.
- Philp J., Kuyukina M., Ivshina I., Dunbar S., Christofi N., Lang S. and Wray V. (2002). Alkanotrophic *Rhodococcus ruber* as a biosurfactant producer. Applied microbiology and biotechnology 59(2-3), 318-324.
- Satpute S., Bhawsar B., Dhakephalkar P. and Chopade B. (2008). Assessment of different screening methods for selecting biosurfactant producing marine bacteria. Indian Journal of Marine Sciences 37(3), 243.
- Schmidt C. (2010). Between the devil and the deep blue sea: dispersants in the gulf of Mexico. Environmental health perspectives 118(8), a338-344.
- Scientific and Environmental Associates (2003). Selection Guide for oil spill applied technologies. Volume 1- Decision making, Virginia, USA.
- Shavandi M., Mohebali G., Haddadi A., Shakarami H. and Nuhi A. (2011). Emulsification potential of a newly isolated biosurfactant-producing bacterium, *Rhodococcus* sp. strain TA6. Colloids and Surfaces B: Biointerfaces 82(2), 477-482.
- Soberón-Chávez G. and Maier R.M. (2011). Biosurfactants, pp. 1-11, Springer.

Joint Conferences:

The 2014 Annual Conference of the International Society for Environmental Information Sciences (ISEIS)

The 2014 Atlantic Symposium of the Canadian Association on Water Quality (CAWQ)

The 2014 Annual General Meeting and 30th Anniversary Celebration of the Canadian Society for Civil Engineering Newfoundland and Labrador Section (CSCE-NL)

The 2nd International Conference of Coastal Biotechnology (ICCB) of the Chinese Society of Marine Biotechnology and Chinese Academy of Sciences (CAS)



Sorial G.A., Venosa A.D., Koran K.M., Holder E. and King D.W. (2004). Oil spill dispersant effectiveness protocol. II: Performance of revised protocol. *Journal of Environmental Engineering* 130(10), 1085-1093.

Tahzibi A., Kamal F. and Assadi M.M. (2004). Improved production of rhamnolipids by a *Pseudomonas aeruginosa* mutant. *Iranian Biomedical Journal* 8(1), 25-31.

The Federal Interagency Solutions Group (2010). Oil Budget Calculator Science and Engineering Team, Oil Budget Calculator: Deepwater Horizon-Technical Documentation, p. 25.

Touchstone J.C. (1992). *Practice of thin layer chromatography*, John Wiley & Sons.

Youssef N.H., Duncan K.E., Nagle D.P., Savage K.N., Knapp R.M. and McInerney M.J. (2004). Comparison of methods to detect biosurfactant production by diverse microorganisms. *Journal of Microbiological Methods* 56(3), 339-347.

Zhao J., Li Y., Zhang C., Yao Z., Zhang L., Bie X., Lu F. and Lu Z. (2012). Genome shuffling of *Bacillus amyloliquefaciens* for improving antimicrobial lipopeptide production and an analysis of relative gene expression using FQ RT-PCR. *Journal of industrial microbiology & biotechnology* 39(6), 889-896.