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Biosurfactant produced by a *Rhodococcus erythropolis* mutant as an oil spill response agent

Qinhong Cai, Baiyu Zhang, Bing Chen, Tong Cao and Ze Lv

ABSTRACT

Biosurfactants have been considered as superior alternatives to currently used surfactants as they are generally more biodegradable, less toxic, and better at enhancing biodegradation. However, the application of biosurfactants is limited by the availability of economic biosurfactants 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 offshore oily water samples. One of its mutants 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 approximately four times those of the parent strain. The results obtained with thin layer chromatography indicated the produced biosurfactant remained unchanged between the mutant and the parent strain. In addition, the produced biosurfactants were recovered with solvent extraction and applied as the oil spill response agents. 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. When compared with Corexit dispersants, it was found that the produced biosurfactants performed better than Corexit 9527 and were comparable with Corexit 9500.

Key words | biosurfactant, hyper producing mutants, oil spill response agent, *Rhodococcus erythropolis*

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INTRODUCTION

Offshore oil spills are of tremendous concern due to the enormous economic loss and the harm to ecological systems that they may cause. Among diverse oil spill response technologies such as *in situ* burning, booming and skimming, absorption and solidification, dispersion has high feasibility and effectiveness in open water (Fingas 2010). In the meantime, it is not restricted by limitation factors such as accessibility, weather conditions, sea states, and oil thickness that other countermeasures require (Scientific & 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 change the interfacial properties, thus breaking down oil slicks into small droplets (Fingas 2010). They have been 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. In total, 4.05 million liters of Corexit dispersants (Corexit 9500 and Corexit 9527) were applied on the surface, while 2,918,550 liters 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 are presented

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

* LC = Liquid chromatography.
** GC = Gas chromatography.
*** MS = Mass spectroscopy.

in Table 1. Among these dispersant chemicals, the surfactant, dioctylsulfosuccinate, sodium salt (DOSS) has the lowest benchmark value and is of the highest toxicity.

The toxicity data used as the reference for the benchmark were basically the LC_{50} (median lethal concentration) of constant exposures, which was regarded as problematic by toxicologists (Schmidt 2010). Moreover, this benchmark used a concentration that is highly possible to be toxic to species at certain stages, especially larvae. Kujawinski *et al.* (2011) published their method and results of monitoring the DOSS associated with the Deepwater Horizon oil spill. The fate analysis of DOSS showed 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 about 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 three to 10 times as much as synthetic surfactants (Mulligan & Gibbs 1993). The development of a hyper producing mutant was proposed as one of the strategies to cope with the economic constraints (Mukherjee *et al.* 2006). The hyper producing mutants in the literature only belonged to a limited genera of *Pseudomonas*, *Bacillus* and *Acinetobacter*, which are the producers of rhamnolipid, surfactin and emulsan, respectively (Mukherjee *et al.* 2006). Previously, no studies have attempted to genetically modify *Rhodococcus* strains for hyper production of biosurfactant. Besides, the dispersing abilities of the above mentioned hyper production mutants in the literature have never been investigated. The capacity of a biosurfactant being used as an oil dispersant is highly dependent on the hydrophilic-lipophilic balance (HLB) value. When the HLB is between 10 and 12, the micelles would stabilize the oil-in-water emulsions, thus breaking down the oil slick and dispersing the oil into the ocean column through the mixing of currents (Pacwa-Płociniczak *et al.* 2011). A *Rhodococcus erythropolis* strain SB-1A was isolated from oily contaminated seawater in Newfoundland (Cai *et al.* 2014) with proper HLB and a potential to disperse crude oil. Therefore, a further investigation of the oil dispersion efficacy of biosurfactants produced by the hyper production *Rhodococcus* mutant is highly desired.

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

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.*

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2014). The strain was cultured with a production medium (PM) composed of $MgSO_4$, 0.2 g; $CaCl_2{\cdot}2H_2O$, 0.05 g; KH_2PO_4, 3.4 g; $K_2HPO_4{\cdot}3H_2O$, 4.4 g; $(NH4)_2NO_3$, 1 g; $FeCl_3$, 0.05 g; glucose, 1 g; nutrient broth 0.1 g; NaCl, 26 g in 1 L of distilled water, with 3% (v/v) n-hexadecane. Incubation was maintained at 30 °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 3,000 cells were plated on PM agar plates. The cells were UV radiated for 45 s with a Thermo scientific 1300 Series Class II, Type A2 Biological Safety Cabinet. This dosage of UV radiation gave around 10–20% survival of the colonies. The UV irradiated cells were then incubated on the agar plates at 30 °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, an oil spreading technique was used to screen hyper producing mutants (Morikawa *et al.* 2000). Ten microliters 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 microliters of bacterial culture was gently added to the center of the oil membrane. A clear zone was formed due to the activity of the surfactants. The area of the clear zone was used to reflect the concentration of the produced surfactants.

Determination of critical micelle dilution

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 critical micelle dilution (CMD). CMD can reflect the concentration of produced biosurfactants. It was defined as the dilution of the culture broth upon reaching the critical micelle concentration (Shavandi *et al.* 2011). After centrifuging at 10,000 rpm for 10 min and discarding the pellet, the 10 mL of cell free broth was diluted with distilled water, while the surface tension of each dilution was measured. The surface tension data were plotted against concentrations of the broth. The CMD values were then found as intersection points of tangential lines (Sheppard & Mulligan 1987). As the broth consists of both aqueous and oil phases, 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 extracted by shaking with methyl tert-butyl ether (MTBE) of the same volume for 24 h. The upper phase was collected and concentrated using rotary evaporation. The concentrated solution was washed with petroleum ether to remove the remaining hexadecane in the solution (Kuyukina *et al.* 2001). After washing, the crude biosurfactant products were collected and stored at −20 °C before analysis and testing.

Thin layer chromatography analysis

After MTBE extraction and removal of the upper phase, the remaining aqueous phase was again extracted with chloroform and methanol 2:1. Ten microliter aliquots of the MTBE extracted solution, chloroform/methanol extracted solution and the remaining aqueous phase were added on the thin layer chromatography (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 was: 100 mL n-butanol dissolves 0.3 g ninhydrin followed by the addition of 3 mL acetic acid. The target compounds were amino acids, which showed red or purple. The recipe of phenol-sulfuric acid was: 3 g phenol and 5 mL concentrated H_2SO_4 were added in 95 mL ethanol (Touchstone 1992). The target compounds were carbohydrates, which showed red or brown.

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

Baffled flask test (BFT) for evaluation of dispersant effectiveness

The baffled flask test was conducted following the protocol proposed by Sorial *et al.* (2004). Briefly, artificial sea water was prepared as 3.5% sea salt solution. One hundred and twenty milliliters of the artificial sea water equilibrated at the desired temperature was added to the baffled flasks. One hundred microliters of crude oil (from a Newfoundland offshore platform) was added to the surface of the synthetic sea water. Then, crude biosurfactants were 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 10 min of settling, the first 2 mL of the sample was drained from the stopcock and discarded, then 30 mL of the sample was collected in a separatory funnel and extracted three times with 5 mL dichloromethane (DCM). The extracts were then diluted to a final volume of 20 mL and subjected to spectroscopy analysis at the wavelengths of 340 nm, 370 nm and 400 nm, respectively, with DCM as blank. The calculation of dispersion efficiency followed the procedure below.

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

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

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

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

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

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, and 300 μL of crude oil-DCM stock were added to 30 mL of synthetic seawater in separatory funnels and extracted three times with DCM. The final DCM volume for each standard solution was adjusted to 20 mL and subjected to spectroscopy analysis with DCM as the blank at 340, 370 and 400 nm. The area of each standard was calculated according to Equation (1). The slope of the calibration curve was thus determined by plotting the area against the concentration of the crude oil in the standards. The concentrations of biosurfactants and Corexit used were based at the same times of their CMCs. The experiments were conducted at room temperature (i.e. around 25 °C).

Data analysis

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

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 an oil spreading technique. In most relevant studies, the high throughput method used to screen the hyper producing mutant was the hemolytic activity (HA) test (Mulligan *et al.* 1989; Iqbal *et al.* 1995) and blue agar plate (BAP) test (Lin *et al.* 1998; Tahzibi *et al.* 2004). However, Youssef *et al.* (2004) demonstrated

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that 38% of biosurfactant producing strains showed no response in the HA tests. In addition, HA tests showed low correlation with surface tension, indicating that HA is not a reliable method to detect biosurfactant. Meanwhile, Satpute *et al.* (2008) used 45 marine biosurfactant producing strains to evaluate the performance of different screening methods and discovered that the HA test was not totally reliable. In their study, only one strain showed a positive response to the BAP test. In both studies, the oil spreading technique was found to be a reliable technique and was recommended by both authors. Good correlation ($r^2 = 0.997$) was found between the concentration of biosurfactant and the diameter of the clear zone (Youssef *et al.* 2004). The oil spreading technique was found to have high sensitivity even when the concentration of biosurfactant was low and water-insoluble (Morikawa *et al.* 2000).

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

Biosurfactant production

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

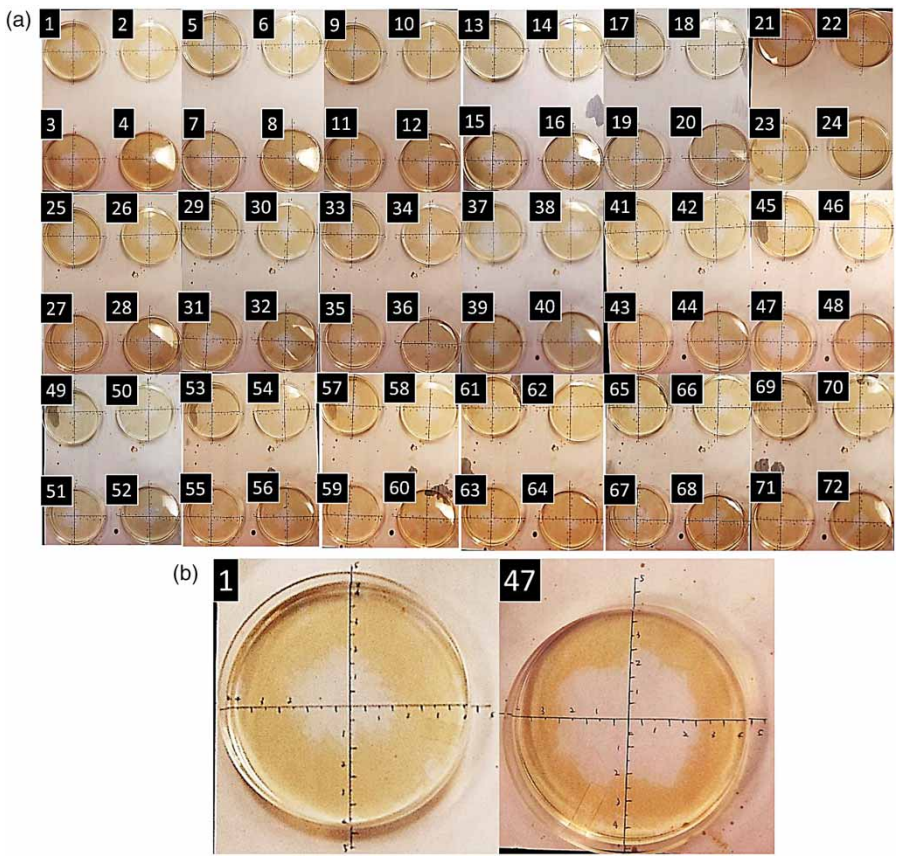

Figure 1 | Results of oil spreading test. (a) Wild strain and the 71 mutants; (b) wild type and mutant #47).

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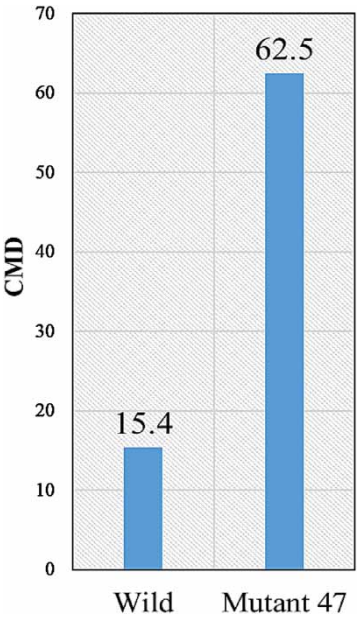

Figure 2 | CMD improvement of mutant #47.

higher rates after the first 48 h. Other studies found the CMD of culture broth of wild type *Rhodococcus* strains was higher than the present study. Philp *et al.* (2002) found that the culture of a *Rhodococcus ruber* strain had a CMD of around 90 after 4 days of incubation. Shavandi *et al.* (2011) found the culture broth of a *Rhococococcus* sp. strain TA6 had a CMD of around 35 under diverse conditions. 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 sonication, would be more efficient than direct dilution of two-phased culture broth. However, sonication before dilution would also lead to lower dilution factors.

Characterization of the produced biosurfactants

Methanol (2:1 v:v) has been commonly used for the extraction of biosurfactants from bacterial culture broth (Franzetti *et al.* 2010). MTBE was later found to be a comparable alternative to the chloroform:methanol solvent system and was recommended, due to its low toxicity and flammability, for large scale application (Kuyukina *et al.* 2001). The biosurfactants are mainly composed of a fatty acid moiety and either a carbohydrate moiety (glycolipid) or an amino acid moiety (lipopeptide) (Soberón-Chávez & Maier 2011). As shown in Figure 3, MTBE extracts contained similar amounts of lipopeptides with chloroform:methanol extracts. However, the MTBE extracts were concentrated for 10 times while the chloroform:methanol extracts were not concentrated. Besides, MTBE was used for the first round extraction while chloroform:methanol was used for the second round. Therefore, MTBE showed poor recovery efficiency for lipopeptides when compared with the chloroform:methanol solvent. In contrast, as shown in Figure 4, MTBE extracts contained many more glycolipids than chloroform:methanol extracts. MTBE showed good efficiency for extracting glycolipids. Moreover, the remaining culture broth after extraction still had a large amount of amino acids and a limited amount of carbohydrate. This might be explained by the following: the original medium contained 1 g glucose (carbohydrates) and 0.1 g nutrient broth (amino acids); after 4 days of microbial transfer, most glucose was consumed, while the nutrient broth was barely used. It is surprising that the culture broth of the wild type had more glycolipids and lipopeptides than mutant #47, while the biosurfactant concentration in the culture broth of mutant #47 was around three times higher. We surmise that mutant #47 produced certain biosurfactants that cannot be effectively extracted by both

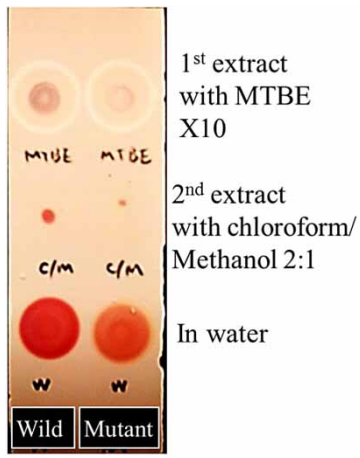

Figure 3 | Ninhydrin stain for amino acid moiety.

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MTBE and the chloroform:methanol solvent. According to the results in Figures 3 and 4, both glycolipids and lipopeptides were presented in the culture broth of the wild type strain and mutant #47.

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

Dispersion efficiency of the *Rhodococcus erythropolis* mutant

The dispersion efficiency of crude biosurfactants from the *R. erythropolis* SB-1A wild type strain and mutant #47 are summarized in Table 2. The dispersion efficiency of crude biosurfactants produced by mutant #47 was 1.35 times that of the biosurfactants produced by the wild type strain. When compared with the Corexit dispersants, both biosurfactants had better performance than the Corexit 9527, while they were not as good as Corexit 9500. The result agreed with the conclusion of Blondina & 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 & Sowby 1997). The biosurfactant from mutant #47 is comparable with the Corexit 9500 for dispersing crude oil produced from Newfoundland offshore. The toxicity and persistency of the produced biosurfactant will be determined in future studies. The productivity could be further improved with technologies such as genome shuffling (Zhao *et al.* 2012) using some superior mutants screened in this study as the parent strains. Moreover, to further reduce the cost of production, the incubation media/conditions and the post-processing process can be further optimized to achieve economic production that may compete with DOSS (Mukherjee *et al.* 2006).

Table 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

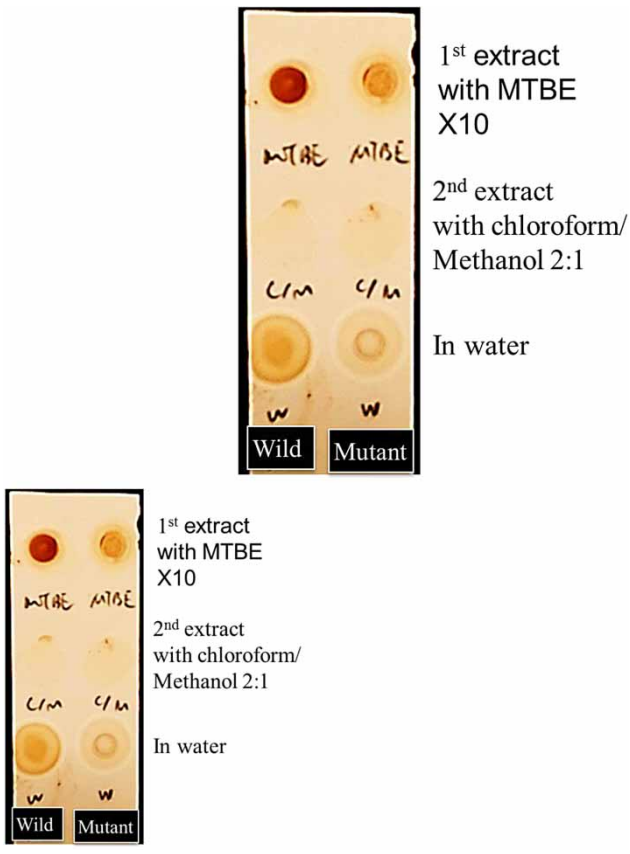

Figure 4 | Phenol-sulfuric acid stain for sugar moiety.

CONCLUSIONS

An *R. erythropolis* SB-1A strain isolated from oily wastewater from Newfoundland offshore was used as the parent strain to develop hyper producing mutants that produced biosurfactants as oil dispersion agents. Genetically improved biosurfactant production was studied for the first time based on a *Rhodococcus* strain, while such a biosurfactant was barely reported as an oil spill response agent. The parent strain was previously found with proper HLB to disperse crude oil in our laboratory. UV induced mutagenesis was conducted to generate possible mutants, and

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subsequently, an oil spreading technique was applied as the high throughput method to screen hyper producing mutants. The oil spreading technique was found to be a reliable and semi-quantitative approach to effectively screen biosurfactant hyper producing strains. Subsequently, mutant #47 was found to be the superior mutant and was subjected to further analysis. The culture broth of both the wild type strain and mutant #47 contained lipopeptides and glycolipid. The lipopeptides in both cultures were the same and of a single component. The dispersion efficiency determined by BFT showed that the dispersion efficiency of mutant #47 was 1.35 times that of the biosurfactants produced by the wild type strain. The dispersion efficiency of mutant #47 is comparable to the Corexit 9500, while it is better than Corexit 9527 when dispersing the Newfoundland offshore crude oil.

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

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