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A NEW HIGH-YIELDING BIO-DISPERSANT PRODUCER MUTATED FROM *RHODOCOCCUS ERYTHROPOLIS* STRAIN P6-4P

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ABSTRACT

Preeminent effectiveness and feasibility of dispersants have been the key reasons for their widely serving as the response agents in oil spill responses. Moreover, dispersants can also overcome the limitation factors of other countermeasures like accessibility, weather conditions, sea states, and oil thickness. However, the public concerns of the usages of the chemically synthetic dispersants are also essential due to their toxicity and persistency in the ecosystem. Bio-dispersants can be a promising alternative as the proven features of lower toxicity and persistency while with high effectiveness, but its broad application prospects are currently restricted by the high production cost that is 3-10 times more than chemical synthetic ones because of the low productivity. Thus, a hyper bio-dispersant producer will be the desired coping strategy.

An isolated bio-dispersant producer from NL offshore, *Rhodococcus erythropolis* strain P6-4P was selected for generating high-yielding producers by mutation. After UV mutagenesis, 21 enhanced mutants were selected through oil spreading screening method. Further productivity quantify test of critical micelle dilution (CMD) with higher resolution was conducted to these mutants. An outstanding mutant showed CMD as high as 225 while 15.4 is the CMD of the wild type strain, which means the new mutant is 14.6 times increase. The 16S rDNA sequencing results revealed that the 16 S ribosomal DNA of the mutant 100% matched with the original strain indicating the mutation occurred on other parts of the genome which will be identified through next-generation sequencing and comparative analysis in the future study. This mutated high-yielding strain was capable to significantly improve the production rate and the total yield of bio-dispersants. The yield of crude bio-dispersant was 54g per liter with 6 days incubation. At 4mg/uL crude product/crude oil ratio, the dispersion effectiveness was found comparable to Corexit 9500A at 1:25 (dispersant/crude oil ratio). Future works on further mutagenesis base on this new high-producing strain by novel mutation methods were also discussed.

Keywords: Bio-dispersant, high-yielding mutant, *Rhodococcus erythropolis*

1. INTRODUCTION

Dispersants are detergent-like products for removing oil from the sea surface and disperse it into the water column, which simulate the degradation of the oil via natural processes (National Research Council 2005). Taking advantage of the energy in even small waves, dispersants have the ability to disperse floating oil slicks into tiny droplets (<70 μm) that allow oil stably drifts apart in the water column (Prince and Butler 2014). Dispersants consist of surfactants dissolved in one or more solvents. They align themselves so that the lipophilic end of the surfactant molecule is attached to the oil phase and the hydrophilic end extends into the water phase. This reduces the interfacial surface

tension between water and oil, allowing oil to mix into the top 5±10 m of the water column as tiny (1±70 lm) droplets (Lessard and DeMarco 2000). This significantly increased surface area that allows microbial access, and finally leads to the broke down of oil by nature processes (Prince and Butler 2014).

Both public and private institutions throughout the world invest enormous economic resources and efforts to prevent offshore oil spill disasters. Despite these efforts, accidents do happen and sometimes large volumes of crude oil are released into marine environments (Zheng et al. 2014). The recent British Petroleum (BP) Deepwater Horizon (DWH) oil spill was reported as one such event that released over 4.9 million barrels (780,000 m3) of crude oil into the Gulf of Mexico (Hemmeret al., 2011). Corexit 9500 was the major dispersant used by emergency responders during the BP oil spill. Zheng (2014) explained that Corexit was expected to break down the crude oil into smaller droplets which would increase the oil–water surface area; also, dispersion can increase mass removal rates through evaporation and photo-oxidation. However, some recent studies indicated that the application of Corexit had led to certain environmental concern (Rico-Martínez and Shearer 2013; Zheng et al. 2014). Therefore, bio-dispersants (i.e., biosurfactants mixed with solvents) can be promising as their proven features of environmental friendly and persistency while with high effectiveness (Cai, et al., 2016). Currently, the production costs of biosurfactants are 3-10 times higher than chemical synthetic ones and the yields of production were relatively low (De et al., 2015; Nguyen et al., 2008). There hence is a need for a more comprehensive study that tackles the development of a high-yielding biosurfactant or bio-dispersant producer and mutation approach can be a coping strategy when questioning for a rapid and efficiency solution (Cai et al. 2016). Previously, there were rarely studies about hyper bio-dispersant producer from genetically modified Rhodococcus strains and the scanty results did not show satisfactory dispersing abilities.

Hence, the primary goal of this study is to develop a high-yielding bio-dispersant producer mutating from *Rhodococcus erythropolis* strain P6-4P. The hyper-producing mutant #25 was screened out by the oil-spreading technique. The improved productivity was confirmed with critical micelle dilution (CMD) test. Subsequently, the mutant (M25) was subjected to 16S rDNA sequencing and compared with the wild type strain. The product yield was quantified and compared with the wild type strain and other biosurfactant producers in the literature. The crude biosurfactant product was subjected to a scale-down Baffled flask dispersion effectiveness test and the obtained results were compared with Corexit 9500A.

2. MATERIALS AND METHODS

2.1 Bacterial strains and growth condition

Rhodococcus erythropolis strain P6-4P was isolated from seawater samples near the offshore platforms (Cai *et al.* 2015). Production medium (PM) was used as the culturing medium for *P6-4P*, which is 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 with shaking at 200 rpm for 6 days (Cai *et al*. 2015).

2.2 Ultraviolet mutagenesis

The *Rhodococcus erythropolis strain P6-4P* was grown for 6 days to logarithmic phase and then serial diluted to deferent concentration levels of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} to find the best result of approximately 3000 cells per plate. Each level was plated 1mL on two PM plates respectively, in which one plate was under UV radiate (Thermo scientific 1300 Series Class II, Type A2 Biological Safety Cabinet) for 45s and the other one for 1m 30s (Cai *et al.* 2016). The survived UV irradiated cells were incubated on the PM agar plates at 30 ºC in the dark until colonies were visible (Mulligan et al. 1989). The mutants were then inoculated in tubes (2 mL Eppendorf) with 1 mL PM and incubated for 48 h shaking at 200 rpm for further test.

2.3 Screening technique for high-yielding mutants

A reliable and high sensitive screening method, Oil Spreading, was used as the throughput method for screening high-yielding UV irradiated mutants (Cai *et al*. 2016). It was formed of a thin oil membrane by gently adding 10µL crude oil to the surface of 40 mL distilled water in a petri dish (D.I. 150 mm). For testing the activity of the surfactants, 10µL bacterial culture was gently added to the center of the oil membrane and the formed clear zone can be measured

then (Morikawa *et al.* 2000). This clear zone area was used to reflect the concentration of the produced surfactants, which shows linear correlation (Cai *et al*. 2016).

2.4 Determination of critical micelle dilution (CMD)

CMD is the dilution of the culture broth upon reaching the critical micelle concentration (CMC), which can reflect the concentration of produced biosurfactants (Shavandi *et al.* 2011). After centrifuging at 10,000 rpm for 10 min and discarding the pellet, the 10 mL cell free broth was diluted with distilled water. Then surface tension (ST) of each dilution was measured (Cai *et al*. 2014). Finally, according to Sheppard and Mulligan's method (1987), the CMD value can be found as the intersection point of tangential line on the figure of ST data plotted against concentrations of the broth. As the broth consists of both aqueous and oil phase, to ensure homogeneity while testing, each dilution was conducted with sonication first and then allowed to stand for 15–20 min to achieve equilibrium before each CMD measurement (Cai *et al*. 2016).

2.5 16S rDNA sequencing

The Mutant25 were subjected to 16S ribosomal DNA (rDNA) sequencing referring to Cai et al. (2016) method, which used universal bacterial primers F27 and R1493 that can be positioned in Escherichia Coli 8-27 and 1512-1493 respectively. DNA template that extracted from pure culture of *Rhodococcus* strains in the polymerase chain reaction (PCR) was an aliquot amount of Mutant25 culture using the primer pair. After the gel electrophoresis confirmation of a successful PCR reaction, PCR products were subjected to a cleanup process and measured by a NanoDrop spectrophotometer for determining the concentrations. Subsequently, the sequencing of cleaned-up PCR product was performed using Applied Biosystems 3130 and/or 3730 systems of the sequencing reactions in the Core Research and Instrument Training Network (CREAIT) of Memorial University (MUN). The final 16S rDNA result of Mutant 25 was aligned with the wild type 16S rDNA partial sequence by using the Basic Local Alignment Search Tool (BLAST).

2.6 Scale-down Baffled flask test

A scale down Baffled flask test was conducted to compare the effectiveness between Corexit 9600A and the frozen-dry crude bio-dispersant products. As shown in Table 1, the seawater: oil: dispersants ratio were kept the same, while the volumes of these components were scaled down. Different dosages of dispersants were used as shown in Table 1.

Table 1: Scale down vessel test of dispersant effectiveness

	Baffled flask test	Scaled down test
Seawater	120mL	30mL
Oil	100μL	25μL
Corexit: 1:10 (oil) & 1:25 (oil)		
Bio-dispersant: 100mg, 80mg, 60mg, 50mg, 40mg, 30mg, 20mg, 10mg		

3. RESULTS AND DISCUSION

3.1 Screening of high-yielding mutant

After 3 rounds of ultraviolet mutagenesis, 77 irradiated mutants of *Rhodococcus ethroypolis* P6-4P were collected for the screening of hyper producing mutants using oil-spreading method. Comparing to the original strain, 18 of the mutants showed a better oil-spreading result with diameters of around 4.5 cm while the wild type strain had a clear zone with a diameter of around 2.5 cm **(**Figure 1**)**. Among these 18 improved mutants, one mutant named M25 showed extraordinary oil-spreading result with a diameter more than 8cm, and the measuring results were limited by the size of the plate. The CMD test was used for further quantifying the productivity.

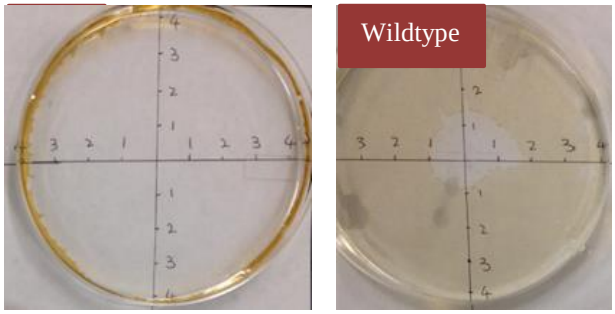

Figure 1: Results of oil spreading test for Mutant (M25) and wild type strain *Rhodococcus ethroypolis* P6-4P

3.2 Improvements in CMD and yield

The CMD was examined to determine biosurfactant concentration. The CMD of the surfactant is generally proportional to the concentration of the biosurfactants, thus it gives an indication on the optimum concentration to be used for maximum performance and minimum cost (Marin et al. 2015). Generally, CMD values have been determined by carrying out ST measurements on a series of biosurfactant solutions with different dilution factors.. The dilution at which the ST begins to sharply increase is termed the CMD (Al-Wahaibi et al. 2014). Thus, higher the CMD, the more industrially promising is the surfactant. Biosurfactant containing broths were used to determine CMD for the biosurfactants produced by both the wild type strain and the mutant. Sonication was applied to ensure each dilution was homogeneous (Cai et al. 2016).

In Cai et al.'s study (2016), CMD value of that improved bio-dispersant producing mutant (Mutant#47) was 62.5. Whereas in this study, the outstanding mutant (M25) showed CMD as high as 225 while 15.4 is the CMD of the wild type strain (Figure 2), which means the biosurfactant yield of the new mutant dramatically increased 14.6 times. The total frozen dried yield of M25 crude bio-dispersant was 54g per liter of broth with 6 days of incubation. Table 2 summarized recent studies of applying molecular engineering tools to improve biosurfactant productions. The current study showed excellent improvements when compared with other studies.

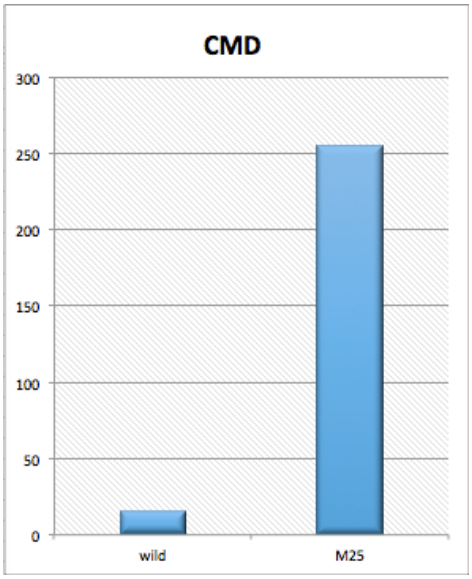

Figure 2: CMD improvement of mutant (M25) comparing to wild strain *Rhodococcus ethroypolis* P6-4P

3.3 16S rDNA sequencing

The 16S rDNA-sequencing results revealed that the 16 S ribosomal DNA of the mutant is identical with the original wild type strain indicating the mutation occurred on other parts of the genome.

3.4 Dispersion efficiency

Scale-down dispersion efficiency test results were demonstrated in Figure 3. The dispersant/crude oil ratio of 1:10 and 1:25 are the recommended dosages for Corexit 9500 dispersant (Wise et al. 2014). When comparing with the Corexit 9500, the dispersant efficiency by adding 100mg crude biosurfactants from mutant (M25) (4mg/uL crude product/crude oil ratio) was found comparable to Corexit 9500A at 1:25 for dispersing a crude oil sample provided by the industrial partner.

Figure 3: Scaled down dispersion efficiency test results (Corexit: 1:10 (oil) & 1:25 (oil); Bio-dispersant: 100mg, 80mg, 60mg, 50mg, 40mg, 30mg, 20mg, 10mg)

Table 2. Comparison of molecular engineered biosurfactant producers and their performances

Mutant and/or recombinant strain	Characteristic feature	Increased yield (g/L)	Refs
Pseudomonas aeruginosa PTCC 1637	Random mutagenesis with N-methyl-N0-nitro-Nnitrosoguanidine	from 1.2 to 12.5	Tahzibi et al., 2004
Bacillus licheniformis KGL11	Random mutagenesis with N-methyl-N0-nitro-Nnitrosoguanidine	from 0.033 to 0.39	Lin et al., 1998
Recombinant *Bacillus subtilis* strain ATCC 21332	Contains recombinantly modified peptide synthetase	N/A (produced less toxic surfactin)	Symmank et al., 2002
Recombinant *Bacillus subtilis*	Whole enzyme module swapping	Same level (changed structure)	Yakimov et al., 2000
Recombinant *Gordonia amarae*	expression of Vitreoscilla hemoglobin gene (vgb)	from 0.021 to 0.084	Dogan et al., 2006
Recombinant *Bacillus amyloliquefaciens* ES-2	Genome shuffle	Roughly 0.028 to 0.130	Zhao et al., 2012
Rhodococcus erythropolis SB-1A	UV mutagenesis	Roughly 4 time improve	Cai et al., 2016
Rhodococcus erythropolis P6-4P	UV mutagenesis	increased roughly 15 times to 54	This study

4. CONCLUSION

An extraordinary high-yielding bio-dispersant mutant M25 (crude bio-dispersant was 54g per liter with 6 days incubation) was found in this study, which was genetically improved from *Rhodococcus ethroypolis* P6-4P strain isolated from Atlantic offshore. UV mutagenesis was conducted for generating possible mutants, and the oil spreading technique was applied as the high throughput method to screen hyper production mutants. Subsequently, M25 was found as the superior mutant and was subjected to further analysis. The CMD as well as dispersion efficiency values of M25 were found to be much better than previous studies. M25 showed a CMD result as high as 225 while the wild type strain was of 15.4, indicating a 14.6 times of increase. The dispersion effectiveness of M25 was found comparable to Corexit 9500A at 1:25 (dispersant/crude oil ratio) when 4mg/μL (crude product/crude oil ratio) crude biosurfactant was applied. In this manner, M25 showed a high potential to overcome the economic bottleneck of biosurfactant production and had promising prospective as an oil spill response agent. All downstream testing were conducted by crude bio-dispersant production from the Mutate25 that was frozen dried. Future work on its separation from the biomass as well as its toxicity and biodegradability tests will be demonstrated.

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