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Enhanced crude oil depletion by constructed bacterial consortium comprising bioemulsifier producer and petroleum hydrocarbon degraders

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Abstract

The aim of this work was to study the production of bioemulsifier by *Rhodococcus erythropolis* OSDS1, and the improvement of crude oil depletion efficiency using a consortium of petroleum hydrocarbon degraders and OSDS1. The results showed that *R. erythropolis* OSDS1 produced highly stable bioemulsifier under various salinity (0–35 g/L NaCl) and pH (5.0–9.0) conditions; more than 90% of the initial emulsification activity was retained after 168 h. Emulsification capacity of the bioemulsifier on different petroleum hydrocarbons was diesel > mineral oil/crude oil > gasoline. A mixed bacterial consortium combining OSDS1 and four other petroleum hydrocarbon degraders was constructed. GC–MS results revealed that the constructed consortium achieved 85.26% depletion efficiency of crude oil in 15 days, which was significantly higher than that of individual strains. During the process, alkane hydroxylase gene (alkB) was successfully amplified from the consortium, confirming presence of crude oil degrading enzymes.

Keywords: Bioemulsifier, *Rhodococcus erythropolis*, Constructed bacterial consortium, Crude oil depletion, *alkB* gene

1. Introduction

Microbial degradation is the primary mechanism for removing petroleum hydrocarbon contaminants from contaminated environment. Among various microorganisms that utilize petroleum hydrocarbons, bacteria are considered to be the most active and important ones (Das & Chandran, 2011). Many different strains of bacteria have been found to be able to utilize one or more petroleum hydrocarbons. For example, *mycobacterial* strains isolated from mangrove sediments by Guo et al. (2010) completely degraded a PAHs mixture comprising Phe (10 mg/L), Fla (10 mg/L) and Pyr (10 mg/L) within 14 days. *Pseudomonas aeruginosa* WatG and Serratia marcescens HokM isolated by Wongsa et al. (2004) from several areas of Hokkaido, Japan (including hot spring water, wastewater, soils and mud contaminated with oil products) exhibited high capacity and wide spectrum in degrading gasoline, kerosene, diesel, and lubricating oil. More than 90% of diesel oil and kerosene were degraded by WatG in 2 to 3 weeks. However, HokM had an

advantage over WatG in utilizing aromatic compounds in gasoline (Wongsa et al., 2004).

In practical applications, biodegradation or bioremediation of oil contaminants containing alkanes, cycloalkanes, and polycyclic aromatic hydrocarbons (PAHs) etc., usually requires a diverse population possessing broad metabolic machineries (Das & Chandran, 2011). Multi-microbe mixed systems (such as a constructed bacterial consortium) with broader suite of enzymes are, therefore, more effective in degrading complex compounds and have received more attention than single strains (Mikesková et al., 2012) (Mikesková et al., 2012). For example, Rahman et al. (2002) prepared a mixed bacterial consortium using five strains isolated from soil contaminated with oil and demonstrated a higher degradation rate than that of individual bacterial strains. For 1% crude oil, the maximum degradation efficiency of 78% was achieved by the consortium, followed by *Pseudomonas* sp. DS10- 129 and *Bacillus* sp. DS6-86, which achieved efficiencies of 66% and 59% respectively. Bacterial consortium combining four different strains isolated from tank bottom oil sludge by Dhote et al. (2018) achieved higher degradation efficiencies of oil (78%) and total petroleum hydrocarbons (75%) than all of the individual strains. Kumari et al. (2018) also reported that when compared against individual strains, a mixture of five selected bacterial strains achieved enhanced degradation rates of naphthalene, fluorene, phenanthrene and benzo(b)fluoranthene in the crude oil.

Several factors limit biodegradation efficiency of petroleum hydrocarbons, low solubility and bioavailability of hydrophobic contaminants are the two most important ones. Biosurfactants, which contain both hydrophobic groups and hydrophilic groups, are able to increase the solubility and, thus, the bioavailability of crude oil, resulting in higher growth of bacteria and greater efficiency of biodegradation (Ron & Rosenberg, 2002). In contrast to chemical surfactants, biosurfactants have some unique advantageous features including low toxicity, high biodegradability, biocompatibility, efficiency, and in situ synthesis (Uzoigwe et al., 2015). These unique features makes them more suitable for deployment in bioremediation processes. Based on the differences in their chemical compositions and functional characteristics, biosurfactants can be classified into bioemulsifiers, which have higher molecular weights, and biosurfactants, whose molecular weights are lower (Ron & Rosenberg, 2002). Low molecular weight biosurfactants can effectively reduce the surface tension as well as the interfacial tension between different phases, forming stable emulsion. Whereas, high molecular weight bioemulsifiers only have the ability to form stable emulsification without reducing surface and interfacial tension.

Aliphatic hydrocarbons constitute a large proportion of crude oil (Liang et al., 2011). Under aerobic conditions, the most common biodegradation pathway of alkanes by microbes is by terminal oxidation, usually initiated by key enzymes such as alkane hydroxylase (alkB) (Paisse et al., 2011) (Abbasian et al., 2015). AlkB was first discovered in *Pseudomonas putida* GPo1 (Kok et al.,

1989), and then numerous publications identified *alkB* genes in different bacterial species worldwide, such as *Pseudomonas* (Tribelli et al., 2018), *Rhodococcus* (Táncsics et al., 2017), and *Bacillus* (Safdari et al., 2017) etc. Thus *alkB* genes were considered as bacterial functional markers for monitoring the hydrocarbon degradation and bioremediation capacities of oil-degrading bacteria (Paisse et al., 2011). For example, *Dietzia* sp. CN-3 isolated by Chen et al. (2017) degraded > 90% of the alkane fraction of crude oil, and *alkB* genes were detected by RT-qPCR. A Gram-negative *Alcanivorax* sp. strain 2B5, isolated by Liu et al. (2010) from oil contaminated sea mud in the Donghai area of China, could degrade C13–C30 *n*-alkanes and branched alkanes (pristane and phytane) in crude oil, *alkB* gene was present in this strain as well.

This study was part of a multidisciplinary project to define a strategy for bioremediation of a site called SWMU in California coastal area, which had been exposed to petroleum hydrocarbon and heavy metal contaminants for more than 30 years (Xia et al., 2017). The goal of this study was to study the production of bioemulsifier by *Rhodococcus erythropolis* OSDS1 previously isolated from the site, and the effect of combining bioemulsifier producer and petroleum hydrocarbon degraders in degrading crude oil contaminants. The specific objectives were: 1) to investigate the bioemulsifier production of *R. erythropolis* OSDS1 under different environmental conditions; 2) to construct a bacterial consortium combining OSDS1 and petroleum hydrocarbon degraders; 3) to test the degradation efficiency of crude oil by the consortium; 4) to detect the functional genes (i.e. alkane hydroxylase gene (*alkB*)) involved in hydrocarbon degradation.

2. Materials and methods

2.1. Bacteria, media and chemicals

Bacterial isolates used in this study were previously isolated from a solid waste management unit (SWMU) contaminated with petroleum hydrocarbons and heavy metals. Detailed information regarding the strains and the unit can be found in our earlier work (Xia et al., 2017).

Mineral salt medium (MSM) used in this study contained 2.42 g/L KH_2PO_4, 5.60 g/L K_2HPO_4, 2.00 g/L $(NH_4)2SO_4$, 0.30 g/L $MgSO_4 \cdot 7H_2O$, 0.04 g/L $CaCl_2 \cdot 2H_2O$, 4.5×10^{-3} g/L $MnSO_4 \cdot 7H_2O$, 0.1×10^{-3} g/L $CuSO_4 \cdot 5H_2O$ and 0.1×10^{-3} g/L $FeSO_4 \cdot 7H_2O$. To adjust its pH to 7.0 ± 0.2 using NaOH/HCl, a pH meter (Orion star A221, Thermo Fisher Scientific, USA) was used to measure the pH. The medium was also sterilized by autoclaving (HVE-50, HMC HIRAYAMA, Japan) (Bury & Miller, 1993). In addition to the MSM, Lysogeny broth (LB) (Thermo Fisher Scientific, USA) was also used.

The crude oil used in this study was sourced from the “North Sea oil field” and stored at room temperature; the mineral oil was acquired from Walgreens (USA); gasoline and diesel were products of Chevron Corporation (USA) and Conoco Philips (USA), respectively.

Unless specified, chemical reagents used in this study were purchased from Sigma-Aldrich, USA, with purity ≥99.0%. Agar was purchased from Becton Dickinson, USA.

2.2. Oil spreading assay

Oil spreading assay was used to detect interfacial activities of biosurfactants: the method was modified from Morikawa et al. (2000). *R. erythropolis* OSDS1 was cultured in the MSM plus 20 g/L of glucose for 3 days under 30 °C at 130 rpm in an incubator (E24, New Brunswick Scientific, USA). The cell free supernatant was then obtained by centrifuging (Avanti J-25, Beckman Coulter, USA) the culture broth at 10,000g for 10 min. 10 µl of crude oil was added to a 100 mm diameter petri dish containing 25 ml of Milli-Q water (produced by Milli-Q® Advantage A10, Millipore Sigma, USA), forming an oil film on the water surface. At the center of this oil film, 10 µl of cell free supernatant was subsequently added. In the presence of biosurfactants, the oil film would disperse, and the diameter of the resultant concentric circle of dispersion would positively relate to the surfactant concentration. The negative and positive controls used in the experiment are Milli-Q water and Triton X-100, respectively.

2.3. Emulsification activity measurement

To measure the emulsification activity, 4 ml of diesel oil and 4 ml of cell free supernatant were added to a graduated round bottom tube, which was then vortexed (Vortex-Genie 2, Scientific Industries Inc., USA) at maximum speed for 2 min and left standing for 24 h at room temperature. To represent the emulsification activity, the emulsification index (E24) was calculated using:

$$\mathrm{E24}(\%) = \frac{HE}{HT} \times 100$$

where HE represents the height of emulsion layer (mm), and HT represents the total height of liquid column (mm). Similarly, E24 can also be evaluated using gasoline, mineral oil or crude oil to determine the emulsification capacity on different petroleum hydrocarbons (Ibrahim, 2018).

2.4. Bioemulsifier production under different culture conditions

2.4.1. Culture time

R. erythropolis OSDS1 was inoculated into the MSM plus 20 g/L glucose at 10% inoculation rate, and then incubated under 30 °C at 130 rpm. Samples were collected every 24 h for a total of 168 h (8 samples) to determine the OD_{600} and emulsification activities of the cell free supernatants.

2.4.2. Carbon sources

Five different carbon sources including 2 g glucose, 2 ml mineral oil, 2 ml gasoline, 2 ml diesel oil, and 2 ml crude oil were added into 100 ml of the MSM separately to obtain media with different carbon sources. *R.*

erythropolis OSDS1 was inoculated into each medium at a 10% rate and then incubated for 15 days at 30 °C and 130 rpm. Subsequently, centrifugation (Avanti J-25, Beckman Coulter, USA) was performed at 10,000 g for 10 min to collect the cell free supernatants, which were then used to determine the emulsification activities of diesel.

2.4.3. Initial salinity and pH levels

In order to determine the effect of initial salinity and pH levels on the bioemulsifier production of OSDS1, different salinity levels of 0, 5, 15, 25, 35, 45, 55, 65, 85, 100 g/L NaCl were obtained by adding different amounts of NaCl into the MSM plus 20 g/L glucose. To obtain different levels of pH, media were adjusted to pH 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 with either 1 N HCl or 1 N NaOH, and filtered through a 0.2 μm membrane filter for sterilization. *R. erythropolis* OSDS1 was inoculated into each medium at a rate of 10% inoculation and then cultured under 30 °C at 130 rpm for 168 h to determine the emulsification activities of cell free supernatants.

2.5. Stability of the bioemulsifier

R. erythropolis OSDS1 was cultured in the MSM plus 20 g/L glucose at 30 °C, 130 rpm for 7 days. Centrifugation was then performed at 10,000 g for 10 min to get cell free supernatant for the stability test. To quantify how temperature affects the stability of the bioemulsifier, cell free supernatants were placed at 4, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 °C, respectively for 2 h, then cooled down to room temperature before measurements, E24 emulsification indexes were calculated using diesel. For the effect of salinity, different amounts of NaCl were added to cell free supernatants to obtain 0, 5, 15, 25, 35, 45, 55, 55, 85, 100 g/ L NaCl concentrations respectively, then the emulsification activities (E24) of each supernatant were measured using diesel. Similarly, the effect of pH was also measured by first adjusting the pH of cell free supernatants to 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0 using 1 N HCl/NaOH respectively, and then recording the emulsification activities for each pH value.

2.6. Construction of a mixed bacterial consortium

2.6.1. Degradation characteristics of selected strains

Degradation characteristics of bacterial strains were analyzed following the method by Xia et al. (2017). More than 180 different compounds in crude oil were detected by GC–MS (SHIMADZU GCMSQP2010, SHIMADZU, Japan) in selected ion mode. Compounds with carbon numbers ranging from 7 to 35 or belonging to various classes (including n-alkanes, alkanes, cycloalkane, BTEX and PAHs) were summarized and analyzed separately to reflect the substrate preferences of selected strains.

2.6.2. Tolerance to the environmental stressors

Cell growth under different salinity and pH levels were tested in 96 well plates. Around 200 μl of LB broth with different salinity (5, 15, 25, 35, 45, 55,

65, 85, 100 g/L NaCl) and pH levels (4.0, 5.0, 6.0, 7.0, 8.0, and 9.0) were added to each well and then inoculated with testing cell at a 10% inoculation rate separately. The plates were cultivated under 30 °C for 72 h, and OD_{600} was measured every 4 h to plot growth curves under different conditions.

2.6.3. Construction of a mixed bacterial consortium

Bacterial strains with different degradation characteristics and bioemulsifier production abilities were selected to construct a mixed bacterial consortium. The survival of each strain in the consortium was tested by inoculating them as a mix into LB broth media and allowing it to grow at 30 °C for 3 days. After 3 days, serially diluted culture broths were streaked on LB agar plates, and different colonies were selected for 16S rRNA gene sequencing as reported earlier (Xia et al., 2017).

2.6.4. Degradation of crude oil by the constructed consortium

The mixed bacterial consortium was cultured in LB broth at 30 °C for 3 days. The cell pellet was washed and resuspended in PBS. With a spectrometer (Thermo Fisher Scientific, US), OD_{600} of cell suspension was adjusted to 0.5 to prepare the bacterial inoculum. Screw-cap vials (60 ml volume) were used to stop the hydrocarbon components from escaping during the degradation experiment. Each vial contained 30 ml of the MSM, 1% (volume to volume) crude oil and 1% bacterial inoculum. The contents of the vials were incubated at 30 °C on a rotary shaker running at 130 rpm. For control, the MSM with only crude oil and no cells was also put through the same procedure. All vials were collected after 15 days, and measured the residual crude oil using GC–MS as described in our previous work (Xia et al., 2017).

2.7. Detection of alkyl hydroxylase gene (alkB)

DNA of the constructed bacterial consortium was extracted using a DNA extraction kit (PureLink™ Genomic DNA Mini Kit, Thermo Fisher Scientific, USA), and the extracted DNA was stored short term at −20 °C prior to testing.

For alkyl hydroxylase gene (*alkB*) amplification: the extracted DNA was used as the template, and the primers *alkB*-1f 5′-AAY CAN GCN CAY GAR CTN GGN CAY AA and *alkB*-1r 5′-GCR TGR TGR TCN GAR TGN CGY TG (Kloos et al., 2006), approximately 550 bp, were used for Polymerase chain reaction (PCR) amplification. PCR was conducted in a thermocycler (c1000 touch thermal cycler, Bio-Rad Laboratories, USA) with the following settings: 94 °C for 5 min; 94 °C for 1 min, 56–62 °C for 1 min, 72 °C for 1 min, 30 cycles; 72 °C for 10 min. The reaction system contained: 23.4 μl of water, 25 μl of master mix (DreamTaq Green Mix, Thermo Fisher Scientific, USA), 0.3 μl of forward primer, 0.3 μl of reversed primer and 1 μl of DNA template. The PCR product was confirmed on 1.5% agarose E-gel (Invitrogen, USA).

2.8. Data analysis

Data was expressed as mean ± standard deviation (SD) of three replicates. Statistical analysis was performed in Excel 2013 and SPSS statistics 22 (Chicago, IL, USA). P-value < 0.05 was considered as significance.

3. Results and discussion

3.1. Bioemulsifier production of R. erythropolis OSDS1

Based on the phenomenon of crude oil dispersion and emulsification in the presence of *R. erythropolis* OSDS1, investigation of its ability to release biosurfactant was conducted. Emulsification activity measurement showed that cell free supernatant of *R. erythropolis* OSDS1 formed highly stable emulsification, with more than 90% of the emulsification activity being maintained for up to 168 h (E24 = 28.66 ± 1.24%, emulsification index after 24 h; E168 = 26.06 ± 1.05%, emulsification index after 168 h). Oil spreading assay showed that the cell free supernatant of OSDS1 did not have the ability to reduce surface tension of petroleum hydrocarbons. Therefore, it can be deduced that the biosurfactant synthesized by *R. erythropolis* OSDS1 is a kind of extracellular bioemulsifier.

Though *R. erythropolis* is known to produce biosurfactants (Cai et al., 2016; Pirog et al., 2013), most of those belong to low molecular weight glycolipids, for example trehalolipeids, decrease both surface and interfacial tension of different phases (Luong et al., 2018; Patil & Pratap, 2018). However, the compound released by *R. erythropolis* OSDS1 described in this study, belongs to the high molecular weight bioemulsifier (extracellular polymer), which forms stable emulsification without causing obvious reduction in surface tension. Thus, *R. erythropolis* OSDS1 is valuable in searching for new bioemulsifiers produced by *Rhodococcus* genus.

3.2. Bioemulsifier production under different culture conditions

The yield of bioemulsifier is highly sensitive to environmental factors. Optimization of the culture conditions is therefore one of the most important ways to effectively boost bioemulsifier production in industry applications.

3.2.1. Culture time

Culture time determines the cell growth phases and also affects the production of metabolites by bacteria. Bioemulsifiers are usually considered as secondary metabolites, but it could also be associated with bacterial growth. Currently, the regulation and physiological role of them are not fully understood yet (Sobrinho et al., 2013). Table 1 showed the bioemulsifier production of *R. erythropolis* OSDS1 over culture time. As can be seen from the table, with the increase in culture time, the changes in emulsification activity of supernatants were consistent with the trend of cell growth. Bioemulsifier production of OSDS1 started during the exponential phase and reached the maximum emulsification index of 65.82 ± 0.38% during the stationary phase. This is similar to what has been reported in the literature. For example, Vasylchenko et al. (2012) described that the maximum

surfactant production may occur from the beginning of initial growth, the stationary phase, or the later cultural development stages. Ron and Rosenberg (2002) also reported that bioemulsifier production usually occurs when cultures reach the stationary stage of growth, probably due to the high concentration of bacteria cells, and large amount of secondary metabolites produced in the stationary phase. Due to the chemical structures and properties, the high molecular weight bioemulsifiers usually include heteropolysaccharides, lipopolysaccharides, lipoproteins, proteins or their complex mixtures (Uzoigwe et al., 2015).

Compared with the emulsification activities reported in the literature, *R. erythropolis* OSDS1 achieved a relatively high emulsification index. For example, Pacheco et al. (2010) reported emulsification indexes ranging from 27% to 60% for a *Rhodococcus erythropolis* biosurfactant producer, which is lower than that was reported in this study. Similarly, the highest emulsification index achieved by *Rhodococcus* sp. reported by Bandyopadhyay et al. (2013) was around 59%. The emulsification indexes of the different oil field strains reported by Mnif et al. (2011) ranged from 45% to 77%, all lower emulsification indexes than findings of this work.

3.2.2. Carbon source

Carbon provides microorganisms with biomass carbon and energy for growth. Carbon also affect the type and amount of metabolites produced by microorganisms. To determine the optimal carbon source for the production of bioemulsifier by *R. erythropolis* OSDS1, various carbon sources were tested and the results were summarized in table 1. As shown in the table, the highest emulsification index of 62.89 ± 1.09% was obtained with glucose as the carbon source. Meanwhile, the second highest emulsification index of 57.97 ± 1.13% was obtained using diesel. Mineral oil and crude oil induced bioemulsifier production with E24 in the mid-range of 35.63 ± 1.99% and 32.95 ± 1.76%, respectively. In case of gasoline as the only carbon source, supernatant had the lowest emulsification index, which suggests that the inductive effect of gasoline on bioemulsifier production of OSDS1 is minimal, gasoline is rarely oxidized completely for energy and biomass carbon.

Most known biosurfactants (for example fatty acids, phospholipids or neutral lipids bioemulsifiers) are reported to be produced in the presence of hydrophobic substrates (Mnif & Ghribi, 2015). Moreover, biosurfactants synthesized by some bacteria like *Y. lipolytica* NCIM 3589 are not viable when soluble substrates like glucose are used (Zinjarde & Pant, 2002). However, in 2001, Sarubbo et al. first reported that *Y. lipolytica* IA 1055 produced biosurfactants when glucose was used as the carbon source (Sarubbo et al., 2001), which indicates that the induction of biosurfactant production is not dependent on the presence of hydrocarbons. *R. erythropolis* OSDS1 used in this study could also utilize glucose in the production of bioemulsifier. The use of a soluble substrate such as glucose for bioemulsifier production potentially reduces the production costs, making

the process more economically and environmentally attractive and thereby, desirable. This is in stark contrast to most biosurfactants synthesized by *Rhodococcus* and related genera, where the biosurfactants are mostly membrane bound and primarily produced when the microbes are grown on hydrocarbons (Franzetti et al., 2010).

Table 1
Preliminary studies of bioemulsifier production by *R. erythropolis* OSDS1 under different culture conditions.

Culture time (d)	OD_{600}	E24 (%)	Carbon sources	E24 (%)	Salinity (g/L NaCl)	E24 (%)	pH	E24 (%)
0	0.00	0.00	Glucose	62.89 ± 1.09	0	65.95 ± 1.24	4.0	0.00
1	0.10 ± 0.01	3.75 ± 0.04	Diesel	57.97 ± 1.13	5	61.79 ± 1.18	5.0	36.95 ± 1.63
2	0.23 ± 0.02	8.12 ± 0.99	Mineral oil	35.63 ± 2.00	15	59.28 ± 1.66	6.0	62.91 ± 0.73
3	0.68 ± 0.02	27.38 ± 1.09	Crude oil	32.95 ± 1.76	25	50.88 ± 1.52	7.0	65.95 ± 1.24
4	1.03 ± 0.06	47.50 ± 0.56	Gasoline	2.26 ± 1.96	35	45.42 ± 1.69	8.0	59.06 ± 1.06
5	1.28 ± 0.02	58.76 ± 1.50	–	–	45	0.00	9.0	47.78 ± 1.92
6	1.31 ± 0.02	61.01 ± 1.09	–	–	55	0.00	–	–
7	1.30 ± 0.02	65.82 ± 0.38	–	–	65	0.00	–	–
–	–	–	–	–	85	0.00	–	–
–	–	–	–	–	100	0.00	–	–

3.2.3. Initial salinity and pH

Effects of initial salinity and pH on bioemulsifier production of *R. erythropolis* OSDS1 were measured and shown in Table 1. As can be seen from the table, with increasing NaCl concentration in the media, emulsification activities of supernatants decreased. When salt concentration was greater than 35 g/L NaCl, supernatant did not show any emulsification ability, which was in accordance with the salinity tolerance of *R. erythropolis* OSDS1 (0–35 g/L NaCl). pH is another important condition affecting cell growth and metabolite production. *R. erythropolis* OSDS1, as shown in Table 1, produced bioemulsifier in the pH range 5.0 to 9.0, which was also in accordance with the pH tolerance of the strain (pH 5.0–9.0). The maximal emulsifier production was achieved at pH 7.0, with an emulsification index of 65.95 ± 1.24%.

3.3. Emulsification capacity on different petroleum hydrocarbons

The surface active substances produced by microorganisms are usually specific and have different emulsifying capacities for different hydrocarbons (Luna-Velasco et al., 2007). In order to determine hydrocarbon specificity of the bioemulsifier produced by *R. erythropolis* OSDS1, four different hydrophobic substrates, including gasoline, diesel, mineral oil and crude oil were investigated. Results showed that the bioemulsifier produced by *R. erythropolis* OSDS1 had the best emulsification effect on diesel (62.89 ± 1.09%), followed by mineral oil (55.13 ± 1.11%) and crude oil (54.69 ± 1.69%), and had the weakest emulsification effect on gasoline (6.63 ± 1.19%). Since gasoline is the least viscous of the four hydrophobic substrates, emulsification made little difference. This is similar to what has been reported by Conlette (2011). Among the hydrocarbons tested, crude oil achieved the highest emulsion turbidity, closely followed by diesel oil. However, hydrocarbons with lower molecular weights (n-Alkanes) recorded lower emulsion turbidities than mixtures of complex hydrocarbons.

3.4. Stability of the bioemulsifier

The effect of heat treatment on the stability of bioemulsifier was determined and shown in Table 2. It can be seen from the table that there was no appreciable change in emulsification activities of bioemulsifier after treatment under different temperatures. Only around 10% of the original emulsification activity was lost when cell free supernatant was treated under 100 °C for 2 h. This loss may be due to the denaturation of proteinaceous compounds in the bioemulsifier during heating (Asfora Sarubbo et al., 2006). Also there was no significant change in emulsification activity at a lower temperature (4 °C), which indicates that the bioemulsifier produced by *R. erythropolis* OSDS1 has good temperature stability.

The effect of added NaCl concentrations on the stability of bioemulsifier was measured and shown in Table 2. No significant change was observed on emulsification activities of supernatant treated with up to 15 g/L NaCl. A sharp reduction of approximately 18% of emulsification activity was observed with the addition of 25 g/L NaCl. Subsequently, despite the continuous increase of NaCl concentration, emulsification activities changed very little. Thus, the bioemulsifier produced by *R. erythropolis* OSDS1 has a relatively good salinity stability.

Table 2 showed the effect of different pH treatments on the emulsification activities of bioemulsifier produced by *R. erythropolis* OSDS1. As can be seen from the table, no appreciable effect on emulsification activity was observed over the pH range from 4.0 to 9.0. The maximum emulsification activity was obtained at pH 6.0.

Stability tests revealed that emulsification capacity of the bioemulsifier remained practically unaltered within a wide pH (4.0–9.0), temperature (4–100 °C), and salinity (0–10% NaCl concentrations) range, which is similar to the results reported by Nitschke and Pastore (2006) and Asfora Sarubbo et al. (2006).

3.5. Bacterial consortium construction

In order to achieve higher efficiency and broader spectrum in degrading complex petroleum hydrocarbon compounds such as crude oil, construction of a mixed bacterial consortium comprising both bioemulsifier producer and petroleum hydrocarbon degraders was attempted. Five bacterial strains with different substrate preferences and environmental tolerance of pH and salinity were selected to form a bacterial consortium, including *Serratia proteamaculans* S1BD1 (substrate preferences: relatively wider spectrum; pH tolerance: 4.0–9.0; salinity tolerance: 0–65 g/L NaCl), *Alcaligenes* sp. OPKDS2 (substrate preferences: alkane & BTEX; pH tolerance: 5.0–9.0; salinity tolerance: 0–45 g/L NaCl), *R. erythropolis* OSDS1 (substrate preferences: alkane; pH tolerance: 5.0–9.0; salinity tolerance: 0–35 g/L NaCl), *Rhizobium* sp. PNS1 (substrate preferences: BTEX; pH tolerance: 6.0–8.0; salinity tolerance: 0–45 g/L NaCl), and *Pseudomonas* sp. BSS9BS1(substrate

preferences: BTEX; pH tolerance: 5.0–9.0; salinity tolerance: 0–55 g/L NaCl) (Xia et al., 2017). All strains were able to survive in the mixed consortium, and growth curves of the constructed consortium under different salinity and pH conditions were measured. The consortium could tolerate salinity up to 65 g/L NaCl, and pH ranging from 4.0 to 9.0. When presented with external osmotic stress caused by increased NaCl concentration, bacterial cells either accumulate or release osmoprotectant solutes (including inorganic ions and organic molecules) to attenuate water fluxes (Wood, 2015). This study used five strains isolated from a seaside saline waste management unit, which has been compacted with oil related compounds for more than 30 years and the salinity of the soil is as high as 10 mmho/cm (~8 g/L) (Xia et al., 2017). Furthermore, due to high wind and high level of solar radiation in local areas that lead to high evaporation rate, salted crusts were observed in some areas (often bare soil areas) and salinity could potentially reach much higher levels. Therefore, all the strains isolated from the site were tolerant to high levels of salinity and petroleum hydrocarbons likely due to adaptation and/or natural selection based on the site characteristics.

Table 2
Stability of bioemulsifier produced by *R. erythropolis* OSDS1 under different temperature, salinity and pH treatments.

Temperature (°C)	E24 (%)	Salinity (g/L NaCl)	E24 (%)	pH	E24 (%)
4	66.67 ± 2.06	0	67.25 ± 1.01	4.0	58.43 ± 1.67
10	65.48 ± 2.06	5	64.33 ± 2.03	5.0	59.32 ± 2.39
20	66.67 ± 2.06	15	65.36 ± 1.13	6.0	68.89 ± 1.93
30	62.07 ± 0.00	25	49.12 ± 1.52	7.0	62.22 ± 1.93
40	62.37 ± 0.52	35	48.15 ± 3.21	8.0	52.22 ± 1.93
50	60.92 ± 2.00	45	48.15 ± 3.21	9.0	51.11 ± 1.93
60	63.11 ± 1.12	55	48.15 ± 3.21	–	–
70	58.13 ± 0.85	65	48.15 ± 3.21	–	–
80	57.64 ± 0.85	85	44.21 ± 0.40	–	–
90	57.85 ± 1.22	100	41.80 ± 0.54	–	–
100	56.46 ± 0.81	–	–	–	–

3.6. Crude oil depletion by the constructed consortium

Depletion efficiencies of crude oil by the constructed bacterial consortium and selected individual strains were measured and compared in Fig. 1. With 1% initial crude oil concentration, constructed bacterial consortium achieved the highest depletion efficiency of 85.26 ± 2.56% in 15 days, higher than that of individual strains (*Serratia proteamaculans* S1BD1: 68.0%, *Alcaligenes* sp. OPKDS2: 63.7%, and *R. erythropolis* OSDS1: 54.9%) (Xia et al., 2017). Current findings are similar with earlier studies such as, findings of Sathishkumar et al. (2008) showed that a mixed bacteria consortium exhibited better growth and degradation efficiency than the single strains. When the initial crude oil concentration was 1%, the consortium had the highest degradation efficiency of 77%, while *Pseudomonas* sp. BPS1-8 and *Bacillus* sp. IOS1-7 achieved efficiencies of 69% and 64% respectively. *Pseudomonas* sp. HPS2-5, along with *Corynebacterium* sp. BPS2-6, had even lower efficiencies (45% and 41%). Using a consortium of five different PAHs degraders, Kumari et al. (2018) were able to increase the degradation efficiencies of naphthalene, phenanthrene, fluorine and benzo(b)fluoranthene in crude oil. With the addition of 40 μg/ml

biosurfactant (rhamnolipid JBR-425), another 10% improvement was observed. In another study by Tao et al. (2017), the indigenous bacterial consortium achieved a degradation ratio of 71.32%. Addition of *Bacillus subtilis* strain ZF3-1 to the consortium enhanced the degradation efficiency to 85.01%. Seven petroleum hydrocarbon degraders isolated from Korean terrestrial environments, were selected by Lee et al. (2018) based on their biodegradation ability, hydrophobicity, and emulsifying activity, to construct a microbial consortium. Lab- and bulk-scale tests both showed the biodegradation of total petroleum hydrocarbons was improved when the microbial consortium was used. Constructed consortium in this study achieved a relatively high depletion efficiency compared with other studies.

Depletion efficiencies of compounds with different numbers of carbon and compounds belonging to different classes in the testing oil are shown in Fig. 1a and b. From Fig. 1a, it can be seen that depletion efficiencies of compounds with different carbon numbers were highly enhanced by the constructed consortium than the individual strains, especially for those compounds with carbon greater than C15. For example, depletion efficiency of C21-C25 by the constructed consortium was 79.6 ± 2.82%, much higher than that of *S. proteamaculans* S1BD1 (44.7 ± 8.9%), *Alcaligenes* sp. OPKDS2 (18.9 ± 4.1%), and *R. erythropolis* OSDS1 (25.7 ± 9.6%). Similarly, depletion efficiencies of compounds belonging to different classes were also enhanced by the consortium (shown in Fig. 1b), especially for compounds belonging to branched alkanes, cycloalkanes and PAHs. The reason for the enhancement may be due to the presence of the bioemulsifier producer *R. erythropolis* OSDS1 in the constructed consortium, which effectively dispersed and emulsified the crude oil, enhancing its bioavailability to the bacteria with different substrate preferences. Similar results were reported by other researchers previously. For example, Abalos et al. (2004) found that the biodegradation of petroleum hydrocarbons can be accelerated by adding rhamnolipid, a biosurfactant produced by strain AT10. The total petroleum hydrocarbon degradation efficiency increased from 32% to 61% in a certain time, while degradation efficiency of alkylated polycyclic aromatic hydrocarbon increased from 9% to 44%.

Though multiple reports have shown that mixed bacterial consortia have higher degradation efficiencies of crude oil than individual strains, few have analyzed the degradation efficiencies of individual compounds in the crude oil. In this work, in addition to observing the enhanced overall depletion efficiency of crude oil by the mixed bacterial consortium, we also measured and reported the improvement on individual compounds > C15, PAHs etc. It is generally believed that unbranched short chain n-alkanes (C8 to C15) are preferentially degraded by microorganisms, rather than C16 to C36 n-alkanes (Sathishkumar et al., 2008), which is consistent with the results obtained from our study. Studies have also shown that low-molecularweight alkanes can be directly absorbed by microbial cells, while medium to long chain alkanes often require the use of biosurfactants to aid microbial uptake

(Rojo, 2009). In this study, the construction of bacterial consortium comprising bioemulsifier producer and petroleum hydrocarbon degraders, effectively increased the depletion efficiencies of relatively complex compounds in the tested crude oil.

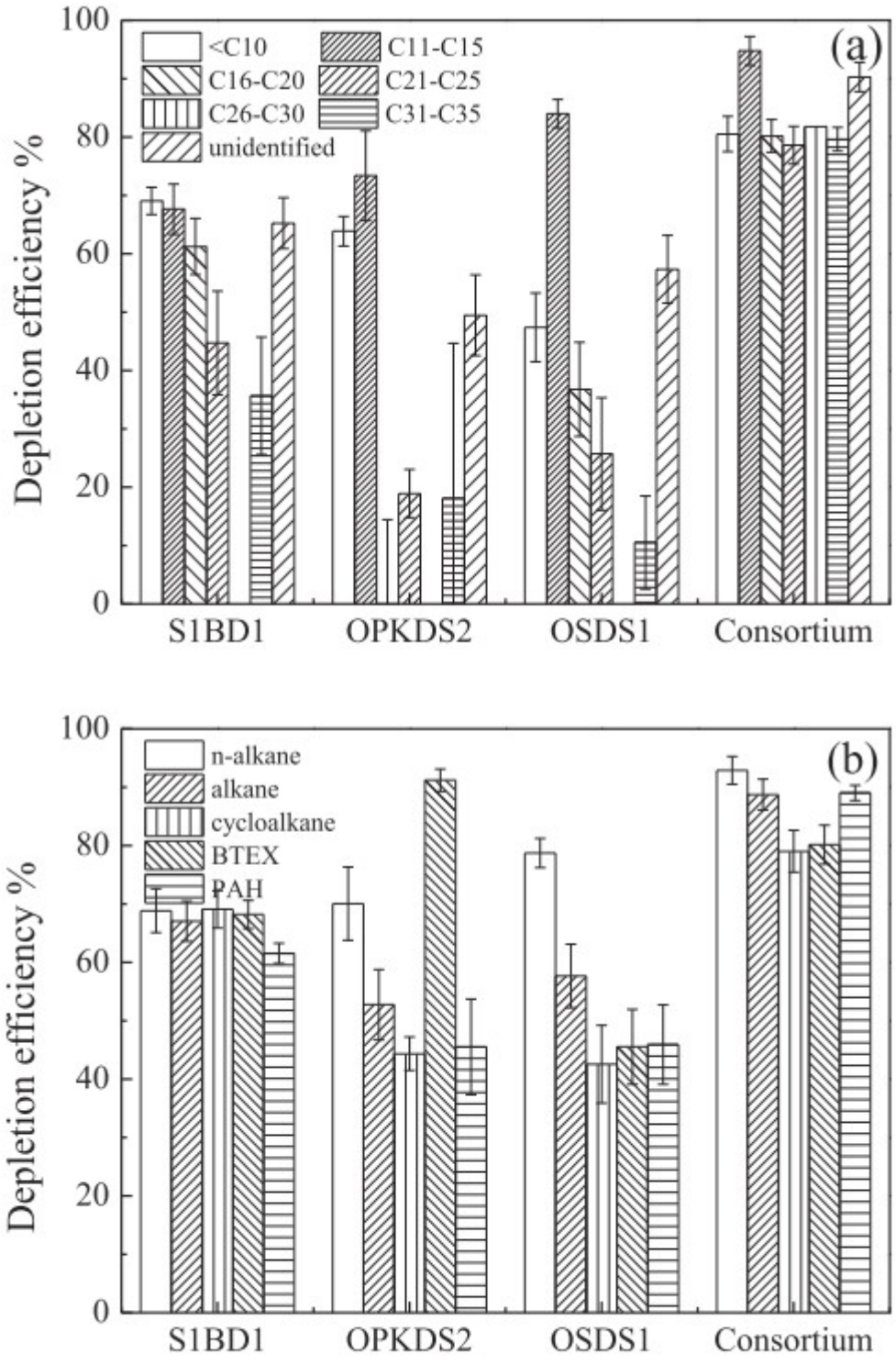

Fig. 1. Depletion efficiencies of crude oil by individual strains and constructed bacterial consortium. (a) Depletion efficiencies of individual compounds in crude oil with different carbon numbers; (b) Depletion efficiencies of individual compounds in crude oil belong to different classes.

In general, bacterial consortium provides a richer mix of enzymes relative to single strains and can thus achieve higher efficiency. Complementing this greater potency of bacterial consortium in degrading hydrocarbons, the bioemulsifier increases bioavailability of the substrates that the enzymes act upon. Therefore, incorporation of bioemulsifier producers into the bacterial consortium results in further enhancement of its overall degradation capacity.

Though microbial biodegradation of petroleum hydrocarbons is economically and environmentally friendly, complete mechanism of the process when applied to in situ bioremediation has not been fully understood yet. Due to variability of the in situ environmental conditions as compared to lab conditions, the survivability of microbes, sustainability of the actual bioremediation, and efficiency of biodegradation in anoxic environment (Macaulay & Rees, 2014) are some of the challenges that need to be considered.

3.7. Alkane hydroxylase gene (*alkB*) detection

Analysis of microbial functional genes, which encode key enzymes involved in contaminant degradation is very important for understanding the mechanism of the major biogeochemical processes. In this study, alkane hydroxylase gene (*alkB*) was successfully detected from the constructed consortium through PCR amplification (a fragment of approximately 500 bp was amplified), which confirmed the presence of crude oil degrading enzymes.

Petroleum hydrocarbons undergo multiple different steps involving various enzymes (Varjani, 2017) during biodegradation. For instance, the conversion of alkane to alcohol starts with the oxidation of methyl group by alkane monooxygenase; cyclic alkanes are converted initially to ketones, by an oxidase system, where mono-oxygenase and lactone hydrolase each carries out a different stage of the conversion pipeline (Abbasian et al., 2015); for aromatic hydrocarbons, which are less amenable to biodegradation than saturated hydrocarbons, cleavage of benzene ring follows an initial oxidative attack, accomplished by different enzymes (Chakraborty et al., 2005). Though alkane hydroxylase (*alkB*) gene was successfully amplified from the consortium degrading crude oil in this study, other genes involved in BTEX and PAHs degradation should also be taken into consideration.

4. Conclusions

Rhodococcus erythropolis OSDS1 produced highly stable bioemulsifier under various salinity (0–35 g/L NaCl) and pH (5.0–9.0) conditions. The bioemulsifier had the strongest emulsification effect on diesel, followed by mineral oil/crude oil, and finally gasoline. A mixed bacterial consortium with bioemulsifier producer and petroleum hydrocarbon degraders was constructed and achieved 85.26% depletion efficiency of crude oil in 15

days, which is promising for use in biodegradation and bioremediation of oil contaminants.

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