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Comparative degradation between heavy and light crude oil mediated by nitrogen-induced cell-surface hydrophobicity

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Abstract

A bacterial strain UKMP-10M2 isolated from a Malaysian petroleum refinery was able to degrade 84% of heavy Khafji sour crude and 68% of light Tapis sweet crude within seven days. Analysis of gas chromatography-flame ionization detector chromatograms show the strain UKMP-10M2 degraded up to 90% pristane and 50% phytane in heavy crude, but significantly lower pristane (50%) and phytane (30%) were degraded from the light crude. A mixture of aliphatic hexadecane and three-ring phenanthrene better supported the growth of isolate UKMP-10M2 compared to using phenanthrene alone, suggesting cometabolism influenced how crude oil with different individual hydrocarbon contents affected the degradation. Peptone as the source of nitrogen increases the emulsifying index in UKMP-10M2 exposed to heavy Khafji sour crude 20% higher than in light Tapis sweet crude. However, BATH assay showed the same nitrogen source increases bacterial cell surface hydrophobicity of UKMP-10M2 up to 14% higher in light Tapis crude oil compared to heavy Khafji. This study suggest the nitrogen source plays a decisive role in elevating UKMP-10M2 bacterial cells hydrophobicity, and in correlation with types of crude oil. Phylogenetic tree analysis based on 16S rDNA sequence results identified the strain to be *Rhodococcus ruber*.

KEYWORDS

biosurfactant, peptone, phylogenetic tree, Rhodococcus ruber, UKMP-10M2

1 | INTRODUCTION

Biodegradation of crude oil using microbes has been widely reported (Adams, Fufeyin, Okoro, & Ehinomen, 2015; Das & Chandran, 2011). The success of crude oil biodegradation depends on numerous factors, particularly the type of crude oil (Das & Chandran, 2011) and nutrient amendments (Adams et al., 2015; Ainon & Kok Kee, 2015; Ainon, Chia-Wei, Nur Faizah, & Kok Kee, 2013).

Ainon, Amir, Raja Farzarul Hanim, and Noor Ainni (2010) reported that both *Pseudomonas aeruginosa* and *Acinetobacter lwoffi* degraded significantly higher percentages of Sumandak light oil than South Angsi heavy oil. Another study by Aoshima et al. (2006) pointed out that *Rhodococcus erythropolis* consumed up to 80% light oil compared to only 60% of heavy oil. The study findings attributed this to the higher hydrophobic hydrocarbon constituents in heavy oil (Aoshima et al., 2006), whereby the fractions of longer carbon chains exhibited higher hydrophobicity that reduced the bioavailability to microbes during the biodegradation process (Kok Kee, Brid, & Salmijah, 2013).

Reviews on nitrogen amendments generally showed that adding a nitrogen source to increase crude oil hydrocarbon degradation. The consensus argument was that the optimal concentration of nitrogen enhanced bacterial growth; thereby, increasing the cells' metabolism and the ensuing hydrocarbon degradation process (Adams et al., 2015; Das & Chandran, 2011). Recent studies by our group showed different bacteria preferred different nitrogen sources, ultimately leading to accelerated crude oil degradation (Ainon et al., 2013; Hamzah, Md. Salleh, Wong, & Sarmani, 2016). One of the mechanisms suggested was the type of nitrogen source affects the generation of undesirable metabolites (nitrite) which suppressed growth (Ainon & Kok Kee, 2015).

Hence, the objective of this study was to identify locally isolated crude oil-degrading bacteria and to evaluate the effect of choosing the optimum nitrogen source to enhance degradation of heavy or light crude oil. In this study, we examined the significance of the nitrogen source on the secretion of biosurfactant and cell surface hydrophobicity of bacteria in response to different types of crude oil.

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2 | MATERIAL AND METHODS

2.1 | Isolation and enrichment of crude oil degrading bacteria

Wastewater samples were collected from an oxidation pond at the effluent treatment system of a petroleum refinery facility in Malacca, Malaysia. Bacteria were isolated and enriched by incubating wastewater samples in mineral salt medium (MSM) supplemented with 1% (volume by volume [v/v]) Khafji sour crude oil (PETRONAS Pte Ltd, Malaysia) as a sole carbon source. The MSM media (Zajic & Supplisson,1979) was fortified with 0.01% trace elements (grams per liter); 0.1 g $MnSO_4 \cdot H_2O$, 0.025 g $CuCl_2$, 0.025 g $(NH_4)_2MoO_4$, $NaB_4\ O_7 \cdot 10H_2O$, 0.025 g $Co(NO_3)_2 \cdot 6H_2O$, 0.025 g $ZnCl_2$, and 0.01 g NH_4NO_3 and nystatin (50 milligrams per liter) to suppress fungal growth (Ainon & Kok Kee, 2015).The pH medium was adjusted to 7.0 and autoclaved at 121°C for 15 minutes (min). After sterilization, the medium was left to cool to 30°C before adding 1% (v/v) Khafji sour crude oil. The culture was then incubated at 37°C in an incubator shaker at 150 revolutions per minute (rpm) for five days. A single isolate was obtained by the method of serial dilution of the enriched culture in MSM, plated on nutrient agar (Oxoid, United Kingdom), and incubated at 30 ± 0.1°C.

2.2 | Identification of isolates using 16S rDNA analysis

DNA of the highest crude oil degrader was extracted using the QIAamp DNA Mini Kit (QIAgen, Germantown, Maryland, USA) according to the manufacturer's instructions. The polymerase chain reactions (PCR) were performed in a Biometra® T-Gradient thermocycler (Beverly, Massachusetts, USA). Amplification of the 16S ribosomal DNA loci was performed in a 50-microliter (μL) reaction mixture using a pair of universal primer (F-5′AGAGTTTGATCCTGGCTCAG 3′, R-5′GGTTACCTGTTACGACTT 3′), and 25 μL Go Taq (Taq polymerase, MgCl, 10x reaction buffer) with the following PCR program: preheating at 95°C for 2 min (one cycle), denaturation 95°C for 1 min 30 sec, annealing at 50°C for 30 sec, elongation at 72°C for 45 min (22 cycles), and extension at 72°C for 10 min. The PCR products were purified with QIAquick PCR Purification commercial kit (QIAgen, Germantown, Maryland, USA), sequenced (ABI PRISM Big Dye™ Terminator V.3; ThermoFisher Scientific, Waltham, Massachusetts), and compared against the National Center for Biotechnology Information (NCBI) nonredundant protein database using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1997). Phylogenetic inferences were made with SEQBOOT (for bootstrap analysis) and NEIGHBOR (for neighbor joining analysis) programs fromMEGA4 version 4.0.2 (Tamura, Dudley, Nei, & Kumar, 2007). Identified isolates were kept in glycerol stock at −80°C.

2.3 | Biodegradation of crude oil

The standard inoculum was prepared according to Hazaimeh, Mutalib, Abdullah, Kok Kee, and Surif (2014) in fresh nutrient broth, and the bacterial cells were harvested by centrifugation and resuspended in 0.85% (weight by volume) sterile normal saline to give a cell concentration of 0.5×10^7 colony-forming units per milliliter [CFU/mL]) at 600 nanometer (nm) wavelength using a spectrophotometer (UVmini-1240, Shimadzu, Kyoto, Japan). A total of 10% (v/v) standard inoculum was transferred into 250 mL glass serum bottles equipped with a butyl rubber stopper that contained 1% (v/v) crude oil, either Tapis light or Khafji sour crude oil, in 50 mL MSM and supplemented with 0.5% peptone as a nitrogen source. The cultures were incubated in an orbital shaker (Infors Multitron, Bottmingen, Switzerland) at 150 rpm and 30°C for seven days. The negative control flasks were incubated without inoculum. After seven days, the culture was analyzed for bacteria growth using the pour plate method (CFU/mL) on nutrient agar (Kok Kee, Mohammad, Sahilah, Palsan, & Salmijah, 2014). The residual crude oil samples were quantified by injecting the samples into a headspace connected to a gas chromatography-flame ionized detector (GC-FID) (Perkin Elmer Model 6000, Waltham, Massachusetts, USA) with a capillary column (60 m × 0.32 mm × 0.25 μm; Supelco, Sigma-Aldrich, Steinheim, Germany). The GC-FID system was programmed as follows: ionization voltage: 70 electron volts; interface temperature: 320°C; oven: 50°C; injector: 200°C; detector: 200°C; and flow rate: 1.00 mL/min.

2.4 | Bacterial adhesion to hydrocarbons (BATH) assay

The hydrophobicity of the bacterial cell was evaluated using BATH assay (Rosenberg, Utnick, & Rosenberg, 1980). Culture grown in MSM and Khafji or Tapis crude oil, supplemented with peptone (1%), was washed twice and resuspended with 0.85% of NaCl to give an optical density of 0.5 at 600 nm. From this bacterial cell suspension, 2 mL was aliquoted into a glass vial and mixed with 2 mL of hexadecane (Sigma-Aldrich, Steinheim, Germany), vortexed for 5 min, and left to stand for 60 min to allow the cells to settle. At the end of the 60 min, 1 mL of the aqueous phase was used to read for bacteria concentration at OD600 nm.

The hydrophobicity of the bacterial cell is expressed as the percentage of cell adherence to hexadecane and calculated as follows:

1 − (OD of the aqueous phase/OD of initial cell suspension) × 100

2.5 | Emulsification index (E24)

The presence of biosurfactant was determined by measuring the ability of the culture filtrate to emulsify hexadecane. The E24 of the culture was determined by adding 2 mL of hexadecane to the same amount of culture filtrate, mixing with a vortex for 2 min, and allowing to stand for 24 hours (hr). The E24 index is given as percentage of height of emulsified layer (millimeters [mm]) divided by total height of the liquid column (mm).

2.6 | Growth of isolate on individual hydrocarbons

Bacteria isolate cultured in nutrient broth after 24 hr was collected via centrifugation (5,000 times gravity). The pelleted bacteria were then rinsed twice using NaCl (0.8%) and then resuspended in NaCl (0.8%) to give a final absorbance of 0.5 at OD600 nm. From this saline suspension, 1 mL was inoculated in MSM supplemented with 1% (v/v) of hexadecane, cyclopentane, phenanthrene, or a mixture of hexadecane with cyclopentane, hexadecane with phenanthrene, or cyclopentane with phenanthrene at ratios of 1:1. Flasks were incubated at 30°C, 100 rpm for seven days. The cultures were filtered using Whatman paper (No. 1) to remove residual hydrocarbons, and 1 mL of the resulting filtrate was then read at OD600 nm using a spectrophotometer. When no difference was observed in the turbidity compared to day-0, it was taken as no growth (–). Significant increase in turbidity was taken as good growth (+) and luxuriant (++) when the turbidity was > four-fold compared to day-0.

2.7 | Statistical analysis

Data were reported as mean values ± standard deviation of the number of replicates. Statistical analysis was performed using SPSS PASW Statistic 17 software (SPSS, Chicago, Illinois, USA) for Windows Vista™ and Student's *t* test with levels of confidence at 95% ($\alpha = 0.05$).

3 | RESULTS AND DISCUSSION

From the wastewater sampled, 14 pure isolates were obtained from the enrichment procedure. Out of the 14, only one isolate, designated as 10M2, reached the optical density of $\times 10^8$ CFU/mL within five days of incubation with crude oil. This isolate was selected for identification, whereby partial sequencing of the 16s rDNA region of the isolate showed 99% similarity to *Rhodococcus ruber* (X80625) (Exhibit 1).

Results of the residual total petroleum hydrocarbons content in heavy Khafji sour crude and light Tapis sweet crude after biodegradation for seven days by *Rhodococcus ruber* UKMP-10M2 are presented in Exhibit 2. GC analysis (Exhibit 3) shows that most of the initial peaks were reduced, particularly the recalcitrant pristane and phytane. However, the percentage degradation of heavy Khafji was higher than light Tapis (Exhibit 2). A closer look also shows that degradation of pristane and phytane were 41% and 20%, respectively, higher in heavy Khafji compared to light Tapis by *R. ruber* UKMP-10M2. This result is interestingly in contrast with most reported studies that showed light oils, with mainly moderate chain aliphatic hydrocarbons and low content of aromatic hydrocarbons, are more rapidly colonized by microorganism, leading to faster degradation compared to heavy oils with high-aromatic content (Yemashova et al., 2007). This is because heavy crude oil, to a large extent, contains higher percentages of long-chain hydrocarbons, with lower solubility that inhibits degradation by bacteria (Santisi et al., 2015).

To understand how individual crude oil contents might play a role in influencing degradation of heavy crude versus light crude by *R. ruber* UKMP-10M2, the bacteria were grown on individual hydrocarbons representing aliphatic (hexadecane), cyclic (cyclopentane), and polycyclic aromatic hydrocarbons (phenanthrene) components. The results of bacteria growth on individual hydrocarbons were compared to growth on mixed hydrocarbons as listed in Exhibit 4. *R. ruber* UKMP-10M2 was observed to grow better on individual hydrocarbons representing aliphatic and cyclic hydrocarbons, with the exception of phenanthrene representing polycyclic aromatic hydrocarbons (PAHs), compared to mixed hydrocarbons. This result is expected as aliphatic and cyclic hydrocarbons with their simple and relatively higher hydrophilic structures were reported to be easily degraded compared to PAHs (Adams et al., 2015; Leahy & Colwell, 1990). However, *R. ruber* UKMP-10M2 cultured in hexadecane mixed with phenanthrene (aliphatic mixed with a PAH) showed better growth that equals growth on either hexadecane or cyclopentane alone. An interesting observation in Exhibit 4 was that phenanthrene (three ring), the most complex hydrocarbon structure tested in this study, better supported the growth of *R. ruber* UKMP-10M2 when it was mixed with hexadecane. Indirectly, this result showed that *R. ruber* UKMP-10M2 was able to degrade phenanthrene more efficiently in the presence of hexadecane.

This observation probably can be attributed to cometabolism, whereby the presence of one hydrocarbon accelerated the degradation of a more recalcitrant hydrocarbon (Hazen, 2010). Patel and Kumar (2017) demonstrated that a microbial consortium degraded 2,4-dichlorophenols better in the presence of monochlorophenol, probably due to the similar structure of both compounds initiating common degradative pathways. Khan, Hamayun, Bibi, and Sherwani (2015) similarly reported a 24% increase in bacterial growth in a culture containing complex pyrene (four ring) with hydrocarbons of similar ring structure such as naphthalene (two ring), anthracene (three ring), and phenanthrene (three ring), compared to growth on pyrene alone. Despite this, in the mixed hydrocarbon study, cyclic cyclopentane of higher structure similarity to phenanthrene did not induce higher growth of *R. ruber* UKMP-10M2. In contrast, hexadecane, an aliphatic hydrocarbon structurally dissimilar to the three-ringed aromatic phenanthrene, induced higher growth of *R. ruber* UKMP-10M2. A study by van Beilen, Funhoff, van Loon, Just, and Kaysser (2006) reported that isolates FOS2B and FOS6A with naphthalene and phenanthrene degradation abilities contained a specific open reading frame (ORF) in the naphthalene and anthracene pathway to degrade aromatic hydrocarbons. However, activation of this ORF was related to cytochrome P450 proteins. The P450-type hydroxylases are commonly used by bacterial isolates during aerobic degradation of medium chain length aliphatic hydrocarbons, that is, hexadecane. So it is possible that *R. ruber* UKMP-10M2 in this study showed better growth when

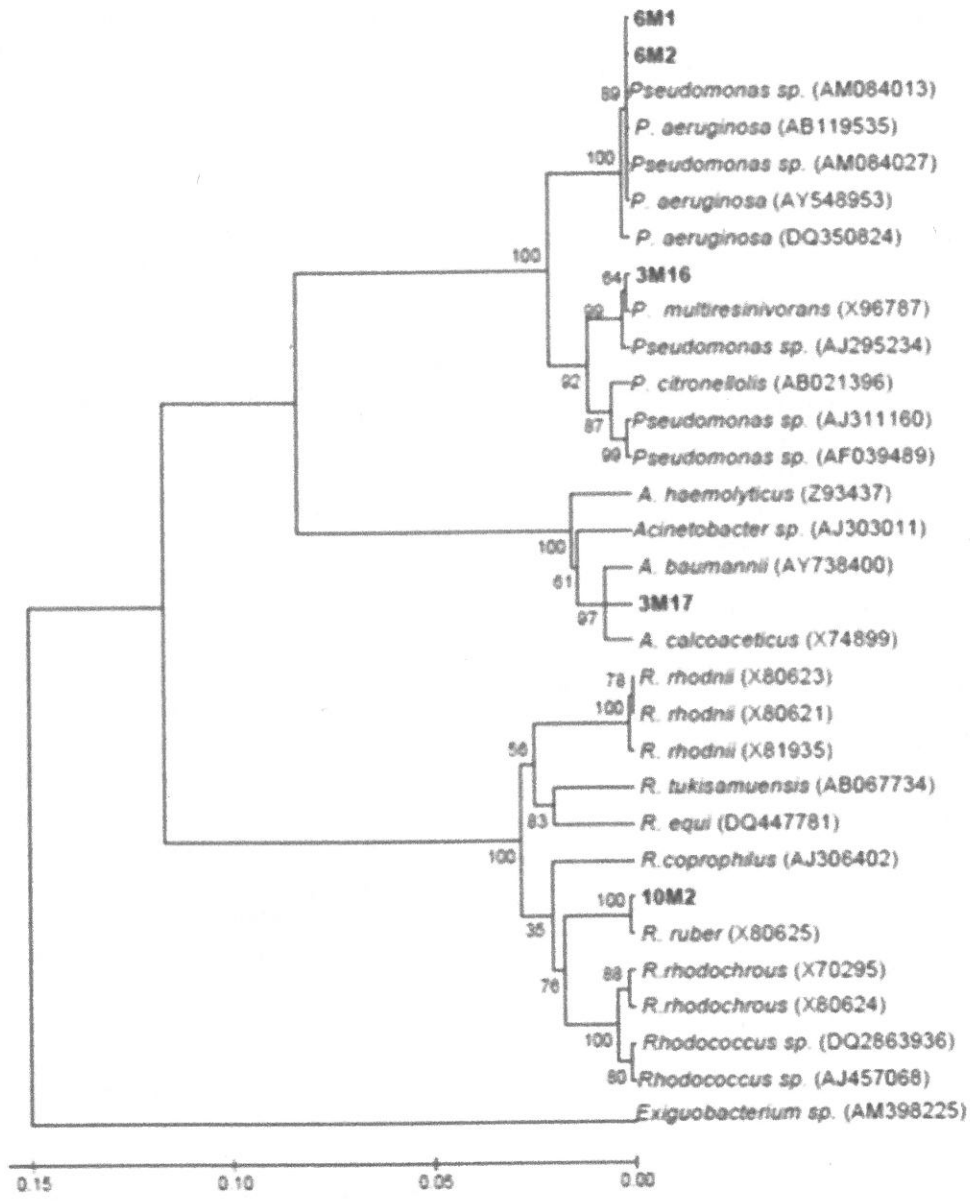

EXHIBIT 1 Phylogenetic neighbors-joining tree of the 16S rDNA gene of isolate UKMP-10M2. The sequence from *Exiguobacterium* sp. was chosen as the outgroup

EXHIBIT 2 Degradation of two different crude oils by *R. ruber* UKMP-10M2 bacteria supplemented with peptone

	Heavy Khafji sour crude (%)			Light Tapis sweet crude (%)		
Isolate	**TPH**	**Pristane**	**Phytane**	**TPH**	**Pristane**	**Phytane**
Rhodococcus ruber	84.31	89.64	48.71	68.19	48.62	29.12

phenanthrene was mixed with hexadecane, because the presence of hexadecane activated the cytochrome P450 hydroxylases, which in turn activated the ORF's aromatic ring structure degradative pathway. Without the presence of hexadecane, the effect of cometabolism was not observed. Thus, the results presented in Exhibit 4 explain the dynamic interaction between different crude oil components, that is, mixture of aliphatics with cyclic hydrocarbons, aliphatics with PAHs, and cyclics with PAHs, affected the degradation of heavy Khaji and light Tapis crude oils, even though biodegradation was performed by the same *R. ruber* UKMP-10M2.

To further understand the mechanism that contributed to different degradation between the two types of crude oil, the ability of *R. ruber* UKMP-10M2 to emulsify crude oil and its cell surface hydrophobicity was studied as shown in Exhibit 5. The cultures were supplemented with peptone because a previous study by our group showed *Rhodococcus* sp. achieved optimum growth on peptone compared to ammonium chloride and yeast (Hamzah et al., 2013), and was expected to stimulate production of biosurfactants. However, results in Exhibit 5 suggest significant differences of the quality of biosurfactant produced. Culture filtrate of *R. ruber* UKMP-10M2 grown on

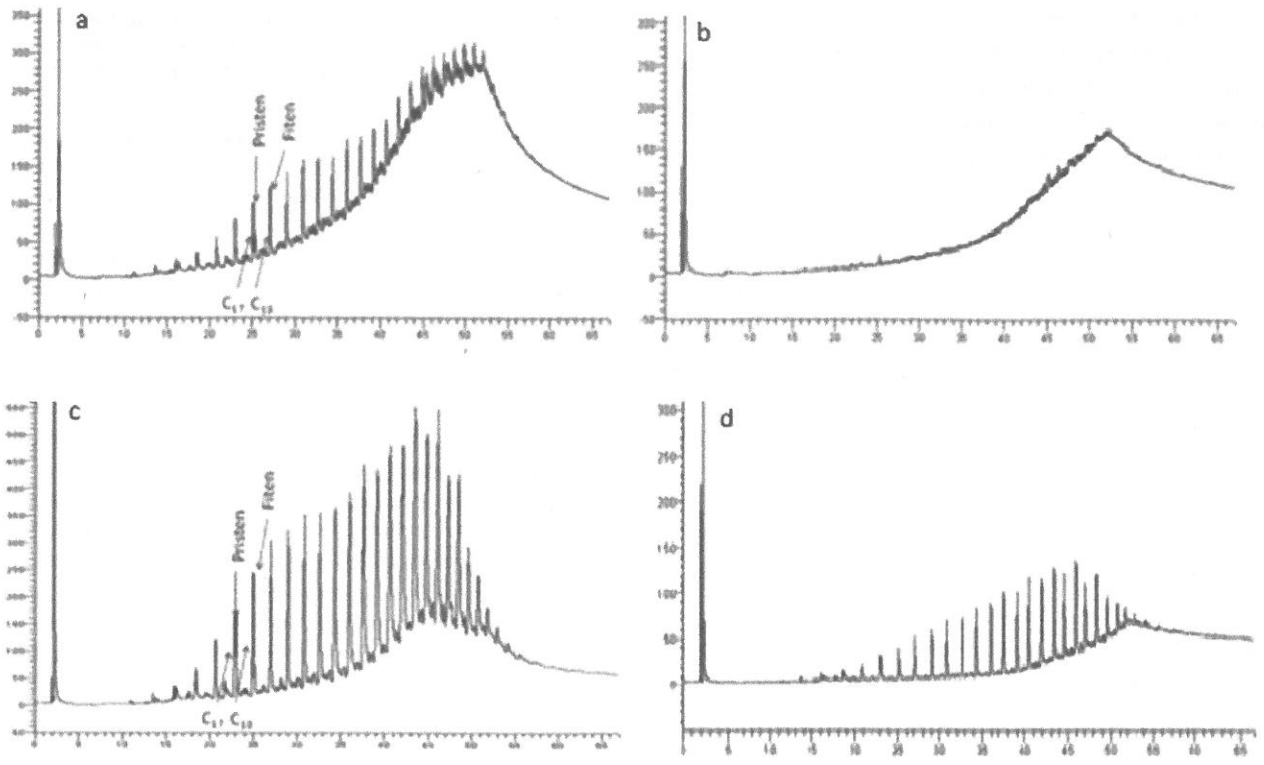

EXHIBIT 3 Representative GC profile of degradation of heavy Khafji sour crude oil at day-0 (a) and day-7 (b), and light Tapis sweet crude at day-0 (c) and day-7 (d) by *Rhodococcus ruber* UKMP-10M2

EXHIBIT 4 Growth of *Rhodococcus ruber* (UKMP-10M2) on representative individual hydrocarbons found in crude oil and mixtures

Hydrocarbons	Growth
Hexadecane	+++
Cyclopentane	+++
Phenanthrene	+
Hexadecane + cyclopentane	++
Hexadecane + phenanthrene	+++
Cyclopentane + phenanthrene	++

Semi-quantitative analysis on the bacterial growth tested on different representative individual hydrocarbons found in crude oil and mixture: + (good), ++ (luxuriant), +++ (above luxuriant).

EXHIBIT 5 Emulsification index and BATH assay of crude biosurfactant from culture filtrate of *R. ruber* UKMP-10M2

Culture filtrate source	Emulsification Index (E24)	BATH Assay (%)
Heavy Khafji sour crude	72.21 ± 0.22	46.3 ± 1.0
Light Tapis sweet crude	50.60 ± 0.71	60.4 ± 0.7

Khafji sour crude exhibited > 20% higher E24 reading. BATH assay, however, showed *R. ruber* UKMP-10M2 cultured in light Tapis showed higher bacterial cell surface hydrophobicity. This result suggested that supplementing nitrogen, in this case peptone, elicited different characteristics of biosurfactant influenced by the type of crude oil.

The presence of heavy crude oil seems to induce higher secretion of biosurfactants, as shown by the higher E24 value, as a mechanism to lower the solubility of pristane/phytane and other aromatic hydrocarbons, making these hydrocarbons highly bioavailable to *R. ruber* UKMP-10M2 to be degraded. Secretion of biosurfactants as a means of solubilizing heavy crude oil is not new, particularly in *Rhodococcus* sp. (Kuyukina & Ivshina 2010). Aoshima et al. (2006) reported *Rhodococcus* sp. secretes biosurfactant that form a stable oil-in-water emulsion in heavy crude oil.

Whereas, in the presence of light Tapis, peptone induced higher cell surface hydrophobicity of *R. ruber* UKMP-10M2 rather than increasing the E24 index through secretion of biosurfactant solely. A pioneering study by Rapp, Bock, Wray, and Wagner (1976) demonstrated that in the presence of aliphatic alkanes that characterize light crude oil, *Rhodococcus erythropolis* HS4 produced trehalose lipids that increased bacterial surface hydrophobicity, usually leading to a general superficial reduction of surface tension.

Although different bacteria are known to select different sources of nitrogen to enhance the production of biosurfactant, as in the case of *Aeromonas* sp grown on soybean, an organic nitrogen source (Ilori, Amobi, & Odocha, 2005) and *Bacillus* sp. producing the four times more biosurfactant when supplemented with ammonium nitrate, compare to using peptone (Joshi & Shekhawat, 2014). However, in this study, the same nitrogen source (peptone) appeared to stimulate different biosurfactant production by the same microbe. Results of this study indicate that it is not a matter of a simple interaction between supplying a particular nitrogen source stimulating the production of homogenous biosurfactant in the same microbial species.

Circumstantial evidence from this study suggests the type of nitrogen source affects biodegradation in two possible ways: (1) induces

secretion of biosurfactant when heavy Khafji crude oil was present and (2) increases the hydrophobicity of the bacterial cell surface when light Tapis crude oil was in the culture. Cooperative action between secretion of biosurfactant to form oil-in-water miscelles and to coat the bacterial cell surface to increase hydrophobicity might be a strategy adopted by *R. ruber* UKMP-10M2 to make the moderately hydrophobic medium chain hydrocarbons present in light crude oil bioavailable for degradation. This distinguishes the possible mode of action by *R. ruber* UKMP-10M2 in secreting large amounts of biosurfactant to emulsify the highly hydrophobic heavy chain hydrocarbons in heavy crude oil. These observations merit further study to better understand the dynamic interaction between nitrogen source, biosurfactant production, and cell surface hydrophobicity in response to different types of crude oil by hydrocarbon degrading bacteria.

4 | CONCLUSION

An isolate identified as *Rhodococcus ruber* (UKMP-10M2) using 16S rDNA and phylogenetic tree comparative analysis was found to biodegrade heavy Khafji sour crude oil more efficiently compared to light Tapis sweet crude oil. A mixture of aliphatic hexadecane increased the degradation of three-ring phenanthrene, compared to phenanthrene alone by *R. ruber* UKMP-10M2, suggesting a cometabolic mechanism. Peptone was found to affect higher biosurfactant secretion as reflected in the E24 index in *R. ruber* UKMP-10M2, as a strategy to effectively degrade heavy crude oil. In contrast, peptone moderately increased biosurfactant secretion by *R. ruber* UKMP-10M2, but significantly increased cell surface hydrophobicity as means to degrade light crude oil.

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