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Comparative degradation of hydrocarbons using bacteria isolated from Angsi light crude oil and Khefji heavy crude oil

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ABSTRACT

Two bacterial species were successfully isolated, namely AL1 from Angsi light crude oil and KH1 from Khefji heavy crude oil. The results from sequencing showed that the isolate AL1 has 97% sequence match with *Acinetobacter baumannii*, whereas the isolate KH1 has 98% similarity to DNA sequences of *Pseudomonas aeruginosa*. Both bacterial species were able to degrade Angsi light crude oil 100% within seven days. However, only *P. aeruginosa* completed the degradation of Khefji heavy crude oil. *A. baumannii* only degraded 67% of the Khefji heavy crude oil. Discussion of the results suggested that *P. aeruginosa* that originated from the Khefji heavy crude oil has less metabolic restriction (eg. biosurfactant secretion and quorum sensing), which allows the bacteria to degrade hydrophobic hydrocarbons predominantly found only in high density crude oil. This advantageous characteristic was not naturally found in *A. baumannii* that was isolated from Angsi light crude oil.

KEYWORDS: degradation, hydrocarbon, crude oil, bacteria.

INTRODUCTION

Biodegradation using microbes has been widely studied because it leads to complete degradation of complex hydrocarbons to carbon dioxide and water [1-4]. Most of the microbes comprising various strains isolated from petroleum-contaminated soil or water have been shown to have oil degradation capabilities. Nevertheless, the success of each field application varied depending on the environmental factors, physical contact between the hydrocarbons and bacteria, metabolic restrictions and duration of the process [5]. However, the abiotic factor of crude oil types and how it affects the hydrocarbons degradation by bacteria were not well explored nor documented.

According to the unit of American Petroleum Institute gravity (API), crude oil with specific gravity more than 40 is classified as high-density heavy crude oil, API between 30-40 as medium-density medium crude oil and API less than 20 as low-density light crude oil [6]. The different density of the crude oil depends on the types of individual hydrocarbons present and the percentage of sulphur. Thus, the toxicity of crude oil also depends on the types and ratio of individual hydrocarbons with different chemical composition and structure present in the crude oil [7]. Due to its complex mixture of hydrocarbons [8], crude oil can inhibit the growth of different bacteria [9]. It has been shown that different bacterial isolates survived in the different types of crude oil [10]. Hamzah *et al*. [11] reported that both *Pseudomonas aeruginosa* and *Acinetobacter lwoffi* were able to resist the toxicity of Sumandak light crude oil and registered a significant growth. However, both bacterial isolates were not resistant towards the South Angsi heavy crude oil. On the other hand, a study by Wong *et al*. [12] pointed out that *Rhodococcus ruber* shows better growth

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in Khefji heavy crude oil compared to the Tapis light crude oil.

Hence, the objective of this study was to identify crude oil-degrading bacteria from two types of crude oil, namely Angsi light and Khefji heavy crude oil. This is followed by the evaluation on how the bacterial isolates were able to biodegrade the opposite crude oil.

MATERIALS AND METHODS

Source of samples

Bacteria were isolated from the wastewater sampled from a treatment pond that was contaminated by Angsi light crude oil and from a sedimentation pond that was contaminated by Khefji heavy crude oil. Both ponds were situated within a petroleum refinery facility in Kuala Terengganu, Malaysia. The sample originated from wastewater contaminated by Angsi light crude oil were incubated in mineral salt medium (MSM) supplemented with 1% (v/v) Angsi light crude oil. The sample sourced from wastewater contaminated by Khefji heavy crude oil were incubated in mineral salt medium (MSM) supplemented with 1% (v/v) Khefji heavy crude oil [3]. Both cultures were then incubated at 37 °C in an incubator shaker at 150 rpm for seven days. At the end of the incubation period, the cultures were serially diluted and plated on nutrient agar and incubated again at 30 ± 0.1 °C for 24 hours. At the end of the 24 hours, a single colony was picked up from each of the plates [12]. The colonies were then streaked onto a MSM agar plate supplemented by the respective Angsi light or Khefji heavy crude oil, followed by incubation at 37 °C for 24 hours. Colonies that were observed to grow within 24 hours were picked and used in the bacterial identification process.

Identification of bacterial species

The DNA from the single colony from each of the two plates (Angsi and Khefji) was extracted using the QIAamp DNA Mini Kit (QIAgen, USA) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was performed to amplify the 16S ribosomal DNA loci in a Biometra® T-Gradient thermocycler (USA) by using a pair of universal primer (F-5'AGAGTTTGATCCTGGCTCAG 3', R-5'GGTTACCTGTTACGACTT 3') [13]. The product of the PCR reaction was then purified using a commercial kit (Qiagen USA) and sequenced using an ABI PRISM Big Dye™ Terminator V.3 sequencer. The nucleic acid sequence obtained from the sequencing was then compared against the nucleotide database in National Center for Biotechnology Information (NCBI) using nucleotide Basic Local Alignment Search Tool (BLAST) to find similar sequences [14].

Biodegradation of Angsi light density and Khefji heavy density crude oil

The biodegradation experiment was conducted by inoculating 1% (v/v) of bacterial inoculum into flasks containing 98 mL of MSM media, followed by either 1% (v/v) of Angsi light or Khefji heavy crude. The bacterial inoculum containing approximately 10^8 colony forming unit of bacterial cell per mL (cfu/mL) was prepared according to Hazaimeh *et al*. [15] in 0.85% (w/v) normal saline solution.

The cultures were incubated in an orbital shaker (Infors Multitron, Switzerland) at 150 rpm and at 30 °C for seven days. After seven days, the residual crude oil samples were quantified by injecting the samples to a gas chromatography-flame ionized detector (GC-FID) (Perkin Elmer Model 6000, US) with a capillary column (60 m × 0.32 mm × 0.25 µm; Supelco, Sigma-Aldrich Germany). The GC-FID system was programmed as follows: ionization voltage: 70 eV; interface temperature, 320 °C; oven: 50 °C; injector: 200 °C; detector: 200 °C; and flow rate: 1.00 mL/min [15].

RESULTS AND DISCUSSION

Table 1 below shows the number of single colonies isolated from the Angsi light and Khefji heavy crude oil wastewater pond. Approximately 40% bacterial isolates were obtained from Angsi light compared to Khefji heavy crude oil. Not only that, there were also a higher number of colonies isolated from Angsi light crude oil that can grow within 24 hours. In comparison, only 1 of the 7 colonies isolated from Khefji heavy crude oil was able to grow in MSM agar within 24 hours of incubation. These results suggest that there is a greater diversity of bacterial species in the Angsi

Table 1. Number of colonies obtained from the enrichment and PCR process.

	Number of colonies obtained		
	MSM broth	**MSM agar**	**PCR**
Angsi light	18	3	1 (AL1)
Khefji heavy	7	1	1 (KH1)

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GCTTAGGAATCTGTCTATTACTGGGGGACCACATCTCTAAAGGGATGCTTATACCGCATA 143
||||||||||||| |||||| |||||||| ||||||| ||||||||||| ||||||||||
GCTTAGGAATCTGCCTATTAGTGGGGGACAACATCTCGAAAGGGATGCTAATACCGCATA 167
CGTCCTACGGGAGAAAGCAGGGGATCTTCGGACCTTGCGCTAATAGATGAGCCTAAGTCG 203
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CGTCCTACGGGAGAAAGCAGGGGATCTTCGGACCTTGCGCTAATAGATGAGCCTAAGTCG 227
GATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCTGTAGCGGGTCTGAGAG 263
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
GATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCTGTAGCGGGTCTGAGAG 287
GATGATCCGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGG 323
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
GATGATCCGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGG 347
GAATATTGGACAATGGGGGGAACCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTT 383
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
GAATATTGGACAATGGGGGGAACCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTT 407
ATGGTTGTAAAGCACTTTAAGCGAGGAGGAGGCTACTTTAGTTAATACCTAGAGATAGTG 443
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ATGGTTGTAAAGCACTTTAAGCGAGGAGGAGGCTACTTTAGTTAATACCTAGAGATAGTG 467
GACGTTACTCGCAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAG 503
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
GACGTTACTCGCAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAG 527
GGTGCGAGCGTTAATCGGATTTACTGGGCGTAAAGCGTGCGTAGGCGGCTTATT-AGTCG 562
|||||||||||||||||||||||||||||||||||||||||||||||||||||| |||||
GGTGCGAGCGTTAATCGGATTTACTGGGCGTAAAGCGTGCGTAGGCGGCTTATTAAGTCG 587
GATGTGAAATCCCCGAGCTTAACTTGGGAATTGCATTCGATACTGGTGAGCTAGAGTATG 622
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
GATGTGAAATCCCCGAGCTTAACTTGGGAATTGCATTCGATACTGGTGAGCTAGAGTATG 647
GGAGAGGATGGTAGAATTCCAGG-GTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACC 681
||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
GGAGAGGATGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACC 707
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Figure 1. Nucleotide sequences of AL1 that matches with the sequence of *A. baumannii* from the NCBI database.

light crude oil, demonstrating that Angsi light crude oil is less toxic than Khefji heavy crude oil to bacteria. Heavy crude oil characteristically contains higher percentage of sulphur and sulphur-containing organic compounds including sulfides and metal-sulphides [16]. These compounds are suspected to inhibit kinetic enzymes in bacteria [17], which can negatively alter the microbial community index, causing lower bacteria diversity [18]. Exposure to heavy crude oil causing a decrease in microbial counts, diversity and proliferation was also observed by Saadoun *et al.* [19] and Ekpo and Ebeagwu [20].

Of the four isolates obtained from MSM agar, only 2 isolates had their 16S ribosomal DNA loci successfully amplified using PCR (Table 1). The results of the DNA sequencing showed that the isolate from Angsi light (AL1) has 97% sequence match with *Acinetobacter baumannii* (Figure 1),

whereas isolate from Khefji heavy (KH1) shows 98% similarity to DNA sequences of *Pseudomonas aeruginosa* (Figure 2).

Table 2 shows the efficiency of *A. baumannii* and *P. aeruginosa* in degrading Angsi light or Khefji heavy crude oil. Both isolates were able to degrade 100% of the Angsi light within seven days. However, only *P. aeruginosa* that was isolated from the Khefji heavy crude oil could almost fully degrade Khefji crude oil. The *A. baumannii* isolated from the Angsi light crude oil was less efficient in degrading Khefji heavy crude oil.

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CGGCGGCCTAACACATGCAAGTCGAGCGGATGAAGGGAGCTTGCTCCTGGATTCAGCGGC 65
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CGGCGGCCTAACACATGCAAGTCGAGCGGATGAAGGGAGCTTGCTCCTGGATTCAGCGGC 60
GGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTCCGGAAACGGG 125
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
GGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTCCGGAAACGGG 120
CGCTAATACCGCATACGTCCTGAGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTATCA 185
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CGCTAATACCGCATACGTCCTGAGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTATCA 180
GATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCG 245
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
GATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCG 240
TAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACG 305
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
TAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACG 300
GGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTG 365
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
GGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTG 360
TGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAA 425
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
TGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAA 420
TACCTTGCTGTTTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCC 485
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
TACCTTGCTGTTTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCC 480
GCGGTAATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGT 545
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
GCGGTAATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGT 540
GGTTCAGCAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTAC 605
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
GGTTCAGCAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTAC 600
TGAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATA 665
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
TGAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATA 660
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Figure 2. Nucleotide sequences of KH1 that matches with the sequence of *P. aeruginosa* from the NCBI data base.

Table 2. Degradation of Angsi light and Khefji heavy crude oil.

Bacterial isolate	**Crude oil degradation (%)**	
	Angsi light	**Khefji heavy**
A. baumannii	100	67
P. aeruginosa	100	98

By looking at the bacterial species and its source from where they are isolated, a few observations can be made. Firstly the *P. aeruginosa* isolated from Khefji heavy crude oil can develop natural resistant against the toxicity exerted by high density hydrocarbons predominantly found in crude oil with higher API. One of the key strategies allowing *P. aeruginosa* to resist Khefji heavy crude oil is its degradation. Since Khefji heavy crude oil contained higher percentages of hydrophobic hydrocarbons, including long-chain aliphatic, monoaromatic and polycyclic aromatic (PAHs) hydrocarbons, it is postulated that *P. aeruginosa* can secrete the biosurfactant that facilitates degradation of Khefji heavy crude oil. Secretion of the biosurfactant was shown to increase the bioavailability of such hydrocarbons to the intracellular enzymes responsible for metabolizing it [21]. Another *Pseudomonas* species, *P. putida*, was observed to be able to mineralize benzene, toluene and phenol [22].

Another possible key factor is the ability of the bacteria to modulate specific hydrocarbon-degrading enzymes *via* quorum sensing [23]. *Pseudomonas putida* AQ8 was reported to activate membrane-bound dioxygenases that are responsible for degrading high-density crude oil *via* the quorum sensing system [24]. It is also reported that other bacterial behaviours regulated by quorum sensing include secretion of biosurfactants [25]. These features are positively involved in hydrocarbon degradation, particularly important to hydrophobic hydrocarbons found in the Khefji heavy crude oil. In fact, a strong correlation between the expression of genes involved in hydrocarbon depletion and quorum sensing has been proposed [25].

In comparison, the *A. baumannii* isolated from the Angsi light crude oil has no such key characteristics. Lower density crude oil consists mostly of shorter aliphatic hydrocarbons, and to a lesser extent a lower percentage of hydrophobic monoaromatics or PAHs [26]. This explains why *A. baumannii* isolated from Angsi light crude oil was not able to secrete the biosurfactant. This will prevent the hydrophobic hydrocarbons inside the Khefji heavy crude oil from degradation. This is because biosurfactant affects the contact, transport and transformation of bacteria to the hydrophobic hydrocarbons [26]. Similar observation was shown in a study using *Acinetobacter* sp., whereby only 69.27% of high-density viscous oil sludge was degraded [27]. Another report showed *A. junni* B6 was able to grow well on media containing Iranian light crude oil, and preferentially degraded aliphatic hydrocarbons, instead of PAHs or monoaromatic hydrocarbons [28]. The same observation was also reported in a recombinant *A. baumannii* pJES strain, whereby the bacteria preferred to degrade alkanes over the more hydrophobic aromatic compounds [29]. Although there is a report of *Acinetobacter* sp. YC-X2 strain that produces the biosurfactant, the production was positively correlated to the cell growth [30]. This means that in the present study, the *A. baumannii* was not isolated from the Khefji heavy crude oil, indirectly showing that the bacteria is unable to grow in Khefji crude oil and hence does not produce the biosurfactant.

Although numerous crude oil-degrading bacterial species were isolated and reported, each of the bacterial species differ in their ability to degrade the different types of crude oil. This study demonstrated the possible reasons why bioremediation of crude oil using bacteria can be inefficient. Ideally, the bacterial species chosen to degrade a particular type of crude oil should be isolated from the same type of crude oil to ensure there is no growth inhibition nor metabolic restriction. The results from the present study suggest the importance of pairing bacterial species with the correct type of crude oil to optimise the process of degradation.

CONCLUSION

From the two wastewater ponds, the enrichment process successfully isolated AL1 from Angsi light crude oil and KH1 from Khefji heavy crude oil. The results of the DNA sequencing showed that the isolate AL1 has 97% sequence match with *Acinetobacter baumannii*, whereas the isolate KH1 shows 98% similarity to DNA sequences of *Pseudomonas aeruginosa*. Both bacterial species were able to degrade Angsi light crude oil, but only *P. aeruginosa* can degrade Khefji heavy crude oil efficiently.

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CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest.

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