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Identification of bacterial diversity from an oil-spill contaminated marine environment in Tanjung Karang, Selangor, Malaysia

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ABSTRACT Bacteria were isolated from crude oil contaminated muddy beach by using the liquid enrichment culture method. Two different types of samples have been taken which are in solidified form and in liquid form (from seawater as oil slick). Colony and cell morphological studies as well as physiological and biochemical studies using classical biochemical test were used to identify some of the isolates as bacteria from the genera *Acinetobacter, Pseudomonas, Moraxella, Neisseria* and *Staphylococcus.*

ABSTRAK Bakteria telah diasingkan dari pantai berlumpur yang tercemar dengan minyak mentah dengan menggunakan kaedah kultur pengkayaan cecair. Dua jenis sample berbeza telah diambil iaitu dalam bentuk pepejal and cecair (dari air laut sebagai lapisan minyak). Kajian morfologi koloni dan sel serta kajian fisiologi and biokimia menggunakan ujian biokimia klasik telah diguna untuk mengenalpasti beberapa isolat sebagai bacteria dari genera *Acinetobacter, Pseudomonas, Moraxella, Neisseria* dan *Staphylococcus.*

(bacterial diversity, crude oil spill, hydrocarbon-utilising bacteria)

INTRODUCTION

The principal forms of petroleum are natural gas, which does not condense at standard temperature and pressure (STP= 760 mmHg, 60°F or 15.6°C), condensate, which is gaseous in the ground but condense at the surface; and crude oil, the liquid part of petroleum. Crude oil can be refined to form various products from gas (C_1 to C_5) to petroleum coke (> C_{20}).

The ecosystem has always been able to cope with naturally occurring amounts of petroleum hydrocarbon. But nowadays it faces a major problem by the great increase in the total input of petroleum hydrocarbons caused by human's activities. With increasing frequency of petroleum transportation, the probability of potential accidental release of petroleum into the environment is also increasing. The other sources of petroleum contamination are service station, crude oil and fuel storage, oil field, refining facilities, broken pipelines, oil-water separator and drilling mud.

Microorganisms with their rapid growth rates, have the most rapid turnover of their DNA and can evolve altered gene to produce novel enzymes for handling " foreign" compounds. It has been well documented that microorganisms are responsible for effective removal of contaminated compounds from the environment. The ability to degrade and/or utilize such compounds is exhibited by at least 25 genera of bacteria including *Achromobacter, Acinetobacter* and *Pseudomonas* and 31 genera of fungi including *Aureobasidium, Candida* and *Penicillium* [1]. In this paper, we report the identification and characterization of bacteria isolated from a beach area at Tanjung Karang, Selangor which had been contaminated by crude oil spill.

MATERIALS AND METHOD

Sampling

Samples that had been contaminated with crude oil were obtained along Kampung Tanjung Karang Lama beach in Tanjung Karang, Selangor

in April 1998 The beach was contaminated by oil spill from December 1997.

The oil-slick was detected at sea through aerial surveillance on December 17, 1997. The authorities decided to allow oil-slick to hit the Selangor coastline before starting clean-up operation on December 25, 1997. This was because the oil patches had spread over a large area and were not easily contained. It would also have been time-consuming and costly to try to deal with the slick at sea especially when the authorities were working with limited resources. The oil-slick which covered a 20 km stretch had obviously been around for quite a long time based on the fact that most of the oil patched had already thickened while some parts has solidified before the oil-slick hit the Selangor coastline. The slick which followed as a result of a tanker collision on October 15, 1997 between a Cyprus-registered tanker, *Evoikos* (carrying 120,000 tonnes of fuel to Singapore) and a Thai Supertanker, the Orapin Global. The collision, which occurred about 13 km off Singapore coastline, has spilled some 25,000 tonnes of fuel oil into the sea. In addition the sinking of a Chinese cargo ship, M.V An Tai (carrying 8485 tonnes of fertilisers) in the North Port, Klang on November 22, 1997 may also be source of contamination because M.V An Tai also had 237 metric tonnes of heavy oil and 27 metric tonnes of diesel on board [5].

Two types of samples were taken from Kampung Tanjung Karang Lama beach: sediment containing oil and seawater containing oil. These sediment samples were placed into sterile plastic bags and sealed while the liquid samples were poured into sterile Schott bottle. The samples were brought to the laboratory and processed on the same day itself to prevent contamination and to ensure that the microorganisms were from indigenous population.

Isolation and purification of microorganisms

The liquid enrichment culture method was used to isolate potential hydrocarbon-utilising microorganisms. The sediment (50 g) was mixed with 100 ml of sterile artificial seawater (per liter) containing NaCl (24.6 g), KCl (0.67 g), $CaCl_2.2H_2O$ (1.36 g), $MgSO_4.7H_2O$ (6.29 g), $MgCl_2.6H_2O$ (4.66 g) and Na_2CO_3 (0.18 g). The pH was adjusted to 8.0 in a sterile 500 ml Erlenmeyer flask. The flask was incubated for 30 min at 37°C on an orbital shaker to make homogenous mixture. The homogenous mixture was centrifuged at 9000 rpm for 10 minutes to separate the particulate from the supernatant. 1 ml of the suspended supernatant (from sediment) and 1 ml of the liquid sample (from seawater) were inoculated separately into 50 ml Erlenmeyer flask containing 1% (v/v) used engine oil and 1% (v/v) diesel oil. The used engine oil was obtained from a service and maintenance workshop at Sentul, Kuala Lumpur while the diesel oil was obtained from a Shell station in Petaling Jaya, Selangor. The used engine oil and the diesel oil were filter-sterilized using Millipore membrane filters (Type HA, 0.45µm), placed in a sterilized swinnex filter holder. Also 1 ml of the suspended supernatant and 1 ml of the liquid sample were separately diluted and 0.1 ml of an appropriate dilution was spread on Basal Salt Media (BSM) agar plates (per liter) containing $CaCl_2$ (0.02 g), $FeCl_3$ (0.02g), KH_2PO_4 (1.0g), K_2HPO_4 (1.0 g), $MgSO_4.7H_2O$ (1.0 g), $(NH_4)_2SO_4$ (1.0g), pH was adjusted to 7.0-7.2; (for agar plates, 2% (w/v) of Bacto Agar was added) containing 1% (v/v) used engine oil and 1% (v/v) diesel oil. All flasks were incubated aerobically at 37°C on an orbital shaker (Innova 4900, New Brunswick Sci., USA) at 220 rpm for four days. After incubation, a loop full of the enrichment culture was streaked onto BSM + 1% (v/v) used engine oil and 1% (v/v) diesel oil (BSM+E.O+D.O) plates. Each colony was picked off randomly from the BSM+E.O+D.O agar plates and patched on TSA plates (per liter): Tryptone (15g), Soya peptone (5 g), NaCl (5 g), Bacto Agar 2% [w/v] as well as BSM+E.O+D.O plates. Each colony was designated by a number. The plates were incubated at 37°C. After 24 hours of incubation, individual colony that showed growth on both plates was picked and streaked on fresh TSA plates and incubated. Each colony was subcultured again on TSA plates to obtain pure culture.

Identification of isolates.

All isolates were examined based on morphological and physiological characteristics. Colony morphologies were observed on TSA plates based on colour, size, shape, edge, elevation and texture. Cell morphologies were

observed by Gram staining method. Motility test was performed using SIM media.

Physiological characteristics were examined based on biochemical tests following the method in Cowan and Steel [2]. Fresh cultures were grown up to 24 hours at 37°C and sufficient inocula were used for the biochemical tests. *Escherichia coli* and *Bacillus megaterium* were used as controls. The biochemical tests included the following: oxidase and catalase activity, sulphide and indole production, oxidation-fermentation, carbohydrate fermentation, lactose fermentation, double sugar fermentation, hydrogen sulphide production and motility test. *Pseudomonas* agar base was also used for selective isolation of Pseudomonad when supplemented with CFC selective Agar supplement SR103 (OXOID Limited, England). The Methyl Red-Voges Proskauer (MRVP) medium was used for differentiation of the *coli-aerogenes* group.

RESULTS AND DISCUSSION

Isolation and purification of bacteria

The liquid enrichment culture technique used in the isolation method is commonly employed as the first step in isolating specific microorganisms from nature and had been considered a reliable method [3]. By favouring the growth, survival and spatial separation from other members of the population, this method was designed to increase the relative numbers of particular organisms. In this study, BSM containing 1% (v/v) diesel oil and 1% (v/v) used engine oil was used as sole carbon and source to select for hydrocarbon-utilising microorganisms. During incubation at 37°C, it was observed that flask containing inoculum from samples turned turbid indicating growth of hydrocarbon-utilising microorganisms. Interestingly, black particles were seen floating in the flask, on the surface of the oily layer. In the beginning, the particles were small and more dispersed. Later they aggregated and formed larger, sticky-brown balls of oil, which sank to the bottom (Figure 1).

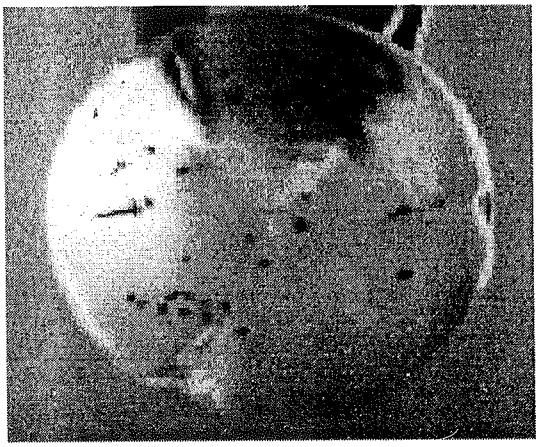

Figure 1. Floating particles (indicated by arrow) in culture flask.

Utilisation of low molecular weight hydrocarbon by the bacteria leaves the higher density fraction in the media, which may form such particle. The production of entrapment of microbes within the oil emulsion may also be possible reason for the formation of the black particles. After 5 days of incubation, the colour of the culture broth turned into white-brownish. After a week of incubation, the oily layer disappeared and the black particles were no more seen but the turbidity still increased. Many colonies were obtained after 24 hours of incubation at 37°C on BSM + E.O + D.O plates. The isolates were individually picked and patched onto BSM + E.O + D.O plates and TSA plates. Colonies that grew on both plates were streaked on TSA plates to obtain pure culture.

There are some limitations in using both kind of oil (used engine oil and diesel oil) in the culture media because some microorganisms cannot withstand the toxicity of used engine oil. This is due to the materials collected from engine that slowed down the growth of bacteria. But there are also some advantages by providing both kind of oil. This will give the microorganisms an alternative to select the composition of carbon compound they prefer most. The diesel oil is expected to undergo faster biodegradation in their susceptibility to microbial attack, since diesel oil is composed of C_{15}-C_{25} carbon compound, while used engine oil is composed of C_{26}-C_{40} carbon compound. Selected isolates were identified using morphological, physiological and molecular analysis.

Identification

The morphological characteristics of 15 selected isolates are presented in Tables 1 and 2. The isolates were differentiated into 2 main groups based on the Gram reaction, which are Gram negative and Gram positive. The isolates were further characterized using classical biochemical tests (Table 3). The results obtained were referred to Cowan and Steel [2] for species identification.

Most of the isolates obtained from the crude oil contaminated samples were *Acinetobacter* sp. (5 out of 15). This particular species is among the bacterial genera most often found in petroleum-contaminated habitat and has been extensively used in studies of n-alkane oxidation. Bacteria belong to this genera have been described as contaminants in petroleum products or even as hydrocarbon-degraders [6]. This species is Gram-negative and coccobacilli or short rods in their shape with size of 1-2 μm. In smear, *Acinetobacter* sp. may show a superficial resemblance to *gonococcus* or *meningococcus*. The members of *Acinetobacter* are non-motile bacteria. Some *Acinetobacter* can breakdown sugar by oxidation but some cannot. *Acinetobacter* sp. does not produce any pigment and gave the negative results for arginine test. The *Acinetobacter* have much common with the *Moraxella* and recent rRNA-DNA hybridisation results lend further support to previous suggestions that the two genera should be united [2].

Table 1: Colony morphology characteristics of isolates on BSM + Engine Oil + Diesel Oil

Isolate	colour	size (μm)	shape	edge	elevation	texture
1	yellow	1-2	circle	entire	convex	smooth
2	whittish	1-2	circle	entire	convex	smooth
3	yellow	1-2	circle	entire	convex	smooth
4	yellow	<1	circle	entire	convex	smooth
5	orange	1-2	circle	entire	convex	smooth
6	yellow	1-2	circle	entire	convex	smooth
7	transparent	1-2	unarranged	undulate	rise	smooth
8	yellow	1-2	circle	entire	convex	Smooth
9	yellow	1-2	circle	entire	convex	Smooth
10	whittish	1-2	circle	entire	convex	Smooth
11	yellow	1-2	circle	entire	convex	Smooth
12	whittish	<	unarranged	undulate	rise	smooth
13	yellow	1-2	circle	entire	convex	smooth
14	yellow	1-2	circle	entire	convex	smooth
15	yellow	1-2	circle	entire	convex	smooth

The other important isolates obtained from this study was *Pseudomonas* sp. Isolation of *Pseudomonas* sp. is not surprising because this genus had been reported to produce many biodegradative enzymes [7] and also their capacity for adaptive changes [4]. The genus *Pseudomonas* is large one, the members of which may be found ubiquitously in the air, soil, fresh and salt water. They are motile by virtue of flagella. Most species of *Pseudomonas* ferment glucose, but not lactose. They are generally capable of reducing nitrate to nitrite, nitrogen or ammonia. *Pseudomonas* sp. is the only hydrocarbon-utilising bacteria that grow readily at 37°C (the optimum temperature for most hydrocarbon-utilising bacteria is actually around 30°C). Their cell morphology is rod, singly, pairs, short chains and 0.5-2.0 μm in size. The other bacteria that have been isolated was *Neisseria* sp.; a Gram-negative coccus shaped bacteria. They breakdown sugar by oxidation. Another isolates was *Moraxella* sp. They are Gram-negative rod bacteria, non-motile and aerobic. They cannot utilise sugar and their growth can be improved by addition of blood or serum but specific growth factors are not unknown. Some Gram-positive bacteria have also been isolated. The Gram-positive bacteria are referred to isolate labelled as numbers (5) and (10).

Table 2. Cell morphology characteristics of isolates on BSM+Engine Oil +Diesel Oil at 37°C.

Isolates	G/ stain	Size (width,µm/length,µm)	Pattern	Shape
1	-	1.6/1.6	Single	Rod
2	-	1.6/3.2	Single	Rod
3	-	1.6/3.2	Single/cluster	Rod
4	-	1.6/3.2	Single/cluster	Rod
5	+	1.6/1.6	Cluster	Coccus
6	-	1.6/1.6	Single	Rod
7	-	1.6/3.3	Single/cluster	Coccus
8	-	1.6/3.2	Single	Rod
9	-	1.6/3.2	Single/cluster	Rod
10	+	1.6/1.6	Single/cluster	Coccus
11	-	1.6/3.2	Single/cluster	Rod
12	-	1.6/3.2	Single/cluster	Rod
13	-	1.6/3.2	Single/cluster	Rod
14	-	1.6/1.6	Single/cluster	Rod
15	-	1.6/3.2	Single/cluster	Rod

Table 3. Physiological characteristics of isolates grown at 37 °C from petroleum contaminated site.

Iso.	oxi	cat	SIM			TSI			MRVP		OF		PRB		CFC	MCA	Most Probable Genus
			H_2S	I	m	s	b	g	MR	VP	ox	fr	pH	g			
1	-	+	-	-	-	A	A	-	-	-	-	-	A	++	W	P	*Acinetobacter* sp.
2	-	+	-	-	+	-	-	+	R	+	+	+	A	+++	W	P	*Xanthomonas* sp.
3	-	+	-	-	-	K	A	+++	Y	+	+	+	A	++++	Y	O	*Acinetobacter* sp.
4	-	+	-	-	-	A	-	-	R	-	+	+	A	++	Y	P	*Pasteurella* sp.
5	-	+	-	+	-	A	-	+	Y	-	-	-	A/K	++	L.O	W.P	*Staphylococcus* sp.
6	-	+	-	-	-	K	K	-	R	-	+	+	A	+++	W	Y	*Acinetobacter* sp.
7	+	d	-	+	-	K	K	+	R	+	-	-	K	++	L.Y	Y	*Neisseria* sp.
8	-	-	-	-	+	A	-	+	Y	-	-	-	A	+	-	P	*Pseudomonas* sp.
9	-	-	-	+	-	A	-	+	Y	-	-	-	d	+	-	P	*Moraxella* sp.
10	-	+	-	-	-	K	A	-	R	-	-	-	A	+	L.Y	Y	*Staphylococcus* sp.
11	-	+	-	+	-	A	-	-	R	+	-	-	A	++	W	Y	*Acinetobacter* sp
12	+	+	-	-	+	K	K	++	R	+	-	-	A	-	W	Y	*Moraxella* sp.
13	+	D	-	+	-	K	K	++	R	+	-	+	A	-	D.Y	Y	*Pasteurella* sp.
14	-	+	-	+	+	K	A	+++	Y	+	+	+	A	+++	L.Y	Y	*Pseudomonas* sp.
15	D	+	-	-	-	A	A	++	R	+	-	-	A	++	W	Y	*Acinetobacter* sp.

Iso = isolate; **oxi**= oxidase; **cat** = catalase; **SIM** = sulphide-indole-motility; **I** = indole; **m** = motility; **TSI** = triple sugar ion; **s** = slant; **b** = butt; **g** = gas; **MR**= methyl-red; **VP** = Voges-Proskauer; **PRB**= Phenol -Red Broth; **MCA** = Mac Conkey Agar; **OF** = oxidation-fermentation; **ox** = oxidation; **fr** = fermentation; **+** = positive result; **-** = negative result; **A** = acidic; **K** = alkaline; **R**= red; **Y**= yellow; **W**=white; **L.O**= light orange; **L.Y** = light yellow; **D.Y**= dark yellow **P**= pink; **O**=orange; **W.P**= whitish pink; **D** = delayed reaction.

They are coccus in shape. Results obtained from morphological and physiological characteristics, suggested that the bacteria that were isolated was *Staphylococcus* sp. Bosset and Bartha [1] reported that Some Gram-positive bacteria have been isolated from contaminated soil and these isolates showed the ability to degrade at least some hydrocarbon.

CONCLUSION

This study have been successful in isolating and identifying bacteria from the Tanjung Karang beach that had been contaminated with crude oil caused by off-shore oil spilled. Morphological, physiological and biochemical analysis suggest that the most of the isolates belongs to the genus *Acinetobacter*.

The others were from the genus *Pseudomonas*, *Neisseria*, *Moraxella* and *Staphylococcus*. The incorporation of used engine oil and diesel oil in BSM media as carbon and energy source provided the opportunity to isolate bacteria, which can utilise/degrade hydrocarbon. These isolates can be use as potential candidates for bioremediation of petroleum contaminated site.

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