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Isolation and characterization of hydrocarbon-utilizing bacteria from soils contaminated with used engine oil and diesel oil

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ABSTRACT Bacteria were isolated from a train workshop in Sentul, Kuala Lumpur (S-I), a car and motorcycle workshop in Kampong Kerinci, Kuala Lumpur (S-II), and a boat workshop in Port Dickson, Negri Sembilan (S-III). All the microorganisms were isolated using Liquid Culture Enrichment Method with dilution streaking method and dilution spreading method. A total of 93 bacteria and 1 strain of yeast were isolated from S-I, 76 bacteria were isolated from S-II and 147 bacteria were isolated from S-III. Further identification using a commercially made identification kit revealed that most isolates are from the genus *Pseudomonas*, *Acinetobacter* and *Aeromonas*.

ABSTRAK Bakteria telah diasingkan dari satu bengkel keretapi di Sentul, Kuala Lumpur (S-I), satu bengkel kereta dan motosikal di Kampong Kerinci, Kuala Lumpur (SII), dan bengkel kapal di Port Dickson, Negri Sembilan (S-III). Semua mikroorganisma diasingkan menggunakan Kaedah Cecair Kultur Pengkayaan dengan Kaedah Corengan Pencairan dan Kaedah Penyebaran Pencairan. Sebanyak 93 bakteria dan 1 strain yis telah diasingkan dari S-I, 76 bakteria diasingkan dari S-II dan 147 bakteria diasingkan dari S-III. Pengecaman lanjut dengan menggunakan kit pengecaman komersial menunjutkan bahawa kebanyakan asingan adalah daripada genus *Pseudomonas, Acinetobacter* dan *Aeromonas.*

(Hydrocarbon-utilizing bacteria, liquid culture enrichment method, dilution streaking method, dilution spreading method)

INTRODUCTION

Oil spills into the environment is a well-recognized problem in today's world. It can affect many species of plants and animals in the environment, as well as humans. The search for effective and efficient method of oil removal from contaminated sites has intensified in recent years. A variety of methods are currently available to treat soils contaminated with hazardous materials. They include excavation, burial, chemically secure landfill, vapour extraction, stabilization and solidification, soil washing, soil flushing, critical fluid extraction, chemical precipitation, vitrication, thermal desorption and incineration [1]. Many of these physico-chemical treatment methods do not destroy the contamination completely and are often costly. One promising method that has been researched is the biological degradation of oil by bacteria. The bacteria can metabolize the oil as it is a compound rich in carbon.

In this study, isolation of microorganisms capable of utilizing hydrocarbons were performed using used engine oil or diesel oil as the carbon and energy source. The isolates were characterized using morphological and physiological characteristics.

MATERIALS AND METHOD

Soil

Details of the sources, sites, characteristics and the designations of the samples used in this study are presented in Table 1.

Table 1. History of sites and characteristics of soils used for isolation of hydrocarbon-utilizing bacteria.

Soil sample	Soil characteristics and history
S-I	Locomotive service and maintenance workshop in Sentul, Kuala Lumpur; spillage of used engine oil and diesel oil; contaminated for over 50 years.
S-II	Car and motorcycle services workshop in Kampong Kerinci, Kuala Lumpur; contaminated for over 10 years.
S-III	Boat service and maintenance area in Port Dickson, Negri Sembilan; contaminated for over 15 years.

The soils samples were taken approximately 0-5 centimetres from the surface layer of the soil. The soil samples were placed into sterile bags and sealed. To prevent contamination and to ensure that the microorganisms were from indigenous population, the samples were brought to the laboratory and were processed on the same day.

Isolation

The Basal Salt Medium (BSM) has all the required minerals for the growth of many microorganisms [2]. 1% (v/v) of diesel oil or used engine oil was supplemented with this medium as the sole carbon source for isolation and maintenance of hydrocarbon-utilizing microorganisms. BSM (per litre): $CaCl_2$, 0.5 g; KH_2PO_4, 0.5 g; K_2HPO_4, 0.5 g; $FeCl_3$, 0.01 g; $(NH_4)_2SO_4$, 0.5 g; pH was adjusted to 7.0-7.2; (for agar plates, 2% of Bacto Agar was added).

Triptone Soya Agar (TSA, OXOID) was used in this study for the purification of the isolates. TSA is a general purpose media which can support the growth of a wide variety of microorganisms.

25 g of soil sample was mixed with 100 ml of sterile distilled water in a 500 ml sterile Erlenmeyer flask. Then the flask was incubated for 30 minutes at 37°C on an orbital shaker to make a homogenous mixture. Following incubation the homogenous mixture was centrifuge at 9,000 rpm for 10 minutes to separate the particulate from the supernatant.

1 ml of the supernatant was inoculated separately into 50 ml Erlenmeyer flasks containing BSM and 1% (v/v) of used engine oil or diesel oil and the flasks were incubated aerobically at 37°C on an orbital shaker (Innova 4900, New Brunswick Sci., USA) at 220 rpm. During the incubation period, observation were made daily and recorded (Table 2) for 12 days. After incubation, a loopfull of the enrichment culture was streaked using dilution streaking method on BSM plates containing diesel oil or used engine oil. Also, 1 ml of the solution was diluted and 0.1 ml of the appropriate dilutions was spread using dilution spreading method on BSM plates plus 1% (v/v) diesel oil or used engine oil.

Purification

Each colony were picked off randomly from BSM containing 1% (v/v) diesel oil (BSM+DO) or 1% (v/v) used engine oil (BSM+EO) agar plates using tooth pick and patched on TSA plates and BSM+EO or BSM+DO agar 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. The cultures were maintained on TSA plates.

Identification

All isolates were examined based on morphological and physiological characteristics. Morphological characteristics were determined based on colony and cell morphology. Physiological characteristics were determined based on classical biochemical test [3] and Microbact 24E Identification System (Medvet, Australia) for bacterial identification and API 20C AUX System (bioMērieux, France) for yeast identification.

Petroleum products

Two kinds of petroleum products, diesel and used engine oil were used as the sole carbon source in culture media. The fresh diesel oil was brown in colour and the used engine oil was black in colour.

Used engine oil were obtained from the locomotive service and maintenance workshop in Sentul, Kuala Lumpur. Diesel oil were obtained from a Shell station in Kuala Lumpur. Both of these substrates were filter-sterilized using

Millipore membrane filter (Type HA, 0.45 μm), placed in a swinnex filter holder.

Bacteria
The bacteria that were used as controls during purification and biochemical test were *Escherichia coli*, *Bacillus cereus* and *Straphylococcus aureus*. They were obtained from the Medical Department, University Hospital, Kuala Lumpur. While *Streptococcus feacalis* (ATCC19433) and *Pseudomonas aeruginosa* (ATCC27853) were purchased from American Type Culture Collection Maryland, USA.

RESULTS AND DISCUSSION

Characterization of contaminated soils
All three soil samples were taken from three different sites that have been used for industrial purposes for over 10 years. Rarely was any fungal or actinomycetes growth observed, and there was no need to add any fungicide to the media for isolation of bacteria.

Presence of bacteria in contaminated soils
The soil samples that were collected had a long historical contamination exposure (Table 1). This exposure may apparently alter the microbial community structure and function which may lead to adaptation to the adverse environment. Possible mechanisms of adaptation includes: genetic adaptation (induction, and/or depression of specific genes) and/or structural adaptation (such as pores in the cell wall for hydrocarbon uptake) [4, 5].

Characterizations of enrichment culture
The characteristics of the liquid enrichment culture is presented in Table 2. The culture media in flasks containing diesel oil showed changes in colour and turbidity after 1 day of incubation compared to the culture media in flasks containing used motor oil which showed the changes after 3 days of incubation. The increased in turbidity of the culture media in experimental flasks and not in control flask indicated that there were growth of hydrocarbon-utilizing microorganisms in the experimental flasks.

The differences in time (i.e. number of days) for colour and turbidity changes observed between the flasks containing diesel oil and used motor oil may be because some microorganisms cannot withstand the toxicity of used motor oil. This may be due to the materials collected from the engine that slowed down the growth of microorganisms in the culture media in the experimental flasks.

Isolation and purification
A total of 316 bacteria were isolated and purified. Among the purified isolates, 93 bacteria and 1yeast were isolated from S-I (Locomotive Service and Maintenance Workshop in Sentul, Kuala Lumpur), 76 bacteria were isolated from S-II (Car and Motorcycle Services Workshop in Kampong Kerinci, Kuala Lumpur) and 147 bacteria were isolated from S-III (Boat Service and Maintenance area in Port Dickson, Negeri Sembilan).

Identification
Table 3 summarized the morphological characteristics of selected bacteria isolates based on colony and cell morphology.

Colony morphology was described based on colour, size (mm), shape, edge, elevation and texture, while cell morphology was described based on Gram stain, size (μm), pattern and shape.

Table 4 summarized the result of physiological studies (biochemical test). The tests conducted include oxidase, catalase, motility (SIM - sulfide-indole-motility agar test), indole, acid from glucose, fermentation of glucose, MacConkey agar (MCA), Pseudomonas Base Agar (PBA), Triple Sugar Iron Medium (TSI) and Methyl Red Voges-Proskaüer test (MRVP) [3].

Among the 316 isolates, 151 isolates were selected for biochemical test. Many isolates were from the genus *Pseudomonas*, *Acinetobacter* and *Aeromonas*. The results shown in Table 5 summarize the number of *Pseudomonas* sp., *Acinetobacter* sp. and *Aeromonas* sp. that were isolated from the soil sample.

The yeast cell that was isolated was identified as *Candida parapsilosis*. *C. parapsilosis* have been reported to degrade hydrocarbon [6].

Table 2. Liquid culture characteristics during 12 days incubation

Day(s)	BSM + 1% (v/v) Diesel		BSM + 1% (v/v) Used Engine Oil	
	Control	Experiment	Control	Experiment
0	Solution was clear; particle of colourless oil on top	Solution was clear; particle of colourless oil on top	Solution was clear; particle of black oil on top	Solution was clear; particle of black oil on top
1	Same as above	Solution become cloudy; milky white colour	Same as above	Same as above
2	Same as above	Solution become cloudier; white particles found at the side of flask	Same as above	Same as above
3	Same as above	Solution become orange colour; white particles found at the side of flask	Same as above	Same as above
4	Same as above	Formation of layer of yellowish white particles on surface of liquid; more particles on side of flask	Same as above	Yellowish and cloudy white particles brownish oil on top, whitish particles found at the side of flask
5	Same as above	Same as above	Same as above	Solution become cloudy; more particles of used engine oil
6-12	Same as above	Same as above	Same as above	Less particles of used engine oil, white particles at the side of flask

Table 3. Morphological characteristics of selected bacterial isolates

Bacterial	Colony morphology	Cell morphology
A-4	Orange, 2 mm, circle, entire, convex, smooth	Gram negative, 1.6 µm, single, rod
A-13(b)	Whitish, 2 mm, circle, entire, convex, smooth	Gram negative, 1.6 µm, cluster, rod
B-5	Yellowish, 2 mm, circle, entire, convex, smooth	Gram negative, 1.6 µm, cluster, coccus
B-15	Green, 2 mm, amoeboid, lobate, effuse, granular	Gram negative, 3.2 µm, cluster, coco-bacilli
B-21	Yellowish, 2 mm, circle, entire, convex, smooth	Gram negative, 1.6 µm, single, rod
15(a)	Whitish, 3 mm, circle, entire, convex, smooth	Gram negative, 1.6 µm, cluster, rod
17 (a)	Whitish, 4 mm, circle, entire, convex, smooth	Gram negative, 1.5 µm, cluster, rod
TS 43	Yellowish, 2 mm, circle, entire, convex, smooth	Gram negative, 1.5 µm, single, rod
BD 8	Yellowish, 2 mm, circle, entire, convex, smooth	Gram negative, 1.4 µm, single, rod

Table 4. Physiological characteristics of selected bacterial isolates.

Isolate	Biochemical test												Most probable genus
	oxi	cat	mot	ind	FG		MCA	PBA	TSI			MRVP	
					A	G			S	B	H_2S		
A-4	-	+	+	-	+	+	p	y	r	y	-	-	*Pseudomonas*
A-13(b)	-	+	+	-	+	+	p	w	r	r	-	-	*Pseudomonas*
B-5	-	+	+	-	+	+	p	w	r	r	-	-	*Pseudomonas*
B-15	+	+	+	-	+	+	g	g	r	r	-	-	*Pseudomonas*
B-21	-	+	-	-	+	+	p	w	r	r	-	-	*Acinetobacter*
15(a)	+	+	+	-	+	+	p	ng	y	y	-	-	*Aeromonas*
17 (a)	+	+	+	-	+	+	p	ng	y	y	-	-	*Aeromonas*
TS43	-	+	-	-	+	+	p	w	y	y	-	-	*Acinetobacter*
BD8	-	+	-	-	+	+	p	w	y	y	-	-	*Acinetobacter*

oxi = oxidase test; **cat** = catalase test; **mot** = motility test; **ind** = indole test; **FG** = fermentation of glucose test, **A** = acid production, **G** = gas production; **MCA** = MacConkey agar, **p** = pink colony, **g** = green colony; **PBA** = Pseudomonas Base Agar, **y** = yellow colony, **w** = white colony, **g** = green colony, **ng** = no growth; **TSI** = Triple Sugar Ion Agar, **S** = slant, **B** = butt, **r** = red (alkaline), **y** = yellow (acid), **H_2S** = hydrogen sulphide production; **MRVP** = Methyl Red Voges-Proskaüer test; **+** = positive result; **-** = negative result.

Table 5. Number of *Pseudomonas* sp., *Acinetobacter* sp. and *Aeromonas* sp. isolated from soil sample.

Isolate	Soil sample		
	S-I	S-II	S-III
Pseudomonas sp.	47	38	19
Acinetobacter sp	0	6	20
Aeromonas sp.	9	0	12

This study has been successful in isolating indigenous hydrocarbon-utilizing bacteria from soil contaminated using used engine oil or diesel oil as the carbon and energy source. These isolates may be used for bioremediation purposes or biosurfactant production.

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