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BIODEGRADATION OF USED LUBRICATING OIL BY MICROBES ISOLATED FROM PRISTINE SOIL ENVIRONMENT

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ABSTRACT Pollution of soil by used lubricating oil is a common phenomenon in most cities in developing countries. This may pose a great threat to the environment and human being at large. Potential of hydrocarbon utilizing bacteria and yeast isolated from pristine (uncontaminated) soil to degrade used lubricating oil was studied in broth culture for 28 days at 30 ± 2^0C. Four isolates (*Pseudomonas aeruginosa, Micrococcus luteus, Trichosporon mucoides* and *Candida tropicalis*) were used for the study. The highest percentage (40.6%) of total petroleum hydrocarbons (TPH) and hydrocarbon fractions degradation was recorded by *C. tropicalis* followed by *T. mucoides* throughout the study period, compared to those recorded by *P. aeruginosa* and *M. luteus.* Thus, pointing out the potential of the yeast species (*Candida tropicalis)* in biodegradation of used lubricating oil from soil environment.

(Keywords: Used lubricating oil, biodegradation, contamination, hydrocarbon)

INTRODUCTION

Hydrocarbons are diverse molecules that are extremely abundant in nature. However, the ability of plants and animals to degrade hydrocarbons is limited. It is within the bacteria, filamentous fungi and yeast that hydrocarbon biodegradation is most common. Several reasons for the limited ability of Eukaryotes to degrade hydrocarbons are: many hydrocarbons are virtually insoluble in water, and thus their bioavailability is limited, hydrocarbons are generally chemically inert but are subject to oxygen additions by various enzymes [1]. The oil industry and other related industries, such as oil extraction and transport, pose a threat to the environment because they can cause a huge influx of petroleum hydrocarbons into the environment [1, 2, 3]. For this reason, there is an increasing interest in finding ways to reverse degradation of ecosystems, which the introduction of petroleum hydrocarbon sources has adversely affected [4, 5, 6, 7]. Currently, microorganisms that have the ability to degrade, remove, or transform organic chemicals are used for the purpose of bioremediation of ecosystems that have been polluted by petroleum oil or its fractional compositions [4, 8]. Many studies have used indigenous microorganisms to remediate polluted soils or wastewater [9, 10, 11, 12].

Microorganisms are equipped with metabolic machinery to use petroleum as a carbon and energy source [13]. Recent interest in biodegradation has focused on the possibility of using these natural processes for the decontamination of soil or water contaminated with complex hydrocarbon mixes. The relative contribution of bacteria and fungi to hydrocarbon mineralization in soil has been reported [14]. Similarly, many other investigators [11, 12, 15] have reported the involvement of bacteria and yeasts in petroleum degradation. The growth of microorganisms on hydrocarbons is often accompanied by the emulsification of the insoluble carbon source in the culture medium. In most cases this has been due to the production of extracellular emulsifying agents during the breakdown of hydrocarbons. These processes aid microorganisms in growing on and metabolizing petroleum. Normal populations of hydrocarbon utilizing microorganisms account for 0.1% of the population but may reach 100% under selective pressure after a spill or prolonged chronic discharges, returning to background levels after the pollutant is removed. Higgins and Gilbert [16] found that in unpolluted water hydrocarbon oxidizing bacteria were low in number but that their number increased by two orders of magnitude in polluted waters. The objective of this study is to isolate and screen microorganisms (bacteria and yeast) from

pristine (uncontaminated) soil environment for potential to degrade used lubricating oil.

MATERIAL AND METHODS

Collection of samples

The soil sample with no history of petroleum contamination used was collected close to the school football field, University of Malaya, Kuala Lumpur. It was collected in a sack and transported to the laboratory for isolation of yeast and bacteria capable of oil degradation. Used lubricating oil was collected from Perodua car service Centre, Petaling Jaya, Malaysia.

Isolation and identification of microorganisms

Soil enrichment technique was used for the isolation of oil degrading microorganisms. In this method, 2 g of soil sample from pristine soil environment was added to 100 ml of sterile mineral salt medium (MSM) [1.8 g K_2HPO_4, 4.0 g NH_4Cl, 0.2 g $MgSO_4.7H_2O$, 1.2 g KH_2PO_4, 0.01 g $FeSO_4.7H_2O$, 0.1 g NaCl, pH 7.4] in 250 ml capacity Erlenmeyer flasks. Two milliliters (2 ml) of used lubricating oil were added to the medium and the flasks were incubated for 7 days at 30 ^{0}C on an incubator shaker (Thermo-line, Japan) operated at 200 rpm. After enrichment (2 times), suspensions of the enriched soil in physiological saline were inoculated into two sets of duplicate oil agar plates [17]. In one set of the plates meant for the isolation of crude oil utilizing bacteria, nystatin was added at a concentration of 50 mg/ml to suppress the growth of fungi. The plates were then incubated at 30 ^{0}C for 3 days. Colonies which appeared on the oil agar plates were randomly picked and pure isolates obtained by repeated sub-culturing on Nutrient agar (Oxoid, Australia). The bacterial isolates were characterized and identified by Gram staining technique and the use of API 20NE for Gram negative bacteria and BBL Crystal rapid identification kit for Gram positive bacteria.

The second sets of plates meant for the isolation of yeasts were added 100 mg of streptomycin to inhibit the growth of bacteria. The plates were then incubated at 30 ^{0}C for 5 days. Suspected yeast colonies which appeared on the oil agar plates were isolated in pure cultures by repeated sub-culturing on sabouraud dextrose agar (Oxoid, Australia). The yeast isolates were Gram stained and identified using API 20 C AUX identification kit as follows: 24 hours old pure culture of yeast colonies were inoculated into an ampoule suspension medium containing 2 ml of 0.85% NaCl to prepare a suspension with turbidity equivalent to 2 McFarland standard, this was followed by transfer of 100µl of the suspension into an ampoule of API C medium and gently homogenized with pipette. The API cupules were filled with the suspension covered with the lids and incubated at 29^0C ± 2^0C for 48 hours. At the end of the incubation period, the growths in each cupule were compared with the 0 cupule, which was used as a negative control. A cupule more turbid than the control indicates a positive reaction. Identification was obtained with the numerical profile entered into apiweb^{TM} identification software.

Biodegradation studies with the microbial isolates

A total of 10 hydrocarbon utilizing bacteria species and 6 hydrocarbon utilizing yeast were isolated from the soil sample obtained from pristine environment. Out of the 16 microbial isolates, 2 bacteria and 2 yeasts were selected for the biodegradation studies due to their rapid growth on oil agar and efficient utilization of oil in the preliminary test in test tubes. The rates and extent of used lubricating oil degradation by these four selected microbial isolates were determined using gravimetric analysis and chromatographic technique [18].

The biodegradation studies was carried out by inoculating 2 ml of 24 hour broth culture of each microbial isolates into 100 ml of sterile MSM [19], that contained 0.5 g of used lubricating oil in an Erlenmeyer flask. The experiment was set up in triplicates with control flasks which contained 100 ml of sterile mineral salts medium plus 0.5 g of used lubricating oil but without added microorganisms. The flasks were incubated in an incubator shaker (Thermo-line, Japan) maintained at 30^0C at 150 rpm for 28 days. At seven days intervals, triplicates flasks per organisms plus control flasks were removed from the incubator shaker and the residual used lubricating oil extracted twice with 150 ml of n-hexane (Merck brand) and dried with anhydrous sodium sulphate. The solvent was removed by rotary evaporator and the weight of the residual oil was measured and recorded, and the percentage biodegradation of the used lubricating oil was calculated using the formula of Ijah and

Ukpe [20]. The residual oil was diluted with n-hexane (Merck brand) and cleaned up with silica gel (HyperSep SI column), 1 microlitre of the extracted oil sample was analyzed using gas chromatography with flame ionization detector (GC/FID). The GC was equipped with cross-linked 5% phenyl methyl siloxane capillary column; HP-5MS. Helium was used as carrier gas. The oven temperature program was started at 50^0C and raised by 25^0C/min until 325 ^{0}C, which was maintained for 11 minutes. The major hydrocarbon fractions were identified on the basis of their retention time and by comparing them to those of analytical standards.

$$\% \text{ biodegradation} = \frac{\text{weight of oil (control)} - \text{weight of oil (degraded)}}{\text{Weight of oil (control)}} \times 100$$

Statistical analysis

Statistical analysis of data was carried out using Analysis of Variance (ANOVA) with SPSS version 17.

RESULT AND DISCUSSION

Based on rapid growth on oil agar and in test tubes of mineral salt medium containing used lubricating oil as the only carbon and energy source, four microbial isolates (two bacterial and two yeast species) were selected out of the sixteen microbial isolates (ten bacteria and six yeasts) for the biodegradation studies. The four microbial isolates were identified as species of *Micrococcus luteus*, *Pseudomonas aeruginosa, Trichosporon mucoides* and *Candida tropicalis.* These four microorganisms have been previously implicated in biodegradation of petroleum hydrocarbons by various authors [17, 21, 22, 23]. The results of the percentage of oil biodegradation by the different microbial isolates are shown in **Table 1**. Compared to the percentage of biodegradation in control flask without microbial inoculation, there was appreciable loss of oil in the flasks inoculated with different microbial isolates. There was rapid biodegradation of oil within the first 7 days of incubation with flask inoculated with *Candida tropicalis* recording 27.8% biodegradation in the seventh day compared to the 1% recorded in un-inoculated flask. At the end of the 28 days, *Candida tropicalis* recorded the highest percentage of oil biodegradation (40.6%) followed closely by *Trichosporon mucoides* (38%), *Micrococcus luteus* (36.8%) and *Pseudomonas aeruginosa* (33.8%). Out of the four microbial isolates *Candida tropicalis* shows higher percentage of oil biodegradation than those of bacterial isolates. The reason for this might be the ability of yeast cells to withstand the toxic effects produced by the oil more than the bacteria; also the reason may be due to the presence of effective degradative enzymes systems in the yeast isolates.

Table 1. Percentage of used lubricating oil biodegradation by microbial isolates

Microbial Isolates (days)	Oil biodegradation (%)			
	7	14	21	28
Pseudomonas aeruginosa	26.4±2.1	28.2±1.8	29.6±3.4	33.8±4.2
Micrococcus luteus	23.8±1.9	32.6±5.1	35.0±2.6	36.8±3.7
Trichosporon mucoides	24.6±3.2	36.2±3.8	38.4±5.1	38.0±4.5
Candida tropicalis	27.8±1.5	36.4±2.7	39.0±3.8	40.6±2.6
Control	1.0±0.8	1.8±1.1	2.4±1.8	2.6±0.9

These results are supported by the findings of Walker et al., [24] who found that *Candida* degraded South Louisiana crude oil more extensively than bacteria *Pseudomonas* and Coryneforms. *Candida tropicalis* recorded the highest percentage (40.6%) of oil breakdown compared to other isolates studied, however, there was no significant difference at $P<0.05$ between the oil perentage degradation by the test organisms. This result is similar to that of Ijah [17] who reported 68.9% crude oil degradation by *Candida tropicalis* in 16 days. Also Palittapongarnpim et al., [25] reported that *Candida tropicalis* degraded 87.3% of total

petroleum hydrocarbon within 7 days of incubation in medium containing crude oil as the sole source of carbon. The difference in results in the percentage of oil biodegradation might probably due to different oil used for the studies, used lubricating oil contains other contaminants like heavy metals which probably inhibits the growth of the organisms and subsequently reduce the rate of oil biodegradation compared to the results of the two different authors above. *Pseudomonas aeruginosa* degraded the least percentage of oil (33.8%) at the end of the 28 days compared to other isolates. This might possibly due to non-production of biosurfactants by the isolated strains. It is reported that *Pseudomonas aeruginosa* strains that produced biosurfactants degrade hydrocarbon faster and better than non-biosurfactants producing strains [26].

Biodegradation of hydrocarbon fractions in used lubricating oil

Biodegradation of hydrocarbons fractions present in the used lubricating oil was determined at seven days intervals for the period of 28 days to determine the extent of biodegradation of different hydrocarbon fractions using GC/FID. The hydrocarbon fractions were divided into four fractions which are: $C_7 – C_9$, $C_{10} – C_{14}$, $C_{15} – C_{28}$ and $C_{29} – C_{36}$.

Table 2 shows the biodegradation of $C_7 – C_9$ hydrocarbon fractions by the four microbial isolates tested for their ability to degrade used lubricating oil within the period of 28 days. The results shows that all the flasks inoculated with different microbial isolates recorded complete biodegradation of $C_7 – C_9$ fractions below the detection limit in the 28 days compared to the un-inoculated control flask. The reason for the complete loss of these hydrocarbon fractions might be due partly to volatilization and because they are short chain hydrocarbons, their degradation might not pose serious challenge to the microbial isolates used for this study. This result is similar to the findings of (Ijah, [17]; Pallasser, [27]; George et al., [28]) who recorded complete degradation of these hydrocarbon fractions (from crude oil) in flasks inoculated with three different microbial isolates within the period of four days.

Table 2. Biodegradation of $C_7 – C_9$ hydrocarbon fractions by microbial isolates

Microbial	Concentration (mg/kg)			
Isolates (days)	7	14	21	28
Pseudomonas aeruginosa	98	85	67	ND
Micrococcus luteus	94	73	64	ND
Trichosporon mucoides	96	71	60	ND
Candida tropicalis	88	65	56	ND
Control	107	96	84	78

ND: Not detected at lowest detection limit of 50 mg/kg

The results of biodegradation of $C_{10} – C_{14}$ hydrocarbon fractions revealed complete degradation of these fractions below the detection level in flask inoculated with *Candida tropicalis* and *Trichosporon mucoides* at the end of 28 days of incubation; however degradation below detection limits was not achieved in the flasks inoculated with *Pseudomonas aeruginosa* and *Micrococcus luteus* (**Table 3**). The efficiency of oil biodegradation demonstrated by *Candida tropicalis* and *Trichosporon mucoides* might be due to their abilities to withstand the inhibitory component of the hydrocarbon fractions or probably due to the fact that the organisms possess an efficient degradative enzyme systems which enable them to degrade the hydrocarbon fractions below the detection limit. This has been supported by different authors [17, 21, 22, 23] who argued that these two microorganisms (*Candida tropicalis* and *Trichosporon mucoides)* were able to degrade C_{10} to C_{14} hydrocarbon fractions because of their efficient degradative enzyme systems.

Table 3. Biodegradation of $C_{10} - C_{14}$ hydrocarbon fractions by microbial isolates

Microbial	Concentration (mg/kg)			
Isolates (days)	7	14	21	28
Pseudomonas aeruginosa	105	91	83	67
Micrococcus luteus	111	93	76	59
Trichosporon mucoides	96	71	64	ND
Candida tropicalis	83	68	53	ND
Control	125	116	109	102

ND: Not detected at lowest detection limit of 50 mg/kg

Biodegradation of $C_{15} - C_{28}$ and $C_{29} - C_{36}$ hydrocarbon fractions within the period of 28 days by different microbial isolates are shown in **Tables 4 and 5**. The results revealed partial degradation of both hydrocarbon fractions, however the flask inoculated with *Candida tropicalis* and *Trichosporon mucoides* recorded the highest degradation of $C_{15} - C_{28}$ and $C_{29} - C_{36}$, respectively. The partial degradation recorded in these fractions of hydrocarbons by the microbial isolates might be due to the complex structural arrangements of these hydrocarbon fractions which possibly made them less susceptible to microbial degradation [28, 29].

Table 4 Biodegradation of $C_{15} - C_{28}$ hydrocarbon fractions by microbial isolates

Microbial	Concentration (mg/kg)			
Isolates (days)	7	14	21	28
Pseudomonas aeruginosa	651	622	591	550
Micrococcus luteus	620	586	573	564
Trichosporon mucoides	623	608	583	526
Candida tropicalis	601	582	541	493
Control	675	667	651	648

Table 5 Biodegradation of $C_{29} - C_{36}$ hydrocarbon fractions by microbial isolates

Microbial	Concentration (mg/kg)			
Isolates (days)	7	14	21	28
Pseudomonas aeruginosa	495	481	462	451
Micrococcus luteus	496	478	466	444
Trichosporon mucoides	492	480	462	426
Candida tropicalis	486	463	458	432
Control	503	495	486	478

CONCLUSION

The results of used oil biodegradation by four microbial isolate (*Pseudomonas aeruginosa, Micrococcus luteus Candida tropicalis* and *Trichosporon mucoides)* from pristine soil, showed *C. tropicalis* and *T. mucoides* ability to degrade used lubricating oil better (4 – 7%) than their bacterial counterpart used for the study within the period of 28 days. This result pointed out clearly the potential of *C. tropicalis* and *T. mucoides* to degrade hydrocarbons in oil contaminated soil within the shortest possible time (28 days) in broth culture. Thus, these yeast isolates can be used either as individual or as a consortium in seeding oil contaminated soil.

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