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Biodegradation of crude oil by soil microorganisms in the tropic

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Abstract

Five microorganisms, three bacteria and two yeasts, capable of degrading Tapis light crude oil were isolated from oil-contaminated soil in Bangkok, Thailand. Soil enrichment culture was done by inoculating the soil in mineral salt medium with 0.5% v/v Tapis crude oil as the sole carbon source. Crude oil biodegradation was measured by gas chromatography method. Five strains of pure microorganisms with petroleum degrading ability were isolated: three were bacteria and the other two were yeasts. *Candida tropicalis* strains 7Y and 15Y were identified as efficient oil degraders. Strain 15Y was more efficient, it was able to reduce 87.3% of the total petroleum or 99.6% of n-alkanes within the 7-day incubation period at room temperature of 25 ± 2 °C.

Abbreviations: MSM – mineral salt medium, TP – total petroleum; MPC – major peak component

Introduction

The increasing demand for petroleum as a source of energy in Thailand has resulted in the import of 1.6 million tons of petroleum per year into the country. The major route of petroleum transportation to refinery plants is by ships. Oil transportation from refinery plants is accounted for 17% of the ships passing through the Chao Phraya River which is opened to the Gulf of Siam. Since 1973 when the oil spills were recorded, more than six million liters of crude oil were spilled into this area and hence affected a number of aquatic lives (Rungreongsilp 1988).

Crude-oil degrading microorganisms, namely, bacteria, yeasts and fungi have been reported as oil degraders (Atlas 1981; Jordan & Payne 1980). Biodegradation of crude oil by these indigenous microorganisms is one of the primary mechanisms by which petroleum and other hydrocarbons are eliminated from the environment (Atlas 1984).

As information concerning the biodegradation of crude oil in Thailand is very scarce, investigations are needed. To gain more and thorough information about light crude-oil biodegradation in the tropic, two objectives of the study were set. Firstly, to isolate and identify indigenous microorganisms that are able of degrading crude oil from oil- contaminated soil, and, secondly, to select the most competitive oil- degrading microorganisms.

Materials and methods

Soil sampling

Soil was collected from an oil-contaminated area in Bangkok. The soil was collected to the depth of 10 cm at 10 random sampling sites. Each of the 10-soil samples were then kept in separate sterilized plastic bags, stored in an ice-bath and transported immediately to the laboraotory. All samples were air-dried, ground and homogenized. Partially, the mixed soil sample was used for microbial culture. The residual homogenized soil sample was again air-dried to reduce more water content and stored at 4 °C if further studies are needed.

Medium

The mineral salt medium (MSM) was modified from Sorkhoh et al. (1991) and Weissenfels et al. (1990). The composition of the medium was: 1.0g K_2HPO_4, 1.0g NH_4NO_3, 0.2 g $mgSO_4.7H_2O$, 0.1 g $CaCl_2.2H_2O$, 0.1 g NaCl, 0.01g $FeCl_3.6H_2O$, 10 ml filter-sterilized vitamin solution (Sorkhoh et al. 1990), 3 ml trace element solution (Krieg 1981) and deionized water to bring the volume up to 1 liter. The vitamin and trace element solutions were filtered-sterilized before added into the autoclaved medium at about 45 °C. The final pH was adjusted to 7.3 by 0.1 m HCl or NaOH.

Cultivation and isolation of petroleum degraders

Ten grams of the homogenized soil sample was inoculated into each of the three replicates of 500 ml Erlenmeyer flasks containing 100 ml MSM and 0.5% v/v Tapis crude oil. The flasks were shaken at a room temperature of 25 ± 2 °C on a rotary shaker at 200 rpm. After seven days of enrichment, 0.1 ml of the supernatant was spread on selective MSM agar plates overlaid with 0.2 ml filtered sterile 1:1 v/v Tapis/CCl_4 by spraying aseptically onto the surface of the MSM agar plates. The plates were incubated at 30 °C for three days. Colonies with different characteristics were then repeatedly transferred to oil/MSM agar plates until pure culture of each kind of colonies was obtained. Sub-culturing of the original culture was performed every seven days. All purified cultures were kept on selective oil/MSM agar slants at 4 °C and was transferred every three months.

Growth measurement and the assessment of crude-oil biodegradation

Growth curves of all microbial isolates were determined by layered plate method (Koch 1981). Nutrient agar and Sabouraud agar were used to cultivate bacterial and yeast strains, respectively.

The five isolated microorganisms were tested for their ability to degrade crude oil. The pre-culture was prepared by inoculating a full loop of each kind of colonies from slants into 100 ml of 0.5% Tapis/MSM broth and shaking for three days. For each kind of colonies, a 500-ml Erlenmeyer flask containing 100 ml of 0.5% oil/MSM broth was inoculated each with pre-culture at an initial concentration of approximately 10^5 viable cells/ml. The pre-inoculum sizes were 5 ml for yeasts and 1 ml for bacteria. The five pure cultures were shaken at 25 ± 2 °C in a rotary shaker at the speed of 200 rpm.

The inoculum from each culture was ten-fold diluted with sterile water in a series to the concentration of 10^{-7} for a plate count technique. The plate count was done on three replicates for each dilution. The incubating temperatures were 30 °C and 37 °C for yeasts and bacteria, respectively. The incubation period was usually three days for yeasts and 24–28 hours for bacteria. Only plates with 30-300 colonies were counted and calulated for the viable microorganisms.

The inoculated and uninoculated control flasks were harvested periodically after 3,5, and 7 days of shaking. The residual oil in the flasks was extracted immediately and analyzed by gas chromatography. The best crude-oil degrader was selected for further experiments. All experiments were done in three replicates.

Petroleum extraction

Biodegraded oil was extracted after 3-day shaking at room temperature at the speed of 200 rpm. Extraction of the residual oil used was modified from those described by Walker & Colwell (1975), Fedorak & Westlake (1981), Bertrand et al. (1983) and Oudot (1984).

The residual oil left in the entire flask was harvested by liquid-liquid extraction method. Briefly, 80 ml of chloroform together with 0.01 g of 1-eicosene in 0.5 ml chloroform, as internal standard, were mixed with culture medium and transferred to a 250 ml separatory funnel. The original culture flask was rinsed with another 20 ml of chloroform which was then transferred to the separatory funnel. The separatory funnel was shaken for five minutes and was placed on a stand until both liquid phases were separated from each other. The chloroform was collected by dropping through a funnel containing Whatman filter paper number 4 and 20 g of anhydrous sodium sulfate to absorb residual water. The extracted aqueous phase was re-extracted by shaking with another 20 ml of chloroform for five minutes in the same separatory funnel. The extracted chloroform was again passed through anhydrous sodium sulfate as described above. All extracted chloroform was pooled together and then evaporated in a vacuum rotary evaporator at 35 °C. The residual degraded oil products were re-dissolved in 10 ml of n-hexane. If the residual samples contain asphalt, it would precipitate in this step since asphalt does not dissolve in n-hexane. If found, the asphalt

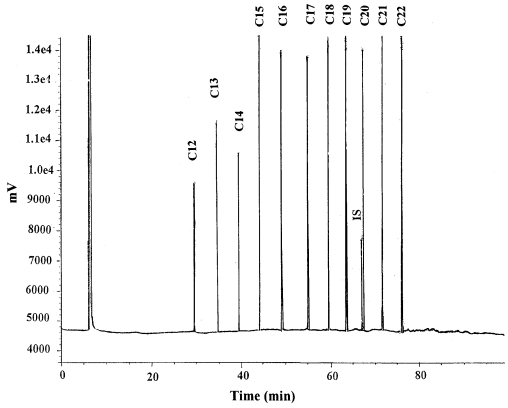

Figure 1. Chromatogram of standard n-alkanes.

component was removed out of the sample by filter through Whatman GF/A glass microfiber filter. The filtered samples were stored in closed rid vials at 20 °C until being analyzed. The filtered samples were injected into the GC-F1D for quantitative analyses. The residual oil was calculated as the percentage of oil left using the chromatogram peak areas. The calculation method is as follows:

$$\%\text{residual oil at day}t = \frac{\text{peak areas of the experiment at day}t}{\text{peak areas after abiotic loss at day}t} \times 100$$

Gas chromatography

Gas chromatographic technique used was modified from the techniques described by Fedorak & Westlake (1981), Oudot (1984), Oudot & Dutrieux (1989), Matisova et al. (1991) and Matisova et al. (1993).

Gas chromatography was performed in a Hewlett Packard gas chromatographic unit model 5890 series II with FID detector, using Hewlett Packard PONA fused silica capillary column. The column internal diameter was 0.2 mm, column length was 50 meter. Helium (99.99%) was used as a carrier gas with a flow rate of 0.7 ml/min, with the split ratio of 1:200 and the septum purge at 3.5 ml/min. The column head pressure was 28 psi. The injection port temperature was 330 °C. Air at the amount of 370 ml/min and hydrogen at the amount of 30 ml/min were used to feed the detector. Helium at the amount of 30 ml/min was also used as make-up gas.

For identification of n-alkane peaks in the Tapis crude-oil gas chromatogram, standard n-alkanes were used as the external standards. Chromatogram of the external standards is shown in Figure 1.

Identification of oil-degrading yeasts

The two yeast strains were identified by morphological and physiological characteristics as described by Lodder (1970); Evans & Richardson (1989); Barnett et al. (1983). Saboraud's 4% glucose was used as the medium for preparation of pre-inoculum. Each pure strain was streaked onto plates and incubated at 30° ± 5 °C for two days. The actively growing pre-inoculum was inoculated to other media or reagents for morphological and physiological tests. The morphology of the vegetative cells grown in both liquid and solid media was studied. The vegetative cells were examined for growth characteristics, shape, mode of reproduction and cellular dimensions, such as the formation of pseudo- or true mycelium and the characteristics of ascospore.

The physiological tests of the yeasts were done on the assimilation of carbon compounds (auxanographic method), assimilation of nitrogen compounds (nitrate), urease test, acid production from glucose and growth on the required media. The yeast strain identification was also confirmed by the Central Bureau voor Shimmelcultures (CBS), Yeast Division, The Netherlands.

No bacterial strain was identified since they were not effective oil degraders.

Results and discussion

Microbial strains

Five different microbial colonies were isolated from soils after seven days of culture. All microorganisms were morphologically examined using a compound microscope. Two were found to be yeasts and were designated as MU7Y and MU15Y. The other three were bacteria and were designated as MU8B, MU11B and MU14B. After eight passages only two strain of yeasts (MU7Y and MU15Y) and one bacterial strain (MU14B) were able to grow in the culture. The MU15Y yeast strain was identified as *Candida tropicalis*. MU7Y, although with slightly different morphology to MU15Y, was also identified as *Candida tropicalis*. Since they behaved differently in growth patterns and in their ability to degrade oil, they were designated as different strains. The two yeast strains identification was confirmed by the Central Bureau voor Shimmelcultures (CBS), Yeast Division, The Netherlands.

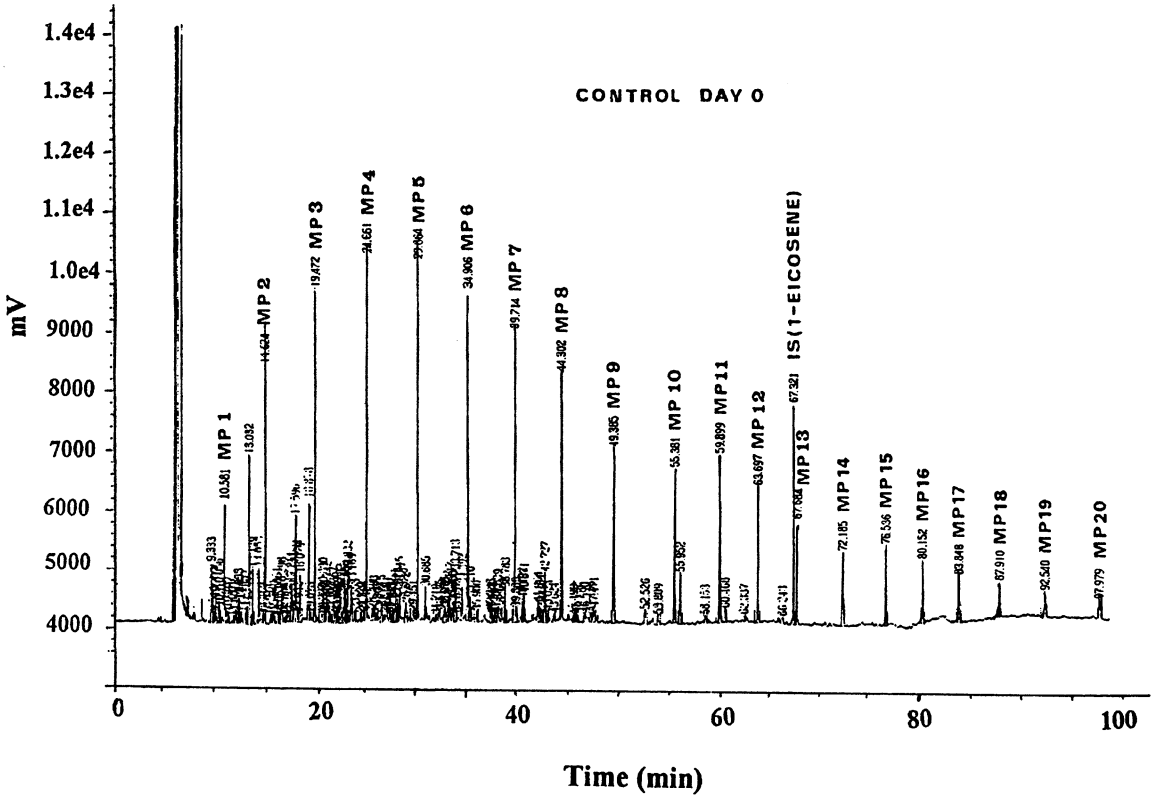

Figure 2. Chromatogram of Tapis crude oil.

Gas chromatography of Tapis crude oil

The chromatogram of Tapis light crude oil is shown in Figure 2. One hundred and twenty nine peaks were found, of which 20 of them were major peak components and were designated as MPC's. The major peak components (MPC's), referred in this study and represented as high and regularly spacing peaks in gas chromatogram, were shown to refer to n-alkanes (Dibble & Bartha 1976; Jobson et al. 1972; Oivieri et al. 1976; Oudot 1990, 1994). Eleven external standards ranging from C_{12} to C_{22} were used as well as the internal standard (IS) of 1-eicosene. When comparing the gas chromatogram of the Tapis crude oil with one of the external standards, it was found that major peaks were presumed to be n-alkanes, with MP1 to MP20 corresponding to n-octane (C_8H_{18}) to n-heptacosane ($C_{27}H_{56}$), respectively. The areas of the MPC's constituted 55.4% of the total petroleum (TP).

Abiotic oil loss

Abiotic oil loss measured the amount of oil loss in the control after incubation. The experimental flasks were sealed tightly with cotton ball stoppers. The aluminum foil was used to wrap around the neck of the flasks including the cotton ball stoppers.

After incubation at 25 ± 2 °C without any added microorganisms, the amount of low molecular weight fraction of crude oil (approximately C_{10} and below) decreased (Figure 3, 4). Most of these fractions disappeared rapidly within the first three days, as 37.6% of total petroleum (TP) and 19.1% of major peak components (MPC) were lost. The rate of oil lost decreased with time. The rates of TP and MPC loss were high during the first three days and decreasing thereafter. The decrease of MPC was slower than TP, indicating that most of the petroleum components lost were unsaturated and volatilized fractions.

Indigenous oil degraders

Results of the residual petroleum content after biodegradation for seven days by the five microorganisms are presented in Table 1. The results clearly show that the two yeast strains demonstrated a better crude oil biodegradation than bacteria, with MU15Y as the best oil degrader. MU15Y reduced 99.6% of MPC or

Table 1. The percentage of residual petroleum after biodegradation by the five microorganisms

Days of incubation	MU 8 B		MU 11B		MU 14B		MU 7Y		MU 15Y	
	TP*	MPC**	TP*	MPC**	TP*	MPC**	TP*	MPC**	TP*	MPC**
0	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
3	64.36 ± 1.05	60.26± 1.25	77.04 ± 2.75	78.38 ± 2.75	43.67 ± 5.0	35.50 ± 1.88	61.43 ± 6.67	53.25 ± 7.92	30.69 ± 7.92	10.26 ± 3.75
5	67.06 ± 4.25	65.90 ± 5.1	73.66 ± 4.13	75.83 ± 3.2	38.68 ± 1.67	31.68 ± 1.46	31.23 ± 7.92	19.32 ± 8.33	14.98 ± 1.25	0.66 ± 0.42
7	60.54 ± 7.5	60.82 ± 8.5	74.47 ± 1.0	75.90 ± 1.0	37.59 ± 1.46	29.35 ± 2.71	19.67 ± 4.2	8.68 ±3.75	12.71 ± 1.5	0.44 ± 0.21

* TP = Total Petroleum; ** MPC = Major Peak Components.

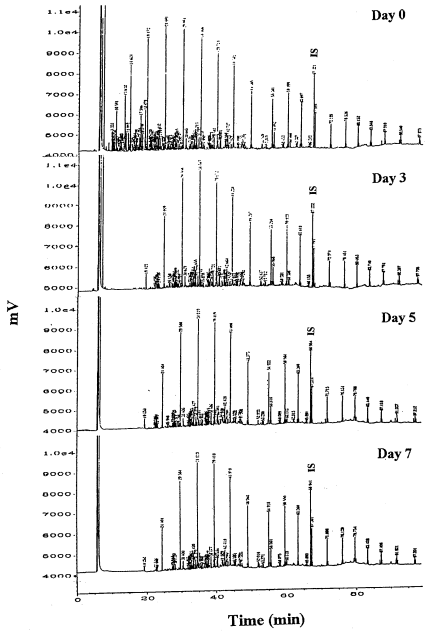

Figure 3. Abiotic loss of crude oil in the control flask at 0, 3, 5 and 7 days.

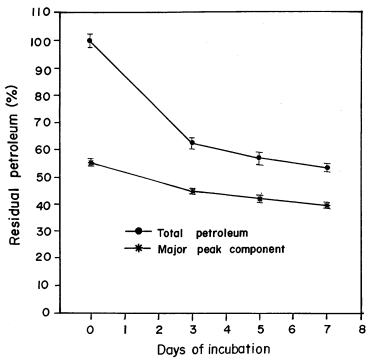

Figure 4. Residual petroleum content after abiotic loss during 7 days at 25 ± 2 °C. The petroleum content is expressed as percentage of the original amount at day 0.

87.3% of TP in seven days. MU7Y degraded 91.3% and 80.3% of alkanes and TP, respectively, within seven days. The bacteria were less efficient as oil degraders. MU8B and MU11B left more than 50% of TP and MPC after seven days.

MU15Y was not only a better microorganism in degrading TP but also a better alkane degrader. It reduced the amount of MPC to a lesser extent than the other strains. MU8B and MU11B reduced TP better than MPC; however, in a much smaller magnitude than MU15Y and MU7Y. The chromatogram of crude oil biodegradation by MU15Y yeast is illustrated in Figure 5.

Although the three bacteria had higher bacterial concentration than the two yeasts, the yeasts were found to degrade oil more rapidly and with the higher extent than the bacteria. Figures 6–8 show the growth curves and percentage of the residual total petroleum and the major peak components of the best three microorganisms, namely, MU15Y, MU7Y and MU14B, respectively, in oil biodegradation. The percentage was calculated after the deduction of the abiotic petroleum loss as was discussed earlier.

Strain MU8B used short-chain alkane fractions of the crude oil to grow; however, this strain had little ability to degrade longer chain alkanes. Both MU8B and MU11B strains degraded little amount of $>C_{14}$ alkanes. Strain MU14B was more capable than the other two bacteria in degrading short and medium chain alkanes; however, they did little in degrading alkanes of $>C_{20}$. MU7Y removed 80.3% and 91.3% of TP and alkanes, respectively. The yeast MU15Y is the best oil degrader among the five microorganisms selected. It degraded 99.6% alkanes and 87.3%

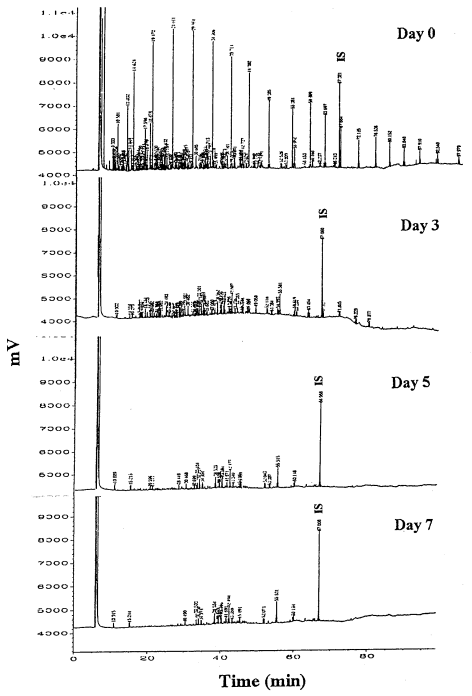

Figure 5. Chromatogram of the crude oil after incubating with the yeast MU15Y at 25 ± 2 °C for 0, 3, 5 and 7 days.

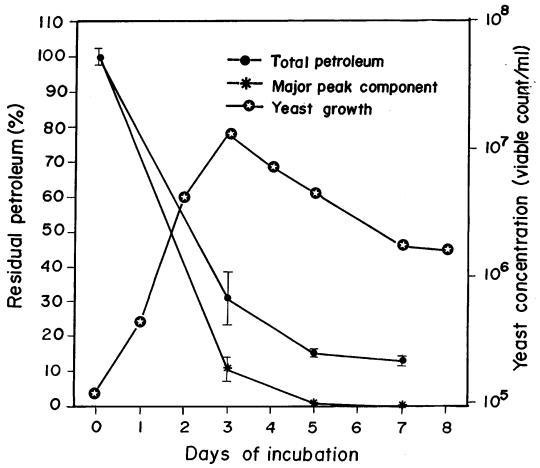

Figure 6. Growth cruve of the yeast MU15Y along with residual petroleum content.

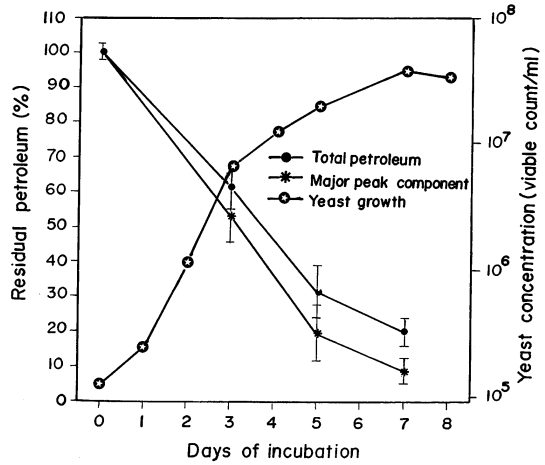

Figure 7. Growth curve of the yeast MU7Y along with residual petroleum content.

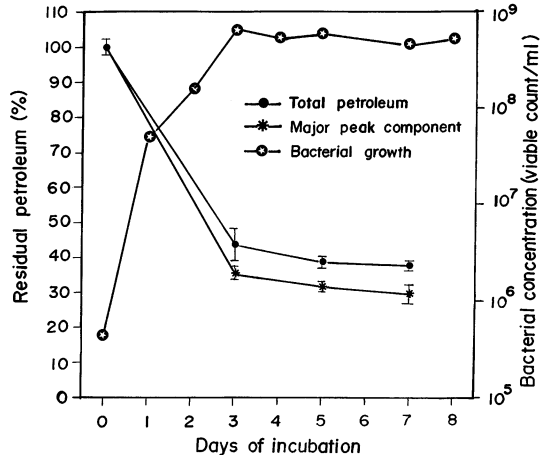

Figure 8. Growth curve of the bacteria MU14B along with residual petroleum content.

TP within seven days as shown in Figure 6. Chromatogram on Figure 5 illustrates an interesting point that all long-chain alkanes were degraded; however, the shorter chain fractions around C_{10} showed up. These fractions can be explained as the products of long-chain biodegradation.

Conclusions

This study demonstrated that n-alkanes up to C_{10} can disappear by a shake- flask technique without added microorganisms. The disappearance of this crude-oil fraction, which is called abiotic loss, may occur via

volatilization. The heavier fractions of hydrocarbons, however, were needed to be eliminated further.

Biologically mediated degradation of petroleum crude oil is a promising method for the heavier hydrocarbon fraction degradation since the microorganisms capable of oil degradation can be found readily in oil-contaminated soil. These microorganisms include both bacteria and yeasts.

The five microorganisms used in this study differed from each other in their ability to degrade crude oil. All organisms, except strain MU11B, degrade major peak components better than the overall total petroleum. Biodegradation of the major peak components reflects the biodegradation of n-alkanes.

The yeast MU15Y is the best oil degrader in this study. After the biochemical identification, it was found to be *Candida tropicalis*.

Appendix 1

The results of physiological tests of the yeast MU7Y and MU15Y.

Tests	MU7Y	MU15Y
Carbon assimilation		
Glucose	+	+
Lactose	–	–
Sucrose	+	+
Maltose	+	+
Dextrose	+	+
Milibiose	–	–
Cellobiose	+	–
Inositol	–	–
Xylose	+	+
Raffinose	–	–
Trehalose	+	+
Dulcitol	–	–
API 20C auxanogram		
Glucose	+	+
Lactose	–	–
Maltose	+	+
Cellobiose	+	–
Inositol	–	–
Xylose	+	+
Raffinose	–	–
Trehalose	+	+
Glycerol	+	+
2-Keto-D-gluconate	+	+
L-Arabinose	–	+
Adonitol	+	+
Xylitol	–	–
Galactose	+	+
Sorbitol	+	+
Methyl-D-glucoside	+	–
N-acetyl-D-glucosamine	+	+
Saccharose	+	+
Melezitose	+	+
Urea hydrolysis	–	–
Nitrate assimilation	–	–
Acid production	–	–
Growth at 37 °C	+	+
Growth at 42 °C	–	–
50% Glucose growth	+	+
10% NaCl growth	+	+

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