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Growth Enhancement of Effective Microorganisms for Bioremediation of Crude Oil Contaminated Waters

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Abstract: The bioremediation of polluted groundwater, wastewater aeration pond and biopond sites was investigated using bacteria isolated from these sites located at the oil refinery Terengganu Malaysia. Out of 62 isolates, only 16 isolates from groundwater (8) and wastewater aeration pond (3) and biopond (5) were chosen based on growth medium containing 1% (v/v) Tapis crude oil. Only four isolates; *Acinetobacter faecalis*, *Staphylococcus* sp., *Pseudomonas putida* and *Neisseria elongata* showed percentage biodegradation of crude oil more than 50% after 5 days using Mineral Salts Medium (MSM). The effect of physical parameters (temperature, pH and agitation) on growth by all four strains showed a maximum growth in MSM medium with 1% Tapis crude oil at 37°C with pH 7 and agitation of 130 rpm.

Key words: Bioremediation, crude oil, contaminated waters

INTRODUCTION

Bioremediation is a process that utilizes biological agents as much as possible for the elimination of environmental pollutants. Growth enhancement of indigenous microorganisms, biostimulation, along with inoculation of foreign oil-degrading bacteria is a promising means of accelerating detoxifying and degrading activities at a polluted site with minimum impact on the ecological systems. This idea has also been reported by Margesin and Schinner (1997). The use of emulsifiers is also a known method for the bioremediation of water contaminated with crude oil (Foght and Westlake, 1988; Janiyani *et al*., 1993; Lebkowska *et al*., 1997). The use of indigenous microorganisms proves to be central for bioremediation of crude oil. Hence, the search for new isolates for its biodegradation potential and enhanced growth requirements (Atlas, 1981; Leahy and Colwell, 1990) particular to the contaminated area needs further research. In this study, we report the isolation and characterization of bacterial strains isolated from an oil refinery in Terengganu Malaysia capable of degrading crude oil, focusing on establishing process parameters for growth enhancement.

MATERIALS AND METHODS

Microorganisms: Bacterial strains were isolated from samples collected from groundwater and wastewater aeration pond and biopond located at the Terengganu oil refinery Malaysia. Ten milliliter of each sample were washed with 90 mL saline and filtered with membrane. Incubation was carried out for 24 h at 37°C on nutrient agar plate.

Media and culture condition: Mineral salts medium (MSM) (Zajic and Supplisson, 1972) was prepared by dissolving 1.8 g K_2HPO_4, 4.0 g NH_4Cl, 0.2 g $MgSO_4.7H_2O$, 0.1 g NaCl, 0.01 g $FeSO_4.7H_2O$ in 1 L of distilled water. Bacteriological agar was added (15 g L^{-1}) to the solution where solid basal medium is required. The pH was adjusted to 6.90 and the medium was autoclaved at 121°C for 15 min. 1.0% (v/v) Tapis crude oil was added as sole carbon source and vitamin solution (1.0 mL L^{-1}) according to manufacturer's suggestion.

Inoculum preparation: Bacterial inoculums were prepared in 50 mL nutrient broth by inoculating a loopful of cells from nutrient agar plate. The cultures were incubated for 24 h at 37°C at agitation of 150 rpm. The cells were then harvested by centrifugation (Appendorf) at 4000 rpm for 10 min at 4°C. The bacterial pellet was resuspended in 10 mL saline to give the inoculums suspension at absorbance of 0.5 and wavelength at 550 nm. Unless specified otherwise, bacterial inoculums was added to give a final concentration of 10% (v/v). The cultures were incubated at 37°C for 5 days. Growth was evaluated by the resulting colony forming unit (cfu mL^{-1}).

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Isolation and identification of microorganisms: All colonies that had grown on crude oil agar was subcultured on Nutrient Agar (NA) and were incubated at 37°C for 24 h. A colony on NA was recultured to obtain a pure culture. All the pure strains were conserved on glycerol and stored at -20°C. Screening of the isolates was carried out based on colony morphology, size, shape, color, Gram staining and biochemical tests, methyl red-Voges proskauer (MR-VP) tests, indole, catalase and oxidase. The isolates were identified according to the descriptions in the Bergeys Manual of Systematic Bacteriology, Vol. 1 (1984), Vol. 2 (1986). API 24 E (Commercial Kit) was used for identification of Gram negative bacteria.

Growth profile study of isolates: For the liquid medium study, 10 mL of the liquid inoculums were added to MSM liquid medium, while for the solid medium study, a loopful of cells from the nutrient agar plate was streaked on the surface of the MSM agar. 1% (v/v) Tapis crude oil was added for both liquid and solid medium. The MSM solid medium was incubated at 37°C for 3 days. The MSM liquid medium cultures were incubated at 37°C with agitation at 150 rpm for 5 days. Spectrophotometer (Hitachi V-1100) reading for optical density at wavelength of 550 nm was used for the growth observation.

Determination of biodegradation activity: The crude oil after biodegradation was determined with reference to sterile control. At the end of an incubation time of 5 days at 37°C, the residual petroleum was recovered by petroleum ether extraction. The total residual oil was fractionated into main molecular classes. After precipitation and filtration using Whatman filters, the maltenes were separated into saturated aromatics and resins by successive elution with petroleum ether on an activated silica gel. The solvents were evaporated and the biodegradation of each fraction was evaluated using the following formula according to Chaillan *et al.* (2004).

$$\text{Biodegradation, B}(\%) = \frac{W_I - W_C}{W_I} \times 100$$

Where:

W_I : Mass of residue in sterile control

W_C : Mass of fraction in culture

Effects of temperature: The effects of temperature on bacterial growth on 1% (v/v) crude oil biodegradation were studied at 30, 37 and 40°C. Duplicate inoculation for all three different temperature studies was performed at the same time using the same batch of medium, bacterial inoculums and crude oil to minimize variations. The growth was measured as cfu.

Effects of agitation: The study of agitation during incubation on growth and crude oil biodegradation by bacterial strains was carried out using orbital shakers (Shelab) at 0 rpm (stationary), 50, 100, 130, 150 and 200 rpm. Incubation was conducted at 37°C and all inoculums preparation, inoculation and sampling times are identical to the procedures described for the other studies (Ghazali *et al.*, 2004). The growth was measured as cfu.

Effects of pH: The influence of medium pH on growth of bacterial strains was investigated at pH 6.0, 6.5, 7.0, 7.5 and 8.0. The initial pH of basal medium was adjusted to the desired pH using 0.1 or 1 M of either NaCl or NaOH. This study was conducted in duplicate and growth was measured as cfu.

RESULTS AND DISCUSSION

Selection of isolates: A total of 62 bacterial strains were isolated from groundwater and wastewater located at the oil refinery Terengganu Malaysia. The results showed that most of the isolates were able to grow on MSM agar with 1% (v/v) Tapis crude oil at 37°C, after 3 days. Only 16 isolates were chosen based on their ability to grow on MSM agar (Table 2) and in liquid medium containing 1% (v/v) Tapis crude oil at 37°C after 5 days (Fig. 1, 2). Only 4 isolates were selected for identification based on the criteria that they were able to display good growth and degradation of crude oil. Verma *et al.* (2006) also reported a 5 day study using 1% (v/v) crude oil for their isolation work and came up with 3 bacterial isolates based on similar selection criteria.

Identification of bacteria: Four isolates (WD2, DD3, TDA 4.2 and TAM 4.4) that exhibited good potential were chosen for further investigation. The results showed that two isolates were short rod Gram Negative

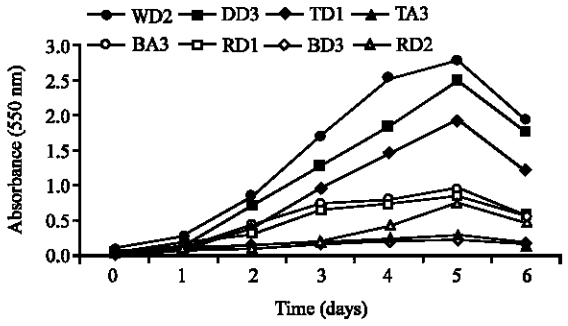

Fig. 1: Growth profiles of groundwater isolates in MSM liquid medium with 1% (v/v) Tapis crude oil at 37°C after 6 days

Table 1: Biochemical and growth characteristics of isolated bacterial cultures

	Isolates			
Characteristics	WD2	DD3	TAM4.4	TDA4.2
Gram stain	-	+	-	+
Cell morphology	Rod	Cocci	Rod	Cocci
Cell size	3 μm	5 μm	2 μm	2 μm
MacConkey	+	-	-	+
Indole test	-	-	-	-
MR test	-	-	-	-
V-P test	-	-	-	-
Citrate test	+	-	+	-
TSI test	K/K	A/A	K/K	K/K
Oxidation/ fermentation	O	F	O	F
Motility	+	-	+	-
Catalase	+	+	+	+
Oxidase	+	+	+	+
Growth at room temperature (29°C)	+	+	+	+
Growth at 37°C	+	+	+	+
Growth at 40°C	+	+	+	+
Colony color	Translucent	White	Creamy	White
Spore forming	+	-	+	-
Genus species	*Acinetobacter faecalis*	*Staphylococcus* sp.	*Pseudomonas putida*	*Nesseria elongata*

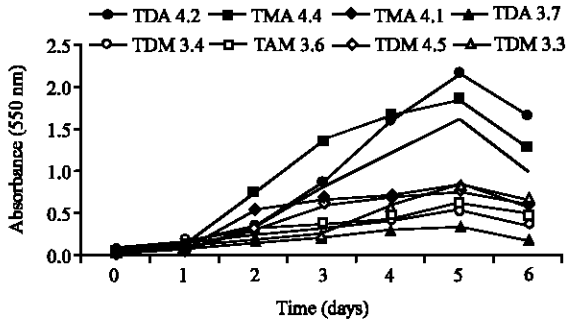

Fig. 2: Growth profiles of wastewater aeration pond and biopond isolates in MSM liquid medium with 1% (v/v) Tapis crude oil at 37°C after 6 days

Acinetobacter faecalis from groundwater sample and *Pseudomonas putida* from aeration pond sample. The other two isolates were coccus Gram Positive *Staphylococcus.* sp. from groundwater sample and *Neisseria elongata* from biopond sample (Table 1).

Growth profile study of selected isolates

Growth study in solid medium: The isolates that demonstrated good and moderate growth in the screening were employed in this study. Qualitative analysis of growth on 1% (v/v) crude oil are shown in Table 2. All 8 isolates from groundwater and 8 isolates from wastewater aeration pond and biopond showed growth in MSM agar plate containing 1% (v/v) Tapis crude oil after three days of incubation at 37°C. Only 4 isolates WD_2, DD3, TD1 and RD1 from groundwater and three isolates TAM 4.4, TDA 4.2 and TDM 4.5 from wastewater aeration

Table 2: Growth of isolates from groundwater, wastewater aeration pond and biopond on MSM agar with 1% (v/v) Tapis a crude oil at 37°C within 3 days

	37°C		
Sites	Day 1	Day 2	Day 3
Groundwater isolates			
TA3	+	+	++
DD3	++	+++	+++
WD2	++	+++	+++
BD3	+	++	++
TD1	++	+++	+++
RD2	+	+	++
BA3	+	+	+
RD1	++	+++	+++
Wastewater isolates			
Aeration pond			
TAM 4.1	+	++	++
TAM 3.6	+	++	++
TAM 4.4	++	+++	+++
Bio pond			
TDM 4.5	++	+++	+++
TDM 3.3	+	+	+
TDM 3.4	+	++	++
TDA 3.7	+	+	+
TDA 4.2	++	+++	+++

+: Acceptable growth, ++: Moderate growth, +++: Good growth

pond and biopond demonstrated moderate (++) growth on day 1 and good (+++) growth on day 2 and day 3. The selected isolates were then screened for the ability to grow on crude oil and/or individual hydrocarbons, as described by Ghazali *et al.* (2004).

Growth of isolates in liquid medium: Figure 1 and 2 show observations made up till day 6 of growth on 1% (v/v) Tapis crude oil at 37°C incubation. A growth rate analysis (based on the gradient of the growth curve) showed that WD2, DD3 and TD1 showed the three fastest rate of growth for isolates from groundwater. Whilst two samples (TDA 4.2 and TAM 4.4) from aeration pond and biopond (Fig. 2) showed comparable good rate of growth. The highest absorbance, hence biomass concentration, was selected on day 5. Present observations are in agreement with Aldrett *et al.* (1997) who reported that the maximum growth on 1%(v/v) oily sludge was after 5 day. There seems to be inhibition factors effecting growth after day 5 as have also been reported by Smith *et al.* (2003). The accumulation of inhibitory factors is an intrinsic property of a batch process and would not pose a problem in field application as an open system would be in operation then.

Biodegradation potential: The highest percentage for crude oil biodegradation was after 5 days for all 4 strains (Table 3). The highest percentage of biodegradation was by WD2 (72%) followed by DD3 (65%), TDA 4.2 (60%) and TAM 4.4 (52%). Verma *et al.* (2006) also reported that *Acinetobacter* strain degrade 75% of the aliphatic and 55% of the aromatic fraction of the oily sludge while the

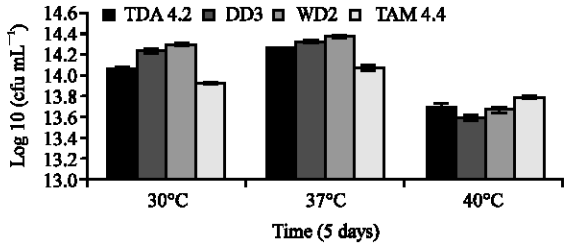

Fig. 3: Growth of isolates TDA 4.2, DD3, WD2 and TAM 4.4 at different temperature in MSM liquid medium with 1% (v/v) Tapis crude oil at 37°C after 5 days

Table 3: Weight losses of Tapis crude oil resulting from the growth of microorganisms, 37°C

Bacterial isolates	Biodegradation percentage (%) after day 5
Acinetobacter faecalis (WD2)	72
Staphylococcus. sp. (DD3)	65
Neisseria elongata (TDA 4.2)	60
Pseudomonas putida (TAM 4.4)	52

Pseudomonas showed 60% biodegradation of both fractions. Our isolates thus exhibited comparable biodegradation potential with published data.

Effect of temperature: The cell count indicated extensive growth in crude oil contaminated water for all 4 isolates. Growth increases with an increase in temperature from 30 to 37°C but not at 40°C (Fig. 3). At 37°C, the maximum growth was achieved at day 5. It would seem that 37°C would be the maximum temperature for enhancing growth of the bacterial isolates functioning as crude oil degraders. This would augurs well with the production temperature at industrial scale as 30-37°C is the normal processing temperature in the industry.

Effect of pH: The results of bacterial growth in pH 6.5, 7, 7.5 and 8 showed that significant growth was observed in medium with pH value between pH 6.5 and 7.5 for all the four isolates with the highest growth at pH 7 after day 5 (Fig. 4). This too augurs well in industrial process as optimal pH for bacterial growth and biodegradation is commonly around neutral (Alexander, 1999). Tyagi (1991) reported that it is imperative to maintain a pH range of between 6.5 and 8.0 in biological systems and this idea of a neutral pH is also supported by Tano-Debrah *et al.* (1999) who reported that most of their isolates grew best around neutral pH.

Effect of agitation: There was marked improvements in cfu mL^{-1} when the cultures were shaken at 100 and 130 rpm (Fig. 5). For all the 4 isolates, the highest growth was recorded when the cultures were incubated with

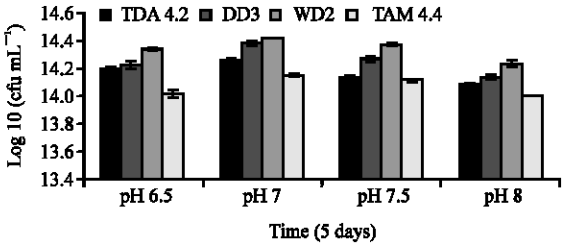

Fig. 4: Growth of isolates TDA 4.2, DD3, WD2 and TAM 4.4 at different pH in MSM liquid medium with 1% (v/v) Tapis crude oil at 37°C after 5 days

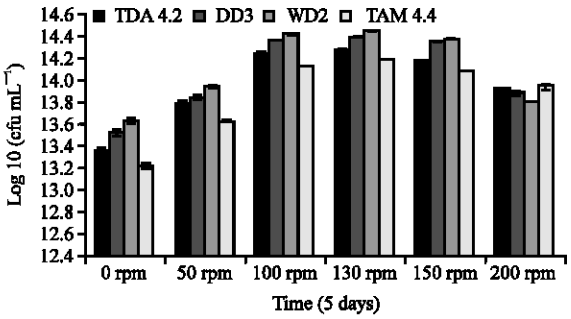

Fig. 5: Growth of isolates TDA 4.2, DD3, WD2 and TAM 4.4 at different agitation in MSM liquid medium with 1% (v/v) Tapis crude oil at 37°C after 5 days

agitation at 130 rpm although agitation at 100 rpm seems not far behind. The lowest increase in cfu mL^{-1} was recorded when incubated with no shaking. Hayase *et al.* (2004) also reported best growth to be at 130 rpm. As all the four isolates grew well at 100 rpm, we would choose an agitation of 100 rpm for an industrial scale production as it would reduce cost when compared to operating at agitation of 130 rpm.

CONCLUSION

Four bacteria isolates (*Acinetobacter faecalis*, *Staphylococcus* sp., *Pseudomonas putida* and *Neisseria elongata*) from contaminated waters that showed good (more than 50%) crude oil biodegradation was obtained from a total of 62 bacteria isolates. The best conditions for growth and biodegradation in 1% Tapis crude oil for the four isolates are at 37°C, pH 7.0 and agitation at 130 rpm.

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