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Comparative Studies on Emulsification and Biodegradation of Indigenous Crude Oils by Enriched Bacterial Culture

D. Kokub, M. Shafeeq, Z.M. Khalid*, A. Hussain* and K.A. Malik**

Hydrocarbon Development Institute of Pakistan, Islamabad
****Nuclear Institute for Agriculture and Biology, Faisalabad, Pakistan***

ABSTRACT

Bacteria from oil contaminated soil and cultured on paraffin oil medium, were adapted to various crude oils. The enriched cultures so obtained were used for emulsification of indigenous crude oils under shaking conditions. Varying degrees of emulsification based on visual observation were noted by different cultures, dependent upon the chemical composition of different crude oils. Tando Alum and Khaskheli crude oils were emulsified in 27 and 33 days of incubation respectively while Joyamair crude oil did not emulsify even after 72 days of incubation. This persistance was found to be mainly due to the low API gravity value i.e., high viscosity, of this oil.

Oil from control (uninoculated) and biodegraded flasks was fractionated and quantified by Adsorption and Gas Liquid Chromatographic (GLC) methods. Comparison of different fractions i.e., saturate, aromatic, NSO (nitrogen, sulphur, oxygen containing hydrocarbons) revealed that the saturate fraction was preferentially utilized during biodegradation. It was observed that crude oils having greater contents of saturate fraction were better emulsified than crude oils low in this fraction. These bacterial strains in mixed culture utilize different fractions of crude oils in the order of saturate >aromatic> NSO and the persistance was found to be mainly due to high viscosity.

INTRODUCTION

Crude oil or petroleum are complicated mixtures of chemical compounds, composed of hydrocarbons together with organic compounds of nitrogen, sulphur and oxygen. The hydrocarbon content may range from 95–98% and as low as 50% for heavy crude oils (Gruse and Stevens, 1966). The

** Corresponding author

hydrocarbons present in crude oils belong to three main classes namely paraffins (alkanes, isoalkanes); cycloparaffins (cycloalkanes) and polar aromatics (mono-, di- and polycyclic polar compounds). The high viscosity of crude oil is due to the lubricant fraction which is characterized by high content of cycloparaffins and aromatic compounds (Gruse and Stevens, 1966).

Accidental oil spillage from tankers, offshore drilling blow outs and other mishaps present considerable oil pollution problems in the sea. The yearly influx of petroleum pollutants in the sea has been estimated to be as high as 10 million tons (Morris, 1971). Large amounts of this crude oil disappear by weathering and microbial degradation (Barth, 1984).

Microorganisms are considered to be the best agents for destruction of organic compounds present in crude oils (Walker & Colwell, 1974; Colwell & Walker, 1977; Atlas, 1981; Rambeloariosa *et al.*, 1984). In nature, microorganisms are present which can utilize paraffins and various aromatics as carbon sources (Jobson *et al.*, 1972; Walker *et al.*, 1975; Fedorack and Westlake, 1981; Rambeloariosa *et al.*, 1984; Teschner and Wehner, 1985). Although hydrocarbon utilizing microoganisms are abundant in nature, tar lumps still occur widely on the surface of sea (Horn *et al.*, 1970; Morris, 1971) and can persist on the site of an oil spill even after two years (Blumer and Saas, 1972). It has been reported that persistance and biodegradability of crude oil is dependent on its chemical composition and the molecular configuration of its hydrocarbon components (Horn *et al.*, 1970; Westlake *et al.*, 1974). The rate of microbial degradation decreases from normal alkanes to isoalkanes and cycloalkanes to aromatics (Blumer and Saas, 1972). Therefore, the present studies were aimed at getting some information about comparative degradation and emulsification abilities of different crude oils available in Pakistan using an enriched bacterial culture of indigenous origin.

MATERIALS AND METHODS

Crude Oils

The crude oil samples were obtained from the oil wells through the courtesy of respective producers as shown in parenthesis: Balkassar, Sakessar Formation (FM) and Joyamair, [Pakistan Oil Fields Ltd. (POL)]; Tando Alum and Fimkassar, [Oil and Gas Development Corporation (OGDC)] and Khaskheli, [Union Taxas Pakistan Inc. (UTP]. Five gram of each crude oil in screw capped tubes was sterilized at 121°C for 15 minutes and was transferred aseptically to 100 ml sterilized medium. The crude oils used in biodegradation studies were analysed for asphaltenes, saturates, aromatics and NSO fractions by the methods given below.

Extraction of residual crude oil

The residual crude oil was extracted from the culture broth after 7, 18 or 26 days of incubation by washing three times with n-hexane in 1:2 ratio. The organic layer was evaporated to dryness by a rotary evaporator under vacuum at 25–30°C and then flushed with a stream of nitrogen until constant weight. The residual oil obtained after different incubation periods was compared with the uninoculated oil obtained through the same procedure.

Fractionation of crude oils

At each incubation period, residual crude oil from inoculated as well as from their uninoculated control flasks was fractionated by Adsorption chromatographic techniques (Jobson *et al.*, 1972) into different fractions of oil, and compared with similar fractions of crude oil i.e., at 0 day incubation.

Packing of the Hyflo supercel celite column

The finely packed chromatographic column is a pre-requisite for better resolution of different fractions. n-Pentane was found unsuitable for packing of Hyflo super cel celite column at room temperature as well as at 4°C. Therefore, the method for packing of the column as described by Jobson *et al.*, (1972) was modified. Small batches of celite suspended in a n-hexane:cyclohexane (1:1) mixture were poured into the column. After removal of the excessive solvent the celite was packed to 370 × 11 mm bed by agitating and pressing with glass rod. Cyclohexane was washed by eluting with n-hexane. The column elute was checked by gas liquid chromatography for complete removal of cyclohexane. One gram of oil suspended in n-hexane was applied to the column and sequentially eluted with 125 ml of n-hexane to elute the deasphaltened oil (containing saturate, aromatic and NSO fractions) and 100 ml benzene to elute benzene soluble asphaltenes (waxy asphalt). These fractions were concentrated by a rotary evaporator and further dried to constant weight by flushing with a stream of nitrogen. The benzene insoluble asphaltenes remaining on the column were calculated by difference.

Adsorption chromatography on silica gel-alumina column

A dual phase column (Jobson *et al.*, 1972) containing, 18 + 0.5 g activated silica gel (70–230 mesh) in the lower half and 20 + 1.0 g activated aluminum oxide–90 (70–230 mesh) in the upper half was used. Both phases were packed after suspending in n-hexane. The n-hexane soluble deasphaltened oil was layered on the top of column and eluted sequentially with 165 ml n-hexane, 250 ml benzene and 250 ml benzene and methanol (1:1) mixture to elute the saturate, aromatic and NSO fractions respectively. These fractions were dried to constant weight.

Gas liquid chromatography of saturate fraction

Saturate fraction of control as well as of residual oil at each incubation period was analysed using Hitachi chromatograph (Model–163) equipped with flame ionization detector (FID) and SE–30 (methyl silicone) glass capillary column (25 m × 0.5 mm i.d) precoated with silanox 101. Other conditions were as follows: Linear temperature programme 50–250°C with 5°C/minute increase; Injector and Detector temperature 300°C; Nitrogen flow rate 4 ml/min.; Hydrogen flow rate 15 ml/min. Reduction in peak height of the components of this fraction in comparison to the control was taken as measure of bacterial utilization of this fraction.

Growth medium

The BH-medium (Bushnell and Haas, 1941) containing 0.2 g $MgSO_4.7H_2O$; 0.02 g $CaCl_2$; 1.0 g KH_2PO_4; 1.0 g K_2HPO_4; 1.0 g NH_4NO_3 per litre (pH = 7.0–7.2) was used throughout these studies.

Microorganisms

The mixed bacterial culture was obtained through enrichment of oil contaminated soil samples collected from various petrol pumps in Faisalabad (Pakistan) in BH mineral medium containing paraffin oil as carbon source. The enriched cultures (10^8–10^9 CFU/ml) (CFU = Colony forming units) were adapted to indigenous crude oils separately by two successive transfers to fresh medium containing 5% (w/v) crude oil and incubated at room temperature (25–30°C), on a rotary shaker (100 rpm.) for 15 days. The mixed culture growing on one type of oil, at mid exponential phase was used

as inoculum (1% v/v) for further studies on the same crude oil only. Thus six different enriched culture were used in duplicate for the six oils used in these studies.

Emulsification studies of crude oils

For emulsification studies, the inoculated as well as the control (un-inoculated) flasks were placed on rotary shaker (100 rpm) at room temperature (25–30°C). At various intervals, the flasks were removed from the shaker and kept undisturbed for two hours. The amount of oil separated out (unmodified) was compared with that of control flasks. The amount of oil miscible in water (Total-separated) was taken as an index of emulsification. Minimum days required for complete emulsification (no oil separated out from the aqueous phase) were recorded for different oils.

Growth behaviour of bacterial culture on different crude oils

For growth studies, stationary phase grown bacterial culture from each crude oil was inoculated in duplicate into 5% (w/v) of respective crude oils and set on rotary shaker at 100 rpm. at room temperature (25–30°C). CFU/ml was calculated by spread plate (Sharpley, 1960) method (using 0.9% NaCl as diluent) at 4 day interval up to 48 days. Growth curve of the log of crude oil utilizing bacteria for each oil was also plotted. Morphologically different types of bacterial colonies were recorded and purified for further use. The changes in pH of the culture broth were also monitored at each incubation period using a pH meter (Corning–130) calibrated with two buffers system.

Dry biomass determination

Oil free culture broth was centrifuged at 15,000 rpm. for 15 minutes. The cells were washed with 30 ml phosphate buffer (pH 7.5) and centrifuged as before. The pellet was resuspended is 2 ml distilled water. The suspension was transferred to a dry preweighed china crucible and dried to constant weight in an oven at 105°C (Berwick, 1984).

RESULTS AND DISCUSSION

Chemical composition of various crude oils

Chemical composition of various crude oils as determined by chromatographic techniques in shown in Table 1. The major component in these oils was found to be the saturate fraction, which ranged from 56–79%.

TABLE 1

Chemical composition of the indigenous crude oils used for biodegradation studies. (expressed as % wt of fractions in oils)

Fraction	Tando Alum	Khaskheli	Sakessar Formation	Balkassar	Fimkassar
Saturates	79	69	60	59	56
Aromatics	14	21	25	20	24
NSOs*	4	8	12	17	17
Benzene sol. Asphaltenes	1	2	2	3	1
Benzene Insol. Asphaltenes**	2	0	1	1	2
DASO (desaphaltened oil) = Sat. + Aro. + NSOs	97	98	97	96	97

* NSOs = Nitrogen, Sulphur and Oxygen containing hydrocarbons.
** Benzene Insoluble Asphaltenes = Total oil – (DASO + Benzene Soluble Asphaltenes)

Emulsification of different crude oils

Time (days) required for complete emulsification and CFU/ml at the time of emulsification is given in Table 2. In the present studies, it has been found that Tando Alum and Khaskheli crude oils were emulsified after 27 and 33 days of incubation respectively (Table 2) while Joyamair crude oil did not emulsify even after 72 days of incubation. It was transformed into a ball like structure which settled down at the bottom of flask and no emulsification of oil in water (medium) was observed. The rapid emulsification of Tando Alum and Khaskheli crude oils might be due to their higher API (American Petroleum Institute) gravity values. It has been reported that higher the API gravity value, lower will be the viscosity (Anonymous, 1986). Therefore

TABLE 2

Growth and emulsification of bacteria on different crude oils. Arranged in the increasing order of time for emulsification.

Location of oil well	API[a] gravity	CFU/ml[b] enriched cultures grown on crude oil used for emulsification	Time required for emulsification (days)
Tando Alum	41.00	1.9×10^{8}(4)	27
Khaskheli	39.72	2.2×10^{9}(3)	33
Sakessar formation	–	2.3×10^{8}(2)	45
Balkassar	25.72	2.8×10^{9}(3)	53
Fimkassar	25.72	3.7×10^{8}(3)	53
Joyamair	15.13	5.0×10^{8}(2)	72[c]

a. American Petroleum Institute.
b. Colony forming units/ml at the time of emulsification. Number in parenthesis represent morphologically different strains.
c. Not emulsified even after 72 days of incubation.
– Not available.

high API gravity values and low viscosities of Tando Alum and Khaskheli crude oils (Table 2) might be responsible for rapid emulsification of these oils. In the present study it has been found that oils with high API gravity values have high weight percentages of saturate fraction. This fraction is reported to be the preferred carbon source for hydrocarbon utilizing bacteria (Jobson *et al.*, 1972; Walker *et al.*, 1975; Masao and Nagata, 1980). It has been further confirmed by chromatographic analyses that Tando Alum and Khaskheli crude oils have greater weight percentage of saturate fractions (79 and 69%) while, Sakessar formation, Balkasser and Fimkassar crude oils have 60, 59 and 56% saturate fractions respectively (Table 1). Therefore, these oils took more time for emulsification. It has been found that morphologically different strains (Number given in parenthesis) were proportionally increased (enriched) in response to various crude oils as shown in Table 2. This differential increase in bacterial population might be dependent upon the chemical composition of oil as well as upon the metabolic capability of these strains to preferentially oxidize different fractions of crude oil. It has been reported that chemical composition of oil had a marked effect on its biodegradability characteristics (Westlake *et al.*, 1974; Atlas, 1975).

Utilization of different crude oils by mixed bacterial culture

The utilization of different crude oils by mixed culture at room temperature is shown by the increase in number of viable cells as function of time as shown in Figure 1. The mixed culture showed no lag phase in utilizing different crude oils, probably because they were pre-grown on oil and may have adapted to utilize the crude oil as carbon source. The absence of lag phase might be due to the catabolism of oil by direct enzyme induction (Van Eyk and Bartels, 1968). As expected, there was no growth in uninoculated control with oil even after 72 days in all oil samples.

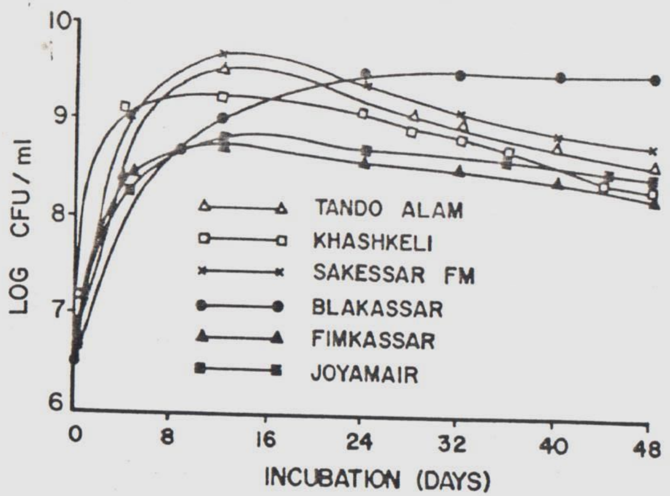

FIGURE 1 Viable counts of enriched bacterial culture inoculated to 5% (w/v) of different crude oils.

Different crude oils metabolized by mixed culture were extracted from the culture broth after 7, 18 or 26 days of incubation. The results showing changes in pH, CFU/ml and dry biomass are shown in Table 3. The pH of the culture broth is important in determining the course of a metabolic sequence and increase or decrease in pH of the culture broth indicated the production of basic or acidic metabolites. In the present study, it was observed that during aerobic oxidation of crude oils from Fimkassar, Sakessar formation and Tando Alum, the pH of the culture broth was lowered by 0.9, 0.8 and 0.5

TABLE 3

Changes in pH, CFU/ml and dry biomass after growing mixed bacterial culture on different crude oils

Crude oil	Incubation period (days)	pH	CFU/ml	Biomass (mg)*
Tando Alum	0	7.0	3.3×10^6	0
	7	6.4	2.2×10^9	233
	18	7.0	5.0×10^9	180
Khaskheli	0	7.1	8.0×10^5	0
	7	6.9	8.9×10^9	133
	18	6.7	1.1×10^{11}	130
Sakessar formation	0	7.1	1.9×10^6	0
	7	6.3	8.5×10^8	210
	18	6.6	9.5×10^8	240
Balkassar	0	7.0	1.7×10^6	0
	7	6.8	2.1×10^8	17
	26	6.6	1.1×10^8	No pellet
Fimkassar	0	7.0	3.2×10^5	0
	7	6.1	2.1×10^9	33
	26	6.8	8.8×10^9	40

* mg/100 ml of BH medium

units at 7 days of incubation respectively (Table 3). While in the oxidation of Balkassar and Khaskheli crude oils by mixed culture, the decrease in pH was found to be lesser than 0.5 units at 7 days of incubation. In this case comparative decrease in pH was observed after 18 and 26 days of incubation (Table 3) and this might be due to the slow rate of metabolism of these strains.

Previous workers have detected fatty acid production from hydrocarbons using the fall in pH as a criterion of acid formation (Bird and Molton, 1970; Abbot and Gledhill, 1971). Some of the carbon from crude oil, during biodegradation is converted into bacterial biomass both living and dead. In the light of the difficulties in separating total bacteria from the sticky oils, both the parameters (CFU & biomass) were considered to have some reliable insight of bacterial growth (Table 3). Generally there was an increase in biomass with time in line with an increase in CFU/ml, however, biomass from Balkassar and Fimkassar cultures did not correspond with CFU/ml. During microbial metabolism of the saturate fraction, extracellular polar compounds are secreted into the medium. These compounds are responsible for the increase in NSO fraction which in turn causes an increase in the viscosity of oil (Table 4). Therefore, extraction of biomass becomes more difficult. The measured biomass from Tando Alum and Khaskheli crude oils is comparatively less after 18 days of incubation. This can be explained on the basis of the fact that in more emulsified oils, oil and water phases are intermixed to such an extent that bacterial cells become entangled between the two phases and the complete separation of biomass became increasingly difficult. Similar observations have been reported previously (Boyles, 1984).

Fractionation of original and biodegraded crude oils

Changes in the chemical composition of various crude oils after growth of mixed bacterial cultures for 7, 18 or 26 days of incubation are shown in Table 4. It has been found that saturate fractions from all crude oils are found to be the preferred carbon source of mixed bacterial cultures. Utilization of the components of saturate fractions from different crude oils by mixed cultures was followed by gas liquid chromatography. Keeping all the conditions of GLC constant the components of the oils were released in the same order for all the oils utilized. The results are presented in Figure 2. The reduction in peak height of various components of the saturate fraction in comparison to similar components of the saturate fraction (same retention time) in uninoculated control flasks indicate bacterial utilization of these components. These results also confirm the results obtained gravimetrically (Table 4).

Identificaton of various peaks present in the metabolized oils are underway and will be presented separately. Decrease in the weight percent of aromatic fraction has also been observed after 18 or 26 days of incubation indicating bacterial utilization of this fraction by mixed culture which has also been confirmed by gravimetric method (Table 4). Decrease in weight percentages of saturate and aromatic fractions indicate bacterial utilization while increase in weight percent of NSO fraction may be due to extracellular accumulation of polar compounds derived from the metabolism of aliphatic hydrocarbons and to lesser extent due to aromatic hydrocarbons (Jobson *et al.*, 1972; Westlake *et al.*, 1974; Walker *et al.*, 1975; Masao and Nagata, 1980).

CONCLUSIONS

From these studies it is concluded that the crude oil utilization by mixed bacteria, enriched on the same indigenous oil, is mainly dependent upon the proportion of the saturate fraction in the oil. The persistance could be

TABLE 4

Changes in various oil fractions as a result of bacterial growth.

	Incubation Period	Saturates g/g oil	% Change*	Aromates g/g oil	% Change*	NSO** fraction g/g oil	% Change*
Tando Alum	0	0.79		0.14		0.04	
	7	0.69	−13	0.14	0	0.05	25
	18	0.59	−25	0.18	29	0.09	125
Khaskheli	0	0.69		0.21		0.08	
	7	0.60	−13	0.13	−38	0.23	188
Sakessar formation	0	0.60		0.25		0.12	
	7	0.54	−10	0.31	24	0.14	17
	18	0.51	−15	0.33	32	0.14	17
Balkassar	0	0.59		0.20		0.17	
	7	0.53	−10	0.23	15	0.19	12
	26	0.26	−56	0.18	−10	0.36	112
Fimkassar	0	0.56		0.24		0.17	
	7	0.52	− 7	0.23	− 4	0.19	12
	26	0.46	−18	0.23	− 4	0.24	41

* = Values with (−) sign indicate 'Utilizaton' and without it are for the 'Production' over 0 day observation.
** NSO = Nitrogen, Sulphur and Oxygen containing hydrocarbons.

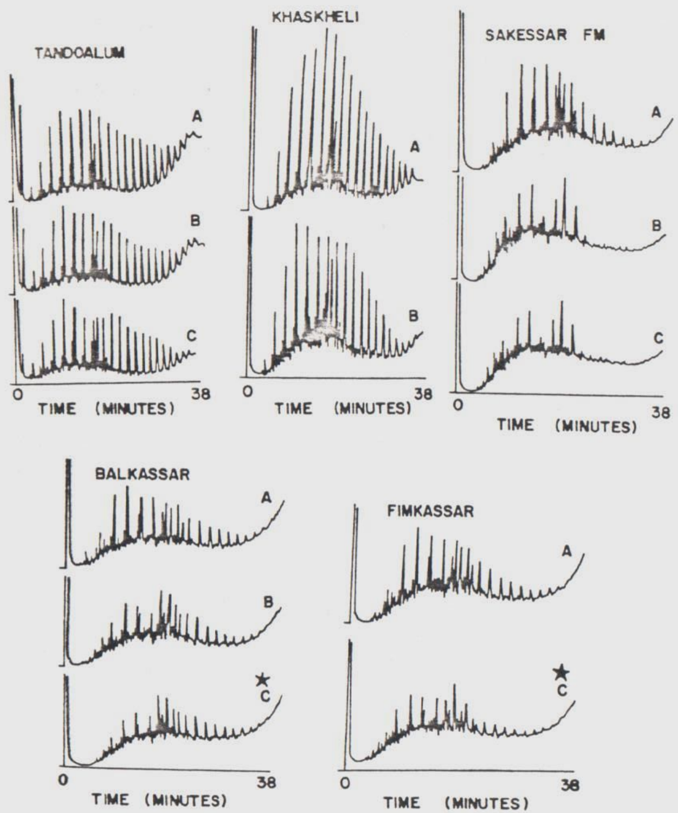

FIGURE 2 Gas liquid chromatographic analyses of residual saturate fractions after sequential utilization of different crude oils, by mixed bacterial culture at room temperature A: Control; B: 7 days; C: 18 days; C*: 26 days of incubation.

correlated to the oil composition mainly to the low API gravity values i.e., high viscosity. Degradation by mixed culture followed the same order of saturate > aromatic> NSO fractions as reported previously by others.

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