Pakistan J. Sci. Ind. Res., Vol. 31, No. 10, October 1988

ISOLATION OF FURNACE OIL UTILIZING BACTERIA CAPABLE OF PRODUCING BIOSURFACTANT

Zafar M. Khalid and Kauser A. Malik

Nuclear Institute for Agriculture and Biology, Faisalabad

(Received August 7, 1988; revised October 26, 1988)

Oil polluted soil samples, collected from various petrol pumps and around furnace oil underground storage tanks, were analysed for their total bacterial population and the frequency of oil utilizing bacteria. It was found that the amount of oil utilizing bacteria ranged from 28.1 % to 71.4 % of the total bacterial population. Air lift percolators having furnace oil impregnated soil, proved useful for enrichment of oil utilizing bacteria and studies on their succession. The individual growth behaviour of six oil utilizing bacteria in air lift percolators is reported. When a mixture of six bacterial strains was employed, furnace oil was used for the production of biosurfactant, which could completely emulsified the oil in the water phase.

Key words: Isolation, Biosurfactants, Oil utilizing bacteria, Furnace oil.

INTRODUCTION

The decomposition of petroleum and petroleum products by microorganisms is of considerable importance. At the petroleum-water interface, bacteria develop in large number and molds and yeasts may also grow [1]. Microorganisms produce a variety of surface active agents (biosurfactants), which alter the conditions prevailing at interfaces [2]. The metabolic pathways and metabolites by which microorganisms produce surface active agents have been thoroughly reviewed [3]. These compounds play a vital role in the adhesion, floculation, and location of microbes at interfaces in bulk media. Biocompounds such as fatty acids, glycolipids, phospholipids, lipopeptides and extracellular lipids are produced either in the exponential or in the stationary growth phase [3]. These compounds have been applied for enhanced oil recovery [4,5] petroleum solubilization in petroleum tanks, in refineries or tanks [6] and for the cleaning of chronically contaminated sites [7]. Culture fluid after growth of an *Arthrobacter* species, RAG-1, on either crude oil or hexadecane has been employed for the dispersion of crude oil [8]. Partially purified emulsifiers have been used for emulsification of heavy oil such as asphaltenes or shale oil [9].

During fractional distillation of crude oil, after the removal of light hydrocarbons a fraction with boiling point 260-371^{o} and comprising different isomers of C_{15}-C_{22} (mole wt. 212-294) is produced which is normally used as heating oil or furnace oil [10]. In Pakistan, this fraction is not fully utilized and a major part is being exported to other countries at a relatively low price. To get some value added product from this fraction, it was subjected to use as a base for biosurfactant production.

The present work was aimed at studying the frequency and succession of oil utilizing bacteria indigeneous to different oil soil eco systems and to isolate furnace oil utilizing bacteria. Growth behaviour of six strains in air lift percolators and biosurfactant production in shake flasks were also examined. The results are presented in this paper.

MATERIALS AND METHODS

Sampling. Soil samples continuously contaminated with oil were collected from various places such as petrol pumps and around furnace oil underground tanks. Surface soil samples were collected with the help of sterile spatula and brought to the laboratory in polyethylene bags.

Medium. The total viable microorganisms were determined using Difco's nutrient agar (NA) plates. The frequency of oil utilizing microorganisms was determined using the BH medium by Bushnell and Haas [11], with the following composition: 0.2 g $MgSO_4 . 7H_2O$, 1 g KH_2PO_4, 1 g K_2HOP_4, 1 g NH_4NO3, 0.02 g $CaCl_2$ and 2 drops of saturated aqueous solution of $FeCl_3$ per litre of medium. The pH of the medium was adjusted between 7.0 – 7.2. Paraffin oil was added as the sole carbon source at a rate of 0.1 ml per plate i.e., 0.4 % V/V. Normal saline (0.85 % NaCl) was used as diluent.

Air lift percolator. An air lift percolation technique was employed to study the succession of oil utilizing bacteria from sand impregnated with furnace oil and inoculated with oil contaminated soil. The same technique was also employed to study the growth of the isolated bacteria.

The details of the percolator are presented in Fig. 1. It consists of a glass column (30 x 210 mm) with side tube having an opening (5mm) for the entry of air. The level of this opening is adjusted in such a way that the stream of air carries the percolating solution in the form of bubbles. After various periods of incubation an aliquot of the percolant was withdrawn from the bottom of the glass column and further diluted for estimating the microbial content.

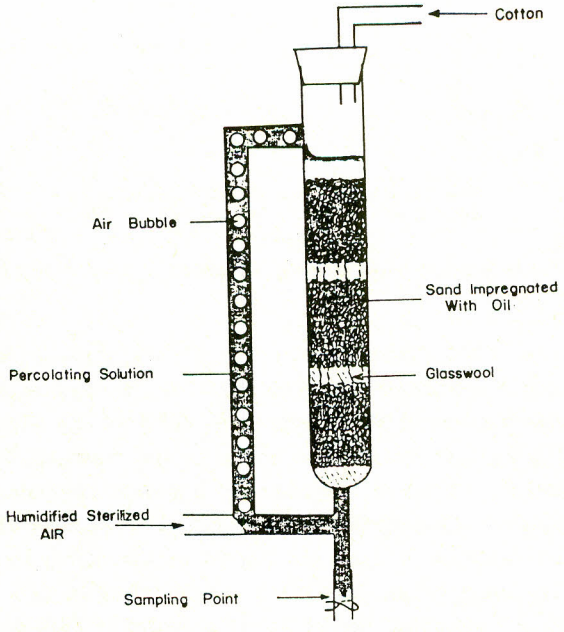

Fig. 1. Air lift percolator.

Washed and dried sand of 0.177 to 0.297 mm size (passed through 50 mesh but retained by 70 mesh sieve) and furnace oil were sterilized by autoclaving at 121° for 15 min. followed by incubating at 30°, for three consecutive days. Furnace oil was then mixed at the rate of 5 g/100 g sand aseptically. This concentration was determined in preliminary experiment and was found to be sufficient to obtain acceptable drainage characteristics in the column. Higher concentration resulted in clogging of the percolator. The sand-oil mixture was filled in the glass column with glass wool after every 40 mm to stop the compact packing of the column. This column was inoculated with the required soil sample or microbial strain under sterile conditions. Air after being filtered through a column of sterile cotton was humidified to reduce the losses by evaporation of water in the column.

Isolation of microorganisms. Aerobic heterotrophic bacteria were isolated by appropriate dilution of soil samples on NA as well as BH paraffin oil medium. From air lift percolating columns, samples of percolating solutions were properly diluted and grown on nutrient agar by pour plate method [1] and incubated at 30°. Colonies appearing on NA plates were transferred to BH oil agar (BHOA) plates. Successive transfer of isolated colonies were made on BHOA plates three times and finally transferred to NA slant for storage.

RESULTS AND DISCUSSION

Using dilution plating technique, it was found that the number of colonies formed per gram of soil on NA plates ranged from 40-140 for various locations whereas the frequency of oil-utilizing microorganism on BHOA plates ranged from 20-100 colonies (Table 1). The amount of oil-utilizing microorganisms varied from 28.1 to 71.4 % of the total microflora of the oil contaminated sample. Similar media were also employed for total microbial count and oil utilizing bacteria by Horowitz and Atlas [12]; Horowitz *et al* [13] and Jamison *et al* [14].

One-gram portion of soil samples from two locations, A and B (Table 1) having 71.4 and 68.3 percent oil-utilizing microorganisms were added to the percolating columns containing oil impregnated sand. The oil-utilizing microbial population in the percolating medium showed an increasing trend (Fig. 2). In the beginning the population determined as colony forming units (CFU)/ml was 38 and 42 CFU/ml of percolating medium. In case of soil sample A, the maximum population (3.4 x 10^6 CFU/ml) was on the 36th day followed by a decrease in further incubation. In the second column with soil sample B, an increase was noted throughout the incubation period of 49 days up to

Table 1. Comparison of number of colony forming units/ gm soil from different locations by soil dilution technique on nutrient agar and BH paraffin oil agar plates. The results are expressed as average of 4 replicates with standard error.

*Location	Soil dilution plating on		Oil utilizers percent of the total bacteria
	Nutrient agar	BH paraffin oil agar	
A	140 ± 12.75	100 ± 13.69	71.4
B	126 ± 10.83	86 ± 10.31	68.3
C	40 ± 7.07	21 ± 3.54	52.5
D	105 ± 10.31	48 ± 7.40	45.7
E	65 ± 9.01	20 ± 3.54	30.8
F	96 ± 11.46	27 ± 4.15	28.1

*Locations are arranged according to the decreasing percentage of oil untilizing microbes. A = S.M. Iqbal Petrol Services, Jaranwala Road, Faisalabad; B = Furnace oil underground storage tank, NIAB, Faisalabad; C = Saeed Petroleum Oil Station (PSO), Jaranwala Road, Faisalabad; D = Rehmat Petroleum Services, D-Ground People Colony, Faisalabad; E = Speedy Service Pump, Jhang Road, Faisalabad; F = Cheema Petrol Station, University Road, Faisalabad.

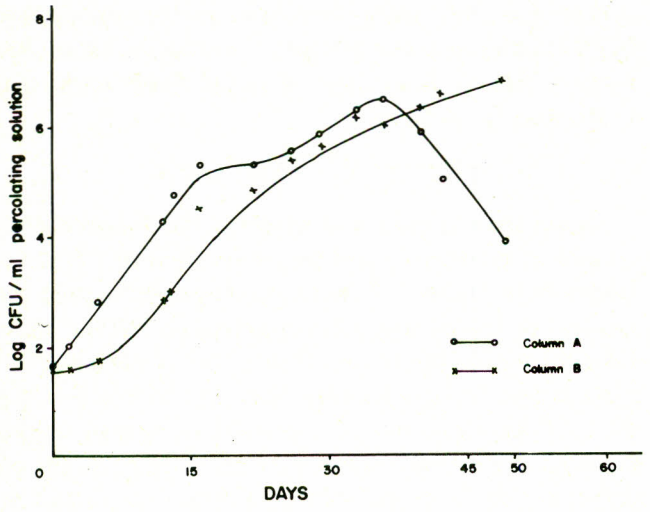

Fig. 2. Growth behaviour of oil utilizing bacteria from soil samples in air lift percolators.

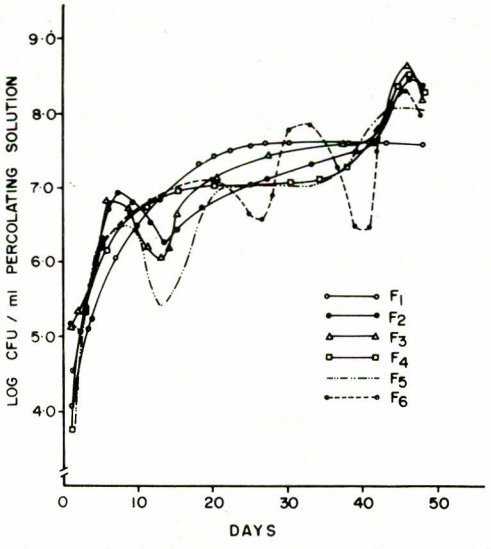

Fig. 3. Growth of six bacterial strains on furnace oil in percolating columns.

7 x 10^6 CFU/ml. Compared to the direct dilution plating technique, where a maximum of 100 CFU/g soil was detected, the percolating columns seem more reliable and practical to isolate much higher numbers as well as variety of microbes. This may be due to the adaptation of the microbes with the surroundings or modified conditions because of the more selective environment. Higher numbers are also achieved because of enrichment and growth of microorganisms in the percolator.

From these enumeration studies, several morphologically different oil utilizing microorganisms were isolated which included some yeasts and bacterial strains. Raymond *et al.* [15] have isolated *Nocardia* sp. from soil by using *n*-paraffins as the sole carbon source.

Out of the strains isolated during the present study, six strains (F_1-F_6) were studied for their individual growth behaviour on furnace oil in percolating columns. These strains were grown in shake flask in BH medium with paraffin oil as carbon source. When sufficient growth turbidity (0.D, 0.60) of medium was obtained, 1 ml of the oil free suspension was added to each column aseptically. Growth studies were carried out for 48 days and the results are presented as log of number of colony forming units per ml (CFU/ml) of percolating medium (Fig. 3). All the microbial strains showed rapid growth during the first 6-10 days, showing direct utilization of furnace oil. In the case of F_1 strain, the increase in CFU stopped after 20 days, indicating the stationary phase. With F_4 the stationary phase started on the 14th day, but an increase was noticed from the 34th day onward. This dioxic growth indicates the use of intermediate products. With F_6 the stationary phase was followed by a decrease on the 26th day. In other strains, namely F_2, F_3 and F_5, the first increase was followed by a decrease for 7-8 days. A slow increase was noted afterwards, in F_2 till 43rd day when a sudden increase occurred in the microbial number. With F_3 and F_5 second increase lead to a stationary phase. In the case of F_3 this stationary phase lasted for 23 days when another increase in bacterial population occurred. In F_5 the stationary phase lasted for a shorter period and the bacterial population showed another increase before the termination of experiment. These changes in growth may be due to an intermediate metabolite repression, which after induction of particular enzyme systems was followed by the utilization of metabolite as substrates.

The variation in the microbial population casts some doubt on the merits of using percolating columns for growth studies. However, this technique has clear advantages in the isolation of a variety of different microbes capable of growing on furnace oil and its by products.

Identification of the bacterial strains. Following Bergey's manual of determinative bacteriology [16] the identification tests revealed (Table 2) that F*1* is *Pseudomonas aeruginosa,* F_3 and F_4 are *Pseudomonas* spp. while F_2 and F_6 are *Micrococcus* spp. and F_5 belongs to *Flavobacterium* family. F_3 and F_4 are different from F_1, because they are unable to denitrify nitrate or liquify gelatin and do not grow at 42°. Being VP positive F_4 differs from F_3 which shows negative reaction. F_2 has darker pink pigments as compared to F_6 strain.

Biosurfactant production. Mix culture of these six bacterial strains was employed for production of biosurfactant from furnace oil (5 % w/v) in BH medium under shaking condition (100 rpm) at 30°. Fig. 4 reveals that as

Table 2. Identification tests for bacterial strains used for growth studies on furnace oil in air lift percolating columns.

Identification tests	Bacterial strains					
	F_1	F_2	F_3	F_4	F_5	F_6
Gram's staining	–	+	–	–	–	+
Shape	Rods	Cocci	Rods	Rods	Rods	Cocci
Motility	+	–	+	+	+	–
Oxidase	+	–	+	+	+	–
Catalase	+	+	+	+	–	+
O/F Glucose*						
O	+	–	+	+	+	–
F	–	–	–	–	–	–
Citrate	+	–	+	+	–	–
Indole	–	–	–	–	–	–
MR	–	+	–	–	–	+
VP	–	–	–	+	+	–
NO_3NO_2	+	+	–	–	+	+
NO_2-N_2	+	–	–	–	–	–
Gelatin liquifaction	+	–	–	–	–	–
Growth at 42°	+	–	–	–	–	–
Water soluble pigment	+	–	+	+	–	–
Water insoluble pigment	–	+	–	–	+	+
Tentative identification	*Pseudomonas aeruginosa*	*Micrococcus* sp.	*Pseudomonas* sp.	*Pseudomonas* sp.	*Flavobacterium* sp.	*Micrococcus* sp.

*Hugh and Leifson's glucose medium.

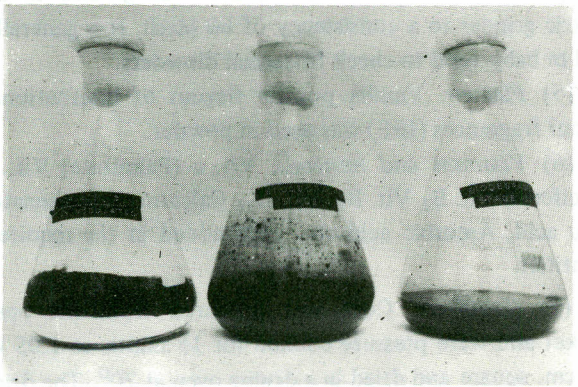

Fig. 4. Photograph comparing the uninoculated control, no change in oil (flask A), with the inoculated samples (mixed culture of F_1-F_6 bacterial strains) incubated at 100 rpm, 30° for 5 days, showing dispersion of furnace oil in water (flask B), and for 10 days, showing complete emulsification, non separable oil water mixture (flask C) because of the formation of biosurfactant.

compared to uninoculated control (flask A) where no change in oil was observed in the inoculated flask, after 5 days of incubation the oil was suspended as small particles in the aqueous phase (flask B). On further incubation, oil and water layers are homogenously mixed (flask C) and did not separate when kept undisturbed (stage II). These studies indicate the formation of surface active compounds (biosurfactants) with the ability to disperse furnace oil in water. Cell free extract from the degraded furnace oil also showed biosurfactance property. These biosurfactants are being analysed for their active compounds.

Thus the indigenous bacteria isolated from oil-soil ecosystem have the ability to produce biosurfactant from furnace oil when applied as mixed culture.

These studies also open up new frontiers of research regarding use of pure culture for biosurfactant production.

REFERENCES

1. J.M. Sharpley, *Elementary Petroleum Microbiology* (Gulf Publishing Company, Houston, Taxas, 1966), pp. 98-149.
2. E. Rosenberg, CRC Critical Rev. Biotecnol., 3(2), 109 (1986).
3. J.E. Zajic and A.Y. Mahomedy, *Petroleum Microbiology* (Macmillan Publishing Company, Macmillan, Inc. N.Y., USA, 1984), pp. 221-298.
4. D.F. Gerson and J.E. Zajic, *Chemistry for Energy* (ACS Symposium Series No. 90. American Chemical Society, Washington, D.C., 1979), pp. 76-79.
5. F. Wagner, V. Behrenslt, H. Bock, A. Kretxhmer, S. Lang and C. Syladtk, *Microbial Enhanced Oil Recovery* (Penwell Tulsa Okla, 1979), pp. 55-109.
6. D.L. Gutnick and E. Rosenberg, Ann. Microbiol., **31**, 379 (1977).
7. R.M. Atlas, Microbiol, Rev., **45**, 180 (1981).
8. A. Reisfeld, E. Rosenberg and D. Gutnick, Appl. Microbiol., **24**, 363 (1972).
9. A.M. Chakrabarty, Trends in Biotechnol., 3(1), 32 (1985).
10. G.D. Hobson and W. Pohl, *Modern Petroleum Technology,* (McGraw-Hill Encyclopedia of Science and Technology, 1973), Vol. 10, 4th ed., p. 75.
11. L.D. Bushnell and H.E. Haas, J. Bact., **41**, 653 (1941).
12. A. Horowitz and R.M. Atlas, Appl. Environ. Microbiol., **33**, 1252 (1977).
13. A. Horowitz, D. Gutnick and E. Rosenberg, Appl. Microbiol., **30**, 10 (1975).
14. V.W. Jamison, R.L. Raymond and J.O. Hudson (Jr.), Dev. Ind. Microbiol., **16**, 305 (1975).
15. R.L. Raymond, J.O. Hudson and V.W. Jamison, Appl. Environ. Microbiol., **31**, 522 (1976).
16. R.E. Buchanan and N.E. Gibbons, *Bergey's Manual of Determinative Bacteriology* (The Williams and Wilkins Company Baltimore, USA, 1974), 8th ed.