JOURNAL OF THE TENNESSEE ACADEMY OF SCIENCE 71(3):73-76 JULY 1996

USE OF A CHEMICALLY DEFINED MEDIUM FOR THE ISOLATION AND IDENTIFICATION OF MOTOR-OIL-DEGRADING MICROORGANISMS

WALTER C. BLACK AND JOHN M. ZAMORA

Department of Biology, Middle Tennessee State University, Murfreesboro, TN 37132

ABSTRACT—This study utilizes a solid medium containing a high concentration of hydrocarbon as the initial screening method for the detection and isolation of hydrocarbon degraders. Soil or water samples were taken from various sites including hydrocarbon-laden environments. These samples were plated onto a 10% 10w-30 motor-oil agar to screen for aliphatic hydrocarbon degraders. When media containing high levels of hydrocarbon were used, 33 identifiable isolates were found to grow. Hydrocarbon-containing media has been used in many previous studies. These previously described media typically contained a low concentration of hydrocarbon (usually 1-2% v/v). However, this concentration of hydrocarbon is not representative of realistic hydrocarbon concentration at or near the source of contamination in the environment. Furthermore, this type of medium would isolate nondegraders as well as degraders and also could facilitate acclimation of some organisms.

Approximately 75% of the >870,000 metric tons of hydrocarbons annually entering freshwater supplies are used automobile crankcase oils and household cooking oils (Loehr and Kukar, 1965; National Academy of Science, 1985). The main sources for these hydrocarbons are oils put into municipal wastewater treatment systems, irresponsibly dumped into sewage drains, poured onto soils, or merely leaked onto roadways (Ali et al., 1979; Amund et al., 1987). Because not all organisms are capable of breaking down aliphatic hydrocarbons, it is advantageous to identify aliphatic hydrocarbon degraders for potential use in reducing the amount of these hydrocarbons reaching freshwater supplies.

The initial isolation technique used by many researchers has involved the inoculation of a liquid medium containing a hydrocarbon as the carbon source coupled with a long incubation period of 5-7 days (Ehlers and Hedrick, 1972; Odu, 1972; Amund et al., 1987; Butt et al., 1988; Okpokwasili and Okorie, 1988). Following incubation, a nutrient-type agar was employed for isolation and identification. Then, the isolated organisms were placed back into a liquid medium with a hydrocarbon carbon source and screened for hydrocarbon-degrading ability. Many microorganisms have the ability to remain in an inactive state in the absence of a suitable food source, especially if water is available (Roszak and Colwell, 1987; Potts, 1994). Using a liquid medium for the initial isolation will result in the isolation of nondegraders on the nutrient-type agar medium. Thus, the use of a solid medium for the initial isolation of hydrocarbon degraders would be a tremendous advantage and could result in reduced time and supplies.

Hydrocarbon-containing agars are already utilized: spirit blue agar contains 3% lipid emulsion (Atlas, 1993). Other methods of incorporating hydrocarbons include adding oil with detergents to agars, spreading a thin layer of oil on the agar, or adsorption of oils to powderized silica gel (Baruah et al., 1967; Walker and Colwell, 1975). Another method uses sonication to emulsify the oil incorporated into the media (Walker and Colwell, 1975, 1976). All of these previous methods employed a low concentration of hydrocarbon (usually 1-2% v/v) and an incubation period of 5-7 days which is less inhibitory and could serve to acclimate organisms.

In the present study, organisms that degrade motor oil were isolated using a solid medium containing a high concentration of hydrocarbon (10%) and a 48-h incubation period. The organisms were subsequently identified.

MATERIALS AND METHODS

Collection—Soil samples were collected from sites of long-term contamination at petroleum terminals in Detroit, Michigan, and Nashville, Tennessee. Water samples were taken from an oxidation lagoon at a petroleum refinery in Catlettsburg, Kentucky; primary and secondary grease tanks at the Central Waste Water Treatment Plant in Nashville, Tennessee; commercial cigarette tobacco extracted with $(NH_4)_2SO_4$ in the microbiology laboratory at Middle Tennessee State University, Murfreesboro, Tennessee; and an aeration tank in the microbiology laboratory at Middle Tennessee State University. One sample was taken from the lubricating grease of a water pump brought into the microbiology laboratory at Middle Tennessee State University for analysis. Organisms known to degrade hydrocarbons were obtained from American Type Culture Collection, Midwest Culture Service, and Carolina Biological Supply.

The soil and water samples were diluted by taking 10 g of soil or 10 ml of water and mixing it with 99 ml of sterile water and then spreading 1 ml of the mixture onto motor-oil agar plates. All inoculated plates were incubated aerobically for 48 h at 37°C.

Medium—The motor-oil agar was prepared by the following steps: 1) 5X minimal salts were prepared by dissolving 9.5 g of KH_2PO_4, 31.5 g of K_2HPO_4, 2.5 g of $(NH_4)_2SO_4$, and 0.25 g of $MgSO_4$ in 500 ml of deionized water; 2) an agar base was prepared by adding 7.5 g of Bacto-Agar to 400 ml of deionized water; 3) the two preparations were autoclaved separately; 4) 100 ml of minimal salts, 50 ml of Valvoline 10w-30 unused motor oil, 5 ml of glycerol, and 0.5 ml of triton X-100 were added to 400 ml of agar and stirred; 5) the mixture was autoclaved, stirred continuously until cooled to 46°C, and then poured. Detection

of nonpigment-producing bacteria was facilitated by addition of 0.003 g/l of phenol red (Walker and Colwell, 1976).

Identification of Organisms—Following the 48-h incubation period, organisms showing growth were isolated by subculturing them to fresh motor-oil agar. Stock cultures of each isolate were maintained on motor-oil agar at 4°C. New plates were subcultured from existing plates monthly. Motor-oil agar slants of the isolates also were maintained at 4°C. All Gram-negative rods were categorized as either oxidase positive or oxidase negative and identified using the API NFT (Analytab Products, Inc. Nonfermentative) and the API 20E (Analytab Products, Inc. Gram-negative rods especially Enterobacteriaceae) test kits, respectively. Prior to API systems inoculation, the Gram-negative rod isolates were subcultured to nutrient agar and incubated 24 h at 37°C. All organisms were identified in accordance with Holt (1989). The identities of the isolates obtained from American Type Culture Collection were not checked. Fungi were identified to genus.

RESULTS

Thirty-three organisms capable of degrading aliphatic hydrocarbons were isolated. Table 1 shows the origin of each isolate and the number of isolates from each location. Most organisms were identified to species based on the results of biochemical tests (Table 2). However, some bacteria could not be identified to species using the biochemical results data bank, i.e., acid-fast bacteria and those physiologically influenced by environmental factors. Many free-living organisms will undergo physiological changes in response to environmental factors (Bartha and Atlas, 1977; Pugh and Reyes, 1991). Responses by these organisms to precise biochemical tests are often outside normal parameters due to adaptation. Therefore, some bacteria were only identified to genus.

Fungi (Table 2) were identified to genus based on morphological characteristics. It was not possible to identify the fungi to species without the consultation of a specialist for each particular genus (W. C. Rosing, pers. comm.).

DISCUSSION

Several of the isolates produced large capsules in response to growing on the hydrocarbon medium used in this study. The enlarged capsule is a result of hydrocarbon incorporation into capsular material (Atlas, 1984; Roszak and Colwell, 1987). When inoculated from motor-oil agar to the API test strips, isolates producing this hydrocarbon-laden capsule yielded unreliable test results. This condition necessitated subculturing the organisms to nutrient agar prior to API testing. It was determined that inoculation to nutrient agar greatly reduced capsule formation and eliminated the possibility of hydrocarbon contamination from capsular material.

Twelve isolates were determined to be pseudomonads which are known to utilize hydrocarbon-metabolized intermediate compounds including long-chain alcohols, aldehydes, and fatty acids. They also are able to use short-chain aliphatic hydrocarbons and cyclic compounds as substrates (Atlas, 1984). Because many of the samples were obtained from areas contaminated with hydrocarbons such as fuels, the number of pseudomonads would be expected to be considerably higher in these soils.

Fourteen of the isolates were of the family Enterobacteriaceae which are characteristically associated with the intestinal tract of mammals, especially man. Farrington and Quinn (1973) reported that the hydrocarbon content of human feces was as high as 33%, mainly in the form of fatty acids. Foods of domesticated mammals (i.e., dogs) are likewise high in fat causing an increased hydrocarbon content in certain animal feces as well. This family of organisms would be expected to possess the enzymes to efficiently catabolize fatty acids.

TABLE 1. Origin of isolates capable of degrading motor oil and number of isolates from each origin.

Isolate	Origin
1-9	Petroleum Terminal, Nashville, Tennessee
10-12	Aeration Tank, Murfreesboro, Tennessee
13	Carolina Biological Supply, Burlington, North Carolina
14	Tobacco Extract, Murfreesboro, Tennessee
15	Water Pump, Murfreesboro, Tennessee
16-20	Petroleum Terminal, Detroit, Michigan
21-23	Oxidation Lagoon, Catlettsburg, Kentucky
24-27	Sewage Plant, Nashville, Tennessee
28-30	Refrigerator Unit, Murfreesboro, Tennessee
31	American Type Culture, Murfreesboro, Tennessee
32-33	American Type Culture Collection, Rockville, Maryland

Most of the isolates (17) came from hydrocarbon-laden soils or a hydrocarbon treatment facility (Table 1). These environments would be very similar to the motor oil that was incorporated into the agar.

Unused 10w-30 motor oil was chosen as the initial screening substrate for several reasons (Guthrie, 1960; King and McKenzie, 1977; Okpokwasili and Okorie, 1988, 1990). Unused motor oil has not been subjected to the thermal stress of an automobile engine which causes cracking of the oil. It contains additives which are chiefly aromatic structures (i.e., biocides which inhibit microbial growth) and are volatilized by the thermal stress of the automobile engine (Table 3). Motor oil (straight chains of carbons) is less easily degraded than cooking oils (long-chain fatty acids). In most car-care manuals, 10w-30 motor oil is recommended.

Known hydrocarbon degraders were obtained from American Type Culture Collection and Midwest Culture Service. These organisms have been reported in previous studies (using a low-hydrocarbon-content isolation medium) as being degraders (Ehlers and Hedrick, 1972; Walker and Colwell, 1976; Amund et al., 1987, Okpokwasili and Okorie, 1988). Two organisms obtained from American Type Culture Collection were able to be isolated from the motor-oil agar used in the present study (Table 2). *Micrococcus luteus* and *Bacillus subtilis* which have been isolated in many previous studies using low-hydrocarbon-content media failed to be isolated on the medium used in this study. *Staphylococcus aureus* and *Proteus mirabilis* which also have been reported in previous studies (to a lesser extent) as being isolated, likewise, failed to be isolated in the present study. Attempts to isolate these organisms were made from both obtained cultures, previously listed agencies, and environmental samples.

The use of a low-hydrocarbon-content liquid medium coupled with a long incubation period for the initial screening process has been shown to culture nondegraders as well as degraders (Ehlers and Hedrick, 1972; Odu, 1972; Amund et al., 1987; Butt et al., 1988; Okpokwasili and Okorie, 1988). Numbers of isolates or bacteria per millimeter also have been shown to be greatly increased when using this type of initial screening process (Walker and Colwell, 1976). It is known that bacteria are able to survive in an environment where there is water but no suitable carbon source (Potts, 1994). Therefore, using low levels of hydrocarbon (1-2% v/v) for lengthy incubation periods (5-7 days) can serve to

TABLE 2. Identity of organisms isolated from motor-oil agar plates.

Isolate	Identification
1	*Pseudomonas fluorescens*
2	*Enterobacter cloacae*
3	*Citrobacter freundii*
4	*P. fluorescens*
5	*E. cloacae*
6	*Serratia liquifaciens*
7	*E. cloacae*
8	*Mucor* sp.
9	*Fusarium* sp.
10	*P. fluorescens*
11	*P. fluorescens*
12	*Pseudomonas* sp.
13	*Serratia marcescens*
14	*Streptococcus faecalis*
15	*Pseudomonas aeruginosa*
16	*Serratia rubidaea*
17	*Pseudomonas* sp.
18	*Pseudomonas putida*
19	*P. fluorescens*
20	*Pseudomonas* sp.
21	*C. freundii*
22	*C. freundii*
23	*P. fluorescens*
24	*Klebsiella pneumoniae*
25	*Geotrichum* sp.
26	*Escherichia coli*
27	*E. coli*
28	*C. freundii*
29	*Mycobacterium* sp.
30	*Enterobacter agglomerans*
31	*Aspergillus niger*
32 (ATCC 9957)	*Acinetobacter* sp. Genospecies 9
33 (ATCC 11172)	*P. putida* Biotype A

TABLE 3. Typical multigrade motor-oil additives and percent volume ranges per quart (0.946 l).

Additive	Volume percent
Antifoaming agents (silicone polymers)	Not applicable
Biocides (benzyl cresols)	0.02
Detergent-dispersant inhibitors (aromatic sulfonate compounds)	3.00-6.50
Oxidation and corrosion inhibitors (sulfur and phosphorus compounds)	0.50-1.50
Pour point depressants (wax naphthalene or phenol condensation products)	0.02-0.20
Viscosity index improvers (polymers or copolymers)	4.50-12.00

acclimate some organisms and allow others to survive through metabolic inactivity.

Using a 48-h incubation period in conjunction with a 10%-hydrocarbon solid medium is less likely to foster acclimation of organisms. This medium with a short incubation period not only served to culture only those organisms readily possessing the ability to utilize hydrocarbons as a substrate but also eliminated the number of steps needed to isolate potential degraders. Thus, the use of this 10%-hydrocarbon solid medium would serve to mimic more realistic environmental conditions.

Previous studies have shown that a high hydrocarbon content (>5%) is inhibitory to some organisms and toxic to others (Walker and Colwell, 1976; Sikkema et al., 1995). The content of hydrocarbons at or near the source of contamination, where the bulk of degradation is desired, is typically >10% and would present a harsh environment to most organisms (Odu, 1972). Likewise, a municipal wastewater treatment system's environment is very competitive making introduction of an organism difficult. Thus, screening for potential hydrocarbon degraders with a solid medium containing a high concentration of a difficult-to-degrade hydrocarbon and a short incubation period would inhance the chances of the survival of the organisms and degradation of the target hydrocarbon contaminant.

ACKNOWLEDGMENTS

We thank W. C. Rosing for identifying the fungal isolates. Thanks also are extended to R. Vigna and T. Kettle of Ashland Oil, Inc., for the materials, cooperation, and assistance they provided. We would like to thank W. Rosing, A. Newsome, and M. de los Reyes for critically reviewing an earlier version of the manuscript.

LITERATURE CITED

ALI, L. H., H. S. ALSALIM, AND R. A. BASHIR. 1979. Water and soil pollution by spent lubricating oils. Environ. Internatl., 2:61-62.

AMUND, O. O., A. A. ADEBOWALE, AND E. O. UGOJI. 1987. Occurrence and characteristics of hydrocarbon-utilizing bacteria in Nigerian soils contaminated with spent motor oil. Indian J. Microbiol., 27:63-67.

ATLAS, R. M. 1984. Petroleum microbiology. MacMillan Publishing Company, New York.

——. 1993. Handbook of microbiological media. CRC Press, Ann Arbor, Michigan.

BARTHA, R., AND R. M. ATLAS. 1977. The microbiology of aquatic oil spills. Advances in applied microbiology. Academic Press, New York.

BARUAH, J. N., Y. ALROY, AND R. I. MATELES. 1967. Incorporation of liquid hydrocarbons into agar media. Applied Microbiol., 15:961.

BUTT, A. I., S. RIAZUDDIN, A. R. SHAKOORI, AND F. JABEEN. 1988. Isolation and identification of petroleum hydrocarbon degrading bacteria from the local environments. Pakistan J. Zool., 20:391-399.

EHLERS, S. E., AND H. G. HEDRICK. 1972. Detection and identification of bacteria utilizing petroleum waste in dilution waters. Developments Industrial Microbiol., 13:283-289.

FARRINGTON, J. W., AND J. G. QUINN. 1973. Petroleum hydrocarbons and fatty acids in wastewater effluents. J. Water Pollution Control Federation, 45:704-711.

GUTHRIE, V. B. (ED.). 1960. Petroleum products handbook. McGraw-Hill Book Company, New York.

HOLT, J. (ED.). 1989. Bergey's manual of systematic bacteriology. Williams and Wilkins Publishers, Baltimore, Maryland.

KING, R. A., AND P. MCKENZIE. 1977. Microbial degradation of marine lubricating oil. Trans. Inst. Marine Eng., 89:33-45.

LOEHR, R. C., AND T. J. KUKAR. 1965. Removal of lipids by conventional waste treatment methods. Internatl. J. Air Water Pollution, 9:479-485.

NATIONAL ACADEMY OF SCIENCE. 1985. Oil in the sea: inputs, fates and effects. National Academy Press, Washington, D. C.

ODU, C. T. I. 1972. Microbiology of soils contaminated with petroleum hydrocarbons. I. Extent of contamination and some soil and microbial properties after contamination. J. Inst. Petroleum, 58:201-208.

OKPOKWASILI, G. C., AND B. B. OKORIE. 1988. Biodeterioration potentials of microorganisms isolated from car engine lubricating oils. Tribiology Internatl., 21:215-221.

——. 1990. Influence of physio-chemical stress on biodegradability of car engine lubricating oil. Internatl. biodeterioration, 27:255-264.

POTTS, M. 1994. Desiccation tolerance of prokaryotes. Microbiol. Rev., 58:755-805.

PUGH, J. R., AND M. L. REYES. 1991. Bacteriological water quality of recreational areas: J. Percy Priest Park, Tennessee. J. Tennessee Acad. Sci., 66:13-18.

ROSZAK, D. B., AND R. R. COLWELL. 1987. Survival strategies of bacteria in the natural environment. Microbiol. Rev., 51:365-379.

SIKKEMA, J., J. A. M. DEBONT, AND B. POOLMAN. 1995. Mechanisms of membrane toxicity of hydrocarbons. Microbiol. Rev., 59:201-222.

WALKER, J. D., AND R. R. COLWELL. 1976. Enumeration of petroleum-degrading microorganisms. Applied Environ. Microbiol., 31:198-207.

——. 1975. Factors affecting enumeration and isolation of Actinomycetes from Chesapeake Bay and Southeastern Atlantic Ocean sediments. Marine Biol., 30:193-201.