MICROBIOLOGICAL STUDIES ON PETROLEUM AND NATURAL GAS

IV. MICROFLORA OF HIGASHIYAMA OIL MINING FIELD

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Microbiology of petroleum and natural gas is included in the category of "geomicrobiology", a new field of applied microbiology. The chief concerns of this field have been microbiological utilization of hydrocarbons, sulfate reducers in oil-layers, microbiological prospecting, etc., and only a few reports have been presented on microflora of petroleum and natural gas fields (1, 2). In the previous papers (3, 4, 5), determination of hydrocarbon-utilizing bacteria and other bacteria, such as *Pseudomonas*, *Achromobacter*, *Alcaligenes*, *Brevibacterium*, *Arthrobacter*, *Micrococcus*, and *Sarcina*, isolated from oil-brines and related materials in petroleum zones in Japan since 1956 was reported. This paper deals with microflora of the world-famous Higashiyama oil mining field where oil is recovered by mining.

MATERIALS AND METHODS

Outline of Higashiyama oil mining field.

The Higashiyama oil mining field is located in the Higashiyama district 8 km east of Nagaoka in Niigata Prefecture. A map of petroleum zones in Japan was previously described by the present authors (3), and a brief paper concerning production of petroleum in Japan was reported by WATERS (6). In the Higashiyama district, it is recorded that oil-outcrops appeared on the surface ground and that an oil well was dug by hand in 1873. During the Second War, the Japanese Government attempted to make an oil-gallery in the Higashiyama district. In 1944, galleries had been built through 300 m of the slope and 300 m of the level. The galleries consisted of two main levels connected to each other through passes. The galleries were tunneled towards oil-layers, and extended about 12,000 m in length. A map of the Higashiyama oil gallery is illustrated in Fig. 1. The galleries were dug under the cap-rock, and oil-seeped sand could be seen by the naked eye under the surface. Crude oil seeped from the sand of the gallery wall, or from leaks which were bored towards the inner parts of the oil-layers. The seeped crude oil was conducted to open trenches (about 30 cm in width and 30 cm in depth), collected in a pool under the

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Fig. 1. Map of Higashiyama oil mining field

ground, then pumped up to an oil-tank outside. A number of wells were also dug to the oil-layers from the surface ground, and a pipe could be seen in the oil-gallery. When the gallery was first opened, about 20 kl of crude oil was produced a day, but production had decreased to about 10 kl a day when inspected. The inside of the oil-gallery was maintained at 14° to 18° and about 90% humidity throughout the year. Fresh air was provided by artificial ventilation. After our inspections, this Higashiyama oil-gallery was closed for economical reasons in 1962.

Sampling

Sampling was carried out in the oil-gallery, and oil-brines, oil-sands, cutting pieces of boring, etc., were aseptically collected. For sampling the inner oil-sands, the surface oil-sand was scraped by sterilized spatula, and a coal-pick hammer employed. Surface soil was obtained for controls to the samples collected in the oil-gallery. The sampling was done three times: in November, 1957; April, 1958; and December, 1959. About one hundred samples were obtained.

Isolation of microorganisms.

Microorganisms were mainly isolated by the agar plate method, and

incubated at 30° for 7 to 10 days. Nutrient, 3% NaCl nutrient, glutamate, synthetic, *p*-hydroxy-benzoate, and kerosene agars were used for isolation of aerobic bacteria. For anaerobic bacteria, 1% glucose nutrient, thioglycolate nutrient, and STARKEY'S agars (7) were used and incubated in vacuum. Soil-extract, CZAPECK'S, and yeast extract-malt extract agars were used for isolation of molds and actinomycetes. The composition of these media was reported in other papers (3, 4). After counting, typical colonies were transferred to slants, and differentiated by the method previously reported (8).

RESULTS

Microorganisms of Higashiyama oil-gallery.

Typical experimental results are shown in Table 1. Considerable numbers of microorganisms were found in the samples from the oil-gallery. The numbers of microorganisms of oil-sands varied from sample to sample. Generally, about 10^4 to 10^6 aerobic bacteria were isolated from one gram of oil-seeped sands and water sands, but few microorganisms were found in oil-exhausted sands and sandy clay, which was dry and pulverous and did not have an oily appearance. The interior distribution of microorganisms was deduced from the microbial numbers in cutting-pieces and brines obtained at boring leaks. For example, the finding of few bacteria showed that the end of the boring-drill had reached an oil-layer where microorganisms had not invaded. Considerable numbers of aerobic bacteria were found in oil-brines obtained directly at the surface of gallery and from the trenches. The aerobic bacteria mainly belonged to *Pseudomonas*, and the fluorescent group (9) was dominant. From a sample which was considered to be rain water leaked from the surface ground, about 10^5 aerobic bacteria per one ml were isolated on nutrient agar, but only a small number of bacteria grew on 3% NaCl nutrient agar. Adaptation to the environment of the oil-gallery was apparent from the facts that nearly the same numbers of colonies appeared on both nutrient and 3% NaCl nutrient agars from the oil-brine samples and that salt concentration of oil-brine was about 3%. Hydrocarbon-utilizing bacteria were also abundantly isolated from several samples. Anaerobic bacteria, including sulfate reducers, were found in oil-brines and oil-sands. Distribution of molds was relatively limited. In oil-rich sands and mud of the trenches, black molds, probably belonging to Dematiaceae, were found, and *Aspergillus versicolor* and *Asp. sydowi* were abundantly isolated from oil-poor sands. However, such molds were not necessarily isolated from all samples. No actinomycetes were recognized in the samples of the oil-gallery, although they were abundantly isolated from the soil of the surface ground. It is noteworthy that the microflora of the Higashiyama oil-gallery was composed of taxonomically restricted microorganisms, whereas many kinds of microorganisms, such as *Bacillus*, *Penicillium*, *Streptomyces*, Fungi Imperfecti, etc., were abundantly isolated

Table. 1. Numbers of microorganisms of samples

No.	Sample	Aerobe					
		Nutrient	3% NaCl nutrient	Glutamate	Synthetic	p-OH-benzoate	Kerosene
8	oil-sand*	$<10^2$	$<10^2$	0	0	0	0
18	oil-sand*	0	0	0		0	0
34	oil-sand*	8×10^5	10^6	3×10^5		3×10^5	$<10^3$
54	oil-sand*	0	0	0		0	0
57	oil-sand*	7×10^5	4.5×10^6	3×10^6		5×10^6	1.5×10^6
59	oil-sand*	$>5 \times 10^6$	2.5×10^6	10^6		6.6×10^5	3×10^5
10	oil-sand**	8×10^4	10^5	$>2 \times 10^5$	$>6 \times 10^5$	$>4 \times 10^5$	2×10^5
39	oil-sand**	$>1.5 \times 10^6$	$>4 \times 10^6$	$>3 \times 10^6$		3×10^5	1.5×10^6
1	oil-sand**	0	0	0		0	0
4	oil-sand**	1.5×10^4	4×10^3	5×10^4	3×10^4	1.5×10^4	5×10^3
17	oil-sand**	0	<10	<10		0	0
58	oil-sand**		4×10^5	1.5×10^5	1.7×10^5	10^5	4×10^3
36	water-sand	$>10^7$	1.5×10^6	$>10^7$		1.5×10^6	4.5×10^6
51	water-sand	$>10^5$	5×10^6	10^5		6×10^4	2×10^5
38	water-sand	$>2 \times 10^6$	$>2 \times 10^6$	3×10^5		4×10^4	7×10^4
29	sandy clay	$<10^2$	$<10^2$	$<10^2$		$<10^2$	$<10^2$
27	cutting pieces of boring	3×10^5	2×10^4	5×10^4	10^5	6×10^4	$<10^3$
7	cutting pieces of boring	8×10^6	7×10^6	7×10^6		3×10^6	5×10^6
30	water of trench (12.5m)	4×10^5	3×10^4	2×10^4		$<10^2$	1.5×10^4
47	water of trench	10^5	8×10^5	10^5		2×10^4	3×10^4
19	water of trench	1.5×10^5	3.5×10^4	6×10^4		$<10^3$	3×10^4
11	water of trench	10^5	$<10^2$	2×10^4		0	$<10^2$
9	water of trench	10^6	2×10^6	7×10^5	2×10^6	5×10^4	7×10^4
60	oil-brine	6×10^5	3×10^4	5×10^5	3.5×10^5	3×10^5	$<10^3$
33	oil-brine	6×10^4	$<10^3$	7×10^4	6×10^4	2.6×10^4	$<10^2$
26	oil-brine	$>1.5 \times 10^6$	3.5×10^5	10^6	10^6	6.5×10^5	2.5×10^5
37	oil-brine	$<10^2$	$<10^2$	$<10^2$	0	0	0
52	oil-brine	$<10^2$	$<10^2$	$<10^2$	$<10^2$	0	0
62	water of pool	$>2 \times 10^5$	4×10^4	4×10^5		1.5×10^5	10^5
61	water of pool	$>10^4$	3×10^4	5×10^4	$>2 \times 10^4$	3×10^4	5×10^3
64	Soil of outside	2×10^4	5×10^4	7×10^5	1.3×10^5	5×10^4	$<10^2$

Numbers indicate microorganisms per 1 g or 1 ml of sample. Numbers of microorganisms include bacteria and molds.

* inner oil-sand

** surface oil-sand

obtained in Higashiyama oil-gallery (1957).

Soil-extract	Czapek	Anaerobe			Remarks
		Glucose nutrient	Thioglycolate nutrient	Starkey	
0	$<10^2$	$<10^2$	$<10^2$	0	<i>Pseudomonas</i> dominant, molds limited.
0	0	0	0	0	
$<10^3$	$<10^3$	$<10^2$	2×10^3	<50	
0	0	$<1.5 \times 10^2$	0	0	Bacteria dominant, molds limited.
10^3	1.5×10^6	7×10^3	1.3×10^3		
1.5×10^5	$<10^3$	1.5×10^2	0		<i>Pseudomonas</i> dominant, mold limited.
5×10^5	3×10^5	$<10^2$	<10	0	<i>Aspergillus</i> dominant, bacteria limited.
$>10^6$	5×10^4	$>10^6$	1.5×10^3	1.7×10^3	<i>Bacillus</i> , black molds.
0	0	0	0	0	<i>Aspergillus</i> , black molds dominant.
4×10^4	2.5×10^3	0		<10	
0	0	<10	0	$<10^2$	Black molds dominant.
1.5×10^5		<10		2×10^4	
4×10^5	1.2×10^5	$>10^5$	$>5 \times 10^4$	6×10^2	Black molds dominant.
10^5	$<10^2$	3×10^3	3.4×10^2	0	<i>Pseudomonas</i> dominant.
3×10^5	$<10^2$	10^3	$<10^2$	5×10^2	
$<10^2$	0	$<10^2$	$<10^2$	0	Chromogenic bacteria and molds.
3×10^4	4×10^4	$>10^3$	3×10^2	0	
6×10^6	6×10^6	8×10^3	6×10^2		Chromogenic bacteria.
$<10^2$	$<10^2$	$>3 \times 10^3$	10^6	4×10^2	Sulfate reducer
3×10^5	$<10^2$	6×10^3	8×10^3	9×10^3	<i>Pseudomonas</i> dominant.
3×10^4	$<10^2$	10^4	4×10^4	1.8×10^4	<i>Pseudomonas</i> dominant.
0	0	10^3	10^4	$>10^3$	<i>Pseudomonas</i> dominant.
5×10^5	2×10^4	$>10^3$	$>2 \times 10^3$	10^4	<i>Pseudomonas</i> dominant.
3×10^4	3×10^4	2×10^2	10^3	$<10^2$	<i>Pseudomonas</i> dominant.
1.6×10^4	1.5×10^4	$>5 \times 10^3$	5×10^2	0	Considered as rain water.
					Fluorescent <i>Pseudo-</i>
2.5×10^4	0	0		$<10^2$	<i>monas</i> .
					Obtained at leak of boring. <i>Pseudomonas</i> dominant.
0	<10	1.5×10^2	1.2×10^3	0	Obtained at leak of boring.
0	0	10^2	3×10^2	0	Obtained at leak of boring.
$>2.5 \times 10^5$	$<10^2$	10^4	1.5×10^4	2×10^3	<i>Pseudomonas</i> dominant.
$>10^2$	5×10^3	$>10^4$		6×10^3	<i>Pseudomonas</i> dominant.
					Mixed several kinds of microorganisms.
$<10^2$	3×10^4	10^4		<10	<i>Bacillus</i> , <i>Streptomyces</i> , <i>Aspergillus</i> , <i>Penicillium</i> , Fungi
					Imperfecti dominant.

Table 2. Relation between microorganisms

No.	Sample	Depth	Aerobe				
			Nutrient	3% NaCl nutrient	Glutamate	<i>p</i> -OH-benzoate	Kerosene
Location A.							
9	oil-sand	surface	7×10 ⁶	3×10 ⁶	4×10 ⁶		
10	oil-sand	20 cm	>3×10 ⁵	>5×10 ⁵	>2×10 ⁵	>10 ⁵	>2×10 ⁵
11	oil-sand	50 cm	>5×10 ⁵	>10 ⁶	>5×10 ⁵		
Location B.							
8	oil-sand	surface	4×10 ⁶	3×10 ⁶	5×10 ⁶	2×10 ⁵	
12	oil-sand	10 cm	<4×10 ³	6×10 ³	2×10 ³		
13	oil-sand	20 cm					
14	oil-sand	40 cm	6×10 ²	5×10 ²	4×10 ²		
New gallery							
3	oil-sand	surface	3×10 ⁵	3×10 ⁵	10 ⁵	10 ⁴	2×10 ⁵
1	oil-sand	surface	3×10 ⁵	10 ⁵	3×10 ⁴	6×10 ⁴	10 ⁵
4	oil-sand	surface	3×10 ⁴	2×10 ⁴	2×10 ⁴	2×10 ⁴	2×10 ⁴
2	oil-sand	5 cm	<10	<10	0	0	<10
5	oil-sand	35 cm	<10	<10	0	0	0

Numbers indicate microorganisms per 1 g of sample.

from the soil of the surface ground.

Microflora and depth from gallery wall.

As mentioned above, distribution of molds in the Higashiyama oil-gallery was characteristic. A relation between microflora and the depth from the gallery wall was determined at locations A and B, because black molds were mainly isolated at A, and aspergilli at location B. Results are shown in Table 2. The oil-rich sands of location A appeared wet and black with oil-seepage. A remarkable number of pseudomonads and black molds were found in oil-sands from the surface to a depth of 50 cm. Almost equal numbers of colonies appeared on each medium, and numbers were not variable with depth. This finding exhibits that similar kinds of microorganisms were distributed from the surface to the interior. However, the oil-poor sands of location B appeared dry and brownish and a moldy appearance could be seen by the naked eye at the surface of the gallery. A large number of *Asp. versicolor* and *Asp. sydowi* were found only on the surface, and a small number of black molds appeared at a depth of

and depth from gallery wall (1958).

Soil-extract	Czapek	Anaerobe		Remarks
		Glucose nutrient	Thiolglycolate nutrient	
3×10^5	7×10^5	3×10^2	3×10^2	<i>Pseudomonas</i> dominant, black molds abundant on Czapek agar.
$>4 \times 10^5$	$>2 \times 10^5$	0	0	<i>Pseudomonas</i> dominant.
$>3 \times 10^5$		0	0	<i>Pseudomonas</i> dominant, black molds abundant on <i>p</i> -OH-benzoate, glutamate, kerosene agars.
5×10^5	4×10^6	$<10^2$	4×10^2	<i>Aspergillus versicolor</i> dominant, bacteria extremely limited.
		$<10^2$	<10	A few microorganisms.
				Few microorganisms, not countable.
3×10^2	2×10^2	<10	<10	
10^5	10^5	$<10^2$	0	Bacteria dominant.
5×10^4	2×10^4	2×10^3	5×10^2	<i>Pseudomonas</i> dominant.
10^4	10^4	$<10^2$	<10	Bacteria dominant.
0	0	0	0	Not countable.
0	0	<10	0	Not countable.

10 cm. A few aerobic bacteria were found at the surface, but the number was greatly decreased at a depth of 10 cm. These findings demonstrate that microflora are controlled by the presence of petroleum. The samples tested were obtained in April, 1958.

Gradual succession of microflora.

During the course of two years' inspection of the Higashiyama oil-gallery, a very slow succession in the microflora was recognized. As the oil-sands of locations A and B exhibited unique microflora, detail observations were made at these places. In comparison with the oil-sands of location A sampled in April, 1958, which demonstrated 10^6 aerobic bacteria per one gram at a depth of 20 cm, the numbers obtained in December, 1959, had decreased to 10^3 per one gram at a depth of 10 cm. As for the molds, the black molds were observed as before, but a few aspergilli had appeared. The color of the oil-sands had changed from black to brownish, and become slightly dry. From the oil-sands of location B which demonstrated the characteristic aspergilli in November, 1957, the number of microorganisms had decreased to 10^3 per one gram at the surface. Moderate numbers of

Asp. sydowi were found, but few aerobic bacteria were isolated from oil-sands obtained in December, 1959. The kinds and numbers of microorganisms in the surface soil had remained almost unchanged during these two years. It seems that the microflora of the oil-gallery had gradually changed to resemble that of the oil-poor sands.

DISCUSSION

At the beginning of this study, the authors thought that microflora of the Higashiyama oil-gallery would be complicated because air was artificially ventilated and work was performed in the gallery by humans who might introduce different kinds of microorganisms. However, experimental results were contrary to the expectations. The microflora of the Higashiyama oil-gallery were relatively simple and stable. As shown in the results, considerable numbers of pseudomonads and black molds were found in the oil-rich sands, even in the interior section. Aspergilli were recognized only on the surface of oil-poor sands. No actinomycetes were found in the oil-gallery. It is of interest that the microflora of the Higashiyama oil-gallery consisted of taxonomically restricted microorganisms. It is obscure whether these microorganisms existed before the opening of the gallery or whether they invaded after its opening. Probably the constants of temperature, humidity and the presence of petroleum control the microflora of the Higashiyama oil-gallery; the presence of petroleum may be the most important factor. However, it seems likely that the microflora of the Higashiyama oil-gallery has been very slowly changing to the oil-poor sand type. As production of petroleum decreases, the microflora eventually will change to those found now in the surface soil. Such a transformation may be of interest from the point of view of petroleum microbiology and microbial ecology.

SUMMARY

Microbiological studies of the Higashiyama oil mining field where oil was recovered by mining were carried out. Microflora of this oil-gallery consisted of taxonomically limited kinds of microorganisms such as *Pseudomonas*, *Aspergillus*, and unidentified black molds which probably belonged to Dematiaceae. In oil-rich sands, considerable numbers of pseudomonads and black molds were found, but aspergilli were isolated only from oil-poor sands. A relationship between depth from the gallery wall and microorganisms and the gradual succession in the microflora was also presented. The presence of petroleum was deduced as the most important factor controlling the microflora.

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