Determination of Bacteria Isolated from the Oil-soaked Sample Materials in the Petroleum Refinery

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In the previous papers[1,2], it was reported that 1405 strains of microorganisms were isolated from 33 oil-soaked sample materials collected in the petroleum refinery and 6 other sample materials, employing hydrocarbons as a sole source of carbon; and the cell production of the kerosene-utilizing yeasts and the chemical transformation of p-cymene by bacteria-isolates were examined.

The present paper describes the determinative studies of bacteria isolated from oil-soaked sample materials and others, employing light naphtha, kerosene, liquid paraffine, or middle oil as a sole source of carbon.

Isolation of bacteria was carried out by the same methods that had been already reported by one of us[1,2]. Sample materials are shown in Table 1. Hydrocarbons employed

Table 1. Sample Materials from Chiba Refinery, Maruzen Oil Co., Ltd.

Sources and properties	Material no.
drain water in a ditch into the waste-treating, plant	6, 7, 8, 9
waste water in the oil separator of the waste-treating plant, in operation	12, 13, 14, 15
waste water in the oil separator of the waste-treating plant, off operation	17, 18, 20, 26
bottom water in the storage tank of crude oil	21, 22, 23
bottom water in the storage tank of middle oil (cracking oil)	33
cooling water in the petrochemical plant	34
soil about the topping unit	1, 3, 4, 5
mud in a ditch in the waste-treating plant	10, 28
soil about the waste-treating plant	11, 16, 25, 41
sediments in the waste-treating plant	19
sand about the storage tank of crude oil	24
waste catalyzing sand in the petrochemical plant	31, 32
crude oil in a drain ditch	2, 27
crude oil (Gach-Saran)	37
crude oil (Khafji)	38
crude oil (Kuwait)	39
crude oil (Blend A)	40
.....	
soil in the perfume plants field	
(Nigoro, Hokkaido)	35
(Engaru, Hokkaido)	36

were light naphtha (d=0.68, 34°-155°C, paraffines 89.2 v %), kerosene (Wako Pure Chemical Industries, Ltd.), liquid paraffine (Wako Pure Chemical Industries, Ltd.), and middle oil (d=0.84, 36°-330°C, benzene, toluene, and xylene 40 w %). 1281 strains of 1405 isolates were bacteria and they were grouped into 713 cultural groups by each substrate with their colony appearances on the isolating culture media. The represetatives of these groups were examined for assimilation of hydrocarbons as a sole source of carbon. 239 representatives of them could utilize each hydrocarbon employed for isolation. These hydrocarbon-utilizing strains were further arranged in 34 cultural groups with their colony appearances on the nutrient agar medium and the 1 % glucose nutrient agar medium, and 34 representatives of these groups served for determinative studies. (Table 2)

Table 2. Isolates by each substrate.

at pH 4.5	yeasts	bacteria	cultural groups of bacteria	hydrocarbon assimilating groups of bacteria
light naphtha	—	53	28	1
kerosene	63	34	31	—
liquid paraffine	23	94	28	7
middle oil	—	—	—	—
total	86	181	87	8
at pH 7.2	yeasts	bacteria	cultural groups of bacteria	hydrocarbon assimilating groups of bacteria
light naphtha	—	335	244	58
kerosene	22	347	216	62
liquid paraffine	16	314	126	95
middle oil	—	104	40	16
total	38	1100	626	231

Determination was carried out mainly according to the Manual of Microbiological Methods[3] and the former report[4]. As shown in Table 3, prevailing microorganisms are as follows: *Pseudomonas aeruginosa* and *Escherichia intermedia* at pH 7.2, and yeasts at pH 4.5, when kerosene was employed as a substrate for isolation; *Corynebacterium bovis*, *Cor. aurantiacum* and *Brevibacterium maris* at 7.2, and *Cor. bovis* and yeasts at pH 4.5, when liquid paraffine employed. There was no tendency to predominance of a certain species, when light naphtha employed, and only gram-negative bacteria were isolated from a small number of sample materials with middle oil. Relation between bacteria-isolates and their origins was not so close as that between hydrocarbon-utilizing yeasts and their origins[1]. Identified species are as follows:

1. *Achromobacter cycloclastes* (Gray and Thornton, 1928) Bergey et al., 1930.
   Strains: Pk-61, Pk-78.
2. *Achr. liquefaciens* (Eisenberg, 1891) Bergey et al., 1923.
   Strain: Pl-11.
3. *Arthrobacter pascens* Lochhead and Burton, 1953.

Table 3. Isolates and their origins

	6	7	8	9	12	13	14	15	17	18	20	26	21	22	23	33	34	1	3	4	5	10	28	11	16	25	41	19	24	31	32	2	27	37	38	39	40	35	36
(light naphtha, pH 4.5)																																							
Achr. cycloclastes	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
(light naphtha, pH 7.2)																																							
Achr. cycloclastes	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	+	−	−	−	−	−	−	−
Achr. liquefaciens	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
Arthr. pascens	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−
Brev. incertum	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Cor. aurantiacum	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	+	−	−	−	−	−	−	+	−	−	+	+	−	−	−	−	+	−
Cor. bovis	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	+	−	−	+	+	−	−	−	−	−	+	+	−	−	−	−	+	−
Mic. luteus	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−
Mic. roseus	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Myc. petroleosocultor	−	+	−	−	−	−	−	−	−	−	+	−	+	+	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−
Noc. polychromogenes	+	−	−	+	+	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Ps. aeruginosa	−	−	−	+	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
(kerosene, pH 7.2)																																							
Achr. cycloclastes	−	−	−	+	−	−	−	+	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
Brev. maris Pk-124	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Brev. maris Pk-60	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Cor. bovis	−	−	−	−	−	−	−	+	−	−	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−
E. intermedia	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Mic roseus	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
Ps. aeruginosa	−	−	−	+	+	+	−	−	+	−	−	−	−	−	−	−	−	+	+	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Sar. flava	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
(liquid paraffinne, pH 4.5)																																							
Cor. bovis	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−	−	+	+	+	−	+	−	+	+	+	−	−	+	−	−	−	−	−	−	−	−	+	−
(liquid paraffine, pH 7.2)																																							
Brev. maris	+	−	−	−	+	+	+	+	+	−	−	+	+	−	−	−	+	+	+	+	+	+	+	−	+	+	+	+	−	+	−	+	+	−	−	−	−	−	−
Cor. aurantiacum	−	−	−	−	−	−	+	−	+	−	−	−	+	−	−	−	−	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Cor. bovis	+	−	+	−	+	−	+	−	+	−	−	+	+	+	−	−	+	+	+	+	+	+	+	−	−	+	−	−	+	+	−	+	+	−	−	−	−	+	+
Cor. pseudotuberculosis	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
Mic. roseus	+	−	−	−	−	−	+	−	−	−	+	+	+	−	−	−	−	+	−	−	−	−	−	−	−	+	−	−	−	−	+	−	−	−	−	−	−	−	−
Noc. polychromogenes	−	−	−	−	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	+	−	−	−	−	−	−	−	−
Sar. flava	−	−	+	−	−	−	−	−	−	−	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
(middle oil, pH 7. 2)																																							
E. intermedia	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Ps. desmolytica	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
Ps. oleovorans	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Ps. ovalis	−	−	−	−	−	−	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

Strain: Pl-191.

This strain is similar to this species, except gelatin liquefaction and hydrogen sulfide production.

4. *Brevibacterium incertum* (Steinhaus, 1941) Breed, 1953.

Strain: Pl-219.

This strain produces acid from lactose, but it seemed to be identical with *Brev. incertum* in respect to other properties than lactose utilization.

5. *Brev. maris* (Harrison, 1929) Breed, 1953.

Strais: Pp-187, pk-124, Pk-60.

These strains are different from *Brev. maris* in encapsulation, but physiologically similar to it. There are differences in morphology among Pp-187, Pk-124, and Pk-60. Pp-187: rods, 0.3-0.4 × 0.6-1.2 μ (heat-fixed), snapping division, elongated cells, 2.0-4.5 μ long, in cultures on the 2% LiCl nutrient agar slant. Pk-124: cocci and short rods, 0.3-0.5 × 0.5-0.7 μ (heat-fixed), rods, 0.7 × 1.6-2.1 μ, in cultures on the 4% LiCl nutrient agar slant, pale orange-colored. Pk-60: cocci, 0.4-0.5 μ, ovoid cells in cultures on the 2% LiCl nutrient agar slant, zigzag chains of ovoid cells and cocci equivalent to a picket formation caused by snapping division, pale brownish orange-colored.

6. *Corynebacterium aurantiacum* Iizuka et Tanabe, 1967.

Strains: Pl-218, Pp-19, Pl-4. (Fig. 1)

7. *Cor. bovis* Bergey et al., 1923.

Strains: Pk-50, Pl-97. (Fig. 2)

These strains should be identified with *Cor. bovis*, but Pk-50 is different from this species in a slight acid production in glucose broth within 2 weeks.

8. *Cor. pseudotuberculosis* (Buchanan, 1911) Eberson, 1918.

Strain: Pp-52. (Fig. 3)

This strain is close to *Cor. aurantiacum* except sugar utilization and source. This strain produces acid from glucose, lactose, sucrose, mannitol, and salicin, oxidatively. No other essential differences than those in source and pathogenicity were found between this strain and *Cor. pseudotuberculosis*. Therefore, this strain was identified with this species.

9. *Escherichia intermedia* (Werkman and Gillen, 1932) Vaughn and Levine, 1942.

Strains: Pk-20, Pk-123, Pm-11.

Pm-11 utilizes acetate, but not succinate.

10. *Micrococcus roseus* Flügge, 1886.

Strains: Pp-30, Pk-152, Pl-3.

11. *Mic. luteus* (Schroeter, 1872) Cohn, 1872.

Strain: Pl-61.

This strain produces no acid from other sugars than glucose.

12. *Mycobacterium petroleosocultor* nov. sp.

Strain: Pl-15. (Fig. 4)

This strain seemed to be closer to *Corynebacterium* sp., especially to *Cor. pseudotuber-*

culosis Pp-52 in respect of morphology, than to every species of the genus *Mycobacterium* listed in Bergey's Manual, but it was weakly acid-fast. Therefore, this strain should be regarded as a new species of the genus *Mycobacterium*, though its pathogenicity was left unascertained.

Type strain: *Mycobacterium petroleosocultor* Pl-15.

pe. tro. le. o. so. cul'tor L. n. m. inhabitant in petroleum-soaked material, *petra* L. rock, stone, *oleum* L. oil, *petroleum* L. petroleum, *oleosus* L. oil-soaked, *oleoso* ablative of *oleosus*, *cultor* L. inhabitant.

Rods, 0.3-0.5 × 0.5-2.0 μ, frequently swollen at one or both ends, staining irregularly, showing club and wedge forms, sometimes tapering at end, or branched, snapping division not certified. Gram positive. Weakly acid fast. Non-motile.

Nutrient broth: Almost moderate growth with pellicle. Nutrient agar plate: pale orange-colored, dry.

Inorganic nitrogen utilized as a sole source of nitrogen. No growth on the nitrogen-free medium. Urease positive. Acid, but no gas from glucose, sucrose, maltose, mannitol, xylose, and salicin, oxidatively. No acid from lactose. Citrate utilized as a sole source of carbon. Aerobic. Nitrates not reduced. Indole not produced. Gelatin not liquefied. Hydrogen sulfide not produced. Acetylmethylcarbinol not produced. Starch not hydrolysed. Non-agarolytic. Milk: Alkaline and litmus reduced. Good growth at 25° to 30°C.

Source: Waste water and mud in a ditch in the Chiba Refinery, Maruzen Oil, Co., bottom water in the storage tank in this refinery, and soil in the perfume plants field (Nigoro, Hokkaido). 1964.

13. *Nocardia polychromogenes* (Vallée, 1903) Waksman and Henrici, 1948.

Strain: Pp-8. (Fg. 5)

According to Bergey's Manual[5], this strain, resembling *Cor. roseum*[4] physiologically, but producing a distinct mycelium in young cultures, seemed to be identical with this species. Description of this strain, except that decribed in Bergey's Manual[5], is as follows:

Inorganic nitrogen utilized as a sole source of nitrogen. No growth on the nitrogen-free medium. Urease negative. Ammonification. No acid from glucose, sucrose, maltose, lactose, mannitol, xylose, and salicin. Citrate utilized as a sole source of carbon. Aerobic. Nitrates reduced further via nitrites. Indole not produced. Hydrogen sulfide not produced. Acetylmethylcarbinol not produced. Starch not hydrolysed. Non-agarolytic.

14. *Pseudomonas aeruginosa* (Schroeter, 1872) Migula, 1900.

Strains: Pk-1, Pk-13, Pk-121, Pk-24, Pk-41, Pk-185.

These strains were identified with *Ps. aeruginosa*, because of the active pyocyanine production, oxidative utilization of glucose, and polar or subpolar flagellation. Pk-1, Pk-13, and Pk-121 produced hydrogen sulfide and yellow insoluble pigments on the yeast extract malt agar.

15. *Ps. desmolytica* Gray and Thornton, 1928.

Strain: Pm-23.

This strain did not utilize acetate, but succinate.

16. *Ps. oleovorans* Lee and Chandler, 1941.

Strain: Pm-14.

This strain did not utilize acetate but succinate.

17. *Ps. ovalis* Chester, 1901.

Strains: Pm-1, Pm-20.

Pm-1 and Pm-20 did not utilize acetate, but succinate, producing yellow diffusible pigment.

18. *Sarcina flava* de Bary, 1887.

Strain: Pk-81.

This strain was identified with this species. This strain differs from urea-decomposing sarcina, *Sar. ureae* in nitrate reduction, gelatin liquefaction, endospore formation, and cultural properties, though it decomposed urea.

Physiological properties of this strain: Inorganic nitrogen utilized as a sole source of nitrogen. No growth on the nitrogen-free medium. Urease positive. Ammonification. No acid from glucose, sucrose, maltose, lactose, mannitol, xylose, and salicin. Citrate not utilized as a sole source of carbon. Aerobic. Nitrates not reduced. Indole not produced. Hydrogen sulfide not produced. Acetylmethylcarbinol not produced. Starch not hydrolysed. Non-agarolytic. Milk: Alkaline, and litmus reduced.

SUMMARY

1405 strains of microorganisms were isolated from 33 oil-soaked sample materials collected in the petroleum refinery and 6 other sample materials, employing hydrocarbons as a sole source of carbon. They were distinguished into 34 cultural groups of hydrocarbon-utilizing bacteria by selection by means of assimilation test and by grouping with their colony appearances. 34 representatives of these groups served for determinative studies. Identified species are as follows: *Achromobacter cycloclastes*, *Achr. liquefaciens*, *Arthrobacter pascens*, *Brevibacterium incertum*, *Brev. maris*, *Corynebacterium aurantiacum*, *Cor. bovis*, *Cor. pseudotuberculosis*, *Escherichia intermedia*, *Micrococcus roseus*, *Mic. luteus*, *Mycobacterium petroleosocultor*, *Nocardia polychromogenes*, *Pseudomonas aeruginosa*, *Ps. desmolytica*, *Ps. oleovorans*, *Ps. ovalis*, and *Sarcina flava*.

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Fig. 1 *Corynebacterium aurantiacum* Pl-218.

a. Normal cells and snapping division on nutrient agar.
b. Branched cells on 2% LiCl nutrient agar.

Fig. 2 *Corynebacterium bovis* Pk-50.

a. Normal cells on nutrient agar.
b. Elongated and branched cells on 2% LiCl nutrient agar.

Fig. 3 *Corynebacterium pseudotuberculosis* Pp-52.

a. Normal cells on nutrient agar.
b. Cells with short branches on 2% LiCl nutrient agar.

Fig. 4 *Mycobacterium petroleosocultor* Pl-15.

Cells on 2% LiCl nutrient agar, similar to cells on nutrient agar.

Fig. 5 *Nocardia polychromogenes* Pp-8.

Mycelium in young culture on nutrient agar.

Fig. 1 a

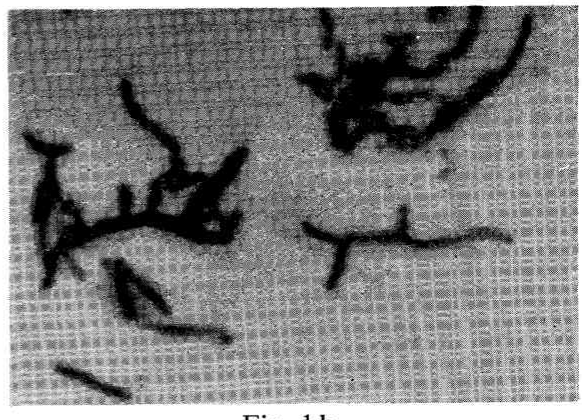

Fig. 1 b

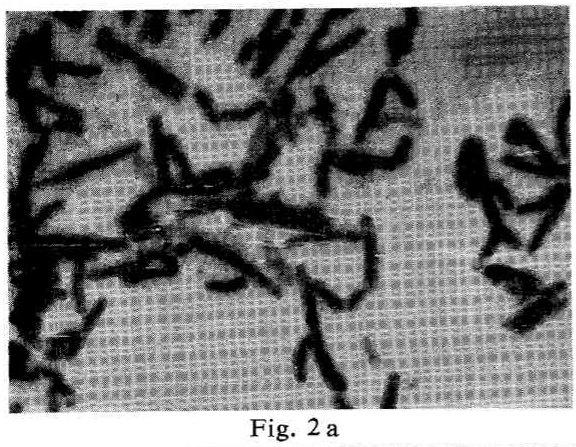

Fig. 2 a

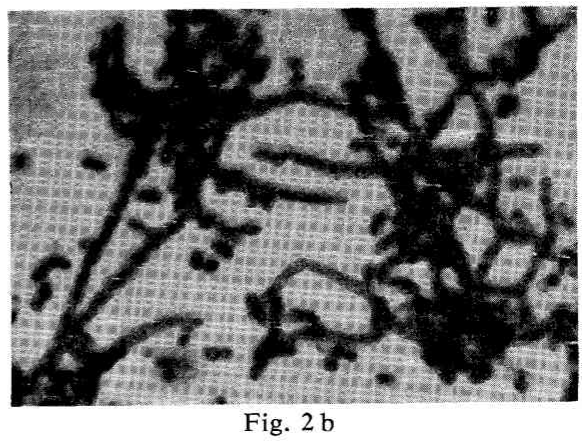

Fig. 2 b

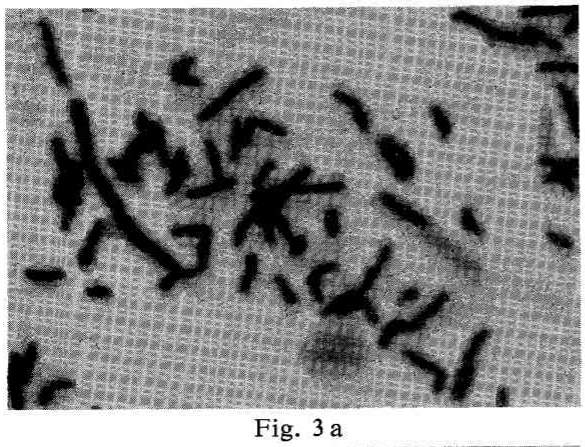

Fig. 3 a

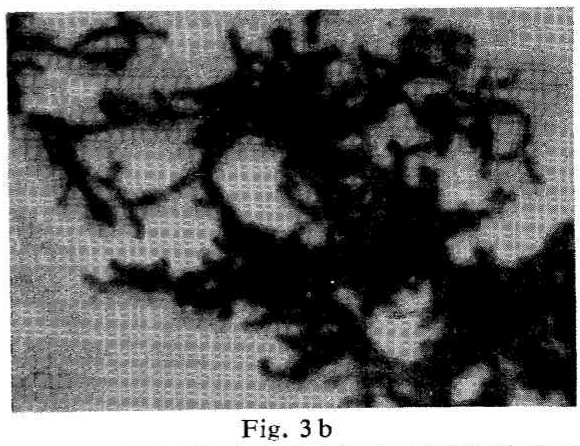

Fig. 3 b

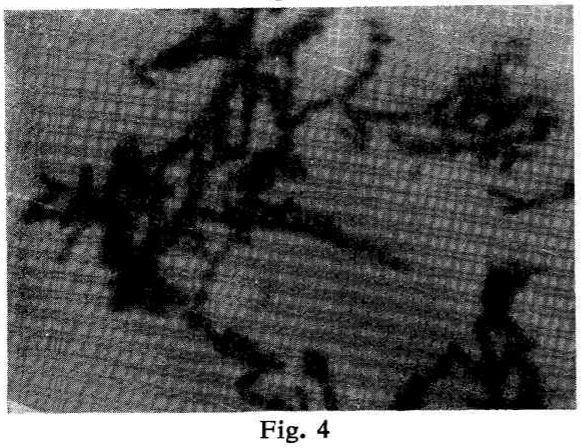

Fig. 4

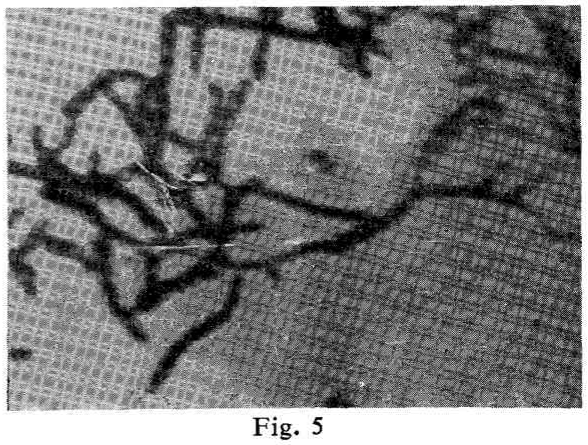

Fig. 5