Microbiology. — "*Oxidation of petroleum, paraffin, paraffinoil and benzine by microbes.*" By Dr. N. L. SÖHNGEN. (Communicated by Prof. M. W. BEIJERINCK).

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In the following it is shown that the hydrocarbons [1]) of the paraffin series, which chemically are so difficult to decompose, are easily oxidised to carbonic acid and water under the action of microbic life.

Most of the fat-splitting moulds do not grow or only very poorly on paraffin. RAHN [2]) has described a white *Penicillium* which can use paraffin as source of carbon whilst, according to this experimenter, bacteria cannot grow on hydrocarbons.

But the latter statement is incorrect. Most of the bacteria which oxidise the hydrocarbons cannot decompose the fatty acids, which in their chemical composition differ little from the paraffins, but some species are also able to split fats by secretion of lipase.

Hence, the paraffin-oxidising bacteria can be classified in two groups: fat-splitting and non-fat-splitting.

To the former belong: *B. fluorescens liquefaciens, B. pyocyaneus, B. punctatus, B. fluorescens non liquefaciens, B. Stutzeri, B. lipolyticum* α, β, γ and δ, and the *Micrococcus paraffinae*, described below. To the second group belong some species of the genus *Mycobacterium*.[3])

Oxidation in crude cultures.

The oxidability of petroleum, paraffin, vaselin and benzine was ascertained as follows.

To 100 cm^3 of a culture liquid consisting of: tapwater 100, ammoniumchlorid 0.05, bikaliumfosfate 0,05, in ERLENMEYER flasks

[1]) For these experiments were used: paraffin (GRÜBLER), paraffinoil (MERCK), vaseline, petroleum (American and Russian), and benzine Beside the common commercial petroleum I often used a more purified product obtained as follows. American petroleum was shaken with sulfuric acid D. 1,84, with repeated refreshing of the acid, then with potash solution; after this again treated with acid and once more with potash; it was then dried on sodium and distilled. The fraction 150°—250° (free from nitrogen) served after removing of a small quantity of sulfuric acid by potash solution as food for the microbes.

[2]) RAHN, Ein Paraffin zersetzender Schimmelpilz. Centralblatt für Bakt. 2 Abt. S. 382, 1906.

[3]) A. WEBER, Ueber die Tuberkelbazillen ähnlichen Stäbchen und die Bazillen des Smegma's. Arbeiten aus dem kaiserlichen Gesundheitsamte 1903. Bd. 19. S. 251. NEUMANN und LEHMANN, Grundrisz der Bakteriologie. 5e Auflage 1912. S. 619.

of ± 450 cm³ capacity, was added about 1% of one of the paraffins; this medium was inoculated with about a gram of garden soil and placed at 20°, 28° and 37° C.

Commonly after two days already growth of microbes is observed in the tubes at 28° and 37°; after about 7 days in those at 20°. The acceleration of the development is then very marked, so that the liquid becomes cloudy in consequence of the great number of microbes growing at the expense of the hydrocarbons. The growth in the cultures, transferred to a similar medium, is also very strong and the droplets of the hydrocarbons are enveloped by a thick slimy layer of microbes. In a short time the hydrocarbons, disappear entirely from the medium.

From the foregoing follows that petroleum, paraffin, paraffinoil, vaselin and benzine are oxidised by bacteria.

This explains the disappearance of the petroleum, daily brought at the surface of canals by motor boats and in other ways, and from the sewage water of the petroleum refineries.

Isolation of the bacteria.

The paraffin-oxidising bacteria were isolated by streaking the above described crude cultures on plates consisting of: washed agar 2 (or gelatin 10), bipotassiumfosfate 0,05, ammoniumchlorid 0,05, magnesiumsulfate 0,05, distilled water 100.

To this medium was added as source of carbon, petroleum in the form of vapour, from a small dish placed on the cover of the inverted culture box.

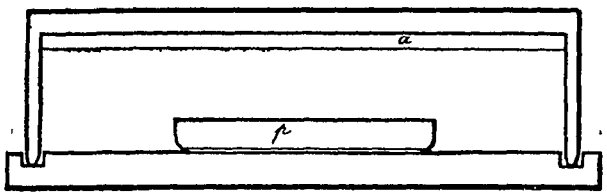

Fig. 1. Culture method on agar a with salts and petroleum as vapour from the dish *p*.

In this way only those bacteria which can oxidise petroleum vapour develop on the agar to colonies and are very easily isolated.

The growth of the microbes is vigorous, the bacteria assimilating, beside the vapour directly taken up, the petroleum

condensed around the colonies and forming an iridescent layer on the agar.

On comparison of the velocity of growth of the various species, much difference between them is observed.

By direct sprinkling of soil, canal water, or other material on the plates, several species which do not accumulate in the described culture liquids, can be isolated. Moreover it is possible in this way to determine the number of paraffin-oxidising microbes in any material. So, in one gram of garden soil at Delft ± 50,000, in one cm³. canalwater ± 8000 paraffin-oxidising microbes were found, which shows that they are very common.

It is clear that this method is also applicable to other volatile compounds.

For a nearer examination the cultures were sown, beside on the above plates, on broth gelatin and broth agar, and on media of other composition.

Accumulation of paraffin-oxidising species at various temperatures.

When the above media, consisting of tapwater, anorganic salts, and one of the hydro-carbons, are placed at temperatures between 15° and 25° C. and the transfers are also cultivated at these temperatures, *B. fluorescens liquefaciens*, *B. punctatus*, and other liquefying species are particularly obvious, but there likewise occur some fat splitting, non-liquefying bacteria and micrococci, which can all be distinguished on broth gelatin.

In the tubes placed at 26°—30° C. the number of liquefying bacteria is still very great, yet, non-liquefying species are more common than at lower temperatures. At the same time the non-fat-splitting group of the paraffin-oxidising species, the mycobacteria, begin to develop, but especially at 30°—37° C. they find their optimum. They are very striking by their morphological properties and pigment formation.

By this method white, brown, red, and red-brown species were isolated. At 37° C., with paraffin as carbon source, a fat-splitting micrococcus developed in almost pure state, which oxidised paraffins vigorously; it was called *Micrococcus paraffinae* and is in its properties, except in shape, similar to *B. lipolyticum* [1]).

If instead of garden soil, sewage water is used for the infection, the growth of fluorescents and of *B. pyocyaneus* may become so intense,

[1]) These Proceedings, 1911.

that the above mentioned species do not wel develop and often quite disappear.

At infection with pasteurised soil (5 minutes at 80° C.) no growth takes place, which shows that to the spore-forming bacteria no paraffin-oxidising species belong.

Under anaerobic conditions paraffins are not broken off by bacteria.

Description of the paraffin-oxidising mycobacteria.

These bacteria are immotile; in young cultures (8 hours on broth agar at 30° C.) they are rod-shaped, length 4 η—10 η, width 0.5 η—1.5 η, after division it often occurs that the two individuals are still joined in one point.

Very characteristic is the appearance of ramifications in these microbes, which remind of bacteroids such as are found in *B. radicicola*.

After some days' culture on broth agar or broth gelatin, these rod-shaped bacteria pass into *Streptococcus*-like organisms, the cells of this form having a diameter equal to the width of the rod form. The *Streptococcus*-form produces, on a new medium, first the rod form, which then again passes into that of the *Streptococcus*.

Spore formation does not occur; heating during 5 minutes at 65° is not resisted.

All species secrete some slime. The growth of the mycobacteria, which after their pigment-forming power on potatoes or on broth gelatin are distinguished in *Mycobacterium phlei* LEHMANN and NEUMANN, *M. lacticola* L. and N., *M. album*, and *M. rubrum*, varies very much on different media as is shown in the table below, where some of the results on growth and pigment formation are given.

On potato these microbes form most pigment [1]) and grow very well; likewise on broth-, malt-, und glucose gelatin. A very good medium is also broth gelatin or broth agar with 3 % glucose.

Besides on the above substances the fat-splitting bacteria and the *Mycobacteria* grow on humus compounds without these being decoloured. The best source of carbon is peptone, then follows asparagin, ammonium chlorid, and potassiumnitrate. Nitrate is reduced to nitrite; denitrification does not take place. In broth, with 3 % peptone, indol is not formed.

In broth with 3 % glucose, no fermentation is observed.

Tyrosin is not changed into melanin.

[1]) The pigment of *Mycobacterium rubrum* is probably carotine; it resists hydrochlorid (38 %), potash solution, and ammonia, dissolves in chloroform and ether and is coloured dark blue by sulfuric acid of density 1,86.

GROWTH AND PIGMENT FORMATION BY *MYCOBACTERIUM* ON VARIOUS MEDIA.

		Broth gelatin	*Potato*	*Malt-gel.*	*Cane sugar gel.*	*Glucose gel.*	*Lactose gel.*	*Maltose gel.*	*Mannite gel.*	*Asparagin gel.*	*Calcium malate gel.*	*Calcium butyrate gel.*	*Calc. acetate gel.*	*Calc. formiate gel.*
Mycobacterium	album	white	whiterose	whiterose	white	white (acid)	white, very slight growth	—	white	white	white	as calc.	white	—
	phleï	red-brown	red-brown	red-brown	—	orange (acid)	—	—	orange, bad growth	orange-yellow	orange-yellow	malate gel. but	red-brown	—
	lacticola	yellow	dark-yellow	yellow	yellow little growth	dark-yellow (acid)	—	—	yellow yellow	yellow little growth	yellow	slighter growth	yellow	—
	rubrum	dark-red	dark-red	dark-red	—	dark-red no acid	—	—	—	red, little growth	red		red	—

Aesculin and indican are not decomposed.

Urea is only splitted by *Mycobacterium album.*

In feebly acid media the growth is inhibited; it is best in neutral or feebly alkaline solutions.

At the oxidation of paraffins, organic acids, probably fatty acids are formed as intermediary products; they are, however, only present in slight quantities and evidently are oxidised almost as quickly as produced. Acid formation from paraffin could, however, be shown with the help of washed agar plates to which a little congo red had been added, or in which some calciumfosfate was precipitated. In the former case blue fields appeared under the inoculation streaks, in the latter clear ones.

Velocity of the petroleum and paraffin oxidation.

The velocity with which *Mycobacterium album, M. rubrum, Micrococcus paraffinae,* and *B. fluorescens liquefaciens* oxidise petroleum, was ascertained by weighing the quantity of carbonic acid formed in a certain time. The diminution of the petroleum could not be directly stated as it always evaporates.

The quantity of the produced carbonic acid was ascertained as follows.

As culture vessel was used a one liter ERLENMEIJER flask provided with a ground glass stopper, bearing a vertical glass tube, reaching to near the bottom, and a side tube. It was filled with ± 200 cm³ of a sterile culture liquid, consisting of distilled water, anorganic salts, and 2 cm³. sterile petroleum.

The vertical glass tube was connected with a large U-tube filled with soda lime; the side tube was joined to an apparatus successively formed by U-tubes, filled with sulfuric acid, beads and paraffinoil (to keep back the petroleum vapour), calciumchlorid, potash solution to weigh the carbonic acid, and calciumchlorid for control, with a KÖRTING pump at the end. When the cock of the pump is opened a current of air, freed from carbonic acid, passes through the fluid and yields the dried carbonic acid, formed in the culture, to the potash tube.

During 24 hours are formed in the culture, if infected and placed at 28° C., Milligrs. carbonic acid by:

Mycobacterium album	55
Mycobacterium rubrum	41
M. paraffinae	34
B. fluorescens liquefaciëns	27
Crude culture	93

About a third part of the weight of the carbonic acid corresponds to the oxidised petroleum.

The velocity with which paraffin is oxidised by these bacteria was estimated by stating the diminution in weight, by the bacterial action, of two grams of paraffin, very minutely mixed with distilled water and anorganic salts, after a month's culture at 28° C.

The rest of the originally added paraffin was dissolved in petroleum-ether; of this solution a certain quantity was evaporated and the remaining quantity of the paraffin was weighed.

So it was found that during a month's culture was oxidised in mgrs. by:

Mycobacterium album	300
„ *rubrum*	330
Micrococcus paraffinae	180
B. fluorescens liquefaciens	180
Crude culture	540

Summary.

1. Paraffins (petroleum, paraffin, benzine) can be used by certain species of microbes as source of carbon and energy, and are oxidised to carbonic acid and water. As intermediary products acid could be indicated.

The bacteria were obtained by means of the accumulation method, with the said substances as source of carbon.

2. The microbes active in this process belong to two groups.

a. Fat-splitting bacteria, very common in nature, as *B. fluorescens liquefaciens, B. pyocyaneus, B. punctatus, B. Stutzeri, B. lipolyticum, M. paraffinae.*

b. Non-fat-splitting bacteria belonging to the genus *Mycobacterium* likewise widely spread, of which the following were distinguished: *Mycobacterium album, M. phlei, M. lacticola,* and *M. rubrum.*

3. The paraffin-oxidising species decompose, on an average, 15 mG. petroleum and 8 mm. paraffin in 24 hours at 28° C. per 2 cm². surface of culture liquid.

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