Microbiology. — *"Oidium lactis, the milkmould, and a simple method to obtain pure cultures of anaërobes by means of it."* By Prof. BEIJERINCK.

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The many methods recommended for the pure culture of anaërobes, — whose multitude proves that none of them quite satisfies the investigators, — may be distinguished in chemical and biological. As to the former, of which NOVY's exsiccator method is certainly the best, everything has been tried. This cannot be said of the biological methods based on the use of living organisms in particular aërobic microbes for the removing of the oxygen. For myself only after using the milkmould to that end I have obtained results worth fixing once more the attention on it.

Some chief points from the life history of *Oidium lactis* important for experiments with this species may precede; a complete description is not necessary here.

Properties of the milkmould.

The milkmould possesses a number of properties which render it very fit for experiments in relation to respiration, nutrition, growth and symbiosis. It unites the character of the moulds to that of the yeasts, in particular with regard to the growth in and upon the substrate which takes place without being accompanied by fermentation, as also without the formation of conidia which currents of air might spread. Within the substrate the long-celled mycelium is found, on the surface the chains of conidia which, even when extending free in the air, cohere and never contaminate the environment as moulds may do.

It is easily obtained. A rich growth results when market milk is left a few days in an open glass in a warm room; the milk then always covers with an *Oidium* film. Lactic acid ferments also develop and by their production of acid further the growth of *Oidium*, whilst they themselves are favoured in their development by *Oidium*, because it oxidises the lactic acid to carbonic acid and

water. In garden soil *Oidium* is generally spread as may be shown by inoculating feebly acidified malt infusion with soil and keeping it at 25° to 30° C. The film which finally covers the liquid contains besides *Mycoderma*, always *Oidium*. Pressed yeast, long whey, sour milk, cheese, the output waters of distilleries and all kinds of acid sewage, are inhabited by *Oidium*. Natural habitats are furthermore the sap flow of many trees caused by *Cossus ligniperda* and allies.

For pure culture acidified malt infusion- or broth-glycerin plates are recommendable. The acid serves to exclude the hay bacteria which have a great disposition to grow in symbiosis with *Oidium* in neutral environment.

The transfers for the collection are kept on malt-agar, but they change thereby in a few months into a tough, leathery mycelium, almost exclusively consisting of long mycelial threads difficult to separate and evenly to mix with the nutriment. To obtain normal material in this case a new isolation from milk or soil is necessary, for the change is an hereditary non-reversible mutation.

Under favourable feeding conditions the growth is remarkably rapid and the respiration and oxygen absorption go parallel with it. This intensity exceeds by far that of the ordinary moulds of the genera *Penicillium* and *Aspergillus*, whilst it equals that of *Mucor*. This holds, however, only good with regard to easily assimilable substances; less decomposable matter such as pectine, cellulose and chitine are not attacked by *Oidium*. Gelatin and agar are neither assimilated. Fermentation phenomena, joined with the evolution of gas, are as said also wanting. Hence, *Oidium* never forms rents or holes in the solid substrata wherein it is cultivated, not even in presence of glucose. This is one of the reasons why it is so well adapted to the culture experiments with the anaërobes to be discussed below.

The products of metabolism are chiefly or only water and carbonic acid; volatile or non-volatile substances noxious to other organisms are not produced.

In regard to carbon-food *Oidium* exhibits a great specialisation. Most hexoses, in particular glucose, levulose and mannose, are readily assimilated and oxidised. Likewise aethylalcohol. Glycerin, too, is a very good carbon source. On the other hand, starch, raffinose, maltose, cane sugar, mannite and all similar substances, are in no way assimilated. Enzymes, as diastase, maltoglucase, invertase, lactase, are hence completely absent. Glucoside enzymes could neither be found. By the absence of these enzymes, *Oidium*, which so easily reacts on the hexoses, is especially fit as a reagent on these enzymes in case they are to be detected in parts

of higher plants or as products of secretion of other microbes; here the auxanographic method may advantageously be applied.

Fats are however split up by *Oidium*, by means of the exoenzyme lipase, active outside the cells. Hence, in presence of fats growth of *Oidium* may be expected at the expense of glycerin and this explains the general occurrence of *Oidium* as well in milk and butter as in other fat-containing materials. For the preparation of lipase the milkmould can afford a good starting material.

As to the nitrogen food *Oidium* resembles the ordinary yeast species and is in this respect rather many-sided. With exception of nitrates and nitrites, and unchanged albuminous substances, the ordinary nitrogen compounds are easily assimilated in presence of good carbon food such as glucose and glycerin. This is in particular true for ammonium salts and urea. Peptones and the higher ammino acids, if alone, are not or very slowly assimilated, but in presence of a good carbon source they may serve as a very good nitrogen food, so that the complete nutrition of *Oidium* in presence of these substances should be called dualistic. Consequently broth bouillon is for *Oidium* an insufficient food and on a broth-agar plate it develops but poorly. This changes however by adding a good carbon source. If this is done locally on a broth-agar plate there results an auxanogram in the diffusion circle of the related matter, which proves at the same time that the other elements required for the growth of *Oidium*, as potassium, magnesium and phosphor, are present in sufficient quantity in the broth. As these elements accumulate in the young cells, either as the same chemical compound found in the substrate or not, such experiments are apt to demonstrate the absorption phenomenon formerly described by me. It is also easy in reversing the experiment, that is by feeding with carbohydrates, to find with the microscope by means of iodine, glycogen accumulated in the so large *Oidium* cells and its disappearing in the auxanograms of nitrogen food, such as ammonium salts or urea, as soon as the carbon food in the substrate is wholly assimilated.

A feebly acid reaction of the medium furthers the growth of *Oidium*, and organic acids, for example acetic and lactic acid, may disappear by oxidation. Other acids as molybdenic and tungstic acid are in good media, such as glucose-broth-agar, reduced by *Oidium* to the well-known blue oxides, which gives rise to beautiful colour experiments. In neutral solutions the salts of these acids are however not affected so that this is a case of reduction in an acid medium. The ordinary alcohol yeasts behave likewise.

Use of the milkmould for the pure culture of anaërobes.

In nature the withdrawing of oxygen from the environment, which is required for the development of anaërobes, is usually caused by aërobic microbes.

They not only absorb the last traces of oxygen from the surroundings but even produce reducing substances in it. In the laboratory this may be imitated by adding to a culture medium containing in small number germs of the anaërobe to be examined, a great number of germs of an appropriate aërobic microbe. How such experiments have hitherto been carried out [1]) may be illustrated by a definite example namely the cultivation of the spore-forming aërobes of the albumin putrefaction; then I will describe the modified method.

A crude culture of putrefaction bacteria is obtained thus. A stoppered bottle is quite filled with a watery infusion of albuminous matter, infected with garden soil and boiled to kill all non-sporogenous microbes. Placed in the incubator the mass soon passes into stinking putrefaction, characteristic by the presence of mercaptans produced by the spore-forming anaërobes. Now to ordinary broth-gelatin or broth-agar an abundant quantity of some intensively growing aërobic bacterium, such as *B. fluorescens* or *B. prodigiosum* is added, together with a little of the to 90° or 100° C. heated material containing the spores of the putrefaction microbes. After solidification in a test tube the aërobes near the bottom will soon absorb the last traces of oxygen and being unable to grow there, not give rise to liquefaction of the gelatin; but they will retain the oxygen penetrating from above and develop strongly in the surface of the gelatin. In the lower part of the tube the spores of the putrefaction bacteria can now germinate and if gelatin is used there will soon appear the large liquefying colonies of the so remarkable *Bacillus septicus*, together with the non-liquefying putrefiers, for the greater part recognisable by the flocculent structure of their colonies, a character related to their sensitiveness to extension and contraction of the substratum wherein they grow, quite as by *B. Zopfii*. For the microscopical examination this method undoubtedly affords good material, but it is hardly possible to reach the single anaërobic colonies without touching others. To this end it is necessary to remove the culture gelatin from the tube by heating it in the flame so that only the outer side of the gelatin melts and the contents may be thrown

[1]) E. Macé, Traité pratique de Bactériologie, 6e Ed. T. 1, pag. 305, Paris 1912. Besson, Technique microbiologique et sérothérapique, 6e Ed., pag. 102, Paris 1914.

out as a whole. One may also with a file make an incision in the glass wall in the neighbourhood of favourably situated colonies. But it is clear that there is much chance that thereby also different colonies intermix so that of a pure culture of anaërobes in the usual sense of the word there is no question in such experiments. For examination with the microscope and for studying the appearance of the colonies the method is useful, but for the culture of pure species it is worthless.

Every good method for pure culture of aërobes and still more of anaërobes should answer the following requirements: the colonies must be situated quite free and at due distances from each other on the surface of the solid plates, they must furthermore be readily attainable with the platinum wire. These requirements can only be satisfied by cultivation in ordinary glass boxes or PETRI dishes, which may take place in the laboratory by means of the exsiccator method of NOVY (see MACÉ, l.c.).

After this method, — the best of the chemical ones, — ordinary culture boxes are placed in an exsiccator filled with pure hydrogen and moreover containing some oxygen-removing substance, such as ferro-ferrocyan or alkaline pyrogallol. But this method also has its drawbacks. It is namely impossible quite to prevent the deposition of vapour at the glass covers, so that drops of water falling down come on the plates; this makes the colonies intermix and spoils the experiment. It is, besides, hardly possible distinctly to see the state of development of the colonies in the closed exsiccator, which may lead to it being opened too early and oblige the experimenter to begin anew. This is very troublesome considering the complication of the experiment.

The *Oidium* method has none of these disadvantages, and if well-managed, produces colonies of the anaërobes situated quite free on the surface of the plates and easily reached with the wire.

The principle of the method is the placing one over the other of two culture plates, separated by a relatively small space of air. One of the plates contains the aërobic microbe which is to absorb the oxygen, while on the surface of the other the anaërobe is to grow. Here, also, I select a definite example for illustration, namely the strictly anaërobic bacilli of the butyric-acid and the butyl-alcoholic fermentations; they have corresponding nutrition conditions and may be isolated in the same way. They are spore-producers, thriving best in malt infusion where they cause strong fermentations accompanied with production of hydrogen and carbonic acid. A crude butyric-acid fermentation is prepared as follows. Wheat- or rye-flour, or better a pap of potatoes infected with soil, is mixed in a glass beaker

with water to which is added some calcium-carbonate, then heated for a few seconds to 90° or 100° C. Kept at 30° to 40° C. there usually results after two days a strong butyric-acid fermentation in which occur various butyric-acid bacteria which are then to be isolated.

For the preparation of a crude butyl-alcoholic fermentation crushed corn [1]) of *Hordeum vulgare nudum* may be used; a pap of potatoes infected with soil and heated not higher than 80° to 85° C. will also do; addition of chalk is not necessary, the butyl bacteria producing no acid. Of course the spores of butyric-acid ferments are still present in such preparations and the surprising fact that by application of the said temperature no butyric-acid but a butylic fermentation ensues, should probably be attributed to the injurious action of the butyl alcohol on the butyric-acid ferments.

The pure culture is effected as follows.

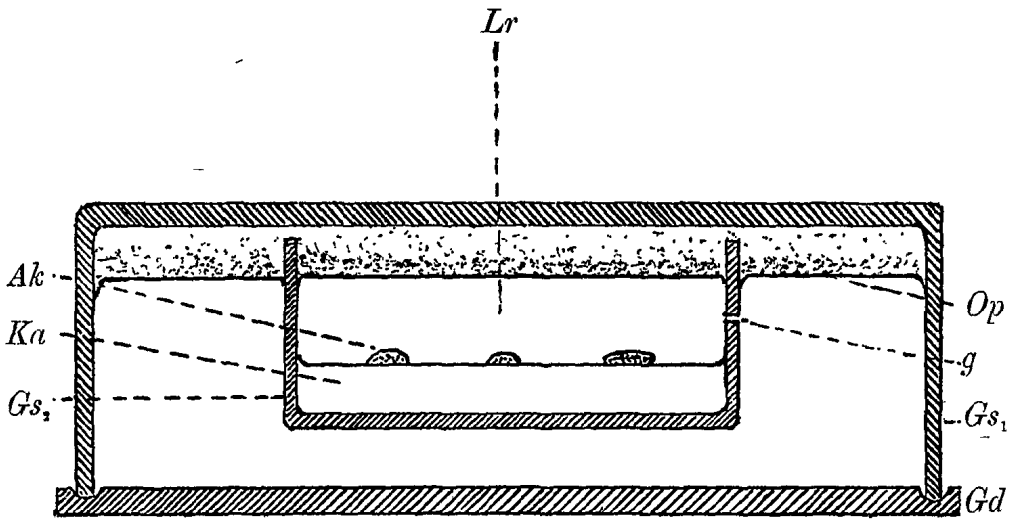
Malt-infusion agar with 5° to 10° glucose is liquefied and after cooling to near solidification and addition of a great quantity of *Oidium lactis* is plated (*Op*) in a large glass dish (Gs_1). At a temperature of 25° to 28° C. the whole surface of the plate is already after 24 hours covered with a thick snow-white film of conidia and the interior of the agar is wholly interwoven with mycelium, which causes a considerable absorption of oxygen.

A second malt-infusion-agar plate (*Ka*) without *Oidium* is now prepared in a glass dish (Gs_2), much smaller than Gs_1. The space between Gs_1 and Gs_2 must be large enough Gs_2 to be caught with the fingers. On its surface a little of the material containing the anaërobes, that is of the crude butyric-acid or butyl-alcoholic fermentations, diluted with sterile water, is spread. Now the lid of the smaller dish (Gs_2) is removed and the plate pressed on the *Oidium* plate the agar side (*Ka*) upward as shown in the figure.

For the escaping of the air from *Lr* a little hole *g* is bored in the glass wall of Gs_2 and closed with a droplet of paraffin introduced with a heated glassrod. At 28° to 30° C. the air in *Lr*, which space can be relatively small, will soon be free from oxygen and the anaërobes on *Ka* can begin to grow. To further the absorption of oxygen from the agar *Ka* in Gs_2, *Oidium* may also be added to it, but then a thin layer of malt agar without *Oidium* should be poured on the surface of *Ka* to obtain a germ-free surface for the sowing of the anaërobes. *Oidium* being strictly aërobic the mycelia do not perceptibly grow through this protecting layer.

[1]) Fermentation et ferments butyliques. Archives Néerlandaises I. 39. Pag. 1. Bactéries actives dans le voisinage du lin. Ibid. Sér. 2. I. 9. p. 418. 1904.

If the glass dishes have good dimensions and the space *Lr* is not too small, one can sideways look through the glass wall and follow



Cultivation of anaërobes by means of *Oidium lactis*. Gs_1 large glass dish with the oxygen-absorbing *Oidium* plate *Op*. Gs_2 smaller dish with the culture plate *Ka* whereon the anaërobic colonies *Ak* grow. *Lr* space between the plates. At *g* the hole in the glass wall of Gs_2 for the escape of the air from *Lr*, which is afterwards closed with paraffin. *Gd* glass lid of the large dish Gs_1. The higher temperature is at the side of *Gd*.

the development of the anaërobic cólonies on *Ak*. So it is easy to decide when the moment for further observation has come without it being necessary to remove plate *Ka* from the *Oidium* plate *Op*, and thus prevent too early opening.

When it is time to open, liquefied malt agar must be at hand to be poured out over the *Oidium* plate, especially in the groove formed by Gs_2, as soon as plate *Ka* is to be restored to its place. The fresh food causes new oxygen absorption by *Oidium* and the growth of the anaërobes can go on.

For the success of the experiment it is essential to mind the following. The placing in the incubator should be managed in such a way that the *Oidium* layer *Op* comes in the cooler, and the cover *Gd* as also plate *Ka* in the warmer part. The vapour in *Lr* will then condense in *Op* and not on the surface of *Ka*. In the reversed position *Ka* will become moist, the colonies intermingle and the experiment fails. Hence the figure is represented in such a position that the colder air is above, the warmer below, as is the actual state in an incubator with bottom-heat. How simple all this may appear, in the execution it will be found necessary to pay special attention to it.

In this way it is possible from the ordinary crude butyric-acid fermentations, obtained as described above, to separate three distinctly different *Amylobacter* species, two of which I described already before (Proceedings Vol. 12, Pag. 973, 1903) under the names *A.* (*Granulobacter*) *saccharobutyricum* and *A.* (*G.*) *pectinovorum*, while from the butyl-alcoholic fermentations two species were isolated, one of which produces large slimy colonies and was described as *A.* (*Gr.*) *butylicum* (Archives Néerlandaises, 1^re^ Série, T. 29, Pag. 2), whereas the other, which secretes no slime, has not yet been investigated. The colonies of all these species colour dark blue with iodine like starch, the staves and clostridia containing a great quantity of granulose.

The butyric-acid and butyl-alcoholic fermentations acquired in other ways than the above mentioned have not yet been examined thoroughly.

As the anaërobic *Sarcina ventriculi* likewise develops very well on malt-infusion agar at 30° tot 37° C. (Proceedings 28 April 1911, Pag. 1412), this species may be isolated just in the same way as the above.

As regards the spore-producing bacteria of the real protein putrefaction the *Oidium*-plate may be prepared just as in the experiment described, only for the cultivation of the anaërobes themselves in Gs_2 it is better to make use of broth agar with 0.5 or 1 % common salt, either with addition of 2 % glucose or not. In this case, too, nutrition with carbohydrates gives in some species rise to production of granulose, in others not.

Another anaërobe isolated by the *Oidium*-method is *Bacillus acidi urici* (Proceedings 23 April 1909, Pag. 990), which ferments uric acid to carbonic acid, ammonium acetate and ammonium carbonate This species also develops best on broth agar at 30° to 35° C.

For beginners it must be noted that on plate *Ka* the facultative anaërobes, such as *Bacterium aërogenes* and *B. coli*, develop quite well, as may be proved by streaking off all the colonies *Ak* on aërobic plates on which the anaërobes only do not grow. This is in accordance with the fact that at the starting of the experiment some oxygen is present in *Lr* sufficient for the very small oxygen want of the facultative, better called temporary anaërobes.