Microbiology. — "*On Bacillus polymyxa*" [1]). By Prof. M. W. BEIJERINCK and L. E. DEN DOOREN DE JONG.

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If the species-conception is taken in a not too limited sense, the closely related, but not identic forms mentioned in Note 1, may be said to comprise the only known aërobic spore-forming bacterium-species, which causes fermentation in a sugar-containing medium. We call it *Bacillus polymyxa.*

It is rather generally spread in fertile soils; its properties are very characteristic and give rise to interesting experiments. The production of aceton first observed by SCHARDINGER, has in the later years drawn attention on this microbe, but the quantity formed is small and from malt or potatoes it does not amount to 1 % of the weight. But the conditions for its formation are not yet well-known and might perhaps be greatly improved as to the quantity. Alcohol is also generated and to a somewhat greater amount than aceton. Besides, a little acetic- and formic acid seem to be produced. Particularly the secretion of the enzyme pectinase and of much slime by the chief variety is of interest.

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[1]) The literature of this Bacterium and its nearest relations is to be found under: *Clostridium polymyxa* PRAZMOWSKI, *Granulobacter polymyxa* BEIJERINCK, *Bacillus macerans* SCHARDINGER and *Bacillus asterosporus* A. MEYER. — A. PRAZMOWSKI, Entwickelung und Fermentwirkung einiger Bacteriën. Dissert. Leipzig 1880, p. 37. — TH. GRUBER, Identifizierung von *Clostridium Polymyxa* PRAZMOWSKI, Centralbl. f. Bakteriol. 2te Abt. Bd. 14, 1905, pag. 353. — F. SCHARDINGER, *Bacillus macerans*, Acetonbildender Rottebacillus, Centralbl. f. Bakt. 2te Abt. Bd. 14, 1905, pag. 772. Zur Biochemie von *B. macerans*. Centralbl. f. Bakt. 2te Abt. Bd. 19, 1907, p. 161. Kristallisierte Polysaccharide aus Stärke durch Mikrobien. Centralbl. f. Bakter. 2te Abt. Bd. 22, 1909, p. 98 and Bd. 29, 1911, p. 189. — A. MEIJER und G. BREDEMANN, Variation und Stickstoffbindung durch *Bacillus asterosporus*. Centralbl. f. Bakteriol. 2te Abt. Bd. 22, 1909, p. 44.

The name *asterosporus* is derived from 9 or 10 rims on the exosporium of the oblong spores, which make the transversal section star-like. By abundant feeding, as on wort-gelatin, many rodlets change into narrow clostridia containing somewhat granulose, colored blue by jodine; so the species may also be called *Granulobacter polymyxa.*

Accumulation and occurrence.

Long ago the following experiment for the accumulation of this species was described [1]).

Coarsely ground rye with some chalk and inoculated with fertile garden soil is mixed with water in a deep beaker to a thick solid paste, boiled during some seconds to kill the non-spore-formers and cultivated at 25° to 30° C. As the spores of *B. polymyxa* soon die at boiling, the heating must last but a short time. After a few days the surface is covered with a coherent film of *B. mesentericus* [2]) and other closely related species, while in the depth a butyric-acid fermentation takes place, usually simultaneously with butylic-alcohol- and polymyxa fermentation.

It is clear that this accumulation reposes essentially on a temporary anaërobiosis of *B. polymyxa*, which can also grow aërobic and so behaves like the alcohol yeast and the *Aërobacter-Coligroup* among the bacteria. The rye produces the sugar causing the fermentation, i.e. the source of energy, which makes the anaërobiosis possible so long as the "excitation oxygen" is still sufficiently present, albeit chemically non-demonstrable, whereas the want of "oxidation oxygen", which is required for aërobiosis in much larger quantity as source of energy, is temporarily excluded. PASTEUR's statement: "la fermentation est la vie sans air" is evidently applicable to *B. polymyxa*.

By sowing out the fermenting matter from the depth on wort-agar, ordinarily already after few days the polymyxa colonies become visible as lumps of slime, together with the unavoidable flat spreading colonies of *B. mesentericus*.

This method can only produce those varieties of *B. polymyxa* which are able to resist a relatively high concentration of the food. Another accumulation method by which also forms adapted to a lower concentration of food are obtained is based on the aërobiosis of our bacterium.

After the observation had been made that flasks of boiled wort, not sufficiently sterilised, were not seldom spoiled at the low temperature of 15° C. by the development of *B. megatherium* and never by *B. mesentericus*, whose germs were certainly also present, the question

[1]) M. W. BEIJERINCK. De butylalcoholgisting en het butylferment. Academy of Sciences. Amsterdam 1893.

[2]) This film may be colourless, brown, red, and even jet black according to the accidentally present varieties of *B. mesentericus*. The black form is rare and sometimes obtained by the "mesentericus experiment" with unwashed currants (boiling with chalk, cultivating at aëration at 30° to 40° C.).

arose: which are the aërobic spore-forming bacteria, which can develop at temperatures of 15° C. or lower and under favorable feeding conditions? We knew already that the obtaining of *B. megatherium* might give an answer to the question, for example in case the spores of this species were only present with those of *B. mesentericus*, but it seemed possible that free competition with the soil bacteria would exclude *B. megatherium* and that some other species could appear. The chief aim of the experiment was to exclude *B. mesentericus*, the common hay bacterium, which produces substances very noxious to other species, and this is to be reached by the low temperature, as the minimum for the growth of this species is at about 20° C. The simultaneous development of *B. megatherium* is of less importance as it is innocuous to other kinds. Of course we had to reckon with the butyric-acid and butylic fermentations, which may very well occur at 15° C, but strong aëration prevents them efficiently.

Although we could expect that the one or more species that were to develop under the chosen conditions would possess a higher temperature optimum than that used by us, we had not to fear a failure if only we cultivated above their minimum.

Knowing that the spores of some spore-formers, for example those of the butylic ferments, and thus perhaps, too, those of the species we sought for, could not or hardly resist boiling, the heating of the culture liquid containing the inoculation material and wanted for killing the non-spore forming species, was not continued much above 85° or 90° C. and only for a few seconds. We used flasks half filled with about 30 cM³ liquid, and in order not to miss somewhat rarer species, we inoculated with so much soil that on the bottom a layer of about 1 cM precipitated. This soil had previously been well-divided and freed from coarse particles. In such a thick layer a beginning of anaërobiosis is possible, but by shaking, butyric-acid or butylic fermentation may easily be stopped.

For food we used at first malt-wort, diluted to 2° to 5° BALLING, later broth-bouillon with 2% to 5% cane sugar, or glucose. Addition of chalk is not absolutely wanted for the success of the experiment but its presence proved favorable.

After we had ascertained with pure cultures of *B. polymyxa* that ammonium salts, nitrates and asparagine are very good sources of nitrogen, we also accumulated with sugars and ammonium sulphate, in a solution of tapwater 100, 2 to 5 % glucose or cane sugar, 0,05 % $(NH_4)_2SO_4$, and 0,02 % K_2HPO_4 with some chalk. The execution of the experiment is as above, but after pasteurising,

the butyric-acid fermentation must be more completely excluded than when using broth-bouillon or malt-wort. For although the latter liquids contain an excellent nitrogen food for *B. polymyxa*, they are of less value for the butyric-acid ferments, for which the ammonium salts are preferable. Hence, in this case it is advisable to use a large ERLENMEIJERflask, as the great volume of soil which sinks to the bottom as inoculation material, can then be better aërated, by which butyric fermentation is prevented.

Although the growth is slow at the low temperature the liquid becomes distinctly turbid and in most cases this is accompanied with fermentation. This fermentation especially awakened our attention as we had expected an accumulation of *B. megatherium*, which causes no fermentation at all.

As the *Coli-* and *Aërogenes*fermentations had been prevented by the previous heating, the butyric-acid and butylic fermentations by the aëration, we now expected that the fermentation of *B. polymyxa* was obtained, and this was confirmed by the pure culture. The fermentation which is chiefly an alcoholic one, proves that our bacterium belongs to the facultative (temporary) anaërobes, and the examination of the gas showed that it is almost pure carbonic acid.

One of the most notable qualities of *B. polymyxa* is its secretion of pectinase, i. e. the enzyme by which some microbes dissolve the central lamellum of plant tissues, thereby disintegrating them into cells. Hence, *B. polymyxa* like *B. mesentericus* may under certain circumstances play a part in the retting of flax, although the real agent in this case is the anaërobic *B. pectinovorum*.

Beans, peas and other plant seeds, left to spontaneous corruption, may change into rich cultures of *B. polymyxa*, the cell-walls of cotyledons and of endosperm being easily attacked by pectinase, whereby the interior of the seeds is changed to a pulpous mass [1]). For the preparation of a pure culture this method is less recommendable than the two foregoing accumulations, on account of the numerous hay bacteria which thereby simultaneously develop; it is, however, a good way to get an initial material for the said accumulations themselves.

It seems to us that the generality of *B. polymyxa* in our surroundings and particularly in the soil should be explained by its pectinase secretion, which must give this species, in combination with its little want of air, a great advantage over the other saprophytes.

[1]) The enzyme seminase, which changes the endosperm of the Leguminosae (*Indigofera*, *Ceratonia*) into mannose, is perhaps identic with the pectinas of *B. polymyxa*.

The very common presence of *B. polymyxa* in the bark of the nodules of the Leguminosae is certainly also a direct consequence of its pectinase production. Its presence there is of so general occurrence, that it reminds more of symbiosis than of saprophytism. In the bacteroïdal tissue *B. polymyxa* is however completely absent.

Properties of the colonies.

The colonies on agar as well as those on gelatin are characteristic. On malt-wort gelatin they resemble at first thin, watery, sideways quickly extending, slowly liquefying layers, which by and by become deeper and cloudy by their strong growth. At length the gelatin is completely liquefied and then these cultures resemble those of common hay bacteria. On malt-wort agar there is a profuse production of slime, whence very distinct voluminous and wrinkled colonies appear. The slime attracts part of the pigment from the wort-agar thereby becoming brown-coloured, which gives a characteristic appearance to the colonies.

On glucose-kalium-phosphate-ammonium-phosphate-agar they become glass-like transparent, somewhat resembling glass globules, so peculiar that at estimating the number of germs in soil samples, they may directly be recognised and counted. Silica plates, saturated with food, also produce such drop-like colonies from soil. Some varieties form much less slime than others and this slime is either tough or soft.

Microscopically those with soft slime consist of much shorter rodlets. Hence, one is at first disposed to think of different species, but further research shows the similarity, which is the more convincing, when beside the natural varieties, the mutation phenomena in the pure cultures are studied. On cane-sugar-asparagine agar many colonies, at first quite homogeneous and soft, when getting older produce small, rather solid, transparent, secundary colonies which, after separation from their surrounding (which is not easy) prove to be constant. On malt-wort agar the variety with tough slime, when growing older produces extensive, flat secundary colonies, showing a hereditary loss of the factors for slime formation.

In liquid nutritive media the form resistent to high concentrations of the food gives remarkable cultures.

In a malt-wort of 10° BALLING at 30° they consist of excessively voluminous slime masses, forming after one or two weeks a thick, coherent, floating film, inflated by carbonic acid, whilst no hydrogen is detectable. Only in the anaërobic butylic fermentation something of the like may be observed but then much hydrogen is present.

Even the most slimy *Aërobacter* forms produce quite different submerged cultures equally dispersed through the solution.

The vigorously fermenting slime varieties of *B. polymyxa* produce aceton, probably after the formula

$$C_6H_{12}O_6 + 2O_2 = C_3H_6O + 3CO_2 + 3H_2O.$$

To the products of the anaërobic fermentation belong in particular aethyl alcohol, with traces of acetic acid and formic acid beside some other products, such as butylic glycol, in small quantities.

The less slimy varieties of *B. polymyxa* can only live in food of lower concentration and spread through the solution as *Bact. aërogenes*. Also in other respects there is similarity between *Bact. aërogenes* and *B. polymyxa*, so that there is cause to conclude to a real relationship. Still there is a great difference in so far as *aërogenes* can assimilate many organic salts, a power quite absent in *B. polymyxa*.

Nutrition.

For the investigation of the substances which can be assimilated by *B. polymyxa*, the auxanographic method is very convenient, particularly in relation to the carbohydrates, *B. polymyxa* being a real "sugar bacterium", which produces much cell-wall matter, which makes the auxanograms very distinct. In judging the latter it should be kept in view that, beside pectinase, *B. polymyxa* produces diastase, invertase and emulsine. In presence of sugar various nitrogen compounds are assimilable, of which, however, only nitrogen is taken up. We preferently used peptone, asparagine ureum, ammonium sulphate and saltpeter. Urease is not secreted; saltpeter is reduced to nitrite, not to nitrogen.

As in absence of sugar the carbon cannot be withdrawn from nitrogen compounds, such as peptone and asparagine, the growth, even on broth-bouillon-agar is but slight and is a criterion for the quantity of sugar present. Hence, if on this medium *B. polymyxa* is densely sown, only small, hardly visible colonies grow, consisting, however, of bacteria with abundant protoplasm and commonly motile. If on such a culture an assimilable carbohydrate is locally distributed, vigorous growth ensues, chiefly reposing on slime formation and a distinct auxanogram results, demarcated by the limit of diffusion of the substance. It is in fact the presence of a small amount of complete food at the starting of the experiment, together with excess of by themselves unassimilable nitrogen compounds, which enables the germs to change into small colonies, which

renders the further growth after addition of the carbohydrate very clear.

Most sugars and polyalcohols are readily assimilated by *B. polymyxa*. This we have ascertained for arabinose, glucose, levulose, mannose, galactose, cane-sugar, maltose, lactose, melibiose, raffinose, rhamnose, glycerin and mannite. On the other hand sorbite, dulcite, erythrite and quercite are not attacked. It is very notable that we did not find any organic salt assimilable by this organism.

The "sugar bacteria", to which *B. polymyxa* belongs, produce from carbohydrates much more visible cell-wall substance than protoplasm, if the carbohydrates exceed the nitrogen food and vice versa.

Hence, *B. polymyxa* may be found, as was observed above, in two microscopically greatly different conditions. At insufficient feeding with carbohydrates, for example on broth agar, it grows as highly motile rodlets, without slime wall; at copious feeding with carbohydrates, as immotile rodlets with a thick slime wall [1]). This circumstance leads to the following experiment, only adapted to the variety of *B. polymyxa* which produces voluminous slime and grows strongly on malt-wort.

The bacterium densely sown on cane-sugar-kaliumphosphate-agar, containing but few nitrogen compounds, may form fairly large colonies consisting, however, almost entirely of the strongly swollen walls of the cells. By addition to the said medium of a few drops of complete food, for example a little broth or malt-wort, containing an excess of sugar, the slime walls grow surprisingly so that the plate covers with a relatively thick slime coat. This slime is built up of the sugars by one or more synthetically acting enzymes, that might be named "cyteses" and should be considered as the genes or factors of the cell-walls.

This slime has the remarkable property of being able to become itself a source of carbon food, but only at the moment when all the cane sugar and all the assimilable nitrogen compounds have been used. If at this time some such nitrogen compound as ammonium-sulphate or asparagin are brought on the slime coat of the plate, the bacteria begin anew to grow and produce new protoplasm from their own cell-walls. This leads to the peculiar consequence, that an auxanogram is produced sinking deep into the layer of slime. For, by the growth the bulk of the bacteria is diminished, because the walls, which chiefly consisted of water and were very voluminous, disappear and are replaced by living protoplasm. So the appearance of the auxa-

[1]) Medici give to the cell-wall of bacteria the singular name of "capsule".

nograms is quite changed when compared with the original state, for by their intense increase the opaque bacteria produce an also opaque auxanogram, whilst the original slime was transparent like glass. This proves that, in this case at least, the biological function of the slime is that of a reserve food.

In this experiment cane sugar was the food for the slime production; as hereby inversion takes place, glucose and levulose are probably the building materials of the slime; that these sugars are assimilated was stated above, and that glucose may also serve for the described experiment we ascertained particularly.

The other sugars have not yet been extensively examined from this point of view, but it seems that all give the same result. This leads to the conclusion that probably no more than two or three factors or genes (endoenzymes) are active in the production of the cell-wall. The problem is evidently of theoretic interest and deserves nearer research.

The wall-substance, which certainly belongs to the cellulose group and therefore may be called cellulan, must have a high power of attraction for water, for else its surprising volume cannot be explained. Nevertheless its molecules cannot be very small as they cannot diffuse at all in water. It is not colored by jodine, nor is it attacked by diastase. But as *B. polymyxa* may use it as a food-substance, this species evidently can excrete an enzyme which dissolves it. It is not improbable that this enzyme is pectinase, but this question is not yet answered. Should this really prove to be true, then the other question arises whether the so-called pectose of the central lamellum of the tissues of the higher plants may not also be a cellulose modification, as it is also easily dissolved by pectinase. This view seems to be much more acceptable than the current hypothesis: the central lamellum should be the calcium salt of an acid, isomeric with arabin-acid.

On the great similarity between pectinase and the seminase of the seeds of the Leguminosae, I already earlier directed the attention. That the latter enzyme does not attack true cellulose is in accordance with the same property of pectinase.

SUMMARY.

With a not too limited species-conception *Clostridium polymyxa*, *Granulobacter polymyxa*, *Bacillus macerans*, and *Bacillus asterosporus* may be brought to one single species: *Bacillus polymyxa*.

It is the only hitherto known aërobic spore-former, which, in

neutral sugar-containing media excites fermentation and thereby proves able to live as a temporary anaërobe.

The chief products of the fermentation are carbonic acid and alcohol. At the aërobic life a little aceton results, evidently from oxidation of sugar.

Anaërobic accumulation is possible in rye paste at 30° C. after short boiling. Aërobic accumulation takes place in dilute malt-wort or broth with 2 % to 5 % sugar, after heating at 85° to 90° C. or short boiling with much garden soil and cultivation at 15° C. by which *B. mesentericus* is excluded, whose growth minimum is at about 20° C.

The general distribution of *B. polymyxa* in decayed plants and its occurrence in the bark of plant roots and of the nodules of the Leguminosae reposes on the production of pectinase, which dissolves the central lamellum of the cellular tissues.

B. polymyxa forms much slime from sugar, which must be considered as cell-wall substance. Without carbohydrates or polyalcohols its growth seems impossible, hence it develops but slightly on broth agar.

The slime may serve as reserve food.

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