Huygens Institute - Royal Netherlands Academy of Arts and Sciences (KNAW)

Citation:

Beijerinck, M.W., Fixation of free atmospheric nitrogen by Azotobacter in pure culture. Distribution of this bacterium, in:
KNAW, Proceedings, 11, 1908-1909, Amsterdam, 1909, pp. 67-74

This PDF was made on 24 September 2010, from the 'Digital Library' of the Dutch History of Science Web Center (www.dwc.knaw.nl) > 'Digital Library > Proceedings of the Royal Netherlands Academy of Arts and Sciences (KNAW), http://www.digitallibrary.nl'

Microbiology. — *"Fixation of free atmospheric nitrogen by Azotobacter in pure culture. Distribution of this bacterium"* By Prof. M. W. BEIJERINCK.

(Communicated in the meeting of May 30, 1908).

When carbon hydrates are used as source of carbon in *Azotobacter* cultures, there existed until now some doubt whether the then occurring fixation of free nitrogen was originally effected by *Azotobacter* itself or by other bacteria found in symbiosis with it, because *Azotobacter* in pure culture with carbon hydrates and free nitrogen only, does not show any considerable development.

For this reason I was formerly of opinion that in such cultures *Bacillus radiobacter*, a species closely allied to the bacteria of the Papilionaceae, and which is never absent in accumulations of *Azotobacter*, would be the real cause of the nitrogen fixation. [1]).

Continued research, however, rendered this supposition more and more improbable, and the facts which are now to be stated have proved beyond any doubt that the said faculty belongs indeed to *Azotobacter* itself.

These facts have regard to the very peculiar relation between *Azotobacter* and the salts of the organic acids, more in particular to calcium malate.

1. *Calcium malate as source of carbon.*

When into a wide ERLENMEYER jar a nutrient liquid is introduced of the composition: 100 tap-water, 2 calcium malate, 0.05 K^2HPO^4, with addition of some 10—20 cM^3 canal-water, or as much soil for infection, care being taken that the layer of liquid in the jar be not thicker than 2—5 cM, on cultivation in a thermostat at 30° C., usually after 2 or 3 days [2]) a floating *Azotobacter* film appears, consisting of strongly motile individuals, and relatively soon obtaining a considerable thickness. Hereby so much calcium carbonate is produced that it forms a closed, floating layer, so to say a cover, on the surface of the liquid.

If some of this film is inoculated into another jar containing the same medium, corresponding phenomena are seen when the culture

[1]) These proceedings of March 1901. Centr.bl. f. Bact. 2te Abt. Bd. 9 pg. 1, 1902. Archives Néerl (2) T. 8 p. 190 and 319, 1903.

[2]) Especially in spring and autumn these experiments succeed. In summer and winter *Azotobacter* seems sometimes absent in the said quantity of water.

conditions are alike. At first sight already, there can be no doubt but under these circumstances fixation of considerable quantities of nitrogen must take place, and chemical analysis proves that this is really the case.

The microscopic image of the *Azotobacter* growth in the malate commonly shows smaller individuals of greater motility than the formerly described forms which are obtained in the mannite solutions. They keep about the middle between *A. chroococcum* and *A. agilis*, and remind strongly of a variety found in America, which has received the name of *A vinlandi*. The plate cultures of such a malate accumulation again prove not to be pure but to consist of the usual mixture of non spore-forming species. They are best grown on a medium of the composition: 100 tapwater, 1 calciummalate, 0.05 K^2HPO^4, 1 to 2 agar, on which the *Azotobacter* colonies become already visible after 12 hours at 30° C., which is not the case with any other species of microbes known to me. As these plates are somewhat cloudy by the produced calciumphosphate and the imperfectly dissolved malate, it is desirable to mix the ingredients in the way as follows. Into a culture tube are first introduced some drops of a neutral, concentrated solution of kaliummalate and herein are dissolved both the calciummalate and the kaliumphosphate, with a little water to dilute, but the smallest quantity possible, as the dissolving power of the kaliummalate is much stronger in the concentrated than in the dilute solution. Then the contents of the tube are mixed with the agar solution.

The malate plates prepared in this way have proved to be better for the growth of *Azotobacter* germs than the mannite and glucose plates, so that of a definite number of germs there develop more to colonies on the former than on the latter. Hence it has become possible more exactly to compute the number of individuals of our species present in a sample of soil than after the old method, to which circumstance we return below.

Before going further I wish to notice the following concerning other salts of organic acids as carbon food for *Azotobacter*.

Except with calciummalate there could be obtained an abundant or moderate growth with calciumlactate, calciumacetate and calciumpropionate, particularly when using canal water for the first infection. It was remarkable that the transport of a malate culture into lactate appeared to succeed nearly as well as of malate into malate, while even relatively rich crude cultures in propionate- or acetatesolutions, obtained directly from soil or water when inoculated into corresponding media, hardly grow on, if at all. This fact is the more remarkable

when we consider that by inoculation of a malate film into propionate or acetate as abundant cultures are obtained as in the said crude cultures in these media. But if it is tried to continue such cultures by re-inoculating anew into propionate or acetate they also soon lose their power of growth. From this we see that the preceding culture conditions to which the inoculation material has been subjected, are by no means indifferent to the vitality of the following generations, which are evidently very easily weakened and then nearly quite lose the faculty of fixing nitrogen. The importance of this fact cannot be denied and certainly deserves a nearer examination.

Calciumcitrate, calciumtartrate and calciumsuccinate, with either garden soil or canal water for infection, give but slowly a moderately developed bacteria film but it grows during a very long time. The film on the citrate is rich in spirilla and the *Azotobacter* form found in it differs in many respects from the ordinary varieties. In all these cases the quantity of bacteria grown during the first 2 or 3 weeks, is still too slight to necessitate a determination of the nitrogen, and could by a rough comparison with former computations be valued at some tenths of milligrams N_2 per gram of the dissolved lime salt. After a long time however, the fixation of nitrogen with these salts is also considerable.

With calciumglycolate in absence of nitrogen compounds no growth of microbes could be observed at all.

2. *Quantity of the fixed nitrogen.*

Neglecting for the moment the volatile acid, to which we shall return below, the analysis of the cultures is performed as follows.

The whole quantity of the liquid, in which are present the calciumcarbonate formed by oxidation from the malate or the other organic salt, besides the as yet not decomposed malate, the salt of the volatile acid, and the bacteria, is treated with a known quantity of normal hydrochloric acid by which the carbonic acid is expelled on heating; a then following titration with normal alkali and phenolphtaleine as indicator, shows how much calciumcarbonate is produced and consequently how much of the organic salt is oxidised.

After addition of a little sulphuric acid the liquid is evaporated to dryness and after KJELDAHL's method examined on nitrogen, while in each of the materials used the rate of nitrogen is stated separately. The calciummalate of MERCK, Darmstadt, proved nearly free from nitrogen.

Now follows a table of some analyses [1]) which give an idea of the amount of nitrogen fixed through *Azotobacter*, when organic salts are used as carbon food. (See table).

These numbers show that the amount of nitrogen which can be fixed in the crude culture is at most 4.9 and 2,8 m.g. per gram of oxidised calciumsalt, obtained respectively with calciumpropionate and calciumacetate (experiment 10 and 11), while, per gram of calciummalate was fixed about 2,6 m.g. (experiment 2), and per gram of lactate 1,8 m.g. (experiment 9). It seems that the fixation goes on more rapidly at the beginning than later in the course of the experiment, whence it follows that when little of the organic salt is used proportionately more nitrogen is fixed than by larger amounts. This should be taken into consideration in judging the favourable results obtained with propionate and acetate, for then solutions were used with only 1% of the salt. As to these salts, they have proved to be in general an unfavourable source of carbon for *Azotobacter* if the *rapidity* of the growth is taken as indicator of the process, and only then to be able to give good results, when for the inoculation, cultures in malate solutions are used, in which a certain variety of our species is present. But also then, as observed above, already at the first passage from acetate into acetate the growth stops almost entirely. Pure cultures of *Azotobacter* develop hardly at all [2]) in solutions of calciumacetate and natriumacetate, whatever may have been the conditions to which these cultures were previonsly subjected. Propionates and lactates still require a nearer investigation.

Of calciummalate, on the other hand, it has decidedly been proved that not only the crude cultures succeed very well and fix much nitrogen even at repeated passages in the same medium, but that this also holds good with regard to the pure cultures of *Azotobacter*. This is the first case in which I got the certainty that no other microbes are wanted, neither in the medium nor in the infection materials, but *Azotobacter* alone to cause the said phenomena. Various authors surely have repeatedly described the fixation of free nitrogen in pure cultures of *Azotobacter*, among others of late with respect to the acetates, but never had I been able to confirm the accuracy of these statements until I made a systematic investigation with calciummalate, a salt which had never before been used to this end, although I had

1) I owe to Mr. D. C. J. MINKMAN, assistant to my laboratory the determinations here referred to.

2) The different varieties behave, however differently and some will begin to grow but the growth soon ceases.

already called attention to it as an excellent source of carbon for *Azotobacter* in my papers of 1902.

It must be allowed that the amount of fixed nitrogen in these pure cultures is not considerable, about 1.5 m.g. for each gram of oxidised malate, but perhaps here too, will be observed a greater production if only the very young cultures are examined; then, however, only little of the salt can be oxidised and the absolute quantities will of course be small.

It seems not superfluous here to call to mind that it is by no means the same whether a known amount of calciummalate be absorbed from a dilute solution or from a more concentrated one. In the latter case the malate will be more easily assimilable for the *Azotobacter* cells, which will induce a stronger oxidation and thus an increased oxygen assimilation in equal times, so that the tension of the oxygen in the liquid will be less than in the less concentrated solutions. As the growth of *Azotobacter* seems favoured by this lower tension, and in any case, a rather strong concentration of the carbon food proves favourable to the process of nitrogen fixation *in absolute quantity* this circumstance has been taken into consideration in all the experiments. Further, we did not always wait for the moment at which the malate had disappeared from the medium, but commonly it was much earlier subjected to the analysis for the reason mentioned above.

The observation that calciummalate can, glucose, cane-sugar and mannite on the other hand, cannot form the starting point for nitrogen fixation in liquid pure cultures, while yet the said carbon hydrates are in the crude cultures much more productive and may even give gains of nitrogen of 7 m.g. per gram of decomposed sugar, gives rise to the supposition that these carbon hydrates must previously be changed by other bacteria into organic acids and that these, at the moment of their production, serve as carbon food for *Azotobacter* and primarily cause the fixation of the nitrogen.

Of course it cannot be malic acid which hereby originates from the sugar; but the important growth of *Azotobacter* to which also the acetates, the propionates and lactates may give rise, suggest the question whether perhaps the acids of these salts may be first produced from the carbon hydrates and then govern the nitrogen fixation.

It is to be remarked that as well in the malate as in the lactate cultures slight amounts occur of a volatile acid, which will perhaps prove to be acetic acid, although it is has not been positively demonstrated by means of BEHRENS' uranylanatrium-acetate reaction. It is of importance to know that this volatile acid is not only found

in the crude, but also in the pure cultures of *Azotobacter*, so that it is certainly a product of this species itself.

In order to ascertain the amount of the volatile acid and the corresponding quantity of decomposed malate, it is supposed in the table to be acetic acid only and produced after the formula:

$$2C^4H^4O^5Ca + 2O^2 = C^4H^6O^4Ca + CaCO^3 + 3CO^2 + H^2O$$

Calciummalate Calciumacetate.

But it may also be formed without access of oxygen. The volatile acid is determined by distillation with sulphuric acid and silver sulphate and titration the distillate with normal alkali.

From the table we see that in the crude cultures nitrogen can without doubt be fixed with calcium acetate as carbon source. In truth we have not succeeded in effecting the same in pure cultures, but now that we have the certainty that *Azotobacter* alone, with malate as carbon food, is able to fix nitrogen, it must be admitted that this also holds good for the acetate cultures, although it is not clear of what nature is the assistance which other bacteria thereby must necessarily lend. Besides it should be noted that the fixation of nitrogen in the pure cultures, also when malate is used as carbon food, is less considerable than when other bacteria, too, can live on this substance at the same time.

3. *Distribution of Azotobacter in the soil.*

Earlier, already, I showed that it is possible to detect a few *Azotobacter* colonies among the thousands of those of the other species, when fertile garden soil is sown on mannite-kalium-phosphate plates. The use of calciummalate instead of sugar has proved to be of importance for the examination of the soil in this direction. First it should, however, be observed that no solid or liquid medium [1]) could be found on which all the germs of *Azotobacter* sown out really develop into colonies. Thus, by sowing about 2400 germs (determined by microscopic counting), on various culture plates, 50, 12, 1, 30, 8, 20, 10, 20 and 75 colonies developed so that the growth in percents was only 2, 0.6, 0.5, 0.3, 0.3, 0.8, 0.4, 0.8 and 0.3. In another experiment were obtained of 10.000 germs sown on glucose-calcium-malate plates, 20, 25 and 48%, and on calcium-kaliummalate-plates 32.5, 36 and 65%. But in other cases, on agar plates with malate only the results were much better. The germs had

[1]) The use of thin layers of liquid media for colony-culture of microbes has been described in Centralblatt f. Bacteriologie, 2te Abt. Bd 20, 1908, p. 641.

been shaken up in sterile tap-water or in malate solutions, of which 1 cm^3 was spread over the plate, care being taken that the water was quite taken up into the agar by its power of imbibition, which is easily effected by softly heating the plate so that the superfluous water evaporates.

We see from these data that *commonly* only a small part of the sown germs comes to growth. Whether perhaps the water itself has a deadly influence on some individuals, or that their death is caused by their passing on the solid medium, could not yet be made out by experiment. Thus, although there be ground to allow that more germs occur in the soil used than are found, the possibility exists that by continued investigation the experiment may be made so as to exclude that source of error.

But in spite of the uncertainty of the method the following result could be stated. By sowing a small quantity, for instance less than $\frac{1}{20}$ gram of garden soil on calciummalate-kaliumphosphate 1% agar, after 24 hours at 30° C. commonly no *Azotobacter* is observed, but a moderate number of moist colonies of about 1 mm. in diameter, first draw attention by their extension and prove to consist of different varieties of *Bacillus megatherium*, containing many spores. They dont cause any considerable oxidation of the malate and as the colonies no more grow after the second day, they evidently develop at the expense of the traces of nitrogen compounds which at first are present in the plates. After the second day a great number of *Streptothrix alba* appear. This microbe is so common in all the examined samples of soil that there can exist no doubt as to its either favourable or pernicious influence on the fertility; but the nature of this influence is as yet wholly unknown.

In a still later stadium the surface of the plate becomes covered with numerous relatively small colonies of bacteria, among which some species immediately draw attention by their extension and commonness.

The oxidation of the malate by all these microbes is slight, so that even after weeks the plates contain but little calcium carbonate, which seems almost entirely produced by the said larger colonies and by *Streptothrix*. All these species seem not to oxidise at all, or perhaps it is more accurate to say, not to oxidise any more after the last traces of fixed nitrogen have been assimilated. As to *Streptothrix*, from its relatively vigorous oxidising power it follows by no means that this should be associated with fixation of nitrogen; this species surely does not possess that faculty. If for the experiment soil is

used shaken from the roots of garden-plants, which are no Papilionaceae, the result is fairly the same; perhaps the number of the above mentioned oxidising forms is more numerous, but this is still doubtful.

Otherwise, however, is the result when the soil is examined which adheres to the roots of clover, pease, and beans when these plants are cautiously dug up. When the soil adhering to such roots is rubbed fine and after dilution in water sown on a malate plate we find, after a period of 2 days at 30° C., first that the said oxidising colonies have very abundantly developed. But, besides, among these colonies much larger ones are distributed, which oxidise much more vigorously and prove to belong to *Azotobacter*, which shows that a distinct relation exists between the distribution of this genus and the said Papilionaceae. Whether this relation will appear to be universal and what may be its signification, further experiments have to decide.

Chemistry. — "*Rapid change in composition of some tropical fruits during their ripening.*" By H. C. PRINSEN GEERLIGS.

(Communicated in the meeting of May 30, 1907).

Some tropical fruits which as a rule are gathered in a green and immature state and allowed to ripen afterwards, accomplish this ripening process so rapidly that within a few days they become tender, well-flavoured and palatable, thus offering a good opportunity for studying the still somewhat mysterious problem of the after-ripening of fruits.

I. Phenomena during after-ripening.

a. Banana (Musa).

As a rule the bunches of bananas, which contain fruits in various stages of maturity, are cut from the plant as a whole when all the fruits are still green and are hung up to ripen. At the moment when the bunch is cut none of the bananas are fit for food; they are hard, tasteless and flavourless, the skin is thick, contains much latex and tannin and adheres to the fleshy part. After a few days the skin becomes thin and yellow and can easily be detached, whilst the edible matter is now tender, sweet and well-flavoured. A couple of days afterwards the fruit is unpalatable again owing to overripeness and decay which change it into a soft mass.