XLIII.—*On an Acetic Ferment which forms Cellulose.*

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DURING my work on the chemical actions of *Bacterium aceti*, described in a previous paper (this vol., p. 172), I met with the peculiar acetic ferment commonly known as the "*vinegar plant*" or "*mother*." This ferment differed so much in appearance from any form of *B. aceti* I had noticed, that it seemed probable it was a distinct organism. In order to ascertain this, and also to enable me to study its chemical actions, I obtained pure cultivations by a combination of the fractional and dilution methods in the way I described in my previous paper. The nutrient solutions used for this purpose were composed of red wine diluted with half its bulk of water, and rendered acid with 1 per cent. of acetic acid in the form of ordinary vinegar. This liquid strongly favours the growth of the acetic ferments, and is at the same time very prejudicial to the growth of most other organisms.*

In order to be more certain of the purity of the culture, inoculations of it were made in gelatin and beerwort. In about ten days, well-defined colonies of the ferment commenced to grow in this solid nutrient mixture. Many of these colonies were transferred separately to suitable sterilised liquids, and in every case the characteristic growth of the "vinegar plant" appeared. There can be no doubt, therefore, that the cultures thus obtained were quite pure.

A pure cultivation of the "vinegar plant," when commencing to grow in a liquid favourable to its free development, is usually first noticed as a jelly-like translucent mass on the surface of the culture fluid; this growth rapidly increases until the whole surface of the liquid is covered with a gelatinous membrane, which, under very favourable circumstances, may attain a thickness of 25 mm. This membrane is slightly heavier than water, and when gently agitated

* In all experiments mentioned in this paper, the same methods were used, and the same precautions taken, with regard to the sterilising of culture fluids, cotton-wool, &c., as have been previously described by me (this vol., p. 173).

sinks until its upper surface is covered with liquid, when another layer of the ferment at once commences to grow above the old one. Frequently as many as five or six layers of growth are thus formed, causing the whole mass to appear striated when observed laterally. In colour, the membrane is white and translucent, unless grown in coloured solutions, in which case it assumes somewhat the colour of the liquid.

On removing the membrane from the liquid, it is found to be very tough, especially if an attempt is made to tear it across its plane of growth; parallel to this plane, however, it is much more easily divided into a number of layers, evidently representing successive stages of growth. In touch and general appearance, the "vinegar plant" has a remarkable resemblance to a soft animal membrane.

If the "vinegar plant" is grown in a liquid unfavourable to its free growth, such as yeast-water, it is first observed as a jelly-like, very transparent mass at the bottom of the solution; and this gradually increases in size until the surface is reached, and the liquid appears to be almost entirely filled with it. Close examination of this mass, however, shows that it is composed of extremely attenuated membranes of the ferment, which ramify through the bulk of the liquid, and actually enclose much of it, thus giving the jelly-like appearance.

When the ferment is grown in solid gelatin and malt-wort, spherical colonies are formed, but these appear only on the surface or a short distance from it. The colonies that grow on the surface gradually spread out into a film, resembling the ordinary membrane which grows on nutrient liquids. Gelatin is not liquefied by the ferment.

The membranous growth of the "vinegar plant" is the only form of growth which I have succeeded in developing. During my work with pure growths, I have made successfully more than 100 cultivations in solutions often varying very much in their chemical composition; but however much the character of the nutrient fluids, or other conditions of growth, were altered, the ferment always reproduced itself in the same very characteristic membranous form described above.

In the many pure cultivations of *B. aceti* I have made, no form of growth in the least resembling in microscopic appearance the tough gelatinous film of the "vinegar plant" was ever observed; the surface zooglœa form of *B. aceti* being thin and easily broken up by the least agitation. The chemical reactions of the two growths are also entirely different; a cold solution of potash at once completely disintegrates the pellicle of *B. aceti*, but the "vinegar plant" membrane will withstand boiling with this solution for many hours without appreciable change. The pellicle of *B. aceti* on treatment with concentrated sulphuric acid and iodine, gives no colour, but the "vinegar plant" is stained of a deep blue, similar reactions to these being also given by iodine dissolved in zinc chloride solution. Thus the membrane of the "vinegar plant" gives the characteristic reactions for *cellulose* (of which I shall show further on it is chiefly composed), whereas the zoogloea pellicle of *B. aceti* does not show any trace of this substance.

Previous writers who have described the membranous growth of the "vinegar plant" (*e.g.*, Zopf, *Die Spaltpilze*, p. 63, who evidently refers to it under the name "Essighautchen," and "Essigmutter"), have considered it to be a zoogloea form of *B. aceti;* but, from what I have said above, it will be seen that this is open to the strongest doubt. The true zoogloea form of *B. aceti* is an entirely different form, as I have just shown. Considering, therefore, that under so many different conditions of growth, the "vinegar plant" adheres to its one distinctive form, without ever approaching to that of *B. aceti*, and considering also that it develops a distinct chemical compound, cellulose, which appears to be entirely absent from *B. aceti*, it is impossible to come to any other conclusion than that the two organisms are specifically distinct.

When a membrane of the "vinegar plant" is examined microscopically, it is found to consist of bacteria, arranged more or less in lines, and lying embedded in a transparent structureless film. These bacteria are most commonly found as rods about 2 μ in length, several often being united together. The divisions, however, are readily seen when the growth is dried and stained with aniline-violet, which brings out the bacteria very distinctly, as the membrane in which they lie remains colourless. In old cultivations, the rods are often to a large extent replaced by micrococci about 0·5 μ in diameter (? spores). Sometimes, and more especially when the ferment has been grown in an unsuitable nourishing medium, like yeast-water, it appears as long twisted threads from 10—30 μ in length, and of a leptothrix nature. I have never observed with this ferment the peculiarly swollen involution forms so frequent in old cultivations of *B. aceti*. In the upper surface of an old membrane of the "vinegar plant," and also in the membrane grown on the surface of solid gelatin, cells are often found whose sides are much distended, and contain one or two highly refrangent nuclei resembling spores in appearance. These nuclei (or spores) are deeply stained by aniline dyes, and are often found free from any envelope.

In the liquid in which a membrane of ferment is growing, a few free swimming cells are always to be found, particularly if the membrane has been shaken, but these cells on cultivation always reproduce the membranous growth.

A temperature of about 28° appears to be most favourable to the growth of the "vinegar plant." Above 36°, it refuses to grow, but still lives.

The fermentations produced by this ferment show the same chemical changes as those produced by *B. aceti*, so far as I have investigated them. Ethylic alcohol is oxidised by it to acetic acid, and the acid so formed is afterwards entirely broken up. Dextrose is oxidised to gluconic acid; and lævulose is also formed from mannitol. Like *B. aceti* also, it has no fermentative action upon cane-sugar, starch, or lævulose. These results were arrived at in a similar manner to those obtained with *B. aceti*, and fully described in my previous paper, it is therefore needless to describe them further.

The great chemical difference between the "vinegar plant" and *B. aceti* is, as we have seen above, the presence of a membrane holding together the cells of the ferment, and which gives the usual reactions for cellulose. In order to ascertain with certainty the chemical composition of this substance, I proceeded in the following manner. A membrane of the ferment was taken, and after well washing with hot water, was boiled for 20 minutes with a 10 per cent. solution of caustic potash. This treatment did not appreciably affect the gelatinous film, but the bacteria embedded in it were disintegrated. After washing the film with dilute hydrochloric acid, and afterwards with water, it was treated with a solution of bromine, according to Müller's process for obtaining pure cellulose. After following out this method, the final product was a colourless semi-transparent film, retaining the shape and gelatinous character of the original membrane. When examined under the microscope, no trace of structural form could be detected. On treating a portion with ammonio-cupric oxide solution, it dissolved with ease, and the filtered solution, when acidified with hydrochloric acid, gave a precipitate exactly similar to that from the cellulose of cotton-wool when treated in a similar manner. Strong sulphuric acid dissolved the membrane without blackening, and on diluting the solution with water and boiling, a sugar was formed which reduced Fehling's solution.

A portion of the membrane was dried at 105°, and 0·2302 gram weighed out, and burnt with chromate of lead. This yielded 0·3737 gram CO_2 and 0·1295 gram OH_2. On calculation this gives—

		Cellulose $(C_6H_{10}O_5)_n$.
C.	44·26	44·44
H	6·25	6·17
O	49·49	49·39
	100·00	100·00

This analysis, together with the reactions mentioned above, leave no doubt that the membrane of the "vinegar plant" is *cellulose.* As the cellulose in a fresh membrane of the ferment is easily dissolved by ammonio-cupric oxide, this, according to Fremy and Urbain (*Compt. rend.*, **93**, 926), shows it to be cellulose proper, like that of cotton-wool; metacellulose, usually found in fungi, is insoluble under similar circumstances, even after treatment with acids. The envelope of yeast cells, usually said to be cellulose, is (according to Liebig) also insoluble in ammonio-cupric oxide.

A different ferment from the one with which we are now concerned, and known as *Leuconostoc mesenterioïdes*, is described as being enveloped in a gelatinous membrane somewhat similar in appearance to the membrane of the "vinegar plant;" but Scheibler has shown that this body is an insoluble modification of the gum, dextran, and decomposable by alkalis into the soluble form. I have examined both the membrane of the "vinegar plant," and also the solution in which it grows, but can find no trace of dextran.

Quantitative estimations (by Müller's process) of the cellulose in membranes of the "vinegar plant," grown under various conditions, show that it varies from 35 to 62 per cent. when calculated on the total weights of the original membranes dried at 100°.

This production of cellulose by a simple cell plant, and its use as a cell connecting medium, seems of great interest in view of the important part which cellulose plays in a similar manner in the more highly organised forms of the vegetable kingdom; and it appeared that any information that could be gained, as to the materials from which cellulose is formed by the "vinegar plant," might perhaps assist in better understanding the complex reactions which go on in the higher plants. To this end, my first experiments were made by inoculating the "vinegar plant" into sterilised solutions of Pasteur's mineral medium, containing respectively 3 per cent. of cane-sugar, dextrose, starch, and alcohol. These solutions were kept at a temperature of 28° for six weeks, but, with the exception of the solution containing dextrose, no growth took place. The dextrose solution developed a small but well-marked growth of the membrane. This set of experiments gives fairly good proof that dextrose can be converted into cellulose by the ferment; but the negative proof concerning cane-sugar, starch, and alcohol, is weak, as some cause other than the inability of these compounds to be converted into cellulose, might have prevented the ferment from growing. In order to get over this difficulty, recourse was had to yeast-water, in which solution the ferment is able to grow, but only, as I have stated above, in a very weak form. 1 gram of each of the carbohydrates to be used (viz., cane-sugar, dextrose, and starch), was dissolved in 100 c.c. of the same preparation of yeast-water, sterilised, and inoculated with the ferment; and at the same time two flasks, containing 100 c.c. each of the yeast-water alone, were inoculated. After 16 days, the flasks were opened and examined; the flask containing dextrose had developed a thick white membrane on the surface, but all the others showed a transparent jelly-like growth in the body of the liquid. The films of ferment in all the experiments were then removed carefully, and after washing with water, were treated with dilute potash and afterwards by Müller's process, in the manner described above. The pure cellulose films thus obtained were then dried at 100°, and weighed, with the following results:—

	Weight of cellulose.
Dextrose and yeast-water	0·0227 gram.
Starch and yeast-water........	0·0067 „
Cane-sugar and yeast-water	0·0080 „
Yeast-water alone No. I........	0·0053 „
„ „ No. II	0·0052 „

In considering these experiments, it is evident, from the extremely close agreement of the weight of cellulose derived from the two solutions of yeast-water alone, that 5·2 mgrms. may safely be taken as the amount due to the yeast-water when the ferment had developed in it. We can therefore fairly deduct this from the weights found in the other experiments, and thus ascertain how far the carbohydrates contained in the solutions may have been converted into cellulose. Thus we find that 17·5 mgrms. of cellulose had been formed from dextrose, whilst only 1·5 mgrms. of cellulose had been formed in the starch solution, and 2·8 mgrms. in the cane-sugar solution. These latter quantities are so very small, that when we consider that it is very difficult to obtain starch quite pure, and that the cane-sugar used is sure to have been slightly inverted by the repeated boilings necessary for sterilisation, I think we may safely conclude that the "vinegar plant" is unable to convert either starch or cane-sugar into cellulose, even when growing freely in their presence. The weight of the cellulose formed in the dextrose solution quite confirms the first experiment with Pasteur's solution, in showing that the ferment can convert this sugar into cellulose. The experiments I have just described are only one series out of two which I made, both of which pointed to a similar conclusion.

The formation of cellulose from dextrose by the ferment is noteworthy, as we have here a case of a simple cell having two totally distinct actions on the same chemical compound; for, as I have before said, the fermentative action of the cell upon dextrose is to produce gluconic acid by a fixing in some way of the oxygen of the air, whilst it also has the power of constructing the more complex molecule, cellulose, from the same substance. It is of course impossible to determine whether any one cell has these two powers at the same time, but I have determined by experiment that during the time that the cellulose membrane is growing, gluconic acid is formed.

The formation of cellulose by the "vinegar plant" from the carbohydrates must not be looked on as an act of fermentation, as it is evidently only a product of assimilation formed for the special use of the plant itself.

A series of experiments carried on in a manner similar to the one I have just described, was made to ascertain the action of the ferment on mannitol and on lævulose, the results of which are shown in the following table. 100 c.c. of the same yeast-water was used in each experiment:—

No.		Total weight of film, dried at 100°.	Cellulose in film, dried at 100°.	Per cent. of cellulose in film.
1	Dextrose ($C_6H_{12}O_6$ + OH_2), 2 grams..................	0·0339	0·0172	50·74
2	Mannitol ($C_6H_{14}O_6$), 2 grams..	0·1755	0·0948	54·02
3	Lævulose from inulin, 2 grams.	0·1777	0·1060	59·65
4	Lævulose from mannitol, 2 grams	0·1643	0·1015	61·17
5	Yeast-water alone............	—	0·0038	—

It is evident, from the above experiments, that mannitol and lævulose are far more favourable to the growth of the ferment than dextrose, and also that cellulose is formed from them more freely.

During the growth of the ferment in lævulose solutions, no trace of fermentative action was observed (neither acid nor alcohol was formed). In Experiment 4, an estimation of the lævulose was made in the solution with the polariscope, both before and after the growth of the membrane. 0·314 gram of lævulose had disappeared, whilst a membrane of ferment weighing 0·1643 gram, and containing 0·1015 gram of cellulose, had grown in the liquid. Not less than 0·113 gram of the sugar disappeared would be required for the formation of the cellulose found, the remainder being probably used for the growth of the ferment cells. The great difference between this experiment with lævulose and the experiment with dextrose—where true fermentation had been going on—will be evident by comparing the results of the analyses of No. 1 experiment with the above.

Here 1·111 gram of dextrose was decomposed, 0·720 gram of which was found as gluconic acid, the remainder having gone for the production of cellulose, food for the cells, &c. Yet—notwithstanding active fermentation having been carried on—the total weight of the ferment formed was only about one-fifth of that found when the ferment grew in a solution of lævulose, although no fermentation was apparent in the latter experiment. This seems to raise a question as to whether the "vinegar plant" derives any benefit itself from the oxidising fermentative power it possesses when grown in presence of dextrose, alcohol, &c., and whether this property may not be something entirely unconnected with the necessities of its growth. My experiments appear to show the possibility of such being the case, and I intend to investigate these actions further.

During the growth of the ferment in presence of mannitol, this substance is converted into lævulose; it is therefore not possible to say that mannitol itself can be converted directly into cellulose.

An experiment was made to ascertain if ethylic alcohol took part in the growth of the cellulose membrane of the "vinegar plant." In this experiment the ferment was grown in yeast-water containing 4 per cent. of alcohol; a cultivation being made in yeast-water alone at the same time. At the end of the experiment, the same weight of cellulose had been formed in both cases, thus showing that alcohol took no part in its formation. In the alcoholic solution, 1·20 gram of acetic acid was found.

The "vinegar plant" is frequently used in country places for the manufacture of home-made vinegar, by introducing a membrane of the ferment into a solution of coarse brown sugar. As a pure cultivation of this ferment has no action on cane-sugar, I procured a membrane that had been used for the purpose described above to examine its action. Microscopic examination showed the ferment to be much contaminated with ordinary yeast cells (*Saccharomyces cerevisiæ*). On introducing a portion into a cane-sugar solution, the yeast cells present inverted and fermented the sugar, forming alcohol which, after the first fermentation had ceased, was oxidised to acetic acid in the ordinary way by the growth of a membrane of the vinegar plant on the surface of the solution.

The "vinegar plant" has no distinctive scientific name, I therefore suggest, in consideration of its power of forming cellulose, that *Bacterium xylinum* would be a suitable name for this ferment.