Microbiology. — *"Emulsion laevulan, the product of the action of viscosaccharase on cane sugar"*. By Prof. M. W. BEIJERINCK.

In the proceedings of the Academy of 9 February 1910 an enzyme was described which produces from cane sugar and raffinose a viscous matter incapable of diffusion. My further investigations, made conjointly with Mr. D. C. J. MINKMAN, proved that this substance is closely related to the laevulan of LIPPMANN [1]) but not identic with it.

Our emulsion laevulan originates in watery nutrient solutions in quite the same way as in the agarplates, so that these solutions change into a milkwhite emulsion; the liquid between the suspending laevulan droplets opalises very strongly. In hot water the substance is fairly soluble and the specific rotation of the polarised light, which, on account of the opalisation can only approximately be determined, is about

$$a_D = -80°$$

whilst LIPPMANN gives for his laevulan

$$a_D = -221°.$$

On account of this considerable difference in its rotating power, a new name, e.g. "sinistran", might seem desirable. But the word laevulan having a collective meaning to which also the more and the less soluble forms of our substance may be brought, we shall here use the general denomination, the more so as it is sure that the laevulan of the literature, like ours, consists of the cell-wall substance of bacteria.

Besides by LIPPMANN the formation of laevulan by bacteria has also been observed by MAASSEN [2]), who does not, however, describe the appearance of the emulsion, so that in this case, too, a modification of our emulsion laevulan seems to be produced. The here concerned microbe is a sporulating fermentation organism, called by MAASSEN *Semiclostridium commune*, but not yet found by us.

Preparation and properties of emulsion laevulan.

We were first of opinion that emulsion laevulan might be best prepared by using *Bacillus emulsionis*, for we had stated that this species does not decompose the once formed laevulan, whilst *B. megatherium* and *B. mesentericus*, which likewise produce emulsion

[1]) Chemie der Zuckerarten 3te Aufl. 1904. Pag. 906, 1312.

[2]) Arbeiten aus dem Kaiserl. Gesundheitsamte. Biol. Abt. Bd. 5, p. 2, 1905.

laevulan, attack this substance and use it as food as soon as the cane sugar fails. We have, however, found that with some precaution it is much easier, especially with *B. mesentericus*, to produce large quantities of laevulan, than with *B. emulsionis*; this reposes on the circumstance that the former species, particularly at high temperatures, about 40°, possesses a very strong vegetative power, whilst the latter always grows slowly and has a relatively low temperature optimum, below or near 30° C.

Hence we used for the preparation of laevulan the common hay bacterium, which is the form of *B. mesentericus* obtained by accumulation methods, such as the method of potato slices and that of malt solutions. But this form is so common in our surroundings and so well adapted to the life in cane sugar solutions of for the rest different composition, that these, after pasteurisation or short boiling and when kept warm, of themselves produce laevulan by the development of the spontaneous spores of the hay bacillus. Such solutions then turn milky and slimy by the formation of the microscopic laevulan emulsion.

For the experiments were used large ERLENMEYER-flasks with 500 cm^3 of a medium of the composition: tapwater, 20 % canesugar, 0.05 % KNO_3, and 0.05 % K_2HPO_4, cultivated at ± 27° C.

This liquid inoculated with *B. mesentericus* very soon obtains the said milky appearance. The same emulsion which to the colonies of *B. mesentericus* and *B. emulsionis* on cane sugar agarplates gives so peculiar a character, is now in large quantity produced in the culture liquid, saturated besides with laevulan in true solution, which causes the strong and characteristic opalisation, not known to us to such a degree in any other substance. Besides, at the bottom of the flasks a thick transparent slime layer is slowly formed, which also proved to consist of laevulan, wherein, however, the bacterial bodies themselves are accumulated, whilst the liquid above it is poor in bacteria but abounds in viscosaccharase and laevulan emulsion. The acid formation in this solution is slight but not absent.

The laevulan may be precipitated with alcohol for which 50 % in the solution is sufficient. Only at a much greater alcohol concentration other substances of the liquid also precipitate. By dissolving in boiling water and again precipitating the further purification is easy. After drying and pulverising a snowwhite nearly tasteless powder results.

From a flask as the above which at first contained 100 G. of cane sugar, 8 G. of pure dry laevulan were obtained after 7 days cultivation, there still being in the liquid 20 G. of invert- and 70 G. of cane sugar; the slime at the bottom not being collected.

From another flask quite alike to the preceding, which also contained 100 G. of cane sugar, were gained 15 G. of laevulan after 17 days, 45 G. of cane sugar and 35 G. of invert sugar still being present.

The slime adhering to the bottom, consisting of *B. mesentericus* with thick cell-walls of laevulan, was used for a new culture for which a solution of 2 % of cane sugar, 0.05 % KNO_3 and 0.05 % K_2HPO_4 was used. After 18 days were obtained 2.25 G. from the 10 G. of original cane sugar, accordingly 22,5 % of laevulan was earned.

Pure laevulan is somewhat soluble in cold water, much better in boiling; all solutions opalise very strongly. It does not reduce FEHLING's coppersolution; only after prolonged boiling a feeble reduction is observed. It is incapable of alcoholic and lactic acid fermentation, but by butyric acid ferments, in absence of air, it gets into as strong a fermentation as cane sugar, whereby hydrogen, carbonic and volatile acid result.

A number of bacteria can feed on it when growing with access of air. *Azotobacter chroococcum* can use it under fixation of free nitrogen and formation of some acid.

By a treatment with acids, especially when warm, it changes readily into laevulose and so becomes fit for alcoholic and lactic-acid fermentation. After the inversion, by heating with resorcine and strong hydrochloric acid, the red colour appears, characteristic of laevulose, whilst with orcine and hydrochloric acid the violet colour, indicating pentose, is completely absent. When distillated and treated with sulphuric acid no perceptible quantity of furfurol can be detected.

As said, the specific rotation, which cannot be exactly determined on account of the strong opalisation is

$$\alpha_D = -80°,$$

and after hydrolysis

$$\alpha_D = -70°.$$

After prolonged heating with acid in the autoclave at 120° the rotation lowered even to

$$\alpha_D = -64°.$$

That of pure laevulose is

$$\alpha_D = -92°.$$

There is some probability that this diminution is due to destruction of part of the laevulose.

As we had found that the slime at the bottom of the flask is less soluble than that obtained by alcohol from the emulsionated liquid above it, we prepared laevulan from this slime also by separate experiments, for we supposed that dextran might occur therein, which is much less soluble in water than laevulan. However, it was found that the laevulan obtained in this way gives no other rotation after inversion than the emulsion laevulan, from which it does not differ. Hence it is sure that hay bacteria produce no dextran at all, but that their cell-wall consists of various modifications of laevulan of different solubility.

Not only in media of the above composition *B. mesentericus* produces laevulan, this happens quite as easily in a yeast decoction with 2 to 20 % of cane sugar, addition of chalk proving favourable. The temperature of cultivation may also vary and even rise to 40° C., but then care should be taken that the laevulan itself be not attacked by the producer.

From the preceding it may be concluded that the large lumps of slime so easily formed on cane sugar agar-plates by *B. mesentericus* and the other emulsionating species consist as well of laevulan as the emulsion which occurs round the colonies of this species in the agar. Hence, it can neither be doubted that the slime of these colonies, which does not diffuse in the agar, is produced by viscosaccharase from cane sugar, and that this enzyme only partly gets out of the bacterial body proper, the cell-wall included. Evidently in the cell-wall itself the enzyme forms new laevulan by converting the cane sugar, with which both cell-wall and agar-plate are imbibed.

The production of cell-wall substance in consequence of the action of an enzyme, which in my former communication was called probable, must now, as regards laevulan, be considered as proved.

Dextran and the dextran bacteria, which we have likewise studied, shall later be treated more thoroughly. For the moment it may be observed that by this substance the polarised light is strongly rotated to the right; we found

$$\alpha_D = + 132°,$$

whilst in the literature by various authors is given for dextran

$$\alpha_D = + 199° \text{ to } 230°.$$

Quite like laevulan it results exclusively from cane sugar. So laevulan as well as dextran are produced by microbes, neither from laevulose, glucose, or any other sugar, but solely from cane sugar and raffinose. The slimy cell-wall substances formed by other microbes from glucose, laevulose and maltose, are of a different nature.