Bacteriology. — "*On Indigo-fermentation*". By Prof. M. W. BEIJERINCK [1]).

At a former occasion it was demonstrated [2]) that the indigo-plants may be brought to two physiologically different groups, viz. indoxyl-plants, to which the woad (*Isatis tinctoria*) belongs and indican-plants. Of the latter, which seem the most numerously represented, were examined *Indigofera leptostachya*, *Polygonum tinctorium* and *Phajus grandiflorus* [3]). The result was that they contain specific enzymes differing from one another, which split the indican into indoxyl and glucose, while in woad there is no such enzyme. Indican can moreover be decomposed by katabolism [4]), i. e. by the direct action of the living protoplasm, which has been observed in some indicanplants, beside enzyme-action. Various microbes, too, can decompose indican and here the decomposition is generally effected by katabolism only; some species, however, contain specific indigo-enzymes. Hence the word "indigo-fermentation" means two quite different processes: a katabolic and an enzymatic process, and the enzymes are of twofold origin, products of higher plants and products of microbes. It is clear that in the formation of indigo from woad, in which no glucoside but free indoxyl occurs, there can be no question of „indigo-fermentation".

1. *Preparation of the Indican as used for the Experiments* [5]).

For the preparation of indican-solutions from indican-plants, a method was described (l. c. p. 122) the principle of which is so quickly to destroy the enzyme that the glucoside can be dissolved without decomposition [6]). This is best done for *Indigofera* and *Polygonum* by immersion in boiling water, by which an extract is obtained of 0.5 to 1 pCt. indican, which as such, or after mixing

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[1]) I am indebted to Mr. J. F. B. VAN HASSELT and Mr. A. VAN DELDEN for assistance in the following study.

[2]) "On Indigo-formation from the Woad (*Isatis tinctoria*)". Proc. Royal Acad. of Sciences. Amsterdam, Sept. 30, 1899, p. 120.

[3]) Received under this name from a horticultural institution.

[4]) For this expression see: Centralbl. f. Bacteriologie 2e Abt. Bd. 6. p. 5, 1900.

[5]) Further informations about the indican and the enzyme of *Indigofera* are found in the recently published interesting paper of Mr. J J. HAZEWINKEL, Maandelijksch Bulletin van het Proefstation voor Indigo, Klaten (Java). Aflevering I, Januari 1900, Samarang.

[6]) For the production of many other glucosides the same method can be applied.

with gelatine or agar is fit for bacteriologic or enzyme experiments.

The leaves of *Phajus grandiflorus* decompose the indican at high temperatures with so much energy, that the extraction by boiling does not produce indican but indoxyl, so that I first took *Phajus* for an indoxyl-plant. In this case, in order to perform the experiment at low temperature without indican decomposition, the preparation should be effected in presence of an enzyme poison which does not act on indican. To this effect the leaves are rubbed down in caustic lime or baryta, then filtered and carbonic acid passed through; after filtering again a very pure indican-solution is obtained [1]). The leaves can also be boiled in diluted ammoniac and the superfluous ammoniac be removed by evaporation. Another method is to crush the leaves under alcohol by which the enzyme, though not destroyed, precipitates in the cells, while the indican dissolves in the alcohol and after evaporation of the latter can be taken up in water.

By evaporating the solutions to dryness, the impure indican results as a brown mass, resembling sealing-wax, which can be powdered and, in dry condition, be kept unchanged an unlimited length of time. The crude, neutralized or feebly alkaline-solutions, when sterilized and preserved from the access of microbes, also remain unchanged for many months [2]).

A purified indican-preparation is obtained from the decoctions by evaporating them to dryness with caustic lime or baryta, dissolving in little water, filtering, passing through carbonic acid or precipitating the baryta with aluminium sulphate, then again filtering and evaporating to dryness. The thus formed preparation contains fewer pigments and fewer proteids than the crude solutions.

The impure or thus purified indican is fit for mixing with a solid medium destined for microbe-cultures. On such "indican agar" or "indican gelatine" poured out to plates, colonies or streaks of microbes produce or do not produce indigo, according to the species. Of this later more.

For our experiments we used the decoction or the crude indican prepared from it, either or not purified with lime, of *Polygonum tinctorium* and *Indigofera leptostachya*, cultivated partly in the garden of the Bacteriological Laboratory at Delft, partly at Wage-

1) The extraction with caustic lime has also been applied by Mr. HAZEWINKEL for *Indigofera*.

2) But after a very long time the amount of indican diminishes when air finds access. When air was excluded I could note no change in the solutions.

ningen, and kindly procured by Mr. VAN LOOKEREN CAMPAGNE. I also received from Mr. HAZEWINKEL of Klaten, Java, perfectly well preserved extracts of *Indigofera* in tins, together with crude enzyme prepared from this plant.

2. *Preparation of the Enzymes.*

For this preparation I followed the method pointed out before (l. c. pag. 124). The plants are rubbed fine in a mortar under alcohol and during the rubbing the alcohol is a few times renewed. In the beginning alcohol of 96 pCt. is taken, which is sufficiently diluted by the juice of the plant, but afterwards some water is added as otherwise the chlorophyll-pigment cannot be completely extracted from the granules. I suppose this must be explained by the strong water-attracting power of the alcohol, which produces from the protoplasm a proteid, impervious to the chlorophyll pigment and possibly to the alcohol itself, but which, by water, becomes again permeable. In this operation the indigo-enzyme is precipitated in the cells and this occurs so quickly that the indican, which is soluble in alcohol has disappeared before its decomposition can set in. As by this method the chlorophyll is completely extracted by alcohol, a colourless product is obtained, which, after drying, first at 37° C. and then at 55° C., is a snow-white powder, directly, or after further pulverising, fit for enzyme experiments. In stoppered bottles I have kept such preparations for months without observing any decrease of activity [1]).

As, in the preparation of the indigo-enzyme from *Polygonum tinctorium* decomposition of the indican occurs much more easily than with *Indigofera*, it is necessary, in order to get colourless preparations from this plant, to proceed with greater precaution and to kill the protoplasm more quickly. This is done by taking only a small quantity of leaf substance at a time for the rubbing in the mortar so that the alcohol can penetrate in a few seconds. With *Indigofera* much larger quantities of leaves may be taken, without fear of obtaining preparations coloured by indigo.

As I could not point out by the ammoniac-experiment, the presence of free indoxyl in *Polygonum* leaves, I thought at first that the

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[1]) The loss of activity in enzyme preparations may be compared to the loss of germinating power in plant seeds. If they are kept in complete absence of water, both, the activity of enzymes and the germinating power of seeds, will last an unlimited length of time.

difference was to be explained by admitting that the enzyme of *Polygonum* is more soluble in water than that of *Indigofera* and so, during the extraction could perhaps in higher concentration act on the indican. But the experiment showed that this is not the case. Neither can the acid reaction of the juice of *Polygonum*, caused by kalium bioxalate, account for this difference, as the addition of this salt, kalium biphosphate, or of a little acid, to the materials used for the preparing of the enzyme from *Indigofera*, produces no change in the course of the phenomena. The addition of asparagine is likewise without effect. Nor is the explanation to be found in the relation of both enzymes to the temperature. I have so come to the conclusion that in *Polygonum* part of the indican is decomposed by the direct action of the living protoplasm itself. This part is however small, and by quickly immersing in boiling water the protoplasm is killed before it causes decomposition.

In the preparation of indigo-enzyme from *Phajus grandiflorus* nothing particular is observed. But we saw before that the decoction method produces no indican but indoxyl from this plant.

As the figure below shows that the enzyme of *Phajus* becomes inactive already at a lower temperature (67° C.) than that of *Indigofera* (75° C.), I must admit that also in the leaves of *Phajus* katabolism exists together with enzyme action and that, at the immersion in boiling water, simultaneously with the dying of the protoplasm, this katabolism causes a vigorous indican decomposition [1]). Hence *Polygonum* and *Phajus* agree in so far as in both indigofermentation is caused by katabolism and by enzymes; but they differ in the fact that in *Phajus* the katabolism is quickened by high, in *Polygonum* by low temperature. In *Indigofera* katabolism seems not to occur at all and the decomposition of indican appears exclusively effected by the enzyme.

From the preparations obtained in the way described, the enzyme itself can but be imperfectly extracted. In water it proves almost quite insoluble, somewhat better in glycerine and best of all in a 10 pCt. solution of common salt, as was already indicated by Mr. HAZEWINKEL, and in a 10 pCt. solution of calcium chloride. In these solutions only a small quantity of enzyme is soluble, for the remaining substance is nearly as strongly active as before the extraction. In the solutions themselves alcohol produces hardly any precipitate, so that more active preparations cannot be procured in

[1]) In § 3 p. 513, will be demonstrated that all the indican is localised in the rotoplasm.

this way. Accordingly the best results in the enzyme experiments are obtained by crude enzyme finely powdered.

3. *On the Distribution of Indican and the Indigo-enzymes in the Plants.*

By the examination of the different parts of indigo- and other plants in the two ways described, the distribution of the indican and the indigo-enzymes was established. It was thus made evident that both commonly occur or lack together.

They are accumulated in the leafy organs, especially in the green leaves; in flowers and flower-buds they are in smaller quantity. In the seeds and germs they fail entirely. The roots and stems of *Polygonum tinctorium* and of *Indigofera leptostachya* are also quite or nearly quite devoid of indican and indigo-enzyme. Only in transverse sections of branches of the latter, kept for some days in strongly diluted indican solution, I could detect traces of indigo-blue particularly in the medulla and the medullary rays and in the bark, which shows that these parts contain some, but very little indigo-enzyme. The absence of enzyme and indican in the stem and roots of *Polygonum tinctorium* can be easily shown as the stems of this plant have a great disposition to form radiculae which are, as the stems, by their herbaceous nature and broad-celled structure, quite fit for such experiments. If the roots are allowed to die off in a chloroform-atmosphere they remain colourless; this is likewise the case when the dying is occasioned by immersion in mercury followed by treatment with ammoniac vapour. But from this follows only that indican and enzyme do not occur together; if but either of them is present it is not detected by this experiment [1]), but may be demonstrated as follows.

If indigo-enzyme is added to a decoction made from the stems or roots of *Polygonum tinctorium*, or if this decoction is boiled with hydrochloric acid and a little ferrichlorid to decompose the indican and oxidise the indoxyl, then no indigo appears; so, indican is absent.

That in the said parts indigo-enzyme, too, is wanting follows from the fact that parts of stems and roots finely crushed in alcohol, after filtering off and drying, produce a powder quite inactive on indican-solution. Even the growing point and the region of growth of the

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[1]) This should be kept in view with regard to the "alcohol-experiment" of Mr. Molisch.

roots contain no enzyme [1]), as thin slices killed in alcohol, remain quite colourless in indican-solution at 45°. The same is the case with entire roots which, after killing in alcohol, are put in indican-solution.

From these facts seems to follow that the growth and development of indican-plants is not in inseparable relation to the presence of indican and enzyme.

To this result we are also led concerning the relation between the development and the presence of indoxyl in the woad, though its distribution in this plant is somewhat different from that of the indican. In woad the indoxyl occurs, besides in the young leaves, and buds, also in the young rootperidermis, in the root-buds and in the growing root-ends [2]). The distribution of the indican agrees with that of the indoxyl in the fact that they are both completely wanting within the thicker stems and all the thicker roots. So there is in woad no indoxyl in the inner part of the stem organs of the leaf-rosettes in spring, when they are ready to elongate and push out the inflorescence which is then in the very period of the most intensive cell-partition and cell-elongation. Likewise, there is no indoxyl in the cambium and the secondary tissues of the woad-roots. Even the flower-buds are in an early period, and when still growing vigorously, free from indoxyl; likewise the embryos, seeds and fruits. First at the germination indoxyl can be pointed out in the seeds and other parts of the germinating plant. So it is very probable that neither indican nor indoxyl are necessarily related to the growth or development of the indigo-plants. But the possibility remains that in certain cases these substances originate as quickly as they disappear. So, in the young leaves of *Indigofera leptostachya*, when kept some days in the dark, a little indoxyl may be detected by means of the ammoniac-experiment, while the normal plant is in all its parts quite free from indoxyl, whence it seems possible, that in normal conditions, there is a continual splitting of indican, which is not observable only because the freed indoxyl directly forms indican again with freshly supplied sugar. For the rest, the woad, of which all full-grown parts are devoid of indoxyl, proves that this substance can relatively quickly disappear.

The appearance of indican, particularly in the peripheric parts of the aerial organs, and the bitter taste it gives them, might suggest

[1]) While in the stem these parts are extremely rich as well in indican as enzyme.

[2]) Which shows that the formation and accumulation of indoxyl is possible in the dark as well as in the light.

the idea that, like tannin, it serves as a defensive against insects and snails. But this supposition would explain only the function of the indican but not that of the splitting products and the indigo-enzyme. If a beneficient influence on the growth in general could be ascribed to indoxyl, then a useful action of this substance on the curing of hurt parts would become probable. And this would also spread more light on the function of the indican and the enzyme, for then it would be clear that the enzyme-action, which operates at the very dying off of the hurt cells, would promote the curing, not only by the formation of indoxyl but also by the production of glucose.

As to the localisation in the cell, I found the leaves of *Phajus grandiflorus* by their broad-celled structure fit for demonstrating microchemically indican as well as indigo-enzyme.

The indican can be precipitated as indigo-blue or indigo-red, and both ways point out that it is present in the protoplasm and wanting in the cell-walls, cell-nuclei, and cell-sap. To demonstrate this a not too thin microscopic transverse section of a leaf is put in living condition in a boiling mixture of strong hydrochloric acid and ferrichloride. The indican is suddenly decomposed and the freed indoxyl as quickly oxidized into indigo-blue, which is easily detected under the microscope as a precipitate in the shape of small blue granules in the colourless protoplasm of the green parenchyma and the epidermis. I could not trace it with certainty in the chlorophyll-granules.

If the sections, in living condition, are put in a boiling mixture of hydrochloric acid and isatine, the indican passes into indigo-red, which sets off in the protoplasm as very characteristic red crystal needles [1]).

The enzyme, on the contrary, is exclusively accumulated in the chlorophyll-granules as is proved by the following.

If living microscopic sections of leaves of *Phajus* are put in an indican-solution (e. g. in a decoct of *Indigofera* or *Polygonum*) they become blackish blue in a short time, which colour is exclusively caused by indigo-blue precipitated in the chlorophyll-granules. In the epidermis much indigo is precipitated only in

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[1]) The presense of indoxyl in urine may be shown with much more certainty and exactness in the form of indigo-red than of indigo-blue. To this end the urine is boiled with hydrochloric acid and isatine by which the colour grows red. At cooling the indigo red crystallises in characteristic microscopic needles. These are easily filtered and dissolve beautifully red in alcohol (best is to boil out the whole filter with alcohol).

the cells of the stomata, elsewhere none at all. If the microscopic sections are beforehand killed and extracted with alcohol, the enzyme spreads in the cell but remains confined within the cell-walls, so that, by putting them into an indican solution they become of a uniform intense blue, in which only the bast bundles remain colourless.

The accumulation of enzyme in the chlorophyll-granules is perhaps connected with the formation of starch from the glucose of the indican.

As to the localisation of indoxyl in the leaves of woad I have acquired no certainty, but I suppose that, like indican, it occurs only in the protoplasm.

The hypothesis of Mr. MOLISCH [1], according to which indoxyl and indican should be in close relation to the decomposition of carbonic acid in the chlorophyll, appears contrary to the great accumulation of indoxyl in the root-peridermis, which is completely free from chlorophyll, and in the colourless root-buds of the woad, which seems unnoticed by Mr. MOLISCH. Nor do I think his arguments and figures convincing for the occurrence of indoxyl and indican in the chlorophyll-granules; moreover was Mr. MOLISCH unacquainted with the existence of indigo-enzymes and their localisation.

Elsewhere than in the indigo-plants indigo-enzymes seem but seldom to occur. Like Dr. VAN ROMBURGH [2] I observed that emulsine of almonds decomposes indican, and in § 6 the intensity of this action is graphically represented in connection with temperature.

The said fact may serve to demonstrate in a simple way the localisation of emulsine in almonds. If thin sections of the seedlobes are put in an indican-solution at 50° C., the vascular bundles will first take a deep blue colour, which shows that there the emulsine is the most accumulated. Then the parenchyma around them grows blue, and finally the more peripheric parenchyma. This points out that the emulsine is nowhere wholly absent but is accumulated about the confines of the central-cylinder, which becomes distinctly visible by this experiment [3].

A rather great number of other plants examined for indigo-enzymes have all given negative results [4].

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[1]) Berichte der deutschen Bot. Gesellschaft, Bd. 17, p. 230, 1899.

[2]) Communicated by Mr. HAZEWINKEL, Maandelijksch Bulletin N°. 1, pag. 8.

[3]) Nearly the same has been found by JOHANNSEN, who examined the decomposition of amygdaline with separate parts of the seedlobes. (Ann. Sci. Nat. Botan. Série 7, T. 6, p. 118, 1887).

[4]) So I could not find indigo-enzymes in: *Indigofera dosua*, *Polygonum persicaria*,

Neither is indican decomposed by sections of branches or leaves of apricots, pears, apples, peaches, while in the kernels of the fruits of these species a feebly decomposing emulsine is found.

Malt, malt-diastase, pancreas, papayotine, pepsine and saliva are inactive; likewise mustard-seed and myrosine prepared from *Tropaeolum majus.*

Glucase, from maize does not decompose indican, which is the more noteworthy as amygdaline is decomposed by it.

4. *Decomposition of Indican by Microbes in general.*

Mr. MOLISCH has drawn attention to the fact, that various species of microbes give rise to indigo-formation from indican and that others do not, which may be rendered useful for differential diagnosis. He experimented with the decoct of *Polygonum tinctorium* or *Indigofera* mixed with agar or gelatine, pouring it out to plates and using these as a solid nutrient. Aerobics and temporary anaerobics from the soil or from canal water sown out on it will develop, and in and around the colonies which split the indican, indigo-blue will separate out in microscopic lumps or globules which often show crystal structure. The „indican microbes" are in this way elegantly distinguished as pigment-microbes among the non-decomposers. [1])

The indican, as a powder, may be added in a percentage of 0.5 to 1 pCt. to solid or liquid nutrients, adapted for the examination of specific mikrobe groups.

P. aviculare, P. fagopyrum, P. bistorta, P. sacchalinense, Trifolium repens, T. pratense, Medicago sativa, Lotus corniculata, Pisum sativum, Vicia faba, Robinia pseudoacacia, Baptisia australis, Melilotus caeruleus, Spiraea filipendula, S. ulmaria, Rubia tinctorium, Asperula odorata, Solanum tuberosum, Amsonia salicifolia, Asclepias cornuti, Scorzonera hispanica, Linaria vulgaris, Stellaria holostea, Cochlearia armoracia, Brassicca oleracea, Isatis tinctoria, Iris germanica.

[1]) Sitz.ber. der Akad. d. Wiss. zu Wien. Math. Naturw.Classe, Bd. 107, p. 758, 1898. Mr. MOLISCH enumerates the following species as decomposing indican: *Bacillus anthracis, B. prodigiosus, Streptothrix odorifera, S. dichotoma, Sarcine lutea, Penicillium sp.* and *Mucor mucedo*; as non-decomposing: *Streptococcus pyogenes, Staphylococcus pyogenes aureus, Bacillus subtilis, B. coli communis, B. fluorescens liquefaciens, B. megatherium* and pressed yeast. Mr. VAN HASSELT and I saw no decomposition with *Acetobacter aceti, A. ranscens, Bacillus cyaneus, B. cyanogenus, B. pyocyaneus, B. diastaticus, B. prodigiosus, B. pseudotuberculosis.* Many spore-forming bacteria, such as *B. subtilis, B. megatherium, B. pulcher, B. mesentericus* and others sometimes decompose and sometimes do not. Further there is no indican splitting by beer-yeast (*Saccharomyces cerevisiae*), wine-yeast (*S. ellipsoideus*), pressed-yeast (*S. panis*), *S. mycoderma, S. passularum, S. uvarum, Schizosaccharomyes octosporus, S. pombe* and by the following moulds: *Aspergillus niger, A. oryzae, Amylomyces rouxii, Mucor oryzae, Oidium lactis, Endomyces magnusii.*

I found that some species decompose indican with extraordinary facility. Especially the common ferment-bacteria of plant infusions, which of late I united in the genus *Aërobacter*[1]), decompose with so much intensity, that they may with some reason claim the name of „indigobacteria"; they will later be discussed in particular. For the species which split with more difficulty this power depends on circumstances not yet quite clear to me. It may occur that in pure cultures colonies of one and the same origin, and separated from the common stock by a few generations only, behave quite differently, so that species, which for a long time I considered as non-decomposing, later proved vigorous indigo-producers. This I observed for instance in the photogenic bacteria of the Northsea. I suppose this fact to be connected with the influence of the sugar freed at the splitting of the indican, as other experiences prove that this influence is not constantly the same for all individuals of a species. That especially glucose acts vigorously on the life of some bacteria, and, even in small quantities, e.g. 0.05 pCt. to 0.1 pCt. may be a violent poison for some photogenic bacteria, I proved before, and this is noteworthy as still smaller quantities are favourable to the same species.

That the different conditions of the bacteria may be of influence on their power for decomposition, follows for instance from the fact that *Bacillus radicicola*, from the tubercles of *Pisum sativum* and *Trifolium*, decomposes the indican, while this is not done by the bacteroids of the tubercles of these plants. Closely allied species may also behave differently; thus, *Bacillus ornithopodis*, from the root-tubercles of *Ornithopus sativus*, does not decompose at all and, among lactic-acid ferments, I observed vigorous decomposition by the rod-shaped ferments used in the yeast-industry (*Lactobacter longus*), and no decomposition by the diplococci and streptococci (*L. lactis*) of the dairy industry. The ease with which this reaction is effected and its clear result recommend it for further research.

The splitting of the indican by microbes is operated in the same way as in indigo-plants, either by katabolism, i. e. by direct ferment-action of the living protoplasm on the indican, or by specific indigo-enzymes. Consequently the forms belonging to the former group decompose the indican in living condition only[2]), those of the latter both living and dead. The experiment, demonstrating this, may be performed as follows.

[1]) Centralbl. f. Bacteriologie, 2e Abth. Bd. 6, N°. 7, pag. 193, 1900.

[2]) The optimum temperature of the decompositon by katabolism agrees, for the examined species, with that of the growth.

Of a culture, grown on a solid nutrient substratum with copious access of air, some material is put on a glass-slide and killed in such a way that eventually present enzyme remains unhurt. This may be done by immersing the material in strong alcohol, in which it should remain at least 24 hours to be quite sure that the microbes are killed, or by exposition to ether-, alcohol- or chloroform-vapour [1]). In the latter case the microbe-material is placed in a glass-box beside a vessel with chloroform, where ferments moulds, and most bacteria die after $^1/_2$ to 1 hour already, while the enzymes in the cells remain unhurt.

If a small lump of killed microbes is put in an indican-solution, poured out to a thin layer in a white porcelain vessel floating on water of circa 45° C., then only those microbes will become blue, which contain indigo-enzyme, while those, acting by katabolism, don't cause decomposition. If in the latter case not all but only most of the microbes have been killed, there will at first be no manifest decomposition, but it will set in as soon as the living individuals have sufficiently multiplied, which is at the same time a good control of the experiment.

The microbes containing enzymes can be dried and powdered after killing and such "crude enzymes", when kept dry, preserve their activity very long. By the little dissolubility of the indigo-enzymes in water, glycerine and salt-solutions, it was not possible by extracting the crude enzymes and precipitating with alcohol, to obtain more active preparations from them.

It has been proved that all examined bacteria, blastomycetes [2]) and moulds, which decompose indican, do not effect this by enzymes but by katabolism, while among alcohol-ferments both cases occur. So indican is decomposed katabolically by *Saccharomyces ludwigi* and *Monilia candida*, while *Saccharomyces sphaericus* [3]), *S. apiculatus*, *S. muciparus* [4]), *S. tyrocola* [5]) contain indigo-enzymes. One of

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[1]) In alcohol vapour many microbes die sooner than in strong alcohol, this having a water absorbing power and thus acting protectingly.

[2]) Blastomycetes have the shape of yeast-cells but produce no alcohol. To these belong e. g. the red "yeasts" *Blastomyces glutinis*, *B. roseus*, *B. granulosus* (of which the last colours deep blue with jodine), and which all decompose indican vigorously.

[3]) Under this name, given by NAGELI, I united the various forms of aethyl-acetate yeast. (Verhandelingen 5e Natuur- en Geneeskundig Congres te Amsterdam, 1895, p. 301).

[4]) This name I give to a saccharose-yeast, very common in pressed yeast and which does not ferment maltose.

[5]) *S. tyrocola* is a lactose-yeast, not rare in Edam cheese. Its cultures on wort-gelatine are sometimes rose-coloured.

these enzymes, that of *S. sphaericus*, which acts the most strongly of all, will be treated in § 6.

Here I wish to remark that indigo-enzymes originate in the yeast-cells only then, when cultured on a solid medium e. g. on wort-gelatine, with abundant access of air. When cultured in nutrient liquids, even with a current of air passing through, they produce no or only very little enzyme.

The indigo-blue, formed by most moulds and yeast-species in the decomposition of indican, is for the greater part confined within the protoplasm, as was already described and figured by Mr. MOLISCH (l. c.); but in those cases when decomposition is very strong, as with many bacteria, the indoxyl streams out and also precipitates outside of the cell in granules of indigo-blue.

5. *Indigo-fermentation by Aërobacter.*

When a decoction of *Indigofera* or *Polygonum* is infected with garden-soil, canal-water or mud, and placed at 28° C., there originates, during a copious formation of indigo, a rich bacteria-flora in which the common gas-producing ferments, which I recently united [1]) in the genus *Aërobacter*, perform the chief part. The first who drew attention to this fact was ALVAREZ, but he went too far by admitting the existence of specific bacteria for indigo-fermentation [2]). By bringing a drop of the first crude fermentation into a second quantity of a decoction and so on, an accumulation, sometimes a pure culture of *Aërobacter* is obtained [3]).

By sowing out an *Aërobacter*-fermentation on indican-gelatine, not only the *Aërobacter*-colonies, but also those of various other bacteria colour deeply blue by indigo. Commonly, however, the *Aërobacter*-species are recognised by their number. But the chief characteristic of *Aërobacter* is its fermenting power and its temporary anaerobiosis, by which the splitting of indican goes on even at temporary exclusion of air, which is not the case with the aerobics. On this characteristic is based the supplanting of the aerobics by *Aërobacter* in liquid cultures and the prevailing part which these bacteria have in the splitting

[1]) Centralblatt fur Bacteriologie. 2e Abt. Bd. 6 N°. 7, 1900.

[2]) Comptes rendus, T. 105, pag. 287, 1887.

[3]) In several other plant-infusions, not from indigo-plants, quite the same is observed. The strongest *Aerobacter*-fermentations are obtained by mixing rye-flour with water to a thick pap and placing it at 28° C After a few hours the development sets in of carbonic acid and hydrogen, caused by the *Aërobacter*-species, never wanting in flour, which in the beginning supplant all other bacteria.

of the indican in the spontaneous indigo-fermentations. In pure cultures this splitting can of course be as well effected by various common aerobics, albeit more slowly.

The decomposition of indican by *Aërobacter* is operated katabolically, as in all other examined bacteria also, so that killed bacteria are inactive and indigo-enzyme cannot be separated out. The optimum temperature for the decomposition agrees with that of the growth and is, for instance, 28° C. for a variety of *A. aërogones* isolated from milk.

The number of *Aërobacter*-forms obtained by sowing out from the decoctions is very great but may be reduced to three chief species, described by me elsewhere (l.c pag. 200). They are *Aërobacter aërogenes*, *A. coli* and *A. liquefaciens*, all represented by many varieties and allied by intermediate forms. Not all varieties are equally active. So, among the forms of *A. coli*, which for the greater part decompose most vigorously, the variety *A. coli* var. *commune*, isolated from the intestines or from faeces, is but feebly active or not active at all and recognisable by this feature.

The products of the decomposition of indican by *Aërobacter* (and by bacteria in general) are the same as by enzyme action, i. e. indoxyl and glucose. If a nutrient liquid containing indican, e. g. decoct of indigo-plants, broth, or yeast-water, is passed into a fermentation tube and infected with *Aërobacter*, indigo-blue is formed in the open end, while in the closed one carbonic acid and hydrogen originate from the glucose of the indican [1]), and indoxyl which remains a long time unchanged.

In proportion as the oxidation of the indoxyl proceeds more slowly, more indigo-red is produced, similarly to the splitting of indican by enzymes and acids. Now the splitting of the indican, and consequently the oxidation of the indoxyl can proceed with much rapidity by the action of enzymes and still more rapidly by acids in presence of ferrichloride, while it is impossible to make the process go on as quickly by bacteria. So it is inevitable that the formation of indigo-red is very great in the case of the bacterial fermentation of the indican, while it is possible to reduce its amount practically to zero in the case of chemical decomposition. As it is besides hardly possible to separate the indigo-blue from the substance of the bacteria,

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1) That the development of gas is due to the sugar of the indican, and not to the free sugar already present in the decoctions or the indican preparations, is proved by the fact that the gas-development is the same when beforehand all free sugar has been removed from the liquid by means of pure beer-yeast, which acts not on indican.

only an impure indigo can be obtained by means of their action.

In consequence of the growth of *Aërobacter* the reaction of plant extractions, particularly of the indigo-plants, first becomes feebly acid, later feebly alkaline by the formation of free alkali. This is also prejudicial to the production of indigo, as in acid solutions the indoxyl oxidises very slowly, by which again much indigo-red is formed, while at the same time part of the indoxyl gets lost in another way.

Worthy of note is the influence of varions sugars on the indican decomposition by *Aërobacter*. Mr. VAN HASSELT found that already $^1/_2$ pCt. glucose, as well in liquid cultures as in gelatine experiments, prevents decomposition, while much larger quantities, even to 10 pCt. of cane-sugar, maltose and lactose have no effect at all and levulose but very little. Evidently the very sugar produced by the splitting counteracts this splitting, while other sugars have not this effect, or in less degree. To this rule mannose makes an exception, as indican decomposition is in the same way counteracted by it as by glucose. This opposing influence gives consequently only partly and not completely the answer to the question after the nature of the sugar separated out of the glucoside by bacteria [1]).

There are however forms of *Aërobacter* which, in ferment-experiments, produce unequal quantities of carbonic acid und hydrogen from glucose and mannose, and by their help it is proved that the sugar formed from indican can only be glucose.

Nitrates, also, have a remarkably opposing influence on the production of indigo by *Aërobacter*. Common saltpetre is active already at $^1/_{20}$ pCt., which is in perfect accordance with the anti-fermenting action of this salt in general, on which reposes its use in the dairy industry, to prevent one of the most important defects of cheese, in Holland called "rijzers".

6. *Indican-decomposition by the Indigo-enzymes.*

The indigo-enzymes prepared from *Indigofera leptostachya*, *Polygonum tinctorium*, *Phajus grandiflorus*, aethyl-acetate-yeast (*Saccharomyces sphaericus*) and emulsine of sweet almonds, have been

1) Mr. VAN HASSELT prepared the osazon from the indican-sugar and found, after recrystallisation from alcohol, the melting point to be at 195° to 199° C., that is nearly the same as that of glucosazon, which is 204° to 205° C. But the melting point of mannosazon is about as high.

compared as to their intensity of action on indican at different temperatures, for which notable differences have been found. No other group of enzymes is known to lead with equal ease and certainty to the determination of these relations as this group of the indigo-enzymes.

The experiments were conducted as follows. Of solutions of about 0.5 pCt. indican [1]) 10 cc. were passed into equal test-tubes selected for the purpose. After heating them to the required temperature in a large beakerglass, arranged as waterbath, with thermoregulator and thermometer, the enzyme was added and the temperature kept constant.

After a few hours the tubes were taken out, alcalised and the indoxyl oxidised by strong shaking, then acidified, by which a very fine, equally divided, purely blue precipitate of indigo-blue is obtained, which allows colorimetrically to establish the intensity of action with sufficient exactness.

It proved wholly indifferent whether in these experiments use was made of the indican of *Indigofera* or of *Polygonum*. Evidently it is the same in both plants.

Great attention should however be paid to the degree of acidity of the indican solutions. The most favourable enzyme action was observed at the rate of about 0.5 cc. normal acid per 100 cc. liquid. An increase of the acid to 2 cc. slackens the reaction notably; likewise the addition of alkali to feebly alkaline. Acid salts, as kaliumbioxalate and kaliumbiphosphate, act in the same way as free acids.

The quantities of the enzymes employed for the experiments amounted to 2—60 milligrams of finely powdered crude enzyme per 10 cc. of the $^1/_2$ pCt. indican-solution.

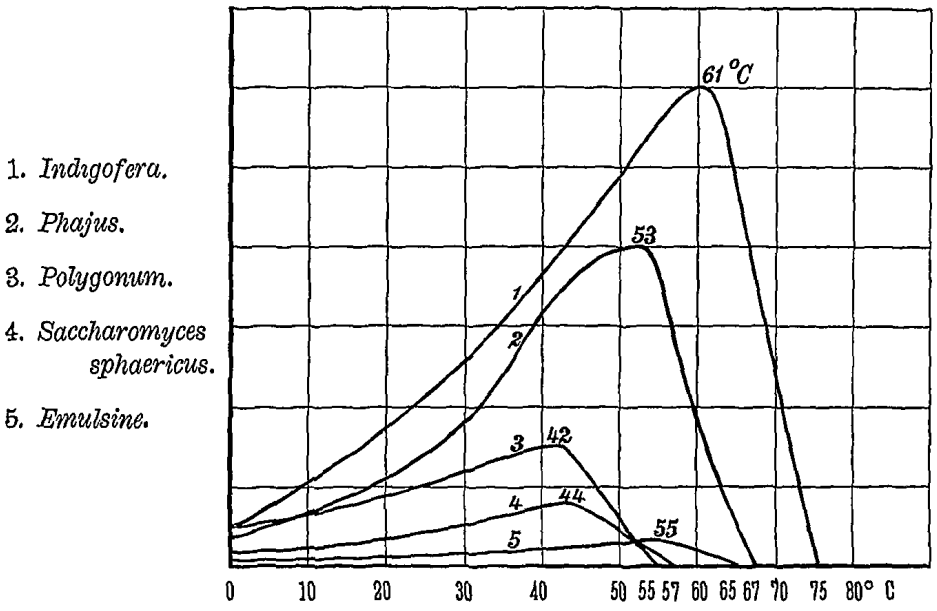
First of all was now established the maximum temperature at which the action of the enzymes ceases entirely, that is, where the enzymes are nearly suddenly destroyed. For *Indigofera* this maximum is at 75° C., but when using a great deal more enzyme a feeble action could still be observed at 78° C. which however quite ceased at 80° C. For *Polygonum* the maximum temperature is at 55° C., and in large quantities a feeble action was still observable at 60° C. For this determination the tubes were placed, for *Indigofera*, at 72°.5, 75°, 78° and 80° C.; for *Polygonum*, at 52°.5, 55°, 58° and 60° C. For both enzymes the action at the rising of the temperature diminishes very quickly near the maximum. In a similar way were

[1]) Stronger solutions give no more exact results.

found as maximum temperatures for *Saccharomyces sphaericus* about 60° C., for *Phajus grandiflorus* 67°, and for emulsine 70° C.

After this the optimum temperatures for the enzyme action were fixed, for *Indigofera* by searching between 55° and 65° the maximum intensity of indigo-formation, testing all temperatures from 55°, 57°, 59° C. and so on. The strongest inversion was found at 61° C. both for powdered crude enzyme and for enzyme-solution in 10 pCt. common salt and 10 pCt. calcium chloride. Changes in the degree of alkalinity or acidity within the narrow confines between which enzyme action is at all possible, deplace the optimum temperature but little [1]). A difference in temperature of 1° C. was only to be observed between 61° and 62°; at 62° C. the decomposition was certainly a little feebler, but between 60° and 61° C. there existed some doubt. At lower temperatures distinct differences in the intensity of the decomposition could only be noted at intervals of 2° C.

The enzyme of *Polygonum*, examined in the same way between 35° and 45° C., gives the most copious production of indigo at 42° C., with a rapid decrease in action above, a slow one below that point.



[1]) Mr. VAN DELDEN found upon addition of acid both for *Indigofera* and *Polygonum* a rising of 1° in optimum, which however disappeared when the employed solutions of crude indican were diluted with an equal volume of water and then, before the addition of enzyme, were brought to the same acidity.

For aethyl-acetate-yeast the optimum lies at 44°, for *Phajus* at 53° C., and for emulsine at 55° C. [1]).

Particularly for emulsine the intensity of action is feeble, and the feebler it is, the more troublesome exactly to fix the temperature-optimum, as is clearly shown by the course of the curved line in the graphic representation.

For the exact determination of the shape of the curved line which indicates the general relation between decomposition and temperature, temperatures above and below the optimum were sought, at which the quantities of indigo, formed after an hour's action, were quite the same. In a system of coordinates with the temperatures as abscisses and the intensity of decomposition as ordinates, these points have then equal ordinates and by determining some such couples the whole course of the curve becomes known. When looking at the curves found in this way we see that the decrease of action above the optimum is much more rapid than the rising below and that the last rising is not proportioned to the temperature.

At the same temperature the indican-decomposition by the various enzymes is operated with very unequal intensity. Proportionate ciphers between them were fixed as follows. In the experiments described before, so much of the enzymes to be compared was added to 10 cc. of indican-solution that at the optimum temperatures effects of equal intensity were observed. This proved to be the case when use was made of the following quantities of crude enzyme in milligrams: *Indigofera* 5, *Polygonum* 20, *Saccharomyces sphaericus* 40, emulsine 100, which numbers stand to one another as 1 : 4 : 8 : 20. When all these quantities were doubled or reduced to the half, the proportions underwent no change. Consequently from these numbers follows that the intensity of decomposition for the different enzymes is expressed thus: *Indigofera* 20, *Polygonum* 5, aethyl-acetate-yeast 2.5, emulsine 1. In the graphic figure the length of the ordinates is taken proportionately with these numbers.

So we find that the curve of the *Polygonum*-enzyme is about uniform with that of the much stronger enzyme of *Indigofera*, but at 0° C. they cross each other, so that at a still lower temperature the action becomes an inverse one.

The great difference in intensity of action is also proved by the fact, that, for the manifest appearance of indigo in 10 cc. of the

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[1]) As is seen, the difference between the optimum and maximum temperature is for all enzymes about 14° C.

indican-solution, there is at least required of the different crude enzymes 2 milligrams of *Indigofera*, 20 of *Polygonum* and *Saccharomyces sphaericus*, and 60 of emulsine.

CONCLUSIONS.

The splitting of the indican by the cell can occur in two ways: by ferment-action of the living protoplasm itself (katabolism), and by enzymes.

All examined bacteria, which act on indican, split by katabolism and hence are in dead condition inactive. The most important among them are the common ferment-bacteria (*Aërobacter*) of sugar-containing plant infusions.

All indican-plants and some species of alcohol-ferments contain indigo-enzymes and consequently can decompose the indican in dead condition too. Indigo-enzymes originate only at abundant access of air. Five of these enzymes proved specifically different, with temperature optima of 61° (*Indigofera*), 55° (emulsine), 53° (*Phajus*), 44° (*Saccharomyces sphaericus*) and 42° (*Polygonum*). For all of them the action is favoured by free acid to an amount of 0 5 cc. normal per 100 cc. of the employed indican-solution; more acid, like alkali, opposes the action.

Indigofera decomposes the indican only by enzyme-action; in the case of *Polygonum tinctorium* and *Phajus grandiflorus* the indican is decomposed partly by katabolism, partly by enzyme-action.

In the leaves of *Phajus grandiflorus* indican is localized in the colourless protoplasm of mesophyll and epidermis, the indigo-enzyme exclusively in the chlorophyll-granules.

Chemistry. — "*Indican — its hydrolysis and the enzyme causing the same.*" By J. J. HAZEWINKEL, Director of the experimental station for Indigo at Klaten (Java). (Communicated by Prof. S. HOOGEWERFF).

The following investigations were done by me in November 1898. After the investigation was closed, I received by the mail the treatise of MARCHLEWSKI and I then went to Buitenzorg to consult Dr. VAN ROMBURGH about these researches.

I first thought it would be better, in the interest of the Javanese indigo growers to keep the results of my work a secret, but having been informed by Prof. BEIJERINCK that he also had taken up the