Experimental Studies on the Variation of Yeast-cells¹.

BY

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I.

IT was the epoch-making works of Darwin that gave the impulse to the tendency which is becoming more and more prevalent in modern zoology and botany, of viewing organisms from the standpoint of variation. As has been generally the case, the beginning was made here also with the higher organisms. Thus, the literature on phanerogamic plants abounds in observations in that direction; the cryptogams came under consideration considerably later, which is also true of yeast-cells and bacteria, or, as we usually style these two groups by a common appellation, micro-organisms. In the case of the Phanerogams, investigators have hitherto generally confined themselves to observations of the forms and phenomena as actually occurring in nature, and to discussions on those observations. It is, however, evident that nothing short of systematic experiments, performed by exact methods, will be able to lead to a real insight into the very complicated problems of variation; in this respect unicellular

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organisms offer the most favourable conditions, everything being here simpler, more easily seen through, and more easily mastered, than in the case of the higher plants. Further, their much stronger powers of multiplication here play an important part, as enabling us to arrive at a definite result within a reasonable time.

As is known, Reess published in 1870 a work, important at that time, on alcoholic fermentation-fungi. Those yeast-cells which give endospore-formation are referred by him to a particular genus, *Saccharomyces*, of which he establishes a series of species. He takes as the basis of his description the form and size of the cells. The large oval cells commonly found in beer-yeast are referred by him to the species *Saccharomyces cerevisiae*; the cells of wine-fermentation, which are also oval, but generally smaller, he considers as constituting a different species, *S. ellipsoideus*; the sausage-shaped cells are termed *S. Pastorianus*, and so forth. In 1883 and the following years I proved that the limits drawn by Reess do not exist in reality, and that we are able from each of the species as conceived by him to develop the rest. If we therefore still use the designations of Reess, it can at most be as denominations of groups of species, and from an essentially different stand-point from his. *It is not, as he and his successors thought, the form and size of the cells per se, in which the distinguishing characters lie, but in the form and size contingent upon particular conditions of cultivation.* Besides shape and size, we now make use of a whole series of other characteristics. When, therefore, we use the old appellations of Reess, it must be borne in mind that they have now very different significations from those in which he used them, and we must have a clear understanding that each of these groups of forms may transform itself pretty readily into each of the others. Several of the best and most vigorous yeast-fungi of wine may, morphologically, be referred as well to *Saccharomyces cerevisiae* as to *S. ellipsoideus*, and in like manner there are beer-yeast fungi which, if we go by the system of Reess, may equally well be looked upon as belonging to *S. ellip-*

soidens as to *S. cerevisiae*; both of these groups again run into *S. Pastorianus*. Large oval cells like those which are characteristic of *S. cerevisiae*, may, under favourable circumstances of nutrition, be developed by wine-yeasts with small oval cells; and, conversely, large typical cells of beer-yeast fungi may form smaller cells resembling wine-yeast. And, again, as regards the sausage-shaped cells the form of which distinguishes those species which, by a name of Reess's, we call *S. Pastorianus*, it must be noted, that there are several ways in which we can produce *S. Pastorianus* in the sense used by Reess, from either of the groups *S. cerevisiae* and *S. ellipsoideus*. These various relations deserve notice at the present moment, because there is now again a certain tendency to speak of *S. cerevisiae*, *S. ellipsoideus*, &c., as if each of these names designated a well-defined species.

If we ask the question, which of the various forms of yeast-cells is the primitive one, there are many reasons which make it probable that it must be the oval form, and that the species classed under the group *S. Pastorianus* have developed from the groups *S. cerevisiae* and *S. ellipsoideus*. Yeast-cells are, upon the whole, of a very plastic nature; yet it is true of at least a large number of the species, that, if cultivated under the same particular circumstances, they appear in the same forms again and again.

As regards the factors which determine the variation of the form of the cells, I have given information in previous papers. Among my former researches on the variations, some experiments are described on the action of the air on some species of beer-yeast, and also on the influence of certain conditions of nutrition with regard to spore-formation. I have shown that in the case of several species there appear suddenly, generally from unknown causes, varieties which have lost, more or less completely, their power of forming endogenous spores. The usual cultivation in a favourable nutritive liquid, as beer-wort, at favourable temperatures, in these cases avails nothing; but in the case of some species, I found that such cells are reduced to their normal state, if some dextrose is

added to the nutritive liquid. Here, therefore, the particular effect exercised by a particular chemical factor has been pointed out. In the instances just mentioned the variations were of a more or less inconstant nature.

In the course of the above researches on the disappearance and recovery of spore-formation, I made in 1889 the discovery that in this domain it is possible to produce very deep-seated transformations, and to render these permanent. It was found that when the cells were cultivated for a length of time in aerated wort, and at a temperature above the maximum for spore-formation and approaching that for vegetative development, they completely lost their power of forming spores.

I have effected this transformation in the case of five of the species described by me in 1883, and also with several beer bottom-fermentation and distillers' top-fermentation yeasts; that is with representatives of the various groups of the *Saccharomycetes* properly so called. Thus we have to do with a common law; yet this seems to apply to the typical and genuine *Saccharomycetes* only; at any rate, I have not hitherto succeeded in producing this transformation in the case of *S. anomalus* and *S. membranae-faciens*, both of them being, indeed, species which, as may be remembered from my papers, differ so widely from the others, that they might well be set up as types of quite new genera.

As might be expected, the law of correlation asserts itself. Thus a closer examination shows that the transformation which we effected does not stand alone, but is accompanied by others. This is especially evident from the fact that the yeast-cells under our treatment at the same time completely lose their former power of forming films on the surface of liquids.

While the loss of spore-formation is attended in all species, without any exception, with loss of film-formation, there appear differences in other directions in the species transformed. Thus in some I observed that in wort-culture they produced a more abundant growth, and at a quicker rate, than

the parent-cells from which they were descended, while in others no such difference could be observed.

The above variation has thus influenced some of the most important morphological and physiological features, and it is of such a constant and radical nature that the resulting cells might well be regarded as constituting new species; however, I described them as varieties in my earliest publications, and will keep this designation for the present. Although they have been cultivated for several years past, under most varying circumstances, through endless generations, still they have kept constant; the newly acquired qualities have proved to be entirely heritable.

II.

The investigations mentioned above are to be found partly in my *Recherches sur la physiologie et la morphologie des ferments alcooliques* (*Comptes-rendus des travaux du laborat. de Carlsberg*, 1881-1891), partly in the *Centralblatt für Bakteriologie und Parasitenkunde*, 1889, p. 638, and partly in the *Annales de Micrographie*, février, 1890. A more detailed summary than the above may be found in my book, *Practical Studies in Fermentation*, which will appear this autumn, published by Messieurs Spon, London. I will therefore dwell no longer on this subject, but pass on to a mention of my recent experiments¹.

About fifteen years ago I published a paper on the biology of *Saccharomyces apiculatus*, in which I pointed out, amongst other facts, that this yeast winters in the earth; later, I found that this also holds good of the true *Saccharomycetes* with endospores. I will, in this connexion, only mention the fact that wine-yeasts belonging to the group *S. ellipsoideus* kept alive in the earth, like *S. apiculatus*, for more than three years. By making comparative investigations on the varieties

¹ In a fuller treatise which I am preparing at present, a detailed account of the phenomena of variation observed by me and of the factors and laws which influence them, will be found.

which had lost their power of spore-formation, I found that, when placed in the earth, they showed a decreased vitality as compared with their original forms, and generally perished within less than a year. In the sugar-solutions, on the other hand, in which they had produced alcoholic fermentation, they kept alive for several years, and, as far as my experiments go, they appear not to be second to their parent-cells in this respect.

Since the varieties no longer possess the power of forming films on the surface of liquids, their life in these becomes quite different from what it used to be. As shown in one of my papers, the cells of the film which develops on fermented beer-wort cause the liquid to become lighter in colour; and from the chemical investigations of Kruis and Rayman (1891) we know that these cells produce a vigorous oxidation, by which the alcohol is converted into carbonic acid and water. Thus the yeast, as long as it is in its normal state, not only produces alcoholic fermentation in the sugar-liquid in which it is present, but when it has carried this fermentation as far as it is able to do, it forthwith takes to transforming the alcohol produced by itself, thus coming to live in a liquid with less and less of alcohol. The varieties not being able to develop any film, they of course have given up this activity. As an example of the contrast between the varieties and their original form in this respect, I will here only mention the result of one of my experiments. Two cultures in the same wort, of which one contained a brewers' bottom-fermentation yeast, the other a variety of the same, which I had produced in the manner described above, were left to stand for six months. The original form had developed a film as usual, and its fluid contained only 1.5 per cent. (by volume) of alcohol, whilst the variety showed 5.5 per cent., that is, the same amount of alcohol as it contained after standing the first month. The beer from the primitive form was lighter in colour than the corresponding beer from the variety. Thus the varieties described manifest in those directions also sharp differences from their primitive forms.

We are now going to mention some experiments, by means of which varieties were produced in another manner than that described above, and with other properties.

If yeast-cells are living on the surface of nutritive gelatine, they are exposed to quite different conditions from those which obtain when, for instance, they are living in wort, and the new generations produced under these special circumstances therefore also acquire a special character. Two varieties, *a* and *e*, of the Carlsberg bottom-yeast, No. 1, were partly cultivated in wort, partly on the surface of wort-gelatine, the cultures being renewed fairly frequently during some months. On the gelatine new varieties developed having a greater fermentative power than their primitive forms. In wort to which cane-sugar had been added, the latter only produced about 13 per cent. (by volume) of alcohol, while the new varieties produced 13.6 per cent. In this experiment only vegetative cells were made use of.

A more marked difference, in the same direction, appeared in some experiments which I made with the top-fermentation yeast *Saccharomyces cerevisiae*, I. They were arranged in the same manner as the foregoing experiments, but the cultures were here made from spores, and instead of wort-gelatine, yeast-water gelatine was used. The growth which was cultivated on gelatine in this case gave 3 per cent. of alcohol more than the corresponding growth which had been cultivated in wort.

Before leaving these experiments, I must emphasize the fact that the gelatine varieties produced were all descended from varieties which, as long as they were cultivated in wort, were very constant, and were distinguished by producing only a comparatively small percentage of alcohol.

Another way in which we may operate on yeast-cells in the direction mentioned, is by the action of antiseptics. In 1887 Biernacki arrived at the conclusion that all antiseptics, when employed in small quantity and under certain conditions, possess the property of accelerating and strengthening alcoholic fermentation. Maercker's and Hayduck's investigations

tend to prove the same. Later, Effront, in his well-known experiments on hydrofluoric acid and alkaline fluorides, arrived at the same general result. In all these cases only transitory transformations are meant.

In the foregoing I have mentioned that a great part of my experiments were carried out to determine the influence which temperature has in respect to the form and the functions of yeast-cells; by this I was also led to make the following researches on the attenuation of the fermenting powers of yeast-cells. Carlsberg bottom-yeast, No. 1, was cultivated in wort at 32° C. through eight cultures in such a manner that each subsequent culture was inoculated from a foregoing one, and left standing at rest until the fermentation had ceased, without being aërated. In the ninth culture a variety was obtained, which gave 1–2 per cent. of alcohol less than its primitive form, when the fermentation was carried out in wort (14 per cent. Ball.) to which was added 10 per cent. of saccharose. Also in other directions changes had taken place; thus the new variety in some brewery-experiments clarified better and gave a more feeble attenuation at the end of the primary fermentation than the growth from which it descended. The variety seems to be constant, and what has been found out for the species in question seems also in the main to hold good for others.

But if the same species was submitted to the same treatment, only with the difference that the growth was strongly aërated and renewed every day, these transformations would not occur; they thus afford an illustration of the fact, that temperature only under certain conditions has the effect described.

Before concluding this survey of the varieties observed by me, I have to record an experiment by which the effect of the chemical composition of the nutrient liquid is shown. *Saccharomyces Pastorianus* I., is one of the disease-yeasts of beer, to which it imparts an offensive odour and a disagreeable bitter taste. According to Mach and Portele's investigations, however, it gives a good wine, and my own experiments have

shown that when this species is cultivated for a number of generations in a solution of cane-sugar in yeast-water, a growth is obtained the cells of which have for a time lost the disagreeable properties referred to. From this it is seen that it is possible to act upon yeast-cells in such a way that they can be made to impart to fermenting liquids a taste and odour different from that originally characteristic of the yeast.

It is a common fact that when bacteria are cultivated in a certain manner they lose their fermenting power. In a very interesting paper, read at the British Association for the Advancement of Science, 1893, Professor Percy Frankland has given an account both of the researches which he himself has undertaken in this direction and of those which at that time had been made by other investigators. He brings into relief the fact that it may frequently be observed that a bacterium which had originally the power of fermenting some particular substance, has lost this power through prolonged culture, and that, indeed, even a single passage through gelatin may apparently destroy the capacity to exercise this function. We do not know any similar instance of such behaviour in the case of the alcoholic yeasts. Their cells may be transitorily very much enfeebled, and, as we have learnt, varieties may be produced which give less alcohol than their primitive forms, but it appears to be impossible to produce a variety which has completely lost its alcoholic fermenting power.

In the Danish edition of my paper on the circulation of *Saccharomyces apiculatus* in nature (1881), I expressed the idea that it might possibly be well to abandon the cultivation of the old culture-yeast (*S. cerevisiae*, as we usually term all these species) for technical purposes and replace it by some of the yeast-species occurring in nature, it being my belief that in this way we should *perhaps* be able to obtain products which were not only different from, but also in certain directions better than those manufactured by means of *S. cerevisiae*. Just at that time there was a great deal of talk about the degeneration of culture-yeast, and there was a general inclination to ascribe to it most of the difficulties occurring in the

brewery-fermentations. But, if we ask, what do we know about this variation which is supposed to have taken place from the primitive form, then the answer must be: Nothing at all. *We have never been able to carry one single Saccharomyces species back to its progenitor!* Some years ago I showed, it is true, that typical Saccharomycetes may appear in forms resembling *Oidium* and *Dematium*, and possessed of a mycelium with distinct transverse septa, but we have not advanced further in that direction. As is well known, Takamine, Juhler and Jörgensen have recently come forward as advocates of the view formerly represented, especially by Bail and Hoffmann, that the Saccharomycetes are merely developmental phases of various common mould-fungi, and they have also all three fallen back upon the idea alluded to above, that there should be a possibility of obtaining, through the original forms, yeast-species that in certain particulars might be better than those actually employed. In their cultivations they started from the moulds themselves, and describe how *Aspergillus* and *Dematium* develop *Saccharomyces*-cells.

Klöcker and Schiønning at the Carlsberg Laboratory repeated the above experiments with *Aspergillus Oryzae*, and in accordance with the previous investigations of Cohn, Büsgen and Wehmer, they were not able to observe any development of yeast-cells. The whole question must at present still be considered as an open one. For the rest we know now so many mould-fungi that develop alcoholic fermentation-fungi, that it would not be a matter of wonder if some should really be found which also develop yeast-cells with endospores. (About this question see the articles of Juhler, Jörgensen, Wehmer and myself in *Centralblatt f. Bakteriologie*, 2^{te} Abt. 1895, pp. 16, 65, 321, 326, and 565.)

In the preceding portion of my paper I have chiefly described the phenomena of variation without inquiring more closely into its mechanism, nor have we examined the agencies and causes to which it is due. I have, however, made extensive experiments in that direction also, but to describe the details of these would on this occasion lead us too far.

Those who would be likely to take an interest in them will find information in the fuller treatises which I am about to publish. I shall, therefore, now only refer to the results in a few words.

Among the above-mentioned variations, those offer the greatest interest in which cells were produced which had entirely lost their power of forming spores and films. In trying to elucidate how this transformation is effected, we have on one hand to examine the external factors, and on the other hand to elucidate how the cells are influenced by them. Of course, we always work with absolute pure cultures; in some cases we experiment with the whole bulk of the vegetation in question, while in others we separate the single cells at the different stages, putting each severally to the test.

We find, that in the cultures under discussion, at the high temperature a vigorous multiplication still takes place, and that even in the first stages a large number of the cells have become transformed; this number increases as the cultivation progresses, but the change is at the beginning of a transitory nature, and it is only through the development of new generations that the newly acquired qualities become fixed, so that at last a constant variety comes out.

As thus shown it is not the temperature alone, which effects the transformation, but only in connexion with the multiplication of the cells. In agreement with this we find, that it is not sufficient to cultivate the cells in water, but that a favourable nutritive substratum and a certain aëration are required.

Of the variations which have been observed in bacteria, in some cases it has been assumed that they were due to chemical changes in the culture-liquid; since this might also apply to my above-mentioned variations in the *Saccharomycetes*, I therefore put the question to an experimental test. The result I obtained was that special chemical compounds do not play any part in that case.

Of the external factors we have then only the nutritive substratum, the aëration and temperature left.

As to the nutritive substratum and aëration my experiments have proved that only this much is required, that they must allow of a vigorous multiplication, and the range within which these factors may vary is a very large one. But this does not apply to temperature. One or two degrees too much or too little is enough to prevent the changes from taking place; if the temperature be a little too low, the effect will not be sufficiently marked, whilst at a somewhat too high temperature the multiplication of the cells ceases too soon, the consequence being that the transformation, which has commenced, does not become fixed. In comparing the temperature with the other external factors we must, in the experiment under discussion, consider it the most influential.

The above described phenomena of variation affect the yeast-cells in very different ways, influencing both morphological and physiological characters. Some of the phenomena still remain isolated as if accidentally caused, others group themselves under certain rules. It is especially the latter, which invite to new investigations in order at last to find out the laws. To discuss what relation there may be between the observed facts and the hitherto established theories of variation would at present only be to waste words. When finishing my experiments I hope in that respect also to be able to give a survey.

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