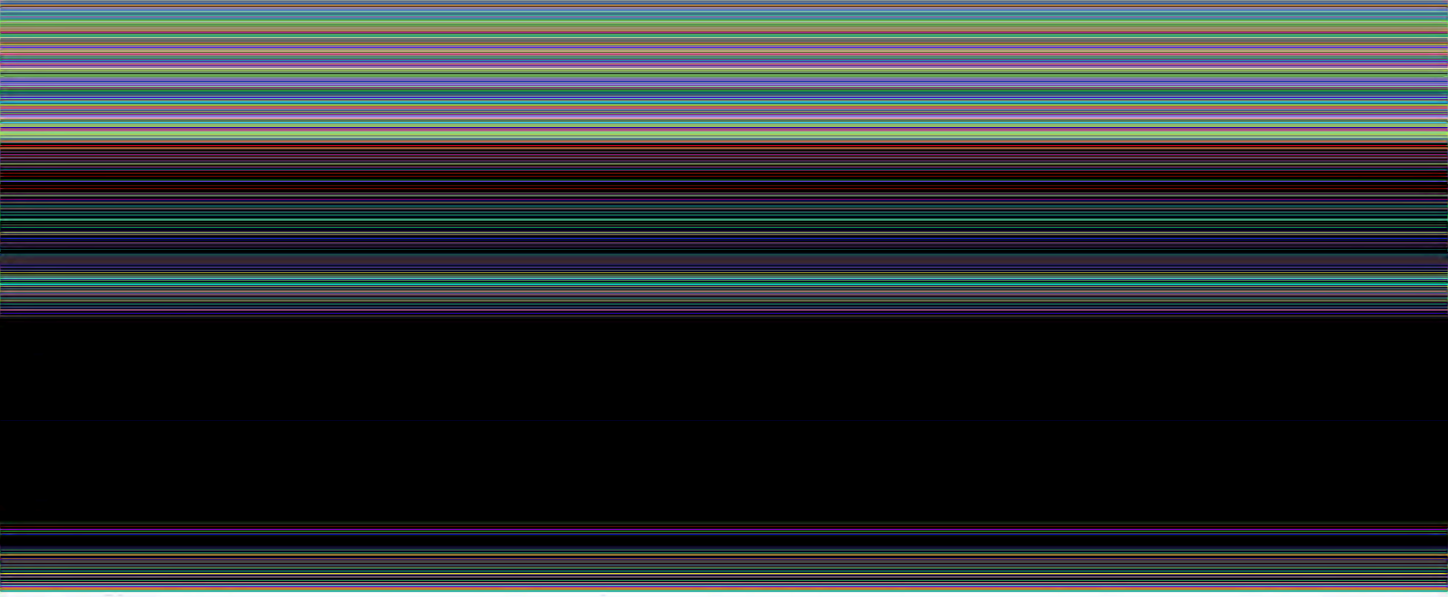

MICRO-ORGANISMS

AND

FERMENTATION.

BY

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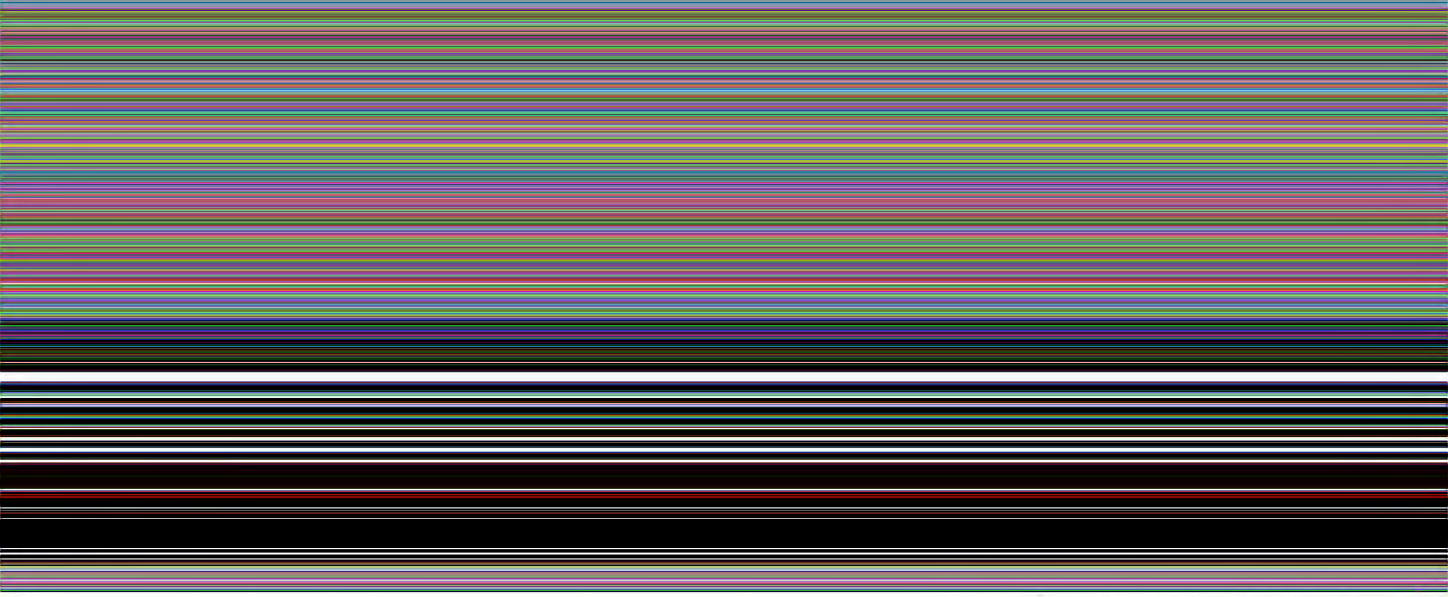

PREFACE.

The present book gives an account of the morphology and biology of the micro-organisms of fermentation, and it thus forms a complement to the text-books which treat mainly of the chemical side of the subject.

I have attempted to give a *general review of all the knowledge we possess in the above-mentioned field*, and have described the various methods of investigation which in the course of time have proved of importance.

In discussing the organisms of fermentation and their relation to industry, there are two names which in a high degree especially attract our attention, namely, *Pasteur* in the older literature, and *Hansen* in the more recent literature of our subject. Since this book is intended to give an account of the *present stand-point of the science*, it is evident that the investigations from the Carlsberg Laboratory must occupy an important position. In Chapters V. and VI. will be found an accurate description of *Hansen's* theoretical investigations on the alcoholic yeasts, of his methods for the pure cultivation and analysis of yeast; likewise an account of the practical employment of his pure yeast, and of the results obtained with it in breweries, distilleries, and pressed yeast factories, and in the preparation of wines from the grape and other fruits.

This book thus appeals to chemists, botanists, and biologists, likewise to those technologists who are engaged in the branches of industry named.

In the bibliographical list I have included all important works of the older and more recent literature which are of interest to the scientist and technologist.

In its present form this book in the main has the same scope and contains the same matter as the third completely revised German and the French editions.

ALFRED JÖRGENSEN.

Copenhagen,
May, 1893.

TABLE OF CONTENTS.

CHAPTER I.

Microscopical and Physiological Examination.

PAGE

1. Microscopical Preparations, Staining, and Microchemical Examination 1–5
2. Biological research by means of the Microscope; moist chambers 6–9
3. Sterilisation (Spallanzani, Scheele, Appert, Schulze, Schwann, Schröder, Dusch) 9–14
4. Disinfection 14–17
5. Flasks: Pasteur, Chamberland, Freudenreich, Hansen, and Carlsberg Flasks 17–21
6. Nutritive Substrata 21–29
7. Preparation of the Pure Culture 29–32
   (*a*) Pure Cultures for Morphological Investigations; Ehrenberg, Mitscherlich, Kützing, F. Schulze, Tulasne, De Bary, Brefeld 30–34
   (*b*) Pure Cultures for Physiological Experiments with Mass-cultures; Pasteur, Cohn, Lister, Hansen, Schroeter, Koch 34–39
8. Counting the Yeast-cells 39–45

CHAPTER II.

Examination of Air and Water.

Tyndall, Miquel, Koch, Hesse, Hueppe, v. Schlen, Frankland, Petri, Pasteur, Hansen 36–47
Hansen's Zymotechnical Analysis of Air and Water .. 47–50

CHAPTER III.

Bacteria.

Forms 51–53
Anatomical Structure, Colouring Matters, Phosphorescent Bacteria, Locomotion 53–54
Multiplication, Growth-forms, Endospores and Arthrospores Bacteria, Zooglœa 54–56
Anærobic and Aërobic Species 56

PAGE

1. Acetic Acid Bacteria 56–61
2. Lactic Acid Bacteria 61–66
3. Butyric Acid Bacteria 66–70
4. Kephir-organisms, Ginger-beer Plant 70–73
5. Slime-forming Bacteria 73–76
6. Bacteria exercising an inverting, diastatic or peptonising action 76–77
7. Saxena Forms 77–80
8. Crenothrix 80–81

CHAPTER IV.

The Mould-Fungi.

Occurrence in nature 82–83
1. Botrytis cinerea 83–86
2. Penicillium glaucum 86–89
3. Eurotium Aspergillus glaucus 89–92
4. Aspergillus Oryzæ 92–96
5. Mucor Mucedo 96–96
Mucor racemosus, Mucor erectus, Mucor circinelloides, spinosus, stolonifer 96–99
Fermentative action of the Mucorinæ 99–100
6. Monilia candida 100–103
7. Oïdium lactis 103–106
8. Fusarium 106
9. Chalara Myroderma 106–107
10. Dematium pullulans 107–109
11. Cladosporium herbarum 109–110

CHAPTER V.

Alcoholic Ferments.

Introduction 111–121
General remarks 111
Earlier Observations of Schwann, de Seynes, Reess, de Bary, Engel, and Brefeld 111–112
Pasteur and his predecessors 113–115
His dispute with Liebig 114
Pasteur's views on the fermentation organisms, his theory of fermentation, and his controversy with Brefeld and Traube 114–118
Aeration experiments of Pedersen, Hansen, and Buchner 118
Nägeli's, Brown's and Hueppe's objections to Pasteur's theory of fermentation; Nägeli's theory 118–120

PAGE

Observations on budding-fungi by Reil, Reess, Zopf, and Brefeld 120

Chemical investigations by Rayman and Kruis 120–121

HANSEN'S INVESTIGATIONS 122–159

General remarks 122

1. Preparation of the Pure Culture 122–123

2. The Analysis 124–158

(a) The Microscopic Appearance of the Sedimentary Yeast 124–125

(b) Formation of Ascospores 125–137

(c) The Formation of Films 137–142

(d) The Temperature Limits for the Saccharomycetes 142–143

(e) Cultivation on a Solid Nutritive Medium .. 143–144

(f) The Behaviour of the Saccharomycetes and Similar Fungi towards the Carbohydrates and other Constituents of the Nutritive Liquid—Diseases in Beer 145–151

(g) Variations in the species of Saccharomycetes .. 151–156

(h) Gelatinous Formation secreted by the Budding-fungi 156–158

The Microscopic Appearance of a Yeast-cell 158–159

Classification of the Genus Saccharomyces 159–188

Saccharomyces Cerevisiæ I. Hansen 159–161

Saccharomyces Pastorianus I. Hansen 161–165

Saccharomyces Pastorianus II. Hansen 165–167

Saccharomyces Pastorianus III. Hansen 167–170

Saccharomyces ellipsoideus I. Hansen 170–172

Saccharomyces ellipsoideus II. Hansen 173–174

Two ellipsoid yeasts described by Will 174–175

Saccharomyces Ilicis. Grönlund 175

Saccharomyces Aquifolii. Grönlund 175

Saccharomyces pyriformis. Marshall Ward 175

Saccharomyces Marxianus. Hansen 176

Saccharomyces exiguus (Reess). Hansen 176–177

Saccharomyces membranæfaciens. Hansen 177–178

Saccharomyces Hansenii. Zopf 178

Saccharomyces Ludwigii. Hansen 179–180

Saccharomyces acidi lactici. Grotenfelt 180

Saccharomyces minor. Engel 180

Saccharomyces anomalus. Hansen 181–182

Saccharomyces conglomeratus. Reess 182–183

Carlsberg bottom-yeast No. 1 and No. 2 184–186

Grouping of cultivated yeasts for practical purposes 186–187

Observations made by Iimisch 187

PAGE

Distillers' yeasts, Pressed yeasts (Delbrück, Lindner, Stenglein, Bébhoubek, Schumacher, Wiesner) 187–188
Other Budding-fungi 188–203
Torula 189–194
Saccharomyces apiculatus 195–200
Mycoderma cerevisiæ and vini 200–203

CHAPTER VI.

The Application of the Results of Scientific Research in Practice.

The Technology of Sterilisation brought about by the earlier investigations on spontaneous generation. Spallanzani, Schwann, Appert, Schulze, Schwann, Schröder, Dusch; Pasteur's results 204–206
Hansen's System and the Reform brought about by it: Diseases in beer; Different Species of Saccharomyces cerevisiæ; Pure Cultivation of brewers' yeast; Constancy of the Characters of yeast-species under the conditions of the brewery; Method for the Practical Analysis of yeast; Yeast-propagating Apparatus; Flasks for the despatch of yeast; Preservation of Pure Cultures 206–214
Accounts of the Results obtained by Hansen's investigations (Lintner, Aubry, Will, Reinke, Bébhoubek, Bungener, Lintner, jun., Frankland, MacCartie, de Bavay, Wilson, Kobisinski, Van Laer, and others) 214–226
Resumé 226–227

Bibliography 228–231
Index 232–237

MICRO-ORGANISMS AND FERMENTATION.

CHAPTER I.

Microscopical and Physiological Examination.

1. Microscopical Preparations, Staining, and Micro-chemical Examination.

The *Microscope* will be for all time of paramount aid in the investigation of micro-organisms, since these, as individuals, are almost always invisible to the naked eye. The earliest important observations in the physiology of fermentation we owe to purely microscopical investigations, and it was not until the last decades that biological and physiological investigations were undertaken. After a certain probability had arisen that the same species of micro-organism did not always occur in the same form, work was eagerly commenced in different laboratories with so-called "*culture experiments*," in which attempts were made, by conditions of growth artificially brought about, to observe the different phases of development in *one and the same* spot, in order to thus determine the entire process of development. The idea was correct, but the way in which it was worked out was at that time so faulty that "culture experiments" threatened in consequence to fall into utter disrepute. The work was carried out without any proper precautions, as the following example shows. Beer yeast was sown on a moist slice of bread; the

culture was carefully covered with a glass shade, and all manner of precautions were observed in order to protect the growth from external contamination. After some days a growth of mould appeared, as is always the case with moist bread; and the conclusion was therefore drawn that the beer yeast was the origin of the mould, and that, consequently, yeast and mould-fungi were different phases of development of one and the same species.

A number of years elapsed before what are now universally acknowledged to be self-evident requirements of such investigations were put in practice, namely, that the first thing to be ascertained, before drawing definite conclusions, must be the *point from which to start*. This requirement was gradually defined with greater precision, and at last, as we shall see later, a point was reached which satisfies this demand in a higher degree than has hitherto been the case in the allied branches of science.

A *microscope* capable of magnifying to the extent of 1,000 diameters is, as a rule, necessary for the investigation of micro-organisms. For the yeast and mould-fungi the only preparation generally required consists in placing a drop of the liquid containing the organisms on an object-glass, and spreading it out in a thin layer by means of a cover-glass. When cultivated on solid substances, a very small portion of the growth is first mixed with a drop of water. At any rate, the preliminary examination of bacteria must always be performed in this manner. In modern bacteriological research, and especially in the case of pathogenic forms, a number of different methods of *drying* and *staining* are employed, partly in order to facilitate observation, and partly with a view to bring out characteristics which would otherwise be observed only with difficulty or not at all. An objection to these methods, urged with unquestionable correctness, is that the violent treatment often alters the proportions of length and thickness, etc., of the bacteria. On the other hand, it must be alleged that certain pathogenic

forms—for instance, the tubercle-bacillus, investigated by *R. Koch*,—could not be determined with certainty until such a preparation had been made; and, indeed, staining is often necessary in order to detect such bacilli. As an example of the methods of staining, we will enter somewhat more closely into the examination of the *tubercle-bacillus*, which led to one of the most important observations made in modern science. *Koch* gave the following method for its examination: The section of the tissue which contains the bacilli is immersed for 24 hours in a mixture of 200 parts of distilled water, 1 part of concentrated alcoholic solution of methylene blue, and 0·2 part of a 10 per cent. potash solution. By this treatment the section is stained dark blue, and is then immersed, for a quarter of an hour, in a concentrated aqueous solution of vesuvin. The section is now rinsed in distilled water until the blue colour disappears, and a more or less strong brown stain remains; finally, the section is treated with alcohol, mounted in clove oil, and examined. The cell-nuclei and most species of micrococci are stained brown by this treatment, whereas the tubercle-bacilli assume an intense blue colour. (Of the known species of bacilli, only the bacilli of leprosy behave in the same way; they differ, however, in other respects from those of tuberculosis.) According to *Koch*, this result depends on the alkaline reaction of the staining solution, since these bacilli never take the stain in acid or neutral solutions; the neutral solution of another colouring matter entirely removes the first stain, except in the case of the tubercle-bacilli, which retain the original staining. Subsequently, various other methods were proposed for the identification of this micro-organism, the most preferable of which is that of *Ehrlich*, who used aniline instead of potash. Aniline is a faintly yellow, oily liquid, the saturated aqueous solution of which has the power of taking up more colouring matter than the solution of potash. *Ehrlich* has also employed mineral acids for decolourising, proceeding on the supposition that the tubercle-

bacilli are surrounded by a cell-wall which is only permeable by alkaline liquids. Therefore, when the bacilli, cell-nuclei, plasma, etc., are stained by the alkaline solution, and the first-named are consequently practically indistinguishable in the mixture, treatment with an acid removes the stain from all the other parts of the section and from all foreign organisms; but, as the presumed envelope of the tubercle-bacilli cannot be penetrated by the acid, these bacilli will remain as the only stained bodies in the otherwise entirely decolorised material. *Ehrlich* carries out the staining in the following manner:—Finely-powdered gentiana-violet is dissolved in a saturated aqueous solution of aniline; 10 to 20 drops of this solution are filtered into a watch-glass, in which the section to be examined is allowed to remain for about 24 hours. It is then rinsed with distilled water and again placed in the watch-glass with a solution of 3 parts of nitric acid in 100 parts of alcohol. After three to five minutes the section is decolourised; it is then transferred to pure alcohol, and finally examined in clove oil.

As is known, photographic illustrations of bacteria have recently come into general use, having been first introduced by *R. Koch*. In order to obtain these, staining and decoloration are quite necessary, partly in order to render the contours of the bacteria sharper, and partly in order to remove all bodies detrimental to the picture.

Staining and decoloration are not generally required in investigations connected with the physiology of fermentation, where the organisms are almost always free, and only seldom mixed with disturbing elements, and only in a few cases has staining led to the discovery of specific characters (Bacterium aceti and B. Pasteurianum, see Chapter III.).

On the other hand, however, it is sometimes necessary in the examination of the organisms of fermentation, and especially of bacteria, to adopt another method of preparation. The particles of organic and inorganic matter which separate from the solutions frequently have a deceptive *similarity to*

various bacterial forms; and, indeed, it is often a matter of the greatest difficulty, if not altogether impossible, even for the most experienced observer to determine with certainty whether the small spherical bodies in the field of the microscope are micrococci or particles deposited by the solution. In such doubtful cases it is advisable, before entering on the physiological examination described later on, to have recourse to *micro-chemical reagents*, which often give good preliminary indications. In beer and in nutritive liquids generally which contain albuminoids, these often separate in spherical and thread-like forms; the starch granules, the dextrins formed from starch, and even some of the hop constituents may also appear as small spherical bodies. The addition of a small quantity of alcohol, ether, chloroform, acetic acid, soda, potash, etc., is often able to throw some light on the nature of these bodies, the resinous substances being dissolved by the former liquids, whilst the albuminoid matter is acted on more or less by the latter solutions; the addition of iodine will impart a blue colour to the starch granules which are present, whilst certain dextrins are coloured red by the same reagent.

In the case of the higher organisms of fermentation—yeast and mould-fungi,—staining is employed for a different purpose, namely, in order to obtain information concerning the *substances which are present in the cell-wall or cell-contents* at different stages of their development. On the addition, for instance, of a solution of iron chloride, or any other salt of iron, to cells which contain tannic acid, a bluish-black or green coloration appears in the cells; in this way it was observed that the cells of Saccharomyces cerevisiæ contain a fairly considerable quantity of tannic acid during the earlier stages of fermentation. If yeast cells are treated with a solution of hæmatoxylin or osmic acid, small, sharply-defined, dark-coloured bodies can be seen, which may be regarded as cell-nuclei of the same nature as those generally observed in the cells of the majority of plants without the aid of this treatment.

2. Biological research by means of the Microscope; moist chambers.

A true and thorough insight into the nature of the organisms of fermentation is not attainable until the *method of physiological investigation* is resorted to. As stated above, endeavours were made long ago to devise methods of this nature; the entire neglect of precautions in carrying out the experiments resulted, however, in complete failure, and a reaction then set in, which found expression, *e.g.* in the work of *Reess* on the Saccharomycetes (1870), in which he expressly stated that he had taken no precautions to obtain pure cultures,—to such a degree had these cultures fallen into discredit. In the course of the following years, however, the matter took a different turn, and it is, perhaps, an almost unique fact in the history of science, that, in so short a time, a new method of investigation not only made its way, but also yielded practical results, both in pathological science and in our own special branch, results which have brought about a revolution in many previously-accepted doctrines.

The aim of physiological investigations of micro-organisms is to gain an insight into their development and vital functions. The means to be employed in order to attain such an insight is naturally to determine such conditions for their growth and propagation which will make it possible to observe the changes gradually taking place in the organism itself and in the substances influenced by it. When the object aimed at is solely to obtain a *knowledge of the various forms* which the organism assumes during its development, the conditions are much more easily attained than when a culture on a large scale of individuals originating from one cell of the species is required for the purpose of gaining an insight, through *physiological*, *chemical*, or *purely practical experiments* with larger quantities of these organisms, into the relations between their forms and external influences and into all their biological functions. In the former case all that is required is a culture

in which the organism is able to develop itself undisturbed, apart from the question whether foreign individuals or species are present in the same preparation. In the latter case, on the contrary, an *absolutely pure culture is required.*

Cultures of the first-mentioned kind may in certain cases be of use in affording information when the case previously mentioned occurs; a nutritive solution in which deposits of various kinds have assumed a more or less deceptive similarity to different bacteria, in consequence of which it is impossible to obtain any certain information by means of an ordinary microscopical examination; the question to be answered by the experiment is accordingly, whether these small bodies are *capable of multiplying.*

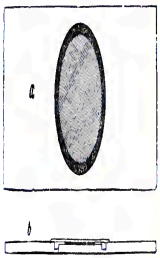

FIG. 1.

Ranvier's Moist Chamber: *a*, seen in plan; *b*, in section.

A drop of the liquid is transferred to the so-called "*moist chamber*," as, for instance, *Ranvier's* (Fig. 1). This apparatus is made by grinding a slight hollow in the middle of a common object-glass; around this hollow a groove is made of greater depth to receive the water; the drop of the nutritive solution, which must be very small, is placed in the middle of the hollow and covered with a cover-glass, which extends beyond the groove; when the cover-glass is in place, it is cemented by means of vaseline, and the drop is thus spread out between the cover-glass and the hollow of the object-glass, and is at the same time protected by the water in the groove from evaporating.

Another kind of moist chamber, invented by *Böttcher*, consists of a glass ring cemented to a common object-glass, upon which, within the ring, some drops of water are placed. A cover-glass, on the under side of which a small drop of nutritive liquid containing the organisms has been placed, is fastened to the edge of the glass ring by means of vaseline.

This apparatus is brought under the microscope, and the changes of the organisms are observed from time to time; or it may be placed in an incubator, maintained at a suitable, constant temperature, and withdrawn at intervals for a thorough microscopical examination.

These forms of apparatus are adapted to morphological or botanical examinations under the microscope. If, on the other hand, a physiological examination is to be carried out, it is

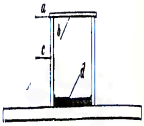

FIG. 2.

Böttcher's Moist Chamber: *a*, thin cover-glass; *b*, layer of nutritive material; *c*, glass-ring; *d*, liquid.

necessary that the pure cultures should be developed on an extensive scale. Among the investigators who have developed the methods in this direction, *Pasteur*, *Lister*, *Koch*, and *Hansen*, deserve special mention. (See "Preparation of the Pure Culture," p. 22.)

Every fermentation, no matter whether the product be beer, wine, spirit, vinegar, or other liquid, is caused by a vegetation of living organisms, "organised ferments," and, in practice, it is endeavoured to obtain, as far as actual circumstances will permit, a pure culture of the forms best suited to the manufacture. Although, in our time, with a better understanding of aims and means, great progress has been made in this direction, yet there must always be limits which, from purely practical reasons, cannot be overstepped; the cultures in the factories will never reach such a perfection

as to keep continually in a state of absolute purity. It is, however, one of the most salient features in the present development of the industry of fermentation, that efforts based upon the right understanding of the paramount importance of the fermentation organisms are being made to emancipate the chief useful species from the action of injurious forms. The very great importance of this was not, however, appreciated until *Hansen*, through methodical selection of certain types of yeast, showed that such a pure growth insures far greater certainty and uniformity than the impure and unknown yeast mixtures hitherto used. We shall come back to this point later on (Chapter VI.).

In experiments in the laboratory, where the object is likewise to prepare cultures of fermentation organisms, greater demands may naturally be made than on a practical scale. In this case it is necessary to work with *absolutely pure cultures*, partly in small quantities, partly in masses so large that they may be transferred at a given point of time from the laboratory to the brewery. Conditions which are wanting in practice are sought to be realised in the laboratory, which is specially arranged for such investigations. We will now briefly mention these requirements and the way in which they are met, and, for purely historical reasons, we will begin with the last link, viz., with the vessels and liquids which receive the originally-prepared small pure cultures, and the expedients to be employed in their cultivation. It is necessary that these vessels and liquids be *sterile* before the inoculating substance is introduced, i.e., they must be freed from all living germs; also that the various utensils and the air in the place where the work is performed should contain as few living germs as possible. The same applies of course to the clothing and hands of the experimenter.

3. Sterilisation.

The principles of the technology of sterilisation as well as the models of the various apparatus appertaining thereto

had been given in the old experiments on spontaneous generation.

As early as the year 1765, *Spallanzani* argued against the doctrine of *Needham* and *Buffon*, that living beings came into existence through spontaneous generation in putrefying liquids or other substances. *Spallanzani* warmed extract of meat in closed flasks, and demonstrated that the contents of the flasks remained unaltered until air is allowed to gain admittance. From this he concluded that the germs which developed in the opened bottles had come in from the air. Later (1782) *Scheele* demonstrated that *vinegar* may be made to keep sound by means of warming. But his discovery was not heeded. In 1810 *Appert* published his book on a means of preserving various foods and liquids through warming. In the 4th edition of his book, which appeared in 1831, he gives directions for the treatment of *wine*, *beer*, and other liquids, his method being essentially the same as that employed in our day (the so-called "Pasteurisation").

To the following period, which was of such great importance for micro-biology, belong the highly meritorious researches of *F. Schulze* and *Th. Schwann*, in which it was shown, that perishable liquids which had been vigorously boiled in flasks would remain sterile if the air subsequently entering were made to pass through sulphuric acid or through red-hot tubes. At the same time *Cagniard-Latour* and *Schwann* described the yeast-cells, and *Kützing* the acetic acid bacteria. *Turpin* started (1838) that most important doctrine: "No decomposition of sugar, no fermentation without the physiological action of vegetation."

Finally, the objection against the experiments of *Schulze* and *Schwann*, that the air entering the flasks had been affected in some manner by the violent treatment to which it had been submitted, that it was no longer able to furnish the conditions of growth required by the germs existing in the liquid, was overcome by the beautiful experiments of

Schröder and *Dusch* (1854), who caused the air to pass through cotton-wool filters, and by this means still obtained the same result.

The principles of the whole technology of sterilisation being thus established, the matter under consideration reached a high state of development and great importance both for science and for industry, more particularly through *Pasteur* and, subsequently, several other eminent scientists, who devoted their energies to these investigations.

1. *Sterilisation of glass and metal articles.*—Sterilisation properly so-called must always be preceded by a thorough mechanical and, in many cases, also a chemical cleansing. Articles of daily use in the laboratory, as, for instance, spatulas, pins, wire, etc., are heated directly in a flame and allowed to cool in a germ-free space. Many pieces of apparatus, however, do not admit of this treatment, and must be sterilised either by heating in steam or in a water-bath, or else in dry air by means of a sterilising oven, in which the objects are heated for one or two hours at a temperature of about 150° C. According to the nature of the objects, some may be put directly into the sterilising oven, whilst others must be previously wrapped in paper. The necks of the flasks are closed by cotton-wool, which is, in addition, often covered by several layers of filter-paper.

2. *Sterilisation of nutritive liquids and solid nutritive substrata.*—Nutritive liquids can be sterilised by filtration or by heat. The former method presents the advantage that the liquids treated undergo less change than when heat is employed, and are, consequently, better suited for the development of many species of micro-organisms. The necessary condition for sterilisation is, that the pores of the filter must be smaller than even the smallest micro-organisms. Gypsum, asbestos, charcoal, and porcelain have been employed for this purpose, the liquids being forced by pressure and suction through thick layers of these substances. The form most generally used is the Chamberland porcelain filter, which,

however, requires frequent cleansing, and must also be frequently sterilised by ignition, it having been proved that the bacteria are able in time to grow through the pores[1].

Liquids and solid nutritive substrata are in most cases sterilised by heat. The way in which this must be done, as well as the duration of the heating process, are dependent on the nature of the substratum in question. Direct boiling on the sand-bath may be employed for the purpose of sterilising, for example, brewery-wort in Pasteur-flasks, otherwise the water-bath may be used. An excellent means of sterilisation is afforded by steam either at 100° C. or under pressure (110–120° C.) by means of *Papin's digestor* (*autoclave*). During cooling, care must be taken that only absolutely pure air comes in contact with the sterilised substance, the air entering the vessel being filtered through cotton-wool or passed through tubes bent several times, if it is drawn in slowly and with no great force[2].

[1] In breweries the *filtration of beer* has been resorted to during the last few years, the filtering media commonly used being paper, cellulose, asbestos, etc. By such filtration brewers sometimes succeed, it is true, in freeing a beer originally sound from deposits of various kinds, and in rendering it bright; but, on the other hand, the fact must be emphasised, that an indiscriminate employment of this method may occasion *great dangers*, as has been directly proved by the experiments made by *Thausing*, *Wichmann*, *Reinke*, and others. If, namely, the filters are not sufficiently effective, it may happen that only the yeast cells are retained, but not the bacteria, which are then enabled to act with much greater energy upon the liquid. Another great danger lies in the fact that a filter, owing to deficient cleansing, may become a seat for the development of different kinds of germs, contaminating all the beer passing through it. If a single cask of a store-room has become infected, and the filter is not effectually *sterilised* after the filtration of this beer, the disease will be communicated to all the other beer.

[2] In the so-called *Pasteurisation of beer*, a merely relative sterilisation is all that is generally aimed at; that is to say, by a cautious treatment of the beer at elevated temperatures, it is sought to check the yeast cells to such a degree that they are capable only to a very limited extent of multiplying and producing fermentation. It is only for transportation to a great distance that it is attempted to kill all living germs contained

Nutritive gelatines must be treated with particular care, as they often lose their power of gelatinising if the heat is too great or if it be applied too long.

If the substance cannot be boiled without suffering great changes or entirely losing its original nature, *fractional sterilisation* must be resorted to.

This, for instance, is the case with blood-serum, which is employed in a gelatinous condition in bacteriological studies. This substance, when heated to 100° C., becomes fluid, and does not again solidify, and it is, therefore, necessary to proceed in a different way in order to get it sterilised in the gelatinous state. It was observed that a temperature of 58° to 62° C. was sufficient to kill the vegetative bacteria which develop in blood-serum. By this treatment of the substance only the *spores* of bacteria remain unkilled. If the gelatinised serum is placed for two or three days in an incubator at a temperature favourable to the development of the spores (30° to 40° C.), the greater number of these germinate, and the new vegetative rods can then be killed by again heating to about 60° C. If this process is repeated several times, the gelatinous mass will remain sterile for an unlimited time. This process, which is also used for the sterilisation of milk, and which was discovered by *Tyndall*, has been further established by *R. Koch*.

A similar method is employed in zymotechnical laboratories for the treatment of nutritive liquids, which, when boiled, are apt to deposit a considerable amount of albuminoid matter, and would thus become comparatively bad nutritive liquids for the alcoholic yeast.[1]

in beer. No general rules can be laid down for a treatment of that kind. The correct procedure depends on the nature of the liquid as well as on the properties of the particular species of yeast, and preliminary experiments must accordingly always be made with regard to temperature as well as to the duration of the action of the particular temperature.

[1] Sterilisation is also attempted in practice, namely, for the purpose *of introducing wort in a sterile condition by means of closed cooling and aerating apparatus into the fermenting-tun*. It is true that the wort

3. *Sterilisation of the Air* is best attained, as stated above, by means of cotton-wool filters; sulphuric acid, salt water baths, cloth filters, etc., are less efficient. In laboratories, where work must often be performed in germ-free air, a glass chamber is employed, the front of which can be raised sufficiently for the operator to introduce his hands. Some time before using the chamber, the whole of its inner surface must be washed, and the chamber then closed. The particles and germs suspended in the air will then settle to the moist bottom and remain there.

4. Disinfection.

Another method of killing disturbing germs is by the use of *disinfectants*, which act as poisons on the micro-organisms. Not a few of these substances have found application in practice. The limit for the employment of such poisonous substances must be determined for each individual case. As manipulations with such poisons may be deleterious to the operator, it is important to ascertain their proper *degree of dilution*.

Investigations having for their object the determination of the *power of resistance of the various species of micro-organisms to poisons* have proved that it varies greatly in different cultures of one and the same species, not only for the spores, but also for vegetative forms. A young culture behaves differently from an old one, and the same applies to individuals belonging to one and the same culture.

cannot keep absolutely free from germs when the fermentation takes place in open tuns, but *a great deal can be effected in this direction by acquiring a true and thorough understanding of the matter.* The expert brewer will always take care that the air in the fermenting room is kept as free from germs as possible, and also that the tuns as well as all utensils that are immersed into the fermenting liquid, *e.g.* thermometers, sample glasses, etc., *are always perfectly clean. As a matter of course*, these precautionary measures could *not acquire any real practical importance until, through Hansen's reform, absolutely pure yeast had been introduced into the fermenting room.*

In all experiments of this kind, organisms which have been tested with some disinfectant should afterwards be brought under the most favourable conditions of growth, otherwise they will not develop, even though they be alive and capable of development. In such cases, the ordinary temperature of the room and solid nutrient substrata are not sufficiently favourable; it is also necessary to allow ample time for the observation of such growths before definitely deciding whether they are dead or not; in fact, it often happens that they have merely been somewhat checked in their development, and that they may develop again, after some time, with their full vigour. Furthermore, the temperature and the medium in which the organisms are present when the disinfectant is employed may be of some importance. Before testing a culture thus treated, great care must be taken to previously free it from all remains of the disinfectant by washing and dilution.

The first information on this subject we owe to *R. Koch*. Subsequently, these researches were continued by *Gruber* and others.

Koch examined several poisons not only with reference to the degree of concentration requisite for destroying bacteria and spores of bacteria, but also with a view to ascertain the particular quantity necessary for checking the micro-organisms in their power of development in suitable nutritive solutions.

I will here briefly state the results obtained by *Koch*. Carbolic acid was found to be a less efficient disinfectant than it is commonly held to be. A solution containing 5 per cent. destroyed the power of development of anthrax spores only after 48 hours, whilst the anthrax bacilli were killed in two minutes by a 1 per cent. solution. A solution of 1 part in 850 proved sufficient to check the growth of the bacillus, and when anthrax spores were moistened 5 to 7 times with a 5 per cent. solution, their development was somewhat retarded. A 5 per cent. solution of carbolic acid in oil or alcohol had

absolutely no effect on the anthrax cells and spores. In the form of vapour, carbolic acid acts more strongly, although even two hours' action of its vapour at 75° C. proved unable to destroy the vitality of these spores. Sulphurous acid is not able to destroy all germs, even under very favourable conditions. On the other hand, chlorine, bromine, and corrosive sublimate are efficient disinfectants. According to *Koch*, corrosive sublimate in the proportion of 1 in 1000 acts fatally on all germs. According to experiments made by *Johan-Olsen*, however, mould-fungi are only destroyed by more concentrated solutions; *e.g.*, Penicillium glaucum only by a solution containing 1 in 400. Several bacteria, the organisms of puerperal fever, abscesses, and putrefaction, likewise germinate and grow, although more slowly than usual, on slices of potato saturated with a solution of sublimate containing 1 part in 500, and their growth is only checked by a concentration of 1 in 300. *Gruber* found from recent investigations, carried out with all precaution, that anthrax spores, for instance, were only killed by 5 parts of sublimate in 1000, 1 part of sublimate hydrochloric acid in 1000, 1 part of sublimate tartaric acid in 1000.

For cleansing pipes, coolers, etc., which often contain very considerable deposits of organic matter liable to decomposition through the agency of micro-organisms, a solution of soda is to be recommended; it acts by dissolving and loosening resinous and albuminoid matters, which can then be removed by means of water. Experiments made by *Aubry* and *Will* have proved that *chloride of lime*, even when strongly diluted (2—5 per cent. of chlorine) is a very efficient disinfectant, owing to its deadly action on fungi. This substance being very cheap is therefore specially suited for cleansing walls, pavements, gutters, etc. Bisulphite of lime is also very efficient (used in solutions containing 2—4 per cent. of sulphurous acid). The filter-bags—which, according to the investigations of *Will*, often contain *very considerable accumulations of wild yeasts and bacteria in the very texture of the material*, and which

in many breweries are never sufficiently cleansed—should be disinfected by treatment with a solution of chloride of lime. *Will*, who experimented with material obtained from a brewery, recommends a solution containing 1 per cent. of active chlorine (corresponding to about 3—3½ kg. of good commercial chloride of lime to 100 liters of water). The filter-bags must be washed with pure water after this treatment. In physiological laboratories, where it is of especial importance to guard against any invasion of foreign germs, an alcoholic solution of salicylic acid will prove of service (it is often used by *Hansen* in the Carlsberg laboratory for cleansing tables). The action of this substance, even in a diluted state, in checking fermentation is generally known.[1] Recently, hydrofluoric acid has also been employed as a disinfectant (see p. 25).

FIG. 8.
Pasteur's Flask.

5. Flasks: Pasteur, Chamberland, Freudenreich, Hansen, and Carlsberg Flasks.

All vessels in which cultures are made must satisfy the

[1] *Biernacki* and others have proved that substances otherwise possessed of antiseptic power can, when very much diluted, act as *stimulants* on yeast-fungi. Thus, alcoholic fermentation is promoted by the addition of corrosive sublimate in a dilution of 1 in 300,000, by salicylic acid solution of the strength 1 in 6000, and by boracic acid solution of the strength 1 in 8000.

condition that they are proof to every contamination from without. *Pasteur's flasks* satisfy this demand in the highest degree.[1] The illustration shows this flask in the slightly modified form employed in the Carlsberg Physiological Laboratory directed by *Hansen*. When the nutritive liquid is boiled, the steam first escapes through the wide straight tube, at the end of which is a piece of India-rubber tubing; when this is closed the only outlet for the steam is through the bent tube. After some time the flask is taken from the sand-bath, and the bent tube may be closed with a plug of asbestos. The sterilisation is then complete, and the contents of the flask can remain for years without suffering alteration. During the cooling and the indraw the air is partially filtered through the asbestos-plug; any germs that are carried further are deposited in the lowest part of the bend, or, at the most, do not pass the enlargement of the thin tube, and therefore do not come into contact with the liquid. Hence, it is evident that the lower part of the bent tube must be heated whenever the flask is to be agitated or emptied through the straight tube, without exposing it to contamination. If the flask is to be opened and placed in connection with another flask, this must be effected either in some small germ-free space, or the opening and connecting must be done in a flame. A Bunsen burner is placed directly in front of the operator, the flask to be emptied to the *left*, and the one that is to receive the liquid or culture to the *right*, close to the burner. Then the tube of the left-hand flask is opened *in the flame* by quickly removing the India-rubber tube with its glass stopper; while the open tube is in the flame, the glass stopper of the flask to the

[1] *Chevreul* and *Hoffmann* had previously found that when vessels employed in sterilising liquids are provided with open but bent tubes their contents will remain sterile. Although *Chevreul* was thus the first to indicate the principle of these flasks, I will not mention them by any other name than that of *Pasteur*, through whom, indeed, they have obtained a wide application.

right is quickly withdrawn, and the hot tube of the first flask is introduced into the India-rubber tube of the second flask. The liquid is now poured into the latter flask, the bent tube of the former flask being at the same time heated. Then the side tube of the left flask is again introduced into the flame, while the stopper of the right flask is heated and put back into its place; finally, the left flask is closed *in the flame* with its tube and stopper. When the operation is quickly performed, there is seldom any danger of contamination.

Pasteur flasks will be found indispensible in certain

Fig. 4.
Chamberland Flask.

operations; as, for instance, in physiological researches where one has to deal with large quantities of liquids.

In recent years various other flasks and vessels have been brought into use, notably the *Chamberland* flask (Fig. 4), the neck of which is closed with a ground cap, which terminates above in a short, open tube; this tube is filled with tightly-packed sterilised cotton-wool.

The *Freudenreich* flask is constructed on exactly the same principle; it has, however, a cylindrical shape.

For certain special purposes *Hansen's* flask (Fig. 5) is employed. The ground cap is provided with a cotton-

wool filter (*a*), and the flask has a small side-tube closed with an asbestos stopper (*d*). This flask is used partly for the preservation of pure cultures, partly for sending small cultures or samples from the propagating apparatus.[1] For the first-named purpose the flask is half filled with a 10 per cent. solution of cane-sugar, to which a trace of the yeast-culture is then added. The asbestos stopper and lower edge of the cap is coated with sealing-wax (*c*). For the last-named purpose the lower part of the flask is filled with cotton-wool (*e*), and some cotton-wool (*b*) is also put into the cap, below the filter. For the mode of employment, see Chapter VI.

FIG. 5.
Hansen Flask.

For the development of very large cultures the Carlsberg vessels (Fig. 6) are employed. They have a capacity of 10 liters, are made of tinned copper, are cylindrical in shape and conical at the top; at the apex of the cone a twice-bent tube (*c d*) with an enlargement (*e*) is either soldered or screwed. At one side of the cone is the inoculating tube and glass stopper (*a*), and at the bottom of the vessel is another tube (*b*) for drawing off the fermented liquid and the yeast. This tube is provided with a pinch-cock. When the liquid is sterilised, the

[1] For the description of this apparatus, see Chapter VI.

bent tube is closed with an asbestos or cotton-wool filter, which is either screwed on or placed over the end (*d*). For further particulars respecting the treatment of these vessels, see *Hansen's* "Untersuchungen aus der Praxis der Gärungsindustrie."

6. Nutritive Substrata.

With regard to the *nutritive substrata*, the problem naturally always consists in finding those which are best suited to the respective organisms. If they also possess the

Fig. 6.
Carlsberg Vessel.

advantage of being *per se* less favourable for the development of competing forms, it is a great point gained. The rule must of course be borne in mind, when comparative investigations are made in different directions, that the nutritive liquid must always remain the same. For the investigation of alcoholic ferments *Hansen* generally uses hopped wort from the filter-bags; in special cases of investigations of this kind yeast-water with an addition of glucose, or a solution of cane-sugar or some other sugar, is employed. If it is desired to use a solid nutritive material, the liquid may be mixed with

5 to 10 per cent. of gelatine. Similar liquids, or more frequently, meat-extract with an addition of peptone, are employed for bacteriological investigations; this mixture is neutralised with sodium carbonate. Either gelatine or agar-agar is used for rendering the medium solid. Solid nutritive substrata are the best for the study of mould-fungi, in most cases preferably sterilised black bread. Where liquids are employed, the most suitable are beer-wort, fruit decoctions, or mixtures of sugar with an addition of tartaric acid or tartrates. *Pasteur* used exclusively liquids as substrata in his investigations on the organisms of fermentation. Later, solid substrata were very extensively employed, and in this respect Koch has given many practical illustrations.

We have now briefly explained how our micro-organisms are cultivated, and guarded against contamination from the liquid itself, from the vessels and apparatus, from the air, and from the experimenter. We have now before us the first and most important question: *How are we to obtain the first absolutely pure culture to be introduced into the flask?* I have, on purely historical grounds, first sketched the *conditions for the preservation of the pure culture*, because these were known long before a certain method for preparing the pure culture itself had been discovered.

In this respect it will be instructive to see how we have advanced step by step, and we will again take up the subject historically, from the moment when really rational endeavours were made for the attainment of this object.

7. Preparation of the Pure Culture.

It is only by starting with one individual cell that we can be certain of obtaining a really pure culture, and such a culture is the indispensable condition for exact scientific investigations of the micro-organisms. These investigations may, as stated above, be carried out for different purposes, namely, with a view to observe the *individual, the isolated cell through its successive phases of development*,—*morpho-*

logical investigation; or the object may be *to study the vital functions of a growth developed from a single cell,—biological and physiological investigation*. As these two *methods* of investigation are of different natures, the means to be employed must likewise differ.

(*a*) *Pure Cultures for Morphological investigations.*—After the discovery had been made, by means of the microscope, that yeast consists of cells, it was not long before the attempt was made to determine, by closely observing one of these cells, the way in which they multiply, and in what forms the new generations occur. In other words, a morphological examination of a pure culture was made. For this purpose it became necessary to guard against such disturbances as would arise from other cells hindering the selected one from multiplying or withdrawing it from the observer's view. On the other hand, it would not matter if foreign cells occurred in other portions of the preparation.

Ehrenberg, as early as 1821, observed the germination of the spores of some fungi by means of investigations of this kind. Later, the propagation of yeast-cells was observed by *Mitscherlich*, *Kützing* (1851), and *F. Schulze* (1860), in the same way. A small quantity of high-fermentation yeast was diluted with beer-wort until it contained only one or two yeast-cells; from a drop of this an ordinary preparation was made, the cover-glass was cemented fast on the glass slide, and the development of the cell was watched under the microscope. The same method was employed, in its main features, by *Tulasne* (1861) and *De Bary* (1866) in their famous researches on the germination of the spores of the fungi. The investigation was carried further by *Brefeld*, who followed the development of the mycelium until it in its turn again formed spores. He sowed the spores on the object-glass. When his investigation was to extend over a longer space of time, during which an ordinary drop of liquid would evaporate, he added gelatine to the liquid, and placed a small shade of paper over the apparatus; this shade was attached to the

tube of the microscope in order to keep out foreign germs as much as possible. When the development took place in ordinary fluid drops, the preparation was placed, in the interval between two observations, under a moist glass-shade; thus, an unbroken observation was not attempted, and was not even possible for the larger fungi. Accordingly, in consequence of the whole arrangement of the experiment, absolutely pure cultures are quite out of the question. As stated above, however, such an investigation may very well be carried on with an impure material.

(b) *Pure Cultures for Physiological experiments with mass-cultures.*—When the object of the pure culture is to employ it for *biological* or *physiological* researches, so that a *mass-culture* of the growth becomes necessary, a direct microscopical control is impossible, and the methods described above cannot be employed. The methods made use of for this purpose may be divided into two groups, namely, the *physiological methods* and the *dilution methods*. In the former, liquids are used, in the latter liquids or gelatines.

(α) *Physiological methods.*—The physiological methods employed by *Pasteur*, *Cohn*, and others, start with the fundamental idea, that the various species occurring in a mixture will multiply unequally according to their different natures, when they are cultivated in one and the same nutritive liquid and at the same temperature, so that those species for which the conditions are unfavourable will be gradually suppressed by the one or more species for which the conditions are favourable. Different liquids have been employed for such cultures in the course of time; as, for instance, alkaline liquids for growths of bacteria, acid liquids for the purpose of freeing yeast-growths from bacteria (lactic, tartaric, hydrofluoric acids, etc.). The weak point of all such methods is, that they *start from an unknown material*, namely, *the impure mixture*. Hence, it is impossible to know what results a treatment of this kind will lead to, because it is

evident that any agency exerted will be hap-hazard, and this does not, properly speaking, constitute a method; in fact, there is always the possibility that the weaker species are not destroyed at all, but merely checked and retarded, so that when the stronger species, after having reached the height of their development, enter into a condition of weakness, other species will get a chance of multiplying. Likewise, there is always the possibility that not one but two or more species thrive equally well in the liquid, and, consequently, develop to the same extent. If we examine, for instance, common brewers' yeast, we may often separate several typically different species of "culture-yeast," as they are termed, from the same yeast-mass by means of *Hansen's* method. The method given by *Pasteur* for the purification of a brewers' yeast may be mentioned as a marked illustration of the dangers connected with the physiological method of treatment. The impure yeast-mass is introduced into a cane-sugar solution to which a small amount of tartaric acid has been added. The object of the method is to free the yeast from any disease germs with which it may be infected. *Hansen's* investigations have, however, proved that, even if the bacteria are suppressed or checked by this treatment, the so-called wild yeasts, and among them *those productive of diseases*, will develop abundantly, and in many cases the culture-yeast becomes totally suppressed instead of being purified. Even if there is primarily only a trace of the wild yeasts, or yeasts of disease, these are apt to develop to such an extent through this treatment that finally they may form the chief portion of the yeast-mass. Thus, this unmethodical treatment of the unknown material has led to an exactly opposite result to that intended. Even when the yeast-mass consists entirely of the so-called wild yeasts, it is not possible by this process of Pasteur's to prepare with certainty a pure culture of a definite species.[1]

[1] According to *Effront's* method, hydrofluoric acid is used as an antiseptic in distilleries. But the use of this material for the purification of

If, now, we ask, whether it is advisable to employ any of the various methods mentioned above for the purification of an unknown impure yeast-mass, the answer must accordingly be in the negative; and this will be the case whether the culture is intended for purely scientific or for industrial purposes, for the danger will always remain of *furthering the growth of species other than the desired one*. And, the starting-point being uncertain, it necessarily follows that the result must be so too. In fact, all such methods must now be regarded as antiquated, and will, whenever resorted to, prove utter failures. Yet in certain cases they may have some value when employed preparatory to the preparation of a pure culture. In the different branches of the fermentation industry there is only one way that will lead to the goal, namely, the application of the same principles which have for many years been followed in agriculture and horticulture —the selection, by means of methodical experiments, of the particular species or type which gives the best results under the circumstances, and which is therefore to be sown alone, without any admixture of other types. The only possible way of effecting this is, however, by the adoption of the methods discovered by *Hansen*, which will come under consideration later on.

(3) *Dilution methods.*—The second group of methods employed for physiological purposes embraces the dilution methods, or the so-called "fractional cultivation," the

an impure yeast-mass, whether brewers' or distillers' yeast, as proposed by Effront, will give rise to the same dangers as were mentioned above in the case of tartaric acid. In fact, a long series of methodical experiments made in the laboratory of the author of this book have shown that by the treatment of impure yeast according to Effront's method the *wild yeasts* and *Mycoderma species* will develop far more actively than cultivated yeast; and our experiments have also shown that in many cases even such a dangerous species as *Bacterium aceti cannot be suppressed at all by this treatment of the yeast-mass*; on the contrary, it was found to *multiply much more actively* when treated with hydrofluoric acid or fluorides.

principle of which is to dilute the material to such a degree that it is ultimately possible to isolate a single cell. In most of these cultures we can only reckon on their probable purity, whereas for the alcoholic ferments *Hansen* has developed the process into an *exact method*.

Lister was the first (1878) who brought methods of this kind into use. In order to prepare pure cultures of lactic acid bacteria he first determined microscopically the number of bacteria in a very small drop of sour milk, counting them in several fields of the preparation, and thus calculating their number in the whole preparation. He then calculated the amount of sterilised water required to be added in order that after dilution there would be on an average less than one bacterium in each drop. With five of these drops he inoculated in one case five glasses containing boiled milk. The result was that the milk in one of these coagulated, showing that it contained Bacterium lactis, whilst the four other glasses remained unaltered and did not show the presence of bacteria. The same method was subsequently employed by *Nägeli* and *Fitz*.

Air has also been made use of for such a dilution (*Pasteur*). A small portion of yeast is dried and ground with powdered gypsum. The resulting fine powder is thrown into the air from a height, a series of vacuum flasks (p. 57) being opened while the particles are falling. Isolated yeast-cells which are distributed in the resulting dust-cloud may then perhaps enter some of the flasks.

In comparison with the physiological methods the dilution method now described is a distinct advance; indeed, we have here approached much nearer to the goal. On the other hand, it is clear that, even if the dilution is carried as far as in the case mentioned, in which only one of several flasks shows development, it is not yet proved that this one flask has received only *one germ*. Thus, there is still great uncertainty, even in such cases where the individuals with which we are working can be counted. Moreover, such

countings are very difficult in the case of the bacteria, and often, indeed, quite impossible. In all cases the accuracy of such calculations is very questionable. Thus, the question remains to be solved: How are we to distinguish the flasks which have only received *one cell* from those which, in spite of the counting, have been infected with *several cells*? For the bacteria, no means has as yet been found of solving this difficulty.

In the case of the yeast, this problem was solved by *Hansen*, who developed the method to such a degree of perfection as to create, in fact, an *exact method* (1881). He employed dilution with *water*, in the following manner:— The yeast developed in the flask is diluted with an arbitrary amount of sterilised water, and the number of cells in a small drop of the vigorously-shaken liquid is found. The counting, in this case, is effected in a very simple manner by transferring a drop to a cover-glass, in the centre of which some small squares are engraved, and this is then connected with a moist chamber (Fig. 2); the drop must not be allowed to extend beyond the limits of the squares. The cells present in the drop are then counted. Suppose, for instance, that 10 cells are found; a drop of similar size is transferred from the liquid, which must first be again vigorously shaken, to a flask containing a known volume, *e.g.*, 20 ccm. of sterilised water. This flask, then, will in all likelihood contain about 10 cells. If it is now vigorously shaken for some length of time, and then 1 ccm. of the liquid introduced into each of 20 flasks containing nutritive liquid, it is probable that half of these 20 flasks have received one cell each. But, here again, as in *Lister's* experiments, it is entirely a calculation of probabilities. If the flasks are left in repose for further development of micro-organisms, there will be a chance of getting a pure culture in some of them. But no certain inferences can be drawn. *Hansen* succeeded, however, in adding a new link, which first gave certainty to this experiment. If, namely, the freshly

inoculated flasks are vigorously shaken, and then left in repose, the individual cells will sink to the bottom, and become deposited on the wall of the flask. It is self-evident that if the flask contains, for instance, three cells, these cells will always, or at least in the majority of cases, be deposited separate from each other and apart, on the bottom. After some days, if the flask is raised carefully, it will be observed that one or more white specks have formed on the bottom of the flask. *If only one such speck be found, we have obtained a pure culture.*

It is evident that by means of this method we are also able to introduce a single cell into the flask with nutritive solution.

It was by this method that *Hansen* prepared all his first pure cultures, with which he carried out his fundamental researches on alcoholic ferments.

Solid nutrient media have also been employed for the preparation of pure cultures for use in physiological investigations. The foundation of such methods was laid by *Schroeter* (1872), who, in his researches on pigment-bacteria, employed slices of potatoes among other nutrients. He had observed that when such slices had been exposed for some time to the air, specks or drops of different form and colour made their appearance. Each of these specks contained most frequently *one* species of micro-organism.

R. Koch subsequently considerably improved this method. He at first prepared his pure cultures by means of streak growths in nutritive gelatine. Afterwards he devised a far better method, the so-called plate-culture method (1883). He proceeds in the following manner. A trace of the crude culture is transferred to a large proportion of sterilised water. From this a small quantity is transferred to a flask containing, for instance, a mixture of meat-broth and gelatine warmed to 30° C. The flask is shaken in order to distribute the germs, and the contents poured on to a large glass plate, which is then covered with a bell-glass. The gelatine quickly sets and the

germs remain enclosed in the solid mass. In a few days they develop to colonies—points or specks which are visible to the naked eye. The purity of the specks of bacteria in the gelatine is ascertained, according to *Koch*, partly by their appearance, colour, form, etc.

When regarded more closely it will be seen, however, that there is no *essential* difference between this distribution of the germs in the liquid gelatine, and the former dilution by means of liquids. The same uncertainty is always present: neither the macroscopical observation of the appearance of the colony nor the microscopical examination of its contents gives any surety of its only containing one species.

The only possibility of securing a really pure culture in the gelatine consists in the direct observation of one individual germ and its development.

Hansen has done this in the case of yeast-cells, and the method which he contrived for the purpose is as follows. *The layer of gelatine formed by the solidified nutritive liquid is arranged in such a way that the position of the isolated germs can be observed under the microscope.* The position of these germs, then, is accurately marked, and the cell can be seen to develop and propagate step by step.

For the glass-plate is substituted a round cover-glass of about 30 mm. diameter. This is fastened to a glass-ring, which again is cemented to a thicker glass, thus forming the moist chamber previously described (Fig. 2), and which is adapted to the purpose, and carries a layer of solid gelatine on its inner upper surface. The essential point in *Hansen's method* is, that the leading principle—"the starting-point of a pure culture must be a single cell"—is consistently carried out, which is not the case in *Koch's* method. The germs must be so sparsely distributed that comparatively few are present in the gelatine layer; the chamber is then either allowed to remain under the microscope, in order that the multiplication of the germs may be directly followed, or the positions of the well-isolated germs are marked, either by

dividing the glass-cover into small squares or by means of the object-marker, and the apparatus is placed in the incubator until the colonies are completely grown. On one cover-glass there may be 50 to 60 well-isolated germs. When the colonies are completely developed, they are transferred to flasks by means of a small piece of platinum wire, which has been previously ignited and cooled. During this transference the culture is for an instant in the air, and is here exposed to contamination. But the danger of contamination at this, the single weak point, is reduced to an insignificant minimum, and disappears if the above-mentioned operation be performed in a small enclosed germ-free space; as, for instance, in a small chamber with glass sides which is sufficiently large to admit the apparatus and the hands of the experimenter (see p. 14). In this way the transference of the colonies is effected with all possible security. From the first flask the culture can be transferred without contamination to a continually increasing number of larger flasks. Thus, *Hansen's* method approaches the desired end as nearly as is possible, and is consequently employed everywhere in exact experiments of this kind.

As early as the year 1883 *Koch's* method of plate-culture was tested by *Hansen*. He prepared a mixture of two species of yeast which can be distinguished from each other microscopically, namely, Saccharomyces apiculatus and a species of the group Saech. cerevisiæ. This mixture was introduced into wort-gelatine, and after shaking was poured on to a glass-plate. Of the specks formed, about one half contained one species exclusively, the other half the other species, and in one of the specks both species were found.

Later (1888) a similar control was carried out for the bacteria by *Miquel*, who introduced 100 colonies from a plate-culture obtained in an air-analysis into 100 flasks containing meat-broth with peptone. The examination of the growths developed in the flasks showed that they contained 134 different species of micro-organisms. The cause

of this evidently depends upon the fact that it is very difficult, and often quite impossible, to separate all germs of bacteria and other organisms from each other by simply shaking the gelatine mixture. This test proves therefore that the plate-culture involves very material errors.

Holm has subjected the method to a thorough analysis (1891), and has experimented with a considerable number of yeast-species, absolutely pure cultures of which were prepared by the above-mentioned method of *Hansen's*. The result of 23 series of experiments with different mixtures was, that only in a single case were 100 colonies developed from 100 cells; that is to say, all the colonies were pure cultures. In all the other series the method proved faulty. In the most unfavourable case 100 colonies were yielded by 135 cells, and the average number obtained was 100 colonies from 108 cells. This proves the plate method to be faulty also in the case of yeast.

Thus, the advantage of *Hansen's* method over *Koch's* for the pure cultivation of yeast is, that it has a certain starting-point. Even if the plate-cultures are repeated several times, one can never be certain whether the desired result has been attained or not. With regard to the bacteria, however, it is generally impossible to secure a starting-point from one individual cell. In such cases *Koch's* plate-culture is still the best method we have.

8. Counting the Yeast-cells.

In the yeast and spirit manufactures it is of importance to determine the *multiplying capacity of the yeast-cells* during the growth of the yeast. This must naturally be effected by a direct counting of the number of cells which occur in a determinate volume of the liquid at different stages of the fermentation. Experiments having this object in view have been undertaken especially by *Delbrück*, *Durst*, *Hansen*, *Heyduck*, *and Petersen*, whilst *Fitz* has applied the method of counting to bacteria.

The counting is performed by means of an apparatus constructed by *Hayem* and *Nachet* (Fig. 7), which was first employed for counting the corpuscles of blood (hence termed *hæmatimeter*). The late Prof. *Powsen*, of Copenhagen, was the first to employ this apparatus for counting micro-organisms, in order to determine their multiplying capacity. The hæmatimeter consists, as shown in Fig. 7, of an object-glass on which a cover-glass of *known thickness* (0·2 mm., for instance) is cemented, and from the centre of which a disc has been cut out. A small drop of the liquid containing the cells is brought into the cavity thus formed, a cover-glass is placed over the opening, and thus rests on the cemented and perforated cover-glass. The drop of liquid must not be

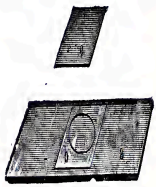

Fig. 7.

Hæmatimeter: *a*, object-glass; *b*, cemented cover-glass with circular opening; *c*, cover-glass.

so large that the pressure of the cover-glass causes it to flow out from the enclosed space, yet it must be high enough to be in contact with the cover-glass. The thickness of the layer of liquid is then known. In order to determine the other two dimensions, and thus be able to work with a given *volume* of liquid, one of the generally known micrometers, *e.g.*, a thin piece of glass on which 16 small squares are engraved, is introduced into the eye-piece of the microscope. The actual value of each of these squares is known when a given system of lenses is employed, and thus, when the square is projected on the object, a small prism of known volume is defined. In certain cases it may be more expedient to make use of an appliance constructed by *Zeiss*, of Jena, from the instructions of *Thoma*, and which consists of a fine

system of squares of known size, engraved on the object-glass itself at the bottom of the cavity. This also improves the microscopic definition of the cells which are on the bottom of the chamber.

When it is merely desired to determine the rapidity with which the cells multiply, or to make repeated observations of the number of cells in the *same* volume, it is quite superfluous to determine the size of this volume; it is then only necessary to work always with the same volume.

It is always necessary that the sample taken should be a fair average one. In most cases it must be diluted and thoroughly agitated for a long time, in order to obtain an equal distribution of the cells; the specific gravity of the liquid must also be such that it will allow the cells to remain suspended in it for a short time. A small drop is then withdrawn in a capillary tube, transferred to the counting apparatus, and covered with the cover-glass. The apparatus is now allowed to remain at rest for some time, in order that the cells may settle to the bottom of the enclosed space, and on this account the specific gravity of the liquid must not be greater than will allow this to take place in a convenient time. Both these requirements are generally satisfied by the wort employed in breweries.

If it is found that the determinate volume contains too many cells to be counted with certainty, the liquid must be diluted. This may also be advisable for other reasons, partly to prevent the formation of froth, which otherwise will generally form abundantly from the violent agitation, and partly to isolate the single cells which are frequently clustered in colonies or large masses in the wort, and are not always separated by shaking, and, finally, in order to bring about a discontinuation of the fermentation and multiplication of the yeast-cells at the beginning of the experiment.

Hansen found that dilute sulphuric acid (1 : 10) on the whole answers these requirements; hydrochloric acid, ammonia and caustic soda may also be used, but they are not

so good. If a very great dilution is required, distilled water can be added, after the addition of one to two volumes of dilute sulphuric acid.

When the different volumes of liquid are measured with accuracy, and particular care taken that the cells are thoroughly distributed by vigorous and prolonged shaking, the determination can be made with great accuracy. Two similar dilutions must always be made, and samples taken from each for counting. As a matter of course, experiments must also be made in order to determine the number of the small squares whose cell contents must be counted in order to arrive at a true average. Such a counting and determination of the average numbers is continued until the number finally obtained is found to have no further influence on the average value. The number of countings necessary, and the accuracy generally, depend on the experience and care of the observer. *Hansen* found that, as a general rule, it was sufficient to count the cells in 48 to 64 small squares.

CHAPTER II.

Examination of Air and Water.

As the water was hitherto regarded as one of the obscure factors in the fermentation industries, and had often to bear the blame of irregularities which could not be explained in any other way, so also many peculiarities in the results obtained at a certain point have at all times been considered to originate from the air. In this was involved a vague misgiving that this invisible air contained substances which act prejudicially to our operations—the nature of these substances, and how it was possible to obtain a closer knowledge of them, was, until the most recent times, involved in obscurity. Chemical investigations of the air, which have been carried out for more than a century, gave no information on this point.

In the course of time a new factor was added; it was incontestably proved that the air is not everywhere equally favourable to the human system; there might possibly be something present which attacked our organism; this unknown matter was called "Miasma" (mixture), the word being taken in a purely chemical sense. Since, however, these miasmata were not traced further, science was thereby not advanced one step.

The discoveries of *Spallanzani* (mentioned in the last chapter), and of later investigators, opened up an entirely new path, namely, the study of micro-organisms. *Pasteur* especially showed that these micro-organisms are of essential importance to the fermentation industries, when he proved that the air contained both bacteria and alcoholic ferments.

The questions then arose: What is the nature of these germs floating in the air? To what degree and extent do they occur in space? Do their number and nature vary with the different seasons of the year? And, finally, are they really able to effectually interfere with technical operations?

It will be of interest to glance at the different methods by which the analyses of air with regard to its germs have been attempted.

The majority of the *analyses of air* have been undertaken with the view to obtain some light on the mysterious obscurity which envelops most contagious diseases, nearly all of which are, as is well-known, attributed to the agency of micro-organisms. With regard to the organisms of fermentation, these have been investigated chiefly by *Pasteur*, and later by *Hansen*. The French savant stated that these germs are always floating about in the air, but that they are present in much larger quantities in the dust which settles on the vessels and apparatus employed. The true alcoholic ferments are present in comparatively small numbers in the air, whilst the germs of mould-fungi are more frequent; he further showed, as was subsequently done by *Tyndall*, that the germ-contents of the air vary both with regard to the quantity and the species. These results were obtained by exposing in open, shallow dishes, in different places, beer-wort, wine-must, or yeast-water containing sugar; after some time their contents were examined for microscopical germs. *Pasteur* also employed for this purpose the so-called vacuum-flasks, containing nutritive liquids and rarefied air. On opening the flask the air with its germs entered.

The most important air-analyses undertaken in recent years are, without doubt, those undertaken by *Miquel*, the director of the laboratory specially arranged for this purpose at Montsouris, near Paris. His fellow-worker, *Freudenreich*, has also added very valuable contributions to our knowledge in this direction.

Miquel performed his first experiments with a so-called *aëroscope* (Fig. 8), which is constructed in the following manner. From the top of a bell, A, proceeds a tube, C, through which air is aspirated, thus causing it to pass through the bell. To the latter is screwed a hollow cone, the mouth, B, of which points downwards; in the apex, D, of this cone there is a very fine opening through which the aspirated air is drawn, and immediately over this opening is fixed a thin glass-plate covered with a mixture of glycerine and glucose. The particles carried in by the air settle to a great extent on the viscous mixture. The micro-organisms here intercepted are distributed as equally as possible on the glass-plate, and counted under the microscope. This method is so

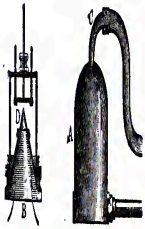

FIG. 8.
Aëroscope.

far defective in that it gives no information on the most important point, namely, which and how many of the intercepted germs are actually capable of development.

In order to determine the number of germs capable of development, and also their nature, *Miquel* employs the following apparatus (Fig. 9). The flask A has fixed into it a tube, B, tapering below and nearly reaching to the bottom; the upper end of this is fitted with a ground cap, H, provided with a narrow filter-tube containing sterilised cotton-wool, asbestos, or glass-wool, ox. On one side of the flask is a tube, *kop*, which is constricted in the middle and is provided with two cotton-wool plugs, *w* and *w*. On the other side is another glass tube, which is connected by rubber, R, with the tube B, which is drawn out to a point, and closed by fusing the

end. The flask is charged with distilled water, and the whole apparatus sterilised. When the apparatus is to be employed, the tube *Asp* is connected with an aspirator; for instance, a bottle filled with water and provided with a cock below; the cap H is taken off, and the air then passes, bubble by bubble, through the opening *a*, through the water *g*, and out through the cotton-wool plugs of the tube *Asp*. Since all the germs of the air are not retained by the water when the air-bubbles ascend through the latter, the cotton-wool plug *w* is intended to catch those which get past the water. When the experiment is finished, the cap H is replaced over the tube R. By blowing through *Asp*, the liquid is made to ascend in R in order that

FIG. 9.

Miquel's Apparatus for Air-Analyses.

any germs which may have settled on the walls of the tube may be washed down into the liquid. Then, by blowing with greater force, the inner cotton-wool plug *w* is driven down into the liquid, and its germs shaken off into the latter. After sterilising the thin tube B in a flame, the point is nipped off, and the liquid is now—by blowing through *Asp*—transferred, drop by drop, into a large number of flasks containing sterilised broth.

The main point here is, by means of preparatory experiments, to obtain such a dilution of the air-infected water that a considerable proportion of the small flasks (one-half for example) remain sterile after inoculation; or several samples of the water may be diluted to different degrees,

and a series of flasks inoculated from each dilution. When a large number of the flasks do not show any development of organisms, there is a *certain probability that in each of the remaining flasks in which growths have developed, only one germ has been sown*. A simple calculation will then show how many germs capable of development in the medium employed were present in the volume of air aspirated through the original flask ("fractional cultivation").

By these methods of investigation *Miquel* found that similar volumes of air in the same locality contained at different times a different number of bacteria. A prolonged rain greatly purifies the air from bacteria, and their number continually diminishes as long as the earth is moist; but when the ground dries, they again gradually increase. In the dry seasons of the year the number of bacteria is thus usually the greatest, whilst the mould-fungi, which thrive best in moisture, and whose organs of reproduction project upwards, are most abundant in the air during the wet seasons. The purest air is found in the winter time; the air of towns is less pure than that outside the towns; germ-free, or nearly germ-free air is found at sea and on high mountains. In certain localities—hospitals, for instance—the air has been found to be very rich in bacteria; in one case even 50 times richer than the air in the garden at Montsouris.

An entirely different method for the examination of the organisms contained in air is that employed in *Koch's* laboratory, and more completely developed by *Hesse*. A glass tube, about 1 meter long and 4 to 5 cm. wide, is closed at one end with a perforated india-rubber membrane, over which another non-perforated cap is bound. A little liquid nutritive gelatine is then poured into the tube, after which the other end of the tube is closed with an india-rubber stopper, through which passes a glass tube plugged with cotton-wool. The whole apparatus is then heated sufficiently to render it sterile, after which the tube is placed in a horizontal position, so that the gelatine sets in a layer in the

lower part of the tube. When the air is to be examined, the outer india-rubber cap is removed, and air slowly drawn through the tube. The germs contained in the air then settle down on the gelatine, and after the aspiration is concluded the tube is again closed and placed in the incubator, where some of the germs then produce visible colonies, which are easily counted. The results show that with a sufficiently slow current of air the bacteria, which are often floating about in the air in larger or smaller aggregations, frequently clinging to dust-particles, settle sooner than the mould-spores; so that in consequence the gelatine in the front part of the tube generally contains the majority of the bacteria colonies, whilst the mould-spores develop further along the tube.

Hueppe, *v. Schlen*, and others, employ liquid gelatine for air-analyses, the air being aspirated through the gelatine, after which the latter is poured on to glass-plates.

Frankland, *Miquel*, *and Petri*, use porous solid substances for the filtration of air for analytical purposes; as, for example, powdered glass, glass-wool, sand, sugar, etc. The sand-filter employed by *Petri* is 3 cm. long and 18 cm. wide. It is filled with sand which has been heated, the size of the grains being 0·25 to 0·5 mm. Two such sand-filters are placed one behind the other, in a glass-tube. The first filter should retain all the dust-particles containing germs, whilst the other filter should remain sterile, and thus serves as a control. The sand charged with germs is distributed in shallow glass-dishes and covered with liquid gelatine. The germs contained in the dust-particles will then develop colonies in the gelatine.

When samples of air are to be sent from one place to another, these air-filters will answer the purpose. On receipt, the sand may be washed into gelatine or, preferably, into sterilised water. After vigorously agitating the water, it is added in drops to flasks containing nutritive liquid, or it may be used in plate-cultures.

Against the employment of gelatine plates for these

purposes, an objection based upon numerous experiments has been raised by *Miquel*, who asserts that many bacteria, when exposed to a temperature of 20° to 22° C., require an incubation of a fortnight before developing distinct colonies in gelatine; on the other hand, however, there are species which will very soon liquefy the gelatine, thus rendering further observation impossible for the next fortnight. The same is the case with the mould-fungi, which will often spread over the whole plate in a few days. Thus, it becomes necessary to count the colonies at such an early stage when many are not to be seen. An additional drawback to the gelatine plates is, that the development cannot take place at a temperature higher than 23° to 24° C, otherwise the gelatine will become liquid; but many species of bacteria give a fair development only at considerably higher temperatures. Other species, moreover, do not develop in gelatine at all, but only in liquids. Finally, it is urged as a very material objection to the gelatine-plates, that many of the colonies consist of several species (see p. 31); *Miquel* proved this by introducing the colonies, one by one, into meat-decoction with peptone, and then again preparing plates from these growths. This is in part due to the fact that bacteria, as shown by *Petri*, often occur in aggregates in the air, and these will either fall directly on to the gelatine-plate or become mixed in the liquid gelatine, where it will always be very difficult to separate the individuals from each other by agitating.

Hansen's investigations of the air were made between 1878 and 1882. The main object of his investigations was to throw light on questions affecting the fermentation industries. As is known, his researches on Saccharomyces apiculatus (1880) were partly based on work of this nature. Since the question concerned the organisms which occur in brewing operations, the choice of a nutritive liquid was easily made, namely, ordinary wort as employed in breweries. The apparatus employed consisted either of ordinary boiling flasks closed with several layers of sterilised filter-paper, the contents of

which were boiled for a certain time, or of flasks of the same kind as Pasteur's vacuum flasks, the necks of which were drawn out to a fine point, and were closed with sealing-wax whilst boiling. A little below the point a notch was made with a file, in order that the point might be easily broken off when it was desired to admit the air.

When these flasks had become filled with the air of the locality to be examined, they were again closed with sealing-wax and thoroughly shaken in order to mix the contents of the infiltrated air with the liquid. The flasks were then put aside for a shorter or longer time, up to six weeks, and their contents examined under the microscope.

In these investigations *Hansen* often found that the wort remained bright and apparently unchanged, even although a growth had taken place. Hence, the examination with the naked eye alone cannot be relied on. He names the following forms which, when present in a feeble state of growth, cannot be detected macroscopically:—Aspergillus, Mucor, Penicillium, Cladosporium, Bacterium aceti and Pasteurianum, and Mycoderma cerevisiæ. Even when these micro-organisms have formed vigorous growths, the above-mentioned nutritive liquid has remained bright.

It was further shown that pure cultures may often be obtained by the use of these flasks, when only one species was drawn into the flask with the air. It very seldom happened that three or four species were found in the same flask. This arises from the fact that only a very small volume of air enters each flask. The advantages of this are evident:—a true knowledge of these germs can only be obtained when they have developed; in cases where several germs penetrate into the same flask, the strongest germ would by its growth, in all probability, prevent the development of the others, so that these would not be detected in a subsequent examination. At the same time, however, this method necessitates the opening of a large number of flasks, which makes the operation cumbersome and costly. As the flasks only show what was present in the

air at the moment of opening, Erlenmeyer flasks were also used to give supplementary information, for which purpose they were allowed to remain in the same locality for some length of time, in some cases as long as 48 hours.

After these preliminary remarks we will give a brief summary of the results obtained by *Hansen*.

He confirms the statement first made by *Pasteur*, that the air *at neighbouring points, and at the same time, may contain different numbers and different varieties of organisms*; and he found that this rule also holds good for places lying close together in the same garden. *Hansen* states, as other characteristics of the distribution of micro-organisms, that those forms, for instance, which in the first half of July commonly occurred under the cherry trees in the garden, were in the latter half of the same month entirely absent from the same place; further, that organisms which at one time were found under the cherry trees, but not under the grape-vines, were to be found later only under the latter; as a proof of the inequality of distribution of the organisms, it is shown that the flasks opened in the same place in the same series of experiments often had the most diverse contents.

The experiments with the vacuum flasks have further taught us that the micro-organisms of the air often occur in *groups* or *clouds*, with intermediate spaces, which are either germ-free or only contain quite isolated germs. As the organisms are not generated in the air, but have their place of growth on the earth, it follows that their presence in the air must be dependent on the condition of the surface of the ground, which again depends, in certain respects, on the weather.

Hansen's numerous analyses have further proved that the *Saccharomycetes occur comparatively seldom in the dust of the air*. Their number in the air increases from June to August in such a way that the flasks at the end of August and the beginning of September are frequently infected

with these organisms, after which a decrease takes place. Those organisms from the air which at other times of the year are found to enter the flasks, must be regarded as unimportant and accidental, and therefore falling outside the principal rule. As most species of the Saccharomycetes have in all probability—like Saccharomyces apiculatus—their winter quarters in the earth and their places of growth on *sweet succulent fruits*, these latter must apparently be considered as the most important source of contamination. At the same times of the year *bacteria* are also found in the largest numbers. This constitutes an important danger in technical operations, since the wort, which is spread in a thin layer on the open coolers, is exposed at the above-named season of the year to a great source of contamination from the germs of the air.

Bacteria are found in the flasks in somewhat greater number than the Saccharomycetes, whilst the *mould-fungi occur in still greater numbers*. Amongst the latter Cladosporium and Dematium are especially prevalent in gardens, and after these Penicillium; whilst Botrytis, Mucor, and Oïdium are less frequent.

After *Hansen* has thus stated which of the micro-organisms existing in *the open air* are capable of developing in flasks with sterilised wort, he proceeds to communicate the results of his examination of *different localities in the brewery*.

When *grains* (draff) are allowed to stand in the open air, they evolve, as is known, acid vapours, and since they always contain a rich growth of bacteria when they remain exposed for a short time, the following question suggests itself:—What is the condition of the air in the neighbourhood of the heaps of grains? It was found that only 30 per cent. of the flasks opened in these vapours became contaminated, and of these 3·6 per cent. with Saccharomycetes and 2·4 per cent. with bacteria, whilst parallel experiments in the garden gave a contamination of about 44 per cent., of which 8·5 per cent.

were bacteria. The air near the grains thus contained fewer bacteria than the air of the garden. The most abundant contamination here was that of mould-fungi, as in all the other localities. After a thorough examination *Hansen* came to the conclusion that, without any doubt, *scarcely a single organism which entered the flasks proceeded from the grains*. At all events the great abundance of bacteria in the grains does not bear any correct relation to the above-stated result, which, with far greater probability, admits of the explanation that the air in this, as in other cases, does not take up any contingent of organisms from *moist surfaces*.

This, however, must not be misunderstood to mean that grains may be accumulated, without risk, in any chosen place, and the remains after removal exposed to the weather. It is clear that this would constitute a great danger. When the remains become dry and are blown about in the air as dust, masses of bacterial germs will be carried up at the same time, and will, without doubt, constitute a source of constant bacterial contamination. For this reason, places where grains have remained for any length of time must be washed with lime-water or, preferably, with chloride of lime.[1]

In a corridor which led to the room where the *barley was turned*, the flasks always received a greater contamination than anywhere else; *bacteria* especially were found in great abundance.

On the *malt floors* the condition of the air was also characteristic; it always contained a very strong *growth of mould*. In the case in question this growth consisted of Eurotium Aspergillus, which was otherwise rare. On the malt itself, as always, Penicillium glaucum occurred the most frequently.

[1] The germs are not killed during the treatment of the grains in the *drying machines*. Such apparatus, therefore, constitute a very great danger in the brewery, in cases where the bacteria can become transported from the dried grains to the open coolers.

The greatest interest, however, attaches to the examination of the different *fermenting-rooms*, partly in "Old Carlsberg" and partly in the brewery "X." In the first-mentioned rooms the air contained fewer organisms than in any of the localities examined in the whole research; in the fermenting-cellars of the brewery "X," on the contrary, a large number of the flasks (55·5 to 100 per cent.) became infected. The organisms which occurred in the air of these cellars were: Saccharomyces cerevisiæ, Mycoderma cerevisiæ, Sacch. Pastorianus, Sacch. ellipsoideus, Torula Pasteur, and other yeast-like forms; further, Penicillium, Dematium, Cladosporium, and rod bacteria. *Hansen* was thus enabled by a favourable chance, to show the following contrast in the state of the air in the most important place in the two above-named breweries: on the one hand an almost germ-free air, on the other hand an atmosphere teeming with germs. That the product of the latter place at this time must have borne the stamp of this condition admits of no doubt, and we find here one of the most important of all facts connected with the practice of the fermentation industries. *The air in the fermenting room itself may contain a world of those germs which are productive of the most calamitous results*; it is, however, possible to keep the air free from these invisible germs, and it admits of no doubt that, on the one hand, the purification of the air entering the fermenting-room by passing it through a salt-water bath, and, on the other hand, the very rigidly maintained order and cleanliness in the cellars of the Old Carlsberg brewery stand in direct relation to the above-mentioned result. *Hansen's* investigations, therefore, here again contain a warning which cannot be repeated too frequently.

Based upon a long series of comparative investigations, Hansen gave the following method for the zymotechnical analysis of air and water.

The principle of this method of air-and water-analysis is as follows:—For brewing purposes it is only necessary to

know *whether the water and the air contain such germs as are capable of developing in wort and beer*. This cannot, as was formerly assumed, be ascertained by means of the meat-decoction peptone gelatine employed in hygienic air- and water-analysis. The zymotechnologist has this great advantage over the hygienist, that he is in a position to *make direct experiments* with the same kind of liquid as that employed in practice, namely wort. *All disease germs that have hitherto been shown with certainty to occur in beer are also capable of developing in wort. Hansen's comparative investigations have proved beyond dispute that the use of gelatines introduces great sources of error.* Thus, for instance, in a series of comparative experiments with corresponding samples of water, the following numbers were obtained:—In *Koch's* nutritive gelatine: 100, 222, 1000, 750, and 1,500 growths were obtained from 1 ccm. of water; in wort 0, 0, 66, 3, and 9 growths; whereas, in beer, none of these water-samples gave any growth. In another series, *Koch's* gelatine gave for 1 ccm. of water 222 growths, wort-gelatine 30; but none of the flasks containing wort and beer, and infected with the water, showed any development of organisms. Thus, only very few, or none at all, of the great number of living germs in the water developed in wort or beer.

Hansen has further shown, that in zymotechnical analyses of water and air, it is a mistake to employ gelatine at the outset, and then to transfer the colonies that have been formed into wort-flasks. Thus, he demonstrated by experiments that several of the bacterial germs existing in atmospheric dust and in water are capable of developing in nutritive gelatine, but not in wort; but several of these species become invigorated to such a degree after having formed a new growth in the gelatine, that they are then enabled to develop in the less favourable medium, wort. In such cases the experimenter is therefore deceived. Another, and a still greater, objection to the gelatine

method is, that several important organisms *do not develop at all* when transferred directly to the gelatine in the enfeebled condition in which they generally occur in atmospheric dust and in water.

Based upon these observations, *Hansen* devised the following method: Small quantities of the water, either in its original state or diluted, are added to a series of *Pasteur*-flasks containing sterilised wort and beer.[1] After incubation at 25° C. for fourteen days the contents of the culture-flasks are submitted to an examination. If only a part of them show any development, the rest remaining sterile, it may be assumed with approximate certainty that each of the flasks belonging to the former set has received only *one germ*. Information is thus gained concerning the number of germs capable of development existing in a determinate volume, and the different germs are also under more favourable conditions for their free development. An exact examination will show to what species these germs belong.

Although, in this method, the wort-cultures give a very small number of growths in comparison to the plate-cultures, yet in many cases the *numbers of wort-growths will be too high*, since these growths are able to develop in the flasks undisturbed and without hindrance from other organisms; when wort is mixed with good culture-yeast in the fermenting vessel, many of these germs will be checked. Further, the flasks which show a formation of mould will have no importance for the brewery, but only for the malt-house. By way of a nearer approach to practical requirements, *Hansen* proposes the following method of procedure. *The flasks containing a development of yeasts and bacteria* are divided into two groups: (1) those in which the growths soon appeared, and (2) the remainder, in which they made their

[1] In the analyses of air the germs are introduced directly, by means of an aspirator, into water, or first into cotton-wool and then into water.

appearance later; as, for instance, after five days. Among the latter growths are those species which develop less readily in wort; and in the brewery these will therefore be generally suppressed by the yeast, and are consequently of less importance in the examination of water or air. Analyses according to this method have been executed by *Hohn*, *Wichmann*, and several others.

For the control of air- and water-filters *Koch's* gelatine method is the best.

CHAPTER III.

Bacteria.

The more our knowledge of these micro-organisms becomes enlarged, the more difficult it is to give a general definition of them. They are known in all forms, from the smallest specks or spheres to green, algæ-like filaments; and they occur very nearly in all possible localities, under the most various conditions, as the cause of putrefaction or decay (Saprophytes), of diseases (pathogenic forms), and of fermentation (zymogenic forms).

The first knowledge of these forms was obtained by placing small quantities of the different substances under the microscope and examining them with high powers. In putrefying meat very small spherical bodies were found, which clearly multiplied by successive divisions; in sour milk short, rod-like bodies occurred; and in putrefying vegetable matter larger spherical bodies and long, fine, thread-like forms; in saliva, on the contrary, very fine, spirally-twisted threads were found, etc. On this account it was convenient to provisionally retain these *forms*, and to describe them as so many distinct *species*. Cohn especially has earned credit in this respect, since to him is due the first systematic classification of bacteria.

We will first consider the various forms and individuals somewhat more closely. As before stated, the bacteria in their simplest form occur as spherical bodies of different sizes, often so small that they can only just be seen even with the strongest powers, and only give evidence of their existence as organisms by their multiplication by division. They are

accordingly divided into *micrococci* and *macrococci* (Fig. 10*a*). When the spheres occur in pairs, they are called *diplococci* (*b*); they also appear in groups consisting of four individuals, *sarcina* (*b*); or of a greater number, arranged irregularly, or in chains, *streptococci* (*c*). From the coccus forms there is a gradual transition to the rod forms—*bacterium*, *bacillus* (*e*),

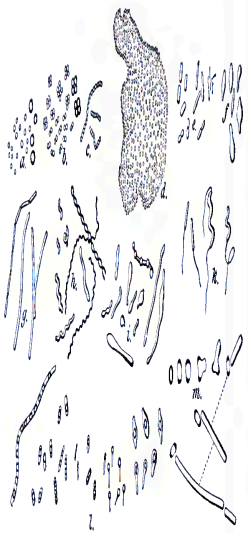

FIG. 10.

Growth-forms of Bacteria: *a*, Cocci; *b*, Diplococci and Sarcina; *c*, Streptococci; *d*, Zooglœa; *e*, Bacteria and Bacilli; *f*, Clostridium; *g*, Pseudo-filament, Leptothrix, Cladothrix; *h*, Vibrio, Spirillum, Spirochæte, and Spirulina; *i*, Involution-forms; *k*, Bacilli and Spirilla with cilia or flagella; *l*, Spore-forming bacteria; *m*, Germination of the Spore.

which vary greatly both in length and thickness. When the rods are enlarged in the middle and taper towards the ends, *i.e.*, spindle-shaped, we have the *clostridium* form (*f*). If the rods are elongated so as to become more or less thread-like, they are called *leptothrix* (*g*), which may also occur as *pseudo-filaments* (*g*), when several rods are arranged length-wise, or as *cladothrix*, when they lie so close to one another and in such a way that they become seemingly ramified; a

true ramification, like that of the mould-fungi, does not occur in bacteria. Rods and filaments frequently assume wavy or spiral forms (fig); when they are only slightly curved, we have the *vibrio* form; when the spirals are more prominent, the *spirillum*, and *spirochæte* forms; when they intertwine like a plait of hair, the form called *spirulina* is produced. To these must be added the remarkable irregular, swollen, or curved forms which many bacteria can assume; the cause of this alteration is, however, not sufficiently known—*involution forms* (i).

We will now select one of these forms and submit it to a thorough examination with a magnifying power of about 1,000 diameters. Like every other cell, it contains protoplasm, a homogeneous, feebly refractive mass, in which infinitesimal particles can be detected here and there, especially if the cell is not in its most active growth. Sometimes a bright spot is found in the middle of the cell, which, from analogy to the higher plants, is considered to be a sap-cavity or vacuole. In some bacteria certain solid substances have been detected, as, for instance, sulphur grains in bacteria which live in water containing sulphur; in some species the plasma can, under certain conditions, be coloured blue by iodine, which indicates the presence of substances resembling starch.

Surrounding this protoplasmic body we find a *cell-wall* or *membrane*. An examination of this by means of staining will generally show that this membrane in its outer layers is swollen up into a gelatinous mass, which becomes especially distinct when masses of bacteria are aggregated together. From a chemical standpoint it must be provisionally assumed that this cell-wall is of a different nature in different species. In some it reminds us of the cellulose of the higher plants, whilst in others it appears rather to resemble the albuminoids in its properties.

Many bacteria contain blue, red, yellow, or green *colouring matters*, which sometimes cause very intense coloration. Under the microscope, however, the individual bacteria

appear only very faintly coloured. It has not yet been determined with certainty in what part of the organism the colouring-matter is situated. Some species of bacteria are *phosphorescent* under certain nutritive conditions.

A remarkable property of many bacteria is their—at least apparent—*free locomotion*. This is either quick or slow, the bacteria rotating about their longitudinal axes, assuming the forms of open or contracted spirals. In some of these motile forms we can observe, under high magnifying power, very fine cilia or flagella (Fig. 10 *k*); whether these are to be considered as organs of locomotion is not yet determined, nor has it been decided whether they issue from the membrane or from the cell contents.

The *multiplication* of bacteria takes place in different ways. In the main, multiplication by *division* and by *spore-formation* in the interior of the cell may be distinguished. The first mode of multiplication has been observed in detail in the larger forms. Fine transverse lines appear, which gradually increase in thickness and become gelatinous; after this the organism separates at these transverse walls into smaller rods (Fig. 10 *g*). Long before a trace of these transverse walls can be observed, a staining of the organism will show that it consists of a series of segments, each of which corresponds to a subsequently-formed member. The newly-formed segment-cells are all in the same plane. A division in two or three directions of space has only been observed in certain micrococci (Sarcina).

It was proved by the investigation of the shapes of bacteria in the above-mentioned manner (especially by *Zopf*), that *the same species of bacterium can occur in very different forms, e.g.*, as spirillum, leptothrix, bacillus, bacterium, and coccus; and we thus obtained the important addition to our knowledge of the history of these plants that the names quoted very often only express *growth forms* of the same species, and not distinct species. The following question, however, remains to be answered:—*Under what conditions*

does a species occur in this or that particular form? Upon this point we know very little at present.

In the case of many bacteria multiplication by *spores* takes place in the following manner. The plasma in the cell becomes darker, and often distinctly granular; after that a small dark body appears, which quickly increases in size, and at the same time becomes strongly refractive; meanwhile by far the greater portion of the plasma of the cell disappears, becoming used up in the formation of the spore; this is seen enclosed in a clear liquid, which gradually disappears; finally, the cell-wall shrivels up, and only remains as a withered appendage to the ripe spore. This organ is often termed a *resting-spore* (Dauerspore), for two reasons, namely, first, because it actually possesses far greater durability and resistance to external influences than the vegetative rods; and, secondly, because the spore formation generally takes place when the nutriment of the organism is either exhausted or unfavourable to the further vegetative growth of the latter; the spores, then, serve to preserve the life of the organism during this critical period.

As soon as favourable conditions of nutriment and temperature again occur, the *spores germinate*. They first increase in size, and the contents lose their strong refractive power. A bacterium then grows out from the spore, the wall of which is sometimes seen to burst or divide into two shells (Figs. 10, 13). The full-grown rod then multiplies in the usual manner.

Bacteria are now sometimes divided into *endosporous* and *arthrosporous* bacteria, of which the first-named form their spores in the interior of the vegetative rods, whilst in the latter group no such interior spore-formation has hitherto been observed; in these forms, members of a series of united generations of vegetative cells separate from the rest and assume the character of spores immediately without previous endogenous rejuvenescence, and become the origin of new vegetative generations (for instance, *Bact. aceti*). Perhaps

by continued investigation endogenous spores may also be found in all species of the last-mentioned group. It is only a supposition that the above-mentioned separated members must be considered as analogous to the spores.

Finally, in the morphology of bacteria, we must mention the so-called *zooglœa* formation (Fig. 10-*d*). It is known that in all branches of the fermentation industries, in places where the cleaning is not strictly attended to, slimy, fatty masses may occur, which gradually increase in thickness. The cause of this is commonly a growth of bacteria occurring in such a manner that the single cells lie very close to each other, whilst at the same time the outer gelatinous layers of the cell-wall greatly swell up. During the continued growth of the bacteria the slimy layer increases in thickness, and often assumes certain characteristic forms. Such slimy masses—known in the sugar manufacture as "frog-spawn"—occur both on solid and in liquid media.

Pasteur made the important discovery, that there are certain bacteria and other micro-organisms which do not require free oxygen, and even produce very active decompositions of the fermenting material when oxygen is excluded. He, therefore, distinguished two classes of micro-organisms, naming the last-mentioned *anaërobic* and the others *aërobic*. More recently *Duclaux* has stated that there are intermediate forms between the two extremes. As an example of anaërobic bacteria, *Pasteur's* bacterium of the butyric-acid fermentation may be mentioned.

We will now pass in review the more important species which are of special interest in the fermentation industries.

1. Acetic Acid Bacteria.

The acetic acid bacteria were first thoroughly described from a morphological standpoint by *Hansen*. The correctness of his investigations was afterwards confirmed by *Zopf*, *de Bary*, and *A. J. Brown*.

As early as in the year 1838 the view was expressed by

Turpin and *Kützing* that the acetic acid fermentation is caused by a micro-organism, which *Kützing* described and delineated under the name of *Ulvina aceti*. Starting from this, *Pasteur*, first in his treatise (1864) and subsequently in his work "Études sur le vinaigre" (1868), furnished experimental proof of the correctness of this view, and also gave a method, based on the results, for the manufacture of vinegar. He assumed that the acetic acid fermentation was caused by a species of micro-organism which he called *Mycoderma aceti*. Subsequent research has, however, shown that there are different species of acetic acid bacteria. *With Pasteur, therefore, it was not at all a question of the employment of one definite, selected species.* His method consists in giving a large surface to the liquid employed—two parts of bright wine to one part of wine-vinegar—and then sowing on the surface of the mixture a young film consisting of "mother of vinegar." When the temperature, the composition of the liquid, and all other conditions are favourable, the formation of acetic acid will proceed more quickly than in the older Orleans process. The installation is claimed to be cheaper, and the loss of alcohol not greater—at all events not to any appreciable extent—than in the last-named process. Yet, as far as I have been able to learn, *Pasteur's* process is never employed. The cause of the uncertainty of the results may be sought in the fact that the composition of the nutritive liquid varies, and especially in the fact that the *bacterial culture was not a pure culture*, and might, therefore, also contain varieties of bacteria which possessed different properties, required different conditions for their growth, and, consequently, would give different products in varying quantities. This will hold good even in those cases in which the composite culture consists only of such varieties which can produce vinegar. As early as 1879 *Hansen* discovered that at least two distinct species are hidden under the name of *Mycoderma aceti*, namely, *Bacterium aceti* and *Bact. Pasteurianum*; and he has shown that also in this branch of industry it is

necessary to start with an absolutely pure culture of a methodically-selected species.—The old Orleans process still prevails in France. In this method the wine which is to be converted into vinegar is placed in tuns, to which atmospheric air has moderately free access. The formation of acetic acid, as in Pasteur's process, takes place in consequence of the liquid becoming covered with a film consisting of "mother of vinegar."—In other countries the German "quick vinegar process" is employed, in which the growth of bacteria, through free access of air and by dividing the liquid

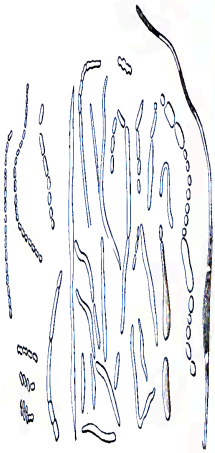

FIG. 11.
Bacterium aceti and Bact. Pasterianum, after Hansen.

into small drops, and distributing these over very large surfaces (shavings), comes into intimate contact with the air. The nature of the micro-organisms taking part in this manufacturing process has not yet been investigated.

While *Pasteur*, in the above-named work, does not explicitly maintain the theory that the oxidation of alcohol to acetic acid is a purely physiological process, yet *Adolf Mayer* expresses this opinion, and *Hansen* emphasises as a certainty the fact that the formation of acetic acid is commonly effected by the action of bacteria. *Hansen's*

researches are, moreover, among the first which proved that a definite fermentation is not induced by one species of bacterium only, but by several; more recently a large number of instances have been discovered. By placing lager-beer in an incubator at 30° to 34° C., he obtained a vigorous film-growth of the acetic-acid bacterium. This consists of long chains of hour-glass-like members, partly as bacterium and bacillus, partly as wavy or curved forms. As a peculiarity of *Bact. aceti* it may be mentioned that this species often yields at a very early stage the different, irregularly swollen involution forms above described, whilst other bacteria do not produce these forms until a very advanced stage, and even then, possibly, only as a consequence of a deficient supply of nutriment; this, however, cannot be the case with the organism under discussion. We have here also one of the first cases, in which it has been shown that the same species can occur in very different forms.

By means of his staining experiments with *Bact. aceti*, Hansen discovered, as already stated, that two distinct species are hidden under this name, of which the one, like most other bacteria, is stained yellow by iodine, whilst the other assumes a blue coloration with the same reagent. For the former he retains the old name *Bact. aceti*, whilst the one stained blue he names after *Pasteur—B. Pasteurianum*. In a lecture he communicated the following new observations:—The film formations on wort and beer, and likewise the growths on wort-gelatine, give a fine blue colour with tincture of iodine, or iodine dissolved in a solution of iodide of potassium, whilst the growths which develop on yeast-water and meat-decoction with peptone and gelatine are coloured yellow; even very old films on beer show a yellow reaction. It is the gelatinous formation secreted from the cell-wall that is coloured blue; it has hitherto not been possible to determine whether the *contents* of the cells are coloured or not. In wort-gelatine, *Bact. Pasteurianum* develops round specks or colonies with a smooth or wavy border, whilst the corresponding

specks of *Bact. aceti* have a tendency to assume stellated forms. From a morphological standpoint the two species behave alike. Spores have not been observed. A fact of practical interest is the observation that pure cultures of the two species of acetic acid bacteria in beer *do not exercise any influence on the colour or brightness of the liquid.* It is therefore possible, to a certain extent, to ascertain whether or not these organisms are alone present, since other bacteria, when present in beer, produce turbidity in the liquid. In order to develop vigorously, *Bact. aceti* not only requires a very plentiful supply of oxygen, but also a fairly high temperature. *Hansen* found that a temperature of about 33° C. was the *most favourable* when Carlsberg lager beer was used. In a well-conducted store-cellar (1° to 3° C.) there is therefore nothing to fear from *Bact. aceti*. But as soon as the beer leaves the cellar, and is exposed to higher temperatures, there is always a danger.

In leaven, especially when it has become old and extremely sour, *Peters* recently discovered an acetic acid bacterium which distinctly differs from *Bact. aceti* and *B. Pasteurianum*. The colonies in ordinary plate-cultures are circular in shape, of a homogeneous appearance, and, when seen in transmitted light, of a strong brown colour; the surface colonies are largely expanded. The single individuals are 1·6 μ long, 0·8 μ broad, truncated at one end, tapering at the other; they occur singly or in pairs, rarely in groups of four. The bacterium does not exhibit any motion. In yeast-water containing 5 per cent. of alcohol it first produces turbidity, then a thin film forms on the surface, and gradually becomes viscous. This bacterium is perhaps identical with the species described by *Duclaux*.

The *Bacillus ethaceticus* discovered by *Percy Frankland* induces a vigorous fermentation in various substances (*e.g.* mannite), the chief products of which are ethyl-alcohol and acetic acid.

Pasteur has shown that, by the oxidation of alcoholic

liquids, ethyl-alcohol is converted into acetic acid, and by further oxidation the latter is converted into carbonic acid and water. This has been recently confirmed by *A. J. Brown*, to whom we are indebted for the most complete researches on the chemical action of acetic acid bacteria.

2. Lactic Acid Bacteria.

When milk is exposed at a temperature of 35° to 42° C. it will soon become sour, and a considerable portion of the acid produced is lactic acid, which is formed by the agency of various species of bacteria. When a certain quantity of lactic acid has been formed, the fermentation ceases. It will recommence, however, if the liquid be neutralised with carbonate of lime, or on the addition of a small quantity of pepsine or pancreatine, which causes the caseïne of the milk to be dissolved.

A method commonly employed for inducing a spontaneous lactic acid fermentation is the following:—To a liter of water are added 100 grams of sugar, 10 grams of caseïne or old cheese, and an abundant quantity of powdered carbonate of lime. This mixture is exposed in an open vessel to a temperature of 35° to 40° C. The liquid is occasionally agitated, or a current of air is passed through it. After completion of the fermentation the liquid is evaporated, when calcium lactate crystallises out, and from this the lactic acid is liberated by treatment with sulphuric acid.

In addition to milk-sugar, lactic acid bacteria are also capable of fermenting cane-sugar, glucose, maltose, and various other substances. According to *Bourquelot's* investigations, a species of lactic acid bacterium, which makes its appearance in the spontaneous acid fermentation of milk, is capable of fermenting cane-sugar without previously inverting it.

In milk-sugar solutions which were free from caseïne, *Fokker* could only obtain feeble lactic acid fermentations,

whilst, on the addition of this substance, the lactic acid was proportionately increased.

In breweries the lactic acid fermentation takes place even in the malting, also in the wort and in the after-fermentation; in the Belgian beers, obtained by spontaneous fermentation, lactic acid is formed in large quantity, and consequently imparts a sharp taste to the beer. In modern low-fermentation breweries it is endeavoured to exclude the lactic acid ferment, and bacteria in general, from the fermentations. "In distilleries," according to *Moercker*, "these are still provisionally regarded as a necessary evil. The production of lactic acid takes place during the yeast-dressing, and its importance appears to be confined to preventing the development of bacteria, and in this way rendering possible the pure fermentation of the alcoholic yeast."

FIG. 12.

Lactic acid bacteria, after Pasteur. In order to give an idea of the size of the bacteria, some yeast-cells are figured amongst them.

We are indebted to *Pasteur* for the first important work on the subject of lactic acid bacteria. In 1858 he described the species which appears when milk spontaneously ferments. In his "Études sur la bière" he figures some bacteria which develop in wort or beer in which lactic fermentation has set in (Fig. 12); he describes them as short rods slightly narrowed in the middle, and commonly occurring singly, rarely united in chains.

Later, *Hueppe* found a bacterium in a spontaneous lactic acid fermentation which converts milk-sugar and other sugars into lactic acid with the simultaneous formation of carbonic acid. It consists of short, plump, motionless cells, the length of which exceeds their breadth by at least one half; they are united in pairs or in groups of four. In sugar solutions and less distinctly

in milk they form spores, which appear as lustrous spheres attached to the end of the rods. In gelatine-plates they form whitish colonies which, as long as they are submerged, are circular, uniformly dark, and have sharp contours; those on the surface have lighter borders. Atmospheric oxygen is necessary for fermentation with this species. It coagulates the caseine of milk.

In recent publications descriptions are found of a large number of lactic acid bacteria; thus, two species of micrococci have been found in saliva and the mucus of teeth; amongst the pigment-forming bacteria, species are also found which, in addition to their pigment-fermentation, are able to produce so much lactic acid from the sugar of milk that the caseine of the milk coagulates; to these belong, according to *Hueppe*, the famous blood-ferment (*Micrococcus prodigiosus*) and, according to *Krause*, a pathogenic form, the *micrococcus* of *osteo-myelitis*.

According to statements made by *Delbrück*, *Zopf* has obtained a lactic acid bacterium by preparing a mash from 200 grams of dry malt and 1000 grams of water, and keeping it for some time at 50° C. The material was then sown in a solution of milk-sugar, on the surface of which the organism formed a film. The filaments consist at first of small rods, later of both rods and cocci.

Peters found a bacterium in leaven, which produces a typical lactic acid fermentation. In plate-cultures it forms circular colonies with concentric stratification. The rods have a rapid sinuous motion; in a neutral solution of sugar in yeast-water at 30° C, this species forms after some time a slimy film; the rods have here developed into long filaments. Spore-formation has not been observed.

The so-called *Pediococcus acidi lactici* examined by *Lindner* gives, when cultivated in a neutral malt-extract solution at 41° C, a strong acid reaction; both in a solution of this kind and in a hay-decoction, which have not been sterilised, this bacterium develops so vigorously that, accord-

ing to *Lindner*, all other organisms are suppressed. It has been proved chemically that the acid, which is abundantly produced, consists for the most part of lactic acid. When a malt-mash or malt-rye mash is maintained at 41° C, the *Pediococcus* develops vigorously, and the rod-shaped lactic acid bacteria are suppressed. In a neutral malt-extract solution, the *Pediococcus* is killed after five minutes' exposure to 62° C. In gelatine it does not thrive well; it is only in puncture-cultivations in neutral malt-extract gelatine, that very vigorous white colonies are formed below the surface. This species appears, on the whole, to thrive better when the air is excluded.

The *Saccharobacillus Pastorianus* described by *Van Laer*, which occurs in the form of rods of different lengths, produces a characteristic disease ("*tourne*") in beer, which manifests itself as follows: the liquid gradually loses its brightness, and when it is agitated filaments of a silky lustre rise from the bottom, and the beer assumes a disagreeable odour and taste. The bacillus, in cultures, develops both in the presence of free oxygen and when this is excluded. In nutrient liquids it ferments the carbohydrates, and amongst them the saccharoses, without previously inverting them. Amongst the fermentation-products lactic acid, acetic acid, and alcohol, are especially mentioned. The acids produced cause the precipitation of nitrogenous compounds in the liquid, and these, mixed with the bacilli, produce the above-mentioned clouds, consisting of lustrous filaments.

Besides the investigators mentioned, several others have likewise carried out researches in this field; as, for instance, *Pasteur's* co-operator *Duclaux*. *Grotenfelt* has recently described some species which must doubtless be regarded as new ones; at any rate he could not identify them with those described by *Hueppe* and *Marpmann*. Some species were observed to yield alcohol in addition to lactic acid by the decomposition of sugar; he expresses the belief that these species take part in the formation of the aroma of butter.

Recently a methodical pure cultivation of certain lactic acid bacteria has been introduced into practice, the principles being the same as those carried out by *Hansen* in the case of alcoholic ferments in breweries, the object being to attain a more rational acidification of the cream employed for making butter. *Weigmann*, *Storch*, and *Qvist* have isolated a series of lactic acid bacteria which, when employed for the acidification of the cream, have imparted to the butter a more or less pure sour taste, and also a more or less aromatic odour, whilst the durability of the butter likewise varies with the different species.

Storch particularly mentions one species which, in experiments on the acidification of cream for the use of dairies, not only gives it a pure and mild slightly sour taste, but also imparts a markedly pure aroma both to the cream and to the butter made from it. In gelatine this species forms very small colonies of a pure white colour and with a smooth border. In milk and whey it occurs as plump, oval, or spherical bacteria, which form flexible chains. It bears some resemblance to *Pasteur's* "ferment lactique." At 28° C. it develops a marked fermentative activity.

Qvist has cultivated another species, which has been employed with still greater success in practice. It occurs both as micrococcus and in other forms, according to the different nutrient media in which it is cultivated. In gelatine it forms small, circular, slowly-growing colonies of a whitish-yellow colour. In puncture-cultivations spherical colonies arise throughout the puncture-channel, and in streak-cultures this organism forms a continuous streak with wavy borders. It was prepared from a sample of butter of remarkable aroma and durability.

On the other hand, several species of bacteria have been discovered in recent years, which cause diseases in milk. Thus *Schmidt-Mülheim* found a micrococcus which occurs in the form of moniliform chains, and causes the milk to become viscous; another species, discovered by *Kutz*, possesses the

same property and also produces a vigorous lactic acid fermentation; other slime-forming species have been described by *Adametz*, *Duclaux*, *Löffler*, and *Guillebeau*; *Weigmann* isolated a species which imparts a bitter taste to milk and secretes a ferment which dissolves caseine. *Jensen* likewise found a species which causes marked abnormal changes in the taste of milk and butter; it has the form of thick, motile rods, of varying length, partly resembling micrococci. *Storch* proved that the disagreeable taste of tallow which butter sometimes has, is caused by a certain species of bacterium, which acidifies and coagulates the milk.

3. Butyric Acid Bacteria.

When milk which has stood for some time, and in which lactic acid bacteria have developed, is neutralised by the addition of lime (chalk), so that calcium lactate is formed, it will, as a rule, enter into a butyric fermentation, which is caused by different species of butyric acid bacteria. This spontaneous butyric acid fermentation takes place most vigorously at 35° to 40° C. Starch, dextrin, cane-sugar, and dextrose, are likewise fermentible by the butyric acid ferments, and these fermentations set in very readily, as the different bacteria belonging to this group are very widely distributed in nature. It is doubtless also such species which take part in the ripening of cheese, and which help to impart the characteristic taste and aroma to the different kinds of cheese. In order to induce a butyric acid fermentation, *Fitz* recommends the employment of a mixture of 2 liters of water, 100 grams of potato-starch or dextrin, 1 gram of ammonium chloride, the ordinary nutrient salts, and 50 grams of chalk; this mixture is to be maintained at 40° C. *Bourquelot* recommends exposing water containing slices of raw potatoes for two or three days at a temperature of 25° to 30° C.

The most important products of the butyric acid fermentation are butyric acid, carbonic acid, and hydrogen.

In the saccharine mashes of breweries, distilleries, and pressed-yeast factories, some species of butyric acid bacteria always occur, and if the mashes are maintained for a lengthened period at certain temperatures, these bacteria develop very rapidly and exercise a retarding influence on the alcoholic ferments. If butyric acid occurs to any extent in beer, it will acquire a very unpleasant taste.

According to *Pasteur's* experiments, the butyric acid ferment can perform its functions without access to the free oxygen of the air. More recent investigations have shown, however, that many butyric acid bacteria exist which not only yield different fermentation products, but also behave differently with regard to free oxygen, in that some are not capable of developing when the latter is present,—so-called anærobic species,—whilst others multiply and induce butyric acid fermentation when they have access to oxygen,—aërobic species.

One of the first species which were minutely described is *Prazmowski's Clostridium butyricum* (*Bacillus butyricus*) (Fig. 13). It occurs in the form of short and long rods, which may be either straight or somewhat curved. Before the formation of spores in the rods, the latter swell and form, as shown in the figure, peculiar spindle- and lemon-shaped, elliptical, or club-like forms; at the same time there is the important fact that they are coloured blue by iodine. On germination the spores burst their outer envelope, and the germ filament grows in the same direction as the longitudinal axis of the spore. *Clostridium butyricum grows most readily at a temperature of about* 40° C., and is especially able to predominate in a solution of sugar if the lactic acid ferment has previously converted a portion of the sugar into lactic acid. This species is decidedly anærobic.

Fitz has described a species belonging to the aërobic forms. It is a bacillus of a short cylindrical form, which is not coloured blue by iodine, is motile in a moderate degree, and forms no spores. It ferments all carbohydrates, with the exception of starch and cellulose.

Hueppe has likewise described a species, which was found in milk, and occurred in the same forms as the species discovered by *Prazmowski*, but was much less sensitive towards oxygen, and must therefore be classed with the

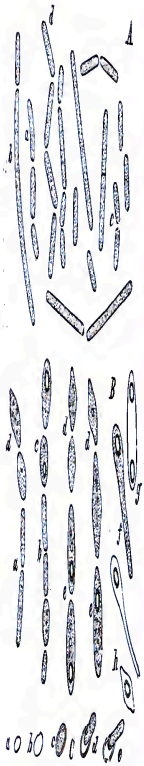

FIG. 13.

Clostridium butyricum Prazm., after Prazmowski: *A*, vegetative state; *a*, short rods; *d*, long rods; at *a* and *b*, rods and filaments curved like vibriones; *B*, formation of "resting-spores" (Dauersporen); *b*, *d*, rods, previous to, *e*, *e*, during, *f*, *g*, *h*, after the formation of resting-spores; *e*, of an elliptical; *d* and *h*, of a lemon-like; *e*, *g*, of a spindle-like; *f*, of a tadpole-like shape; at *a*, rods still in their vegetative state; *C*, germination of resting-spores: the spore *a* expands into *b*; *c* shows the differentiation of the membrane into exo- and endosporium. The contents surrounded by the endosporium issue from the polar fissure of the spore in the form of a short rod (*d*), which appears prolonged at *e*.

aërobic species. *Grotter* found associated under the name of *Clostridium butyricum* three well-defined species, two of which are exclusively anaërobic. One of these last-mentioned species consists of straight or slightly-curved rods, which become spindle- or barrel-shaped during the formation of spores. In nutrient gelatine it forms colonies which, when seen in transmitted light, appear blackish-brown or black. The second species consists of strongly-curved vegetative rods, in which the spores are formed at the end; it forms yellowish or yellowish-brown colonies. The third species is also capable of growth and of causing fermentation in the absence of oxygen; its development is, however, decidedly promoted by the presence of oxygen, and it is only then able to produce spores. The vegetative rods are cylindrical; with the formation of spores the rods become spindle-shaped, and in the centre of the spindle the large spore is formed. The colonies in nutritive gelatine are of a yellow colour. All three species form butyric acid and butyl-alcohol.

According to *Fitz* the spores of butyric acid bacteria can withstand a boiling temperature for a period of time which is naturally dependent, as in all cases, on their condition and on the nature of the substratum; *Fitz* gives three to twenty minutes as the limits. They can, however, also be killed by a lower temperature, if continued long enough; thus they are killed by being heated for six hours at 90° C. in a solution of grape-sugar; but in glycerine, at the same temperature, only after six to eleven hours.

Thus the same holds good for butyric acid fermentation as for lactic acid fermentation, namely, that it is not produced exclusively by one species. When butyric acid fermentation occurs in distilleries, breweries, and pressed-yeast factories, bacteria are frequently found which are entirely different from those described above.

Clostridium butyricum, and various other species, are capable of dissolving cellulose, and therefore play an im-

portant part in the *cellulose fermentation*, which is employed in various branches of industry.

4. Kephir-organisms.

The so-called "*Kephir*," on which the investigations of *Kern* have thrown some light, is an effervescent alcoholic and sour milk, which is prepared by the inhabitants of the Caucasus from cows', goats', or sheep's milk. It is prepared by adding a peculiar ferment, "kephir-grains," to milk. These are white or yellowish, irregularly-shaped, uneven grains, about the size of a walnut and of a tough gelatinous consistency, and when dried become cartilaginous and brittle. The most essential part of these grains consists of rod-like bacteria, which are connected in threads and have developed gelatinous membranes. *Kern* calls this bacterium *Dispora Caucasica*. Yeast-like fungi are also found in kephir-grains, and among these different varieties of true *Saccharomycetes*. In the preparation of kephir a little milk is first poured on the grains and allowed to stand for twenty-four hours; the milk is then poured off, and the grains preserved for future use. This milk is now mixed with fresh milk, and poured into bottles which are corked, or into leather sacks which are tied; after some days a fermentation has taken place. It now contains about two per cent. of alcohol. This result is probably brought about by the simultaneous action of the above-mentioned *Dispora* and the yeast cells in combination with the lactic acid ferments which are probably always present in milk. These ferments convert a portion of the milk-sugar into lactic acid; the alcohol and a part of the carbonic acid probably result from the action of the yeast cells. Then, as the fermented milk contains considerably less coagulated caseine than ordinary sour milk, it may further be assumed that the above-mentioned *Dispora* is also able to partly liquefy (peptonise) the coagulated caseine, perhaps with the help of the gelatinous mass secreted by the bacterium and which is found in the kephir-grains, but is not present in the

fermenting milk. If one of the above-mentioned kephir-grains is allowed to remain in milk, it will grow very slowly and only attain, according to the researches of *de Bary*, a double size after the lapse of several weeks. This author considers it probable that under such conditions single *Dispora* cells separate themselves and give rise to new kephir-grains. According to the mode of preparation published by *A. Lesy*, kephir can also be obtained without the addition of *Kern's* ferments. When milk which is becoming sour is repeatedly and violently shaken, an effervescent alcoholic kephir-like drink is obtained, which, as regards taste, etc., does not perceptibly differ from kephir prepared with kephir-grains. According to *de Bary* the kephir obtained by shaking contained about one per cent. by volume of alcohol, whilst a sample of the ordinary kephir contained only 0·4 per cent. by volume (Schmiedeberg). According to the recent investigations of *Duclaux*, *Grotenfelt*, *Adametz*, and others, there are also certain yeast-fungi which are capable of fermenting milk-sugar by themselves, without the aid of bacteria (see Chapter V.).

The Ginger-beer Plant, which presents morphological resemblances to the *Kephir* ferment, has been examined from a botanical and biological point of view by Professor *Marshall Ward*. If this ferment is introduced into saccharine solutions to which ginger has been added, it transforms them into an acid effervescing beverage, ginger-beer. When fresh, it occurs as solid, white, semi-transparent, irregular, lumpy masses, brittle like firm jelly, their size varying from that of a pin's head to that of a large plum. It induces an alcoholic fermentation in the saccharine solution, which at the same time becomes viscous. *Marshall Ward* isolated the numerous micro-organisms existing in the masses described above, and gave accurate descriptions of a series of yeast-fungi, bacteria and moulds, among which two organisms proved to be essentially concerned in the fermentation of ginger-beer. One of these is a Saccharomyces,

belonging to the ellipsoid group of this genus, and probably originating from the ginger and brown sugar employed in ordinary practice; the author has named it *Saccharomyces pyriformis*. It inverts cane-sugar, actively ferments the products, and forms a white pasty deposit at the bottom of the flasks. It yields spores on gypsum blocks at 25° C. in 40 to 50 hours; it also forms spores on gelatine.

In hopped wort it induces a not very vigorous fermentation, and it forms a film on the surface; the cells in this film are usually pyriform or sausage-shaped.

The other constant and essential form is a Schizomycete, *Bacterium vermiforme*, which, according to Professor *Ward*, originates from the ginger. It is a peculiarly vermiform organism, enclosed in hyaline, swollen, gelatinous sheaths, and imprisoning the yeast cells in brain-like masses formed by its convolutions. It is the swollen sheaths of this organism which constitute the jelly-like matrix of the "plant." It also appears without the sheaths, and with all the various growth-forms which we meet with among the bacteria. It is a markedly anærobic bacterium. The gelatinous sheaths are only developed when the saccharine liquid is acid, and free from oxygen.

Of the other organisms which occur in the ginger-beer plant, a *Mycoderma* species and *Bacterium aceti* were found in all the specimens examined, and a variety of other bacteria and fungi also occurred as casual intruders.

The author has proved experimentally that *Saccharomyces pyriformis* and *Bacterium vermiforme* are the only two essential species in the ginger-beer fermentation, since it was only by inducing a fermentation with these two species that he was able to produce an effect of the same kind as that obtained when the ordinary ginger-beer plant is employed. But it is only when both species develop together in the liquid that they bring about this result, and the author's experiments point to the view that the relations between the yeast and the bacterium are those of true

symbiosis, so that the two species form a lichen-like compound organism, which induces a "*symbiotic fermentation.*"

5. Slime-forming Bacteria.

Among the various species of slime-forming bacteria there are several which are of peculiar interest in the fermentation industries, as they occur in wine and fermenting wort, in which they cause morbid changes. According to all analogy, this slime formation may be regarded as a phenomenon closely related to the commonly-occurring zoogloea formation of certain bacteria (see p. 56). In the case of certain species the slime is, however, also regarded as a product of the decomposition of sugar, and not as a substance separated from the organism itself.

In the viscous fermentations examined by *Béchamp* a kind of gum termed viscose was formed together with carbonic acid, and frequently also mannite.

In his "Etudes sur la bière" (Plate 1, Fig. 4) *Pasteur* describes bead-like chains of spherical organisms, which render wine, beer, and wort so viscous that they can be drawn out in threads.

In Berlin "Weissbier" (white beer), which had become ropy, *Lindner* found a strong development of a certain *Pediococcus*. The disease could be produced by adding pure cultures to sterilised white-beer wort. On the other hand, this organism had no action on hopped beer-wort or low-fermentation beers.

In *ropy* Belgian beer *Van Laer* found the cause of this disease to be small, very thin rods (1·6 to 2·4 micro-millimeters long), which were partly isolated and partly united in pairs by means of a zoogloea-like substance. When added to beer-wort, this first became turbid, and afterwards ropy. On meat decoction with gelatine these rods gave colonies with concentric rings of different colours and with a hollow in the middle; streak cultures give broad, white bands, with a

sinuous border; puncture-cultivations give a white stripe, which soon extends to the bottom of the glass; the gelatine forms fissures which become filled with the growth, while at the same time a speck is formed on the surface. Experiments carried out with pure cultures of this bacterium in beer-wort have shown that one and the same form includes several species, which have a somewhat different action on wort.

FIG. 14.

Leuconostoc mesenteroides Cienkowski, after Zopf. *A*, cell cluster of the variety with no envelopes, taken from a potato cultivation; *B*, series showing the development of a cultivation grown in gelatine, free from sugar; *B a*, has no envelopes; *B b*, the same after 24 hours' growth in a solution of molasses, the envelopes are already seen but are not strongly developed; *B c*, after 48 hours' growth in molasses, the envelopes are more strongly developed and partly encased in each other; *C*, a small gelatinous mass from which the cells have been expelled.

They are all included under the name *Bacillus viscosus*. If sterilised wort is infected with this bacterium and alcoholic yeast added after the lapse of some hours, the liquid becomes

viscous. If the wort be infected with a mixture of absolutely pure yeast and bacteria, the disease will develop in a varying degree, according to the proportion of bacteria. If, however, these are only added after the completion of the primary fermentation, the disease will not appear at all. The greater the proportion of *nitrogenous* matter in the liquid, the sooner it will become viscous; even liquids which do not contain sugar can be made ropy by these species; on the other hand, the phenomenon does not occur in pure sugar solutions.

The so-called *frog-spawn fungus* (*Leuconostoc mesenteroides*) was investigated by *Cienkowski* and *van Tieghem*, and more recently by *Zopf* and *Lisenberg*. Both the European form and the variety found by *Winter* in Java occur spontaneously in beet-root sap, and in the molasses from the manufacture of sugar, in which they form large slimy masses (frog-spawn) and multiply vigorously. The fungus forms chains of cocci, two of which are always more closely united; in opposition to earlier observations, *Zopf* found that these cocci present no differences with reference either to their morphology or physiology; spore formation could in no case be proved. Consequently, the analogy which was formerly assumed to exist between this fungus and the algal genus *Nostoc* (implied in the name *Leuconostoc*) falls through.

Under certain conditions the cells become enclosed in a strong gelatinous envelope, which consists of a mucilaginous carbohydrate, the so-called *dextran*. This formation—a product of assimilation—only occurs in the presence of grape sugar, and not in solutions of milk-sugar, maltose, and dextrin, because these carbohydrates and likewise glycerine cannot be assimilated. Under certain conditions of cultivation, *e.g.*, in potato-cultures, the species develops quite a different form, in which the gelatinous envelope is completely absent.

The *Leuconostoc* ferments grape-sugar, cane-sugar (after previous inversion), milk-sugar, maltose, and dextrin, with

production of gas and acid. The fungus secretes an enzyme which inverts cane-sugar; but no other enzymes could be detected.

Especially characteristic of this fungus is its power of resisting elevated temperatures, the *younger* growths possessing this power in a higher degree than older cultures.

It is also remarkable that the growth and fermentative action of the fungus are very favourably affected by the presence of considerable quantities of calcium chloride.

6. Bacteria exercising an inverting, diastatic, or peptonising action.

Bacteria play a very great part in the formation of soluble chemical ferments. This constitutes one of the chief means by which these organisms exercise such an important activity in the economy of nature.

According to statements made by *Hansen*, many species of the bacteria which generally occur in *beer* secrete *invertive ferments*. Amongst these species are a number of bacteria which exhibit an invertive action in a pure cane-sugar solution but lose this property when yeast-water is added. Similar properties were observed by *Wortmann* in the case of bacteria which develop *diastatic ferments*. He found these on putrefying beans and potatoes, and grew the cultures in mixtures of nutritive salts and wheat-starch. *Morenno* also found a species which exercises diastatic action and which frequently occurs in the outer envelope of maize. *Peters* found a bacillus in leaven which brought about the solution of starch. In ordinary gelatine-plate cultures this fungus forms peculiar curved colonies, consisting of long filaments about 0·5 μ thick; in young colonies the filaments are shorter and motile. In beer-wort the bacillus forms rods which exhibit very active movements, and which gradually produce a film on the surface. The spores are rod-like, and their highly refractive contents are for the most part situated at the ends.

Peters described another bacillus, which he found among the organisms occurring in leaven, and which possesses a *peptonising power*. Small rods grow out of the spores, and these rods increase to long filaments, which in their turn divide into rods. In ordinary nutritive gelatine this species does not thrive well, if at all; whilst, on the other hand, it thrives readily and vigorously when "soluble starch" is added; the gelatine rapidly becomes liquefied. Spores appear abundantly in cultures in neutralised yeast-water. In suspended drop-cultures it was found that small pieces of boiled white of egg were much acted on or completely dissolved by this species.

7. Sarcina Forms.

In addition to the above-mentioned *Pediococcus acidi lactici* there also occur in fermenting liquids a number of

Fig. 15; Sarcina.

other spherical bacteria, the life-histories of which are only very imperfectly known. Both in bottom-fermentation and in top-fermentation (especially in distilleries and pressed-yeast factories) different varieties of *Micrococci* occur, the injurious action of which is strongly emphasised in the journals relating to these industries. This has, however, only been satisfactorily demonstrated by direct experiment in a single case (see Section on "Slime-forming bacteria"). In bottom-fermentation lager-beer these forms appear as small, more or less spherical, water-grey bodies, sometimes isolated, sometimes arranged in groups, generally in groups of four. They were described by *Hansen* under the name of *Sarcina* (Fig. 15). Organisms belonging to this group are found in very different localities. The true places of growth of the individual species are, however, not yet known.

Reinke often observed such forms, both in bottom- and

top-fermentation beers. He found that lager-beer, when so attacked, soon yielded a considerable sediment, and developed a bad odour and taste. The Berlin white beer often assumed a red colour, and it was then always found to be infested with many *Sarcina* forms, the growth of which increased considerably after a few days at a somewhat higher temperature; temperatures between 10° and 14° C. are stated by *Reinckes* to be particularly favourable in this case. However, he correctly lays stress on the fact that it is not certain whether *Sarcina* or the rod bacteria, which are also present, are the actual cause of the disease; it is only known that in red beer the presence of *Sarcina* is a symptom of abnormal conditions; whether it is the cause or the result can only be determined by exact investigations.

In the fresh residues from the distillation of spirit, which are employed as fodder, *Brüstlein* found a sarcina-like micrococcus, which possesses pathogenic properties. It has not yet been determined by direct experiments whether the so-called "mahlanders" or "greasy heels" of domestic animals is caused by this organism.

Lindner examined a series of sarcina-like organisms, and contributed largely to our knowledge of the life-histories of these organisms. The so-called *Pediococcus cerevisiæ* appears in cultures in the form of *cocci*, *diplococci*, or *tetrads*. Cultures made on meat broth with pepione gelatine, and partially covered with thin plates of gypsum, showed that the access of air is favourable to the growth of the colonies of this bacterium; during the first days all the colonies were found to be colourless; subsequently a yellowish, or yellowish-brown, tinge began to appear. The gelatine was not liquefied. On meat-broth gelatine this organism gave, in streak cultures, a greyish white, moist streak, with nearly smooth borders, and which, in thin layers, was strongly iridescent. In puncture-cultivations it developed throughout the length of the puncture, forming a white tuft on the surface of the gelatine, which spread out like a leaf. On boiled slices of

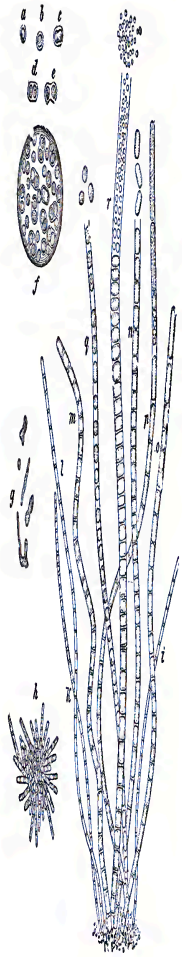

FIG. 18.

Crenothrix Kühniana, after Zopf: a—e (600 : 1), cocci in different stages of division; f (600 : 1), small, round coccogonea; g (natural size), zoogloea; h (600 : 1), colony of short filaments composed of pill-like cells, originating from the germination of a small collection of cocci: i—r, filaments, partly straight, partly spirally curved (i, ss), of very varying thickness, with more or less pronounced contrast between the the base and apex, and different stages of the division of their members and shortin; the sheathed filament r shows short rods at the base, which higher up are divided into small cylindrical pieces; at the apex the cocci are seen arising from the longitudinal divisions of the cylindrical discs.

potato this species thrives but poorly; in other cultures of this kind peculiar involution forms appear. In meat-broth gelatine the organism was killed after eight minutes' heating at 60° C., but not at 59° to 53° C. after the lapse of 12 minutes. In hopped beer-wort it yields a sediment, and subsequently forms a film. The formation of acid in the liquid after the action of this *Pediococcus* is very slight, and the author assumes that traces of lactic acid are formed. *Lindner* states that in no case was he able to produce any real disease in wort or beer by inoculating these liquids with a vigorous growth of this bacterium; he therefore remarks that the change in the flavour of the beer may not be caused by this species, but by other bacteria co-existing in the infected beer; on the other hand, he states that *Pediococcus cerevisiæ* causes a *turbidity*. The slime-forming species described by *Lindner* has been mentioned above.

A. Petersen observed that an abundant development of a Sarcina could take place in bottom-fermentation lager-beer without causing any disease; on the contrary, the beer was bright and stable, and had an agreeable taste and odour.

Thus, Sarcina species exist which are not productive of any disturbance in the brewery. The investigation of this problem has not, however, as yet been carried further.

8. Crenothrix.

In microscopical examinations of water we often meet the very typical forms of *Crenothrix Kühniana* (Fig. 16).

This ferment (frequently associated with *Beggiatoa alba*) occurs in every water which contains organic matter; sometimes it multiplies to such an extent that it may make the water unfit for use. Thus, according to *Zopf*, great calamities have been caused by this fungus in the water supplies of Berlin, Lille, and certain Russian towns. In consequence of its power of storing iron compounds in its walls, it forms red or brown flocks in water. Its forms are very beautiful; it occurs in the form of cocci (*a*–*f*), which

by partition and formation of viscous matter form zoogloea; these cocci frequently grow to articulate filaments, which are provided with distinct sheaths (*h*, *i—r*); they increase in thickness towards the apex; when they have arrived at a certain age, they divide within the sheath into smaller pieces, which become round and issue forth as rods, macro- or micrococci; these are sometimes seen floating about in water. We do not yet possess a more exact knowledge of the life-history of this beautiful bacterium.

CHAPTER IV.

The Mould-Fungi.

The mould-fungi ordinarily affect the fermentation industries in a somewhat different manner from the bacteria. Whilst the latter—in distilleries as a rule, in breweries only exceptionally—make their appearance in great force during the fermentation, and are therefore able to bring about important changes in the course of the fermentation, and in the resulting products, the mould-fungi, on the contrary, usually occur outside the true field of the fermentation in that they select as places of growth the vessels, tools, rooms, the green malt, and the quiescent masses of yeast, especially top-fermentation yeast. Accordingly the mould-fungi have a more subordinate, but nevertheless very real, importance. If we only sufficiently examine a growth of mould which has developed on the ceiling or walls of a fermenting room, or on the sides of a vessel, it will very soon be found that we have practically never to do with a mould growth *alone*; in nearly every case bacteria and yeast-like cells are found amongst the filaments of mould. These filaments extend upwards, and thereby raise the foreign bodies which in this exposed position are more readily carried away, partly by the workmen, and partly by the air.

During malting, all sorts of microscopic organisms are present on the raw materials containing starch. The mould-fungi are usually regarded as the most dangerous enemies, and this is certainly due to the fact that they are visible to the naked eye during development, and thus obtrude themselves upon our notice in an unmistakable manner. If,

however, *numerical superiority* be taken into account, the bacteria, which are always present in large numbers on green malt, must certainly be placed in the front rank. Judged from this side, it may even be considered doubtful whether the greatest influence on the product must be attributed to the mould-fungi (*Penicillium*, *Aspergillus*, etc.,) when these are met with in a state of vigorous development on the malt, or whether it is not far more probable that it is the numerous other organisms accompanying them which here play the most important part.

I have often found on the surface of pieces of pressed yeast a fine white parasitic growth, which most frequently consists of a mould *mycelium*, belonging principally to forms resembling *Chalara* and *Dematium*. It is very possible that when these plants form a thick layer on the surface of the yeast-mass, they retain by their respiration a portion of the free oxygen which is necessary to enable the quiescent yeast to remain alive for a longer time. Even here I always, without exception, found bacterial growths.

The truth is, that from observations made in breweries and elsewhere, a growth of mould nearly always serves to indicate that other organisms of a doubtless more injurious and more active character are present in the growth. It is, therefore, of great importance that the walls of fermenting-rooms should be *smooth*; this is effected with the greatest certainty by employing the enamel paint now so much in use.

The following is a review of the most important mould forms which are of interest for the fermentation industries.

1. Botrytis cinerea

forms small greyish-yellow patches on moist, decaying vegetable matter, and can also occur on wort. From the greyish-brown mycelium the conidiophores are thrown up; these are perpendicular, articulated filaments, generally arranged in tufts. They grow to the height of 1 mm.,

after which the apical cell throws out near its point, and almost at right angles, two to six small branches (C'). The

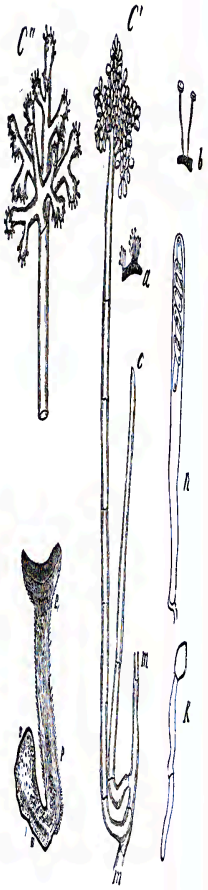

FIG. 17.

Botrytis Cinerea, after de Bary; a, b (natural size), Sclerotia, from which at c the conidiophores, at d the apothecia (fruits with asci), are shown out; e, C, conidiophores (C', with conidia just ripe), springing from the mycelium filament m; C'', end of a conidiophore with the first commencement of formation of conidia from the ends of the branches; k, germinating conidium (× 300); g, s (slightly magnified), section through a sclerotium s, from which a very small apothecium (g, g) is thrown up; n, single asens, with eight ripe spores (× 300).

lowest of these branchings are the longest; these again develop below their ends one or more short side branches. The topmost branches are almost as wide as they are long. Thus a system of branchings is formed which is shaped like a raceme or a bunch of grapes. When the longitudinal growth is at an end, the inner space of the branches becomes separated from the main stem by the formation of a transverse wall close to the latter. At the same time the ends of the branches and of the main stem swell, and on the upper half of each swelling several small papillæ now appear near together; these quickly increase to oval blisters, filled with plasma, and become narrowed, stalk-like, at their base. When these conidia (*C*) are completely developed, the walls of the branches carrying them are shrivelled up, and the conidia are consequently brought so closely together that they form a loose, irregular aggregation which readily falls off. If these clusters are placed in water, the conidia become detached from their stalks, and the envelopes of the branches, devoid of plasma, shrivel up or are only to be found in traces; their former place of attachment to the main filament appears only as a slightly raised scar. The member next below can now throw on one side the shrivelled apex, grow upwards, and form a new cluster; this can be repeated several times, whereby the conidiophores attain a considerable length.

Under certain conditions this mould can assume a peculiar *state of rest*, the so-called *sclerotium* (*sklēros* = hard) (*a*, *b*, *ss*). The hyphal threads branch extremely freely, and the branches intertwine themselves into a continuous body of diverse shape, circular to narrow spindle-shape, and of varying size up to a few lines; the extreme ends of the filaments are brown to black, and the ripe, solid *sclerotium* thus consists of an outer black rind and an inner colourless tissue. Such bodies are capable, after a long period of rest—at least one year—of forming a new growth, and may in so far be compared with the bulbs and roots of the higher plants. If the *sclerotium* is brought into a moist place soon after it comes to maturity,

the inner colourless branches break through the black outer rind and throw up the conidiophores (*a*). If, however, the *sclerotium* is not brought into a moist place until after it has been in rest for some time, a large tuft of filaments develops from the inner tissue, and these shoot up perpendicularly and finally spread out to a flat, plate-shaped disc (*b* and *ps*); the ends of the filaments appear parallel on the free upper surface of the disc; some of them remain thin, others swell up to club-shaped asci, and each of these asci forms in its interior eight oval spores (*s*). The mould has now entered upon the stage in which the formation of *apothecia* takes place. The spores germinate when they are set free, and the germ tubes grow into conidiophores.

According to *Bersch*, *Pitz*, and *Roese*, this organism is the cause of one of the diseases of wine, which manifests itself as an unpleasant smoky taste and smell. Similar cases of disease have been occasionally observed in breweries; it has, however, not yet been determined with certainty whether they are caused by this mould.

2. Penicillium glaucum.

A mould which is far more widely distributed in the fermentation industries, especially in green malt, is *Penicillium glaucum*. It forms a felt-like mass on the substratum, is at first white, then greenish or bluish-grey, and spreads with great rapidity. The mycelium consists of transparent branched and divided filaments, which, when immersed in liquids, are able to swell somewhat irregularly. From these filaments the conidiophores (*f*) are thrown up perpendicularly. They consist of elongated cylindrical cells, the terminal cell of which soon stops in its longitudinal growth and becomes tapering and pointed; the cell next below throws out one or more opposite branches, which rise up close to the terminal cell and, like this, consist of one pointed cell. In more vigorous individuals the branches may again ramify (compare Fig. 184, above),

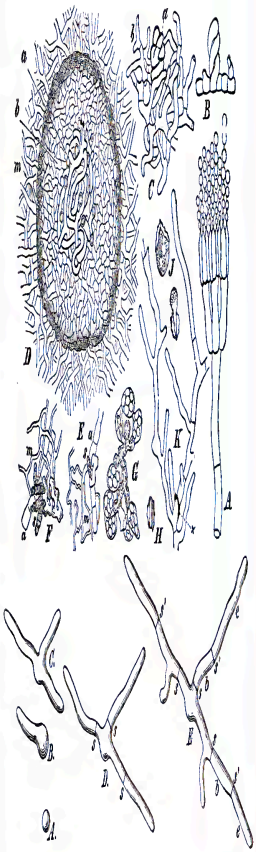

Fig. 13.

Penicillium glaucum, after Brefeld and Zopf: *A*, conidiophore; *B*, organs of generation; *C*, first development of the sclerotium (*a*, ascus-forming hyphæ; *b*, sterile filaments); *D*, very young sclerotium in section (*a*, ascus-forming hyphæ; *b*, sterile portion of the sclerotium; *m*, mycelium); *E* and *F*, ascus-forming hyphæ (*a*) with young asci (*b*) and sterile mycelium threads (*m*) from a more developed sclerotium; *G*, group of asci with spores; *H*, spore; *I*, germinating spores; *K*, young mycelium (with spore at *x*); *A—B* (below), germination of a conidium, after Zopf (more highly magnified); *A*, conidium before germination; *B*, it has thrown out a germ tube; *C*, three germ tubes have been formed; *D*, each germ tube shows towards the spore a transverse septum (*s*); *E*, each germ tube has become divided by another septum (*s'*) into a terminal cell (*c*) and an inner cell (*b*).

or similar branches may also spring from the next cells, and these again ramify and become pointed as described above. In this tuft of branches each pointed cell (*sterigma*) breaks up into a series of spherical conidia, and finally the tuft carries a large number of conidia, arranged in series, which, when ripe, are readily scattered. These round, smooth conidia give to the patches of mould their greyish-blue colour; when they fall upon moist surfaces, they are able to germinate at once.

In culture experiments with this fungus, *Brefeld* made the interesting observation that *Penicillium* can occur under certain conditions with an entirely different form of growth. He enclosed cultures of this mould-fungus on slices of coarse, non-sterilised bread, between glass-plates, and allowed the culture to further develop with the greatest possible exclusion of atmospheric air. There then appear in pairs on the mycelium short, thick branchings, which become entwined (*B*, above); one part of this spiral throws out short, thick filaments (*C*), whilst the hyphal thread carrying the spiral develops numerous fine branchings, which envelop the spiral and form a covering (*D*), consisting of an inner solid and an outer felt-like layer; the inner cells gradually become coloured yellow, and the outer loose cells are cast off. In this small yellow ball—*sclerotium*—a formation of swollen cells (*E*, *F*, *G*) gradually takes place by the continued branching of the above-mentioned spiral filaments, and in each of these new cells eight large and lenticular spores are produced, which have a circular furrow on the margin, and three or four slight ridges on the outer membrane (*Exosporium*). After the collapse and absorption of all the remaining interior elements the spores are at last set free, and the small yellow ball is then filled with the spore-dust. The entire development requires six to eight weeks. The *sclerotia* may be preserved in a dry state for several years without losing their power of germination. When the spores (*H*) are sown, the exosporium bursts open like a shell at the circular furrow, and the endo-

sporium swells and emerges (*f*), and elongates itself to a germ tube, which quickly develops conidiophores.

Penicillium possesses the power of secreting an *inverting ferment*, which is able to convert cane-sugar into other sugars.

3. Eurotium Aspergillus Glaucus.

The development of this fungus was first thoroughly described by the celebrated *de Bary*. It forms a fine felty, greyish or greyish-green covering on various materials, and is able to grow with the greatest luxuriance on green malt.

The mycelium consists, as in the case of *Penicillium*, of fine transparent and branched threads, provided with transverse septa. Some of the hyphal threads are thrown up perpendicularly, are thicker than the rest, and very rarely branched or divided by septa. Their upper ends swell to spherical flask-shaped heads (*c*), and these throw out from their entire upper portion radially divergent *pripilla* of an oblong form; these *sterigmata* then throw out at their apex small round protuberances, which are attached to the *sterigmata* by greatly contracted bases, and after some time are defined from the former as independent cells (spores, or *conidia*). Below the base of the first spore, a second begins to form from the crown of the *sterigma*, and pushes the first upwards; a third then forms, and so on. Each *sterigma* thus carries a chain of spores, the youngest of which is closest to the *sterigma*. This occurs at the same time over the whole surface of the enlarged ends of the conidiophore, which is thus finally covered with a thick head of radially-arranged chains of spores. These masses of spores form the greyish-green dust which covers the mycelium.

Finally, the *conidia* separate from one another; they have then a warty appearance on their outer surface. These small bodies are able to germinate (*p*) directly after they have become detached, and quickly develop a new mould-fungus; on this fact depends the rapidity with which the plant spreads. Under certain conditions, which are not yet

Fig. 19.

sufficiently known, but which in every case appear to be connected with a free supply of nutriment, the mould develops *perithecia*. These appear at first as tender branches, which, at the termination of their longitudinal growth, begin to twine their free ends in the form of a spiral of four to six turns (*f*); the threads of the spiral gradually approach nearer together, until finally they are brought into contact, so that the entire end of the filament takes the form of a helix (the *ascogonium*). There then grow from the lowest turn of the helix two or more small branches, which cling closely to the spiral. One of these small branchings (*S, T, p*) quickly outstrips the others in growth, and its upper extremity reaches the uppermost turn of the helix, and becomes fused with it. The other branch or branches likewise grow upwards along the spirals, shoot out into new branches, and gradually become so interlaced that finally the spiral becomes surrounded by an unbroken envelope (*W*). These branches become divided by septa perpendicular to the surface, and the envelope consequently consists of short, angular cells, in which new septa appear parallel to the surface, so that the envelope becomes thicker and composed of many layers (*V, X, F*). The small sphere now formed is about one-quarter mm. in diameter; the outermost layer is yellow, whilst the inner

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FIG. 19.

Eurotium Aspergillus Glaucus de Bary: *m, m*, hyphal thread, carrying a conidiophore *c* (from which the conidia have fallen), a perithecium *F*, and the first rudiments of an ascogonium, *f* (× 140); *s*, three stigmata from the crown of a conidiophore, showing the conidia-constrictions; *p*, germinating conidium (× 250—300); *A*, Ascus; *s*, germinating ascospore; *k*, germ tubes; *S*, spiral ascogonium; at *p* the commencement of the growth of one of the enveloping hyphæ; *T*, older stage; *W*, ascogonium, already surrounded by the envelope; *V*, longitudinal section of an older stage; in the centre the ascogonium, surrounded by the envelope, which now consists of several layers; *X*, longitudinal section of a later stage of development; the ascogonium is enveloped in a sheath of many layers, and it has loosened its convolutions, and commences to throw out the asci-forming branches; *M*, portion of an older asci-bearing branch; *a*, a young ascus; *a'*, an older ascus which has burst.

layers remain soft, and later are dissolved. The spiral after a time extends and throws out on all sides branched filaments which dislodge the interior layers of the envelope. These branchings finally take the form of an *ascus* (*M*, and *A*), and in each eight spores are formed. After the breaking up of the *ascus* the spores lie loose in the interior of the *perithecium*, and are liberated by the rupture of the now fragile wall of the latter. The spores, as in the case of *Penicillium*, are bi-convex, warty, and possess an outer stout membrane and an inner one, which, on germination, bursts the outer membrane into two valves (*r*).

This mould-fungus contains a *diastatic* ferment, which converts starch into dextrin and maltose.

In addition to this species, several others, closely related, occur in nature, and also find their way to the places mentioned here. In the greater number only the conidia stage is known.

4. Aspergillus Oryzæ.

In the preparation of the strong fermented *Japanese rice wine* ("saké"), the so-called *Aspergillus Oryzæ* is systematically employed.[1] The rice grains, freed from the hulls, are steamed, but the aggregation and gelatinisation of the grains are avoided. In order to prepare a malt serviceable for the brewer from these grains, which are not capable of germination, and from which the ordinary diastatic action is consequently excluded, the mass of grains is mixed with the so-called "Tane koji"—rice grains, which are coated over with the mycelium and conidia of *Aspergillus Oryzæ*; or the yellowish-brown spores of the fungus are mixed with the steamed rice grains. In the moist and warm air there develops on the rice at the end of about three days a white velvety mycelium, which gives to the whole mass an agreeable odour, resembling apples or pine-apples. Before the fructifi-

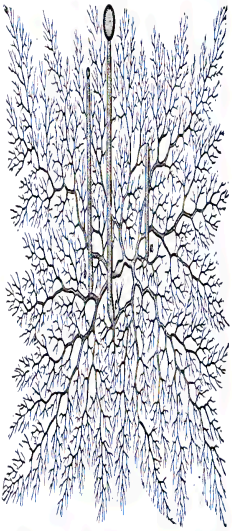
[1] Researches on this ferment were made by *Atkinson*, *Bingen*, *Cohn*, *Hoehn*, *Kellner*, *Mori*, and *Nagaoka*.

cation of the fungus takes place, a fresh quantity of steamed rice is introduced, and this also becomes coated over with mycelium; this process is repeated several times. In the koji-mass thus produced a part of the starch has been converted, and some of the albuminoids, which before were insoluble in water, have become soluble. The koji-mass is mashed, 21 parts of koji being mixed with 68 parts of rice boiled by steam, and with 72 parts of water. This pasty mass is allowed to remain at about 20° C.; after some days it becomes clear, the saccharification of the starch and dextrin continually progresses, and at the same time a spontaneous and very violent fermentation sets in, being caused by a yeast-like fungus, which does not stand in any genetic relation to the *Aspergillus*, and about which nothing is known. At the end of two or three weeks the fermentation is finished, and the product, after being filtered, is a clear, yellow, sherry-like liquid, containing 13 to 14 per cent. of alcohol. It is then pasteurised at 44° C. in iron vessels.

Atkinson found a ferment in koji which is soluble in water, and which inverts cane-sugar and converts maltose, dextrin, and starch-paste into dextrose. The researches of *Kellner Mori*, and *Nagaoka*, likewise showed that the koji-mass possesses a strongly invertive ferment, which converts cane-sugar into dextrose and levulose, maltose into dextrose, starch into dextrin, maltose, and dextrose. The various micro-organisms which occur in the koji-mass in all likelihood possess different invertive ferments. The presence of such different invertive ferments has before been pointed out by *Bourquelot*.

5. Mucor.

The genus *Mucor* belongs to the most interesting of the groups of mould-fungi with which we have to deal, since it embraces species with very marked fermentative action. These generally occur as a grey or brown felt-like mass, sometimes of very considerable height—even several inches



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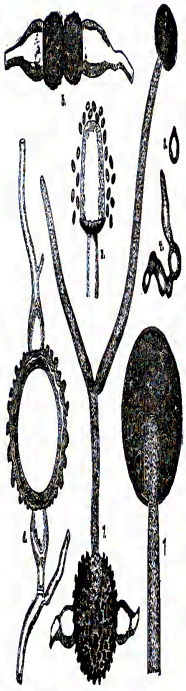

Fig. 80.

—in which small yellow, brown, or black spherules can be distinguished by the naked eye.

We give a description of the most frequently occurring species.

Mucor Mucedo (Fig. 20), one of the most beautiful mould-fungi, and one which occurs very generally on the excreta of phytophagous animals, has a transparent white mycelium, which develops numerous and delicate ramifications on the surface of and within the substratum, and which, in its earliest stages of development, and until the sporangia begin to form, is without transverse septa, and therefore unicellular. From the mycelium are thrown up single vigorous branches, the sporangium-carriers; the points of these branches which, according to *Zopf*, contain a reddish-yellow fatty colouring matter, swell greatly, and below the swelling a transverse septum is finally formed, whereby the sporangium is marked off from the sporangium-carrier. The transverse wall becomes arched upwards, and forms a short column—termed the *columella*—in the interior of the spherical head, whereby an inner space of peculiar form (1) results. The protoplasm of this space breaks up into a number of small portions, which become surrounded with a membrane and are rounded off; these are the spores. At the same time the sporangium becomes coated on its outer surface with small needle-shaped crystals of calcium oxalate. As soon as the ripe black sporangium takes up moisture, the wall is dissolved, and the spores with their yellowish contents are scattered on all sides along with the swelling contents of the sporangium. The columella, which projected upwards in the sporangium still remains at the

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FIG. 20.

Mucor Mucedo, after Brefeld and Kny: *A*, tree-like ramified mycelium with isolated thicker upright branches (*a*, *b*, *c*). 1, Sporangium; 2, columella and spores; 3, 4, germinating spores; 5, 6, development of the zygospore; 7, germinating zygospore with sporangium.

end of the sporangium-carrier; this is now surrounded at its base by a collar (2), the remains of the outer wall of the sporangium. When the refractive spores fall on a favourable substratum, they swell very considerably and send out one or two germ tubes (3, 4), which quickly develop to a vigorous mycelium.[1]

In addition to this mode of reproduction, *Mucor Mucedo* and the other species possess also a sexual method of reproduction, which takes place by means of a conjugation of two branches of the same mycelium. Two such short branches, filled with plasma, and growing towards each other, form club-like swellings and come in contact at their free ends, which become flattened (5). Each of the branches is then divided into two cells by a septum, and the end cells, which are in contact (the conjugating cells), coalesce by the dissolution of the originally double wall which separated them. The two conjugated cells are either equal in size, as in *Mucor Mucedo*, or unequal, as in *Mucor stolonifer*. The new cell thus formed—*zygospore* (6)—quickly increases in size and expands to the shape of a ball (in *Mucor stolonifer* to the shape of a barrel), after which the wall becomes thickened and stratified; externally it is coloured dark and covered with wart-like excrescences. These outer layers are very resistant to the action of acids. The contents possess an abundance of reserve substances (fat). The zygospores are generally able to germinate only after a long period of rest; the germ tube, after bursting the outer layers, quickly develops the above-mentioned sporangia (7). In the zygospore we thus find a *resting-stage* of the plant, an organ which by its structure enables the mould to preserve its life during periods which are unfavourable for its growth.

Mucor racemosus, which occurs especially on bread and decaying vegetable matter, has a branched, many-celled sporangium-carrier, which can also attain to a consider-

[1] Many of the above-stated botanical characters do not apply to M. Mucedo alone, but must rather be considered as generic characters.

able height. The brownish sporangia are developed at the ends of the branches. The spores are colourless. When this fungus is cultivated in wort, the submerged mycelium swells irregularly, and a large number of transverse septa appear, which divide it into large barrel-shaped or irregular cells filled with highly refractive plasma. These cells—*gemmæ*—are readily separated, and then assume a spherical shape (compare Fig. 21, 7), as was first observed by *Bail*, and *multiply by budding like the true yeast-fungi; the same takes place with the submerged spores (Mucor-yeast, spherical yeast)*. The mycelium produces a similar characteristic formation of gemmæ when cultivated on solid substrata. The plasma of the filaments collects in certain places in a compact mass, and is then enclosed at both ends by a transverse wall. At the same time the cell swells, the walls become thickened, and fatty substances are stored in the interior. The intermediate portions of the hyphæ gradually lose their contents.

Mucor erectus occurs, for example, on decaying potatoes and has the same microscopic appearance as *Mucor racemosus*; physiologically, however, it differs from this.

Mucor circinelloides (Fig. 21) has a very characteristic appearance. The mycelium (1) shows the remarkable branching which occurs in some of the species of *Mucor*:—the main branches (*b*) send out short, root-like, repeatedly-forked branches (*c*); at the base of these grow new mycelial branches (*r*), which become erect, and are able to form sporangia (2 to 5); the sporangium-carrier is branched. During its development it becomes considerably curved, and to this the species owes its name of *circinelloides*. In this form, as in *Mucor spinosus*, whose chocolate-brown sporangia are distinguished by the columella being studded on its uppermost part with pointed, thorn-like protuberances, the mycelium, when submerged in a saccharine liquid, produces a similar formation of gemmæ, as *Mucor racemosus* and *Mucor erectus*.

Mucor stolonifer (*Rhizopus nigricans*) attains a very considerable size, and occurs very commonly, for instance, on succulent fruits. This mould is easily recognised, since the brownish-yellow mycelium sends aslant into the air thick hyphæ without septa. These attain a length of about 1 cm., then sink their points to the surface of the substratum, and send out fine, greatly ramified hyphæ, resembling rootlets,

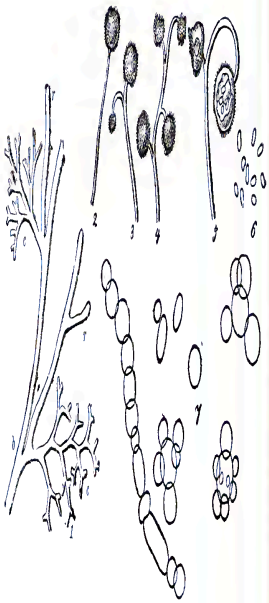

FIG. 21.

MUCOR CIRCINELLOIDES, after van Tieghem and Gayon: 1, Mycelium; 2, main branch; 4, root-like branches; r, axillary branches; 2–4, development of sporangia; 5, opened sporangia; 6, spores; 7, submerged mycelium and budding cells.

into the latter, whilst other hyphæ rise perpendicularly and develop sporangia; other branches again form new "runners." The black sporangium possesses a high, dome-shaped columella, and develops a number of dark-brown round or angular spores. When these become free by the absorption of the sporangium wall, the columella is turned over on the

sporangium-carrier like an umbrella, the line of junction of the external wall remaining in evidence in the form of a collar.

The species of *Mucor* have, considered from one point of view, very considerable interest, since they are able to act, in different degrees, as true *alcoholic ferments*. As previously mentioned, some of the species of *Mucor*, when immersed in a fermentable saccharine liquid, very quickly change their appearance; and whilst the mould thus approaches the yeast-like fungi in its appearance, it at the same time causes an actual alcoholic fermentation, yielding alcohol and carbonic acid as the chief products. If then the above-mentioned free cells of the mould-fungus are brought to the surface of the liquid by the bubbles of carbonic acid, they are able to again develop the mould form. The power of bringing about an alcoholic fermentation is possessed by the majority of the species of *Mucor*, but in a different degree; still the fermentative power is not exclusively connected with the formation of the above-mentioned budding gemmæ, since these have not been observed in *Mucor Mucedo* and *stolonifer*.

According to the recent investigations of *Hansen*, the various species, as far as they really are alcoholic ferments, induce fermentation not only in solutions of dextrose and invert-sugar, but also in solutions of maltose. Of all the species which he investigated, *Mucor racemosus* is *the only one that is capable of inverting a cane-sugar solution*; the others are consequently unable to bring about fermentation in a solution of this sugar.

The most active fermentative power is possessed by *Mucor erectus*. In beer-wort of ordinary concentration—14 to 15° Balling—it yields up to 8 per cent. by volume of alcohol. It also induces alcoholic fermentation in dextrin solutions, and converts starch into reducing sugar. *Mucor spinosus* yielded up to 5·5 per cent. by volume of alcohol in beer-wort. In maltose solutions distinct fermentation phenomena were observed, and at the end of eight months the liquid con-

tained 3·4 per cent. by volume of alcohol. *Mucor Mucedo* has only a comparatively feeble fermentative power both in wort (up to 3 per cent. by volume of alcohol) and in maltose and dextrose solutions. *Mucor racemosus* produces in wort as much as 7 per cent. by volume of alcohol, develops invertase, and ferments the inverted cane-sugar; thus, as mentioned above, it stands quite alone.

Mucor circinelloides is, according to *Gayon*, without action on cane-sugar, whilst it exercises a very powerful action on invert-sugar (yielding 5·5 per cent. by volume of alcohol). *Gayon* concluded from this that this mould might with advantage be employed to extract the cane-sugar from the molasses in the manufacture of sugar. So far, however, as I have been able to learn, this observation has not yet received any practical application.

6. Monilia.

Under this name are found described in works on mycology a large number of different fungi of comparatively simple structure; from a mycelium, the colour of which varies according to the species, branches are thrown up, which give rise to series of egg-shaped or elliptical spores. The genus has lately attracted interest on account of one of its species, which *Hansen* has provisionally named *Monilia candida*, from *Bonorden's* description, and which shows very remarkable physiological properties. It occurs in nature in the form of a white layer covering fresh cow-dung, and on sweet, succulent fruits. When introduced into wort, it develops a copious growth of yeast-like cells, which resemble *Saccharomyces ellipsoideus*, or *cerevisiæ*. At the same time it excites a vigorous alcoholic fermentation, and whilst this is progressing forms a mycoderma-like film on the liquid; the cells of this film extend more and more, and finally form a complete mycelium. In the first period the fungus produced only 1·1 per cent. by volume of alcohol, whilst *Sacch. cerevisiæ* gave 6 per cent.; but the *Monilia* continued the fermentation,

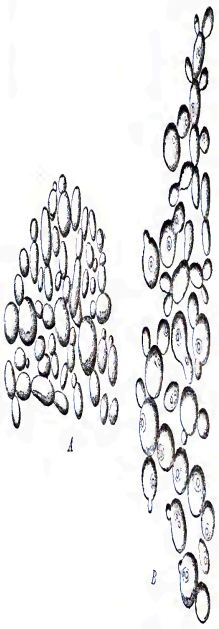

FIG. 42.

Monilia candida, after Hansen: *A*, growth in beer-wort or other saccharine nutritive liquids; *B*, cells of a young film-formation; *C* (p. 102), growth of mould: forms like *a* are frequent; they consist of chains of elongated more or less thread-like cells, rather loosely united; at each joint there is generally a verticil of oval cells, which readily fall off; *b* represents another form, also very frequently occurring, but distinguished from the former by having no verticillate cells; instead of these there generally issues from every joint a branch of the same form as the mother cell, but shorter; the links of these chains are not seldom closely united together, the constrictions in many cases disappear, and a very typical mycelium, with distinct transverse septa (*c*), is produced; the forms *b* and *c* occur in the nutritive medium, *a* commonly on the surface. Forms like *d* have much resemblance to Oidium lactis. *e* shows a chain of pear-shaped cells with verticils of yeast-cells resembling Saccharomyces exiguus; the chain of lemon-shaped cells represented at *f* closely resembles Ehrenberg's figures of Oidium fructigenum. Between the principal forms here described there are numerous yeast-cells of different forms, and differently arranged in colonies; as is usually the case, there also appear forms like Saccharomyces conglomeratus Reess.

Fig. 92 c.
Monilia candida, after Hansen. (For description see preceding page.)

and produced, at the end of six months, 5 per cent. by volume of alcohol, whilst the culture-yeast did not give more than the above-mentioned quantity.

Further experiments with this fungus led to the remarkable discovery that it *does not possess the power of secreting the soluble chemical ferment invertase*, and *yet ferments cane-sugar as cane-sugar*. As is known, cane-sugar has heretofore been considered to be *not directly fermentable*; *Hansen* has thus proved that this statement is not universally applicable.

His investigations have likewise proved that this species also *ferments maltose*. Since *Monilia* does not form invertase and is yet able to excite a fermentation in maltose solutions, it follows that a *previous conversion of maltose into dextrose is not necessary in order to bring about a fermentation of this sugar*.

The liquids containing the above-mentioned sugars showed during fermentation the presence of carbonic acid and ethyl-alcohol.

Finally, it is worthy of mention that this fungus is distinguished by its power of withstanding high temperatures. In beer-wort and cane-sugar solutions it develops vigorously at 40° C., and induces an active fermentation at this temperature.

7. Oidium Lactis.

A mould-fungus which has played an important part in the literature of the physiology of fermentation and in that of medicine is *Oidium lactis*, the so-called lactic acid yeast.

Some authors have sought to establish the theory that this fungus is a stage in the development of species which, under other circumstances, occur in entirely other forms, and with quite different properties. It was thus brought into genetic relation with Bacteria, Chalara (see below), Saccharomyces, etc. Both *Brefeld* and *Hansen* have carried out numerous investigations with this fungus, and have undertaken culture experiments, which were continued for a long time without producing any other than the ordinary Oidium-form. Recently,

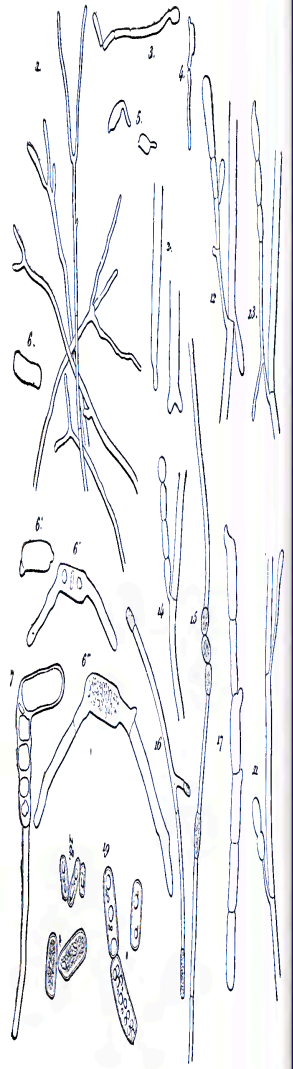

FIG. 23.

Oïdium lactis, after Hansen: 1, Hyphæ with forked partitions; 2, two ends of hyphæ—one with forked partition, the other with commencement of development of a spherical link; 3–5, germinating conidia; 6–6⁴, germination of a conidium, sown in hopped beer-wort in Reinvic's chamber, and represented at several stages; at each end germ tubes

it is true, *Brefeld* has discovered, in several higher fungi, a formation of conidia resembling chains of Oïdium cells; but it has not yet been determined whether this also includes that particular species which we designate *Oïdium lactis*.

Fresenius correctly gave to this species the specific name *lactis* (of milk); for universal experience goes to show that it has its ordinary place of abode in milk, where it can in the majority of cases be found. Up to now, however, no evidence has been brought forward that this mould-fungus stands in causal relation to the acid fermentations of milk. Further, it occurs spontaneously in various other liquids, and among these in the saccharine mixtures which find employment in the fermentation industries, and in these it is able to induce a feeble alcoholic fermentation.

The often forked, branched, thin-walled, transparent hyphæ (1) form a thick white felt; in the uppermost portions of the filaments transverse septa are formed close together, after which the single cells, filled with very refractive plasma, become detached as conidia (3 to 7, 11 to 14, 17 to 19). When the fungus grows on solid substrata, the hyphæ unite and form remarkable conical bodies. As a rule, the conidia in longitudinal section are rectangular with rounded corners (3, 6, 17 to 19); in a growth of this mould-fungus, spherical, roundish, pear-shaped, and quite irregular conidia (4, 5, 11 to 14) are, however, also nearly always found. These organs of multiplication, the only ones known, send out one or more germ-tubes. The fungus may occur in beer, especially when poor in alcohol. As the amount of alcohol increases, the conditions for its growth become more unfavourable; still, neither wort nor beer is exposed to the danger of being attacked to any

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have developed; after 9 hours (6′′) these have formed transverse septa and the first indications of branchings; 11–14, abnormal forms; 15, 16, hyphæ with interstitial cells, filled with plasma; 17, chain of germinating conidia; 18, conidia which have lain for some time in a sugar-solution; the contents show globules of oil; 19, old conidia.

extent by *Oïdium*, since it is not able to compete in the struggle with the concourse of organisms which at once appear when fermentable liquids are exposed to the germs of the air.

In numerous investigations with top-fermentation yeast, I found that this offers a very favourable nutritive material for this fungus, especially when the yeast is in a quiescent state at the end of the fermentation. Sometimes a microscopic examination showed an enormous number of conidia. It is not known what influence such a growth exercises on the quality of the yeast and the beer; without doubt it is advisable to avoid the fungus as much as possible.

8. *C. G. Matthews* observed that the red colour which appears on grains of malt, and more particularly when the quality is not very good, is produced by a *Fusarium* (probably *graminearum*). He cultivated this mould on various substrata. The fasicular spores are spindle-shaped, curved, and uni- or multi-cellular; they are colourless, or only very slightly tinted, but were embedded in the preparations in a strongly-coloured mass. The formation of mould commences at the germinal end of the corn, and spreads from thence more or less over the surface. When such corns germinate at all, they show an abnormal development, since they either send out only single rootlets with a sickly appearance, or the plumule only. Whilst the spores of *Penicillium*, *Mucor*, *Aspergillus*, etc., are easily distributed over the malt heaps by the air, the grains attacked by the *Fusarium* can, according to *Matthews*, only communicate the mould to the neighbouring grains, probably because the spores of this mould have a greater weight, and more closely adhere to the original mould-growth than do the spores of the other organisms.

9. *Chalara Mycoderma* (Fig. 24) is described in *Pasteur's* "Études sur la bière" as one of the habitants on the surface of grapes. The mycelium forms a film on liquids, and consists of branched, greyish filaments, often filled with highly refractive plasma, and which develop at different points conidia

of unequal form and size. *Cienkowski*, in his memoir on the fungi occurring in films, first gave a detailed description of *Chalara*. *Hansen* found that this mould-fungus develops in ordinary wort and lager beer.

10. A mould-fungus about which a great deal has been written in the literature of our subject, but whose practical

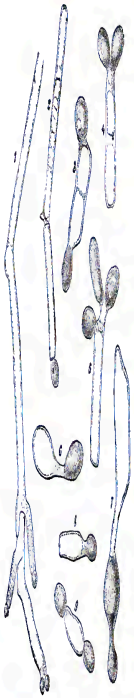

FIG. 24.

Chalara Myoderma, after Hansen: 1, a branched hypha, the terminal limb of which is throwing off conidia; 2, a hypha, at the upper cell of which a sterigma, which has thrown off conidia; 3–9, various forms of links of hyphæ, which are separating conidia.

importance certainly stands in inverse ratio to the attention bestowed on it, is *Demadium pullulans* (Fig. 25), which was first described by *de Bary*, and later more minutely by *Loew*. It frequently occurs on fruits, especially grapes, and has a

branched mycelium, from which buds are thrown out; these have a striking resemblance to ordinary yeast-cells (4), and are able either to propagate through many generations by

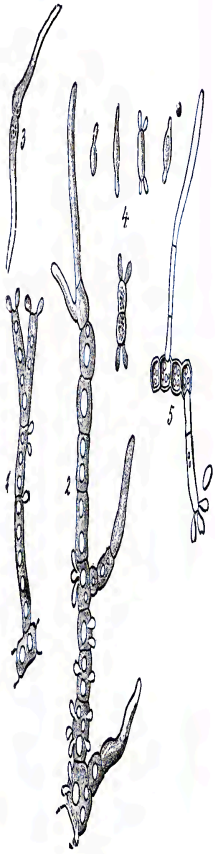

FIG. 55.

Dematium pullulans, after Loew: 1, 2, full-grown mycelial threads with yeast-like cells; 3, cells of the latter kind developing to mycelial threads; 4, cells with yeast-like buds; 5, appearance of yeast-like cells on the germ tubes of the brown-walled cells.

yeast-like budding, or to produce germinating threads, which give rise to a mycelium (3). When this has attained a certain age, it forms numerous closely-situated transverse

septa, and gradually becomes brownish or olive-green (5); in this we have the resting stage of the plant. In *Hansen's* air-analyses *Dematium* was very frequently found, from spring until late autumn, in wort to which the air had access; he observed that when the mould was sown in a saccharine liquid, it at first only developed mycelial threads; after some time, however, the yeast-like cells were separated, without inducing alcoholic fermentation. *Pasteur* has very fully treated of this organism in his "Études sur la bière." Since it occurs so abundantly on the surface of grapes, where the wine-yeast is developed, and since this often has exactly the same appearance as the yeast-like cells thrown off by *Dematium*, it might be imagined that the conidia of the latter were identical with the wine-yeast cells (*Saccharomyces*). In different parts of the above-named work *Pasteur* expresses himself differently on this point; in certain relations he only puts forward this connection as a supposition, whilst in other places he regards it as a matter of fact. Here again we have an example of the attempts previously mentioned to connect the yeast-fungi (*Saccharomycetes*) with the mould-fungi. According to the present methods of research, the question no longer admits of doubt. The true wine-yeasts can, under certain conditions, which have now been thoroughly investigated, produce spores in their interior; under the same conditions the conidia of *Dematium* develop no spores, and are thus distinguished from the wine-yeast.

11. Finally, we have to mention a mould which may occur, for example, in fermentable liquids and in fermenting rooms,—*Cladosporium herbarum*. This organism sometimes occurs in very large quantities in fermenting rooms; some years ago I found, in a bottom-fermentation room, the ceiling and a portion of the walls thickly covered with small black patches; these consisted of this mould, whose conidia I consequently always found in the yeast. The plant consists of a yellowish-brown mycelium, with short, straight, stiff, and brittle filaments, of which those growing erect can produce

at their upper extremities conidia of very varying forms—spherical, oval, cylindrical, straight, or curved. The systematic position of the mould and its possible genetic connection with other known fungi is just as little established as its influence on nutritive liquids. *Eriksson* states that rye is sometimes attacked by Cladosporium, and that the mould, consumed in bread made from rye, or in beer, may give rise to diseases in the human being.

Concerning these, or at least closely-related forms, *Zopf* described exact morphological investigations with numerous illustrations in his memoir on Fumago, and also in his work on the fungi. These last-mentioned black, dew-like fungi occur very frequently on parts of plants. *Frank* correctly says:—"We are still quite in the dark with regard to specific differences, the reason of which is especially to be found in the frequent *polymorphism* of these organisms, and in the fact that the different evolution-forms are scarcely ever found together."

CHAPTER V.

Alcoholic Ferments.

INTRODUCTION.

It does not lie within the scope of a work of this description to give a detailed summary of the knowledge of bygone times, and it will suffice to pass in review as much only as is necessary for the proper understanding of the present position of the subject under discussion. As the investigations of the last decade originated essentially from questions connected more or less directly with practice, the results obtained are also fully entitled to a practical application. It is evident, however, that this can only be brought about when the essential results of these scientific investigations are thoroughly appreciated; and it is with the object of facilitating this that the following *résumé* is given.

The term *alcoholic ferment*, as commonly used, is very comprehensive. Mould-fungi, as well as bacteria and budding-fungi, are able to induce alcoholic fermentation; but here we have only to deal with the last-mentioned. Amongst these *budding-fungi* are *some which also develop mycelium*, whilst *with others this form of growth does not as a rule occur; among these latter yet another group is included under the name Saccharomycetes, on account of the property which its members possess of forming endogenous spores.*

In the year 1839 *Schwann* found that in the case of certain yeast cells new cells were formed in their interior, and that these were liberated through the bursting of the walls of the mother-cells. *J. de Seynes* (1868) was,

however, the first who distinctly described the spores in yeast-cells. Shortly afterwards, in the year 1870, *Reess* proved that the formation of spores occurred in several species of yeast, and stated that the germination of these endogenous cells took place by budding. As far as the, at that time, very imperfect methods of experimenting permitted a conclusion being drawn, it appeared probable that there was a separate group of such budding-fungi, and to this group *Reess* gave the name *Saccharomycetes*.[1] The conditions favourable to the formation of such reproductive organs in the cells were, however, unknown; there was no definite method by means of which their formation could be insured, and experiments having this for their object were made at random. In the work already quoted *Reess* also proposed a system for the classification of the *Saccharomycetes*, which he based solely upon the size and form of the cells. Such a classification founded upon purely microscopical appearances, has, however, proved to be useless, and it is impossible to distinguish between the different species by means of the characters indicated by *Reess*. His work has consequently been of no real practical importance; and since the essential conditions for the formation of spores were unknown to him and to his successor *Engel*—so that it was purely a matter of chance whether, in a culture of *Saccharomycetes*, spore-forming cells were obtained or not—it is easy to understand the doubt subsequently expressed as to the existence of spores, and the disputes which also followed as to whether the yeast used in practice had or had not lost the property of forming spores. Finally, *Brefeld* believed that he had definitely proved that cultivated yeast was completely deficient in this property. This confusion was at last dissipated and order established

[1] The same author was however less consistent when he admitted into this group other kinds which did not yield spores, and in this he was also followed by *de Bary* in "Comparative Morphology and Biology of the Fungi, Mycetozoa and Bacteria," Oxford, 1887. *Reess* himself thus at once destroyed the very system, the construction of which he had just taken in hand.

when *Hansen* discovered the conditions regulating the formation of spores, and upon this basis for the first time devised a *method* for obtaining them.

Pasteur's "Études sur la bière" was published in the year 1876, and this work advanced in many directions our knowledge of the phenomena connected with fermentation. The main portion of this book is devoted to the doctrine, that every fermentation and every putrefaction is brought about by micro-organisms, a doctrine which he had defended with great force in earlier papers. *Pasteur's* name is with justice associated with this important doctrine, since it was mainly through his experiments that its truth has been confirmed and recognised. The idea, however, can be traced much further back. *Lémé* and others expressed the belief that the processes of fermentation and putrefaction were caused by living microscopic organisms; but proof was not forthcoming until much later. It has already been mentioned that in the year 1836 *Cagniard-Latour* proved that the yeast of beer and wine consists of cells which reproduce themselves by budding, and that these cells bring about alcoholic fermentation. Shortly afterwards *Schwann* arrived at the same conclusion. In the year 1838 the view was expressed that different fermentations were caused by different micro-organisms; and it was about this time that *Turpin* stated that there was "no decomposition of sugar, no fermentation without the physiological activity of vegetation." I would refer the reader to the above exposition of this doctrine, which in its historical development is so closely related to the doctrine of spontaneous generation (see *Sterilisation*).

Important discoveries never originate from a single man, but are really the result of the work of many investigators; it is, however, in general much easier to conceive the idea of some truth than to furnish sufficient proof of its correctness. Thus, although the doctrine was not new, when in 1857 *Pasteur* commenced his experiments, some very essential

connecting links were wanting, as is evident from the fact that *Liebig* again gave preference to *Stohl's* experiments in support of the chemical theory of fermentation. The victory gained by *Pasteur* in this dispute constitutes the foundation of his great fame.

In his "Études sur la bière" *Pasteur* clearly and incontestably proves the *significance of the micro-organisms*, and he lays much stress upon the marked influence which bacteria are capable of exercising upon fermentation and on the character of the resulting beer. He also treats of the budding-fungi; and in the case of some imperfectly described members of this group, he intimates, as *Batl* and others had done previously, that they affect the character of the products of fermentation in various ways. In this *Pasteur* is merely repeating the indistinct views of previous investigators, and *his suggestions take two opposite directions*. This is distinctly seen in his observations on the so-called essous yeast and the aërobic yeast. It is possible that in this case he may have been dealing with distinct kinds of yeast, but it is also possible that they were merely forms of ordinary brewers' yeast modified by some treatment to which they had been subjected. It must not, however, be overlooked that Pasteur was clear as to his position, and even pointed out the reason why the question could not be decided, namely, that *it was not then possible to determine whether at the starting point he was dealing with only one or with several species. An accurate method for the pure cultivation of the different kinds of yeast had not then been discovered* (compare Chapter I., 7. Preparation of the pure culture). A true orientation in the world of micro-organisms is consequently not found in this work, and it is not possible in any part of *Pasteur's* statements to find such characteristics for the budding-fungi on which a scheme of analysis could be based. *Pasteur* classed with the *Saccharomycetes* all the budding-fungi which showed any marked power of producing alcoholic fermentation, and it is nowhere clear whether his

descriptions apply to *true Saccharomycetes* or to *other budding-fungi*. These yeast-fungi, which in our present system may belong to very different classes, were further regarded as stages of development of mould-fungi resembling *Dematicum*, but no evidence was given in proof of this view. Whether or not there are different species of these budding-fungi (*Saccharomycetes*, *Torula*, *Dematicum*), *Pasteur* leaves undetermined. His treatment of the botanical problems mentioned must on the whole be regarded as having broken down in the essential points.

The reason above all others why this work was not able to bring about the reform in brewing indicated in its preface was,—as will be clear from what has been said above—that from the position of science at that time, it was not possible to see clearly into the relations of the different alcoholic ferments during the process of fermentation. *Pasteur* was therefore unable to get beyond the indefinite conjectures and contradictory views of his predecessors. In his review of the micro-organisms which cause diseases in beer, he speaks only of *bacteria*; and the view that these are the only causes of diseases in beer has since been repeatedly expressed by *Duclaux* in 1883, and by other French, English, and German writers. *Pasteur*, basing his views on these studies, recommended brewers to purify their yeast; and in order to free it from bacteria, advised its cultivation in a sugar solution containing tartaric acid, or in wort containing a little phenol (see below).

In contradistinction to this, *Hansen*, in the year 1883, brought forward his doctrine that some of the *most dangerous and most common diseases of low-fermentation beer were caused, not by bacteria, but by certain species of Saccharomyces*, and that each of the names employed by *Reess*, namely, *Saccharomyces cerevisiæ*, *Saech. Pastorianus*, *Saech. ellipsoideus*, represented not one but several different kinds or races. He showed that varieties which until then had been incorrectly grouped under the one name

Saccharomyces cerevisiæ gave in the brewery products having different characters. Starting from this, *Hansen* elaborated his method, by means of which a pitching-yeast, consisting of *only one species*, is employed. After some resistance this system has been recognised and introduced into practice in all countries where the brewing industry is carried on. *Velten* of Marseilles, who formerly worked with *Pasteur*, has, however, recently attacked this system, the mistake of which he deems to be that *Hansen's* yeast consists only of one species. He considers it an advantage in *Pasteur's* purified yeast that the latter consists of several different kinds, and regards this combination of various species as necessary in order that the beer may acquire the desired taste and bouquet. *Hansen's* latest investigations (see Chapter I., 7) show how completely this doctrine breaks down. *Hansen* proved by experiment that when yeast is treated with tartaric acid, according to *Pasteur's* method, the *conditions are so favourable for the development of the yeasts which produce disease, that finally the culture-yeast becomes completely suppressed.* *Pasteur* consequently greeted *Hansen's* method as an advance, in that he wrote, "*Hansen* was the first to perceive that beer-yeast should be pure, and not only as regards microbes and disease-ferments in the narrower sense, but that it should also be free from the cells of wild yeasts."

As, however, *Pasteur's* work always retains its technical importance, on account of the force with which the influence of bacteria in the fermentation industries is asserted, so it also possesses great theoretical interest, especially from the new theory of fermentation enunciated therein, and which at the time rightly attracted much attention.

Contrary to *Brefeld*, who asserted that yeast could not multiply without free oxygen, and *Traube*, who indeed granted that yeast was able to develop without free oxygen, but maintained that it then required for its cell-formation the soluble albuminoids in the liquid, *Pasteur* stated that the organisms

of fermentation constitute a group of living beings, whose function as ferments is directly "a necessary consequence of life without air, of life without free oxygen"; and further, that such a fermentation can also take place in a pure sugar solution. He maintains that the reason why *Brefeld* could not get yeast to develop in a moist chamber in an atmosphere of carbonic acid, was because he was working with old yeast-cells, whilst it is only possible for yeast to multiply in the absence of free oxygen when the cells are very young. The minute quantity of free oxygen which is present in the liquid to which the yeast is added "rejuvenates the cells and makes it possible for them to again resume the power to bud, to preserve life, and to carry on their multiplication without access of air."

Hence *Pasteur* makes a distinction between two classes of organisms: *aërobic*, those which cannot live without the presence of free air; and *anaërobic*, those which can exist in the absence of air. According to his view, these latter constitute "ferments in the true sense of the word."

It would be incorrect to assume that the presence of alcohol and carbonic acid amongst the products of a fermentation unconditionally presupposes the influence of "organisms of alcoholic fermentation in the true sense of the term." The researches of *Lechartier* and *Bellamy*, which were subsequently extended by *Pasteur*, showed, namely, that when grapes, oranges, and other fruits on which no yeast-cells were present, were preserved in vessels filled with carbonic acid, a development of alcohol and carbonic acid took place. "The fermentative character is consequently not a condition of the existence of yeast; the fermentative power is not peculiar to cells of a special nature, is no fixed structural characteristic, but is a property which is dependent upon external conditions and upon the mode of nutrition of the organism."

"In short, fermentation is a very general phenomenon. It is *life without air*, *life without free oxygen*; or more

generally still, it is the necessary result of chemical work carried out on a fermentable substance, which by its decomposition is capable of evolving heat; the heat necessary to effect this work being borrowed from a part of that which is liberated by the decomposition of the fermentable substance. The class of fermentations properly so-called is limited by the small number of substances which are capable of evolving heat on decomposition, and which will serve as nourishment for the lower organisms when the admission of air is excluded" "(Études sur la bière," page 261). This is briefly Pasteur's famous theory of fermentation.

Fermentations dependent upon oxidation,—such as the acetic acid fermentation, which, as *Pasteur* himself had observed, requires an abundant supply of air,—were consequently not regarded by him as true fermentations. It is seen, moreover, that he does not strictly adhere to his definition, in that he emphasises the fact that yeast also possesses fermentative properties when air is present, although to a less degree than when oxygen is excluded. The correctness of this under certain conditions has been confirmed in the case of bottom yeast by *Pedersen* (1878), and by *Hansen* (1879), who came to the conclusion that the amount of substance in a wort which a definite quantity of yeast can convert into alcohol and carbonic acid is smaller when the liquid is aërated during fermentation than when no aëration takes place. *Ed. Buchner* (1885) obtained a similar result in his experiments with bacteria.

Hansen arranged his experiments in such a way that a rotatory motion was imparted to the liquid which was being aërated, and the cells thus brought into continual contact with the vigorous current of air which was blown through the fluid. Nevertheless, there was a distinct alcoholic fermentation, and it certainly follows that this was not induced by life without air.

In *Nägeli's* "Theorie der Gärung" (1879) it is shown that the admission of oxygen is highly favourable to alcoholic

fermentation in a sugar solution when no other nourishment is present, and consequently the yeast does not multiply, or does so only to a small extent. *Nägeli* therefore states (p. 26) that "*Pasteur's* theory, that fermentation is induced through want of oxygen, in that the yeast cells are forced to take the necessary supply of oxygen from the fermentable substance, is refuted by all the facts which bear upon this question."

A. J. Brown, who also holds this view, made a series of experiments in which fermentations were conducted in presence of an abundant supply of oxygen, whilst in a duplicate set of experiments conducted simultaneously, oxygen was excluded; the same number of non-multiplying yeast cells were present in both cases, and all the other conditions were kept constant. These experiments showed—contrary to *Pasteur's* theory—that the yeast cells exercised a greater fermentative power in the presence of oxygen than when the latter was excluded.

Recently *Hueppe* and his pupils have also opposed *Pasteur's* theory, and have brought forward examples of fermentation organisms "which can induce the specific fermentations mostly even more readily when atmospheric oxygen is present."

Of *Nägeli's* manifold work on the lower organisms, we will only mention, as connected with the foregoing, the "molecular-physical" theory of fermentation put forward by him, and which is essentially a modification of *Liebig's* theory. Whilst *Pasteur* explains fermentation as the result of activity occurring within the cell, *Nägeli* defines fermentation as a transference of the vibrations of the molecules, groups of atoms and atoms of different compounds (which themselves suffer no change), contained in the living plasma to the fermentable substance, whereby the equilibrium of its molecules becomes disturbed and their decomposition brought about. In the process of fermentation, the vibrations of the plasma molecules are thus transferred to the fermentable

substance. The cause of fermentation is present in the living plasma, and therefore in the interior of the cells; but it operates at a moderate distance outside the cell. The *decomposition of sugar* into alcohol and carbonic acid takes place to a small extent within, but *mainly outside the yeast cells*. This theory is thus distinctly opposed to that of *Pasteur*, and follows on the lines of the theories propounded by *Stahl* and *Liebig*.[1]

Rayman and *Kruis* added to our knowledge of the biology of yeast-fungi by their experiments on beers which, during a period of several years, had undergone fermentation with absolutely pure cultures, prepared by *Hansen's* method. These investigators found that the fermentation product obtained by means of pure cultures of *Saccharomycetes*—the normal conditions of temperature, etc., obtaining in the brewery being maintained—is a single alcohol, namely, *ethyl-alcohol*. This alcohol remains together with the living yeast for years in the beer when the latter is preserved at a low temperature and air is excluded; when, on the other hand, a yeast film is allowed to form on the surface through the admission of air, a vigorous oxidation sets in, and the *alcohol becomes converted into carbonic acid and water*. In prolonged fermentations the *Saccharomycetes* hydrolyse to a variable extent the albuminoids present in the nutrient fluid, and they can also oxidise the products to formic and valerianic acids. The same authors distinguish two reactions in normal fermentations, namely, a sugar-hydrolysing reaction taking place in the nutrient medium, and a synthetic (albuminoid)

[1] In *Brefeld's* numerous mycological treatises the budding-fungi occupy a somewhat prominent position; thus this naturalist showed that, many *Uredineæ*, *Basidiomycetes*, and other fungi can assume a budding-fungus stage. This had also been previously shown by *Bail*, *Rees*, *Zopf*, and others; and since *Brefeld* did not prove whether these forms exhibit the property of forming endogenous spores, which is characteristic of the *Saccharomycetes*, nor whether they possess any marked fermentative activity, his indefinite statements that they are identical with the *Saccharomycetes* lost all weight.

reaction taking place in the interior of the organism. They regard fermentation as an alternate hydration and dehydration.

In all these different theories of fermentation, the main point of all questions relating to the subject is not touched upon:—How comes it that, in these microscopic cells, the *plasma*, which has the same appearance in the different species, yet in one cell induces an acetic acid fermentation, in another butyric acid fermentation; in a third it induces a direct fermentation of cane-sugar, whilst in a fourth the cane-sugar becomes first hydrolysed and then fermented? The cause of these different kinds of activity of *plasma* is still an unsolved problem.

The theories of fermentation hitherto put forward fail to give any comprehensive explanation of known facts, and consequently they have here only an historical interest.

From the above *résumé* it will be seen that, at the time when *Hansen* commenced his investigations, our knowledge of the alcoholic ferments was very deficient and untrustworthy. Consequently the problem had to be attacked experimentally from the very foundations. *Hansen* has done this in the work which he has now carried on for many years.

The previous investigators had certainly gone as far as was possible along the paths which they had marked out. When we compare their investigations—especially those of *Pasteur* and *Reess*—with those of *Hansen*, we find that the latter attacked the problem from new points of view and with new methods. He extended his investigations on this subject far and wide in all directions. His researches have not only opened up new paths from the scientific standpoint, but they have also brought about a reform in the fermentation industry. For these reasons it is but right that they should form the ground-work of the following section of my book.

HANSEN'S INVESTIGATIONS.

When *Hansen* published, in the year 1878, his treatise on "Micro-Organisms in Beer and Wort," he pointed out the uncertainty which prevailed in the works of earlier writers, concerning the true *Saccharomycetes*; and he emphasised the fact that it was not possible to proceed further along the path which they had pursued, but that the investigations, and especially those commenced by *Pasteur* and *Reess*, must, if they were to be carried further, *be attacked from a totally different point of view*. It was only in the latter end of the year 1881 that he succeeded in finding the key to the solution of the problem. The problem was, in the first place, to devise a method by means of which one could obtain growths, each of which was derived from *a single cell*, in order to determine by experiment *whether these guaranteed pure cultures exhibited constant characters*—that is to say, how far the *Saccharomycetes* occur as species, varieties, or races—and, should this prove to be the case, to determine what these characters are. When this problem was solved, the next was to devise a method for the analysis of yeast and to study in different directions the conditions of life of these organisms.

1. Preparation of the Pure Culture.

In the first chapter of this book it was pointed out that the idea had been expressed on various sides, that the only condition for an exact knowledge of the micro-organisms, hundreds or thousands of which we find in every drop when examined under the microscope, consists in the isolation of a single cell, and in working with a pure growth obtained from this cell. The different methods which had been employed were also briefly described.

Hansen has repeatedly pointed out in his papers that *the only method which is certain in all cases is to start from the individual cell and to secure the beginning from this*. He has devised two different methods for this purpose. In

his first method a liquid medium was employed, and in his second method a solid medium, for the cultivation; in both cases the culture was previously diluted as already described (Chapter I, 7. Preparation of the pure culture).

With the help of the acquired knowledge of the species it was possible to submit these methods to a searching examination, with the result that they proved to be reliable.

If it is desired to isolate from a mixed *growth* of different species those which are in an *enfeebled condition* it is necessary, as *Hansen* points out, to employ the dilution method, using a suitable nutrient fluid, as, for example, wort, the conditions being then favourable for the growth of the organisms in question.

If, on the other hand, we wish to separate from a mixed growth a species which is *in a vigorous state of development*, and whose further growth is consequently not dependent upon specially favourable conditions of nutriment, we can attain our object more readily and in a shorter time by the employment of a solid nutrient medium—in this case gelatine and wort. It has been proved that the addition of gelatine to wort diminishes its value as a nutritive material for the yeast-fungi. A series of experiments carried out by *Holm* show in fact that, if at the commencement of a fermentation when the yeast-cells are in their most vigorous state of development, some of these cells be introduced into wort-gelatine, about 4 per cent. of those sown do not develop; if, on the other hand, the yeast-cells are taken at the conclusion of a fermentation, when they are enfeebled, about 25 per cent. of them give no colonies in wort-gelatine.

The advantage of this method, as employed by *Hansen*, for the study of the budding-fungi is that it makes it possible to *directly observe the individual cells under the microscope* and to *follow their further development*, since the gelatine plate is enclosed in a moist chamber (compare page 26, Dilution methods).

2. The Analysis.

Throughout the entire series of *Hansen's* researches a leading idea obtains, namely, that *the shape, the relative size, and the appearance of the cells, taken by themselves, are not sufficient to characterise a species*, since the same species, when exposed to different external conditions, can occur in very different forms and quite different in appearance. On the other hand, the forms of development of the cells, regarded from another point of view, constitute very important distinctive characters for different species. Thus it is found that *different species under the same treatment behave differently and assume different forms*. This can only be explained by assuming that there are intrinsic, indwelling characters in the special cells which exert an influence of their own.

In the following we give a brief account of the various means by which *Hansen* determined the characteristics of different species. These investigations form at the same time contributions to the general physiology of the budding-fungi.

(*a*) *The Microscopic Appearance of the Sedimentary Yeast.*—The first examination of a yeast will generally consist in observing under the microscope the appearance of the *sediment*. As examples illustrating what can be ascertained in this way, we may call attention to the following figures (Figs. 34, 37, 39, 41, 43, 45), representing the young sedimentary forms of the six species of *Saccharomycetes* which have been specially investigated by *Hansen*. The growths were obtained by cultivating the cells for some time in wort, then introducing fresh wort, and by maintaining a temperature of 25° to 27° C. for 24 hours a vigorous growth was developed. If we now compare, for instance, the figures representing *Saccharomyces cerevisiæ I.*, with those which illustrate the three *Pastorianus* species, we find that, *taken as a whole, they show marked differences*. *Saccharomyces cerevisiæ* consists mainly of large round or oval cells, the *Pastorianus* species form

mostly elongated sausage-shaped cells. It is, however, a very different matter when cells of the first species are mixed with cells of one of the other species; it is not then possible, judging from the form alone, to distinguish the larger and smaller oval and round cells of the *Pasteurianus* species from many of the cells of *Saccb. cerevisiæ*. The two species *Saccb. ellipsoideus I.* and *II.* consist mainly of oval and round cells; sausage-shaped cells, however, also occur; and consequently it is in this case likewise impossible to determine the species by the form of the cells when these are mixed with *Saccb. cerevisiæ* or *Saccb. Pasterianus*.

Neither can any conclusions be arrived at by direct measurements of these sedimentary forms.

A glance at these six groups of figures of pure cultures shows that we have here *three different classes of budding-fungi*, one of which is represented by *Saccb. cerevisiæ*, whilst the second includes the three *Pasterianus species*, and the third the two *ellipsoid species*. This much, but only this much, is possible from a purely microscopical examination, and, it must be pointed out, only under the conditions of cultivation indicated.

(b) *Formation of Ascospores.*—By *Hansen's* investigations on the formation of endogenous spores in the *Saccharomycetes* the first essential link of an *analytical method* for the examination of yeast was found. We will give a brief account of the experimental method adopted and of the general results obtained.

The formation of spores in yeast-cells has been investigated by various naturalists; of the many, and in part contradictory statements, however, the only result which remained as correct was the fact that *Saccharomyces cells could, under certain unknown conditions, form spores in their interior.*

After making a large number of experiments, *Hansen* was able to determine the following *conditions regulating the formation of spores in the Saccharomycetes*:—

1. *The cells must be placed on a moist surface and have a plentiful supply of air.*
2. *Only young, vigorous cells can exercise this function.*
3. *The most favourable temperature for most of the species as yet examined is about 25° C. This temperature favours spore-formation in all known species.*
4. A few *Saccharomycetes* likewise form spores when they are present in fermenting nutrient fluids.

A growth of yeast is developed in the manner described on page 124. A small quantity is transferred to a previously sterilised gypsum block; this block is enclosed in a flat

FIG. 38.

The first stages of development of the spores of Sacch. cerevisiæ I., after Hansen: *a*, *b*, *c*, *d*, *e*, rudiments of spores, where the walls are not yet distinct; *f*, *g*, *h*, *i*, *j*, completely-developed spores with distinct walls.

covered glass and is maintained moist by half filling the glass with water.[1] If it be desired merely to bring about the formation of spores, the apparatus may be allowed to remain at the ordinary room-temperature.

Hansen was the first to give an accurate description of *the structure of spores* and a detailed account of their *evolution, founded upon observations of individual spores*; and he distinguished *three typically different groups of Saccharo-*

[1] *Ascospores* can also be obtained when yeast is spread upon sterilised solidified gelatine, prepared with or without a nutrient solution, kept in a damp place; likewise in yeast-water and in sterilised water; finally, spore-forming cells also occur in the films of the *Saccharomycetes*. The method is evidently not dependent upon these different substrata but upon the knowledge of the factors which render it possible for the cells to exercise this function of forming spores.

sugestes which are distinguished by the mode of germination or by the form of the spores.

After a certain lapse of time, which varies with the different species, roundish plasma-particles appear in the cells, and these are the *first indications of spores* (Fig. 26). In their further development, they become surrounded by a *wall*, which is seen more or less distinctly in the different species.

In the *first type*, to which *Saccharomyces cerevisiæ I.* belongs, the spores can expand during the first stages of germination to such an extent that the pressure which they exert on each other whilst they are still enclosed in the mother cell, brings about the formation of the so-called partition walls (Fig. 27). This causes more or less plasma

Fig. 27.

Spores of Sacch. cerevisiæ I. in the first stages of germination, after Hansen: at *a*, *d*, *e*, and *g*, formation of partition-walls; at *e*, *f*, and *g*, the walls of the mother cells have become ruptured; at *g* a compound spore divided into several chambers, the coherent wall is ruptured in three places.

to become squeezed or wedged between the spores, or the walls of the spores may be brought into contact. During the further development, a complete union of the walls may take place, so that a true *partition wall* results; the cell then becomes a compound spore divided into several chambers.

During *germination* (Fig. 28) the spores swell and the wall of the mother-cell, which was originally moderately thick and elastic, becomes stretched and consequently thinner. Finally it becomes ruptured, and then remains as a loose or shrivelled skin, partially covering the spores; or it becomes gradually dissolved during germination.

Budding can occur at any point on the surface of the swollen spores; this budding usually takes place after the wall of the mother-cell has become ruptured or dissolved, but it also occasionally takes place within the mother-cell. After the buds have formed, the spores can still remain connected, or they can soon become detached from each other.

An especially curious and exceptional case is when the wall separating two spores becomes dissolved, so that a *fusion*

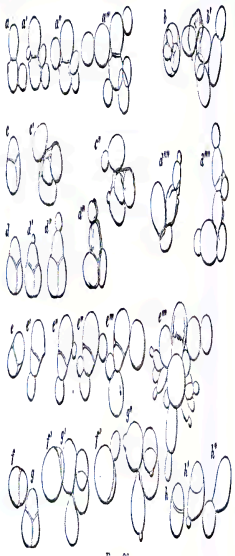

Fig. 28.

Budding of the spores in Saccharomyces cerevisiæ I., after Hansen: *a*, three spores without the wall of mother-cell; *b*, cell with four spores; at *b'* the wall of mother-cell is ruptured; *c*, cell with four spores, three of which are visible; at *c'* and *c''* the ruptured wall of mother-cell is seen; *d*, cell with three spores, at *d''* the ruptured wall of mother-cell; *e*—*e'''* development of a very strong colony; *f*—*k*, other forms of development; at *h''* the wall between the two spores has disappeared.

of the spores results (see Fig. 28, *e*—*e'''* *and* *h*—*h''*). *Hansen* assumes that the biological significance of this phenomenon is that the spores, placed under unfavourable conditions, have a greater chance of forming buds. One spore plays the part of a parasite to the other. The growing

together of the spores mentioned above is perhaps the beginning of this process.

The germination of the spores of such species of the groups *Saccharomyces Pastorianus* and *Saccharomyces ellipsoideus* as have been examined takes place in essentially the same way as above described.

A *second* and quite different type occurs in the case of

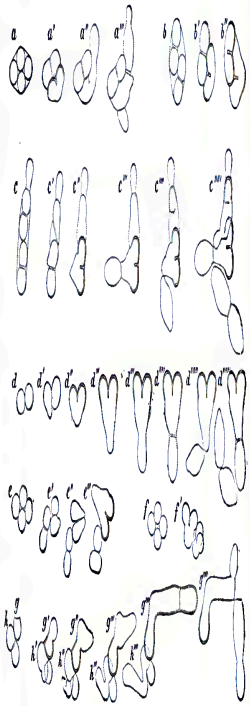

Fig. 30.

Germination of the spores of Saccharomyces Ludwigii, after Hansen: a—e represent a gypsum-block culture 12 days old; f—k, a similar culture, one-and-a-half months old.

Saccharomyces Ludwigii (Fig. 29), where *the fusion takes place in the very first stages of germination*; in this case, however, it is the *new formations* and not the spores which grow together. These new formations are further distinguished

from the previous type in that they are not yeast cells, but mycelium-like growths,—*promycelium*. The development of yeast cells takes place from this *promycelium*, a *sharp*

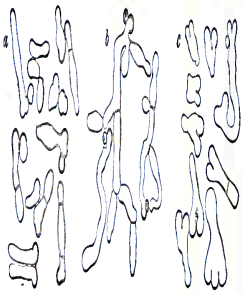

FIG. 30.

Saccharomyces Ludwigii, after Hansen. Germinating spores from old gypsum block cultures. At *a* and *b* each spore has developed a germ-filament; at *c* are shown different forms produced by fusion.

partition wall being first formed; the cell then becomes detached, and finally its ends become rounded. At the ends of these cells buds are developed, and these also become detached at the partition walls.

FIG. 31.

Germination of spores of Saccharomyces anomalus, after Hansen.

In the case of older spores this curious fusion is more uncommon (Fig. 30). Some germ-filaments develop into a branched mycelium (group *b*).

The *third type*, which occurs in *Saccharomyces anomalus*

(Fig. 31, see also description of the species), is distinguished from the last-mentioned in that the spores are of a *quite different shape*, and resemble the spores of *Endomyces decipiens*.[1] They somewhat resemble a half sphere with a rim round the base.

During germination the spore swells and the projecting rim can either remain or disappear. Buds then make their appearance at different points on the surface of the spore.

One of the objects of *Hansen's* investigations was also to determine in what way the formation of spores was influenced by different *temperatures*, with the view to ascertain whether the different species behave alike, or whether it might not be possible in this way to discover different characteristics. It was, therefore, necessary to determine: 1, the limits of temperature, i.e., the highest and lowest temperatures at which spores could be formed; 2, the most favourable temperature, i.e., the temperature at which spores appeared in the shortest time; and, finally, 3, the relation of the intermediate temperatures.

In determining the desired intervals of time, the moment was registered at which *the cells showed distinct indications of the formation of spores* (compare Figs. 26 and 32). It is not possible to make use of ripe spores in these determinations, since no criterion exists for complete ripeness.

The results obtained by *Hansen* are as follows:—

The formation of spores takes place slowly at low temperatures, more rapidly as the temperature is raised to a certain point; when this point is passed their development is again retarded, until finally a temperature is reached at which it ceases altogether.

The lowest limit of temperature for the six species first investigated was found to be 0·5 to 3° C., and the highest limit 37·5° C. *Hansen* also determined the intermediate temperature and time relations for these six species, and

[1] A fungus which is parasitic on the lamellæ of certain mushrooms.

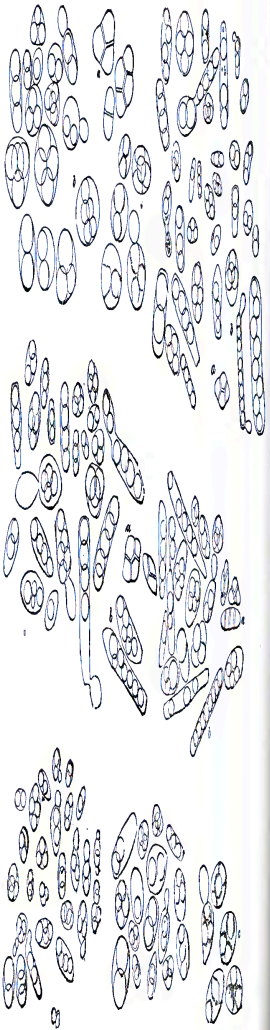

FIG. 32.

Saccharomycetes with asco-spores, after Hansen: 1, Saccb. cerevisiæ I.; 2, Saccb. Pastorianus I.; 3, Saccb. Past. II.; 4, Saccb. Past. III.; 5, Saccb. ellipsoideus I.; 6, Saccb. ellips. II.; a, cells with partition-wall formation; b, cells containing a larger number of spores than usual; c, cells showing distinct rudiments of spores.

found that when these two values are graphically represented with the degrees of temperature as abscisse and the time intervals as ordinates, the curves obtained for all six species had essentially the same form. They sink from the ordinates of the lowest temperatures towards the axes of the abscisse, and then rise from these; at the same time, however, it is seen from these curves that the *cardinal points determined more especially from the highest and lowest temperatures, give characteristic distinctions for the different species*; that is to say, *that the limits of temperature within which the formation of spores can take place are different for the various species* (compare classification of the genus Saccharomyces).

With regard to the time required for the appearance of the first indications of spore-formation in *the six species* investigated under the same conditions of temperature, the following was observed: At the highest temperature the time required for the development was in all the species about 30 hours; at 25° there was also no great difference in the time required; *at the lower temperatures, however, very evident differences occurred*. Thus, in the case of *Sacch. cerevisiæ I.*, the first indications of spore-formation at 11·5° C. are only found after ten days; in the case of *Sacch. Pastorianus II.* after 77 hours, and so on.

In all determinations of this kind *a very considerable influence is exerted by the condition of the cells*, according to whether they have been grown at a high or low temperature, whether they were old or young, feeble or vigorous, etc., etc. It follows from this that the composition of the nutrient fluid also exercises an influence. In methodical, comparative experiments of this nature, a necessary condition is, therefore, that the previous cultivation of the cells should always be carried out in the same manner. If these external conditions be varied, the limits for the reactions of the species corresponding to such varied conditions must likewise be determined.

By means of these experiments *Hansen* has adduced an important character for the determination of the *Saccharomycetes*.

A new distinctive characteristic for the species has been discovered by the same author in the *different anatomical structure of the spores*. Both these characters and others which are described in the following pages (*e.g.*, film formation, etc.,) must necessarily be considered in a complete examination of a *Saccharomyces* species.

The method given below for the *analysis of low brewery-yeast from a practical standpoint* was based by *Hansen* on certain observations of the temperature curves for the development of spores and on the structure of the spores. Thus it was found that at certain temperatures the species employed in the brewery, the so-called *cultivated yeasts*, *develop their spores later than the so-called wild yeasts*, several species of which also occur as *disease-germs* in the brewery. *Hansen* also found that *the structure of the spores in these two groups is generally different*, in that the young spore of a cultivated yeast has a distinct wall or membrane, and the contents are not homogeneous, are granular, and exhibit vacuoles; in the case of wild yeast, on the other hand, the wall of the young spore is most frequently indistinct and the contents are homogeneous and strongly refract light. It should also be added that the spores of cultivated yeasts are usually larger than those of wild yeasts.

1. For the *continual, daily control of brewery-yeast* as regards contamination with wild species, the following very convenient method is made use of:—At the conclusion of the primary fermentation, a small quantity of the fermenting liquid is removed from the fermenting-vessel in a sterilised flask; this is set aside for some hours until the yeast has settled to the bottom, and the sediment is then spread upon a gypsum block in the manner described on page 126. This is then introduced into a thermostat maintained at a temperature of 25° C. or 15° C.

It was found namely—and it has been subsequently confirmed by the elaborate investigations of *Holm* and *Poulsen*—that the species of cultivated yeasts employed in low-fermentation breweries can be divided into two groups. *One group* yields spores later than wild yeast when a temperature of 25° C. is maintained; *the other group*, on the contrary, gives spores in about the same time as wild yeast at the above temperature, but at a temperature of 15° C. the cells of wild yeast show spore-formation considerably sooner than the cells of these cultivated yeasts.

The cultures maintained at 25° C. are examined after an interval of 40 hours, and those maintained at 15° C. after an interval of three days.

Experiments of my own show that *high brewery-yeasts* can be analysed in a similar manner.

By means of experiments which were undertaken with the view to determine to what extent *Hansen's* analytical method can be relied on for technical purposes, *Holm* and *Poulsen* came to the conclusion that *a very small admixture of wild yeast, about* 1-200*th of the entire mass* (Carlsberg bottom-yeast No. I.), *can be detected with certainty in this manner*. *Hansen's* previous researches had shown that when, for instance, the two species, *Sacch. Pastorianus III.* and *Sacch. ellipsoideus II.*,—which are capable of producing yeast-turbidity in beer—are present to the extent of only 1 part in 41 of the pitching-yeast, the disease is *not* developed, provided that the normal conditions of fermentation and storage have been maintained; further, that *Sacch. Pastorianus I.*, which imparts to beer a disagreeable odour and an unpleasant bitter taste, can, under the same conditions, scarcely exert its injurious influence when the admixture of this yeast amounts to less than 1 part in 22 of the pitching-yeast. Consequently *Hansen's* method for the analysis of yeast by means of ascospore formation gives ample information as to the presence of these disease ferments.

This method likewise possesses the advantage that *the*

analysis can be performed with mixtures such as ordinary pitching-yeast, and that it can be performed in a short time.

When the object of the analysis is to more accurately characterise the different species present in the sample, a number of cells are isolated by fractionation, and each of the growths obtained is separately examined.

In an investigation on bottom-yeast during the different stages of the primary fermentation, published by *Hansen* in 1883, it was shown that as a rule the wild yeasts are present in largest amount during the last stages of primary fermentation in the upper layers of the liquid. *The samples of the liquid which are taken from the fermenting vessel for the analysis of the yeast, must therefore, as stated above, be taken during the last days of the primary fermentation.* If much time elapses before an analysis is commenced, the yeast must be introduced into wort, and one or more fermentations carried out; and this applies whether the yeast to be examined was in a dry or a liquid state.[1]

It is evident, however, that, valuable as the analysis of yeast is, it must always remain of secondary importance in the brewery; the most important link in the system will,

[1] The observation mentioned above with reference to low-fermentation yeast has been confirmed by *J. Vuylsteke's* experiments, in which fermentations were carried out with mixtures of different *Saccharomycetes* in cylindrical glass vessels of about two litres capacity; by counting the cells and by means of cultures the relative proportions of the different species were determined. It is found, however, from the experiments hitherto conducted by *Vuylsteke* that the rule mentioned is not of general application in the case of mixtures of high-fermentation yeasts with wild yeasts. In some experiments with mixtures of *Saccharomyces cerevisiæ I, Hansen*, and *Saccharomyces Pastorianus I, Hansen*, the wild yeast was found to have increased towards the end of the primary fermentation, whilst in other experiments a diminution of the wild yeast was observed. On the other hand, all the experiments with mixtures of *Saccharomyces cerevisiæ I*, and *Saccharomyces Past. III*, showed that the impurity was greater in the upper layers of the liquid at the end of the primary fermentation than at the commencement, just as in the case of bottom-fermentation.

under all conditions, be the employment of a pure cultivation of a selected species of yeast.

2. The analysis of the yeast in the *propagating apparatus*, which *must be absolutely pure*, is carried out as follows:—At the conclusion of fermentation, samples are withdrawn, with every precaution, into Pasteur flasks or into the Hansen flasks employed for sending yeast samples; from these, small quantities are introduced into flasks containing yeast extract, and these are maintained at a temperature of 25° C., the object being to test the yeast for bacteria. The remainder is set aside for the yeast to settle, the beer is decanted, and a sample portion of the sediment is introduced into a sugar solution containing some tartaric acid. After three or four cultivations in such a solution it is further cultivated a few times in beer-wort, and then tested for spore-formation. The smallest traces of wild yeast in the apparatus are brought into a state of vigorous development by this treatment (see Chapter I., 7. Physiological methods).

(c) *The Formation of Films.*—By the observation of the formation of films, *Hansen* has found characteristics for the *Saccharomycetes* in a manner quite different from that given above. A new path for the study of these fungi was thus again opened up, since the statements hitherto made by different authors in this direction are not in accordance with their true behaviour.

It is a very generally-known phenomenon, that *fermented liquids become coated with films*. It is also well-known that the films formed by the budding fungi—*Mycoderma aceti*, *Mycoderma vini*—have especially attracted attention; and the frequent mention of such films in the literature of the subject led to a result well-known also in other branches of science; they were spoken of as if well understood for so long that at last the belief in the actual existence of this knowledge became firmly rooted. After *Hansen* had submitted this question to an experimental investigation, he showed, however, that this view was erroneous.

Hansen has treated a large number of films, and amongst them several forms which are most closely related to different species of *Saccharomyces*. *Mycoderma*, which do not produce endogenous spores. According to *de Seynes*, *Reess*, and *Cienkowski*, these Mycoderma-species do yield ascospores; it is, however, highly probable that these investigators were dealing with impure films, containing an admixture of true *Saccharomycetes*. It is, indeed, a matter of no little difficulty to determine the purity of such a culture if one does not start from a single cell; for if *Mycoderma cerevisiæ* is cultivated as sedimentary yeast, the cells assume an entirely different appearance; they become filled to a greater extent with plasma, whilst the cells of the film are, as is known, poor in plasma and contain strongly-developed vacuoles. Such forms, which are generally regarded as *Mycoderma cerevisiæ*, readily and quickly form films; some simultaneously exhibit distinct signs of fermentation, whilst others do not. On beer and wort these films are grey and dry in appearance; afterwards they become wrinkled and lighter in colour; air is found freely intermixed between the cells. Some of the varieties of *Torula* investigated by *Hansen* yield similar films; the film of *Chalara Mycoderma*, on the other hand, is gluinous, tough, and slightly lustrous; in the case of *Monilia*—which, as previously mentioned, can occur with budding cells, and directly ferments cane-sugar—the film formation is peculiar: even during vigorous fermentation a film forms on the bubbles of foam, spreads gradually over the whole surface, and sometimes becomes wrinkled. Thus, the cells in the flask first sink to the bottom as sedimentary yeast, set up a vigorous fermentation, and again rise with the bubbles of carbonic acid to the surface, where they enter upon a new phase of development. If sterilised lager-beer is infected with this fungus, no fermentation sets in, and only a thin film resembling dust is developed; under other conditions the fungus forms a white, flowry, wool-like layer, as in the case of *Oidium*.

The true Saccharomycetes also form films, which, however, *differ somewhat from those mentioned above; and this is also the case with some of Pasteur's Torula and with Saccharomyces apiculatus.* From these observations it is evident that the formation of films is not a peculiarity of certain species, but must be regarded as *a general phenomenon common to micro-organisms.*

In the case of the *Saccharomycetes* this phenomenon generally occurs in the following manner: If cultures in wort are left undisturbed for a shorter or longer time at the ordinary room temperature, small specks of yeast gradually appear on the surface of the liquid after the termination of the primary fermentation; these can afterwards coalesce to figures of different forms and sizes, to isolated patches, the upper surfaces of which are flat and the under surfaces arched. Finally, they become united to a coherent and generally light greyish-yellow, glutinous film, which may extend to the walls of the glass vessel, forming a complete ring. Such a perfect film-formation only occurs after the primary fermentation is at an end. If the flask be shaken, pieces of the film become detached and *sink to the bottom*; and in this way a complete layer can gradually collect at the bottom, whilst the film becomes continually renewed and assumes a marbled appearance owing to the younger portions being thin and dark, whilst the older parts are thick and light.

The conditions under which a film can be formed are a *free, still surface*, with direct access of air; and a vigorous film-formation presupposes an abundant supply of air. It follows from this that a far more rapid and vigorous development will take place in a *Chamberland* flask, or in an ordinary boiling flask with filter-paper tied over the mouth, than in a *Pasteur* flask where the admission of air is more limited. The function of film-formation is thus in this respect subject to the same conditions as obtain in the case of endogenous spore-formation.

Simultaneously with the formation of a film, a decolorization of the wort takes place, the latter becoming of a pale yellow colour. This reaction takes place most quickly at the higher temperatures, and occurs most markedly with those species which give rise to the most vigorous film-formation.

The preliminary cultivation of the cells is the same as that previously described (page 124). The liquid is removed from the growth obtained, and fresh sterilised wort is added; the mixture of yeast and wort is agitated, and a drop is transferred—with the usual precautions—to an ordinary flask of about 150 c.cm. capacity, half filled with wort, and a piece of filter-paper is then tied over its mouth. *Hansen* exposed flasks treated in this way to different temperatures, and determined:—

1. The limits of temperature for the formation of films;

2. The approximate length of time required for their formation at different temperatures; and

3. The microscopic appearance of the growths at different temperatures.

The main point in these investigations of the six species previously mentioned is *the microscopic appearance of the films of these species, formed at the same temperature*; and here again, when regarded from a different point of view to that considered in the last section, we have a complete investigation of the relation between the external interfering factors and the forms, which proves that we have to do with so many perfectly distinct types or species.

The examination of the films was made, except when otherwise stated, when they were so far developed that they could just be seen with the naked eye.

A glance at the illustrations representing these film-growths (see page 161 and following pages) will show that their general character is usually different from that of the sedimentary forms. For instance, the sedimentary form of *Saccharomyces ellipsoideus I.* is egg-shaped or spherical, whilst in the

film, elongated cells quickly appear, and the growth gradually assumes an appearance perfectly different from that of the sedimentary yeast.

If we compare the film-formations of the six species, we find that the films developed at the higher temperatures offer very few points of difference which are of use in their examination, *Sacch. cerevisiæ I.* and *Sacch. ellipsoideus II.* being alone distinguishable from the remainder. It is quite otherwise, however, when *young films developed at a temperature of* 13° to 15° C. are examined. The two species, *Sacch. Pastorianus II.* and *Sacch. Pastorianus III.*—which are both top-fermentation yeasts, and which in the ordinary cultures cannot be distinguished from each other with certainty by the form of their cells—exhibit in this case entirely different forms of growth; and an equally striking difference is likewise found between the otherwise similar species *Sacch. ellipsoideus I.* and *II.*

An examination of the *limits of temperature* for the formation of films shows that for *Sacch. cerevisiæ I.* and *Sacch. ellipsoideus I.*, these lie within about 38° and 3° to 6° C.; the limits for the three *Pastorianus* species are 34° and 3° C.; *Sacch. ellipsoideus II.* has the same lower limit as the last mentioned species, whilst its maximum temperature, however, is 38° to 40° C.

The time limits, when compared with those previously given for ascospore-formation, show that in both cases the development takes place more slowly at low than at the higher temperatures; at temperatures near to the minimum and maximum limits only a very slight and imperfect film-formation is ever obtained.

At temperatures above 13° C. *the film of Sacch. ellipsoideus II. develops so rapidly and vigorously* that the flasks with this yeast can be recognised by this alone. Thus, at 22° to 23° C. the film completely covered the surface at the end of six to twelve days, whilst in the case of the other five species a period three times as long

was required for the formation of films which were generally more feebly developed. This species and *Saccb. Pasterianus III.* also develop a vigorous film comparatively rapidly at the ordinary room-temperature, whilst in the same time the other species are left far behind.

As mentioned above, the film-formations have different maximum temperatures. This is related to the fact *that the maximum temperature for budding is not the same for the different species.* It was proved that *budding and fermentation can take place at temperatures at which film-formation no longer occurs.* Thus, in the case of *Saccb. cerevisiæ I., Saccb. ellipsoideus I.* and *Saccb. ellipsoideus II. Hansen* still observed a vigorous fermentation and budding at 38° to 40° C., and at 34° C. also in the case of the three species of the group *Saccb. Pasterianus.* A relationship is thus shown to exist between the influence of temperature on budding and fermentation on the one hand, and film-formation on the other.

(*d*) *The Temperature Limits for the Saccharomycetes.*—Just as the influence of temperature on the development of spores and films varies with the different species, so it has also been shown by *Hansen's* investigations (1883) that both spores and vegetative cells of different species likewise possess unequal powers of resistance to hot water. In this respect the spores have a greater resisting power than the vegetative cells.

In experiments of this nature, as in the cases previously mentioned, the condition of the cells has a very marked effect on the results, which are especially influenced according as old or young cells have been employed. Thus, it was found that the cells of *Saccb. ellipsoideus II.*, which had been cultivated in wort for two days at a temperature of 27° C., were killed in five minutes when heated to 56° C. in sterilised distilled water, whilst cells of a similar culture but 2½ months old were able, under similar conditions, to withstand five minutes' heating to 60° C. without being killed.

Ripe spores of the same species, which had been developed

at a temperature of 17° to 18° C., and in the course of eight days at the same temperature had become partially dried, withstood a temperature of 62° C. for five minutes, but were killed at 66° C.

In the case of *Sacch. cerevisiæ I.* the vegetative cells are, under similar conditions, killed by five minutes' heating at 54° C., whilst at 62° C. the spores are killed.

An interesting grouping of *Hansen's* six species with reference to a fixed temperature is also found when they are cultivated in wort under conditions favourable to film-formation (see page 139). When, for instance, a temperature of 36° to 38° C. is employed for the development, the three *Pastorianus* species will be dead at the end of eleven days, whilst *Sacch. cerevisiæ I.* and the two ellipsoid species will still be living. From this result it is also evident that the rule formerly given that top-fermentation yeasts can develop at higher temperatures than bottom yeasts is incorrect.

Later experiments made by *Kayser* in some of the directions mentioned above confirm these results, and they also show that the yeasts can resist a considerably higher temperature when in a dry state than in the presence of moisture. For instance, a pale ale yeast was killed when exposed for five minutes in a moist condition to a temperature of 60° to 65° C., whilst when dry it withstood a temperature of 95° to 105° C.; in the case of a wine yeast (St. Emilion) the temperatures were 55° to 60° C. and 103° to 110° C. The resisting power of the spores was 10° to 20° higher.

Vegetative cells which had developed from the heated spores exhibited a somewhat greater power of resistance than normal vegetative cells. This increased resistive power was, however, not transmitted further, and, on cultivation in beer-wort, disappeared even in the second generation.

(c) Cultivation on a Solid Nutritive Medium.—*Hansen* discovered distinct characteristics for several species of the *Saccharomycetes* by suitable cultivation on a solid nutritive medium. For this purpose he employed small flasks contain-

ing wort, to which about 5·5 per cent. of gelatine had been added, the flasks being closed by means of cotton-wool plugs. When these flasks are inoculated with the six known species (*Sacch. cerevisiæ I*, *Sacch. Pastorianus I*, *II*, *III*, *Sacch. ellipsoideus I*, *II*), and set aside at a temperature of 25° C., the growths which develop show in the course of eleven to fourteen days such macroscopic differences that four groups may be distinguished more or less sharply. *Sacch. ellipsoideus I.* stands alone, in that its growth exhibits on the surface a characteristic net-like structure, which enables this species to be distinguished by the unaided eye from the other five species. When gelatine with yeast-water is employed for such cultures and the experiment conducted at 15° C., *Sacch. Pastorianus II*, after sixteen days gives growths, the edges of which are comparatively smooth, whilst the growths obtained from *Sacch. Pastorianus III.* are distinctly hairy at the edges. A microscopical examination shows that in this case the two species are also distinguishable morphologically. This is not, however, by any means always the case with cultures in solid media; in fact, the differences are often less marked under such conditions than when nutritive liquids are employed.

For the *Mycoderma species* and *Sacch. membranæfaciens* *Hansen* has discovered an important characteristic in their behaviour in wort-gelatine, in which they form shield-like colonies readily distinguishable from those of the *Saccharomycetes*.

In connection with this we may also mention *Hansen's* observation that some species, *e.g.*, *Sacch. Marxianus* and *Sacch. Ludwigii*, can develop a *mycelium* when grown in a solid medium, whilst others are not able to do so.

In the case of some cultivated yeasts *P. Lindner* found distinct differences in their growths on gelatine.

Will and others have also shown that the characteristics exhibited by cultures on nutritive gelatine are often very variable.

(*f*) *The Behaviour of the Saccharomycetes and Similar Fungi towards the Carbohydrates and other Constituents of the Nutritive Liquid. Diseases in Beer.*—The first striking proof of the fact that *Saccharomyces* species can perform very different work in the nutritive liquid, was obtained after *Hansen's* discoveries, by means of *pure cultures of the yeasts* prepared in the Carlsberg laboratory, and afterwards in many other laboratories, and *which were subsequently tested in practice*. There are in fact breweries in which a large number of different species of yeast have been tried on a large scale and under the same conditions, and where the attenuation, taste, odour, time of clarifying, and permanence as regards yeast turbidity, &c., &c., have been found to differ for each individual species.

Hansen's epoch-making researches on the *disease-yeasts* (1883) again showed, from another point of view, the marked differences amongst the *Saccharomyces* species in their action on the nutritive liquid; he discovered, namely, groups of the so-called wild yeasts, which *bring about detrimental changes in beer*, whilst others were found to be *harmless*. Amongst the former, again, there are some which communicate a *bitter taste* and *disagreeable odour* to the beer (*Sacch. Pastorianus I.*) without as a rule producing turbidity; whilst others only fully develop their activity in a late stage of the secondary fermentation, and cause the *beer to become turbid* (*Sacch. Pastorianus III.* and *Sacch. ellipsoideus II.*), in that an abundant yeast deposit forms in a comparatively short time in the finished beer after it has been drawn off. It is only when these species—*Sacch. Pastorianus I.*, *Sacch. Pastorianus III.*, and *Sacch. ellipsoideus II.*—are introduced into the wort *at the commencement of fermentation* that they are able to induce disease. The addition of disease-yeast to the beer in the store casks or to the drawn-off beer has no appreciable effect; the inoculation of bottled beer with *Sacch. ellipsoideus II.* will only take effect when the beer has been very strongly infected. The main result is that the danger

of infection *lies in the pitching-yeast*. These diseases have led to very great difficulties and have caused considerable losses in breweries. *Hansen's* observations on the disease yeasts have been confirmed by *Grönlund*, *Will*, *Lindner*, *Kolesinsky*, *Krieger*, *Windisch*, and *P. Lindner*, and extended by new examples. The wild yeasts can also bring about disturbing effects in top-fermentation breweries. For instance, the so-called "summer-cloud" of Australian beers is caused, according to *de Bary*, by a wild *Saccharomyces* species. This organism causes turbidity and imparts to the beer an acid, bitter taste.

Recently *Pichi* has also detected disease-yeasts in wine.

Just as the mould-fungi exhibit a different behaviour *towards the carbohydrates* (see Penicillium, Mucor, Monilia), so the different *Saccharomycetes* and similar fungi have been shown *by Hansen's* comprehensive investigations to also exhibit *pronounced characteristics* in the same direction. In addition to the true *Saccharomycetes* we will here also review *Mycoderma cerevisiæ*, *Saccharomyces apiculatus*, the *Torula* forms, and *Monilia*.

Hansen examined the behaviour of a large number of *Saccharomycetes towards the four carbohydrates—saccharose (cane-sugar), maltose, lactose, and dextrose.*

His known six species of Saccharomycetes (*Saccharomyces cerevisiæ I.*, *Saccharomyces Pastorianus I.*, *II.*, and *III.*, *Saccharomyces ellipsoideus I.* and *II.*, see page 159) behave as follows:—They all develop invertase; they convert cane-sugar into invert-sugar, which they then ferment; they ferment maltose and dextrose, but not lactose. All the bottom-yeasts used in practice show the same behaviour towards the four sugars mentioned.

Saccharomyces Marxianus (page 176), *Saccharomyces Ludwigii* (page 179), and *Saccharomyces exiguus* (page 176) do not ferment maltose and lactose; they invert saccharose and ferment nutritive solutions of invert-sugar and dextrose.

Saccharomyces membranæfaciens (page 177) and *Mycoderma*

cerevisiæ (page 200) can neither invert nor ferment the above four carbohydrates.[1]

Sacch. apiculatus (page 195) does not invert saccharose, and of the four sugars mentioned it only ferments dextrose. It therefore only induces a feeble alcoholic fermentation in beer wort.

Amongst the *Torula forms* (page 189) examined by *Hansen* there are many which do not secrete invertase, do not ferment maltose, and which only yield about 1 per cent. of alcohol (*by* volume) in beer wort. Other species invert saccharose. In nutritive dextrose solutions the different species induce a more or less vigorous fermentation.

Monilia candida (page 100) possesses no invertive action, but ferments saccharose (without hydrolysing it), maltose, and dextrose. It ferments beer-wort, but at the ordinary room-temperature it only very slowly yields the higher percentages of alcohol, as compared with the *Saccharomycetes*.

If we now review all these different properties of the *Saccharomycetes* we shall see that they fall into two groups:—

I. Those which develop invertase, and induce alcoholic fermentation. This group is further sub-divided into
   (*a*) those which not only ferment saccharose and dextrose, but also vigorously ferment maltose (*Hansen's* first described six species, and the yeasts employed in the brewing industry).
   (*b*) those which ferment saccharose and dextrose, but not maltose (*Sacch. Marxianus, Ludwigii* and *exiguus*).

II. Those which do not develop invertase, and do not induce alcoholic fermentation (*Sacch. membranæfaciens*).

The *budding fungi which do not form endospores* (*non-Saccharomycetes*) show, with reference to the properties of inversion and fermentation, the most varied characters.

[1] According to *Loeb's* experiments, some species of *Mycoderma* present in beer are capable of inducing alcoholic fermentation.

I. The greater majority do *not ferment maltose*. Many of these induce a more or less vigorous fermentation in solutions of dextrose and invert-sugar. Some (*Torula* forms) invert saccharose, and many possess no invertive ferment (*Mycoderma cerevisiæ*, *Torula* forms, *Sacch. apiculatus*).

II. Only one species (*Monilia candida*) ferments maltose, saccharose (direct), and dextrose, without however possessing any invertive action.

From the above it is clear that, as pointed out by *Hansen*, the *Saccharomycetes* cannot be characterised merely as alcoholic ferments.

When we consider the behaviour of the above-named fungi in the fermentation industries, it is at once seen that it is only amongst the *Saccharomycetes* that species occur which rapidly and vigorously ferment maltose. The yeasts for breweries and distilleries must therefore be looked for from among the true *Saccharomycetes*. The fungi not included in the genus *Saccharomyces*, of which by far the greater majority do not ferment maltose, are scarcely capable of playing any important part in these industries; on the other hand they can be employed in the manufacture of wines from grapes, berries, and fruits, since several of them are able to induce just as vigorous a fermentation in solutions of dextrose and invert-sugar, as the *Saccharomycetes*.

It is perfectly clear from the above that *a suitable species must always be selected*.

These different properties of the various species of budding-fungi are of special importance in analytical chemistry in cases where solutions containing several different carbohydrates are under examination. In fact *Hansen* expressed the opinion that it will be possible in this way to arrive at a more exact quantitative determination of the different carbohydrates in wort. Several chemists have been recently engaged on this problem, but a true solution has not yet been found.

The discovery of isomaltose by *C. J. Lintner, jun.*, has opened up a new path in the study of the composition of wort, whilst his investigations are of great importance, in that they also give us a more intimate knowledge of the process of fermentation.

Very recently the budding-fungi have been also eagerly looked for in *milk*. *Grotenfelt* discovered a *Saccharomyces* (page 189), whilst various budding-fungi (pages 192–194) not belonging to this genus were found by *Duclaux*, *Adametz*, *Kayser*, and *Beyerinck*; they all hydrolyse milk-sugar. These species have not yet been found in breweries.

Ferwi found that certain white and red species of yeast are capable of exercising a *diastatic* action. *Morris*, in experiments with pressed yeast, arrived at similar results.

The different action of the *Saccharomyces* species on the same nutritive liquid (*e.g.*, wort, must) and under the same conditions, has been further studied by *Borgmann*, *Amthor* and *Marx*.

According to *Borgmann's* experiments, the chemical changes brought about in wort by the two Carlsberg bottom-yeasts, No. 1 and No. 2, show a pronounced difference. These two species—which had been in use for some time in the fermenting-room, and were still practically pure—were employed for pitching two fermenting vessels containing wort from the same brew; the fermentation took place under conditions which enabled a true comparison to be made, and the resulting beer was stored as usual. The differences in the chemical products were especially pronounced in the proportion of free acid (No. 1 contained in 100 c.c., 0·086, and No. 2, 0·144 acid, calculated as lactic acid), and glycerine (No. 1 contained 0·109 and No. 2, 0·137).

As a result of these experiments, *Borgmann* points out that the ratio between the alcohol and glycerine in these two beers differs from that previously found in beers, the ratio obtained from previous analyses being:—

	Alcohol.	Glycerine.
Maximum	100	5·497
Minimum	100	4·140

whilst the Carlsberg beers gave the following numbers:—

No. 1.		No. 2.	
Alcohol.	Glycerine.	Alcohol.	Glycerine.
100	2·63	100	3·24

It is thus seen, that, as *Borgmann* also points out, good beer may be produced in which the ratio of glycerine to alcohol is lower than the previously-admitted minimum.

A series of *eight different species of Saccharomyces*, and amongst them six "cultivated" yeasts, all in absolutely pure cultures, were investigated by *Amthor* with reference to their chemical action on beer-wort. His results again confirmed *Hansen's* principle, that in practice a selection must always be made. The fermentations were conducted in Pasteur flasks of one liter capacity under the same conditions and in two series, the first of which corresponded to the primary fermentation in the brewery, and the second at the same time also to the secondary fermentation. The alcohol, extract, specific gravity, attenuation, glycerine, nitrogen, reducing substance, and the degree of colour, were determined in the fermented worts. The tables show, as pointed out by the author, palpable differences in the work accomplished by the different species. The percentage of alcohol (by volume) varied within the limits 4·34 and 6·02 (3·55 to 5·94 at the end of the primary fermentation), the extract between 8·27 and 11·23 (8·49 to 12·61 at end of primary fermentation), the attenuation between 36·7 and 53·3 (28·8 to 52·1 at end of primary fermentation); the percentage of glycerine showed very striking differences and fluctuated between 0·08 and 0·15; and likewise the amounts of nitrogen and reducing substance, and to some extent also the degree of colour, showed considerable differences.

A very considerable number of *Saccharomycetes* occurring

in *must*—absolutely pure cultures of which were prepared by *Hansen's* method—were investigated by *Mayr*, both from a botanical point of view and with reference to their chemical action on the nutritive liquid. The time required for spore-formation was very different for the different species, and likewise the number of spore-forming cells and the number of spores in individual cells exhibited striking and constant differences. In connection with this, it is especially of interest that the pure cultivated species show distinct differences in fermentative power and in the production of volatile substances, which impart a special bouquet to wine, and finally in their power of resistance to different acids and to elevated temperatures. As marked differences in taste are produced by not a few species, *Mayr* is justified in emphasising the practical importance of such investigations, since it thus becomes possible, by the addition of yeasts of known properties to wine-must, to produce wines having definite characters as regards taste, &c., independent of the locality.

More recently *Author* has also investigated a number of absolutely pure cultures of wine yeasts, and has detected typical differences both with regard to spore-formation and to the time of duration of the fermentation, finally also in the chemical composition of the wines produced. Similar results have also been obtained by *Jacquemin*, *Roumieer*, *Martinaud*, and *Rietsch*, in France; *Müller-Thurgau*, in Switzerland; *Nathan* and *Wortmann*, in Germany; *Mach* and *Portele*, in Austria; *Forti* and *Pichi*, in Italy; the comparative experiments conducted by these authors having been partly carried out on a large practical scale.

(*g*) *Variations in the species of the Saccharomycetes.*—*Hansen's* numerous investigations have proved that the *Saccharomycetes* are affected in various ways by external influences. From the results recorded in the previous sections, we are perfectly justified in saying that there are a number of species, not only of the so-called wild yeasts (species which

were formerly described under the general names *Sacch. Pastorianus*, *Sacch. ellipsoideus*, &c.), but also of well-characterised top- and bottom-yeasts, which are employed in practice. It is a point of great practical interest that species cultivated in beer-wort, the cultivation of which has been uninterruptedly continued for several years, have shown no, or at most but slight, changes. At the same time that *Hansen* arrived at these results, he also discovered that it was possible, by suitable treatment, to produce variations in different directions; also the individual peculiarities of the cells in an absolutely pure culture can here assert themselves. Some of these changes are only temporary, and disappear under suitable treatment when the species re-assumes its original character. Others become more deeply rooted, and it is then only under especially favourable conditions that the culture can be deprived of its newly-acquired properties. In certain cases it was not possible, even after years of methodical treatment, to re-convert a culture to its original state.

1. As is known, the data regarding the time required for *the appearance of the first indications of spore-formation* in the previously-described six species, are subject to the condition that the growth has been previously cultivated in wort for 24 *hours* at a temperature of 25° C. At the same time that *Hansen* published (1883) the temperature curves for these six species, he also found that cultures which had been grown in wort at the above temperature, but for *two* days instead of one, developed spores more slowly and more sparingly than usual. If, however, such cultures are subsequently treated in the manner first described, the culture again assumes its normal condition. We have here, therefore, an example of a very feebly-rooted variation.

2. In a gelatine culture of "*Carlsberg bottom-yeast* No. 1" both *oval and elongated sausage-shaped cells* are often found, so that according to *Reess* the presence of two

species must be assumed. If colonies of each kind are separately introduced into flasks containing wort, growths are again obtained which consist partly of egg-shaped and partly of "*Pasterianus*" cells. *Hansen's* experiments showed that when these latter cultures were repeatedly re-cultivated in fresh flasks the cells still partly retained their sausage-shape for a lengthened period. When such a culture was introduced into a yeast-propagating apparatus, the growth obtained from it still showed an admixture of these cells; these disappeared, however, after the yeast from the propagating apparatus had been introduced into an ordinary fermenting vessel. In this case, therefore, the variation is more strongly rooted, and only disappears after the yeast has been propagated through a series of fermentations.

Another example in the same direction is that of a species of *Saccharomyces cerevisiæ* (a bottom-yeast) which, after a lengthened and difficult development, was subsequently cultivated in wort at a temperature of about 27° C. when the cells obtained exhibited their *ordinary appearance*; when cultivated at 7·5° C., however, *grouped colonies with mycelium-like branches* were obtained. This is an interesting example of the influence of temperature on the form of yeast cells.

3. As an example of a much more deeply-rooted change in the nature of the cells, *Hansen's* observations on *Saccharomyces Ludwigii* may be mentioned. When single individuals taken from an absolutely pure culture are again separately cultivated as pure cultures, it is possible to obtain growths which exhibit *great differences in their power of forming spores*. By a methodical choice of single cells *Hansen* succeeded in obtaining growths which, under the known conditions, completely failed to yield spores; on the other hand, he found that when, starting from the same original growth, a yeast speck which had sprung from a spore-yielding cell was chosen and further developed, a growth was obtained which was forthwith capable of yielding an abundance of spores. By such methodical selection, three varieties were separated from this species, one

of which was characterised by its high capacity of forming spores; in the second this property had nearly disappeared, whilst the third did not form spores at all. After numerous cultivations in wort, the third form returned, but only slowly, to its original condition, in which it was able to form spores; when it was cultivated in a solution of dextrose in yeast-water, however, this property was immediately re-acquired.

Another example of physiological transformation is the following: The three species described under the group *Saccharomyces Pastorianus* form under certain conditions a *dough-like sediment* similar to those of the other *Saccharomycetes*, under other conditions, however, a *film-like, wrinkled*, or a *caseous sediment* consisting of small lumps (*Pasteur's levure caséeuse*), that is to say, sediments of very different appearance, and yet produced by the same species. In the last-mentioned case, the fermenting wort also assumes a very characteristic appearance, and, contrary to what ordinarily occurs, remains bright throughout the fermentation, so that the yeast flakes can be observed to rise from the bottom to the surface and to again sink. If this curious sedimentary yeast is repeatedly cultivated by new fermentations in wort, it can be again transformed into the dough-like form.

A similar physiological transformation occurs in the *film-formations of the Saccharomycetes* (p. 137).

4. At the beginning of the year 1889, *Hansen* published[1] the results of a series of experiments which were undertaken with the view to discover the conditions causing variation, and by experiment to bring about the formation of new races, and if possible new species. These studies are being continued in his laboratory. The following account is taken partly from the source mentioned and partly from more recent publications.

Hansen found in the case of several *Saccharomyces* species, that when their cells were cultivated in aërated wort *at a*

[1] Centralbl. f. Bakt. u. Parasitenk. Bd. V., p. 665, 1889.

temperature approaching their maximum temperature, they became affected in such a manner that *they lost their power of forming spores*, and the same applies also to the innumerable generations gradually formed in new cultures at the optimum temperature. Yet the cells had a vigorous appearance and were further cultivated under very varied conditions.

Similar changes were also brought about by cultivation on a solid nutritive medium. These newly-formed varieties, as *Hansen* provisionally calls them, have not only lost their power of spore-formation, but at the same time also their property of forming films.

These investigations also have a practical bearing on the *brewing* industry, although in a direction different from that of *Hansen's* earlier researches. The Carlsberg bottom-yeast, No. 2, well-known to the brewing world, is one of the species which loses the property of spore-formation when it is subjected to the above-mentioned treatment. In the case of this yeast, it has been proved by numerous experiments that, simultaneously with the change mentioned, the plasma of the cells also undergoes transformation in other directions. The newly-formed growth gives a more feeble fermentation, the higher percentages of alcohol being produced more slowly than usual; in short, after the yeast has been treated as described, it works in a different manner than before such treatment.

No objection can be raised to the view that we are here possibly dealing with the formation of new species. We know in fact that the species are not fixed and unchangeable, as was generally assumed in *Linné's* time, but that the characters of a species are only constant under certain conditions. The complete elucidation of these important and intricate problems can, however, only be effected by a series of experiments carried on through a long period of time and varied in different directions.

In order to guard against any misunderstanding, it may not be superfluous to remind the reader that these remarkable changes are only brought about by a long-continued and

violent interference with the vital process of the cells, and that they do not occur so long as the development takes place in the normal manner.

An example of the persistence with which *Saccharomyces* cells retain, under normal conditions, the property of spore-formation, is met with in breweries and distilleries. We have here species of yeasts which have lived through hundreds of years, and have developed an infinite number of generations under conditions which, as a rule, have not permitted the exercise of the above-named function, and yet the power to do so has always been persistently retained.

(k) *Gelatinous Formation secreted by the Budding-fungi.*—Under certain but as yet undetermined conditions, the colonies produced by the budding of yeast cells can unite to irregular masses which sink to the bottom more quickly than the single yeast cells (breaking and clarifying in the brewery). This behaviour is undoubtedly related to a phase in the development of yeast cells which *Hansen* discovered in 1884. He discovered that not only the *Saccharomycetes* but also other budding-fungi are able to secrete a *gelatinous network*, which can be seen as threads or plates, and in which the cells lie imbedded (Fig. 33, A, B). If, for example, some moderately thick brewery yeast is placed in a glass and allowed to remain covered up in such a manner that it slowly dries, and then a trace of this yeast mixed with water, the network can be clearly seen (Fig. 33, A). The formation also occurs in the gypsum block and gelatine cultures. I have myself very frequently observed this remarkable formation, after *Hansen* had called my attention to its nature, in the yeast samples which are sent to my laboratory in filter-paper enclosed in envelopes.[1] *Hansen* also found it in the

[1] This method of preserving a sample of yeast for some time is very convenient. A small piece of filter-paper is rapidly passed through a flame several times, a few drops of yeast are poured on to it, and it is then folded up, and afterwards wrapped in several layers of paper which have been similarly treated.

film-formations of nearly all species. An ordinary microscopic examination of the pitching-yeast in a brewery does not show this formation; with the help of staining, however, its presence can be readily detected (Fig. B). When the yeast was repeatedly washed, it was no longer possible to detect the

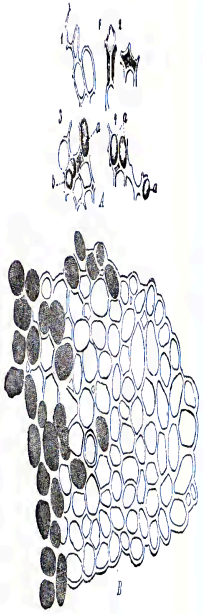

Fig. 93.

Yeast cells with gelatinous network, after Hansen: *A*, network obtained by partial drying; 1, portion formed of threads, from which the cells have become detached; 2 and 3 show that the network can also form complete walls; such a formation is seen between *a* and *b*; *a* is a vegetative cell, 4 is a cell with two spores; 4, shows three cells, *a*, imbedded in the network. *B*, network with yeast cells; the latter were stained by methyl-violet; the network is not stained. Some of the yeast cells are still in the meshes, but most have become detached.

network by staining; but if the water was removed, and the yeast set aside for a time, the gelatinous masses could after suitable treatment be readily seen. By varying the conditions of nourishment of the cells, the development could be promoted or hindered, and the chemical composition modified.

The whole behaviour suggests the zoogloea-formation of bacteria.

The Microscopic Appearance of a Yeast-cell.—As an introduction to the systematic description of the separate species of *Saccharomyces*, we give the following general description of the *Saccharomyces* cell.

The microscopic appearance of a yeast cell as it most frequently occurs in a fermenting liquid is a spherical or oval figure, which, by the swelling out of its wall, gives rise to one or more buds, which detach themselves sooner or later from the mother cell. This cell is consequently surrounded by a membrane which can vary somewhat in the different stages of the development of the cell, but rarely in a noticeable degree. It is otherwise, however, with the contents of the cell. The contents present the simplest appearance when the cell is observed in its most vigorous state of growth. The cell-contents then consist of clear homogeneous plasma. As the processes of multiplication and fermentation continue, different bodies appear in this plasma; partly clear portions filled with sap (vacuoles), partly larger and smaller particles, some of which can be shown to be fat globules, whilst others appear to be of a similar nature to the plasma. These granules have been minutely described by *Rouin*. This granular appearance of the plasma increases with the further development of the cell, and at a very advanced stage of the fermentation, when the cell has almost come to a state of rest, the plasma may become reduced to a thin layer on the inner side of the wall, whilst a large vacuole occupies the remaining space, and contains numerous small and large grains, many of a fatty nature. If such cells are again brought into a fermentable liquid, they soon exhibit a highly characteristic appearance during the period which precedes the macroscopic phenomena of fermentation. The grains disappear, and numerous fine plasma-threads appear in the clear cell-sap, and gradually circumscribe rounded vacuoles; finally these disappear, and the cell again becomes filled with clear homogeneous plasma.

As in most vegetable cells, a *cell-nucleus* (first discovered by *Schmitz*) is also found in the yeast cell, and its presence can be proved by staining with osmic acid or with picric acid and hæmatoxylin. According to *Hansen* this cell-nucleus is either spherical or disc-shaped. In old film-formations of *Saccharomycetes*, he found cells which distinctly showed the nucleus without any treatment.—*Janssens* observed the partition of the cell-nucleus both in the budding and in the spore-formation of the Saccharomycetes.

Classification of the Genus Saccharomyces.

Budding fungi, *mostly without a mycelium, the individual species of which occur with cells of different form and size. Under certain treatment, and sometimes also without any previous treatment, cell-nuclei are seen. Under certain conditions the cells develop* endogenous spores; *the germinating spores of most species grow to budding cells; in exceptional cases a promycelium is first formed. Number of spores* 1 to 10, *most frequently* 1 to 4. *Under favourable conditions the cells secrete a gelatinous network, in which they lie imbedded.*

The greater number of the species induce alcoholic fermentation.

Saccharomyces Cerevisiæ I, Hansen.[1]

(Figs. 34–36.)

This and the five following species (*Saech. Pastorianus I., II., and III., Saech. ellipsoideus I. and II.*), all develop invertase; with this they effect the hydrolysis of saccharose to invert-sugar, and they ferment the latter. They produce a vigorous fermentation in dextrose solutions, and likewise in maltose solutions, especially when a nutrient liquid such as yeast-water is added. All are vigorous alcoholic ferments

[1] This top-fermentation yeast must not be confused with *Hansen's* Carlsberg bottom-yeast No. I.

which in the course of fourteen days at the ordinary room-temperature, readily produce 4 to 6 per cent (by volume) of alcohol in beer wort. They are unable to ferment lactose.

Saccharomyces cerevisiæ I., is an old English top-fermentation yeast, which is employed in breweries in London and Edinburgh.

The young growth of sedimentary yeast (Fig. 34) developed in wort, consists essentially of large round and oval cells; really elongated cells do not occur under these conditions.

FIG. 34.

Saccharomyces cerevisiæ I. Hansen. Cell-forms of young sedimentary yeast, after Hansen.

Ascospore-formation (Figs. 26–28, 32, 1):—[1]

At	37·5° C.	no ascospores are developed.						
„	36–37°	„	the first indications are seen after				29	hours.
„	35	„	„	„	„	„	25	„
„	33·5	„	„	„	„	„	23	„
„	30	„	„	„	„	„	20	„
„	25	„	„	„	„	„	23	„
„	23	„	„	„	„	„	27	„
„	17·5	„	„	„	„	„	50	„
„	16·5	„	„	„	„	„	65	„
„	11–12	„	„	„	„	„	10	days.
„	9	„	no ascospores are developed.					

[1] The preparation of the growth of a *Saccharomyces* species for these investigations must be made in the following manner:—After the cells have been cultivated for some time in ordinary wort (about 14 per cent.

Spores strongly refractive to light. Wall of spores very distinct. Size of spores 2·5–6 μ.

Film-formation:—

At 38° C. no film-formation occurs,

„ 33–34° „ feebly-developed film-specks are seen after 9–18 days.

„ 26–28 „ „ „ „ „ 7–11 „

„ 20–22 „ „ „ „ „ 7–10 „

„ 13–15 „ } (Fig. 35) { „ „ 15–30 „

„ 6– 7 „ } (Fig. 35) { „ „ 2– 3 months.

„ 5 „ no film-formation occurs.

Microscopic appearance of the cells in the films:—

At 20–34° C.: Colonies frequent; sausage-shaped and curiously formed cells occur.

Fig. 35.

Saccharomyces cerevisiæ I. Hansen. Film-forms at 15–6° C., after Hansen.

At 15–6° C. (Fig. 35). The greater number of the cells resemble the original cells; isolated cells of different form.

In old cultures of films all forms occur, including greatly elongated mycelium-like cells (Fig. 36, p. 162).

Saccharomyces Pastorianus I. Hansen.

(Figs. 37, 38.)

Bottom-fermentation yeast.

Sedimentary forms grown in wort:—Mainly elongated,

Ball) at the ordinary room-temperature, the young vigorous cells obtained are introduced into fresh wort, in which they are allowed to develop for about twenty-four hours at 25° to 27° C. This growth is used for the gypsum-block culture.

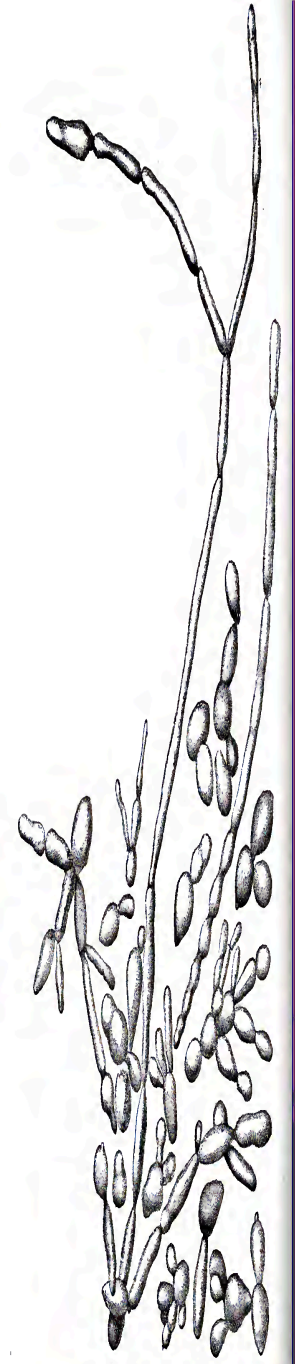

Fig. 98. Sacch. cerevisiæ I. Hansen. Cell-forms in old cultures of film, after Hansen.

sausage-shaped cells, but also large and small round and oval cells (Fig. 37).

It frequently occurs in the air of the fermenting-rooms. It imparts to the beer a disagreeable *bitter* taste and unpleasant odour; it can also produce turbidity, and can interfere with the clarification of the beer in the fermenting vessel.

FIG. 37.

Saccharomyces Pastorianus I. Hansen. Cell-forms of young sedimentary yeast, after Hansen.

Ascospore formation (Fig. 32, 2):—

At	31·5° C.	no ascospores are developed.					
„	29·5—30·5°	„	the first indications are seen after			30	hours
„	29	„	„	„	„	27	„
„	27·5	„	„	„	„	24	„
„	23·5	„	„	„	„	26	„
„	18	„	„	„	„	35	„
„	15	„	„	„	„	50	„
„	10	„	„	„	„	89	„
„	8·5	„	„	„	„	5	days
„	7	„	„	„	„	7	„
„	3 — 4	„	„	„	„	14	„
„	0·5	„	no ascospores are developed.				

Size of spores 1·5—5 μ.

Film-formation:—

At 34° C. no film-formation occurs.

„ 26–28° „ feebly-developed film-specks are seen after 7–10 days.

„ 20–22 „ „ „ „ „ „ 8–15 „

„ 13–15 „ „ „ „ „ „ 15–30 „

„ 6– 7 „ } (Fig. 38) { „ „ 1– 2 months.

„ 3– 5 „ „ „ „ „ „ 3– 6 „

like Fig. 38, but without the large colonies.

„ 2– 3 „ no film-formation occurs.

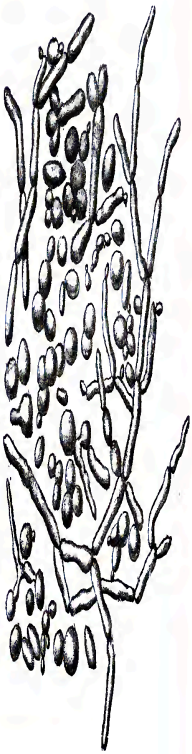

FIG. 38.

Saccharomyces Pastorianus I. Hansen. Film-forms at 13–15° C, from Holst's drawing in Hansen's Memoir.

Microscopic appearance of the cells in the films:—

At 20—28° C. nearly the same forms as in the sedimentary yeast.

At 13—15° C. strongly-developed mycelium-like colonies of very elongated sausage-shaped cells (Fig. 38) are moderately frequent.

In *old cultures of films* the cells are smaller than in the sediment; very irregular and sometimes almost thread-like cells are found.

Saccharomyces Pastorianus II. Hansen.

(Figs. 39, 40.)

Fig. 39.

Saccharomyces Pastorianus II. Hansen. Cell-forms of young sedimentary yeast, after Hansen.

Feeble top-fermentation yeast.

Sedimentary forms grown in wort:—Mainly elongated sausage-shaped cells, but also large and small oval and round cells (Fig. 39).

It frequently occurred in *Hansen's* analyses of the air in the brewery; appears to belong to the species which do not cause diseases in beer.

Ascospore-formation (Fig. 32, 3):—

At 29° C. no ascospores are developed.
„ 27–28° „ the first indications are seen after 24 hours.
„ 25 „ „ „ „ „ 25 „
„ 23 „ „ „ „ „ 27 „
„ 17 „ „ „ „ „ 36 „
„ 15 „ „ „ „ „ 48 „
„ 11·5 „ „ „ „ „ 77 „
„ 7 „ „ „ „ „ 7 days.
„ 3–4 „ „ „ „ „ 17 „
„ 0·5 „ no ascospores are developed.

Size of the spores 2–5 μ.

FIG. 40.

Saccharomyces Pastorianus II. Hansen. Film-forms at 15–9° C., after Holm's drawing in Hansen's Memoir.

Film-formation:—

At 34° C. no film-formation occurs.
„ 26–28° „ feebly-developed film-specks are seen after 7–10 days.
„ 20–22 „ „ „ „ „ 8–15 „
„ 13–15 „ „ „ „ „ 10–25 „
„ 6–7 „ „ „ (Fig. 40) „ „ 1–2 months.
„ 3–5 „ „ „ „ „ 5–6 „
„ 2–3 „ no film-formation occurs.

Microscopic appearance of the cells in the films:—

At 20—28° C.: Nearly the same forms as in the sedimentary yeast; also irregular sausage-shaped cells.

At 15—9° C.: Mostly oval and round cells.

In *old cultures of films* the cells are smaller than in the sediment; very irregular and sometimes almost thread-like cells are found.

Streak cultures of this species in *gelatine yeast-water* give, after sixteen days at 15° C, growths with comparatively *smooth edges*, and in this respect it also differs from the following species.

Saccharomyces Pastorianus III. Hansen.

(Figs. 41, 42.)

Fig. 41.

Saccharomyces Pastorianus III. Hansen. Cell-forms of young sedimentary yeast, after Hansen.

Top-fermentation yeast.

Sedimentary forms grown in wort:—mostly elongated, sausage-shaped, but also large and small oval and round cells (Fig. 41).

It was separated from a bottom-fermentation beer which showed *yeast-turbidity*, and has been proved by *Hansen* to be

one of the species which produce this disease. Recent experiments of *Hansen* show that this disease-yeast possesses another peculiar property; namely, when the fermenting wort has an opalescent appearance, the addition of Sacch. *Pastorianus III.* will in certain cases effect a clarification.

Ascospore formation (Fig. 32, 4):—

At 29° C. no ascospores are developed.
„ 27–28° „ the first indications are seen after 35 hours.
„ 26·5 „ „ „ „ „ 30 „
„ 25 „ „ „ „ „ 28 „
„ 22 „ „ „ „ „ 29 „
„ 17 „ „ „ „ „ 44 „
„ 16 „ „ „ „ „ 53 „
„ 10·5 „ „ „ „ „ 7 days.
„ 8·5 „ „ „ „ „ 9 „
„ 4 „ no ascospores are developed.

Size of the spores 2–5 μ.

Film-formation:—

At 34° C. no film-formation occurs.
„ 30–28° „ feebly-developed film-specks are seen after 7–10 days.
„ 20–22 „ „ „ „ „ 9–12 „
„ 13–15 „ „ „ „ „ 10–20 „
„ 6– 7 „ (Fig. 42) „ „ 1– 2 months.
„ 3– 5 „ „ „ „ „ 5– 6 „
„ 2– 3 „ no film-formation occurs.

Microscopic appearance of the cells in the films:—

At 20–28° C.: Nearly the same forms as in the sediment-ary yeast.

At 15–3° C.: Strongly-developed colonies of elongated sausage-shaped or thread-like cells, which closely resemble a mycelium in appearance (Fig. 42).

In *old cultures of films*, the cells have the same forms as at 15–3° C, and are often still thinner and more thread-like.

FIG. 42.
Saccharomyces Pastorianus III. Hansen. Film forms at 16—30° C., after Hansen.

Streak cultures of this species in *gelatine yeast-water* give, after sixteen days at 15° C, growths with distinctly *hairy edges*.

Saccharomyces ellipsoideus I. Hansen.

(Figs. 43, 44.)

Bottom-fermentation yeast.

Sedimentary forms grown in wort:—mostly oval and round cells; sausage-shaped cells rare (Fig. 43).

Fig. 43.

Saccharomyces ellipsoideus I. Hansen. Cell-forms of young sedimentary yeast, after Hansen.

Occurs on the surface of *ripe grapes*.

Ascospore-formation (Fig. 32, 5):—

At	32·5° C.	no ascospores are developed.						
„	30·5—31·5°	„	the first indications are seen after				36	hours.
„	29·5	„	„	„	„	„	23	„
„	25	„	„	„	„	„	21	„
„	18	„	„	„	„	„	38	„
„	15	„	„	„	„	„	45	„
„	10·5	„	„	„	„	„	4½	days.
„	7·5	„	„	„	„	„	11	„
„	4	„	no ascospores are developed.					

Size of the spores 2—4 μ.

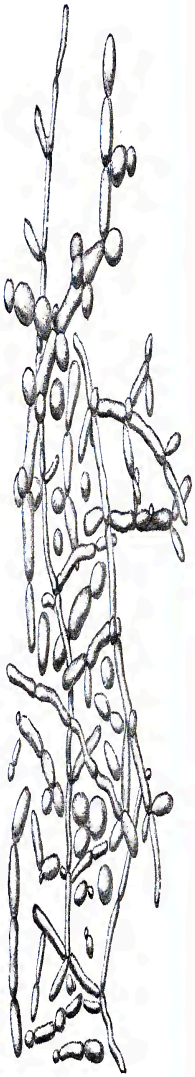

FIG. 44.

Saccharomyces ellipsoideus I. Hansen. Film-forms at 13–15° C. from Holm's drawing in Hansen's Memoir.

Film-formation:—

At 38° C. no film-formation occurs.
„ 33–34° „ feebly-developed film-specks are seen after 8–12 days.
„ 26–28 „ „ „ „ „ 9–16 „
„ 20–22 „ „ „ „ „ 10–17 „
„ 13–15 „ (Fig. 44) „ „ „ 15–30 „
„ 6– 7 „ „ „ „ „ 2– 3 months.
„ 5 „ no film-formation occurs.

Microscopic appearance of the cells in the films:—

At 20–24° C. and 6–7° C., the cells are smaller and more sausage-shaped than in the sedimentary yeast.

FIG. 45.

Saccharomyces ellipsoideus H. Hansen. Cell-forms of young sedimentary yeast, after Hansen.

At 13–15° C., freely-branched and strongly-developed colonies of short or long sausage-shaped cells, often with verticillated branches (Fig. 44).

In *old cultures of films*, the cell forms are the same as at 13–15° C.

Streak cultures of this species in *wort-gelatine* (wort with the addition of about 5·5 per cent. of gelatine) give—in contradistinction to the other five species—in the course of eleven to fourteen days at 25° C. a characteristic *net-like structure*, by means of which it can be distinguished by the naked eye from other species.

Saccharomyces ellipsoideus II. Hansen.

(Figs. 45, 46.)

Usually bottom-fermentation yeast.

Sedimentary forms grown in wort:—mostly oval and round cells; sausage-shaped cells rare (Fig. 45).

It was separated from beers which showed yeast-turbidity; is a species which *causes yeast-turbidity*, and has been shown by *Hansen's* experiments to be more dangerous than *Saccl. Pastorianus III.*

Ascospore-formation (Fig. 32, 6).

At 35° C. no ascospores are developed.
„ 36–34° „ the first indications are seen after 31 hours.
„ 33 „ „ „ „ „ 27 „
„ 31·5 „ „ „ „ „ 23 „
„ 29 „ „ „ „ „ 22 „
„ 25 „ „ „ „ „ 27 „
„ 18 „ „ „ „ „ 42 „
„ 11 „ „ „ „ „ 5½ days.
„ 8 „ „ „ „ „ 9 „
„ 4 no ascospores are developed.

Size of spores 2–5 μ.

Film-formation:

At 40° C. no film-formation occurs.
„ 36–38° „ feebly-developed film-specks are seen after 8–12 days.
„ 33–34 „ „ „ „ „ 3– 4 „
„ 26–28 „ „ „ „ „ 4– 5 „
„ 20–22 „ „ „ „ „ 4– 6 „
„ 13–15 „ „ „ „ „ 8–10 „
„ 6– 7 „ „ „ „ „ 1– 2 months.
„ 3– 5 „ „ „ „ „ 5– 6 „
(Fig. 46.)
„ 2– 3 „ no film-formation occurs.

Microscopic appearance of the cells in the films:—

At all temperatures, the same forms as in the sediment; at and below 15° C. the cells are only slightly more elongated (Fig. 46).

In *old cultures of films* there are colonies of short and long sausage-shaped cells, often with verticillated branches.

Related to this species are two ellipsoid species, described by *Will*, and which are also *disease-yeasts*. One of these, a bottom-fermentation yeast, gives colonies in wort-gelatine, which when young form—whether on the surface or embedded in the gelatine—a network with large meshes; afterwards they become denser in the middle, with irregularly-fringed

FIG. 46.

Saccharomyces ellipsoideus II. Hansen. Film-form at 22–8°, after Hansen.

edges; sometimes, however, compact colonies with regular outline are formed under the same conditions. The maximum temperature for spore-formation is 30° C.; at the optimum temperature (24° C.), the first indications of spores are seen after eleven hours. The lower limit for spore-formation is 4 to 5° C. The vegetative cells are killed when heated in sterilised wort for half an hour at 70° C. The temperature limits for film-formation are 41° and 4° C. In old films especially are found numerously-branched clusters, consisting of very much elongated cells. This species imparts a *rough bitter after-taste* to beer and also causes *turbidity*.

The second ellipsoid species which was obtained from a beer showing yeast-turbidity, gives colonies in wort-gelatine,

some of which are sharply defined, whilst in others the outline is indistinct. The temperature limits for spore-formation are 32° and 0·5° C, the optimum temperature being 24° C. The temperature limit for the vitality of the vegetative cells in wort is 70° C. In old films very numerously-branched clusters occur. Besides causing *yeast-turbidity*, this species also imparts a sweetish, *disagreeable, aromatic taste* to beer, and a *bitter, astringent after-taste*. The yeast sediment always has a dark colour.

SACCHAROMYCES ILICIS. GRÖNLUND

which was found on the fruit of *Ilex Aquifolium*, is a bottom-fermentation yeast, consisting mainly of spherical cells. The temperature limits for spore-formation are 8° and 38° C. The spores have no vacuoles. In the films, slightly-elongated cells are found. Streak cultures on gelatine have a floury, but otherwise a variable, appearance. This species, grown in wort, imparts a disagreeable, bitter taste. According to *Schjerning* it contains invertase, and induces alcoholic fermentation in solutions of saccharose, dextrose, and maltose. In ordinary beer wort it can produce about 2·8 per cent. alcohol (by volume).

SACCHAROMYCES AQUIFOLII. GRÖNLUND

was also found on the fruit of *Ilex Aquifolium*. It is a top-fermentation yeast, and consists of large round cells. The temperature limits for spore-formation are 8° and 31° C.; the spores contain vacuoles. In the films, spherical and egg-shaped cells alone occur. Streak cultures in gelatine vary in appearance, some being glossy and some floury. This species imparts to wort a disagreeable, sweet taste, with a bitter after-taste. It inverts saccharose and induces alcoholic fermentation in solutions of saccharose, dextrose, and maltose. In ordinary beer-wort it can produce about 3·7 per cent. alcohol (by volume).

SACCHAROMYCES PYRIFORMIS. MARSHALL WARD

(see ginger-beer plant, page 71).

Saccharomyces Marxianus. Hansen.

This species, which was discovered by *Marx* on grapes, and described by *Hansen*, develops in beer-wort in the form of small oval cells, essentially similar to those of *Sacch. ellipsoideus* and *ellipsoideus*. Elongated, sausage-shaped cells, often in colonies, soon appear, however, and if the culture be set aside for some time small mould-like particles are formed, and partly swim in the liquid, and partly settle to the bottom. These particles consist of mycelium-like colonies of essentially the same nature as the film formations of the six species previously described; they are also built up of cells, which are readily separated at the point of union. The ascospores are kidney-shaped, spherical, or oval. After cultivation for two to three months in wort contained in the two-necked flasks, there were only traces of film-formation with only a few sausage-shaped and oval cells.

This yeast is one of those species which develop a mycelium under certain conditions of culture on a solid nutritive medium.

In beer-wort it yields only 1 to 1·3 per cent. (by volume) of alcohol, even after long standing. It does not ferment maltose; it inverts saccharose; and in nutritive solutions of the latter, and of dextrose, it yields considerable quantities of alcohol.

Saccharomyces exiguus (Reess). Hansen

develops in wort a growth, the cell-forms of which most closely correspond to the species described by *Reess* under the above name. It is, however, impossible to determine whether *Reess* was really dealing with this species, since any *Saccharomyces* species may, under certain conditions, form a preponderating number of similar small cells.

This species only gives scanty spore-formation and weak film-formation, but it yields a well-developed yeast-ring. The cells of the film resemble those of the sedimentary yeast, but short sausage-shaped and small cells are more frequent.

Hansen found this species in pressed yeast. Its behaviour towards the sugars is similar to that of the last species, though it develops a greater fermentative activity in solutions of saccharose and dextrose. In wort, it also yields only small quantities of alcohol. It does not ferment maltose solutions. It inverts saccharose.

Experiments of a practical nature, which were conducted by *Hansen*, have shown that this species does not produce any disease in beer, even when present in considerable quantities either at the beginning or end of the primary fermentation, or when it is added after storage of the beer.[1]

Some other species examined by *Hansen* can likewise ferment saccharose and dextrose, but not maltose and lactose.

Saccharomyces Joergensenii, described by *Lindet*, also belongs to the group of the *Saccharomycetes*, which may be termed *Sacch. exiguus*. The growth consists of small round and oval cells. The optimum temperature for spore-formation is 25° C., the temperature limits being 8° and 30° C. At temperatures above 30° C. the growth rapidly dies. A true film-formation was not observed; in old cultures only a very feeble yeast ring was formed, and this consisted of round and oval cells. In gelatine it yields colonies which resemble those of low-fermentation brewery yeast. Wort-gelatine becomes slowly liquefied. The streak-culture is dirty grey in appearance, with smooth edges. This species ferments saccharose and dextrose, but not maltose. When it is mixed with cultivated yeasts and grown in wort, it consequently becomes suppressed and cannot therefore produce any disease in beer.

Saccharomyces membranæfaciens. Hansen.

This peculiar species, which occupies a special place amongst the *Saccharomycetes*, when grown in wort, yields a strongly-developed light grey, wrinkled film, which very quickly covers

[1] This is of special interest, as *Sacch. exiguus* was formerly regarded as a disease-producing species.

the whole surface of the liquid, and which consists mainly of sausage-shaped and elongated oval cells; these have strongly-developed vacuoles, and have a more or less emptied appearance. Between the colonies is an abundant admixture of air.

The spores are very abundantly developed, not only under the ordinary conditions of cultivation, but also in the films. They are irregular in form, and at the ordinary room-temperature they germinate in a Ranvier chamber after ten to nineteen hours.

On wort-gelatine, the cells form dull grey specks, often with a faint, reddish tinge, which are rounded, flat and spread out, and wrinkled. The colonies embedded in the gelatine present, however, a quite different appearance. The gelatine becomes liquefied by this fungus, although only slowly.

This species is incapable of fermenting either saccharose, dextrose, maltose, or lactose, and neither does it invert saccharose. It was found in the slimy secretion on the roots of the elm, and shows considerable resemblance to the species *Mycoderma cerevisiæ* and *Mycoderma vini*, but it is a true *Saccharomyces*.

Koehler found this species in very impure well-water. *Pichi* has described two species which very closely resemble *Sacch. membranæfaciens*.

Saccharomyces Hansenii Zopf

was discovered by *Zopf* amongst the fungi of cotton-seed flour. It forms very small spherical spores, which are mostly developed singly, and at most in pairs, in the mother-cell. It does not induce alcoholic fermentation in fermentable nutrient sugar solutions, but on the other hand crystals of calcium oxalate are observed in the sediment. *Zopf* found such crystals in nutrient solutions of galactose, grape-sugar, cane-sugar, milk-sugar, maltose, dulcite, glycerine, and mannite.

Saccharomyces Ludwigii. Hansen.

(Figs. 29, 30.)

This characteristic species, which was discovered by *Ludwig* in the viscous secretion of the living oak, is the only one of the known *Saccharomycetes* which can be recognised solely by means of a microscopic examination. The following description is from *Hansen's* investigations. The cells are very variable in size, are elliptical, bottle-shaped, sausage- or frequently lemon-shaped. Partition walls can occur in all the complex cell-combinations. The vegetative growths in wort-gelatine are—like those of nearly all the *Saccharomycetes*—round, light grey, or faintly yellow. In wort it only yields 1·2 per cent. (volume) of alcohol after a long continued fermentation; and this is in accordance with the fact that maltose is not fermented by this species. In dextrose solutions, on the other hand, it yields alcohol up to 10 per cent. by volume. It inverts saccharose, but does not ferment solutions of lactose and dextrin, and it does not saccharify solutions of starch. It readily develops spores in aqueous solutions of saccharose, in wort-gelatine, in yeast-water, and in wort; in the latter case even when no film has formed.

Spore-formation (Figs. 29, 30) occurs most rapidly at a temperature of 25° C. It is characteristic of this species that, especially in the case of the young spores, *a fusion of the germinated spores* often occurs, and *these new formations develop germ-filaments* (*promycelium*), from which new yeast-cells become gradually marked off by sharp transverse septa. At the ends of these cells, buds are developed, and these again become marked off by transverse septa.

In old cultures there is often a strong tendency to form *mycelium*, but portions are only exceptionally found the cells of which are firmly united together, and which show only slight constrictions; these portions have distinct, straight, transverse walls. Each cell of such colonies can

form buds and spores. Amongst them are also found irregular cells, and very large many-branched cells.

It is also characteristic of this species that when kept in a solution of saccharose the cells die within two years, whilst most of the other *Saccharomycetes* examined can be preserved in this liquid for a much greater length of time.

Saccharomyces acidi lactici. Grotenfelt.

Grotenfelt has described under this name a species of *Saccharomyces* which, when added to sterilised milk, produces an intense curdling with formation of acid; on gelatine and agar-agar it forms white porcelain-like colonies, and on potatoes it yields broad, moist, whitish-grey patches, which soon become brown. In puncture cultivations in gelatine short flask-shaped growths develop from the point of inoculation into the gelatine. The cells are elliptical, 2·0 to 4·35 μ in length, and 1·50—2·90 μ in breadth.

When a solution of milk-sugar was inoculated, with the addition of calcium carbonate, and the product distilled, alcohol could be detected. In a neutral 3 per cent. solution of milk-sugar, *Saccharomyces acidi lactici* yielded 0·108 per cent. of acid in eight days.

Saccharomyces minor. Engel.

The vegetative cells are completely spherical, in size up to 6 μ in diameter, and are united in chains or in groups composed of but few cells (6 to 9). Spore-forming cells 7 to 8 μ, and containing 2 to 4 spores of 3 μ in diameter.

This organism is, according to the above author, the most active ferment in the fermentation of bread.[1]

[1] Decisive experiments on the *essential active factors in the fermentation of bread* have not yet been made. In the manufacture of white bread, ordinarily "*pressed-yeast*" is used; this consists in the main of alcoholic ferments, and according to the generally-accepted view the yeast is the only active ferment. In the manufacture of black bread, and in some countries also of white bread, so-called *leaven* is employed;

Saccharomyces anomalus. Hansen.

(Figs. 31 and 47).

This very curious species was found by *Hansen* in an impure brewery yeast from Bavaria. It gives a rapid and vigorous fermentation in wort, and even at the beginning of the fermentation develops a dull grey film. During fermentation the liquid acquires an ethereal, fruity odour.

this is made by kneading together flour, bran, and water, and allowing the mass to undergo spontaneous fermentation. It contains bacteria in large numbers, and also yeast-like cells, and amongst the latter alcoholic ferments. Very opposite views have, however, been expressed with regard to the importance of these different organisms in the fermentation of black bread.

According to *Chicoisbert* (1888) and *Marcano* the active ferment is a bacterium, *Boutroux* attributed the fermentation to the activity of both bacteria and budding fungi; later he regarded alcoholic yeast as the chief cause. *Laurent* regarded the so-called *Bacillus panificus* as the main cause of the fermentation of bread. *Düsmenberger's* investigations led to the conclusion that the budding fungi must be looked upon as the only essential organisms of fermentation in bread. The rising of the dough is accordingly caused in the first place by the carbonic acid liberated by the alcoholic fermentation; further by the expansion of the air and the vapourisation of the alcohol, water, and volatile fatty acids formed by the bacteria. *Peters* found four different budding fungi in leaven, and one of these has been identified with *Saccharomyces minor Engel*. The second is of about the same size as *Saccharomyces minor*; the cells are egg-shaped, and in nutrient liquids develop to moderately large, many-branched colonies; it yields spores abundantly. In addition to the above, a species of *Mycoderma* and a species related to *Saccharomyces cerevisiæ* were also found. *Peters* describes several species of bacteria occurring in leaven, but none of them has all the properties of *Laurent's Bacillus panificus*; on the contrary, these properties were found divided amongst various bacteria. *Laurent*, therefore, was probably dealing with impure cultivations. These bacteria gave no alcoholic fermentation, and no appreciable evolution of gas in sterilised dough.

The above experiments constitute a good preliminary to the decisive experiments on the cause and action of the rising of dough.

The diseases of black bread which have been investigated by *Uffelmann*, *Kretschmer*, and *Nencki&Sieber*, *e.g.*, vigorous growth of mould, sliminess caused by an exuberant growth of bacteria, may no doubt be partly attributed to impure leaven, in which the most various organisms will thrive.

The cells grown in wort are small, oval, and sometimes sausage-shaped, and in their microscopic appearance they resemble the *Torula* species. When the development has gone on for some time many of the cells both in the sediment and in the film are found to contain spores.

The spores are developed on various substrata, both liquid and solid, and also under conditions where abundant nutriment is present. In an ordinary gypsum-block culture a moderately abundant development of spores is obtained after forty hours at 25° C.

The *form of the spores* is highly characteristic (Fig. 47); it resembles a hemisphere with a projecting rim round the base. On germination the spores swell and develop buds (see Fig. 31).

FIG. 47.

Spores of Saccharomyces anomalus, after Hansen. Some spores are free, others enclosed in the mother-cells. At the bottom, on the right-hand side, are three spores, surrounded by the burst wall of the mother-cell.

After *Hansen* had drawn attention to the above curious *Saccharomyces* species, it, and probably other allied species, were also observed by *Holm*, *Lindner*, and *Will*, who likewise found it in impure brewery yeast. Yeasts yielding hat-shaped spores appear in fact to be by no means uncommon.

As was previously mentioned, the spores of this fungus resemble those of *Endomyces decipiens*, and a relationship perhaps exists between this *Saccharomyces* and the fungus named. As yet, however, no proof has been forthcoming in support of this.

SACCHAROMYCES CONGLOMERATUS, REESS.

This species is described by *Reess* as follows:—"Round budding cells, of 5 to 6 μ diameter, united in clusters, which

are formed from two old cells which, before budding in the direction of their common longitudinal axis, usually simultaneously throw out several buds as branches. The asci very frequently remain united in pairs, or each united to a vegetative cell. Spores 2 to 4, which on germination again give rise to clusters. Occurs on decaying grapes and in wine-yeast at the commencement of fermentation. Fermentative action doubtful."

In *Hansen's* cultures of film-formations of the *Saccharomycetes*, colonies of the above-mentioned appearance were found in old films of the six species first investigated by him. And since *Hansen* never found a definite species among his cultivations which could be identified as *Rees's Saccharomyces conglomeratus*, he is inclined to assume that the cell-colonies mentioned of the different *Saccharomycetes* are identical with this species.

The different races or species of yeast may be divided into two groups, according to the kind of fermentation to which they give rise, namely:—*bottom-yeasts* and *top-yeasts*. In spite of many assertions to the contrary, it has not hitherto been possible to bring about an actual conversion of top-yeast into bottom-yeast, or vice versa. The investigations of *Hansen* and *Kühle* show that it is certainly possible for a bottom-fermentation yeast to produce transitory top-fermentation phenomena; these, however, quickly disappear with the progressive development of the yeast. When formerly it was stated that by the continued cultivation of, *e.g.*, bottom-yeast at an elevated temperature, this could be converted into top-yeast, these old experiments can only be explained on the assumption that the bottom-yeast employed was impure and contained an admixture of top-yeast, which at the elevated temperature gradually developed at the expense of the bottom-yeast, until it finally constituted the chief portion of the yeast.

As examples of two different bottom-fermentation species of yeast, the *Carlsberg bottom-yeasts* "No. 1" (Fig. 48) and "No. 2" (Fig. 49) employed in the *Old Carlsberg* brewery at

Fig. 48.

Carlsberg bottom-yeast No. 1, after Hansen.

Copenhagen, may be more minutely described. Distinct differences are noticeable even in the ordinary microscopic examination.

The cells of the species No. 1 (Fig. 48) are mostly somewhat elongated, but there are also smaller characteristic

Fig. 49.

Carlsberg bottom-yeast No. 2, some cells with spores, after Hansen.

pointed cells. When the yeast taken from the fermenting-vessel is washed with water and placed for a short time under ice, the contents of all the cells will quickly assume a granular appearance, and if the yeast is kept in this manner for several days the number of dead cells will very rapidly increase. The cells of the species No. 2 are, under normal conditions, roundish oval, some being almost spherical. Here and there giant-cells occur (left-hand side of figure). In a yeast-mass

washed with water the cell-contents long remain clear, and only slightly granular, and if the yeast be kept for a long time in this way only very few dead cells will be found.

The gelatine cultures of both species form colonies, having the ordinary appearance of the *Saccharomycetes*.

On gypsum blocks the species No. 2 develops spores much more quickly and abundantly than No. 1 species.

The fermentation phenomena also differ. No. 2 gives thick, high foam and a dense, firm layer on the surface; No. 1 gives a low foam and the liquor is often exposed in places. No. 2 clarifies comparatively quickly; No. 1 clarifies slowly. No. 2 forms a firm layer at the bottom of the fermenting-vessel, whilst No. 1 gives a fluid sediment. In the primary and secondary fermentations No. 2 gives a more feeble fermentation than No. 1.

The finished beer obtained with the two yeasts in the same brewery shows marked differences. With regard to taste, the beer obtained with No. 2 yeast is preferred by most; but this is a matter of opinion; at all events the taste is different in the two cases. Finally the two species give very different results as to the stability of the beers with regard to yeast turbidity. The beer prepared with the No. 1 yeast is decidedly more stable in this respect than that prepared with No. 2 yeast. Consequently No. 1 is especially suited for lager and export beers, and No. 2 for running beers. These characteristics have always been found to remain unchanged for years.

From the above description of the microscopic relationships of these two types of bottom-yeast it must on no account be assumed that we are able, by means of a microscopic examination of an unknown species of yeast, to determine whether it will give a high or a low attenuation, or whether it will clarify slowly or quickly, etc., etc. *Hansen's* investigations have, on the contrary, proved that it is *impossible to establish any general rule by this means*, since species which give a high attenuation may have the same

microscopic appearance as species which give a low attenuation. It will only be possible to form an opinion in this direction when our knowledge of the structure of the plasma is much more advanced. Statements to the contrary which have hitherto appeared in the literature of the subject are simply erroneous assertions.

A preliminary *grouping for practical purposes* of the different species or races of *bottom-* and *top-fermentation beer-yeasts* which have been prepared in pure culture by *Hansen's* method in my laboratory, is as follows:—

A.—Bottom-fermentation Species.

1. Species which clarify very quickly and give a feeble fermentation in the fermenting-vessel; the beer holds a strong head. The beer, if kept long, is liable to yeast-turbidity. Such yeasts are only suitable for draught-beer.
2. Species which clarify fairly quickly and do not give a vigorous fermentation; the beer holds a strong head; high foam; the yeast settles to a firm layer in the fermenting-vessel. The beer is not particularly stable as regards yeast-turbidity. These yeasts are suitable for draught-beer and partly for lager beer.
3. Species which clarify slowly and attenuate more strongly; the beer has a good taste and odour; the yeast deposit is less firm in the fermenting vessel. The beer is very stable against yeast-turbidity. These yeasts are suitable for lager beer, and especially for export beers which are not pasteurised or treated with antiseptics.

B.—Top-fermentation species.

1. Species which attenuate slightly and clarify quickly. The beer has a sweet taste.
2. Species which attenuate strongly and clarify quickly. Taste of beer more pronounced.

3. Species which attenuate strongly, clarify slowly, and *give a normal after-fermentation*. The beer is stable against yeast-turbidity.

As a very significant result of practical experience, and one which shows how pronounced are the characters of many species of cultivated yeast, the fact may here be mentioned that generally speaking *the above grouping holds good, even under the different practical conditions obtaining in widely separated countries*. For instance, the Carlsberg yeast No. 1 gives everywhere a beer which is very stable as regards yeast-turbidity; other species, which clarify more rapidly, have been found to retain this property everywhere under normal brewery-conditions.

An example of the permanence of the specific properties under very different external conditions has also been given by *Iwaisch* in a comparative examination of two bottom-yeasts. One of the species gave a low attenuation and multiplied to a very small extent in the wort, whilst the other, on the contrary, gave a high attenuation and possessed the power of multiplication in a high degree; the course of the fermentation in the two cases also showed marked differences. These differences still obtain on varying the concentration of the wort or the quantity of the yeast, at very different temperatures, also when cultures are employed which have been grown in wort containing diastase, under various conditions of aëration with ordinary wort, and with a specially prepared wort very poor in maltose, in the presence of grains during fermentation, and in solutions of cane-sugar. Likewise in fermentations which were carried on for six months, an examination of the product showed that the typical differences of the two species had not disappeared.

Besides the beer top-yeasts, there are also certain high-fermentation species which are employed in *distilleries* and in *yeast factories*. In recent years a number of distillery yeasts have been prepared in the author's laboratory in

pure culture. They exhibited marked differences in their sedimentary forms and in ascospore-formation. The species which were introduced into practice also differed in this respect. *Delbrück*, *P. Lindner*, and *Stenglein* have also had the same experience.

Béchamp, *Schumacher* and *Wiesner*, have carried out microscopical and chemical investigations of yeasts of the kind last mentioned, and *Béchamp's* "Studien über Presshefe" (Prague, 1870) especially contains accurate descriptions of the appearance under the microscope of ordinary pressed yeast in the different stages of its development, and observations on the microscopic indications of the quality of the manufactured yeast, so far as can be judged from the contents of the cells. The decomposing yeast cells show a change in the colour and consistence of their plasma; this gradually becomes darker and liquid, the vacuoles become larger, and the sharp outlines between the vacuoles and the plasma gradually disappear, the plasma shrinks from the cell-wall and finally collects in irregular masses in the cell-fluid; these also disappear at last, and finally the cell-wall is dissolved. According to the above authors, there also occur in pressed-yeast, cells which suddenly develop a number of small vacuoles; these "abnormal vacuolar" cells quickly perish.

Other Budding-Fungi.

(*Torula*, *Saccharomyces apiculatus*, *Mycoderma cerevisiæ* and *vini*.)

In the following pages we give a review of some other fungi, which are of more or less importance in the fermentation industries, and which resemble the *Saccharomycetes* in that they multiply by budding; these species develop a *mycelium* only exceptionally. On the other hand they are all distinguished from the *Saccharomycetes* by the absence of the property of forming endogenous spores which characterises the latter.

The forms examined by *Hansen*, and which produce a mycelium, must strictly be classed with the mould-fungi. Since, however, their position amongst the moulds has not yet been systematically determined, these species may, on practical grounds, be described in this place.

TORULA.

The yeast-like forms which *Pasteur* figured and described under the name *Torula*, are widely distributed and therefore not unfrequently occur in physiological analyses connected with fermentation. They occur in both spherical and more or less elongated forms, and are distinguished from the genus *Saccharomyces*, as was first pointed out by *Hansen*, in that they are unable to form endogenous spores. In most cases they multiply only by budding, in some few cases also by the formation of mycelium.

Hansen has observed many different species, and has described the following more in detail:—

The *first* occurs in wort, the cells being either single or in colonies of a few cells. Some cells have a large vacuole in the middle, and this sometimes contains a small strongly-refractive particle. The size of the cells varies considerably (1·5 to 4·5 μ). The species does not secrete invertase, and causes a scarcely perceptible alcoholic fermentation in beer-wort.

The *second species* has, under the same conditions, larger cells (3 to 8 μ) than the first; they resemble the foregoing, except that the contents of the cells grown in wort are often very granular.

The *third species* which, under the microscope, resembles the last, produces under the same conditions as much as 0·88 per cent. by volume of alcohol; it gives a distinct head with evolution of carbonic acid, but it cannot invert cane-sugar.

The *fourth species* (2 to 6 μ) inverts cane-sugar and produces a little more than 1 per cent. by volume of alcohol

in wort with considerable frothing; it does not, however, ferment maltose.

The *fifth species*, which in the form and size of its cells resembles the first, develops a uniform, dull grey film on wort

FIG. 50.

Torula, after Hansen: sedimentary forms after one day's cultivation in beer-wort at 35° C.

and yeast-water at the ordinary room temperature, likewise on lager beer and even on liquids containing as much as 10 per cent. of alcohol. It inverts cane-sugar and forms a slight film on the solution. It does not, however, excite any appreciable alcoholic fermentation.

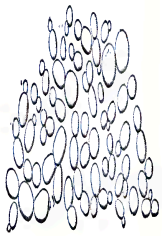

FIG. 51.

Torula, after Hansen: sedimentary forms after one day's growth in beer-wort at 35° C.

A *sixth species* (Fig. 50), which forms spherical and oval cells, gives a distinct fermentation in beer-wort, yielding as much as 1·3 per cent. by volume of alcohol. It does not ferment maltose solutions. It inverts cane-sugar and in 10 per cent. and 15 per cent. solutions of this sugar in yeast-water it yields respectively 5·1 and 6·2 per cent. (volume) of alcohol after fourteen days' cultivation at 25° C.; the last growth yielded 7 per cent. (volume) of alcohol after two

months. Dextrose solutions of the above concentration and under similar conditions gave 6·6 and 8·5 per cent. of alcohol by volume.

The *seventh species* (Figs. 51 and 52) was found in the soil under grape-vines. The sedimentary cells are most frequently oval and in part larger than those of the last species. The cells of the films are partly very irregular in form. This *Torula* produces only 1 per cent. (volume) of alcohol in wort, does not ferment maltose, and neither ferments nor inverts cane-sugar. In 10 per cent. and 15 per cent. solutions of dextrose in yeast-water it gives 4·6 and 4·5 per

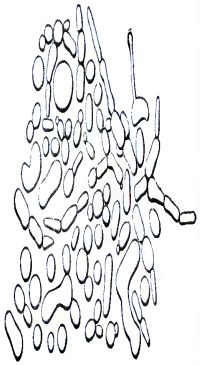

FIG. 52.

Torula, after Hansen; same species as Fig. 51. Film-formation on a wort culture ten months old.

cent. by volume of alcohol after 15 days at 25° C., and 4·8 and 4·7 per cent. after 26 days. In two other flasks 4·8 and 5·3 per cent. of alcohol had been produced after long standing. *Hansen* assumes that this species takes part in vinous fermentation, and considers it probable that species such as the sixth and seventh, which produce a vigorous fermentation in dextrose solutions, take part in the fermentation of grape-juice and other fruits. On the other hand they have probably little importance in breweries and distilleries since they are unable to ferment maltose.

Another species of *Torula* (*Torula Nove Carlsbergiæ*),

the cells of which exhibit very different forms, has been described by *Grönlund*. It imparts a disagreeable bitter taste to wort. According to *Schjerning's* investigations it inverts cane-sugar, and induces alcoholic fermentation in solutions of cane-sugar, dextrose, and maltose. In ordinary brewery-wort it can produce about 4·7 per cent. (volume) of alcohol.

Torula species which contain no invertase, yield only about 1 per cent. (volume) of alcohol and do not ferment maltose, are found widely distributed in nature. Those which have been examined ferment solutions of dextrose.

Related to the above are the red-coloured budding-fungi (the pink yeast of medicinal bacteriology) universally distributed in atmospheric dust; several species of these are known; *Kremer*, for instance, found in must a top-fermentation torula-yeast which produces a red colouring-matter soluble in water. It ferments dextrose and in a 10 per cent. solution it yields 4·5 per cent. by volume of alcohol; it inverts cane-sugar, directly ferments maltose, but has no action on lactose.

These different species cannot be distinguished by the microscope alone either from each other or from the round cells of the *Saccharomycetes*. *Pasteur* distinguished the *Torula*-forms from the other yeasts, because the species which he examined exited only a very feeble alcoholic fermentation. It will be seen, however, from the above that there are also species with pronounced fermentative activity.

Hansen assumes with some degree of probability that they are derived from the higher fungi, and in his cultivation experiments he has, as mentioned above, in a few cases observed the development of a *mycelium*.

Duclaux found a yeast-fungus in milk which induces *alcoholic fermentation in a solution of lactose*. A conversion of lactose into galactose was not observed. This fungus appears to be most closely related to the *Torula*

species. The cells are 1·5 to 2·5 μ in diameter, and almost spherical. According to *Duclaux's* experiments, this yeast is more aërobic than the ordinary alcoholic yeasts. Even with strong aëration of the liquid, the whole of the milk-sugar is used up in the alcoholic fermentation. In a 5 per cent. solution of milk-sugar 2·5 per cent. of alcohol was formed in eleven days at 23° C. The most favourable temperature for the fermentation of a neutral solution is 25° to 32° C., whilst at 37° to 40° C. the fermentation ceases. Small quantities of acid have a retarding influence on the fermentative activity of this yeast.

Adametz likewise describes a budding-fungus which ferments *milk-sugar*. Since this fungus does not yield endogenous spores by *Hansen's* method, it is classed in the group of *non-Saccharomycetes*. The cells are of about the same size as those of *Saccharomyces cerevisiæ*, and are spherical and elliptical. The colonies grown on peptone-gelatine are round with slightly jagged borders and are of a dark brown colour. A puncture-cultivation in wort-gelatine yields a dull, flat moss on the surface and a vigorous growth in the punctured channel, and from this numerous rays penetrate into the gelatine. In sterilised milk, this fungus induces fermentation phenomena within 24 hours at 50° C., in 48 hours at 38° C., and in about four days at 25° C. In this fermentation, the milk-sugar is alone decomposed.

Both of the species mentioned above have been more closely investigated by *Kayser*, together with a *new species* which likewise ferments lactose and also belongs to the *non-Saccharomycetes*. All three yield colonies on gelatine, which are more spread out than those of beer- and wine-yeasts; in the middle of the colonies there is a thicker portion, whilst the border resembles mycelium. In milk and in neutral liquids, when sufficiently aërated, they induce an appreciable fermentation at 25° to 30° C. The milk does not coagulate or become viscous during the

alcoholic fermentation. All three species ferment lactose, galactose, cane-sugar, glucose, invert-sugar, and finally maltose, but the last only with great difficulty. In the fermentation of milk-sugar with these yeasts, the resulting liquids are as rich in alcohol as the strongest beers. *Kayser* remarks that it may perhaps be possible to make practical use of this observation and by means of these fungi to convert the large quantities of whey, obtained in the manufacture of cheese, into an alcoholic liquor.

Beyerinck has described two yeasts which also ferment milk-sugar, and which must be provisionally regarded as *non-Saccharomycetes*; these are *Saccharomyces Kephir*, which occurs in kephir-grains and consists of longish, variously-formed cells, and forms slightly jagged colonies on gelatine; and *Saccharomyces Tyrocola*, which consists of small roundish cells, and forms snow-white colonies on gelatine. *Beyerinck* found that these two species secrete a particular invertive ferment (*lactase*) which inverts not only cane-sugar but also milk-sugar; it does not, however, invert maltose. Lactase can be prepared as follows:—A five per cent. solution of milk-sugar, containing also nutrient salts and asparagine, is fermented with kephir-yeast; the product is filtered and the ferment is precipitated from the filtrate by the addition of alcohol. According to *Schuurmans Stekhoven*, however, the enzyme of *Beyerinck's* kephir-yeast does not invert milk-sugar.

It was previously proved by *Bourquelot* that *Aspergillus niger* contains a soluble chemical ferment which has some similarity to the invertase of beer-yeast, but is distinguished from the latter by its property of converting maltose into dextrose. He points out, however, that two ferments may possibly be present, just as diastase is now regarded as a mixture of several ferments. A doubt of this nature will always arise when a soluble chemical ferment is found to vary in its action.

SACCHAROMYCES APICULATUS. REESS.

(Fig. 53.)

As already pointed out, the name of this ferment is incorrect according to our present views, for only those budding-fungi which yield endogenous spores are considered to belong to the *Saccharomycetes*, and the fungus in question does not possess this property. We will, however, provisionally retain the old generic name, as has been done by *Hansen*, until the systematic classification has been further developed.

As is known, this ferment was the subject of one of the finest and most thorough biological investigations of our time, for *Hansen* was enabled, after several years' work, to determine both its habitat in nature and its regular migrations at the different seasons of the year. The reason that this species was selected for the investigation was that, whilst other species occur in very varied and uncertain forms, which makes the study of their occurrence in different localities very difficult, this ferment can be recognised with certainty by its form, since it always occurs in cultures with lemon-shaped cells; this is the typical form of the species.

Sacch. apiculatus occurs abundantly in wine-yeast, especially during the first stages of the fermentation, also in Belgian spontaneously-fermented beer; in nature it is found abundantly on ripe, sweet, succulent fruits.

If a little of such a growth in a drop of nutritive liquid is examined under the microscope, the development of the fungus can be followed. This is, as was first pointed out by *Hansen*, very characteristic (compare Fig. 53). It is seen that the buds formed from the typical lemon-shaped cells may be either lemon-shaped (*a*, *b*, *c*, *e*, *f*) or oval (*a*—*c*); it is also noticed that the oval cells must first form one or more buds before they are able to assume the lemon-shape (*c*—*f*), and finally, that the lemon-shape of a cell attained by budding (*k*, *k'*, *k''*) may be lost again on the formation of a new bud (*k'''*). Under other conditions the cells can

FIG. 58.

Saccharomyces apiculatus, after Hansen. Budding cells: *a*–*a*‴, a cell which in the course of 2½ hours developed a bud at its lower extremity; *b*–*b*‴, a similar series, showing the development of a bud at the upper extremity of the mother cell, whilst a bud had been previously formed at the opposite end; *c* is a chain of cells, *c*′ is the same three-quarters of an hour later; the lowest bud had, like those above it, assumed the typical form of the species, but in the figure it is seen from the end, so that its longitudinal axis is at right angles to the plate of the paper; *d*–*d*‴, development during 1½ hours; *e*–*e*‴, during 2½ hours; *f*–*f*‴, during 9 hours; in *e*–*f*, it is seen that the oval cells first develop a bud and only subsequently assume the typical lemon-shape; *g*–*m*, abnormal cells and series of development.

assume quite different forms, sausage-shaped, half-moon-shaped, bacteria-like, &c. (*g*–*m*). Is there now any rule in this apparent confusion of forms? It was shown above that the fungus can form two kinds of buds, and that the oval buds must develop one or more new buds before they can assume the typical form. The question then is: Under what conditions are those two kinds of buds developed? It was shown by means of culture experiments that the lemon-shaped buds are developed especially during the first stages of the culture, but are afterwards crowded out by the oval forms.

We will now give a further description of the fungus from a physiological and biological standpoint.

Saccharomyces apiculatus is a bottom-fermentation yeast, which is capable of exciting alcoholic fermentation in beer-wort; the fermentation in this liquid is, however, a feeble one, only 1 per cent. (volume) of alcohol being produced, whilst *Saccharomyces cerevisiæ* (bottom-yeast) under the same conditions gives 6 per cent. by volume of alcohol. This arises from the fact that *Sacch. apiculatus cannot ferment maltose*. *Hansen* also found that it *does not secrete invertase*. On the other hand it excites a vigorous fermentation in 15 per cent. and 10 per cent. solutions of dextrose in yeast water, and in one experiment as much as 3 per cent. (volume) of alcohol was formed. After three months the liquid still contained sugar, whilst the amount of alcohol had not increased during the last one and a half months. The fungus was thus unable to complete the fermentation. In another of *Hansen's* experiments as much as 4·3 per cent. (volume) of alcohol was produced.

It was found from experiments, in which a mixture of this fungus with *Saccharomyces cerevisiæ* was grown in beer-wort, that, being the weaker species, it was crowded out by the latter, although it retarded the *Sacch. cerevisiæ* to no small degree.

In flasks with the same beer-wort, and at the same tem-

perature, and each containing one species, *Saccharomyces apiculatus* will multiply to a greater extent than *Saccharomyces cerevisiæ* in a given interval of time.

At the critical time of the year, the ferment, if present in the wort in considerable quantities, may exist for a length of time, side by side with *Saccharomyces cerevisiæ*, and will no doubt retard its action a little; but when the beer is transferred to the lager cellar, the fungus remains inactive in the alcoholic liquid and frequently perishes.

The most interesting phases in the life of this ferment are the *conditions of its occurrence in nature*, which have also been explained by *Hansen*. Microscopic investigations and culture-experiments showed that in the summer the ferment was *abundantly developed on sweet, succulent fruits* (cherries, gooseberries, strawberries, grapes, plums, &c.) *during their ripening*. On the contrary it was only very exceptionally found on the same fruits so long as these were unripe. Since it is found in a vigorous condition of budding on the above-mentioned ripe fruits, but is never or only exceptionally found on other fruits, leaves, twigs, &c., it is perfectly clear that *Saccharomyces apiculatus* has its true habitat on the ripe fruits named. This was also further proved by the fact that it always, without exception, occurs in the soil under the cherry trees, plum trees, vines, and other plants on the fruits of which it is found; whilst, on the other hand, it was only extremely seldom that it was found in the numerous samples of soil from other and most diverse localities. The ferment is brought on to and into the earth at such places by the fallen fruit and by the rain, and the question then arises does it also winter there? The answer was obtained in two ways: partly by taking numerous samples of soil from these places during winter and spring—these, when introduced into flasks containing wort, gave in by far the greater number of cases a vigorous growth of our ferment

—partly by introducing, with every precaution, cultures of *Saccharomyces apiculatus* into the soil and leaving them during the winter. In the spring and early summer the soil was again examined, and culture experiments proved that the ferment was still alive in all the samples. Thus, it was proved that the ferment *can* winter in the soil, and it was also previously shown that it practically *only* occurs at the stated places in the soil. In some more recent experiments of *Hansen's*, vigorous growths of the ferment in well-closed Chamberland filter-tubes were placed below the surface of the earth. After three years, the contents of the tubes were introduced into sterilised wort, and a vigorous development of the ferment was obtained. The period of its life can thus extend beyond a year.

Finally, it remained to be proved that the soil is its *true* habitat during the winter; in order to prove this, *Hansen* examined the dust from the most diverse places from January to June, also the dried fallen fruits of many plants, and finally also various excrements. The seventy-one analyses gave a negative result, and thus furnished the proof that *the true winter habitat of the ferment is the soil under the previously mentioned plants*. It retains its ordinary appearance during the long winter-time, and in the summer it is again carried into the air by the united action of insects and the wind, and, through these two means of transport, it becomes further distributed from fruit to fruit.

It is evident that at the time when the ferment appears in abundance on the ripe fruits mentioned, it may also be carried by the wind to other places, and thus also on to unripe fruits. Even in his first memoir, *Hansen* stated that the reason of *the rare occurrence on unripe fruits* might be that the ferment *quickly perishes, partly from want of nourishment and partly from the drying up of its cells*. Subsequently he proved by experiment the correctness of this view. He stirred up with water partly old and partly young cells, and placed them in thin layers, either on object-glasses

or on thin pulled-out tufts of cotton-wool, which were then allowed to dry, protected from the sun. In less than twenty-four hours all the cells had perished. It is self-evident that the isolated cells lying on the unripe fruits are still more unfavourably placed than in the experiment. If, however, thick layers of the cells are wrapped in cotton-wool or filter-paper, they will continue to live for a long time, as they do in the earth—in filter-paper, for instance, over eight months.

No complete investigations have been published on the life-history of other alcoholic ferments. *Saccharomycetes occur very generally on fruits containing a sweet juice.* For several years *Hansen* has carried on experiments similar to those described, with species of *Saccharomycetes* which often occur in fruit gardens, namely *Sacch. Pastorianus I.*, *Sacch. ellipsoideus I.*, also with Carlsberg bottom-yeast No. 1, and with some top-fermentation beer-yeasts. He always found that the yeasts *sown in the soil in September were still alive after a year.* Some species had formed spores at the surface of the soil. Further experiments will probably show that the true *Saccharomycetes* also have their habitat on fruits during the summer and in the soil during the winter.

In antithesis to these direct observations of *Hansen*, is *Pasteur's* statement that the wine-yeasts are unable to live in the soil during the period from one season to the next. Where the yeasts come from which are found on the grapes at the time of ripening, *Pasteur* was unable to say.

Mycoderma cerevisiæ and vini.

It is characteristic of these species that they very readily form films on various alcoholic liquids. Under the above names are included a number of different species, some of which can excite a feeble alcoholic fermentation; they behave differently towards lager beer, some causing disease whilst others do not.

The *Mycoderma cerevisiæ* (Fig. 54) examined by *Hansen*, and which is very generally met with in the Copenhagen

breweries, forms variously-shaped cells. The cells are usually transparent and less refractive than the true *Saccharomycetes*; in each cell there are generally one, two, or three highly refractive particles, which often have a quivering, rolling motion. This micro-organism forms a dull, greyish, wrinkled film on wort and beer, and does not excite alcoholic fermentation; neither does it invert solutions of cane-sugar.

The colonies on the surface of the gelatine are *bright, grey, dull*, and *spread out like a film* or *hollowed like a shell*. By means of this macroscopic appearance *Mycoderma* is readily distinguished from the ordinary *Saccharomycetes*

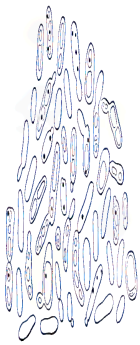

FIG. 34.

Mycoderma cerevisiæ from Copenhagen breweries. Drawn from nature by *Holm*.

which, on the same medium, form bright greyish-yellow colonies with a dry or lustrous surface, and of a more or less arched form. *Saccb. membranæfaciens* (page 177), which differs so markedly in its biological behaviour, and which very rapidly gives a strong film on the liquid, alone resembles *Mycoderma* also in its behaviour on plate cultures.

The form of film described above was obtained by *Hansen* when lager beer was exposed in open vessels at temperatures between 2° and 15° C.; at 33° C. a development still occurred, but at temperatures above 15° C. this species gave place more and more to competing forms. Therefore, since low temperatures are favourable to its development, it will

readily thrive in the storage cellar, especially as lager beer forms a much more favourable medium for its growth than wort. This is seen to be the case when traces of a pure film are introduced into lager beer and wort contained in open vessels and left to develop; the culture in the lager beer nearly always remained pure, whilst in the wort various other species made their appearance.

In *Hansen's* comprehensive series of experiments on Carlsberg beer, it was always found that both lager and export beers were attacked by this fungus; but there was never the smallest sign that the beer had acquired any disease from this source. The fungus was widely distributed just at those periods when the beer was found to be particularly stable and of good flavour. This has also been confirmed by numerous experiments on lager and export beers conducted by *Grönlund* and *A. Petersen*, and those carried out in the laboratory of the author. It is self-evident that we are here only speaking of beer which has been properly treated. In imperfectly closed bottles and casks *Mycoderma cerevisiæ* will of course rapidly develop a film, which alone is sufficient to destroy the product.

Bělohoubek was the first who found that under certain conditions *Mycoderma* can cause considerable injury in the brewery. Subsequently *Kühle* described a curious cloudiness in lager beer, which had the appearance of a cloud of fine dust in the liquid, and which manifested itself either during storage or after tapping; he attributes this disease to *Mycoderma*, and he further assumes that it is the weak wort and also a particular composition which specially favour the development of *Mycoderma*. It is to be hoped that further investigations will throw more light on this subject.

Hansen had previously expressed the opinion that the name *Mycoderma cerevisiæ* denotes not one, but several different species, and *Lindner's* experiments have confirmed this. The latter investigator describes four different species which he isolated from cloudy beers. They are distinguished

from the species described by *Hansen* by the fact that they produce alcohol in beer-wort; one yields 0·26 per cent. by volume, two yield 0·79 per cent., and the fourth produces 2·51 per cent. *Laiché* concludes from his experiments that these four species cause diseases in beer, namely, turbidity and changes in taste and odour; in this respect they also differ from *Hansen's Mycoderma*. *Laiché* is inclined to assume that the chemical composition of the wort has no influence on the disease caused by *Mycoderma*, since, in his experiments, the disease was produced in worts of high extract and in worts of low extract, in worts which were rich in sugar and in worts containing little sugar.

It is frequently stated that the chemical activity of certain species of *Mycoderma* on the surface of vinous liquids is a process of oxidation by which alcohol is converted in some cases into carbonic acid and water, in others into acetic acid; fatty acids are also said to be formed, and these are oxidised and ethereal salts produced (*Schulz*).

Winogradsky found that the *Mycoderma* occurring on *wine*, prepared in pure culture by *Hansen's* method, *changes its form with the composition of the nutritive solution*; he experimented partly with solutions, the mineral constituents of which remained constant whilst the organic substances varied, and partly with solutions in which the reverse was the case.

Although *De Seynes*, *Reess*, *Engel*, and *Cienkowski* claimed to have found ascospores in *Mycoderma*, it has not since been possible to bring about this formation. It would appear from the figures given that the fat globules which occur in many unicellular fungi during the resting stage had been mistaken for spores; in some cases the mistake appears to have arisen through the presence of an admixture of true *Saccharomycetes*. The old name *Mycoderma* is therefore more appropriate to this fungus than the new term *Saccharomyces*.

CHAPTER VI.

The Application of the Results of Scientific Research in Practice.

It is universally acknowledged that the process of fermentation plays a very important part in all branches of the fermentation industries. The better insight which has been gradually gained in this direction has been brought about through the development of the science of the organisms of fermentation. This gradual development may be divided into three great periods.

The investigations of the *first* period all relate to the important question, whether living organisms can come into existence by *spontaneous generation*. The *second* period is noted for *Pasteur's* classical researches. In the *third* period, dating from 1879, and which was founded by *Hansen*, a reform was for the first time carried out.

1. The first period (1745 to 1857) gave rise to the *theory of sterilisation and its foundation in practice* (see page 9).

Spallanzani's discoveries in connection with spontaneous generation formed not only the starting-point of modern bacteriology (compare p. 10), but they also acquired great importance in practical life. In 1782 *Scheele* proved that vinegar can be preserved unchanged after it has been heated, and *Appert* (1810) likewise showed that beer, wine, and other liquids can be preserved by similar treatment. It was further shown that air can be purified by passing it through a strongly-heated tube (*Schwann*) or through a cotton-wool

filter (*Schröder* and *Dusch*). From this, the result was also arrived at that water can be purified by a similar process, provided the filter is sufficiently dense.

2. The *Pasteur* period dates from 1857. The great merit of this investigator was that he proved that bacteria exert a disturbing influence in different fermentations, and that they can produce diseases in liquids which are undergoing alcoholic fermentation.

It is therefore necessary to proceed in such a manner that infection of this nature is avoided, and this is best attained by preventing the access of impure air to the liquids. The consequence of this doctrine—as regards the brewery—is the abandonment of open coolers and refrigerators, the aëration of the wort by air which has been previously sterilised, and the purification of the air in the fermenting-rooms.

The statements in Chapter VII. of Pasteur's "Études sur la bière" (1876), regarding the importance of the *oxidation of the wort* during cooling, are also of practical value. By means of direct determinations of the amount of oxygen in the wort, *Pasteur* showed that a certain quantity of oxygen, partly in the free state, and partly combined in the wort, exerts an influence on the course of the fermentation and on the brightening, but that when the proportion of oxygen in the wort exceeds certain limits it can act injuriously on the character (*force et arome*) of the beer (page 377).

Although several investigators have undertaken elaborate researches in this direction, it has not hitherto been possible to establish any fixed rules for practical guidance. These must be determined by trial experiments for each individual case.

Scheele's and *Appert's* method for the treatment of vinegar, wine, and beer, at elevated temperatures, was taken up by *Pasteur*, and through his great authority obtained a wide application (the so-called Pasteurising). Recently milk has also been treated in this manner, especially since *Koch* proved that the tuberculosis bacillus is so widely distributed.

The experiments on *aëration* described in "Études sur la bière" gave rise to extensive series of investigations which yielded valuable information on the *fermentative* and *reproductive* power of yeast in the presence of varying amounts of air. This relation plays an important part in the *distillery* and in *pressed-yeast works*. No results, however, have as yet been obtained, which can be directly applied in practice.

The reason why the method proposed by *Pasteur* for the purification of yeast has acquired no real importance for practical purposes, has been already stated (page 24).

3. With *Hansen's* investigations on the alcoholic ferments there began, as *Amthor* says, a new era in the history of the fermentation industries. In the year 1883, *Hansen* demonstrated that the universally dreaded yeast-turbidity, and likewise the disagreeable changes in taste and odour, in fact some of the commonest and worst *diseases of beer*, are not caused by bacteria, by the water, by the malt, by the particular method of brewing, &c., as was then commonly believed, but that these diseases *must be attributed to the yeast itself*; for in such cases the pitching-yeast contains, in addition to the cultivated species, *other Saccharomycetes, which act as disease germs* (*Sacch. Pastorianus I. and III., Sacch. ellipsoideus II.*). *A basis was thus formed for the new system.*

He subsequently showed that the *name Saccharomyces cerevisiæ embraces many different—both bottom- and top-fermentation—races or species*, which communicate very different characters to beer.

As the rational result of these scientific investigations he completed the third link of his new system—his method for the *pure cultivation of yeast*. If it were possible to free the impure yeast mass from wild yeasts as well as from bacteria and mould-fungi, we should still not attain all we desire; for *if the purified yeast contains several species of Saccharomyces cerevisiæ, we are still dealing with a mixture which*

as just as uncertain as before the purification, and, in addition, the composition of such a yeast-mass is always liable to change during fermentation. In fact, *Hansen* has shown in recent investigations that cases occur in which *two yeasts, each of which by itself gives a faultless product, will when mixed give rise to disease in the beer*. He made these experiments with the two species of Carlsberg bottom-yeast No. 1 and No. 2 (see page 184); in one set of experiments the pitching-yeast consisted chiefly of No. 1 with a small admixture of No. 2, and in the other set, the reverse was the case. It was found that in all cases the small quantity of the admixed yeast, whether No. 1 or No. 2, made the beer less stable as regards yeast turbidity, than when the chief constituent of the pitching-yeast was employed alone. Thus the two cultivated yeasts under these conditions behaved in such a manner as to produce effects similar to those brought about by the wild yeasts (*Saech. Pastorianus III.* and *Saech. ellipsoideus II.*). We can therefore only obtain true *uniformity in working* when *a suitable species* has been obtained from the yeast-mass *by systematic selection, and cultivated by itself* (compare pages 26–32).

The systematic studies which *Hansen* has carried on for many years on the *constancy of the characters of different species of yeast*, have proved that, under the conditions of the brewery, they only undergo slight changes, which are of no importance in practice, and this result has been confirmed by various investigators.

On the other hand, he found that, when the conditions of the life of the yeast are disturbed by a systematic and more vigorous treatment, it was possible *to produce varieties* (see page 151), which remained more or less constant in their properties, and even to produce new species. As a result of these investigations *Hansen* obtained useful varieties of some brewery yeasts.

The biological and physiological characteristics of the species discovered by *Hansen* led him also to a *method*

for the practical analysis of brewery yeast (page 134), by means of which it is possible to ensure in time against foreign yeasts prevailing. It was previously proved by his experiments on a large scale that the forms which produce yeast turbidity may be present to the extent of one part in forty-one of the pitching-yeast, and the species (*Sacch. Pastorianus I.*) which produces a disagreeable odour and an objectionable bitter taste to the extent of one part in twenty-two, without exercising any injurious influence, provided the brewing operations are conducted under normal conditions. It has been found, however (by the experiments of *Holm* and *Poulsen*), that it is possible, by *Hansen's* analytical method, to detect with certainty the presence of 1-200th part of wild yeast.

From numerous analyses carried out by this method, it has been shown that the rules which were formerly generally accepted for judging a sound fermentation do not suffice for determining the presence of disease-germs, since both the head of the liquid, and the attenuation, breaking, and brightening may be satisfactory in spite of the yeast being strongly contaminated with disease-germs.

The question—how long will a pure culture remain in its original good state?—can evidently not be answered in a general way. *Hansen* found that different races *differ in their power of resisting infection*; likewise the length of time during which a yeast will remain pure and good will vary for the same species in dissimilar fermenting rooms. We also know that the season plays an important part, and that the time of year when wild yeasts, bacteria, and moulds are most abundant in the air, is especially dangerous. Infection is also known to occur at other times of the year, especially from utensils, &c.; disease-germs often gain admission to the brewery through the open coolers; cask sediments form another source of contamination. Most frequently, however, brewers introduce disease-germs into the fermenting vessels with the pitching-yeast which they obtain from other

breweries. The analysis will, however, always indicate infection long before it has become dangerous, so that a new, pure cultivation of the same yeast can be introduced in good time. A still greater certainty is attained by the continuous working of the yeast-propagating apparatus described below. The main result achieved is that we now *no longer proceed hap-hazard, and are not compelled to leave the fermentations to take their chance, as was formerly the case.*

Since the various species of yeast differ in their power of resisting competing disease-germs, it is in many cases of great importance to be able, at short intervals, to introduce into the brewery a considerable quantity of a suitable and absolutely pure species of yeast, which has been previously selected by systematic experiments. This object is attained by means of the *yeast-propagating apparatus* devised by *Hansen* and *Kühle*, and which, when once charged with an absolutely pure cultivation, will work continuously for years. The apparatus (Fig. 55) is described in detail with directions for use in *Hansen's* "Untersuchungen aus der Praxis der Gärungsindustrie"[1]; it consists of three principal parts with the connecting tubes, namely:—1, the arrangement for aërating the wort, consisting of the air-pump (*A*) and air-vessel (*B*); 2, the fermenting-cylinder (*C*), and 3, the wort-cylinder (*D*).

The air, which is partially purified by previous filtration, is pumped into the air-vessel, from which it can be passed to the wort-cylinder or to the fermenting-cylinder. In both cases it has to pass through sterilised cotton-wool filters (*g*, *w*). The *wort-cylinder* is connected by piping directly with the copper, from which the hopped wort is run boiling hot into it; it is then aërated in the closed cylinder and cooled by water from *p*. The wort is then forced into the *fermenting-cylinder*, which, like the wort cylinder, is constructed on the

[1] Published by R. Oldenbourg, Munich: 1st part, 2nd edition, 1890; 2nd part, 1892. In this work will also be found a description of *Hansen's* complete system.

same principle as the ordinary two-necked flask. It is fitted with a doubly-bent tube (*c*, *d*), which dips into a vessel containing water, a vertical glass tube (*f*, *i*, *f*) for measuring the height of the liquid in the cylinder, an appliance (*b*, *b*) for

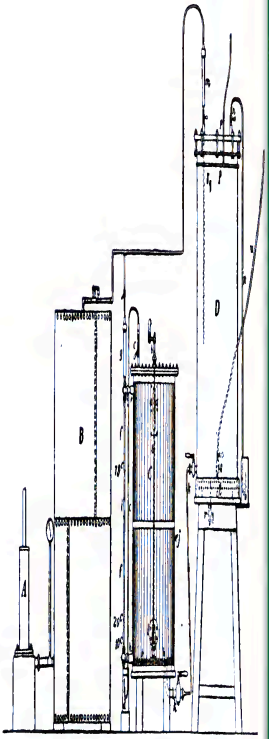

FIG. 33.

Yeast-propagating apparatus devised by *Hansen* and *Kühle*: *A*, *air-pump*; *B*, *air-vessel*; *C*, *fermenting-cylinder*; *a*, window; *b b b*, stirrer; *c c*, doubly-bent tube; *d*, vessel containing water; *l*, cock for drawing off the beer and yeast; *f f*, glass tube connected at *c* and *k* with the cylinder, and graduated for the measurement of fixed quantities of liquid; *g*, filter; *i*, india-rubber tube placed at the middle of the glass tube; *j*, tube with rubber connection for introducing the pure culture; *k k*, connection with the *wort-cylinder D*; *m*, filter; *n n*, doubly-bent tube; *o*, vessel containing water; *p p*, coil water tube; *q*, tube with cock (*r*) for introducing wort; *t*, outlet-tube for the water used in cooling; *y*, cock (the wort is allowed to rise to this height); *r*, cock for drawing off wort.

stirring up the settled yeast, and a cock (*i*) for drawing off the beer and the yeast. At about the middle of the cylinder there is a small side-tube (*j*) fitted with india-rubber connection, pinch-cock, and glass-stopper. When a portion of the wort has been forced into the fermenting-vessel, the pure yeast—which is forwarded to the brewery in a flask specially constructed for this purpose—is introduced through the rubber tube at *j*; this is again closed, and the remainder of the wort may then be added either at once or after the lapse of a few days, according to the quantity of yeast which has been introduced.

Where it is necessary to regulate the temperature during fermentation, the fermenting-vessel is surrounded by a copper water-jacket.

By means of this simple apparatus it is possible to obtain, at short intervals, absolutely pure pitching-yeast sufficient for about eight hectoliters of wort. As already stated, the apparatus, when once started, works continuously. For further details I refer the reader to the exact description in *Hansen's* work mentioned above.

A modification of the propagating apparatus has been devised by *Bergh* and *Jörgensen* (Fig. 56). The filtered air passes through the three-way cocks at *A*, *B*, and *C*, into the two cylinders *A* and *B*. The upper cylinder holds about 50 liters, and the lower cylinder 160 liters. *A* is provided with a stirrer (*E*), a tube (*a*) for introducing the yeast and withdrawing samples. The bent tube *F* is for the exit of the carbonic acid. The tube *G P* connects the two cylinders, and the connection can be made or unmade by means of the cock *G*. *H* is the outlet for the water used in cleaning *A*.

The cylinder *B* is surrounded by a cast-iron jacket made in two parts; the upper portion serves as a water-jacket for cooling the wort and for regulating the fermentation; the lower portion is used as a steam-jacket, and is provided with a cock at *O* for the entrance of the steam, and another at *S* for the outlet. *M* is a ring-shaped tube provided with small

holes; this is connected with the cold-water main during the cooling of the wort; the water flows out at *N*. The stirrer *J* is set in motion by means of toothed wheels. The height of the liquid in the cylinder is indicated by means of a float,

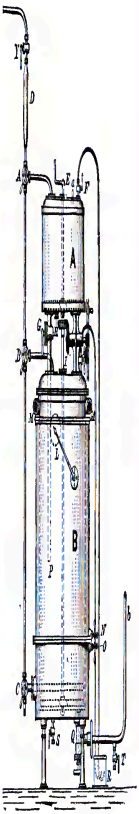

Fig. 58.

Yeast-propagating apparatus devised by *Borgh* and *Jörgensen*.

connected with which is a pointer and arc *L*. Connected with the top of the cylinder is the bent tube *K*. At the bottom is the cock *Q*, which is in connection with the pipe *b*. Both the bent tubes dip into the vessel *R*, which is filled with water.

The wort is introduced into the lower cylinder, where it is treated in the ordinary manner. The pure culture is introduced into the upper cylinder, and is then washed down into the lower cylinder by means of a little wort, which is forced from *B* into *A*, and then back again into *B*. When a vigorous multiplication of the yeast has set in, the liquid is stirred up and a portion forced into *A*; this is to be used to start the next fermentation. The cylinder *B* thus serves alternately as fermenting-cylinder and wort-cylinder.[1]

Other modifications have been devised by *Brown* and *Morris*, *Elion*, *Kolosinski*, and *Van Laer*; more widely different are the forms devised by *P. Lindner* and *Mayr*.[2]

In order to be able to send to a distance the selected *pure cultures in a liquid condition*, a special form of flask was devised by *Hansen*. The yeast can be sent to great distances in these flasks, and there is no difficulty in safely transferring it from the flask to the cylinder of the propagating apparatus.

In sending small quantities of pure cultures, and in such a manner that they may be safely and readily employed for further *cultivation*, the small *Hansen flasks* (page 20) are employed. They are connected, in the flame, with the Pasteur flask in which the pure culture has developed. A

[1] Both the above described forms of apparatus are manufactured by Messrs. *Burmeister* and *Wain*, of Copenhagen; *Hansen* and *Kühle's* apparatus is also made by *W. L. Jensen*, of Copenhagen.

[2] An apparatus which has now become of considerable importance as a link in *Hansen's* system of pure yeast cultivation is the *closed cooler* mentioned above, by means of which it is possible to introduce the wort into the fermenting-vessel absolutely pure and properly aerated. Appliances having this for their object were devised by *Velten* shortly after the publication of *Pasteur's* "Études," and were constructed in accordance with *Pasteur's* theoretical views, but hitherto they could not acquire any practical importance; for what was the use of having a pure wort when the disease-germs were again introduced with the yeast? The conditions which render such appliances useful are only now attained through the introduction of pure yeast, and the open coolers will therefore gradually disappear in the future.

trace of the yeast is transferred to the cotton-wool, and the flask is again closed in the flame with the asbestos stopper, which is then coated over with sealing-wax. When the culture is to be used, the flask is again connected with a Pasteur flask containing wort, and the yeast is rinsed into the latter.

This method has proved especially valuable for sending absolutely pure yeast to tropical countries. In many cases it would otherwise have been impossible to send pure cultivations to Australia, South America, and the most distant countries of Asia.[1]

It is a fact of the greatest importance, that even after the lapse of years, *the perfectly identical yeast once selected can always be had again*, a sample of the pure culture being preserved in the laboratory in a 10 per cent. solution of cane-sugar (page 20). In such a solution, the cultivated yeasts can be kept alive and unchanged in their properties for years.

With regard to the preservation of micro-organisms on solid substrata, *Percy Frankland* found that bacterial ferments sometimes completely lose their fermentative power after repeated cultivation in solid media. In some cases the fermentative power disappeared after a single plate-cultivation.

The absolutely pure and systematically selected races of yeast, prepared in large quantities for industrial purposes, are now—*Hansen* having made his first experiment in 1883

[1] The use of sterilised filter-paper for sending yeast samples is for quite a different object. This method is used either for sending an impure brewery-yeast to a laboratory in order that a pure culture may be prepared from it, or a sample of a pure culture may be conveniently sent in this manner, enclosed in an envelope; it is clear, however, that a sample sent in this way can no longer be depended on as absolutely pure, and the sample can therefore only serve as material for a new pure cultivation.

in the well-known Old Carlsberg brewery at Copenhagen—employed in numerous breweries in all beer-producing countries, not only in Europe, but also in America, Asia, and Australia.

Since *Hansen's system* was first carried out in a bottom-fermentation brewery, it naturally first found application in breweries of the same kind, and it is here that it has reached the highest degree of perfection. *Any one who wishes to become acquainted with the application of this system* to one or the other branch of the fermentation industries, should therefore *take as the starting-point of his studies the results achieved in bottom-fermentation brewing.*

The advance was subsequently introduced into *top-fermentation brewing*, and it was found that here also the system offers the same advantages. At first the same objection was again raised which had been brought forward against pure cultivations of bottom-yeast, namely, that it could not produce a secondary fermentation, as the latter was supposed to be caused by the so-called wild species of yeast; the objection, however, again fell through when the question was submitted to the practical test of experiment. *There are, in fact, top-fermentation yeasts which give a vigorous secondary fermentation, and others which produce only a feble after-fermentation. A species should therefore be systematically selected which will answer the required conditions.*

The new system afterwards continually spread, and has now forced its way into *distilleries, pressed-yeast factories*, and into the different *vinous fermentations.*

Hansen's epoch-making investigations of the last decade have given rise to a very extensive literature, the contents of which may be summed up under three headings:—Treatises of a purely ephemeral nature, the only object of which is clearly to oppose the work of the Danish investigator and to bring obstacles against the introduction of

this reform into science and practice; other treatises, which discuss the subject, but which show a misunderstanding of the separate links or of the kernel of the system; and, finally, a number of valuable investigations, the object of which is to throw light on the system from various sides and thus to facilitate a true conception and the practical application of the system.

It would lead us too far to discuss these different sides of the literature. In concluding this description, I will confine myself to a few quotations from the highest authorities who have thrown light on the subject from various points of view, and have merited the greatest praise in helping the extension of the system in different countries.

Professor *Lintner*, sen., gives the following review of the situation in 1885[1]:—

"Now that different breweries have employed pure cultivations of the Carlsberg yeasts, and that the Scientific Station at Munich has also introduced pure cultivations of Munich yeasts into various breweries, the results obtained may be summarised as follows:—

"1. By contamination with so-called wild yeasts, a brewery-yeast, normal in other respects, may gradually become incapable of producing a beer of good flavour and with good keeping properties.

"2. A contamination of this kind can occur through wild yeasts present in atmospheric dust during summer and autumn, or the wild yeast may be introduced with the pitching-yeast or with cask sediment.

"3. By means of *Hansen's* methods of analysis and pure cultivation, it is possible to isolate from a contaminated yeast, the desired brewery-yeast in a good and pure condition.

[1] Zeitschrift f. d. ges. Brauw., 1885, p. 393.

"4. The pure cultivated yeast possesses in a marked degree the properties of the original yeast previous to contamination, both as regards the degree of attenuation, and the taste and keeping properties of the beer.

"5. Different races of normal bottom-fermentation yeast (*Sacch. cerevisiæ*) exist with specific properties, which are constant for each race and form distinctive characteristics."

Professor *Aubry*, director of the Scientific Brewing Station at Munich, wrote (1885)[1]:—"In addition to the breweries mentioned (Spatenbräu and Leistbräu in Munich), a large number of breweries at home and abroad have carried out experimental fermentations with pure Carlsberg yeast. The results which were expected were naturally not attained in all cases, the degree of attenuation was found to be too low in the greater number of cases,[2] the taste was not the one locally desired, &c., &c., but *all the reports which reached us were favourable as regards the keeping properties, brilliance, and the freedom of the beer from any taste of yeast.* The good properties of the yeast have brought about its permanent introduction into many breweries, as, for instance, the Liesinger brewery, at Liesing, near Vienna. In the present brewing season the Spaten brewery in Munich has made extensive use of yeast obtained from Carlsberg, and a great part of the pitching-yeast used in the brewery of the Franziskanerkeller in Munich, during the winter, was also derived from pure cultures of Carlsberg yeast. The course of fermentation and the results with regard to the taste, condition, and keeping properties of the beer, answered all requirements. The property of giving a somewhat low attenuation appears to be characteristic of the yeast, for it remains constant. The taste of the beer at first differs somewhat from the

[1] Zeitschrift f. d. ges. Brauw., 1885.

[2] Carlsberg bottom-yeast No. 2, a quick clarifying species.

ordinary Munich taste, but approaches more nearly to this with later generations; it remains, however, soft and agreeable.

Dr. Will writes (1885)[1]:—"If now, as I trust I have made clear, it is possible to detect with certainty the species of yeast which have an injurious influence in the brewery, we must make practical use of this knowledge, and only employ pitching-yeasts which do not show the above-mentioned characteristics for the injurious species which so frequently and actively exert a disturbing influence in the brewery. This, however, will only be possible when yeast cells endowed with the properties of normal bottom-yeast are isolated from the ordinary brewing yeast, and further cultivated with the exclusion of every contamination; in other words, *when only pure cultivated yeast* is employed in the brewery. *Hansen* is entitled to the greatest praise in this particular direction, since he has pointed out a way and devised a method which enabled him to attain the desired end. The far-reaching results which were obtained in Old Carlsberg with pure cultivated yeast have already caused many other breweries to employ only pure cultivated yeast, and the results in general have given satisfaction when varieties of normal bottom-yeast were chosen which corresponded with the requirements as regards attenuation and taste.

"It is to be hoped, therefore, that the value of pure cultivated yeast may become recognised in ever increasing circles, and many old prejudices regarding the yeast overcome; also that the smaller breweries, which have besides many difficulties to contend with, will not resist the conviction that a number of calamities may be avoided by the introduction of pure cultivated yeast into an otherwise well-conducted brewery. The amount expended will yield a liberal interest."

Dr. Reinke, who is at the head of the experimental

[1] Allgem. Brauer- und Hopfenzeitung, 1885.

brewing station of the Royal Agricultural College in Berlin, made the following significant statement of the situation in 1888[1]:—

"Without the exact study of *Hansen's* pioneering investigations, and without their utilisation, no one at the present time is able to permanently resist the competition in the brewing industry. *Hansen's* researches have brought about a revolution in the brewery, especially with regard to the treatment of the yeast."

Professor *Bělohoubek*, of the Bohemian Polytechnic at Prague, says in his well-known biography of Hansen, 1889[2]:

"No one will be surprised that the establishment of the principle of pure yeast-cultivation, and the truly crushing criticism concerning the general custom of leaving fermentations in the brewery to chance which until then prevailed, and, above all, that the actual introduction into the brewery of pure cultivated yeast prepared by *Hansen's* method, produced at first astonishment amongst practical men—with some honourable exceptions—then ridicule, and finally provoked hostile opposition; for it is known to the initiated what obstinate conservatism there is in brewing circles, where all innovations and reformatory efforts are not only met with passiveness and mistrust, but are sometimes most tenaciously resisted. Fortunately many important factors were united in the struggle against the opposition, which finally suffered a decided defeat in spite of the support of some theoretical specialists, more particularly in North Germany and Austria-Hungary. It was chiefly the correctness of *Hansen's* views which contributed to this victory, and which completely convinced the most eminent authorities of Europe on the science of fermentation; secondly, the fact that able experts also outside Denmark began to experiment with pure yeast-cultivation; thirdly, the

[1] Chemiker-Zeitung, 20 Dec., 1888, p. 1749.
[2] Zeitschrift f. d. ges. Brauw., Munich, 1889, p. 605.

highly favourable results which were obtained in the brewery with pure yeast; and, finally, also the fact that in 1887 Professor *Hansen*, in conjunction with Captain *Kühle*, succeeded in devising a pure yeast apparatus which enabled them to produce large quantities of the pure yeast which had been prepared on a comparatively small scale in the laboratory. At the present time hundreds of breweries obtain a pure cultivated standard yeast from institutions in which pure cultivations of beer-yeast are prepared, and thousands of breweries do the same indirectly in that they obtain their pitching-yeast from the above breweries. *The universal employment of pure yeast in the brewing industry is therefore now only a question of time.*

"If we now weigh with the most complete objectiveness the significance of these facts as applied to the conditions obtaining in European bottom-fermentation breweries, we are compelled to acknowledge that the reform introduced by Professor *Hansen* is still more far-reaching than is generally assumed. A result of this reform is already being discussed in brewing circles, namely, the abandonment of open coolers in all breweries where pure yeast is employed, as these freely permit of the contamination of the wort with micro-organisms and especially with bacteria and the so-called "wild" yeasts. It is therefore proposed to filter the hopped wort, or to separate the suspended matter (cooler-deposits) by another method, to saturate the wort with filtered air, and to cool it by artificial means. But these are by no means all the precautions which must be adopted in order to guard against further infection of the wort in the fermenting-rooms and in the lager-cellar. Only when these questions have been solved—perhaps by means of closed fermenting-vessels of a suitable material, by the sterilisation of the fermenting and storage vessels, by a more rational arrangement of the fermenting-rooms and lager-cellars, and by the ventilation of these by means of filtered air, &c.—only then will it be possible amongst beer producers and consumers to enjoy to

the full the great advantages of having a beer of better quality and keeping properties than the present beer, advantages which are a result of the employment of pure yeast.

"The above statements concerning the importance of pure cultivated yeast, refer throughout to beer bottom-yeast only. There could be no doubt even from the first that it would also be possible to employ *Hansen's* method of pure yeast-culture to top-fermentation yeast, and with the same result; this has since been proved experimentally by *Alfred Jörgensen*, and pure top-yeast has proved just as successful in the brewery as pure bottom-yeast. The writer of these lines is convinced that the introduction of pure cultivated species of yeast into distilleries, and especially into pressed-yeast factories, will give very advantageous results. In distilleries—other conditions being maintained the same—better fermentations and a greater yield of alcohol in comparison with the average now attained are to be expected, whilst in pressed-yeast factories a better yield of yeast should result from a successful selection of a pure cultivated species, and possibly the employment of clear mashes will then be found preferable to mashes containing the grains as now employed."

In *Dr. H. Bungener's* treatise "La levure de la bière" 1890,[1] the following statement occurs, contrasting the old with the new period:—"In France, *Hansen's* system has been eagerly taken up by *L. Marx*, *A. Fiddler*, and *Kokosinski*. In some breweries, it has been recently introduced, and it will soon be adopted by others. We are convinced that its introduction into all the larger breweries of France, and in fact everywhere else, will only be a question of time. It has in fact been established, that it ensures regular working and a good result in one of the most important stages of the manufacture, where hitherto chance and, in consequence, also uncertainty prevailed."

[1] Moniteur scientifique du Dr. Quesneville, Juillet-Août, Paris, 1890.

Prof. *C. Lintner*, jun., of the Technical College, Munich, writes (1891)[1]:—"In the abstracts relating to advances in the brewing industry, the epoch-making investigations of the Danish savant *Emil Chr. Hansen*, and their application in the breweries, have been frequently reported. A connected account of *Hansen's* reform and methods, however, has not yet appeared in this journal, though such an account would be by no means undesirable, considering the great importance which the subject has acquired during the seven years since its introduction into the brewing industry. Hitherto, the brewery has mainly benefited from *Hansen's* system, which, however, has also already found its way into the distillery and pressed-yeast factory, and these branches of the fermentation industry will also be greatly benefited by its introduction."

In England some of the most celebrated authorities have frankly acknowledged the value of *Hansen's* investigations. Amongst these is Professor *Percy Frankland*, who has expressed himself as follows[2]:—

"*Emil Christian Hansen*, of Copenhagen, has *enormously extended our knowledge of the alcohol-producing organisms or yeasts*; he has shown that there are a number of distinct forms, differing indeed but little amongst themselves in shape, but possessing very distinct properties, more especially in respect of the nature of certain minute quantities of secondary products to which they give rise, and which are highly important as giving particular characters to the beers produced. *Hansen* has shown how these various kinds of yeast may be grown or cultivated in a state of purity even on the industrial scale, and has in this manner revolutionised the practice of brewing on the continent. For during the past few years these pure yeasts, each endowed with particular properties, have been grown with scrupulous care in laboratories equipped ex-

[1] Dingl. Polytechn. Journal, Jahrg. 72, Bd. 279, Heft 9.

[2] Royal Institution of Great Britain. Meeting, February 19, 1892.

pressly for this purpose, and these pure growths are thence despatched to breweries in all parts of the world, particular yeasts being provided for the production of particular varieties of beer. In this manner scientific accuracy and the certainty of success are introduced into an industry in which before much was a matter of chance, and in which nearly everything was subordinated to tradition and blind empiricism."

The system has now been introduced into *top-fermentation breweries* in all countries. After its adoption some time ago by various American and Australian breweries, which are worked on the English system, *W. R. Wilson* succeeded (1892) in carrying out this important reform in a London brewery, both primary and secondary fermentation being effected by a single selected species of yeast. According to the reports in English journals numerous breweries in Great Britain have successfully adopted *Hansen's* system of pure cultivated yeast.

The following is taken from a report by *J. C. MacCarthy* of Melbourne[1]:—

"The Burton yeast[2] yields a mild 'round' flavoured beer of great brilliancy and stability, and one that is excellently suited for bottling. I now come to a matter that should be of interest to your readers. Mr. *de Baccay* and I read with some astonishment the statements made by certain scientific gentlemen in England, concerning the difficulty or impossibility of obtaining after- or secondary fermentation when *one* type of yeast alone—say *Sacch. cerevisiæ*—is used; for there has not been the slightest difficulty in obtaining secondary fermentation in 'stock' or bottled ales, where the Australian or 'Burton' yeasts have been used here.

"I have with Mr. *de Baccay* over and over again examined both 'stock' and bottled ales fermented with pure Burton yeast, and that secondary fermentation was vigorous in them,

[1] The Brewers' Journal, 1889, No. 391, p. 480.

[2] A "Burton" species obtained in pure cultivation in the laboratory of the author of this book from English yeast.

any one who saw the foam and 'head' on the beers could not doubt.

"Mr. *de Barsy* tells me that he frequently obtains a well-marked secondary cask-fermentation in a fortnight from racking the beer, and this in cases where the yeast used was fresh from the laboratory, and therefore practically free from the slightest intermixture of other types of yeast." (*De Barsy's* brewery in Melbourne is worked on the top-fermentation system.)

MacDarte, therefore, basing his opinion on these facts, has no doubt but that within a few years *Hansen's* system will also be adopted in all the important *top-fermentation* breweries of the world. His detailed account is of great interest, since it again affords proof, obtained in actual practice, that there are differences in the species of *Saccharomyces cerevisiæ*, and thus it also proves the necessity for making a selection from these species with reference to practical requirements.

W. R. Wilson writes[1]:—

"Some gyles have been prepared with pure yeast, and have been compared against ale brewed at the same time, but pitched with ordinary yeast. It is admitted on all hands that, so far as can be at present ascertained, the *pure-yeast ale* is *immeasurably superior* to the other. *Hansen's pure yeast system* applied to English high fermentations, appears to be *equally suitable* for *ales*, *porters*, and *stouts*."

With regard to the employment of the system in top-fermentation breweries in North France and Belgium, the following statements may be quoted.

Dr. E. Kokosinski, director of the laboratory at Lille, writes[2]:—

"In August, 1888, after three years' preparatory study,

[1] The Brewers' Journal, 1892, p. 327.

[2] Application industrielle de la méthode Hansen à la fermentation haute dans le Nord de la France. Compt. rend. de la Station scientifique de Brasserie, Gand, 1890.

I introduced pure cultivated yeast for the first time into a top-fermentation brewery in Lille. Shortly afterwards, at the beginning of 1889, I also introduced it into some other breweries in Lille, Roubaix, Douai, and St. Omer, and at the present time there are fifteen breweries in North France in which pure yeast is employed, and *all, without a single exception, obtain excellent results with it.*"

He summarises the results of his *practical experience with beers obtained with the help of pure cultivated top-yeast*, as follows:—

"1. They have the particular flavour which the brewer wishes to obtain;

"2. This taste is uniform and always remains constant; it is characterised by great pureness;

"3. The clarification takes place more readily and more quickly;

"4. The beers are more resistant to the action of bacteria and have a greater stability.

"It follows from the above that if *Hansen's* method has rendered great service in bottom-fermentation, it is now about to do the same also for top-fermentation, and in the latter case it will be of far greater value, for in top-fermentation we have not the advantage of the low temperature which obtains in bottom-fermentation, and which tends to check the action and the development of disease germs."

Professor *van Laer*, of the scientific station at Ghent, expresses himself in a similar manner[1]:—

"If, to the practical results obtained by myself, we add those obtained by Messrs. *Alfred Jörgensen* and *Kobosinski*, we are justified in stating that the pure yeast question has been solved both for top-fermentation and bottom-fermentation, and that its general application is merely a question of time."

[1] Application industrielle de la méthode Hansen à la fermentation haute en Belgique, 1890. Compt. rend. de la Station scientifique de Brasserie, Gand, 1890.

Similar opinions on the importance of *Hansen's* work have been expressed by other famous zymotechnologists such as *Delbrück* (Berlin), *Pfuhler* (Lyon), *Griessmayer* (Munich), *Lauger* (Mödling), *Moercker* (Halle), *Marx* (Marseilles), *Schrenckhofer* (Vienna), *Thausing* (Vienna), and others. Several of his former opponents have become his warmest supporters.

Finally it may be mentioned that the numerous and very different types of top-yeast which have been prepared in pure culture in the analytical section of my laboratory since 1884, have given just as satisfactory practical results as the pure bottom-yeasts, when the yeast has been treated with the necessary care.

The French opponents to the employment of *Hansen's* system have in fact taken up their position on the old standpoint of 1876, when *neither the wild yeasts nor the very different types of cultivated yeasts* were known.

In the above we have only spoken of the brewing industry. *Hansen's* discoveries are, however, already being applied to other branches of industry in which alcoholic fermentation plays a part. Thus experiments have been made at many places in *pressed-yeast factories* and in *distilleries*, and in a great number the system has been already introduced with success. In *wine-fermentation* a beginning was made, as stated above, in 1888, by a pupil of *Hansen's*, *L. Marx*, of Marseilles; subsequently other investigators have worked in the same direction in France, and also *Müller-Thurgau* in Switzerland, *Forti* and *Pichi* in Italy, *Mach* and *Portele* in Austria, and *Wortmann* in Germany. *Nathan* (Würtemberg) and *Kayser* (France) have likewise made extensive experiments in connection with the fermentation of fruit-wines.

A result of *Hansen's* epoch-making discoveries was the establishment of *special laboratories*, the object of which is to prepare absolutely pure material for employment in practice, to carry out control analyses for the brewery, and

to instruct the younger generation in the true understanding and the proper application of his discoveries. Such institutions have now been established in almost all countries, and are partly private and partly supported by state; they have already produced a number of able teachers, analysts, and technologists, who are working with energy and judgment with a view to more widely circulate in science and in practice the views of the Danish investigator.

Hansen's investigations have indirectly exercised an influence in connection with the *dairy*; as was mentioned in a previous chapter, pure cultures of lactic acid bacteria have been successfully employed for the souring of cream. Finally, also, in the *tobacco fermentation*, *Schloesing* and *Suchsland* have made experiments with the view to produce a definite aroma in tobacco leaves by the addition, during the fermentation, of pure cultures of certain species of bacteria.

The idea which underlies all these reformatory investigations, is the principle which has been recognised and carried out for centuries in horticulture and agriculture, namely, that in order to obtain the desired species of plant, the pure seed should be sown free from the seeds of all other plants.

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INDEX.

Acetic acid bacteria, 56
Acetic acid fermentation, 57
Achmeta, 69, 149, 193
Aërobic species, 56
Aëroscope, 38
Air analysis, Hansen's zymotechnic, 47
  analysis, Koch and Hesse, 40
  analysis, Miquel, 37—40
  examination of, 36
  examination of air and water, 36
  filtration of, 41
  sterilisation of, 14
Alcoholic ferments, 111
Amthor's investigations, 150, 151
Anaërobic species, 56
Analysis (Hansen's methods), 124
  Hansen's, of brewers' yeast, 124
  of the yeast in propagating apparatus, 137
  withdrawal of samples from fermenting-vessel for, 134
Anatomical structure of bacteria, 53
Appert, 10, 204, 206
Application of the results of scientific research in practice, 204
Arthrosporous bacteria, 55
Ascospores, anatomical structure of, 120, 124
  budding of, 126
  formation of, 125
  germination of, 127
  temperature limits for the formation of, 131, 160—173
Aspergillus Oryzæ, 92
Aubry, 16, 217

Bacillus butyricus, 67
  ethiocticus, 60
Bacteria, 51
  anatomical structure of, 53
  arthrosporous, 55
  endosporous, 55
Bacteria, exercising invertive, diastatic, and peptonising action, 76
  growth forms of, 51—53
  involution forms of, 53
  multiplication of, 54
  slime-forming, 73
  staining of, 2
  zoogloea, formation of, 56
Bacterium acet, 57—60
  Pasterianum, 57—60
  vermiforme, 72
Ball, 97, 120
Barny, 148, 223
Beer, filtration of, 12
Bellamy, 117
Belohoubek, 188, 202, 210
Berenick, 149, 194
Biermacht, 17
Bisulphite of lime, 16
Boettcher's moist chamber, 8
Borgmann's investigations, 149
Botrytis cinerea, 89
Bottom-fermentation yeast, 183
  species of, 189
Bourgelot, 194
Bouteux, 181
Bread-fermentation, 190
Brefeld, 33, 112, 116
  Brown's, 119
  Nägeli's, 118
  Pasteur's, 117
  Traube's, 116
Brewers' bottom-fermentation and top-fermentation yeast, practical analysis of, 134
Brown, A. J., 56, 119
Buchner's aëration experiments, 118
Budding of spores, 126
Budding fungi, gelatinous formation, 136
Bungener (on pure cultivated yeast), 221
Burton yeast, 223
Butyric acid ferments, 66

Cagniard-Latour, 10, 113
Cane-sugar solution, 204
Carlsberg vessels, 20
  low-fermentation yeast, No. 1, No. 2, 184
Carrie, Mac (on pure cultivated yeast), 229
Cellulose fermentation, 70
Chalara Mycoderma, 106
Chamberland flasks, 19
Chevreul, 18
Chicandart, 181
Chloride of Lime for cleansing purposes, 16
Cienkowski, 107
Cladosporium herbarum, 109
Classification of genus Saccharomyces, 130
Clostridium butyricum, 67–69
Cohn, 24, 51
Control of yeast in practice, 194
Cooling of wort, 215
Counting of yeast-cells, 32–35
Crenothrix Kühniana, 80
Culture experiments, 1
  fractional, 26
  of Saccharomycetes on solid medium, 149

Dairy, 227
De Bary, 23, 71, 107
Delbrück, 32, 68, 226
Dematium pullulans, 107
Dilution methods, 26–32
Diseases in beer, caused by yeast, 25, 145, 163, 167, 173, 174, 206, 207
  in milk, 65
Disinfection, 14–17
Dispora caucasica, 70
Distillers' yeast, 187
Druff, 46
Drying-machines, 46
Duclaux, 66, 64, 66, 115, 192
Dunnenberger, 181
Durst, 32
Dusch, 11, 205

Effront, 35–36
Ehrlich, 3
Ehrenberg, 23
Endomyces decipiens, 181
Endosporous bacteria, 55
Engel, 112
Eriksson, 110
Eurotium Aspergillus glaucus, 89

Ferment, alcoholic, 111
  acetic acid, 56
  butyric acid, 66
  lactic acid, 61
Fermentation, alcoholic, 111
  acetic acid, 56
  butyric acid, 66
  cellulose, 70
  lactic, 70
  lactic acid, 61
  rice-wine, 92
  viscous, 73
  products, chemical examination of, 149
  theory of, Brefeld's, 116
Fermentative action of the Mucor species, 99
Fermenting-rooms, Hansen's examination of the air in, 47
Film formation, 137
Filter-bags, 16
Filtration of beer, 12
  of air, 41
Fitz, 37, 67, 69, 86
Flasks, Carlsberg, 20
  Chamberland's, 19
  for forwarding small and large cultures, 213
  Freudenreich's, 19
  Hansen's, 19
  Pasteur's, 18
  vacuum, 37, 43
Fluhler (on pure cultivated yeast), 221, 226
Fokker, 61
Forel, 161, 226
Fractional cultivation, 26
  sterilisation, 13
Frank, 110
Frankland, 41, 60, 222
Fresenius, 105
Freudenreich's flasks, 19
Frogs' spawn fungus, 75
Fruit-wine fermentation, 226
Fumago, 110
Fusarium graminearum, 106

Gelatine cultures, Hansen's, 30
Koch's 29
Gelatinous formation secreted by the budding fungi, 156
Gemmæ, 97
Generation, spontaneous, 10
Germination of spores, 127—130
Ginger-beer plant, 71
Glycerine, proportion of, in the fermentation products of various Saccharomycetes, 149—150
Grains (draff), 45
Grossmayer, 226
Grotenfelt, 64, 77, 149, 180
Growth forms of bacteria, 51—53
Gruber, 15, 16, 69
butyric acid bacteria, 69
Grönlund, 146, 173, 202
Guillebeau, 66

Hæmatimeter, 33
Hansen's acetic acid bacterium, 57
aëration experiments, 118
air analysis, 47
analysis of brewery yeast, 194
analytical methods, 124
counting of yeast-cells, 32
cultivation of Saccharomycetes on solid medium, 143
examination of the air, 42
flasks, 19
Koch's plate cultures examined by, 91
method for symotechnical analysis of air and water, 47
preparation of pure cultures, 28—31
reform in scientific and practical fermentation brought about by, 206
Saccharomyces species, 130
water analysis, 49
and Kühle's yeast-propagating apparatus, 200
Hayduck, 82
Hesse, 40
High-fermentation yeast, 183, 186, 221
Hoffmann, 18
Hohn, 82, 50, 123
and Poulsen's investigations, 186
Hueppe, 41, 62, 64, 68, 119
Hydrofluoric acid, 17, 35

Introduction to alcoholic ferments, 111
Involution forms of bacteria, 53
Irmisch, 187
Isomaltose, 149

Jacquemain, 151
Jalowetz, 159
Jensen, 66
Jorgensen, 211, 221

Kayser, 145, 146, 198—194, 226
Kephir, 70
Kern, 70
Koch, 3, 15, 22, 26, 50, 205
Koehler, 176
Kolesinski, 146, 221, 224
Kramer, 192
Krause, 63
Krieger, 146
Krüs, 120
Kühle, 188, 202
Kützing, 28, 57

Lactic-acid bacteria, 61
fermentation, 61
Lactose, yeasts fermenting, 192—193
Laer, Van, 64, 73, 225
Langer, 226
Laschk, 146, 147, 177, 202—203
Laurent, 151
Leaven, 180—181
Lechartier, 117
Leuconostoc mesenterioides, 73
Liebig, 114
Lime, bisulphite of, 16
Lindner, 63, 73, 75, 144, 146
Lintner, jun., 149, 222
Lintner, sen. (on pure cultivated yeast), 216
Lister, 27
Löffler, 66
Low-fermentation yeast, 183, 186
Ludwig, 170

MacCarrie, 223, 224
Mach, 151, 226
Maercker, 82, 226
Marcano, 151
Marpmann, 64

Marshall Ward, 71
Martinaud, 151
Marx, 151, 176, 221, 226
Mass-cultures, pure cultures for physiological experiments with, 24
Matthews, C. G., 106
Mayer, 58
Micrococcus prodigiosus, 68
Micro-organisms of air and water, 36
Microchemical examination, 1
  reagents, 9
Microscope, 1
Microscopic appearance of sedimentary yeast, 124
  preparations, 1
  and physiological examination, 1
Milk, diseases in, 66
  -sugar, yeasts fermenting, 102—106
Mignel's air analysis, 37—40
  Koch's plate cultures examined by, 31
Mischeliteh, 39
Mixtures of Saccharomycetes, 136
Moist chamber, Boetchier's, 8
  Ranvier's, 7
Monilia candida, 100
Morris, 149
Mould-fungi, 52
Mucor species, action on the various sugars, 99
  fermentative action of, 99
  circinelloides, 97
  erectus, 97
  Mucedo, 96
  racemosus, 96
  spinosus, 97
  stolonifer, 98
  yeast, 97
Multiplication of bacteria, 64
  yeast, 158
Multiplying capacity of yeast cells, 82
Müller-Thurgau, 151, 226
Mycoderma cerevisiæ and vini, 200

Nägeli's theory of fermentation, 118
Nathan, 151, 226
Net-work, gelatinous, 156
Nucleus in yeast cell, 159
Nutritive substrata, 21
  medium, cultivation of Saccharomycetes on solid, 143

Odium, 103
Orleans process, 57

Panum, 33
Pasteur, 25, 27, 37, 66, 116, 205
  acetic acid bacteria, 57
  "Études sur la bière," 62, 113—114, 205
  theory of fermentation, 117
  flasks, 18
  practical results, 205
Pasteurisation of beer, 12
Pedersen's dilution experiments, 118
Pediococcus acidi lactici, 69
  cerevisiæ, 78, 80
  causing ropiness in white beer, 73
Penicillium glaucum, 86
Peptonising bacillus, 77
Peters, 60, 68, 76, 181
Petersen (Sarcina), 80, 202
Petri (Filter), 41
Physiological methods, 24 [1
  and microscopical examination, 1
Pichi, 148, 151, 226
Plate cultures, determination of errors made in, 31—32
Portele, 151, 226
Ponfick, 136
Practice, application of the results of scientific investigation in, 204
  control of yeast in, 194
Pramowski, 67
Preparations, microscopical, 1
Preservation of pure cultures, 214
Pressed yeast, 180
Propagating apparatus, Hansen and Kühle's, 209
  Bergh and Jörgensen's, 211
  analysis of yeast in, 187
Pure culture, preparation of, 22, 122
  Hansen's methods, 28—31
  Koch's method, 26
  Pasteur's methods, 24, 27
  preservation of, 214
Pure cultures for morphological investigation, 23
  for physiological experiments with mass cultures, 24
  sending of, 213

Quick vinegar process, 58
Quist's lactic acid bacterium, 65

Rawitzer's moist chamber, 7
Ratz, 65
Rayman and Kruis' examination of beers, 120
Rum, 158
Reagents, micro-chemical, 5
Reess, 86, 112, 130
Reinke (on pure cultivated yeast), 218
Resistance to infection of the different yeast types, 208
Rest, state of, of mould-fungi, 85, 88
Resting spores, 55
Rietsch, 151
Rommel, 151

Saccharomyces acidi lactici, 180
anomalus, 181
apiculatus, 147, 195
Aquifolii, 173
Saccharobacillus Pastorianus, 64
Saccharomyces cerevisiæ, I., 137, 139–161
conglomeratus, 182
ellipsoideus, I., 170
ellipsoideus, II., 173
exiguus, 176
Hansenii, 178
Ilicis, 175
Jörgensenii, 177
Kayser, 194
Ludwigii, 139, 179
Marxianus, 146, 176
membranæfaciens, 146, 177
minor, 180
Pastorianus, I., 161
II., 165
III., 167
pyriformis, 72, 175
Tyrocola, 194
cell of, described, 168
variations in the species, 151
Saccharomycetes, analysis of, 124
ascospores, 125
behaviour towards the different sugars and the other constituents of the nutritive liquid, 146
classification of, 139
culture on solid nutritive medium, 149
diseases in beer caused by, 145
film formation, 137
germination of spores, 127
Saccharomycetes, microscopic appearance of sedimentary yeasts, 124
pure cultures, 122
temperature limits, 142
Sakè, 92
Samples, forwarding of, in Hansen's flasks, 213
in filter-paper, 214
withdrawal of, from fermenting-vessel for analysis of yeasts, 134
Saxena forms, 77
Schiele, 10, 204, 205
Schlen, 41
Schlossing, 227
Schmitt-Mülheim, 65
Schmitz, 130
Schröder, 11, 205
Schroeter, 29
Schulze, 10
Schumacher, 188
Schwackhöfer, 226
Schwann, 10, 111, 204
Sclerotium, 85
Sedimentary yeast, 124
Slime-forming bacteria, 78
Soda solution for cleansing purposes, 16
Solid nutritive medium, cultivation of Saccharomycetes on, 149
Spallanzani, 10, 93, 204
Spherical yeast, 97
Spores, budding of, 198
germination of, 127
conditions regulating formation of, 126
structure of, 126, 134
temperature limits of formation, 131
Stahl, 114, 120
Staining method, 2
Ehrlich's, 3
tubercle bacillus 3
Starch-resolving bacterium, 76
Sterilisation, 9–14
of glass and metal articles, 11
of nutritive liquids and solid nutritive substrata, 11
of air, 14
Storch's lactic acid bacteria, 65, 69
Sublimate, disinfection by means of, 16
Suckstorf, 227
Sugar, behaviour of Mucor species towards the various kinds of, 99

Sugar, behaviour of Saccharomycetes, etc., towards the various kinds of (review), 146–149

Tartaric acid, 24–25
Temperature limits for the Saccharomycetes, 142
Thawing, 226
Tobacco fermentation, 227
Top-fermentation yeast, 188, 221
Torula, 139
Traube, 116
Tubercle bacillus, staining, 3
Turpin, 87, 113
Tyndall, 13, 37

Vacuum flasks, 37, 49
Variations in the species of Saccharomyces, 151
Velten, 116
Vuylsteke, 196

Ward, Marshall, 71
Water analysis, Hansen's zymotechnical, 47
examination of air and, 68–70
Weigmann, 65, 66
Wichmann, 70
Wiesner, 188
Will, 17, 144, 149, 174, 218
Wilson, W. R., 222–224
Windisch, 146
Wine yeast, investigations by Marx, Amthor, and others, 151
Winogradsky, 209
Winter, 75
Wort, introduced into fermenting-vessels in state of sterility, 19
Wortmann, 76, 151

Yeast, analysis of, 124
ascospores, 125
Burton, 223
bottom-fermentation, 189
cell, microscopic appearance of, 168
cells, counting of, 82–85
cells, partition wall formation, 127
culture on solid nutritive medium, 143
diseases in beer caused by, 25, 145, 163, 167, 173, 174, 206–207
film-formation of, 137
high-fermentation, 188
low-fermentation, 189
Mucor, 97
network formation of, 156
pure cultivation, by Hansen's method, 93–94, 122
purifying by Pasteur's method, 24–25
pure cultivation in practice, 214
grouping of the species for practical purposes, 186
pressed, 180
propagating apparatus, Hansen and Kühle, 208
propagating apparatus, Bergh and Jörgensen, 211
properties of the different species in practice, 185
sedimentary, 124
spherical, 97
top-fermentation, 186
treatment of, with hydrofluoric acid, 25
varieties of, 151

Zoogloea, formation of bacteria, 76
Zopf, 68, 80, 110, 120, 178
Zygospores, 96

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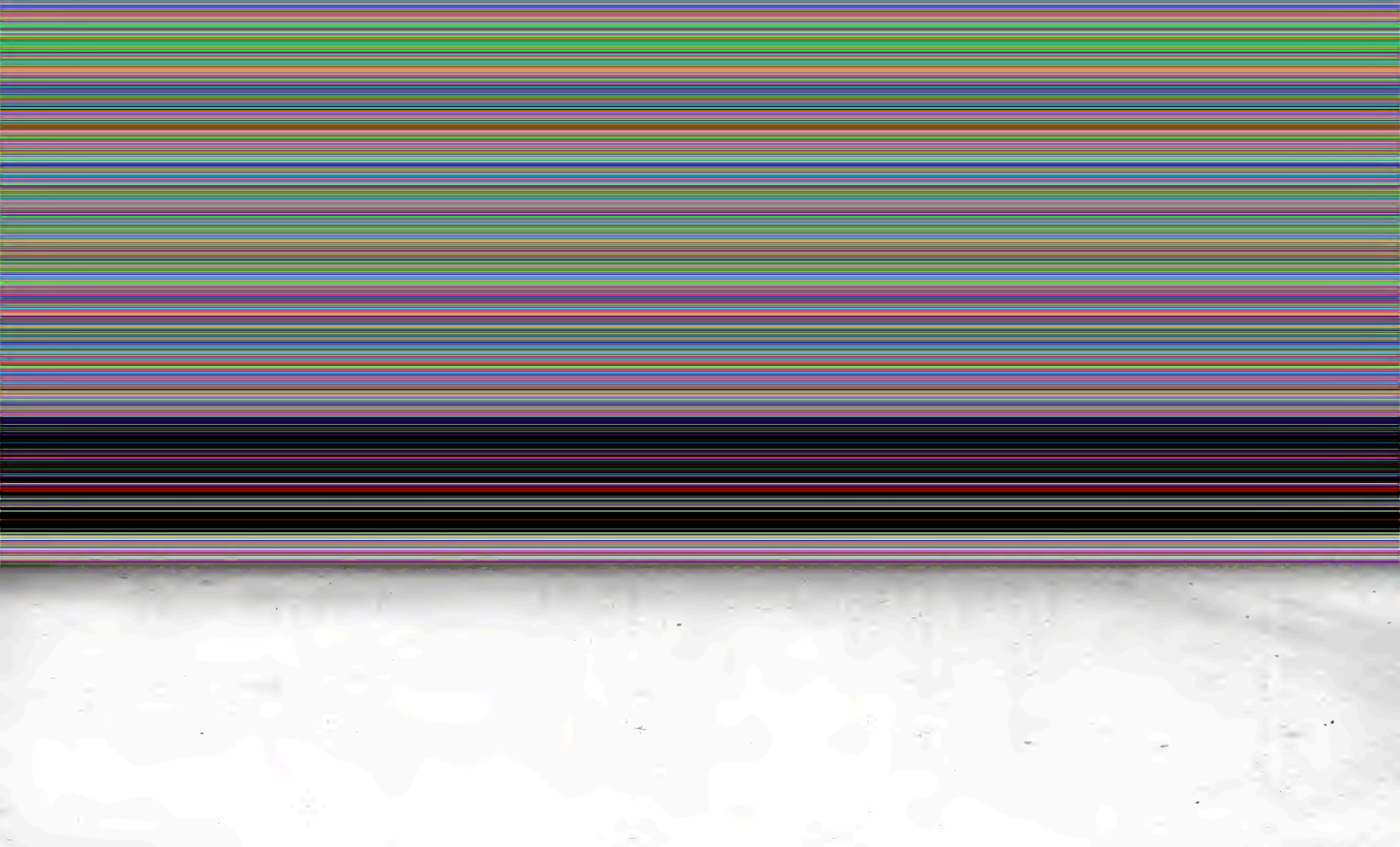

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