THE YEASTS

GENETICS, CYTOLOGY, VARIATION, CLASSIFICATION AND IDENTIFICATION

ARTHUR T. HENRICI

Department of Bacteriology and Immunology, University of Minnesota, Minneapolis, Minn.

CONTENTS

This review is concerned with the cytology and genetics of yeasts, with their induced and spontaneous variations and mutations, with their classification and identification. It is limited to papers appearing during the past decade, save that some older papers have been cited for the sake of continuity or clearness. No attempt has been made to review the vast literature on the physiology and biochemistry of the yeasts.

For some years there has been maintained an international type culture collection of yeasts at the Technische Hoogeschool in Delft, a section of the Centraalbureau voor Schimmelcultures. This collection of cultures from a variety of materials and from

all parts of the world has been carefully and systematically worked over by several persons with the direction of Prof. A. J. Kluyver, and the results are being published as a series of monographs. The first appeared in 1931—"Die Sporogenen Hefen" by Fr. N. M. Stelling-Dekker. A second division, "Die Anaskosporogenen Hefen" by Frl. Jacomina Lodder, is in two portions. The first half, dealing with asporogenous yeasts which do not form mycelium or pseudomycelium, was published in 1934. The second half, dealing with asporogenous yeasts which do form mycelium, the *Mycotoruloideae*, has not yet been published. This review has largely been built up around these two monographs. Partly because the second half of Miss Lodder's monograph is lacking, and partly to keep the review within a reasonable size, the *Mycotoruloideae* will not be reviewed.

Any discussion of yeasts must be preceded by a definition of the limits of the group, or hopeless confusion will result. Many bacteriologists with little experience in studying yeasts think that they know very precisely what a yeast is, and define it as a unicellular fungus multiplying by budding. Actually such a definition will apply only to a small proportion of the organisms usually classified as yeasts, and only to these when they are maintained under constant conditions and not studied too closely. Kohl (1908) begins his book, "Die Hefepilze", with the statement that "Die Hefepilze umfassen die Sprosshefen, die Spalthefen und Hefeähnliche Pilze". The "Sprosshefen" are the common unicellular yeasts which multiply by budding; the "Spalthefen" are a series of tropical yeasts, producing alcoholic fermentation and forming multiple endogenous spores like the budding yeasts, but which multiply by binary transverse fission; and the "Hefeähnliche Pilze" are fungi which regularly and obviously produce true mycelium (*i.e.*, they are not strictly unicellular) but in which a unicellular yeast-like growth form is usually dominant. One might with reason exclude this last group, and define yeasts as unicellular fungi, if it were not for the fact that a number of yeasts normally unicellular have been found to give rise to mycelium in certain media, or to give rise to variants that permanently produce mycelium in all media. Even the common

industrial yeast, *Saccharomyces cerevisiae*, the type organism about which our conception of the category "yeast" has grown, will often produce a fringe of true mycelium burrowing into the agar at the edge of giant colonies 4 to 6 months old. Yeasts, then, are not exclusively unicellular, nor do they multiply exclusively by budding. The closest one may come to a satisfactory definition is to state that they are fungi with nuclei in which the usual and dominant growth-form is unicellular.

While the central concept of this category is clear, to anyone who will study large numbers of yeasts and yeast-like fungi, it must become obvious that this group of microörganisms shades off by such slight transitions into various groups of higher fungi that they cannot be considered a natural phylogenetic group. And while it is equally clear that the bulk of the organisms which we call yeasts are lower Ascomycetes or imperfect forms of these, it is by no means certain that all of them are; there is strong evidence that some of them are Basidiomycetes. It is well to keep in mind, therefore, that the term "yeast" does not have the precise botanical significance that such terms as "mushroom" or "moss" or "fern" have.

The true relationships of the yeasts must be derived, as with other fungi, from a study of their sexual reproduction. Such a study also involves cytology and genetics, which in turn have an important bearing upon the problems of variation that must be considered in any discussion of taxonomy. Our knowledge of the cytology and sexual reproduction of the yeasts we owe very largely to Guilliermond, though in recent years important knowledge has been contributed by others, notably Winge and Laustsen. It will not be necessary to review all of this work in detail, because this has recently been done by Guilliermond (1940).

GENETICS OF YEASTS

Sexual reproduction in yeasts was first clearly recognized by Guilliermond in 1902 in *Schizosaccharomyces* and by Barker in *Zygosaccharomyces*. These observations were amply confirmed, and in addition, heterogamic conjugation between cells differ-

entiated by size was observed in various budding yeasts, notably members of the genera *Debaryomyces* and *Nadsonia*. These studies left no doubt that some of the unicellular fungi which we call yeasts exhibit sexual reproduction and that the sexual spores are ascospores.

In a large number of yeasts, especially those of industrial importance, however, spores are formed without any trace of conjugation preceding spore-formation, and these yeasts have until very recently been considered to be parthenogenetic, *i. e.*, devoid of sexuality. Since in yeasts which reproduce sexually, Guilliermond had observed some cells forming fusion tubes which failed to fuse, such cells proceeding to form spores parthenogenetically, and in other cases he observed many cells forming fusion-tubes without actual fusion taking place, he postulated that sexuality in yeasts is undergoing a retrograde evolution in which the various steps are: Conjugation → Fusion tubes without conjugation → Parthenogenesis. One might well extend this concept to include as the last stage the complete loss of the power to form spores. The asporogenous or imperfect yeasts would thus represent the end of this evolutionary series.

Guilliermond described an additional type of sexuality in *Saccharomycodes Ludwigii* observed by Hansen as early as 1893. This yeast forms usually four spores without previous conjugation. On germination, however, the spores conjugate within the mother cell, two by two, so that only two vegetative cells emerge from each ascus containing four spores. This process Guilliermond designated parthenogamy, and he considered it as further evidence of degradation in sexuality, placing this process just ahead of parthenogenesis in the retrograde evolution.

Guilliermond's observations and theories have been accepted generally by those working with yeasts. Beginning in 1935, however, a series of papers by Winge, and by Winge and Laustsen have extended greatly our knowledge of sexuality in the yeasts, and have presented facts of wide significance and importance. Briefly, they have shown that the process which Guilliermond called parthenogamy (*i.e.*, conjugation of spores, or of cells derived from spores) occurs in the industrial yeasts of the genus *Sac-*

charomyces, as well as in *Saccharomycodes*, and that it is doubtful that any of the spore-forming yeasts are entirely parthenogenetic. The vegetative cells of such yeasts are normally diploid, but haploid cells may be derived from single spores, and such haploid cells from different races may be caused to fuse and give rise to hybrids.

Winge (1935) described the germination of spores of *Saccharomyces cerevisiae* (race Johannisberg II, varieties *ellipsoideus* and *marchalianus*) and of *Saccharomyces validus*. In all of them spores were found to fuse by pairs on germination, but not all of the spores fused. Spores which germinated without fusion gave rise to small cells tending toward a globular form, whereas fused spores produced larger elongated cells. The small globular cells were recognized as haploid, the larger elongated ones as diploid. Haploid vegetative cells derived from a single spore often fuse with other haploid vegetative cells to produce a large diploid cell. Cytological studies showed that the fusion of spores or of haploid vegetative cells was followed by nuclear fusion. "A striking feature is the absence of an established system governing the zygote formation. Sometimes two spores unite to form a zygote before or after they have germinated singly, sometimes a spore unites with a haploid vegetative cell; or two vegetative cells conjugate, and they may be sister-cells, or mother-cell and daughter-cell, or more distantly related cells."

These observations indicate that with regard to sexual behavior, yeasts may be divided into two great classes. In one (the genera *Schizosaccharomyces, Zygosaccharomyces, Zygopichia, Debaryomyces, Nadsonia, Nematospora*) the vegetative cells are *haploid.* The diploid phase extends only through the short period following nuclear fusion. This is immediately followed by meiosis, the spores being haploid, and giving rise to haploid vegetative cells on germination. In the other group (*Saccharomyces, Saccharomycodes, Hansenula*) the vegetative cells are *diploid.* Meiosis occurs during spore formation, and the spores are haploid. These may give rise to haploid vegetative cells, but the latter are small and not vigorous. The spores, or vegetative cells derived from them, fuse to give rise to the diploid form producing large

and vigorous cells. Guilliermond (1940) applied the term "haplobiontic" to those yeasts whose vegetative cells are normally haploid, and "diplobiontic" to those whose vegetative cells are normally diploid; but a note by B. O. Dodge appended to Guilliermond's article points out that these terms have been used in a different sense.

Winge and Laustsen (1937) developed a micromanipulative technique by which the four spores of an ascus could be separated, isolated, and studied in pure form. Working with a culture derived from a single cell isolated from a strain of *Saccharomyces cerevisiae* used in commercial pressed yeast manufacture, they found that sometimes a single spore gave rise to a pure haploid culture, but in other cases the cells derived from the single spore were diploid from the beginning. They explain the latter cases by assuming that an intracellular nuclear fusion takes place immediately following the first division of the spore nucleus. Diploid cells derived from a single spore readily formed spores when transferred to a suitable substrate. Haploid cells derived from a single spore either failed to form spores at all, or did so only after fusion had taken place between two vegetative cells. Thus a haploid race derived from a single spore may behave either like *Zygosaccharomyces, i. e.*, a yeast of the type which Guilliermond designates "haplobiontic", or like an asporogenous yeast of the genus *Torulopsis*. Since yeasts of the latter type are usually small globular forms, it was natural to assume that such yeasts might be haploid and self-incompatible, and that if they were brought into cultures of other races or species, diploidization and spore formation might occur. Accordingly they tested the species of *Torulopsis* in the Carlsberg collection, but in no case did spore formation occur. Nevertheless, in the opinion of the reviewer, this still remains an attractive field for investigation.

Winge and Laustsen (1937), discussing the occurrence of genetic segregation in the ascus, point out that since the vegetative cells of *Saccharomyces* species are normally diploid, a single-cell culture of one of these yeasts will not be necessarily a "pure" culture; since in spore formation segregation of new types may occur,

and spore formation may be easily overlooked. That such segregations actually occur was observed repeatedly when giant colonies were started from each of the four spores of an ascus. These differed in contour and texture. The parent culture was therefore heterozygous. That cultures from isolated spores were homozygous was shown by producing new giant colonies from spores produced from a single spore culture. These "bred true". Obviously, to study mutations in these yeasts it would be better to have a single-spore culture than a single-cell culture.

A third paper (Winge and Laustsen, 1938) is not available to the reviewer but has been abstracted briefly in the fourth paper (1939a). The authors state that they showed "how hybridization of different yeasts is arranged by placing in a droplet of culture solution two (haploid) spores of different species so as to enable them to copulate, forming a (diploid) zygote from which the hybrid yeast germinates." Moreover, this procedure now makes it possible to undertake rational breeding work with yeasts, and possibly to produce new yeasts of commercial value.

Winge and Laustsen (1939a) then proceeded with such breeding experiments. Single spores were isolated from several strains of *Saccharomyces cerevisiae*, and from *S. italicus*, *S. validus*, *S. mandshuricus* and *Zygosaccharomyces priorianus*. These spores were brought together in pairs and when conjugation had been observed to take place, subcultures were made and various characters, particularly giant colonies, were observed. Giant colonies of 14 new hybrids differed in texture and contour from both of the parent types. The hybrids were also compared with the parental types as regards their ability to ferment sugars. In all cases, ability to ferment was a dominant character; if one of the parent types fermented sucrose, for instance, and the other not, the hybrid invariably fermented sucrose.

Crosses were obtained not only between different races of *Saccharomyces cerevisiae*, but also between different species of *Saccharomyces*, and in one instance an intergeneric hybrid (*Zygosaccharomyces priorianus* x *Saccharomyces cerevisiae* Rasse II) was obtained. The relation of such inter-fertility to problems of taxonomy is discussed. When the spores of the parental

types were tested, 43 to 90 per cent of them were capable of germination. Spores derived from hybrids fell into two groups, one showing germination of 0 to 13 per cent of the spores, the other 50 to 94 per cent. The latter are considered to be examples of intraspecific hybrids, the former of interspecific hybrids. Hybrids between two species may be propagated by budding, but tend toward sterility in sexual reproduction. This provides a method for testing specific relationships. In all cases where the parent types differed in fermentation characters, the hybrids showed reduced germination of the spores. Strains showing the same fermentation characters are not necessarily specifically identical; one cross between two yeasts giving identical fermentations yielded hybrids with very poor spore germination.

The use of these facts in breeding and selecting better industrial yeasts is pointed out by the authors. They found that a typical top yeast may segregate out a typical bottom yeast through its spores, and concluded that there is no definite line of demarcation between these two types of brewing yeasts. One of their hybrids proved to be superior in compressed yeast manufacture.

Saccharomycodes Ludwigii is an interesting yeast whose four spores occur in two pairs, often the two spores in each pair attached, and whose spores fuse in germination. Winge and Laustsen (1939b) reported on genetic studies with this yeast. When the four spores are isolated, and forced to germinate without fusing, they show differences in growth. Some spores yield small, globular buds, others long cylindrical ones. In some cases growth continues normally, in others growth ceases after a very small number of buds have formed. These characters depend upon the Mendelian segregation of two pairs of genes. Gene *N* gives rise to normal growth, while *n* causes a cessation after a single short hypha has formed; gene *L* produces long cylindrical cells, *l* short globular ones. These characters may be segregated as *Nl* and *nL*, and this is a matter of chance. But in each pair of spores in an ascus, one has one set of genes and the other the other; either *Nl* and *nL*, or *NL* and *nl*.

If the isolated spore is capable of growth, (*i.e.*, if it contains *N* and not *n*) it will grow continuously as a haploid yeast; it never

diploidizes and therefore never forms spores. Spores having the formula *NL* give rise to elongated cells which form giant colonies "characteristically scaly or rather lobed, under the lens tufty." Spores having the formula *Nl* give rise to smooth colonies. Colonies derived from diploid cells (*Nn Ll*) are characteristically rough. Consequently the rough or lobed colony character is dominant. The authors also obtained a mutant which produced a new race having the formula *NNLl*, in which each of the four spores gives rise to continuous growth.

CYTOLOGY OF YEASTS

The researches of Winge and Laustsen indicate that yeasts behave like higher organisms with regard to the transmission of hereditary characters in sexual reproduction, and this observation naturally gives rise to renewed interest in the cytology of yeasts, especially in the behavior of the nucleus during vegetative and sexual reproduction. Bacteriologists have been interested in the nuclei of yeasts because these microbes have seemed to be the next step above the bacteria in scale of size and complexity, and it has seemed that methods which would certainly demonstrate nuclei in yeasts might be applicable to bacteria. Yeast cells are usually packed with a variety of stainable granules or globules, and earlier studies did not distinguish these clearly. It is probable that Moeller in 1893 saw and photographed the true nucleus of yeasts, but the certain existence of a nucleus was not definitely established until the researches of Guilliermond in 1901. Earlier literature on the nuclei of yeasts has been reviewed extensively by Kohl (1908) and Guilliermond (1920).

Yeasts, especially *Saccharomyces cerevisiae*, are rich in volutin partly deposited in granules in the cytoplasm and partly dissolved in the fluid of the vacuoles. Early workers did not always distinguish this material from the chromatin of the nucleus, since both stain with the basic dyes. In particular Wager (1898), one of whose drawings has been widely reproduced in textbooks, mistook the vacuole and its granules for the nucleus, as was pointed out by Guilliermond (1920). Although means for differentiating volutin from chromatin (vital staining with neutral red, solubility

in hot water) were available earlier, the development of the Feulgen method for the microchemical demonstration of thymonucleic acid *in situ* has provided a more certain method for this differentiation. The application of the Feulgen method has demonstrated the occurrence of thymonucleic acid in the nuclei of yeasts, and it is now certain that yeasts produce two nucleic acids: "yeast" nucleic acid, abundant, probably identical with the volutin granules; and thymonucleic acid, scant, contained in the nucleus.

Though Feulgen failed to obtain a positive "nuklealreaktion" in yeasts, Margolena (1932), and Pietschmann and Rippel (1932) obtained positive reactions which were, however, so faint that a satisfactory microscopic picture was not obtained. Imšenecki (1936) obtained a positive reaction with masses of yeast cells, but could not see the color in the individual yeast cells. Rochlin (1933) on the other hand, obtained a very definite picture with *Saccharomyces cerevisiae.* The Feulgen method gave an intense and beautiful staining of the nucleus, no other structure was colored. The nucleus, 0.8 to 1.3 μ, was vesicular, with the chromatin arranged as a crescent along one side. A similar picture, with less shrinkage, was observed in slides stained with iron hematoxylin. Winge (1935) states that Laustsen also developed a modification of the Feulgen method which gave a better differentiation of the nuclei than is obtained with hematoxylin. Badian (1937) used Rochlin's technique, and obtained identical results, which also checked the results of his modified Giemsa stain.

Although it has long been certain that yeasts show single, discrete nuclei, it is still not certain how these behave during budding or fission, conjugation and spore formation. These nuclei are about 1 μ in diameter, and in attempting to see what goes on in such a small body there is always a temptation to strain the resolving power of the microscope to the utmost. It is my opinion that descriptions of details in morphologic structures less than 1 μ in diameter should always be taken *cum grano salis.* It is to be hoped that the development of the electronic microscope will solve some of these riddles.

Earlier investigators were about evenly divided between those

who saw only amitotic division of the nucleus, and those who pretended to observe the formation of chromosomes and division by karyokinesis, at least during spore formation. This work has been reviewed by Badian (1937). Guilliermond maintained until recently that division during budding is amitotic, but in 1917 described a sort of mitosis in *Schizosaccharomyces octosporus*. This yeast produces 4 or 8 ascospores following conjugation. The nucleus of the zygote is large and vesicular, containing a nucleolus and several granules of chromatin. On division, an achromatic spindle is formed within the nuclear membrane with the chromatin granules (very small) gathered as an equatorial plate in the center. The nuclear membrane is absorbed, the chromatin granules move toward the two poles, the spindle elongates, and the chromatin at the two poles gathers together to form the two daughter nuclei. The nucleolus, left in the middle of the cell, disappears. Second and third divisions take place in a similar manner, the daughter nuclei dividing simultaneously. The chromatin granules, considered to be chromosomes, are too small to count. The entire process is very difficult to demonstrate due to the small size of the nucleus. Guilliermond (1940) stated that he observed mitosis-like features during budding in *Saccharomyces paradoxus*.

Kater (1927) ascribed the widespread acceptance of amitotic division as the normal process in budding to the difficulties encountered in attempting to study nuclear division in such small organisms, and pointed out that the development of genetics makes it now impossible to accept amitotic division as a normal process. He studied *Saccharomyces cerevisiae* by fixing wet smears in Bouin's fluid. If these are stained in iron hematoxylin before all of the picric acid has been washed out, the nuclear structures are revealed without interference from the metachromatic material. The resting nucleus contains a nucleolus, with chromatin as granules at the periphery. In budding, this chromatin becomes arranged upon linin strands that form an achromatic spindle, and becomes divided into chromosomes, probably at least 8 in number. Both the resting nucleus and the mitotic figures resemble those of *Phaseolus*. There is no description of the nucleus in sporulation.

Badian approached the study of nuclear phenomena in yeasts

following a series of investigations, dating from 1930, on nuclei in bacteria, myxobacteria, and actinomycetes. In these primitive organisms he claimed to observe free in the protoplasm rod-shaped chromosomes which divide longitudinally at cell division. Fertilization occurs by autogamy, the chromosomes fusing to form bivalent chromosomes. A return to the haploid phase is accomplished by a reduction of the chromatin, part of the chromosomes disappearing after two successive divisions of the bivalent chromosome. These papers are quoted by Badian (1937).

Badian studied the nuclei of *Schizosaccharomyces pombé*, *Saccharomyces cerevisiae*, *Saccharomycodes Ludwigii*, and *Rhodotorula glutinis*. Wet smears were fixed in osmic acid vapor and stained by a modified Giemsa method, or by the Feulgen method according to Rochlin. In *Schizosaccharomyces pombé*, the nucleus of the resting cells is globular with the chromatin arranged as a crescent at one side. In fission, this chromatin is arranged as two rod-shaped chromosomes, which divide transversely to form four, two moving to each pole of the cell; after this a cross-wall is formed, and the two chromosomes in each cell fuse to form a globular nucleus. In fertilization, the chromatin in each of the cells about to fuse also appears as two rod-shaped chromosomes. Upon fusion their chromosomes join at the ends, to form two long (bivalent) chromosomes. These become shorter and fatter, and occupy a position in the center of the cell at right angles to its long axis. These now divide longitudinally to form four, and one pair passes to each end of the zygote, where they divide again (transversely) to form now four pairs of chromosomes, each pair becoming a spore nucleus. In some cases some of the spore nuclei degenerate, so that the number of spores may be less than four.

In *S. cerevisiae*, the resting nucleus appears the same as in the preceding yeast, and forms two chromosomes. The division of the nucleus may precede the appearance of a bud, and takes place in the middle of the mother-cell. The two chromosomes divide longitudinally and one pair passes to the bud where they become transformed into a new resting nucleus. In spore formation, the pair of chromosomes undergoes two divisions, yielding four pairs of chromosomes, each pair forming a spore nucleus. Again, some

chromosomes may degenerate without forming spores. In spore germination, the spores fuse and immediately the zygote commences to bud. In the fused spores the two pairs of chromosomes fuse end-to-end, then divide longitudinally, one of the two pairs thus formed passing into the bud.

The vegetative division of the nuclei in *Saccharomycodes Ludwigii* and *Rhodotorula glutinis* proceeds in the same manner as in *S. cerevisiae.*

Badian points out the similarity between nuclear division as he observed it in the yeasts, and the same process observed in higher fungi, and also as he observed it in bacteria. It is difficult to evaluate Badian's work without repeating it, and it involves a delicate and painstaking staining method. One's first reaction is that he has seen more than the microscope will reveal in such minute bodies as bacteria and yeasts. Guilliermond (1940) states that "His figures, however, are too diagrammatic to be taken into consideration; they fit neither with our nor with his own photomicrographs." This criticism is largely true, though several of Badian's photomicrographs clearly show paired bodies similar to the chromosomes he describes. At any rate, Badian's observations are so striking and important that they deserve serious consideration and careful repetition. Certainly the two chromosomes described by him are more definite than the vague accumulations of minute chromatin granules described by Guilliermond. The occurrence of bivalent chromosomes rather than a doubling of the chromosomes in the diploid phase is noteworthy.

Renaud (1938), with *Saccharomyces ellipsoideus* fixed in Bouin's fluid and stained with iron hematoxylin, reported that in budding, a centrosome appears on the surface of the nucleus facing the bud. This divides, and one part enters the bud. The membrane dissolves, an achromatic spindle is formed, and the chromatin becomes arranged in chromosomes that form two masses in the middle of the spindle. The number of chromosomes could not be counted because of their small size, but Renaud believes that there are more than four. He states that mitosis is similar to that observed in higher Basidiomycetes, and much more complex than the process described by Badian.

Richards (1938) attacked the problem of mitosis in yeasts by

the use of colchicine, which is known to arrest mitosis in the metaphase with higher organisms. He believed that if yeast nuclei divide by mitosis, the addition of colchicine to the medium should retard growth, and the chances of finding cells in mitosis would be increased. Instead, colchicine stimulated growth and no chromosomes were found in preparations stained with iron hematoxylin, by the Feulgen method, or examined by ultraviolet microscopy.

Winge and Laustsen have not described in any detail cytologic studies to accompany the genetic studies which they have published, though they promise such a paper in the future. Winge (1935) described and illustrated unequivocally the nuclei and their fusion in the zygotes of *Saccharomyces ellipsoideus* (strain Johannisberg II). In the higher Ascomycetes and Basidiomycetes a characteristic feature is a delay between cell fusion and nuclear fusion, the zygote giving rise to binucleate mycelium. Guilliermond (1940) claims that yeasts show a tendency in this direction. In *S. paradoxus*, the nuclei of the zygote may fail to fuse, but show "conjugate division," the two nuclei dividing simultaneously to provide two nuclei for the first bud, in which they fuse. Renaud (1938) observed the same thing in a strain of *S. ellipsoideus*, two or three buddings with conjugate mitosis preceding fusion of the nucleus.

We may summarize the newer developments in the cytology of yeasts by stating that there can no longer remain any doubt that they possess single discrete nuclei. There can remain a legitimate doubt regarding the method of nuclear division, but the balance of evidence favors the conclusion that both in budding and spore formation the nucleus divides by mitosis as in higher organisms. This is supported especially by the papers of Winge and Laustsen which show that in genetic behavior the yeasts behave precisely like higher organisms, and must depend, therefore, upon a chromosomal apparatus. In one case (Winge and Laustsen, 1939b) they were able to show by segregations in the spores of *Saccharomycodes Ludwigii* in which planes the nuclei divided, and in which divisions the characters were segregated.

VARIATIONS IN YEASTS

In addition to the investigations of Winge and Laustsen on the genetics of yeasts, there have appeared during the last decade a number of papers on the occurrence of spontaneous and induced variations in yeasts and yeast-like fungi. Scattered earlier observations had indicated that such variations do occur, particularly the development of mycelial races from normally unicellular yeasts, and of asporogenous races from normally spore-forming yeasts. Much of the more recent work has been done by Nadson and his associates in Leningrad, and since many of the publications are in Russian, they have been available to me only as abstracts and quotations. While some purely spontaneous variations have been observed, most of the observations have concerned variations induced by exposure of the yeasts to injurious agents,—chloroform, cyanide, lithium chloride, immune serum, x-rays, and radium emanation. Some of the variants have been relatively stable for a time, but have eventually reverted; these Nadson designates "dauermodifikationen." Others have remained permanent, and these he designates as "saltants." It is noteworthy that Nadson recorded the production of variants in lower fungi under the influence of x-rays previous to the publication of Muller's fruitful researches upon x-ray mutants of *Drosophila.*

Nadson and Philippov (1928) exposed a "rose yeast" to x-rays. Subcultures plated from the exposed cells showed sector mutants in the colonies. In the original culture the colonies were smooth to mucoid, the cells round, encapsulated, and filled with fat. A variant was obtained which showed the same color, but the growth was dry, the surface matted and folded. The cells were cylindrical with little oil and no capsules, and they reproduced by exogenous spores borne upon sterigmata, precisely as in the yeast *Sporobolomyces roseus,* previously described by Kluyver and van Niel. A second variant was similar, but the color was light orange rather than rose. These variants remained stable over three years (80 culture generations) and could not be caused to revert by renewed exposure to x-rays. They note also the

development of asporogenous races from the sporogenous yeast *Nadsonia fulvescens* under the influence of x-rays.

Derx (1930) observed spontaneous variations in yeasts of this same group. When freshly isolated from nature (leaves of plants) species of *Sporobolomyces* grow as pink mucilaginous colonies. On these colonies, firm rough secondary colonies may appear. The mucilaginous colonies show only budding globular or oval cells; the secondary colonies show elongated cells or mycelium, and form the characteristic sporobolomyces type of spore, which give the surface of the colony its powdery appearance. By repeated reinoculation of these spores there may be obtained a race purely rough and sporulating, without any mucilaginous stage. The same transformation was observed in liquid media, the rough sporulating type appearing as a pellicle. If cultures are transferred at frequent intervals using the vegetative cells rather than spores, a reversion of the rough form to the mucilaginous type may be obtained. Derx also records another type of variation in *Sporobolomyces*, a sudden appearance of a single white colony among many (normal) pigmented ones. This mutant was identical with the parent type in all characters save for the lack of pigment, and the variation was permanent.

Nadson and Krassilnikov (1932) recorded spontaneous variations which occurred over a period of three years in a yeast-like fungus, *Guilliermondella selenospora*, belonging to the *Endomycetaceae*. Five races emerged. One was characterized by abundant typical mycelium and parthenogenetic asci. A second showed poorly developed mycelium and many yeast-like cells, with spore formation preceded by conjugation. A third showed prickly mycelium and no spores. A fourth yielded colonies with tufts of mycelium on the surface and showed no spores. The fifth was entirely yeast-like with no mycelium, and showed conjugation between the bud and mother-cell. This last race also differed from the parent fungus by its ability to ferment mannose, glucose, and fructose.

Philippov (1932) studied the influence of x-rays upon the red yeast, *Rhodotorula glutinis*. New races were obtained which differed in the fat content of the cells, the character of the budding, the production of gum, and pigmentation.

Punkari and Henrici (1933) described spontaneous variations in an asporogenous yeast, *Torulopsis pulcherrima.* This was an old laboratory culture originally isolated by Grosbusch, which had become unstable. Variants appeared as sectors or as secondary colonies in giant colonies, and resulted from aging of the colonies, or spontaneously. Variations in color (red and white) and in texture (smooth and rough) were observed to occur independently. Roughness was associated with the development of mycelium in both red and white variants. The authors emphasized that these variations occurred in a yeast which had never formed spores, and compared them in significance to the variations occurring in haploid cultures of smut sporidia described by Christensen. The later researches of Winge and Laustsen support the view that asporogenous yeasts may be haploid and self-infertile. Such variations then must be regarded as mutational and not the result of segregation or of hybridization. In a second paper (Punkari and Henrici, 1935) further observations of the same yeast are recorded. It was noted that white variants from the original red yeast are much more stable than the parent stock; that white variants arise from red usually as sectors, *i.e.*, at the edge of the growing colony; while in those rare cases where white reverted to red, this occurred as secondary colonies in the center of the colony. The contention that the variations were independent of any sexual process is not certain in view of the later observation by Windisch (1938) that *Torulopsis pulcherrima* does form ascospores by isogamous conjugation. Spore formation occurred in the presence of a species of *Penicillium,* and in very old agar cultures dried to such a degree that the growth adhered to the agar.

Nadson and Rochlin (1933) reported upon variations induced by exposing cells of *S. cerevisiae* to radium emanation (radon). This was applied in glass capillaries in doses of 19.6 to 23.3 millicuries, placed in bottles containing 10 ml. of beer wort. The capillary was then crushed, and the liquid inoculated with the yeast. After 24 to 48 hours the cultures were plated on wort agar, and the resulting colonies compared with control colonies from cultures in wort without radon. From the normally smooth type parent strain, two rough variants were obtained, one with

small globular cells, the other with large cylindrical cells, resembling species of *Torulopsis* and of *Mycoderma* respectively. Another variant was characterized by abundant spore formation, producing spores readily in beer-wort, which the parent strain would not do. On re-exposing this sporulating variant to the radon, a new type with restricted rugose colonies and distorted cells was obtained. The sporulating race is considered a "dauermodifikation," but the other three variants, irreversible in many generations, are considered "saltants," the equivalent of "mutants" in higher organisms. These new races differed quantitatively in ability to ferment sugars, and in volume of crop. One was an improvement over the parent strain in producing more alcohol, a larger crop, and in settling more quickly.

Meissel (1933) reported on variants of *S. cerevisiae* induced by the action of potassium cyanide. The yeast is relatively resistant to this poison, surviving an exposure of 24 hours to a 5 per cent solution. Plates made from suspensions so treated yielded a great variety of variants, both morphological and physiological, 40 in all, some of which reverted after a time, others appeared to be permanent. Rugose colonies in some cases showed globular cells, in other cases elongated cells. Variants re-exposed to KCN underwent renewed variations. A noteworthy feature was the development of variants which, after growing for a time, underwent spontaneous autolysis accompanied by a dark brown discoloration of the colony. From these autolyzed areas secondary colonies sometimes grew, which showed new variations. These autolytic variants may be related to the lethal variants noted by later authors.

Krassilnikov (1934) observed spontaneous variations in *S. cerevisiae*. These developed in old (1½ to 2 months) cultures. Some 25 new races were obtained, divided into five groups differing from the parent culture in stability, in cell form, in colony form, and in physiological characters. A noteworthy variant was a "lethal" form, with brown colonies and reduced resistance to unfavorable influences, a tendency to die if not frequently transplanted. Variants also appeared in 10 to 12 days in cultures grown at high (37°) temperature, appearing either as sectors or

as secondary colonies. These resembled the variants obtained spontaneously by growth at a lower temperature over a longer period of time.

Rochlina (1934) obtained variants of *S. cerevisiae* through the action of radium. Wort agar was thickly seeded with the yeast, and poured into Petri plates with a capillary tube of radium in the center. Immediately about the tube there was no growth, but in the inhibitory zone, some colonies appeared which were variants. Among these was a stable asporogenous race with ameboid cells, cells with numerous projections which are considered to be undeveloped buds with thin walls. Colonies were rough and cells showed cytological differences from the parent type.

Fabian and McCullough (1934) reported on induced variations in yeasts; in this report, obviously, they were influenced greatly by the work of Hadley and others on dissociation in bacteria. They worked with *S. cerevisiae* (strains Saaz and Frohberg), *Hansenula anomala*, and *Zygosaccharomyces mandshuricus*. Variations induced by aging or by serial transfers in broth containing lithium chloride, broth containing brilliant green and picric acid, broth containing alcohol, or by desiccation on gypsum blocks or by growth at different temperatures produced three main types: the *S* or smooth form, with globular cells and smooth colonies; the *R* or rough form with elongated cells and rugose colonies; and the *G* or gonidial form, with small bacteria-like colonies. Growth in lithium chloride and brilliant green broths gave rise to both *R* and *G* forms. Desiccation on gypsum blocks gave rise to *G* forms but no *R* forms. The production of *G* forms was stimulated by growth in broth containing alcohol.

R forms in general showed the same biochemical characters as the *S* forms from which they were derived, but produced pellicles on liquid media. The *G* forms, however, differed markedly, failing entirely to produce alcoholic fermentation, but producing acid without gas from some sugars.

R and *G* forms reverted to the *S* forms on repeated transfers in malt extract broth. *G* forms were said to arise from both *S* and *R* forms, either by a gradual shrinkage in the size of the cells,

or suddenly by the production of multiple small buds. A transitional, or *T* form, is also described as preceding the appearance of *G* forms. The reversion of *G* forms to *S* occurred suddenly without any transitions.

The *G* forms described and illustrated by Fabian and McCullough are obviously bacteria, and it is difficult for the reviewer not to believe that they are bacteria which have contaminated the cultures rather than offspring of the yeasts. There is further evidence of contamination in experiments reported by these authors. Thus the *G* forms of the Saaz yeast are described as changing to a pink mucoid yeast, obviously *Rhodotorula glutinis*, and to an *R* form with black wrinkled colonies, obviously *Monilia nigra*, two of the common air contaminants which appear frequently on yeast media. Though the uncritical character of the work makes it difficult to evaluate, the *R* forms described have been observed repeatedly by others.

The experiments of Nadson and his coworkers were continued with *Zygosaccharomyces mandshuricus* by Olenov (1935). He retained the radon in capillary tubes which were placed upon the surface of agar plates seeded with the yeast; only the beta and gamma rays were used. After 1 to 4 days the radon tube was removed. Each colony which developed was examined microscopically and grossly, and an approximately equal number of control colonies (not exposed to radon) were similarly examined. This yeast proved more stable under radon treatment than did the one studied by Nadson and Rochlin, and single exposures gave rise to only slight variations. By exposing such variants again to radon, new variants were produced, and after 4 such re-exposures, 15 new races were obtained. It is noted that this amount of exposure would be lethal if continuous. In three instances new variants developed spontaneously in subcultures from races previously exposed to radon; but no variants developed spontaneously in the parent strain which had never been exposed to radon. The variants differed from the parent race in many characters which varied independently. The parent type gave smooth giant colonies, while giant colonies of most of the variants were rough. Cell forms varied from globular to

cylindrical, in one case irregular or ameba-like cells. While spore formation in the parent race was preceded by isogamous conjugation, in some of the variants it was parthenogenetic, and others were asporogenous. Though the parent strain fermented only glucose, twelve of the variants fermented galactose, sucrose and maltose as well.

In further studies (Olenov, 1936) it was shown that one of the induced variants, asporogenous and capable of fermenting maltose, was more successful than, and would crowd out, the parent race in beer wort. This was attributed to the ability of the new race to ferment maltose, but it was found capable of overgrowing the parent race in other media. Thus the author claims to have carried out a degree of experimental evolution, involving the development of new races by induced mutation, the adaptation of one of these to, and its selection by, the environment (beer wort).

Wickerham and Fabian (1936) described differences in the morphology of the *S* forms, and of *R* forms induced by cultivation in media containing lithium chloride. They worked with *Saccharomyces aceris-sacchari* Fabian and Hall, and with *Pichia alcoholophila*. The cells of the *R* forms were characterized by elongated form, abundance of large oil drops and larger vacuoles. Both cells and colonies were larger than in the *S* forms. In cultures of the *R* form, daughter cells separate immediately, while they tend to remain attached to the mother cells in the *S* cultures. The *R* cells tend to float to the top of the liquid media; *S* cells tend to sink.

Nadson (1937) presented generalizations drawn from the previous papers by himself and his students. It is noted that the effect of x-rays is not apparent immediately, and that variations may not appear until subcultures of the exposed culture are studied. Induced variations may or may not be hereditarily transmissible. But it is noteworthy that temporary modifications are of the same character as permanent ones, and whether they become hereditary or not depends largely upon the dosage of the inciting agent. Certain observed variations are considered to be atavistic in character, for example the development of the

spore-forming *Sporobolomyces* from *Torulopsis* types. No incontestably progressive or evolutionary changes have been observed. The age of the culture treated is of importance, young and old cultures are more likely to give rise to variants when exposed to radiation than are cultures of middle age, and old cultures are more likely to form variants than young ones. There is an optimal dosage of the inciting agent. Both radiation and chemical agents incite the development of variations of the same character. According to dosage such agents produce effects which may be indicated by the range: No effect → Stimulation → Depression → Necrobiosis → Death. Variations may appear in cells exposed to the range, Stimulation → Necrobiosis, which Nadson calls the "field of action." From a single strain of *S. cerevisiae*, stable races were obtained which corresponded in morphology with seven other species. The review concludes with a complete bibliography of the papers from Nadson's laboratory, only some of which have been cited here.

Throughout this series of studies on genetics and variation of the yeasts there runs a common thread. Whether arising by genetic segregation or by hybridization, whether by spontaneous or induced mutation, or by unknown mechanism, there is a tendency for the normally globular yeast with smooth colonies to give rise to forms with cylindrical or sausage-shaped cells, then to pseudo-mycelium, and finally to true mycelium, accompanied by increasing roughness of the colonies. Other variations are noted,—the loss of pigment, the "lethal" variants, and losses in fermentative powers and spore formation noted by various workers—but these characters all vary independently of the *S-R* transformation. The *S-R* variation in yeasts and yeast-like fungi shows such a close parallelism to this type of variation in bacteria that one can hardly escape the conclusion that they are identical phenomena. As with the bacteria, the formation of rough variants is prone to take place spontaneously in old laboratory strains, and may be induced by such agents as lithium chloride, immune serum, x-rays, and radium emanation. The rough variants of both groups of microbes are characterized by the tendency to form pellicles on broth, to spontaneous agglutina-

tion or nonspecific agglutination by various agents, to a loss of pathogenicity. But there are also morphological parallels. The tendency of the cells of the rough forms of yeasts to become sausage-shaped has its parallel in the bacillary forms observed in the rough forms of the pneumococcus, for instance, or in the long filamentous cells found in rough colonies of the gram-negative bacilli. The transformation of yeasts to mycelial fungi has its counterpart in the observation recorded by Novak and Henrici (1933) of a coccus transformed into branched mycelium characteristic of actinomycetes. One may find in earlier literature, notably in the work of Lieske (1921), a suggestion that unicellular bacteria bear a relation to the actinomycetes similar to that of the yeasts to the molds. This is as yet rather vague and conjectural, but it is obvious that it behooves the bacteriologist interested in problems of cytology, genetics and variation to follow the development of these fields in connection with the yeasts and lower fungi.

PHYLOGENY OF YEASTS

As early as 1897 Schröter had pointed out the similarity between the spore-forming yeasts and mycelial fungi of the family *Endomycetaceae*. Following his earlier studies of sexuality in yeasts, Guilliermond in 1909 began to trace the origin of the yeasts in the higher fungi, and subscribed to the idea that they were derived from the *Endomycetaceae*, a family of primitive Ascomycetes which produce mycelium that may give rise to yeast-like cells by budding or by fragmentation of the mycelium into oidia, and which produce ascospores by isogamous conjugation of contiguous cells in the mycelium. In the genus *Endomycopsis* yeast-like cells are formed by budding, and it is a simple transition from such a fungus to budding yeasts of the genus *Zygosaccharomyces*, which form ascospores by isogamous conjugation but produce no mycelium. In the genus *Endomyces* free cells are produced as oidia which multiply by fission, and again it is a simple transition from such fungi to yeasts of the genus *Schizosaccharomyces*.

The relationship of the ascospore-forming yeasts to the *Endomycetaceae* is so obvious that it has been accepted by all

mycologists, and the spore-forming yeasts have been generally classified as a family, *Saccharomycetaceae*, placed next to the *Endomycetaceae* in the order *Plectascales*, the two families differing only in the formation of mycelium. But we have already seen how easy is the transformation from a unicellular yeast-type to a filamentous mold-type. Stelling-Dekker (1931) points out how difficult it often is to determine whether a given species is sufficiently mycelial to be placed in the *Endomycetaceae* rather than the *Saccharomycetaceae*, and that the same species has been placed in both families by different authors. She considers that a separation of the families is unwarranted, and includes the ascospore-forming yeasts in the family *Endomycetaceae*, of the order *Plectascales* of the Ascomycetes.

In his earlier writings Guilliermond derived all of the yeasts from the *Endomycetaceae* through the process of retrograde evolution in sexuality. With further development of knowledge of sexuality in those yeasts whose spores conjugate on germination ("diplobiontic") or whose sexuality is manifested by a conjugation of haploid buds derived directly from spores ("haplo-diplobiontic"), Guilliermond has developed a new concept of the phylogeny of yeasts involving two lines of descent; the haplobiontic yeasts which form spores following isogamous or heterogamous conjugation and derived from the *Endomycetaceae* of the order *Plectascales*, and the diplobiontic and haplo-diplobiontic yeasts derived from the *Exoascales*, especially through the genus *Taphrina*. This double phylogeny would warrant a sharp separation of the spore-forming yeasts into two groups. We will not attempt to review in detail the numerous papers in this field which have appeared from Guilliermond's laboratory since 1931, the following statements being taken from his review (Guilliermond, 1940).

In the *Endomycetaceae*, ascospores are formed by the conjugation of two neighboring cells in the filament of mycelium, which are haploid; the zygote gives rise to ascospores which are diploid. These on germination give rise to mycelium, which in turn may give rise to yeast-like cells by budding or by fragmentation. The mycelium and the yeast-like cells are haploid. In this family

Guilliermond places the yeasts which he calls haplobiontic (*Schizosaccharomyces, Zygosaccharomyces, Zygopichia, Debaryomyces, Nadsonia, Nematospora*).

In the remaining genera the vegetative cell is diploid, only the spores being haploid in the "diplobiontic" *Saccharomycodes Ludwigii*, or the spores and the first few buds in the "haplodiplobiontic" genera (*Saccharomyces, Hansenula*). These Guilliermond compares to fungi of the *Exoascales*, and particularly to the genus *Taphrina*, the life cycle of which was reported by Wieben (1927). In the parasitic fungi of this genus, the haploid ascospores give rise to haploid budding yeast-cells on germination. These soon conjugate, but the nuclei do not at once fuse; instead there is produced a binucleate mycelium, as in higher Ascomycetes and Basidiomycetes. Eventually the nuclei fuse to give rise to asci. Guilliermond considers this cycle to be similar to that of the diplobiontic yeasts, the diploid phase of the yeasts being represented by the binucleate phase of *Taphrina*; instead of a 2n nucleus, there are two "n-chromosome" nuclei. As was mentioned in a preceding section, Guilliermond and Renaud found some tendency toward a binucleate stage in the life histories of some yeasts.

Guilliermond's concept of the phylogeny of the yeasts would make the genera *Saccharomyces* and *Zygosaccharomyces* only distantly related, belonging to separate orders of the Ascomycetes. Stelling-Dekker's classification makes these two genera but subgenera of a single genus, *Saccharomyces*, in the broader sense. In this she is following a suggestion of Klöcker. She justifies her arrangement on the ground that the *Zygosaccharomyces* species closely parallel the *Saccharomyces* (*sensu strictu*) species in physiological and other characters; that parthenogenesis is common in *Zygosaccharomyces* and conjugation is occasionally seen in yeasts placed in the genus *Saccharomyces*. Winge and Laustsen (1937) also point out that "The circumstance that *Saccharomyces* may be cultivated in the haploid state even though it is inclined to revert to the diploid phase by zygote formation shows that there is only a slight biological difference between *Saccharomycetes* in the strict sense of the term and *Zygosaccharomycetes*." The

fact that these authors (1939) were able to obtain hybrids by crossing a species of *Saccharomyces* with a species of *Zygosaccharomyces* indicates that these genera cannot be very distantly related.

Guilliermond (1940) further speculates upon a possible relationship between the yeasts and the smut fungi (*Ustilaginales*). The latter, although Basidiomycetes rather than Ascomycetes, show similarities in their life history to the *Exoascales* such as *Taphrina*. The smut spore may be considered to be homologous with the asci, containing nuclei which fuse. On germination the smut spore produces a short filament of mycelium which gives rise to large numbers of yeast-cells (sporidia) which may multiply as such indefinitely by budding. Such smut sporidia, isolated in pure culture, have often been mistaken for true yeasts. Guilliermond compares the life cycles of various types of *Ustilaginales* with the various groups of yeasts.

There is, however, better evidence that some yeasts are related to Basidiomycetes in a consideration of those belonging to the genus *Sporobolomyces*, the nature of which was first clearly recognized by Kluyver and van Niel (1925). They are yeasts which produce a rose or salmon-colored pigment. The colonies become powdery on the surface, and if colonies in this stage are inverted in position, a faint mirror image of the colonies appears on the lid of the Petri dish in which they are contained. This image is composed of spores which are forcibly discharged from the surface of the colony. The vegatative cells are oval to cylindrical in form and multiply by budding like other yeasts, and a pseudomycelium may be formed. Cells on the surface of the colony give rise to fine stalks, or sterigmata, upon which the kidney or sickle-shaped spores are formed. When mature these spores are discharged forcibly, the discharge being preceded by the appearance of a droplet of moisture. The authors note that the shape, formation and mode of discharge are precisely as Buller had previously described for mushrooms, and considered this evidence that yeasts of this group might be considered to be related to the Basidiomycetes.

This was rejected by Lohwag (1926) on the ground that a similar method of spore-discharge is observed in other fungi not related to the Basidiomycetes. Lohwag stated that only a cyto-

logical study to determine whether the pretended basidiospores result from a fusion of two nuclei would establish the true nature of the spores. Such cytological studies by Guilliermond (1927) revealed but one nucleus in the vegetative cells and the cells which give rise to the spores. He concluded, therefore, that the spores are not basidiospores, but asexual conidia. This argument was opposed by Kluyver and van Niel (1927) and Buller (1933), who point out that a failure of nuclear fusion does not exclude the possibility that the spores of *Sporobolomyces* are basidiospores because parthenogenetic basidiospores are known in higher Basidiomycetes, and because the ascospores of many yeasts are known to be parthenogenetic in origin. Buller, who had studied so carefully the mode of spore discharge in the mushrooms, was fully convinced that the spores of *Sporobolomyces* were typical basidiospores, and this position was also supported by Derx.

Derx (1930) found the natural habitat of these yeasts to be leaves and straw, and showed that when freshly isolated they give rise to colonies which are smooth and mucoid, spore-formation first appearing in secondary colonies, or in floating islands of growth upon liquid media. He discovered a new group of yeasts which discharge their spores, colorless or slightly yellowish, and with globular or ovoid, symmetrical basidiospores; they are discharged by the same mechanism as in *Sporobolomyces*. This yeast Derx placed in a new genus, *Bullera* (which, according to Ciferri and Verona (1938), should be spelled *Bulleria*, in order to conform with the International Botanical Rules of Nomenclature).

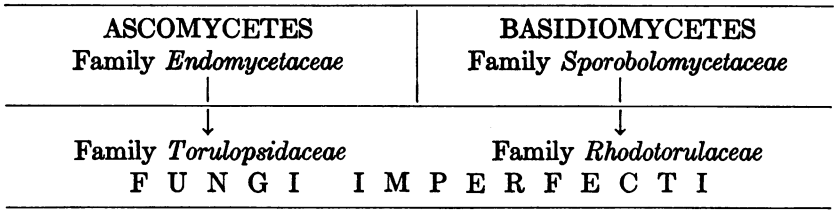
Ciferri and Redaelli (1935) considered the spores of *Sporobolomyces* to be conidia rather than basidiospores, and placed these yeasts together with *Nectaromyces Reukaufii* in a family of conidia-forming yeasts, the *Nectaromycetaceae*. *Nectaromyces Reukaufii* is a peculiar yeast occurring in the nectar of flowers in groups of four cells in the form of a cross or an "aeroplane." In cultures ordinary budding cells without spores are formed. Nadson and Krassilnikov (1927) recorded a mutant which gave rise to mycelium that produced conidia. On the basis of this single observation, Ciferri and Redaelli proposed to unite this genus with *Sporobolomyces*. It seems to the reviewer that this position

is untenable, and that the genus *Sporobolomyces* represents a group of yeasts derived from the Basidiomycetes.

The phylogenetic relationships of the yeasts which form no spores cannot be determined with certainty, as with other imperfect fungi. It has been widely assumed in the past that all of them are Ascomycetes which have permanently lost the power to form ascospores. Among the asporogenous yeasts, however, the group forming rose, red or orange pigments show characteristics which separate them from the others. These have been especially noted by Lodder (1934) who proposed to place them in a separate family, the *Rhodotorulaceae*. We shall consider the characteristics of this family in a later section. Nadson and Philippov (1928) observed the origin of yeasts resembling *Sporobolomyces* species from asporogenous mucoid rose yeasts indistinguishable from *Rhodotorula* species, and Derx (1930) observed the same sort of transformation to occur. Lodder (1934) records that cultures of *Sporobolomyces* maintained in the type culture collection at Delft have irreversibly lost the power to form basidiospores and become indistinguishable from the ordinary rose asporogenous yeasts. These facts indicate strongly that the common red yeasts are to be considered as imperfect forms of the basidiospore-forming yeasts of the genus *Sporobolomyces*.

Unicellular growth forms appearing either as budding yeast-like cells or as oidia multiplying by fission occur so frequently in the growth of many different species of fungi of all classes that it seems improbable that those which are permanently or dominantly unicellular, and which we call yeasts, can be phylogenetically homogeneous. Many species of the *Mucorales* give rise to budding yeast-like cells when grown submerged in liquid media, and it is noteworthy that such species also produce an alcoholic fermentation. Nevertheless there are no true yeasts known to show relationships to the Phycomycetes. Many of the fungi pathogenic to man, as *Blastomyces, Coccidioides, Histoplasma, Sporotrichum,* grow as yeast-like unicellular fungi in the tissues, but grow as mycelial fungi in artificial cultures, unless (in some cases) they are cultured in the presence of blood or serum under reduced oxygen tension.

The known yeasts appear to fall rather sharply into two narrow and apparently monophyletic groups. Those which produce carotinoid pigments fall either into the basidiomycetous family of *Sporobolomycetaceae* or the imperfect family of *Rhodotorulaceae*. Those without carotinoid pigments fall in the ascomycetous family of *Endomycetaceae* or the imperfect family of *Torulopsidaceae*. The systematic relationships of the yeasts may be indicated by the following diagram:



CLASSIFICATION OF YEASTS

The remainder of this review will deal with the classification and identification of yeasts, presenting first an outline of the systems of Stelling-Dekker (1931) for the ascospore-forming yeasts, and of Lodder (1934) for the asporogenous yeasts. This will be followed by discussions of selected groups of yeasts in which important taxonomic work has been done during the past decade. The review cannot be considered complete, since the technological literature has not been covered adequately. Undoubtedly, in the literature on dairy products, on silage, on foodstuffs, on the various fermentation industries, there may be found important taxonomic data which the reviewer has overlooked. Before considering the classification and identification of yeasts it is necessary to take some note of the various characteristics of yeasts by which they are classified and identified, and of the technical procedures which must be used to demonstrate these characteristics.

Technical Procedures for the Identification of Yeasts

The identification of yeasts involves a determination of both morphological and cultural characters. Of the morphological

characters it is necessary to determine the relative size and shape of the vegetative cells; whether they multiply by fission or by budding; if by budding, whether buds are formed at any place on the cell (multipolar) or only at the ends of elongated cells (bipolar); whether the buds are separated from the mother cell by abstriction or by the formation of a cross-wall followed by fission. Further, it is necessary to determine whether the buds separate quickly after they are formed, giving rise to free cells or groups of only two or three, or whether they tend to remain attached forming clusters ("sprossverbände"); in the latter case the cells, if elongated, may give rise to a branched structure resembling closely true mycelium ("sprossmyzel," pseudomycelium); in some cases true mycelium with cross-walls is formed. With yeasts forming pseudo- or true mycelium, clusters of yeast-like cells may arise by budding, usually at the nodes ("sprosskonidien," "blastospores," "appareil sporifère").

Since these vegetative characters do vary with the composition of the medium, it would be desirable if yeast workers would agree upon a standard reproducible medium upon which to grow yeasts for morphological descriptions. The most widely used medium is beer wort, or beer wort agar. This is not, however, a medium of standard composition, and not readily available to the laboratory which only occasionally works with yeasts. Some workers have used hopped wort, others unhopped. Workers in wineries have used must (grape juice), or must agar; Japanese workers have used "Koji" preparations. Stelling-Dekker (1931) gives the following recipe for wort: 1 kg. of malt-meal (dried "Langmalz") is added to 2.6 liters of tap water and heated to 45°C. for three hours with continuous stirring; it is then heated to 60°C. for an additional hour, filtered, and the filtrate heated to 120°C. for 15 minutes in the autoclave. The filtrate is brought to a concentration of 15° Balling, again filtered, and finally sterilized. The Digestive Ferments Company markets dehydrated wort agar which presumably is fairly constant in composition, but it is, in my opinion, too acid (pH 4.8) and when autoclaved, it is hydrolyzed partially and forms a soft, mushy medium. I have compared the morphology of a limited number of yeasts

on wort agar and on agar containing 5 per cent pure glucose and 1 per cent Bacto peptone, and have found but slight differences. The latter is a readily reproducible medium.

The development of mycelium or pseudomycelium depends upon many factors, but especially upon the age of the culture and the nutrient value of the medium. Talice (1930) has studied the conditions affecting the formation of pseudomycelium in the *Mycotoruloideae*. He recommended especially the use of a dilute potato infusion (20 grams potato pulp to 1 liter of water). Three successive transfers in this medium should be studied before a yeast is to be considered free of mycelium formation. Lodder (1934) used this method, incubating the cultures three weeks at 25°. Rivalier and Seydel (1932) described a method for slide cultures which was also used by Lodder. The medium consisted of 2 per cent dextrose, 1 per cent peptone, 2 per cent agar. Martin, Jones, Yao and Lee (1937) used corn-meal agar in slide cultures. Soriano (1938) used thin blocks of agar on the underside of coverslips sealed over the depression of concave-ground slides. Some sort of slide culture is almost essential in clearly observing the morphology of the *Mycotoruloideae*.

Benham (1931) found that corn-meal agar is an important medium in studying the morphology of the *Mycotoruloideae*, especially for the development of the diagnostic chlamydospores of *Candida albicans*. This medium is made of 62.5 grams of corn meal heated in 1500 ml. water at 60° for an hour, filtered, 19 grams agar added, the mixture steamed in the Arnold sterilizer 1¼ hrs., filtered through cotton, tubed and autoclaved. The reaction is pH 6 to 7.

In addition to the morphology of the vegetative cells, the presence or absence of spores, their mode of formation, and the number and shape or structure of the mature spores are important diagnostic characters. Basidiospores of *Sporobolomycetaceae* are readily detected, appearing as a powdery coat on the surface of the colony, and forming the typical mirror colony on the lid of the culture dish. From the mirror colony a pure preparation of spores may be obtained for microscopic examination. The production of ascospores by *Endomycetaceae* requires the use of special

media or methods. Although ascospores may appear abundantly in ordinary cultures on rich media, especially with strains recently isolated from nature, they cannot be considered as absent until repeated attempts have been made to force their development upon special media.

The use of blocks of plaster of Paris moistened with water or with dilute peptone solution, which has been the standard method for inducing spore formation in yeasts since Hansen, has been replaced often in recent years by Gorodkowa's agar (glucose 0.25 per cent, meat extract 1 per cent, NaCl 0.5 per cent). Stelling-Dekker (1931) reviewed the literature on various methods for inducing spore formation, and concluded that no one procedure is adequate; a number of methods must be used in succession before a yeast may be called asporogenous. In addition to the plaster block and Gorodkowa's agar, she used potato and carrot plugs, Kufferath's "gelose farhydre," exposure to ultraviolet light, raisins and raisin agar, and for *Debaryomyces* species, growth on sterilized sausage. The medium of Kufferath (1928, 1930) is prepared from malt hydrolyzed with H_2SO_4, neutralized with $CaCO_3$, to which is added agar, and NaOH to give an alkaline reaction; a series of such media of varying degrees of alkalinity are prepared, and the unknown yeast is inoculated on all of them. Mrak and McClung (1940) noted that in some instances spores were formed on cucumber wedges, when they failed to develop on other media. The reviewer has used with much success McKelvey's (1926) agar (dilute carrot infusion with $CaSO_4$) which has repeatedly revealed spores that failed to develop on Gorodkowa's agar or plaster blocks. Niehaus (1932) used soil extract gelatin (1000 to 1500 grams garden soil extracted with 1000 ml. water, filtered, 15 per cent gelatin).

Ochmann (1929) made an extensive study of the influence of the nitrogen sources in the medium upon spore formation in six yeasts (*Saccharomyces cerevisiae* (Johannisberg strain), *S. turbidans*, *S. pastorianus*, *S. validus*, *Schizosaccharomyces octosporus*, and *Schizosaccharomyces pombé*). Spore formation was induced by transferring the yeasts from the media studied to blocks of soaked and sterilized beechwood. The basal medium was 10 per cent dextrose, 0.5 per cent KH_2PO_4 and 0.25 per cent

$MgSO_4$. To this was added 26 different nitrogenous compounds. Wort was used as a control medium. It was found that with each species spore formation was stimulated by certain N compounds which would not necessarily permit of spore formation with other species. Thus nitrates favored spore formation by *S. pastorianus* but not by the others, and so on. This paper is cited to indicate the difficulties inherent in a classification of yeasts based upon spore formation. Probably with many of the asporogenous yeasts we have not yet found the environmental conditions necessary for sporulation.

Since spore-formation is often delayed, cultures should be examined repeatedly over a period of several weeks before they are to be labelled as asporogenous. Windisch (1938) reported spore formation in *Torulopsis pulcherrima* only after the agar cultures had dried considerably, and Todd and Herrmann (1936) had the same experience with *Debaryomyces neoformans*. Fuchs (1935) found that with old laboratory strains which had quit forming spores this character could be revived by growing them on wort agar, transferring the young growth to plaster blocks, then back to wort agar, and so on. Usually after three such treatments, spore formation will begin again.

By using aerated solutions, Stantial (1935) showed that washed cells of *S. cerevisiae* could be made to sporulate in the presence of certain sugars; mannose or maltose gave up to 85 percent ascus formation. Acetate also favored the process, especially in the presence of mannose or dextrose. The yield was found dependent upon the ratio of cell to solute concentration; also, prior cultivation of the yeast in a bios-containing medium was a prerequisite for sporulation in the above procedure.

The presence or absence of spores is determined by microscopic examination of wet preparations and of stained slides. Recognition of spores in the living cells requires experience. Often fat-globules or water-vacuoles have been mistaken for spores. Spores may be differentially stained by one or another modification of Moeller's spore stain, widely used to stain bacterial spores. The following method has given the best results in my hands: 5 per cent chromic acid solution 5 minutes, Ziehl's carbol fuchsin steaming 5 minutes, decolorization for 2 minutes in 1 per cent

aqueous H_2SO_4, counterstain with Loeffler's methylene blue 1 minute. Spores are red, asci and vegetative cells blue. Ochmann (1929) stained the spores of *Schizosaccharomyces* species with methylene blue followed by Bismarck brown (spores blue, cells brown) and by Gram's method (spores blue-black, cells red). Kufferath (1928) insisted that differential staining should be the only criterion of spores, but many others have noted that in some yeasts the spores may fail to show the differential staining. This has been my experience with *Nadsonia fulvescens* and with *Debaryomyces* species.

In the genus *Debaryomyces* an important diagnostic character is the warty character of the wall of the mature spore. This is not always present, and may be difficult to observe, largely because the single spore completely fills the ascus, and so the outer surface of the spore wall is closely applied to the inner surface of the ascus wall. Mrak and Bonar (1938b) have observed that if the cultures inoculated in Gorodkowa agar with yeasts of this genus are incubated at 16°, or lower, the asci are larger in relation to the size of the ascospores, and the warty walls may be readily observed.

The mode of growth of yeasts in liquid media is of greater diagnostic importance than in bacteria. The formation of a dry, matte, pellicle from the beginning of growth is characteristic of the genera *Pichia*, *Hansenula*, some species of *Debaryomyces* and *Mycoderma*. A mucoid scum may develop late with other genera. This pellicle may be observed on the liquid media used for sugar fermentations. Most recent descriptions of yeasts have been accompanied by illustrations of their giant colonies, usually grown upon wort gelatin. It has been repeatedly demonstrated that the characteristics of these colonies are very easily changed by slight variations in the composition of the medium, especially the consistency of the substrate. Here a standardized medium is especially desirable. Stelling-Dekker (1931) rejected the giant colony in favor of the wort-agar stroke culture, as giving essentially the same sort of information. The growth may be mucoid, pasty or tenacious; smooth, matte, or wrinkled; white, creamy, buff, or definitely colored.

Lodder (1934) separated the asporogenous yeasts with carotinoid pigments in a family, the *Rhodotorulaceae*, and used a method devised by Molisch for the determination of the carotinoid pigment. A considerable amount of growth is brought into a test-tube containing 2 ml. of a 20 per cent solution of KOH in 40 per cent alcohol, and allowed to stand in the dark for some days. Some of the sediment is removed with a pipette and examined microscopically for red or organge-red crystals. In all cases where definitely red, orange or yellow yeasts were investigated, such crystals were found. Mrak and McClung (1940) experienced considerable difficulty with this method, and found that storage of the tubes for six weeks was necessary to give consistent results. However, it is hardly necessary to resort to microchemical methods. Lodder found no crystals from old cultures of nonchromogenic yeasts giving the yellowish-brown color that often appears with age. The carotinoid pigments appear early in growth and are usually distinctive enough to be recognized by simple inspection of the culture tubes. The "iron-red" pigment formed by *Torulopsis pulcherrima* and some other species is to be distinguished from the carotinoid pigments. It is definitely maroon rather than pink, coral, or rose, as in the *Rhodotorulaceae*; it usually diffuses into the medium, and often fails to color the cells, which remain white. It will not develop on synthetic media free from iron.

In a number of genera of yeasts the species are identified by sugar fermentations. Stelling-Dekker (1931) has discussed extensively methods for determining sugar fermentations. She used two methods: growth in Einhorn fermentation tubes containing 2 per cent of the sugar to be tested in yeast infusion (200 grams compressed yeast extracted in 1 liter of water in the autoclave), and quantitative determinations by used of the van Iterson-Kluyver apparatus, the latter method especially in studying the fermentation of raffinose.

The fermentation of raffinose occupies a special place in Stelling-Dekker's system. Some yeasts split this trisaccharide to levulose and the disaccharide melibiose, fermenting the levulose but not attacking the melibiose; such yeasts are described as fermenting

"⅓ of raffinose." Other yeasts completely hydrolyze raffinose to its component monosaccharides, and are said to ferment raffinose completely. This procedure is therefore a method of separating yeasts upon the basis of their ability to ferment melibiose. Stelling-Dekker's method requires the use of quantitative apparatus, an inconvenience in routine work. I have been unable to determine why she does not study the fermentation of melibiose directly; its cost is considerably higher than that of raffinose, but not prohibitive. Zimmermann (1938), to use Stelling-Dekker's keys, determined the character of the fermentation of raffinose by titrating the remaining sugar after 14 days.

Generally, 2 to 3 per cent solutions of sugars and large volumes are used rather than the 1 per cent solution in small fermentation tubes commonly used to study fermentations by bacteria. I have been unable to find any discussion of this, but there is a real reason for it. I have repeatedly observed yeasts to give a vigorous fermentation in tubes containing 10 to 12 ml. of liquid, when they gave no or slight fermentation in small tubes containing only 2 to 3 ml. I suspect this to be due to more complete diffusion of oxygen in the smaller volume of liquid, favoring oxidative rather than fermentative respiration. Martin, Jones, Yao and Lee (1937) recommend a vaseline seal on the sugar fermentation tubes to maintain anaerobiosis. Langeron and Guerra (1938) used paraffin with paraffin oil.

The reviewer uses, for fermentation tests, tubes of large diameter (25 x 150 mm.) with inverted Wassermann tubes (10 x 75 mm.) for gas-traps, containing about 20 ml. of medium. With pellicle-forming yeasts it is well to shake the pellicle free after a day or two, so that some cells will sink to the botton of the tube. One should shake the tubes vigorously before reading the results, as sometimes the solution may be supersaturated with gas, yet show none in the gas-trap. On shaking, effervescence is seen. Yeasts often form acid without gas from sugars; these fermentations have so far found no place in the systematics of yeasts, save that numerous authors have noted that *Candida albicans* regularly produces acid from sucrose, though it produces alcohol and gas from dextrose and levulose.

In 1914 Kluyver published certain generalizations concerning sugar fermentations by yeasts which were restated by Stelling-Dekker, and are referred to by Langeron and Guerra (1938) as the "Kluyver-Dekker laws"; 1. A yeast which cannot ferment glucose cannot ferment any other sugar. 2. A yeast which can ferment glucose can also ferment fructose and mannose. 3. A yeast cannot ferment both lactose and maltose.

In addition to fermentations, it is necessary with some groups of yeasts to determine what substances they may utilize as sources of nitrogen or carbon. One prepares a basal medium containing all ingredients necessary for growth, including in one case a universally available source of carbon (all yeasts can utilize dextrose), in the other case a universally utilizable source of nitrogen (ammonium sulphate could be utilized in the presence of dextrose by all of the yeasts studied by Lodder). Other salts as potassium phosphate and magnesium sulphate are necessary. If liquid media are used, the trace elements and growth-accessory substances ("bios" complex) must be added, which are easily provided by adding a trace of yeast extract to the medium.

Lodder (1934) made use of Beijerinck's "auxanographic" method for testing the utilization of sugars by yeasts. A basal medium is made, composed of 0.05 per cent $(NH_4)_2SO_4$, 0.1 per cent KH_2PO_4, 0.05 per cent $MgSO_4$, and 2 per cent of washed agar. This is heavily seeded while molten with the yeast to be tested, and poured into a Petri dish. The heavy seeding obviates the necessity of providing "bios". Small quantities of the sugars to be tested are deposited upon the surface of the agar in dry form, including dextrose on each plate as a control. Where the sugar diffuses into the agar, growth will occur if this sugar can be utilized by the yeast being studied. For testing the utilization of N-sources by the auxanographic method, Lodder's basal medium is: 2 per cent dextrose, 0.1 per cent KH_2PO_4, 0.05 per cent $MgSO_4$ and 2 per cent washed agar. Peptone, ammonium sulphate, asparagin, urea and potassium nitrate were tested as sources of nitrogen. Zimmermann (1938) found the auxanographic method to give doubtful results, and preferred to use liquid synthetic media, as did also Mrak and McClung (1940).

Stelling-Dekker (1931) stressed especially the utilization of nitrates and of alcohol in identifying the spore-forming yeasts. The utilization of alcohol was tested in a liquid medium containing: 3 per cent ethyl alcohol, 0.1 per cent $(NH_4)_2SO$, 0.1 per cent KH_2PO_4 and 0.05 per cent $MgSO_4$. The utilization of nitrates was tested by inoculating simultaneously two tubes of agar, one composed of: 2 per cent dextrose, 0.1 per cent KNO_3, 0.1 per cent KH_2PO_4, 0.05 per cent $MgSO_4$, and 2 per cent washed agar; the other tube of the same composition with nitrate omitted. A slight growth almost always occurs in the control tube, but a definitely heavier growth in the tube containing nitrate indicates that this substance can be utilized.

Classification of the Sporobolomycetaceae

The basidiospore-forming yeasts were united by Derx (1930) into a family of "Sporobolomycetes" described as follows: "Microscopic fungi which mostly propagate by budding like *Saccharomycetes*; from part of the vegetative cells there are produced single or rarely branched, often bifurcated sterigmata projecting into the air, on which are formed light, hyaline, distinctly apiculate spores, which when mature are projected in a manner similar to the multiple spores of true Basidiomycetes. The spores, which in form are similar to multiple basidiospores, may form secondary spores like the multiple basidiospores of *Protobasidiomycetes*." Within this family Derx included two genera, *Sporobolomyces* and *Bullera* (amended to *Bulleria* by Ciferri and Verona, 1938).

The genus *Sporobolomyces* is defined by Derx as follows: "Vegetative growth rose, red or salmon. Spores more or less compressed laterally, kidney-shaped, pear-shaped, asymmetric." The genus *Bulleria* is defined as: "Vegetative growth white, whitish, cream, straw or yellow, without any trace of red. Spores round, ovoid, globular, symmetrical." Derx recognized seven species of *Sporobolomyces* in a collection of 40-odd strains. These were distinguished by the color of the vegetative growth, the shape of the spores, the texture of the colonies, and the odor of the cultures. Ciferri and Verona (1938) proposed to subdivide

the genus into two groups, the subgenus *Blastoderma* with pseudomycelium, and the subgenus *Eusporobolomyces* without pseudomycelium. They added several additional species. There are but two species of the genus *Bulleria* described, differing in the size of the vegetative cells, and the size and shape of the spores.

Classification of the Ascosporogenous Yeasts

Stelling-Dekker includes all of the yeasts which form ascospores, together with the related yeast-like (mycelial) forms in a single family, the *Endomycetaceae*, which is subdivided into four subfamilies, the *Eremascoideae*, the *Endomycoideae*, the *Saccharomycoideae* and the *Nematosporeae*. The common spore-forming yeasts are mostly included in the third subfamily, which is further divided into three tribes, the *Endomycopseae*, the *Saccharomyceteae* and the *Nadsonieae*. The distinguishing characters of these subfamilies and tribes are as follows:

Family Endomycetaceae

Growth-forms mycelium, pseudomycelium, oidia or yeast cells ("conidia"), together or singly. Vegetative multiplication by transverse fission or by budding. Naked asci result from isogamous or heterogamous conjugation or parthenogenetic. Ascospores spherical, hemispherical, angular, sickle- or spindle-shaped, smooth, warty, or with an encircling rim. Both oxidative and fermentative species.

Subfamily A. *Eremascoideae*

Growth-form only mycelium. Vegetative multiplication by transverse fission. Spores hat-shaped, result from isogamous conjugation. Dissimilation exclusively oxidative. But one genus *Eremascus*.

Subfamily B. *Endomycoideae*

Growth-form either mycelium with oidia or only oidia. Vegetative multiplication by transverse fission. Spores round, oval or hat-shaped, result from isogamous or heterogamous conjugation. Dissimilation oxidative or fermentative. There are two genera, *Endomyces* with both mycelium and oidia, and both oxidative and fermentative respiration; and *Schizosaccharomyces* with no mycelium, only oidia multiplying by transverse fission, and with dominantly fermentative respiration.

Subfamily C. *Saccharomycoideae.* Growth-form either mycelium with yeast cells ("conidia"), occasionally also oidia, or only budding yeast cells and then often pseudomycelium. Vegetative multiplication by transverse fission, by multipolar budding or by bipolar budding, the latter upon a broad base. Spores spherical, hemispherical, angular or sickle-shaped, or with an encircling ridge, formed by isogamous or heterogamous copulation or parthenogenetic. All transitions between oxidative and fermentative dissimilation. There are three tribes in this subfamily:

Tribe A. *Endomycopseae*

Growth-form mycelium with buds ("conidia"), at times oidia. Vegetative multiplication by transverse fission and by multipolar budding. Spores parthenogenetic or following isogamous copulation. But one genus, *Endomycopsis.* Dissimilation is dominantly oxidative, at times also fermentative.

Tribe B. *Saccharomyceteae*

No mycelium, only budding yeast cells or pseudomycelium. Vegetative multiplication by multipolar budding. Spores produced by isogamous or heterogamous conjugation, or parthenogenetic. This tribe includes the largest number of species of spore-forming yeasts, contained in the genera *Saccharomyces, Torulaspora, Pichia, Hansenula, Debaryomyces,* and *Schwanniomyces.*

Tribe C. *Nadsonieae*

No mycelium, only budding yeast cells, at times pseudomycelium. Vegetative multiplication by bipolar budding, more or less upon a broad base. Spores parthenogenetic or following heterogamous conjugation. There are three genera: *Saccharomycodes* with round spores that conjugate during germination; *Hansienaspora* with parthenogenetic spores; and *Nadsonia,* in which following heterogamous conjugation between a bud and the mother cell, a second bud develops into an ascus.

Subfamily D. *Nematosporoideae.* Growth-form mycelium and budding yeast cells. Vegetative multiplication by multipolar budding. Spores needle- or spindle-shaped, with or without flagella, parthenogenetic or formed after isogamous conjugation. Both oxidative and fermentative. There are three genera: *Monosporella* with one needle-shaped spore; *Nematospora* with 2 to 8 flagellated spindle-shaped spores produced parthenogenetically; and *Coccidiascus* with 8 spindle-shaped non-flagellate spores produced by isogamous conjugation.

We cannot, of course, take space to indicate the characteristics and activities of all of the genera of ascospore-forming yeasts, but a translation of Stelling-Dekker's key will serve to indicate the characters upon which their identification is largely based.

Key to the genera of Endomycetaceae

1. a. Spores fusiform [2]
   b. Spores not fusiform [4]
2. a. Spores always single *Monosporella*
   b. At least 4 spores per ascus [3]
3. a. Spores with one non-motile flagellum *Nematospora*
   b. Spores without flagella *Coccidiascus*
4. a. Vegetative multiplication by transverse fission [5]
   b. Vegetative multiplication by transverse fission, by multipolar budding, or both [6]
   c. Vegetative multiplication by bipolar budding with more or less broad bases to the buds [11]
5. a. True mycelium and oidia *Endomyces*
   b. Only oidia, no mycelium *Schizosaccharomyces*
6. a. True mycelium with crosswalls and "bud-conidia," at times oidia *Endomycopsis*
   b. No mycelium, at times pseudomycelium; "bud conidia," no oidia [7]
7. a. In wort a dry matte pellicle from the beginning [8]
   b. In wort no pellicle, or a soft slimy pellicle after some time [10]
8. a. Nitrates utilized *Hansenula*
   (Lodder (1932) described a new yeast having the characters of *Hansenula*, but producing hat-shaped spores after isogamous conjugation; she therefore created a new subgenus, *Zygohansenula*, the type species being *Z. californica*)
   b. Nitrates not utilized [9]

9. a. Spores round, angular or hat-shaped, smooth; cells in young cultures long-oval to filamentous *Pichia*
   aa. Spore-formation parthenogenetic. Subgenus *Pichia sensu strictu*
   bb. Spore-formation sexual. Subgenus *Zygopichia*
   b. Spores round with a warty wall; cells in young cultures round to short-oval. *Debaryomyces*[1]
10. a. Cells short-oval to elongated, spores round, kidney-shaped or hat-shaped, smooth, 1 to 4 per ascus. *Saccharomyces*
   aa. Spore-formation parthenogenetic. Subgenus *Saccharomyces sensu strictu*
   bb. Spore-formation sexual. Subgenus *Zygosaccharomyces*
   b. Cells round, spores round and smooth, with an oil drop in the middle; copulation tubes formed before spore-formation *Torulaspora*
   (Krumbholz (1933) questions the desirability of retaining the genus *Torulaspora*)
   c. Cells mostly round, but also oval, small. Spores round with a warty wall, almost always 1, rarely 2 per ascus, formed sexually. *Debaryomyces*[1]
   d. Cells oval, rather large, spores round, with a warty wall and a ridge around the middle, copulation tubes formed *Schwanniomyces*
11. a. Cells small, bipolar budding, spores hat-shaped, smooth, 2 to 4 per ascus, parthenogenetic *Hanseniaspora*
   b. Cells large, bipolar budding on a broad base, spores round to oval, smooth, 1 to 4 per ascus. Spores conjugate on germination *Saccharomycodes*

[1] This name was spelled *Debaromyces* in Tanner's translation of Guilliermond (1920) and this spelling has been used by American authors.

c. Cells large, bipolar budding on a broad base, spores round with a warty wall, one spore per ascus. Conjugation before spore-formation; the ascus is formed by budding from the fertilized cell. *Nadsonia*

In order to indicate the characters upon which species are separated in Stelling-Dekker's system, the reviewer has also translated her key to the most important group, the subgenus *Saccharomyces* in the strict sense.

Key to the species of Saccharomyces sensu strictu

1. a. Fermentation of dextrose and galactose only [2]
   b. Fermentation of dextrose, sucrose, and ⅓ raffinose only [4]
   c. Fermentation of dextrose, sucrose and raffinose (completely) only *S. microellipsodes*
   d. Fermentation of dextrose, galactose, sucrose and ⅓ raffinose only [5]
   e. Fermentation of dextrose, sucrose and maltose only *S. heterogenicus*
   f. Fermentation of dextrose, sucrose, maltose, and ⅓ raffinose only [6]
   g. Fermentation of dextrose, galactose, sucrose (weakly) and maltose only *S. Chodati*
   h. Fermentation of dextrose, galactose, sucrose, maltose and ⅓ raffinose only [7]
   i. Fermentation of dextrose, galactose, sucrose, maltose and raffinose (completely) only [8]
   j. Fermentation of dextrose, galactose, sucrose, ⅓ raffinose, and lactose only *S. fragilis*
2. a. Rather long clusters of cells in wort from the beginning *S. dairensis*
   b. Cells in wort singly or in pairs [3]
3. a. But one spore per ascus *S. unisporus*
   b. Several spores per ascus *S. globosus*

4. a. Cells elongated, in clusters — *S. muciparus*
   b. Cells round or oval — *S. Chevalieri* and varieties *Lindneri, torulosus*
5. a. Cells in young wort culture small, round to oval (3.5 to 5.5) μ x (4 to 7) μ — *S. exiguus*
   b. Cells in young wort culture oval (3.5 to 5) μ x (5 to 10) μ — *S. mangini* and variety *tetrasporus*
6. a. Cells oval or egg-shaped — *S. oviformis*
   b. Cells long-oval to elongated — *S. Bayánus*
7. a. Cells in young wort culture round, oval, egg-shaped or pear-shaped, (3 to 7) μ x (5 to 14) μ; length to breadth ratio, 1 to 2 — *S. cerevisiae* and varieties *ellipsoideus, turbidans, Marchalianus, pulmonalis, festinans*
   b. Cells in young wort culture oval to sausage-shaped, (2.5 to 4) μ x (9 to 11) μ; length to breadth ratio, 3 to 4. — *S. intermedius*
   c. Cells in young wort agar short to long oval, (4 to 8) μ x (9 to 18) μ — *S. Willianus*
   d. Cells in young wort culture oval, (3 to 5.5) μ x (5 to 9) μ, but elongated on wort agar (2 to 4.5) μ x (6 to 14) μ — *S. odessa*
   e. Cells in young wort culture elliptical or elongated, (3 to 5) μ x (7 to 10) μ, on wort agar after a longer time very long, up to 30 μ; fermentation of maltose weak — *S. tubiformis*
   f. Cells in young wort culture round to oval, (3 to 6) μ x (4.5 to 9) μ, in older cultures elongated, in old wort agar cultures forming irregularly shaped cell-complexes. — *S. paradoxus*

8. a. In young wort cultures long clusters of long-oval cells — *S. pastorianus*
   b. Cells in young wort cultures single or in pairs or threes — [9]
9. a. Cells in young wort culture oval, (3 to 5) μ x (7 to 10) μ — *S. carlsbergensis* and varieties *monacensis, valdensis, mandshuricus, polymorphus*
   b. Cells in young wort cultures filamentous; length to breadth ratio, 4 to 6 — *S. validus*
   c. Cells in young wort cultures long-oval (4 to 5.5) μ x (9 to 13) μ — *S. Logos*
   d. Cells in young wort cultures oval to elongated, (3 to 6) μ x (7 to 15) μ, on wort agar oval to filamentous, (2 to 5) μ x (4.5 to 20) μ — *S. uvarum*

Classification of the Asporogenous Yeasts

Lodder (1934), excluding from the asporogenous yeasts all of those yeast-like fungi which form true mycelium, includes those which form pseudomycelium. She distinguishes these structures as follows: "*True mycelium* is composed either of long, non-septate filamentous, sometimes branched cells, or if septate, often branched filaments, in which the separate elements arise by the formation of cross walls in the filaments. By pseudomycelium, I understand septate, often branched filaments, in which the usually somewhat elongated cells have arisen, one from another, by budding."

In practice this is going to be an exceedingly difficult distinction to make. One may readily recognize typical true mycelium and typical pseudomycelium, but one encounters many types of filamentous structures where the origin of the cells (by budding or by cross-walls) could be determined only by watching the filament grow. Further, one frequently finds both true and pseudomycelium in a single pure culture, sometimes one and sometimes the other dominating, according to the age of the culture or the

composition of the medium. We have noted already that the rough variants of yeasts may form true mycelium. Stelling-Dekker makes no such distinction, frankly including with the yeasts fungi that form true mycelium but which multiply dominantly as yeast-like growth forms; and in a later publication, Diddens and Lodder (1939a) admit of some true mycelium in the *Mycotoruloideae.*

Lodder subdivides the asporogenous yeasts into three families: The family *Nectaromycetaceae* of Ciferri and Redaelli (1935) is retained to include the single genus *Nectaromyces*, the peculiar yeast found in the nectaries of flowers which sometimes forms conidia on the surface of the colony. She excludes from this family the genera *Sporobolomyces* and *Bulleria* which Ciferri and Redaelli had included, on the ground that these yeasts form basidiospores, not conidia.

The *Rhodotorulaceae* form a new family created to include all of those asporogenous yeasts which form carotinoid pigments. As has been indicated, these may be imperfect forms of the *Sporobolomycetaceae.*

The remaining asporogenous yeasts are contained in the family *Torulopsidaceae.* These parallel to a considerable extent the ascospore-forming yeasts, and undoubtedly are for the most part imperfect forms of them. A number of the genera of the asporogenous yeasts find a counterpart in the sporogenous group. Thus the asporogenous lemon-shaped yeasts of the genus *Kloeckera* differ from the apiculate sporogenous yeasts of the genus *Hanseniaspora* only in the matter of spores; and the elongated pellicle-forming yeasts of the genus *Mycoderma* are obviously the asporogenous forms of the sporogenous genus *Pichia.* From time to time, as with other imperfect fungi, spores are found in yeasts formerly believed to be asporogenous. Recent observations of this sort are the discovery of spores in a strain of *Torulopsis pulcherrima* by Windisch (1938), and the observation by Niehaus (1932) and by Dvornik (1938) that all strains of *Kloeckera* form spores when freshly isolated. Diddens and Lodder (1939b) record the discovery of spores in the following yeasts formerly reported as asporogenous, with the changes in nomenclature in-

dicated: *Monilia pinoyisimilis* and *Monilia pseudotropicalis* = *Saccharomyces fragilis*; *Monilia macedoniensis* = *Saccharomyces macedoniensis*; *Monilia javanica* = *Hansenula anomala*; *Candida pelliculosa* = *Hansenula javanica*. Todd and Herrmann (1936), confirmed by Giordano (1939), noted that many strains of pathogenic yeasts formerly referred to as *Torula histolytica*, *Torula hominis*, etc. actually form spores of such a character as to warrant referring them to the genus *Debaryomyces*.

Balțatu (1939) claimed that he could induce sporulation in all of the strains of *Mycoderma* which he studied (6 from the Botanical Institute of Geisenheim, 7 from the Centraalbureau voor Schimmelcultures, and one isolated by himself) by growth in grape must, or on plaster blocks moistened with grape must, especially if the medium was acidified. One to eight round spores were formed, usually two or four according to the species. At times spore-formation was preceded by copulation between two vegetative cell; at times the spores fused on germination. In the latter case fusion of four or more spores resulting in but one vegetative cell was observed! He would transfer the yeasts designated *Mycoderma* to the ascosporogenous group, and would include all of the pellicle-forming yeasts in one genus, *Mycoderma*, with three subgenera: *Mycoderma sensu strictu* with round spores, *Pichia* with hemispherical spores, and *Willia* (= *Hansenula*) with hat-shaped spores. A number of species are described, some with new names.

The *Torulopsidaceae* are subdivided into two sub-families: The *Torulopsidoideae*[2] include those yeasts with no (or only primitive) pseudomycelium, and no "appareil sporifère" (clusters of buds arising at the nodes of the pseudomycelium). The *Mycotoruloideae* form pseudomycelium and an "appareil sporifère". The latter subfamily is to be the subject matter of part II of her study. It includes yeast-like fungi which have been the subject of an enormous, confused and contradictory literature. These yeasts will be omitted from this review, hoping that soon the publication of Miss Lodder's second volume will provide a sound basis for the study of this group.

[2] Miss Lodder in her work of 1934 used the name *Torulopsoideae*, but Diddens and Lodder (1939a) point out the correct spelling.

The *Torulopsidoideae* comprise the majority of the "wild yeasts", common as contaminants in all sorts of bacteriological work.

Key to the genera of Torulopsidoideae

1. a. Cells mostly lemon-shaped, bipolar budding *Kloeckera*
   b. Cells mostly triangular, budding at the three angles *Trigonopsis*
   c. Cells mostly flask-shaped, budding often upon a broad base *Pityrosporum*
   d. Cells otherwise, usually round, oval or cylindrical [2]
2. a. No pellicle in wort cultures, or only a soft slimy pellicle after some time [3]
   b. A matte, dry pellicle in wort cultures from the beginning [4]
3. a. Formation of long slender tubular processes resembling the copulation tubes of *Zygosaccharomyces* on Gorodkowa agar *Asporomyces*
   b. No tubular processes formed *Torulopsis*
4. a. Cells often cylindrical, multiplication by budding, the buds not separated from the mother-cell by fission *Mycoderma*
   b. Cells polymorphous, multiplication by budding, the buds often separating from the mother-cell by fission *Schizoblastosporion*

The largest number of species of the *Torulopsidoideae* are to be found in the genus *Torulopsis*. There has been some argument regarding the proper scientific name of this genus. The name *Torula* widely applied since the time of Pasteur and Hansen is invalid since it had been applied previously by Persoon in 1801 to an entirely different sort of fungus. The name *Cryptococcus mollis* was given by Kützing in 1833 to an organism which he found on moist window panes and which he classified with the algae. Later he included in this genus *Cryptococcus fermentum* and *Cryptococcus cerevisiae*, obviously yeasts. This name was not, however, applied generally to yeasts until it was revived in 1901 by Vuillemin, who redefined the genus to include only

pathogenic yeasts, since which time it has been widely used by medical mycologists. It is agreed, however, that pathogenicity is too indeterminate and variable a character for generic determination. Benham (1935) reëxamined some of the herbarium material of Kützing deposited in the New York Botanical Garden, and found a variety of organisms, including budding cells which she considers to be yeasts. She therefore accepted *Cryptococcus* as having priority as a generic name for asporogenous yeasts. Kützing had not described budding.

Lodder (1938) examined some of Kützing's original material deposited in the Rijksherbarium at Leyden and also found a mixture of organisms with some budding cells. She pointed out, however, that these do not fit Kützing's description, and rejected *Cryptococcus* as being both a *nomen dubium* and *nomen confusum*, and accepted the name *Torulopsis* introduced by Berlese in 1895 as a substitute for the inadmissible name *Torula*.

The following key to the species of *Torulopsis* will indicate the characters by which the species are distinguished.

Key to the species of Torulopsis

1. a. Fermenting sugars [2]
   b. Not fermenting [8]
2. a. Fermenting only dextrose [3]
   b. Fermenting dextrose and sucrose [4]
   c. Fermenting dextrose, galactose and sucrose *T. Holmii*
   d. Fermenting dextrose, sucrose and maltose *T. colliculosa*
   e. Fermenting dextrose, sucrose and lactose; cells long-oval *T. kefyr*
   f. Fermenting dextrose, galactose (very weakly), sucrose and lactose; cells round *T. sphaerica*
3. a. Agar stroke culture not slimy; formation of a reddish pigment in the presence of iron *T. pulcherrima* and var. *variabilis*
   b. Agar stroke culture slimy; no red pigment *T. Molischiana*

4. a. Cells relatively large (3.5 to 5.5) μ x (5 to 12) μ [5]
   b. Cells small (1.5 to 4) μ x (3 to 5) μ [6]
5. a. Nitrates utilized *T. utilis*
   b. Nitrates not utilized *T. dattila*
6. a. Cells in wort cultures in clusters; only peptone utilized [7]
   b. Cells in wort cultures single or in pairs; ammonium sulphate and asparagin utilized as well as peptone *T. Gropengiesseri*
7. a. Cells mostly oval to long-oval *T. bacillaris*
   b. Cells mostly round to oval *T. stellata*
8. a. Nitrates undoubtedly utilized [9]
   b. Nitrates utilized but slightly or not at all [11]
9. a. Wort agar stroke culture slimy [10]
   b. Wort agar stroke culture not slimy *T. aeria*
10. a. Cells in wort and wort agar culture round to short-oval; stroke culture yellowish *T. alba* and var. *japonica*
    b. Cells in wort round to short-oval, in wort agar oval; stroke culture yellowish tending toward red *T. rotundata*
11. a. Wort agar stroke culture slimy [12]
    b. Wort agar stroke culture not slimy [15]
12. a. Cells round to short-oval *T. neoformans*
    b. Cells oval or long-oval [13]
13. a. Cells in young wort culture mostly oval, length to breadth ratio <2 [14]
    b. Cells in young wort culture mostly long-oval, length to breadth ratio >2 *T. flavescens*
14. a. Lactose utilized; no growth with alcohol as source of carbon *T. Laurentii*
    b. Lactose not utilized; good growth with pellicle when alcohol is source of carbon *T. luteola*
15. a. Dextrose only utilized *T. uvae*
    b. Other sugars utilized [16]
16. a. Cells round *T. candida*
    b. Cells oval [17]

17. a. Cells relatively large (3.5 to 5) μ x (5 to 11) μ *T. lipofera*
    b. Cells relatively small (2.5 to 4) μ x (3.5 to 5) μ *T. minor*

The Rhodotorulaceae

Lodder justified the creation of a new family to include the red yeasts on the ground that these form a homogeneous group, all producing carotinoid pigments which are lacking in other yeasts, none fermentative, and apparently related to the *Sporobolomycetaceae* rather than to the ascosporogenous yeasts, as has been indicated in an earlier section.

Red or pink yeasts occur as air contaminants in bacteriological work probably more frequently than any other yeasts. The reason for their abundance in the air is not known, for they certainly are not common in soil. The observation of Derx that the Sporobolomycetes are abundant on leaves and straw may explain their origin. Being rather inert biochemically, they are of little practical importance. They are a cause of discoloration of sauerkraut, as reported by Pederson and Kelly (1938), who found that factors which tended to inhibit the normal bacterial sequence (high temperature, high salt content, high acidity, low nitrogen content) favored the growth of pink yeasts. Schnegg and Weigand (1936) made the surprising observation that pink yeasts were present in numbers from 48 to 38,400 per ml. in 11 samples of boric acid solution (3%) purchased in pharmacies.

Lodder includes all of the *Rhodotorulaceae* in one genus, *Rhodotorula*, in which she recognizes 13 species and 10 varieties. These are subdivided into two groups on the basis of their ability to utilize nitrates. Within these groups the species are differentiated according to the shade of the pigment, the size and shape of the cells, and the sliminess of the growth on agar, characters which the reviewer considers to be highly variable. *Rhodotorula glutinis* is the type, and most common species.

Industrial Yeasts

Ever since Hansen applied the name *Saccharomyces cerevisiae* to a brewing yeast, and *Saccharomyces ellipsoideus* to a wine

yeast, these two names have been applied traditionally to nearly all of the industrial yeasts used in baking, brewing, wine-making and in distilleries. Hansen's original descriptions separated these species mainly upon the shapes of the cells, especially in the scums of old cultures grown at low temperatures on hopped beer wort. *S. cerevisiae* showed round to oval cells, *S. ellipsoideus* clumps of elongated cells. By tradition there has developed a concept of these two yeasts associated with their uses. *S. cerevisiae* has been looked upon as a low-alcohol species with characteristic flavors, a cultivated yeast occurring rarely if at all in nature; while *S. ellipsoideus* has been considered to be a high-alcohol yeast producing different flavors, and occurring almost universally upon ripe grapes. That there is a real difference in industrial yeasts must become apparent to anyone who tries to make wine by inoculating grape juice with commerical yeast cakes! It is now evident that these differences are not of a character to warrant the separation of species.

Stelling-Dekker has pointed out that the morphological differences between *S. cerevisiae* and *S. ellipsoideus* are too slight and too dependent upon the duration of growth to be considered valid. She therefore reduced *S. ellipsoideus* to the rank of a variety of *S. cerevisiae*, the variety differing from the species in a tendency toward ellipsoid rather than oval cells; and a "fairly good" growth with ethyl alcohol as the source of carbon, as compared with a "scant to fairly good" growth with *S. cerevisiae*. In practice one finds so many gradients in these characters that it is almost impossible to decide where to draw the line. This difficulty was noted by Zimmermann (1938) in a study of yeasts isolated from turbid wines. Mrak and McClung (1940) identified 95 strains from grapes as *S. cerevisiae*, and only 4 as *S. cerevisiae* var. *ellipsoideus*! They state that "since many of the cultures identified as *S. cerevisiae* were isolated as the predominating organism from naturally fermenting musts it is quite possible that they are enologically different from the type cultures of *S. cerevisiae* and *S. cerevisiae* var. *ellipsoideus*, although the physiological characteristics used in taxonomy are similar."

This statement by Mrak and McClung may well be applied

generally to industrial yeasts. From the wide variety of types present in nature, there have been selected races having particular desirable characters,—high alcohol production in the distillery yeasts, baking quality and high yield from cheap media in the compressed yeasts, flavor and readiness of clearing and other characters in the brewing and wine yeasts. It now seems to be conceded that the flavors of wines depend in part upon the yeasts used, and there are available a variety of strains named for different types of wines. It is even claimed that rum can be made successfully only with special rum yeasts. These various races or strains have been selected in the past partly direct from nature by the industrial processes in which they are used, and undoubtedly in part from mutants appearing during their industrial uses. Since the introduction of pure culture methods into the fermentation industries, there has been a continuous search for better yeasts by scientific artificial selection, and as has been noted, a start has been made toward actually breeding new races. The characters by which these different cultivated races or strains of yeasts are distinguished do not lend themselves to species characterization. Nevertheless industrial yeasts are classified in a practical way by such characters, and it will be worth while to note some of the newer developments in this field.

For many years industrial yeasts have been divided into two groups, top yeasts and bottom yeasts, according to the character of their fermentation in industrial use. Top yeasts tend to be uniformly distributed in the wort, and producing abundant gas early in the fermentation, are carried to the top: while bottom yeasts tend to sediment rapidly, and the main fermentation takes place at the bottom of the vats. Hansen first applied the name *S. cerevisiae* to a yeast which had been cultivated for many years in England, and which is a typical top yeast. As a typical bottom yeast he described "*S. cerevisiae Carlsberg No. 1*", which he later recognized as a separate species, *S. calsbergensis*. Top yeasts are said to used in the production of English ales, bottom yeasts in most lager beer brewing. Baking yeasts and some distillery yeasts are top yeasts. Trautwein and Wassermann (1930) indicate that the essential difference between top and bottom yeasts

is in their rates of fermentation and respiration. In a study of 12 strains of top yeasts and 10 strains of bottom yeasts, they found the repiratory rate of the former to be on the average 77 per cent greater than the latter. There was a constant ratio between the respiratory rate and the fermentation rate. They consider these to be constant racial characteristics.

As early as 1894 Bau had noted that various races of brewery yeasts differed with respect to their ability to hydrolyze and ferment melibiose, top yeasts failing to produce gas from this disaccharide while bottom yeasts do. Stelling-Dekker (1931) confirmed this, and divided the industrial yeasts into two groups upon this basis. As has been mentioned, instead of melibiose she uses raffinose and quantitative methods. Top yeasts, of which *S. cerevisiae* is the type, ferment raffinose only one-third; bottom yeasts, of which *S. carlsbergensis* is the type, ferment this sugar completely.

Hansen had noted as early as 1905 that top and bottom fermentation are variable characters. He succeeded in developing top yeasts from bottom yeasts by growth at low temperatures, but could not accomplish the reverse change. Winge and Laustsen (1939) obtained bottom yeasts from top yeasts by spore segregation. They state that "it appears quite unjustified to use the ability to ferment the whole or one-third of raffinose as standards for distinguishing bottom and top yeasts respectively, as there is no regular connexion between these characters", and that "the categories of top yeast and bottom yeast have undoubtedly been interpreted too literally both in practice and in the literature."

Stockhausen has recently published a series of papers on what he calls "high-fermenting" and "low-fermenting" yeasts. By these terms he apparently means something different from top and bottom yeasts, since he speaks (Stockhausen and Koch, 1936) of "hoch und niedrigvergärenden untergärige Bierhefen". The type of the high-fermenting yeasts is the Frohberg yeast (which Stelling-Dekker places in *S. carlsbergensis*); the type of the low-fermenting yeasts is the Saaz yeast, which on the basis of raffinose fermentation is listed by Stelling-Dekker as *S. cerevisiae*. Stockhausen (1935) stated that the Saaz yeast is not a true cultivated

yeast, but a wild yeast, *S. cratericus*, that gives beer a raw flavor and other undesirable characters.

Stockhausen and Koch (1936) stated that the low-fermenting yeasts leave much more sediment when the brewing vat is drained than do high-fermenting yeasts. This is apparently due to the flocculation and sedimentation of the former ("bruchhefen") and the fine distribution of the latter ("staubhefen"). High-fermenting yeasts are carried over into the after-fermentation vats to a greater extent. In laboratory tests no differences in the total yeast production could be noted. Stockhausen (1935) stated that while one may speak of high-fermentation and low-fermentation as racial characteristics, these are strongly influenced by external factors. Protein-rich wort leads to high fermentation. Low-fermentation yeasts are rich in phosphorus and magnesium, high-fermenting strains contain more calcium. In mixtures the high-fermenting yeasts predominate.

Guilliermond (1920) stated that top yeasts ferment at higher temperatures than bottom yeasts. This classification of the brewery yeasts may thus be parallel to the division of wine yeasts into "normal-fermenting" and "cold-fermenting" types. "Cold" yeasts have been recently studied by Porchet (1936) and by Percher (1938). A slow but complete fermentation of must may be carried out by some of these yeasts even at 0°C. The resulting wines are no different from those produced by normal wine yeasts. Osterwalder (1934) made an extensive study of one such yeast, which he identified as *Saccharomyces intermedius*, var. *turicensis*. It fermented dextrose, levulose, galactose, sucrose, maltose and raffinose. Zimmermann (1936) described a cold-fermenting yeast, and later (1938) two more, which differed from the "normal" wine yeasts in fermenting raffinose completely rather than one-third. Mrak and McClung (1940) found but one of 195 strains of *Saccharomyces* from grapes, must or pomace which fermented raffinose completely.

Several other procedures for differentiating industrial yeasts have been proposed. Fink (1932) noted that yeasts of different sources vary in their cytochrome spectra. Bakery yeasts, *i.e.*, compressed yeasts of the "respiratory" type, present the typical

4-band spectrum of reduced cytochrome. Brewery yeasts, or yeasts of the fermentative type, present incomplete, usually 2-band spectra. The test is easily and quickly carried out and can be used to detect the adulteration of commercial bakery yeast with brewery yeast. Scum-forming yeasts show spectra of the "respiratory" type, wine and distillery yeasts those of the fermentative type. The character of the spectrum depends upon the degree of aeration during the previous cultivation of the yeast. Baker's yeast grown anaerobically was easily changed to one with an incomplete cytochrome spectrum, and wine and distillery yeasts yielded a 4-band spectrum when strongly aerated, but this was not observed with brewery yeast except after prolonged cultivation with a high degree of aeration (Fink and Berwold, 1933).

Stockhausen (1935) noted variations in the amount of gum produced by different types of industrial yeasts. Beer yeasts produced 4 to 6 per cent, wine yeasts 6 to 8 per cent, and baking yeasts 8 to 12 per cent. Drews (1937) noted that compressed yeast made from brewery yeasts did not keep as well as that made from selected baking strains. This was due to the variations in pH optima for autolysis. For a bottom brewing yeast this was 5.0; for a top brewing yeast, 4.7; for a top distillery yeast (grown under aeration as for compressed yeast manufacture) 4.25; and for a top baker's yeast grown on grain mash, 4.35.

Schultz, Atkin and Frey (1940) propose to subdivide industrial yeasts according to their requirements for certain growth accessory substances. They studied 44 strains, varieties or races of *S. cerevisiae* and *S. carlsbergensis*, and found that these fell into three groups. The yeasts are grown upon a basal synthetic medium containing the bios ingredients (inositol, β-alanine, and Bios III); the same medium plus thiamin; and the same medium plus thiamin and vitamin B_6. Type A yeasts give a low crop on basal medium, increased by thiamin, and further increased by vitamin B_6. Type B yeasts give a high crop on the basal medium, which is depressed not more than 50 per cent by thiamin, and normal with both thiamin and vitamin B_6. Type C yeasts are depressed more than half by thiamin, but give a high crop with

both substances present. They found all three types in both species, *S. cerevisiae* (top) and *S. carlsbergensis* (bottom).

The Genus Brettanomyces

Although properly belonging to the *Mycotoruloideae*, brief notice will be taken of the yeasts of the genus *Brettanomyces* because of their industrial importance. They are concerned with the after-fermentation of English "stock" beers (porter, stout, pale ale) and are very different from the *Saccharomyces cerevisiae* responsible for the main fermentation. Similar yeasts are used in the production of Belgian lambic beer. Such yeasts were first studied in 1904 by Claussen who observed that they produce unusually high percentages of alcohol, and large amounts of both non-volatile and volatile acid. They were further studied by Schiønning in 1907–1909, who recognized two types differing in morphology and cultural characters. These two types were also recognized by Kufferath and van Laer in 1921 who proposed the generic name *Brettanomyces*, correcting "Brittanomyces" which had been used by Claussen. Kufferath and van Laer described two species, *B. bruxellensis* with moist, shiny colonies and *B. lambicus*, with dull colonies like those of film yeasts. Both were isolated from Belgian lambic beer.

Very recently these yeasts have been intensively investigated by Custers (1940). He believed that such yeasts have been obtained only from English and Belgian beers, excepting one strain isolated by Krumbholz and Tauschanoff (1933) from a spontaneously fermenting French grape must, and named by them *Mycotorula intermedia*. Custers considered this to be a species of *Brettanomyces* close to *B. bruxellensis*, but differing from the others in a greater tendency to form pseudomycelium in all media; he suggests that it may be the "wild" form of these yeasts, the others having been modified by cultivation.

Custers studied 17 strains, of which he isolated 7 from lambic beer, and 2 from English beer. The remaining strains had been isolated by Schiønning, Kufferath, and by Krumbholz and Tauschanoff. These strains were subjected to taxonomic analysis according to the procedures described by Stelling-Dekker and

Lodder. The 17 strains fell into 4 species and two varieties. Morphologically they are variable, but a characteristic feature is the "ogive" cell, elongated cylindrical cells pointed at the ends. All tend to form some pseudomycelium in potato infusion. Physiologically they differ from most yeasts in their ability to oxidize alcohol to acetic acid under aerobic conditions, producing enough acid to kill the cultures, so that stock cultures can be maintained readily only in media containing calcium carbonate. They grow very slowly, taking nearly six months for the complete fermentation of beer wort, and producing about 10 per cent of alcohol.

Custers proposes to include *Brettanomyces* as a genus of the tribe *Mycotoruloideae* which he defines as follows (in translation):

"Cells ovoid or globular, often elongated or "ogive" shaped, pointed at the ends. Budding from all parts of the cell, forming irregular clusters. Tendency to form a poorly developed pseudomycelium with only a primitive blastospore apparatus. Ascospores are not formed. In malt extract slow growth usually accompanied by slow, long drawn out fermentation; sediment, sometimes a pellicle. In this medium and in malt agar, formation of a characteristic aroma. Under aerobic conditions, strong production of acid from sugars. Slow growth, relatively rapid death of cells in malt agar, but can be maintained longer in malt agar or yeast-extract glucose agar if calcium carbonate is added. Can ferment a variety of sugars. Potassium nitrate and nitrite (in dilute solutions), ammonium sulphate, urea and peptone can be utilized as sources of nitrogen."

The four species are *Brettanomyces anomalus* n.s., *B. Claussenii* n.s., *B. lambicus*, and *B. bruxellensis*; there are two varieties of the last species, *non-membranaefaciens* and *lentus*. These species and varieties are differentiated by sugar fermentations, by the character of the growth on wort agar, and by the presence or absence of pellicles.

Custers made extensive chemical studies of the fermentations caused by species of *Brettanomyces*. Under anaerobic conditions, only an alcoholic fermentation occurs; no appreciable amounts of acid are formed. The amount of alcohol exceeds that of CO_2 in the classical formula for fermentation. Under aerobic con-

ditions considerable acetic acid is formed. This is thought to be due to an oxidation of the alcohol by the yeast. In similar experiments with *Saccharomyces cerevisiae* and *S. carlsbergensis* no acetic acid was formed. At pH 6.4 the oxidation of the alcohol stops at acetic acid; at pH 4.35 and 3.77 the acetic acid is further oxidized to carbon dioxide and water. It was further found that under aerobic conditions *B. Claussenii* produces more alcoholic fermentation than under anaerobic conditions, *i.e.*, a negative Pasteur effect.

Apiculate Yeasts

The apiculate yeasts are those with lemon-shaped cells (ellipsoidal cells with a small button-like projection at each pole). Such yeasts are common on grapes, and growing more rapidly than the true wine yeasts, are abundant at the beginning of fermentation. They may contribute unfavorably to the flavor of the wine. They have been studied considerably with rather confusing results as regards classification. There has been dispute concerning spore formation, and we consequently find them listed with both the sporogenous and the asporogenous yeasts.

Stelling-Dekker (1931) recognizes *Hanseniaspora* as the valid generic name for the sporogenous apiculate yeasts. She defines the genus as follows: "Cells lemon-shaped or long-oval, vegetative multiplication by bipolar budding. No pellicle on wort. Spores at first spherical, later hat-shaped, which is not always distinct. Ability to form spores easily lost on prolonged cultivation. Fermentation of dextrose, levulose and mannose only, at times weak. Nitrate assimilation negative. Almost no growth with ethyl alcohol as a source of carbon." She includes two species, *H. valbyensis*, strongly fermenting and with never more than 2 spores per ascus, and *H. Guilliermondi* with 4 spores per ascus.

Lodder (1934) accepts *Kloeckera* as the valid generic name for the asporogenous apiculate yeasts, which she defines as: "Cells lemon-shaped, short-oval, long-oval, or sausage-shaped. Vegetative multiplication by bipolar budding. Strong fermentation of dextrose (levulose and mannose) or of dextrose and sucrose. Of the N-compounds tried, only peptone was utilized. With

ethyl alcohol as the growth substrate, almost no growth." She describes ten species and one variety. These are subdivided first on the basis of sucrose fermentation, further on the size and shape of the cells and the appearance of the agar-stroke culture.

Niehaus (1932) published an extensive study of 81 strains of apiculate yeasts from widely distributed vineyards. He noted, as had other previously, that the apiculate form is characteristic of young cultures, and that in older cultures ellipsoid or cylindrical cells are found. When hanging-drop cultures were studied it was observed, however that newly-formed cells were ellipsoid, and only became apiculate when about to bud. He explains the relative absence of apiculate cells in old cultures as due to the inhibition of budding by the accumulation of alcohol, to which the yeast is very sensitive; excepting one strain, only from $\frac{1}{2}$ to about 3 per cent of alcohol being produced.

Each of the 79 freshly isolated strains readily produced spores on plaster blocks. One other formed spores on Kufferath's medium. The spores were almost always single, only occasionally were two spores found. Spore formation was parthenogenetic. Nuclei were demonstrated in the spores, and they were observed to germinate, sending out a short promycelium from which vegetative cells were budded. Since the form of the spores (no hat-shaped ones observed) and their germination appeared to be different from that described by Stelling-Dekker for *Hansenia spora* he created a new genus, *Kloeckeraspora*, to include the yeasts which he studied. To the reviewer this seems to be hair-splitting with a vengeance. Castelli (1935) failed to confirm the observations of Niehaus. He observed globular bodies corresponding to the single spores described by Niehaus, but his microscopic studies indicated that they were fat globules, and germination studies were negative.

Dvornik (1938) reported (from the same laboratory as Niehaus) new extensive studies and fully corroborated the findings of Niehaus. Each of 50 freshly isolated strains produced spores. Only one spore per cell was formed parthenogenetically. The identity of the spores was established by spore-staining, by the staining of nuclei within the spores, and by germination. The promycelium described by Niehaus could not be confirmed.

Saccheti (1939) also confirmed the regular formation of spores by 120 strains of apiculate yeasts freshly isolated from various fruits. The ability to form spores was quickly lost on continued cultivation. The spores could not be differentially stained.

My curiosity piqued by reading these papers during the preparation of this review, I have recently obtained three samples of table grapes (Concord, white seedless, and Tokay) and allowed them to ferment after crushing. At the beginning of fermentation plates were poured, and the majority of the colonies in all three samples proved to be apiculate yeasts. Four or five were subcultured from each sample, and all of the 14 subcultures readily formed spores varying from 1 to 4 in number on Gorodkowa's agar. They were all identified as *Hanseniaspora Guilliermondi*. From 241 yeasts isolated from grapes, must or pomace, Mrak and McClung (1940) obtained 11 diagnosed as *Hanseniaspora*, 1 as *Kloeckeraspora*, and 16 as *Kloeckera*. They observed that apiculate yeasts were isolated more frequently from grapes than from must or wine, and Zimmermann (1938) obtained only one apiculate yeast in 45 strains isolated from wines which had become turbid in storage. The abundance of apiculate yeasts in must at the beginning of fermentation and their relative absence in the finished wine may be attributed to their sensitivity to alcohol; Niehaus (1932) found that most of them were inhibited by a concentration of about 3.7 per cent of alcohol in must.

The existing confusion regarding the classification of the apiculate yeasts indicates clearly a difficulty frequently encountered when yeasts must be placed in different genera on the basis of spore-formation. The imperfect yeasts are obviously an artificial (though necessary) category. To the reviewer the balance of evidence at the present time indicates that all apiculate yeasts are potentially sporogenous, but that the ability to form spores is rapidly lost in artificial cultivation.

Film-Forming Yeasts

Pellicle-forming yeasts fall in the genera *Hansenula*, *Pichia*, *Zygopichia* and *Debaryomyces* of the sporogenous yeasts, and in the genus *Mycoderma* of the asporogenous yeasts. Growing as a

scum on the surface of liquid media, they tend to be oxidative rather than fermentative in the dissimilation of sugars. If fermentative, they produce but little alcohol, tending to produce considerable amounts of esters. Many of them utilize alcohol readily, and may be a cause of spoilage of fermented beverages, or may contribute to the development of special flavors, as in sherry. Such yeasts also appear frequently in pickling solutions of various sorts, especially brines.

The film of yeasts concerned in the development of flavor in sherry (and in certain Arbois wines of France) is called the "flor." Hohl and Cruess (1939) isolated 15 strains of film-forming yeasts from the mixed flora of samples of "flor" obtained from Jerez de la Frontera and from the Arbois district. Three strains fell in the genus *Pichia*, very close to *P. membranaefaciens*. Seven strains fell in the genus *Saccharomyces*, very close to *S. cerevisiae* var. *ellipsoideus*. These strains differ from the typical *Saccharomyces* species in forming a more or less heavy film, after fermentation is complete. One strain proved to be *Hansenula saturna*. Four strains were species of *Torulopsis* (closest to *T. dattila*). They were actively fermentative, and could perhaps be strains of *S. cerevisiae* which failed to form spores. This paper presents an extensive bibliography on "flor" yeasts and discusses some of the physiological properties of the strains isolated. Unlike the apiculate yeasts, they show a high alcohol tolerance, and tend to reduce the alcohol content of the wine.

Scrivani (1939) also investigated the taxonomy of the "flor" yeasts. He isolated 119 strains from 54 samples of Italian wines. The following species were obtained: *Zygopichia chantigrana*, *Pichia Derossii*, *Pichia membranaefaciens*, and its variety *acidificans*, *Mycoderma cerevisiae*, *Mycoderma Lafarii*, *Mycoderma vini*, *Mycoderma acidificans* var. *degradans*, and 4 undetermined species of *Mycoderma*.

Castelli (1933) found species of *Hansenula* to be the dominant yeasts in a sour-dough ferment used in household baking in Italy, and described two new species of the genus.

Mrak and Bonar (1939) studied film-forming yeasts from pickle brines. Yeasts were isolated from 29 food brines (dill

pickle, cucumber salt stock, Zucca melon, green olive, Sicilian olives, cauliflower and ham). The yeasts isolated were identified as *Pichia membranaefaciens, Mycoderma decolorans, Debaryomyces Guilliermondi* var. *nova zeelandicus, Debaryomyces membranaefaciens,* and *D. membranaefaciens* var. *hollandicus.* The salt tolerance of these yeasts was determined, and compared with that of related yeasts from type culture collections. The *Debaryomyces* species were found to be more widely distributed in brines, and to show a higher salt tolerance than the other yeasts studied. Though *Debaryomyces* species could grow in pickle brines containing up to 24 per cent salt, the *Mycoderma* and *Pichia* species were limited to brines containing 15 per cent or less. The paper includes a bibliography on brine yeasts and their salt tolerances.

Otani (1939) found that film-forming yeasts were the dominant species in "Nukamiso" pickles, a naturally fermented food prepared from raw vegetables in Japan. Such yeasts were more frequent in the less acid samples; no yeasts were obtained from a highly acid sample. Of the film-forming yeasts, *Hansenula anomala* and two unnamed species of *Mycoderma* are described. In addition certain yeasts that did not form scums were isolated, among them *Zygosaccharomyces nukamiso,* n.s., and *Torulopsis nukamiso,* n.s.

Mrak and Bonar (1938a) studied yeasts forming a slime on sausages. Those isolated best fitted the description of *Debaryomyces Guilliermondi* var. *nova zeelandicus.* Difficulties were encountered in distinguishing between species of *D. Guilliermondi* and *D. membranaefaciens.*

Osmophilic Yeasts

The term "osmophilic" was applied by Richter in 1912 to microörganisms which can multiply in solutions of high osmotic pressure. The brine yeasts just mentioned could be well designated by this term. In recent years there have been described another group of yeasts having a tolerance for high osmotic pressures, those growing in concentrated musts, honey or syrups.

Krumbholz was concerned with yeasts which grow in concentrated musts. In the Rhine wine region there occurs at times

a warm, dry, sunny autumn, when the grapes attain a characteristic over-ripeness designated as "edelreif." Such grapes may become infested with *Botrytis cinerea*, in which case they are designated as "edelfaul." From "edelreif" or "edelfaul" grapes there is obtained a must of unusually high sugar content, 30 to 40 per cent, sometimes as high as 60 per cent. From such concentrated must there is produced an exceptional type of wine, the "rheinischen Ausleseweine." The fermentation is slower and less complete than that of ordinary wines. Kroemer and Krumbholz (1931) presented a preliminary report upon yeasts isolated from such wines, and showed that the fermentation is brought about by species different from the *ellipsoideus* variety of *S. cerevisiae* commonly concerned in wine production. Yeasts were collected from naturally fermenting "Trockenbeerenauslesen," and grapes from various sources were placed in sterile concentrated must from which yeasts were isolated after fermentation had begun. It was found that osmophilic yeasts were widely distributed upon "edelreif" and "edelfaul" grapes. From 39 samples species of *Zygosaccharomyces* were isolated, from 14 samples small-celled budding yeasts, from 3 samples both of the preceding groups, from 2 samples apiculate yeasts, from 2 samples species of *Hansenula*, and from 2 samples species of *Rhodotorula*. It was concluded that the yeasts active in the fermentation of the concentrated musts fall in the first two groups.

The species of *Zygosaccharomyces* were divided into two specific groups: one, related to *Z. priorianus*, contained three new species, *Z. polymorphus*, *Z. variabilis*, and *Z. amoeboideus*[3]; the other, related to *Z. Nadsonii*, contained one new species, *Z. globiformis*. Species in the first group were found to be especially resistant to high osmotic pressures, growth continuing up to a concentration of 90 grams of sugar in 100 ml. They grew and fermented best at temperatures above 20°, in sugar concentrations of 40 to 50 per cent, gave a slow fermentation with but little alcohol and no volatile acid. *Z. globiformis* was less tolerant of the higher osmotic pressures, equally weak in fermentation, and produced

[3] Lodder (1932) considers *Z. amoeboideus* to be very close to *Z. cavarae* var. *beauwerie* and suggests renaming it *Z. cavarae* var. *amoeboideus*.

some volatile acid. The small-celled budding yeasts were designated as species of *Saccharomyces* though no spores were found. They fell into three groups designated as *S. stellatus*, *S. bacillaris*, and *S. granulatus* (all new species). *S. stellatus* grew in sugar concentrations up to 90 per cent with the optimum between 30 and 40 per cent, and produced up to 10 per cent alcohol. The other two species gave weaker fermentations. Extensive studies on the activities of these various yeasts in natural wine fermentations are presented.

Krumbholz (1931a) described the morphology and cultural characters of the four new species of *Zygosaccharomyces*. Several varieties or races of *Z. polymorphus* were recognized. Diagnostic characters of this species are the form of the giant colonies and the occurrence of long copulation tubes at the time of spore formation. The isogamous (occasionally heterogamous) conjugation and the relationship of the cell form and colony form to the nature of the sugar in the medium are discussed. It was noted that these yeasts fermented sucrose only after crushing the cells. The normal cells fermented only glucose, fructose, mannose and maltose. *Z. globiformis* is especially characterized by a greater tendency to heterogamous conjugation, the formation of nonfunctioning copulation tubes, and parthenogamy. This species ferments sucrose and raffinose ($\frac{1}{3}$) in addition to the sugars fermented by *Z. polymorphus*. Later, Krumbholz (1933) described the formation of copulation tubes without copulation in *Z. globiformis* and discussed the relationship of the genus *Zygosaccharomyces* to *Torulaspora*.

The small-celled osmophilic yeasts are described in detail by Krumbholz (1931b). Although no spores were found, these yeasts are referred to the genus *Saccharomyces* because of their morphological characters. Lodder (1934) transfers two of the species of Kroemer and Krumbholz (1931) to the genus *Torulopsis*, as *T. bacillaris* and *T. stellata*, but fails to describe the third species, *T. granulatus*. All three of these yeasts tend to grow in clusters as small, oval to globular budding cells. They ferment glucose and sucrose, but not lactose or maltose. *T. bacillaris* and *T. stellata* also ferment raffinose ($\frac{1}{3}$).

Karamboloff and Krumbholz (1931) described another osmophilic species, *Zygosaccharomyces gracilis*, from a Portuguese wine. It ferments glucose, fructose and maltose, galactose slightly, and sucrose only after the cells have been crushed. It is very resistant to high acidities.

The relative osmotic tolerances of a variety of yeasts are discussed by Kroemer and Krumbholz (1932). Twenty species were tested in nutrient media containing various concentrations of NaCl, $NaNO_3$, KCl, KNO_3, and glycerol. The yeasts studied included a variety of common yeasts in addition to the osmophilic yeasts which the authors had previously isolated. Most of the species studied showed rather high degrees of tolerance to the concentrated solutions, especially the species of *Hansenula* and *Zygosaccharomyces*. Some of them grew in media containing 40 to 50 per cent glycerol. Potassium nitrate was tolerated better than the other mineral salts, many of the yeasts growing in a saturated solution.

Saccheti (1932) also studied yeasts in concentrated must, and isolated two species of *Zygosaccharomyces*, *Z. gracilis* var. *italicus* and *Z. felsineus*, n.s.

The role of osmophilic yeasts in the fermentation of honey was studied by Fabian and Quinet (1928). They isolated 25 strains from 20 samples of honey, among them *Zygosaccharomyces japonicus*, *Z. Barkeri*, and *Z. priorianus*. A new species, *Z. mellis* (not *Z. mellis acidi* Richter) is described. An asporogenous yeast was named *Torula mellis*. Yeasts were isolated from honey which had not fermented, as well as from fermented honey, and it was suggested that fermentation depends upon absorption of moisture by the honey from the air. Thermal death points of the yeasts and their spores were determined, and pasteurization of the honey at 62.5° for 30 minutes was recommended as a preventive measure.

Honey-fermenting yeasts were also studied by Marvin (1928) who found species of *Zygosaccharomyces*. Wilson and Marvin (1929) found yeasts capable of fermenting 80 per cent honey on all parts of the bee, in the honey sack, in stored pollen, and on the skins of grapes. Marvin, Peterson, Fred and Wilson (1931)

noted that when honey crystallizes, the water extruded from the crystallized sugar tends to dilute the solution and so reduce the sugar concentration that yeasts may grow. They isolated four types of *Zygosaccharomyces*, *Z. mellis*; *Z. Nussbaumeri*, and two which they considered to be new species, but which were not named.

Lochhead and Heron (1929) initiated a series of papers dealing with the role of yeasts in honey spoilage. From 13 samples of fermented honey yeasts were isolated which not only tolerated high percentages of sugar, but failed to grow with concentrations of honey less than 32 per cent, *i.e.*, they are truly osmophilic rather than merely tolerant of high concentrations. Four types were isolated, *Zygosaccharomyces Barkeri*, the *Z. mellis* of Fabian and Quinet, *Z. Nussbaumeri* and *Z. Richteri*. Seeking the source of these yeasts, the nectar[4] of 44 flowers was examined, and yeasts capable of growing in 80 per cent honey medium were found in all but three. These fell into 11 species, of which three were *Zygosaccharomyces* species and the remainder *Torulopsis* species. Sugar-tolerant yeasts were also isolated from nectar in the bee-hives, from honey tanks and from air in the extracting house. The various yeasts are clearly described and illustrated. Factors influencing honey fermentation, such as temperature, soluble nitrogen content, and degree of inversion of the sugar are discussed.

Lochhead and Farrell (1930) searched for osmophilic yeasts in soil by inoculating soil samples into an 80 per cent honey medium. No such yeasts were found in garden, orchard, clover field or cereal field soils, but were obtained from soil about apiaries, more abundantly in old ground. Five species of *Zygosaccharomyces* and two of *Torulopsis* were isolated and described. Lochhead and McMaster (1931) found yeasts to be present in all of 191 samples of normal honey from all parts of Canada. A method of counting osmophilic yeasts in honey by serial dilutions in a 66 per cent honey medium is described.

Lochhead and Farrell (1931a) counted the yeast colonies in

[4] For a discussion of non-osmophilic yeasts in nectar and a review of literature on nectar yeasts, see Zinkernagel (1929).

normal honey by plating on 60 per cent honey agar. *Zygosaccharomyces Richteri* was found to be dominant in fermenting samples, but not necessarily so before fermentation begins. Species of *Zygosaccharomyces* were the most frequent in occurrence in nearly all samples of normal honey. *Schizosaccharomyces octosporus* was obtained from one sample.

Lochhead and Farrell (1931b) found a "bioactivator" in honey which stimulated fermentation by osmophilic yeasts of the genus *Zygosaccharomyces* in synthetic media. This substance is dialyzable, insoluble in ether and acetone, not precipitated by 85 per cent alcohol, non-volatile. It is resistant to heat in acid solution but not in alkaline. The substance is separable into two parts by adsorption with charcoal. The two fractions are inert alone. Farrell and Lochhead (1931) studied this "bioactivator" further in comparison with "bios." Complementary fractions from treatment with charcoal were found to exert effects similar to bios I (inositol) and bios II, when tested with the Toronto strain of *Saccharomyces cerevisiae*. Charcoal treatment of honey removes by adsorption bios II leaving a residue relatively inert by itself, containing inositol. Inositol, however, is not the active substance for the strain of osmophilic *Zygosaccharomyces* tested, the growth of this yeast being dependent upon the presence of another substance which, though not essential for the Toronto yeast, appears to be present in crude bios II.

Lochhead (1933) concluded that not only the moisture content of the honey but also the initial yeast contamination (which may vary from one to a million per gram) are factors in determining the keeping qualities of honey. With a moisture content of 17 per cent or less the honey will keep irrespective of the yeast count; with 18 to 19 per cent the honey is safe if the count does not exceed 10 per gram; with more than 20 per cent there is always danger of fermentation. The content of yeast "bioactivators" did not seem to influence the keeping quality of the honey. Lochhead and Farrell (1936) studied the effects of various preservatives on osmophilic yeasts. In freshly extracted honey, 0.025 per cent sodium benzoate and 0.01 per cent of sodium sulphite or bisulphite prevented fermentation.

In addition to the publications on sugar-tolerant yeasts in concentrated must and in honey, older literature mentions the presence of yeasts and their activities in cream candies, sugar and syrups. More recently Fabian and Hall (1933) have studied yeasts causing fermentation in maple syrup. They isolated a number of species: *Saccharomyces aceris-sacchari* n.s., *S. Behrensianus, S. monacensis, Zygosaccharomyces mellis, Z. japonicus, Z. Barkeri,* and *Z. Nussbaumeri. Saccharomyces aceris-sacchari* produces 4 spores which conjugate in pairs on germination, as in *Saccharomycodes Ludwigii,* and indeed appears to correspond very closely with *S. Ludwigii* in all characters save the failure to form a cross-wall at the base of the buds. *Saccharomyces Behrensianus* also forms spores which conjugate on germination, as did the strain identified as *Saccharomyces monacensis.* The latter was compared with a yeast labelled *S. monacensis* from Dr. Tanner's collection, and found to be identical. Fabian and Hall discussed the heat resistance of the yeasts they isolated, and the moisture content of the fermented syrups.

Melliger (1931) studied yeasts fermenting dates, which may well be considered with the osmophilic yeasts since he found 60 per cent of sugar (invert, not sucrose) in "amhat" dates which could be exported from Egypt to Europe; "haajani" or red dates contain less sugar. These dates were placed on moistened cotton in flasks until fermentation occurred, then cultures were made. Curves for the rate and degree of fermentation of grape must were determined for each of the 89 yeasts isolated. These curves fell into 6 groups; in particular curves for the *Saccharomyces* species could be distinguished easily from curves for the *Zygosaccharomyces* species. Eight species were recognized, two of *Zygosaccharomyces,* two of *Torulopsis,* and one each of *Hanseniaspora* and *Mycoderma.* The *Hanseniaspora* species was described by Lodder (1932) as a new one, *H. Melligeri.*

Pathogenic Yeasts

Since yeasts from many sources may lodge on the skin or be carried into the body in food or air, and since the human body in health and disease has been so thoroughly studied by bacteriolo-

gists, it is not surprising that almost all of the yeasts in the catalogue have been reported at one time or another as human parasites, often as pathogens. Moreover, since yeast infections are encountered but rarely and usually reported by medical bacteriologists having but little acquaintance with this group of organisms, it is not surprising that the same species have been described under many different names. It would not only take too much space, but would be almost futile to review here even the recent papers. The reader interested in a complete description of all of the yeasts that have been isolated from the human body is referred to Dodge (1935).

Fortunately, with increased knowledge and a free exchange of cultures among experts, it is becoming daily more clear that the truly pathogenic yeasts of man and animals are represented by only a very few species. These may be divided into two groups: the yeasts which cause deep-seated infections endangering life (European blastomycosis and American "torula meningitis") caused by a yeast which has been known generally in Europe as *Cryptococcus hominis*, in America as *Torula histolytica*; and the yeasts which cause superficial infections of the skin and mucous membranes, members of the *Mycotoruloideae* and commonly referred to as the "medical Monilias."[5]

Recognition that yeasts isolated from cases of European blastomycosis and from American torula meningitis are identical apparently was made independently by Benham and by Lodder in 1934. Benham (1934) briefly reported on the identity of strains called *Cryptococcus hominis* received from Europe with strains of *Torula histolytica* from America. Later, Benham (1935) compared 22 strains of this type, 12 from sources which indicated that the yeast was the primary pathogen, 7 from normal skin, 2 from feces, and 1 from a sarcoma. These proved alike in morphological and cultural characters. The 12 pathogenic strains produced lesions in rats, the other 10 did not except in vitamin-deficient

[5] We are, of course, including in this discussion only those pathogenic fungi which may legitimately be called yeasts, and excluding those with yeast-like growth forms in the body which grow as mycelial molds in cultures (*Blastomyces*, *Coccidioides*, *Paracoccidioides*, *Sporotrichum*, *Histoplasma*). The *Mycotoruloideae* are omitted from this review.

rats. She concluded that the difference in pathogenicity is one of degree only. Strains from deep-seated lesions grew well at 37°, while strains from the skin surface grew poorly or not at all at this temperature. Agglutination and absorption of agglutinin tests indicated complete identity of the Busse-Buschke strain of *Cryptococcus hominis* with the Freeman-Weidmann strain of *Torula histolytica*. Benham recommended that all of these yeasts be designated *Cryptococcus hominis*.

Lodder (1934) found the following yeasts in the Centraalbureau collection to be identical: the original yeast of Busse from European blastomycosis (*Cryptococcus hominis*); the yeast (*Saccharomyces neoformans*) which Sanfelice described in 1894 as capable of producing experimental tumors in animals; the Freeman and Weidmann strain of *Torula histolytica*; a yeast isolated by Arzt from an ulcer of the tongue and named by him *Blastomyces neoformans*; a yeast isolated by Meyer in 1912 from a nasal swelling in a horse, and named by Harrison in 1928 *Torula nasalis*; a yeast isolated from a case of cutaneous "blastomycosis" by Castellani and labelled *Torulopsis hominis* var. *honduriana*. Since the name used by Sanfelice has priority, Lodder designated all of these pathogenic yeasts as *Torulopsis neoformans*, which she described as follows:

"Cells round or short oval (4 to 6.5) μ x (4 to 7.5) μ, single or in pairs. A sediment in wort, with a ring and after a long time a thin pellicle. No fermentation. Dextrose, levulose, mannose, galactose, saccharose and maltose may be utilized as the sole sources of carbon in synthetic media. Of the nitrogen sources tested, ammonium sulphate, asparagin, urea and peptone were utilized. Good growth with ethyl alcohol as the growth substrate. Agar stroke cultures (75 days at 15°C.) yellowish, soft, moist, shiny, mucoid, smooth, with a smooth border."

The reviewer would like to add to this description the occurrence of a light tan color with a slightly rosy cast on 5 per cent dextrose, 1 per cent Bacto-peptone agar. Such a color may appear in old cultures of other yeasts, but has been present after a few days in all of the strains of *Torulopsis neoformans* which I have studied. Another important diagnostic character is the development of voluminous capsules about the yeast in infected tissues.

A further step in establishing the identity of this yeast was made by Todd and Herrmann (1936) who described the formation of ascospores. Studying two strains (one isolated at the University of Iowa, the other from Minnesota) from cases of torula meningitis, they observed spore formation in cultures aged until the medium began to dry (3 to 6 weeks) using Sabouraud's glucose or maltose agar. The single spores and their asci are somewhat different from those found in any other described yeasts. The spore is contained within a cell with greatly thickened walls, and is excentric in position. The ascus may become heart-shaped, with the spore occupying the apex of the heart, from which it is discharged at maturity. They noted the development of two sorts of cells in the aging cultures, a small thin-walled cell, and a large thick-walled cell, and observed the fusion of these in hanging drop cultures. A life cycle is described and illustrated, rather complicated in that the ascospore appears to bud off several cells before it finally throws off the "hull" of the ascus. Since the yeasts they studied formed single spores by heterogamous conjugation they referred them to the genus *Debaryomyces* and suggested that the sporogenous pathogenic yeasts be called *Debaryomyces hominis*.

I had no difficulty in verifying the observations of Todd and Herrmann with the strain which I had furnished them, and with another strain isolated later from a case of "torula meningitis." No such structures appeared, however, in a yeast otherwise identical isolated from a myxoma-like lesion of the thigh. Todd and Herrmann reported finding spores in 8 other strains, including the Freeman-Weidmann strain which had been studied by Benham and by Lodder.

The observations of Todd and Herrmann were confirmed by Redaelli, Ciferri and Giordano (1937). They studied 19 strains from cases of meningitis and encephalitis (all originally labelled *Torula histolytica*), Sanfelice's original strain of *Cryptococcus neoformans*, the *Cryptococcus psichrophilicus* of Nino, and the *Torula nasalis* of Harrison. With all of these the formation of single spores was observed essentially as described by Todd and Herrmann, except that conjugation appeared at times to be

isogamous as well as heterogamous. The formation and liberation of buds by the ascospore while still within the ascus they consider to be a procedure for extruding excess fat ("exosmose par blastospores"). The Italian authors also noted with some strains a warty wall characteristic of the spores of *Debaryomyces*. Taking recognition of the synonymy previously established by Benham and by Lodder, they propose *Debaryomyces neoformans* as the proper scientific name for these pathogenic yeasts, and this appears to me to be the valid name for most of them.

This yeast was studied further *in extenso* by Giordano (1938) from the standpoint of taxonomy, physiology and pathogenicity. Some 29 strains were examined. Sixty-eight synonyms are listed! The formation of ascospores is reconfirmed. Giordano points out the analogy between the pathogenic yeasts in question, and the apiculate yeasts; in both groups some strains form spores, while other strains otherwise identical do not. Are they to be listed in separate genera? Giordano believes that a careful study will show fewer and fewer asporogenous strains. A large part of this paper is devoted to a study of the lesions in experimentally inoculated animals.

Of the yeasts described as pathogenic which do not fall in synonymy with *Debaryomyces neoformans*, nearly all may be dismissed (excepting the "medical Monilias") as lacking proof of pathogenicity. They have been isolated from superficial lesions where they may well have been simple contaminants, and are not pathogenic to laboratory animals. One further species, however, deserves some consideration. This is a yeast first isolated by Anderson in 1917 from feces and named by him *Cryptococcus glabratus*. It was reisolated from the intestines by Benham (1935) who not only found identical cultural characters but also complete reciprocal absorption of agglutinins with Anderson's strain. It was nonpathogenic for rabbits. Black and Fisher (1937) reported isolating this yeast (in large numbers) from the nasopharynx of a boy suffering from bronchopneumonia; the cultures were made to obtain pneumococci for typing, since no sputum was obtained. Their strain did not kill rats, but produced lesions in the omentum from which the yeast was reisolated.

Lodder and de Vries (1939) studied five strains of this yeast: Anderson's original strain; one which had been described by Ota in 1924 and was derived originally from the Dermatologic Clinic at Bern; one from an ulcer of the vulva; one from urine of a diabetic; and finally one from sputum in a patient suspected of tuberculosis. It should be noted that the patient with the ulcer of the vulva gave a positive skin test to this yeast while other persons gave negative reactions; and that the yeast was isolated from the sputum of the pulmonary case in large numbers on three occasions over six months; and that in spite of extensive x-ray findings no tubercle bacilli were found. Five rats were inoculated intracardially with each of the five strains. Of these 25 rats, five died after some months, with pleural lesions, and the yeasts were reisolated from the lungs. This yeast therefore appears to be of frequent occurrence in man and may be pathogenic. It should be kept in mind by those who have occasion to study yeast infections. Lodder proposed changing the name to *Torulopsis glabrata*. It differs from *Debaryomyces neoformans* especially in its ability to ferment dextrose, levulose and mannose with the production of gas; galactose, sucrose, maltose and lactose are not fermented. Alcohol is not utilized.

Pityrosporum

In spite of the fact that this organism has been known since 1874 to be commonly present in the skin, especially of the scalp, and thought by some to be the cause of seborrhoeic dermatitis, it remains somewhat of a mystery. Commonly called the "bottle bacillus" it is said to have been named *Pityrosporum Malassezi* by Sabouraud in 1895, but according to Lodder (1934) the original publication in which this name was given cannot be found. A number of microörganisms have been cultivated from the skin and have been said to be the true *Pityrosporum*, but they differ among themselves. Lodder (1934) describes three species in the Centraalbureau collection: *P. Malassezi*, isolated by Benedek from a case of pityriasis capitis; *P. pachydermatis* isolated by Weidman from the skin of a rhinoceros; and *P. rhinoserum* originally from Sabouraud's laboratory. The latter was found to be identical with *P. pachydermatis*.

The organism which Sabouraud is said to have named *Pityrosporum Malassezi* is most frequently referred to as *Pityrosporum ovale* since it is thought to be identical with one which had previously been described by Bizzozzero as *Saccharomyces ovalis.*

The organism isolated by Benedek (1930) from dandruff scales in maltose broth hanging-drop cultures is described by Lodder (1934) as follows: "Growth very slow. Cells short-oval, oval, or flask-shaped (2.5 to 3.8) μ x (4 to 5.5) μ, single or in pairs or rarely in threes. Budding frequently on a broad base. In wort no ring or sediment. No fermentation. No growth on ethyl alcohol medium. Wort-agar stroke culture hardly visible, matte, not differentiated in color from the agar."

Ota and Huang (1933) isolated an organism which may be identical with that of Benedek. Scales were soaked in 60 per cent alcohol and after washing in the condensation water, placed on the surface of agar slants containing butter or lecithin in the medium.

Moore (1935) rejected the organism described by Benedek because it is too large. The organism as it occurs in dandruff scales was given dimensions of (0.8 to 1.5) μ x (2 to 3) μ by Benham (1939). Moore made cultures on Difco wort agar. A variety of common molds grew as well as the yeast which he describes. The latter varied considerably in morphology, and the morphology was different on different media. The growth on agar was pinkish to buff. Cultural characters on a variety of media are described. Acid, no gas, was formed from galactose, dextrose, d-mannose, levulose, maltose, sucrose and melitose.

Moore, Kile, Engman and Engman (1936) reported further on the organism which Moore had isolated. They note that the organism is also variable in morphology in the scales of epidermis according to the acuteness or chronicity of the disease. Inoculation by scarification gave reactions in patients, rabbits and guinea pigs.

Benham (1939) studied cultures isolated by Moore and found them to fall in her group III of "Cryptococci" which we have noted previously appears to be identical with *Debaryomyces neoformans.* The organism of Moore fell in the nonpathogenic subgroup, not growing at 37°. Benham isolated a variety of yeasts

from seborrhoeic scalps on wort agar, among them a very small oval one which failed at first to grow on subcultures. When ether washings of the scalp and ether extracts of comedones were added to the medium, growth continued. With this clue a variety of fatty materials were studied. Eight strains of the yeast were isolated from 30 cases. Although on ordinary wort agar growth is slight and transparent, as described by Lodder, a rich growth is obtained on media containing fatty materials. Lanolin, butter and stearic acid were especially favorable; pork fat, chicken fat and linseed oil gave a lesser growth, while a number of other oily substances gave only poor or no growth. The cells are small (2 to 3) μ x (4 to 5) μ, often flask-shaped, the buds with a broad base, often separated from the parent cell by a cross wall as in *Saccharomycodes* or *Schizoblastosporion.*

Emmons (1940) found that an organism identical with that of Benham and of Ota and Huang could be readily isolated in dextrose broth containing glycerol. Bacterial growth is entirely inhibited at 28 per cent glycerol, while *P. ovale* continues growth up to 48 per cent. In these primary cultures the organism grows on nutrients provided by the epidermal scales. Subcultures would not grow in any concentration of the glycerol-broth. Subcultures grew readily on acid dextrose agar over which an ether extract of lanolin or oleic acid had been spread. Morphologically and culturally the strains isolated by Emmons appeared to be the same as those described by Benham, not at all like those isolated by Moore.

It appears that the organisms isolated by Benedek, Ota and Huang, Benham and Emmons are sufficiently alike to be considered identical, and to be so like the organism that is found in dandruff scales that they may be considered to be the true *Pityrosporum ovale.* Whether this organism is the cause of seborrhoeic dermatitis is, however, doubtful. The disease itself is so vague and variable as seriously to limit critical experimentation. Apparently identical lesions have been produced by scarification inoculation of widely different organisms. *Pityrosporum* may be found on normal skin, especially in the sebaceous glands.

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