A Microbiological Process Report

Yeasts: I. Morphology

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The yellowish gray, clay-like sediment that collects at the bottom of the casks or barrels in which beer or wine is fermented must have been associated with the cause of fermentation by the ancient brewmasters and wine-makers, for its name in most languages is linked to some phenomena of fermentation.

In 1680, Loewenhook, the Dutch inventor of the microscope, was the first man to see, with the aid of his new instrument, the spherical and egg-shaped tiny cells contained in the yeast sediment. But, by not elaborating on what he had seen, he left the actual discovery, that the yeast sediment is composed mainly of a unicellular fungus, to Schwann, Cagnard-Latour, Kützing, and others around the years 1825 to 1830. These early investigators applied the name "yeast" or "yeast fungi" only to the living part of the sediment and named the newly discovered plant *"Saccharomyces"* (sugar fungus). Beginning at the latter part of the 19th century up until the present time, yeast became one of the most extensively studied substances.

It soon became evident that the species often considered most typical of the yeasts, *Saccharomyces cerevisiae*, a species employed widely in making beer, wine, whiskey, grain- and molasses-alcohol, bread, and so on, has many relatives. These relatives, mainly because of their different and frequently unwanted behavior in the production of beer, were closely described and distinguished from the typical yeast. The number of fungi studied which exhibited similarity in morphological, physiological, or genetical characteristics to *S. cerevisiae* or its closest relatives increased with the years. Today the word "yeasts" as a collective name, denotes a group of fungi with loosely defined borderlines. The number of yeast strains described in the literature provided with names, and maintained and registered in culture collections, is above 1300. This number does not include the many strains used or maintained in the private culture collections of industrial establishments. The number of recognized species and varieties is about 185. Many of the excessive names are simply synonyms of the recognized species or refer to subvarieties, forms, physiological races, special forms, and so on.

The purpose of this and the following articles in this series is to present condensed introductory information in the ecology, morphology, genetics, taxonomy, technics of cultivation and observation, nutrition, biochemistry, metabolism, and practical utilization of yeasts for the use of persons who come in contact with yeasts in the field of applied microbiology and have a limited formal training on the subject. Special attention will be paid to the technical terms used by the various groups of this science, so that the processing engineer or the production manager should become familiar with the language of the mycologist, geneticist, or biochemist.

These articles will serve their purpose if the reader will be able to see yeasts as they are and form a coherent picture of their activities and manifold applications.

ECOLOGY

The place of yeasts in this world and their relation to the rest of the living creatures may be divided into two chapters. In the first chapter, their life in nature, yeasts are well distributed over warm and temperate zones of the surface of the earth and carry on life, utilizing the remnants and excretions of plants and animals. They live principally on sugar-containing liquids such as juices of damaged fruits, leaves, stalks, roots, and in the nectars of flowers. They may be present in the intestinal tracts and in the excrements of insects and higher animals. From the soil, where they carry on a seasonal vegetation in dilute sugar solutions washed from the trees and where they mate, sporulate, or hibernate, yeasts are carried by the wind or insects to the places where ample food is available. There a colony develops, filling the life-supporting commodity in a day or two, with millions of newly reproduced yeast individuals. Some of these cells, by way of another wind, insect, bird, or fruit-eating animal, will be disseminated to another life-supporting environment. The bulk of the new cells, without a chance for establishing another colony, is washed by rain back to the soil. In the temperate zone the main season for the propagation of free living yeasts is from early summer until late fall, while, in subtropical or tropical climates they will colonize the year round.

The soil in vineyards may contain live yeasts as deep as two feet. The surface of 100 healthy grape berries

may contain as high as 22 million yeast cells and the surface of damaged berries up to 800 million yeast cells. The juice of grapes received at the wineries contains up to 15 million yeast cells per ml.

Besides the fruit-to-fruit, or flower-to-flower life, many yeasts are natural habitants of food products. Milk, cheese, outer surface of salami and other meat products, syrups, and so on, offer living space to yeasts. A few yeasts and yeast-like fungi are known to carry on parasitic life preying on live plants, animals, or humans.

In the second chapter, the real opportunity to live and multiply in a scientifically calculated and controlled environment, and usually the year round in quantities impossible in nature, is given to yeast by man.

The efficiency of several yeasts to perform certain tasks has prompted man to provide the yeasts with the necessary environment. This harmony of interest resulted in the scientific domestication of a large number of strains belonging to a few species.

Man utilizes almost every type of yeast activity. By anaerobic respiration, the fermentation of sugar into carbon dioxide and alcohol far beyond their need, yeasts, especially the strains of genus *Saccharomyces*, became the key agents in the production of wine, beer, rum, distilled liquors, alcohol, bread, and so on. Glycerol is produced if the chain of reactions is blocked halfway by adding certain chemicals to the medium.

When air is present, yeasts like *S. cerevisiae* or *Candida utilis* (formerly *Torula utilis* or *Torulopsis utilis*) assimilate sugar and cheap inorganic nitrogen compounds into their body substance, doubling the cell number (and the weight) about every two hours. Their bodies contain about 50 per cent proteins and are nourishing for both humans and animals. Besides proteins, yeasts synthesize nearly all vitamins of the B group, ergosterol (provitamin D), and highly valued unidentified growth factors in quantities far above their needs. *Trichosporon pullulans* (formerly *Endomyces vernalis* or *Endomycopsis vernalis*) and *Endomyces lactis* (formerly *Oospora lactis*) synthesize fat and accumulate it in their bodies far above their possible need. Fat may be produced on commercial scale by the use of these yeasts. *Eremothecium ashbyii* and *Ashbya gossypii*, two most distant relatives of *S. cerevisiae* (in fact, not truly yeasts), under proper treatment, synthesize riboflavin up to several per cent of their body weight and secrete it into the surrounding medium. They are used in the commercial production of riboflavin. The enzyme invertase, produced in excess by yeasts, obtains application in candy and food industry. In the preparation of certain dairy products and pharmaceuticals, in sewage disposal, and so on, yeasts play essential roles.

Yeasts are also used as scientific tools, in assaying vitamins, in identifying sugars, and in genetic studies.

Owing to such diversified utilization, the newly grown yeast in the various fields of their utilization in the United States amounts to many hundred million pounds per year; each pound represents about 20,000 billion cells.

Yeasts, obeying biological forces, follow very distinctly the general patterns of life motivation of all living creatures. They pursue self-development and maintenance, race preservation, space (environment) domination and strive for perfection.

Self-development and maintenance, as the prerequisites to fulfillment of the other life programs, are performed by yeasts with speed and efficiency. Utilizing the surrounding nutrients and the biological power of its parent cell, a yeast cell may grow within an hour or two from inception into full maturity. Although an optimum exists for each environmental factor, most yeasts can develop or maintain life under extreme conditions.

In reproducing a new cell, the parent cell rejuvenates itself. A cell may reproduce for countless generations and may remain young, while another cell, having had no chance to reproduce, may grow old quite rapidly. Age in the life of yeast does not mean the time spent in hours or days. It means changes due to the effect of the environmental conditions on the cell. A several-hour-old cell may be killed or permanently crippled, brought into sporulation, or forced to dormant (aged) state by environmental changes. On the other hand, the author found a 14-year-old yeast culture kept on agar at 4 C perfectly viable, capable of reproducing with the ease it did when it existed as a few-day-old culture.

Race preservation is probably the strongest among the biological forces, for most of the yeast activities seem to strive for this purpose.

Space domination actually serves both factors, self-maintenance and the race preservation. It involves the spoiling of the environment for the existence of competing or harmful organisms. By acidifying the medium, yeast eliminates organisms preferring alkalinity; by forming CO_2, they exclude aerobic organisms, especially molds and acetic acid bacteria; by using up far more sugar than they need in fermenting it into alcohol, they reduce the food available for other organisms and store up a food (alcohol) which they may utilize in an aerobic life after all the sugar is gone.

Improvement of the individual and the race operates through several traits. They are studied under such titles as adaptation, mutation, and genetic processes. Effort for improvement goes as far as stopping the life continuance, through lethal genes, of individuals becoming unprepared to produce offsprings with a minimum standard.

The most prominent activities of yeasts, many of which are used in their identification, classification and/or in industrial utilization are as follows: 1) extracellular

digestion of the nutrient, 2) assimilation of the nutrient, 3) dissimilation of the nutrient, 4) dissimilation of internal reserves, 5) conditioning of the extracellular environment, 6) production of biologically active substances like vitamins, growth factors, enzymes, 7) vegetative reproduction, 8) spore formation and germination, 9) resting cell formation and germination, 10) sexual activities, 11) internal reorganization of the cell structure, in connection with some major biological activity, and 12) self-inflicted death.

Activities 1, 2, 3, 4, 5, 6, and 11 are motivated principally by self-development and maintenance; 7, 8, 9, and 12 lead to species preservation; 1, 3, and 4 partially relate to space domination, and 8, 10, and 12 are directed towards self and/or species improvement.

MORPHOLOGY OF YEASTS

The morphology essential in the classification and identification of yeasts includes the description of the shape, size, and internal structure of the yeast cells; the changes during the reproduction of vegetative cells and the position of the newly formed cells to their parent; the changes the cells undergo during sexual activities, when forming resting cells, ballistospores or ascospores; the size, shape, surface of spores, their number per ascus, and mode of germination; and the appearance of a yeast growth visible to the naked eye, referred to as macroscopical appearance. The morphological changes connected with ascospore formation and sexual activities will be discussed here separate from their genetic importance.

The yeast cell. The shape of the yeast cell and its structural parts are alternatively described as tri-dimensional objects, such as sphere, globe, egg shaped, cylindrical, olive shaped, and so on, or as two dimensional pictures as they appear through the microscope, like circular, elliptical, triangular, bottle shaped, and so on.

Our present knowledge on yeast morphology was fashioned by countless microbiologists. These observations complemented with each author's own observations were critically evaluated, commented and illustrated with drawings, photographs, and micro-photographs in the books of such microbiologists as Szilagyi (1890), Guilliermond (1920), Henneberg (1926), Henrici (1930), Stelling-Dekker (1931), Lindner (1930), Lodder (1934), Prescott and Dunn (1949), Diddens and Lodder (1942), Jörgensen (1948), Lindegren (1949), Skinner et al., (1947), and Lodder and Kreger Van Rij (1952).

The size and shape of cells within a strain are the same when the yeast is propagated under identical conditions; they may change with the nutrient and the environment.

Some strains produce overwhelmingly one cell form. Others may, as a rule, propagate in several shapes,

which characteristic is often referred to as dimorphism if two shapes predominate and polymorphism if more than two cell forms occur.

The standard vegetative cells of *S. cerevisiae,* the most typical in appearance and most widely used domesticated yeast, are egg shaped, elliptical, or occasionally spherical with typical dimensions of 4 to 8 μ in diameter for spheres, and 4 to 7 by 7 to 10 μ for the elliptical cells. In old cultures, especially in pellicles grown on liquid surface, they also form filament-like, elongated sausage shaped cells.

The other yeasts are either more spherical or more elongated than *S. cerevisiae* or have different shapes. Species of genus *Torulopsis* (Torula) form perfect spheres; *Saccharomyces ellipsoideus* (a variant of *S. cerevisiae*), *Saccharomyces pastorianus,* the species of genus *Schizosaccharomyces,* and many others usually form somewhat lengthier and more cylindrical shaped cells than *S. cerevisiae;* species of *Saccharomycodes, Nadsonia, Hanseniaspora* and *Kloeckera,* produce mainly lemon-shaped cells; the cells of yeasts belonging to genus *Trigonopsis* are usually triangular; those of *Pityrosporum* are club or bottle shaped; species of many *Candida, Eremothecium, Ashbya,* and *Eremascus* principally form elongated threads (the last three genera are not true yeasts).

The various shaped cells, with regard to the mode of their production and the place on the mother cell where they are formed, are named by special terms. The nomenclature used by the various authors is not quite uniform. The perfectly round cells are called "torula." The elliptical, egg-shaped or elongated spheres if produced by budding are called "yeast" cells, sometimes referred to as "Y" form. The more elongated cells are termed as "filamentous" or "F" form. The short cylindrical cell if produced by fission is called "oidium" or "arthrospore." The term oidium refers to short, sharp edged cylinders produced by splitting a mycelium into fragments, and arthrospores to cells produced by fission of a short cylinder. The sharp edges of arthrospores usually round up to give a more elliptical shape to the cell.

"Mycelium" or "true mycelium" is the name of a long thread in which the cells are formed by *fission,* in distinction from the pseudomycelium which denotes the threads formed by the *budding* process. These threads are sometimes signified by the symbol "M."

The short oval cells formed by budding, usually in abundance, at the ends of the long pseudomycelial cells are the "blastospores;" and those developed on the sides of pseudomycelia are the "blastoconidia." Some authors term all the typical yeast cells as "blastospores" and the blastoconidia as "conidia." The lemon-shaped yeast cells are sometimes referred to as "apiculatus yeasts." The occurrence of these forms is of diagnostic value.

The special resting cells (dormant or durable cells) with thickened cell walls are frequently called "chlamidospores." The sexual spores are the "ascospores," and those grown on sterigmata are referred to as "ballistospores" or occasionally as "basidiospores."

The size of the vegetative cells of the same strain increases with the ploidy. Mundkur (1954) puts the mean cell volume of haploid cells from 4.64 to 14.42 μ^3, of diploid cells from 18.73 to 37.71 μ^3, of triploid cells from 19.38 to 48.01 μ^3, and of tetraploid cells from 24.10 to 53.81 μ^3.

A few yeasts are quite small, 1 x 3 μ in diameter, and a few quite large, 8 to 12 x 14 to 20 μ; the "pseudo" and "true mycelium" cells with a 7 to 10 μ diameter may grow to 70 μ long.

Some molds under anaerobic conditions produce cells which look and reproduce like budding yeast cells. However, these yeast-shaped molds regain their mold-like appearance when grown in surface culture.

Structure of the yeast cell. The interior of the actively propagating yeast cells does not show internal structure without staining, while the older cells, the resting cells, and the cells forming or containing ascospores show some internal structure without staining. By various staining technics many structural parts can be demonstrated in all yeast cells.

Because of quick changes in the interior appearance of cells, which in many cases only means the concentration or dispersion of certain compounds, the cytologists have not as yet reached agreement in interpreting the cell structure of the yeast. Some points of the arguments are over 75 years old.

The three main parts of the cell are the cell wall, the cytoplasm, and bedded into the cytoplasm, the *nucleus*. The cell wall, a protective skin, thin and flexible in the young and thicker and more rigid in older cells, is permeable for many compounds in solution. It is the place of many metabolic activities where the living substance of the cytoplast and its acting tools, the enzymes, meet with and act upon the substrate of the environment. The surface area of the cells contained in one pound of yeast covers approximately 20,000 square feet. Although smooth in appearance, the cell wall, obviously, consists of a very fine network of active surface which in reality is much larger than the measurable surface area. This may explain the extreme speed of the biological activities of yeasts.

Using an optical or electron microscope to observe the cell wall, numerous circular to elliptical scars, where the daughter cells broke off, can be demonstrated.

The total content inside the cell wall is the protoplasm. The living substances of the cell are the cytoplasm and the nucleus, both of which may contain non-living reserve substances in the form of granulated bodies, droplets in dissolved or dispersed state besides nutrients and/or metabolites.

The thin compact layer of the cytoplasm surrounding the cell inside the cell wall, the cytoplasmic membrane, is semipermeable and it determines the kinds and the amounts of substances which may enter or leave the cell. The cytoplasm, which in young, actively reproducing cells occupies most of the interior and looks homogeneous or somewhat granular, is composed of a thick sap of water in which proteins, nutrients, salts, metabolites, and reserve substances are dissolved or dispersed. Depending on the physiological state of the cell, the cytoplasm contains several enclosures with varying shapes and sizes and of different importance.

Among enclosures, besides the nucleus, are the vacuole or vacuoles, oil droplets, dancing bodies, glycogen, chromatin substances, metachromatic granules, volutin, basophilic bodies, mitochondria, crystals of sulfur or calcium oxalate, and so on.

The vacuoles, so called because at first glance they look empty, one, two or occasionally three in a cell, are spherical or distorted spherical containers filled with a transparent homogeneous liquid containing bodies that may be demonstrated by special stains. Their sizes vary from about $\frac{1}{20}$ to $\frac{8}{10}$ of the cell content, small and hardly visible in young cells and large and quite conspicuous in older or resting cells. In old cells, a few vacuoles may fill the majority of the space within the cell wall.

According to one school of cytologists, the central vacuole is part of the nuclear structure, but most cytologists consider vacuoles as mere containers of reserve substances independent from the nucleus. Longer cells of certain species usually contain two vacuoles of equal size symmetrically located toward the two ends.

Some authors consider the sharply refracting, occasionally numerous oil drops as vacuoles. They coalesce as the cell ages into one or a few large oil drops. Size and location of the oil drops in the cells or in the spores are quite characteristic to a few genera or species.

Metachromatic bodies or voluntin granules, visible after staining, are located in certain vacuoles and are believed to play an essential role in the cell life. Dancing bodies, visible without staining, are particles inside the vacuole exhibiting Brownian movement. They may dissolve in the vacuolar sap, thus disappear, and again reappear after they become condensed.

Mitochondria, first reported by Henneberg (1926) then confirmed by Lindegren (1949) in the cytoplasm of *S. cerevisiae* are small granules consisting of ribose nucleic acid with small amounts of desoxyribonucleic acid besides some lipoidal substances. Their number in resting cells may be as high as 50, but they disappear before cell division; they obviously serve as reserve material. Some geneticists believe they play a part in the extra-nuclear heredity mechanism.

Basophilic granules visible in older cells when stained with basic dyes are obviously reserve materials and probably identical with the mitochondria.

Glycogen, the complex reserve carbohydrate, usually

located in special vacuoles, becomes visible with brown color when stained with iodine.

Occasionally other bodies may be found in the cytoplasm, such as typical octaedor crystals of calcium oxalate or sulfur, and so on.

Nucleus, the center of the heredity and cell organization, is the most important and most argued part of the yeast cell. Various authors call the same structural parts of the nucleus by different names or apply the same name to different structures. These structural parts are visible only after complicated stainings. Five concepts concerning yeast nucleus are summarized here in brief:

According to Guilliermond, the nucleus, during cell reproduction, undergoes an amitotic division—probably the nuclear division in the ascus of *Schizosaccharomyces octosporus* is mitotic; chromosomes could not be defined for being too small. Renoud, a student of Guilliermond (Ranganathan and Subramanian, 1947), demonstrated the existence of minute centrosomes and substantiated the occurrence of true mitosis. This concept is essentially the same as initiated by Dangerard (1893) and accepted and further developed by de Lamater (1950).

Badian (1937) claimed that the yeast nucleus contains a small nuclear vesicle, at one end of which lies the chromatin material consisting of two chromosomes which divide longitudinally.

Wager (1898) and Wager and Peniston (1910) described the yeast nucleus as a very elaborate structure. The drawing of the yeast nucleus according to their concept has been republished in several books and has become the subject of many comments and much criticism. Their nucleus consists of the nuclear vacuole or central vacuole of the cell to which at one side a heavily staining body, the nucleolus, is attached. The nucleolus is surrounded by a peripheral layer of chromatin with a dense chromatin patch in it. The center of the vacuole is occupied by a small central volutin granule. This granule and the nucleolus are connected to the membrane of the vacuole with a fine chromatin network. The importance of this conception is that the Lindegren (1949) school, in principle, accepted and used it for the basis of their interpretation of the structure of the yeast nucleus.

Lindegren (1949) changed the name of most of the Wager, Wager and Peniston structures. He calls the central volutin granule as nucleolus, the nucleolus as centrosome, the peripheral layer of chromatin as heterochromatin, the chromatin patch as centriole, and the chromatin network as chromosomes. Lindegren, interpreting his microphotographs, claims that diploid yeast cells have six pairs of chromosomes floating in the sap of the nuclear vacuole. They aggregate into two dense bars during division, and the bars divide longitudinally to form four bars. Then the vacuole pushes an extension into the base of the bud, the four bars disintegrate into their component chromosomes and one of the two chromosome clusters migrates into the daughter cell. The nucleus undergoes a regular mitosis with each cell reproduction.

De Lamater (1950) concludes—in accordance with Guilliermond, Renaud, Ranganathan and Subramanian, Winge and others—that the true nucleus of the yeast is the body considered by Lindegren as centrosome; and that the true nucleus lies outside the membrane of the central vacuole and has no contact to or any structure within the vacuole. De Lamater's interpretation of his own microphotographs is that cell reproduction is a mitotic process. He considers the chromosome number in diplophase cells of *S. cerevisiae* to be four, or probably more and confirms the presence and activity of centriole, which is different from Lindegren's centriole.

The latest, but obviously not as yet the final, word on yeast nucleus came from Mundkur (1954). His is in direct opposition, on many points, to the former concepts. He investigated haploid, diploid, triploid and tetraploid yeast strains in frozen-dried cytological preparations. The cells supposedly suffer no structural deformation as occurs in the older fixing and staining methods. He believes the distorted structures misled the observers in their conclusions.

He concludes that the nucleus is an extravacuolar optically empty vesicle having no detectable chromosomes or chromatin network at any period; the Foulgen positive particles are in a uniform submicroscopic dispersion in the nucleus; there are no recognizable nucleoli; the nuclear division, an elementary process involving the elongation of the nuclear vesicle and simple closure of the medial constriction, is not mitotic and is not necessarily synchronized with the budding process; presence of centrioles, which may orient the nuclear division, is not obligatory. The mean nuclear diameters of the cells he observed were 0.85 μ in haploid, 1.33 μ in diploid, 1.78 μ in triploid, and 1.98 μ in tetraploid cells.

Quite recently Lindegren et al. (1955) listed the following 16 cytological structures of the yeast cell: The cell wall, plasma membrane, bud scar, cytoplasm, nuclear membrane, spindle, centriole, centrosome, spindle chromatin, centrochromatin, chromosomes, nucleolus, nuclear sap, mitochrondria, glycogen located in cytoplasm, and metaphosphate in the nuclear vacuole.

Changes in Cells

The yeast cells are subject to visible changes in connection with specific physiological functions such as growth, vegetative reproduction, conjugation, ascospore formation, ballistospore formation, chlamidospore formation, germination of spores, and so on.

Growth. During reproduction the bud grows until it reaches the size of the mother cell. The cells of the fission yeasts grow longitudinally before cell division. The word growth is also used in referring to the formation of the colony or culture. Alternate terms are propagation or reproduction.

The average sized cells may also double their size (weight) when aerated in a medium containing proper nutrients in quantities less than sufficient for reproduction. Such cells are called over-fed cells. Cell reproduction may be interrupted artificially with little or no effect on the growth. This means that the cells continue growing above their normal size. Spoerl et al. (1954) showed that S. cerevisiae exposed to α-irradiation, x-irradiation or γ-irradiation stopped reproducing new cells, but the existing cells kept on growing, forming large cells. Loveless et al. (1954) reported similar effect when he exposed the same organism to special chemicals, "mitotic inhibitors", as methyl-bis-(beta-chloroethyl) amine or triethylenemelamine. He contributes the inhibition of cell reproduction to the formation of carbonium ions.

No microscopic measurements are reported by these investigators, but judging from the weight measurements the size of the increased cells was about twice that of the normal cells.

Townsend and Lindegren (1954), in a series of microphotographs, showed the appearance of single cells and the budding characteristics of S. cerevisiae of various ploidy.

Haploid cells are usually spherical and small in size. The third and fourth buds frequently appear near the first bud, forming typical rosettes. The cells tend to clump in liquid cultures. The small colonies are round and rough.

Homozygous (illegitimate) diploids are more elliptical and larger than haploid cells. They too produce rosette formation, by mother and daughter cells budding close to the base of their junction, the same way as do haploid cells.

Heterozygous (legitimate) diploid cells are elliptical, larger than the haploids. They predominantly reproduce by terminal or subterminal budding. The second bud appears opposite the first bud on the mother cell. The cells are generally uniform in size and seldom aggregate. The colonies are smooth.

The triploid cells are elliptical and larger in size than diploids. They form large vacuoles in liquid medium. End to end budding in chains is frequent on solid medium. Tetraploid cells are even larger than triploids.

Reproduction

Reproduction is the process in which a new individual, identical to the original, is brought into existence. In the life of yeast it means the bringing forth of a new cell by an existing cell. The meaning of reproduction is synonymous with propagation or multiplication. It is customary to divide the ways of yeast reproduction into vegetative reproduction and sexual reproduction. The term sexual reproduction is interchangeably used with the term ascospore formation. Such designations are quite correct in connection with the higher Asco-

mycetes, for the ascospores of many higher fungi, by their large number, are effective means of multiplication or reproduction. For yeasts, however, spore formation, as well as sexuality, suggests a different, more intricate and more complex purpose than the reproduction or multiplication of the individual, which for yeast is accomplished very effectively by the vegetative way of reproduction. In fact, vegetative reproduction is the only means of multiplication for about one half of the recognized species. Spore formation and sexuality in the life of yeasts should be dealt with according to their merits, not according to analogy borrowed from higher fungi, and should not be considered merely as a means of reproduction. The purpose of ascospore formation for yeast goes well beyond the production of individuals resistant to adverse conditions. The most significant purpose is the rearrangement of the heritable qualities; it is nature's big game of chance to recombine heritable elements to make up the characteristics of the progeny.

Yeast heredity, its relation to sexuality and ascospore formation will be presented in a review on genetics. At this place, spore formation is considered principally from the morphological point of view.

Vegetative or true reproduction. The conventional yeast cells produced in abundance during the actively growing phase of a culture are the vegetative cells, and the process by which they come to existence is the vegetative reproduction. Reproduction of a cell, from the beginning until completion, can be observed microscopically within a few hours' time.

Yeast cells reproduce in three distinct ways: By budding, by fission, and by a process that is between budding and fission. Each way of reproduction is characteristic to a number of genera, not necessarily closely related to each other. A few genera reproduce by both budding and fission.

Reproduction by budding. The wall of the well developed cell at a point becomes thin and flexible. Forced by the internal pressure of the cell content, the flexible part of the cell wall begins to bulge out in the form of a tiny sphere. This bulge, called bud or sprout, grows constantly until it reaches the size and shape of the parent cell, which retains its original size during the process. The nucleus of the parent cell divides just prior to or simultaneously with the budding process, and, as the bud reaches about one-third to one-half its final size, one of the twin nuclei moves in. The mother cell may also share a part of one of its vacuoles; the two nuclei organize themselves in their respective cells, and by narrowing the gate to nil, the cells become independent from each other. The two usually equal-sized cells are equal in maturity and, with the exception of their cell walls, are equally young.

The budding is called *polar* if the new cell appears at one of the shorter ends of the mother cell, *bipolar* if a bud appears at each end of the mother cell, and *multi-*

lateral if the buds appear at any place on a mother cell.

The cells may hang together for a shorter or longer time before becoming completely separated. While hanging together both cells may enter into a new process of reproduction, and, by constant repetition of the process, 30, 50 or more cells may hang together forming arborescent clusters. In the strains used as bakers' yeast and molasses yeast an in many strains of bottom fermenting yeasts, only two cells stay together in liquid media; they separate before starting a new bud. In some strains of distillers' yeast, four cells stay together in a bead-like fashion connected end to end. Several cells may engage in forming two buds at the same time.

The staying together of the cells in one fashion or another or the splitting right away is characteristic only of the strain (not of the species or genus) and only when propagated under specific environmental conditions. Some buds, after reaching the size of the parent, keep on growing in length thus forming elongated cells. These, after reaching a certain length, are the pseudomycelium cells.

Reproduction by fission. Reproduction by fission is similar to the pattern used by bacteria. The elliptical or cylindrical cell elongates by growing toward its longer axis until it almost doubles its length. During this longitudinal growth, the nucleus divides and the two nuclei move toward the two ends; around the middle of the cell two new cell walls develop, dividing the parent cell into two short, equally young arthrospores. Occasionally one of the sister cells divides its nucleus before the separating cell walls are completed and begins a new fission. Two, three or more cells may stay together for a while before the individual cells fall apart. The ends of the cells where they separate are flat. They, however, round up in most cases, and the cells attain a more or less elliptical shape. *Schizosaccharomyces* is the type genus reproducing by fission.

If cells elongate many times the length of a normal cell, and such cells stay together in end to end position, a thread-like structure, a true mycelium is formed. At maturation, usually due to changes in the environment, some mycelia may fall into many short oidium cells with lengths about twice the width. This is typical to the genera of *Endomyces* and *Oospora*.

Reproduction by the intermediate process. Lemon-shaped yeasts, like *Nadsonia* and *Kloeckera*, begin to reproduce by terminal budding at a broad base with a wide inter-cell connection. After the new cell obtained its part of the divided nucleus and reached its full size, two cell walls are formed on the broad connecting base in a fashion resembling fission. Bipolar budding is frequent for lemon-shaped cells.

Reproduction by combination. Species of genera *Trichosporon*, *Candida*, and *Endomycopsis* may produce new cells by both processes, budding and fission, side by side. A single cell usually starts out with budding; after a while a long filament-like cell grows out which eventually falls into short oidia. In *trichosporon*, the true mycelia may form blastospores by budding.

Resting cells. Induced by environmental conditions some yeast cells convert themselves into resting cells, frequently termed as *chlamidospores* or in the German literature Dauerzellen. Resting cells are conspicuous by their larger size, giant cells, thick cell wall, and highly refractive oil droplets. They are frequent in the pellicles of old liquid cultures, in the bottom layer of giant colonies, in bottom yeasts located on the walls of beer casks or on the walls of breweries. Resting cells resist adverse conditions more than vegetative cells; they will germinate and reproduce by budding when placed into favorable environment.

Spore Formation

The cell forms referred to as chlamidospores, blastospores, and arthrospores are not true spores. By processes resembling those used by some higher fungi, about one half of the recognized yeast species produce spores with a certain regularity as part (but not a compulsory part) of their life cycle. These yeasts are called spore-forming or sporogeneous yeast in distinction from the nonspore forming or the asporogeneous yeasts. The spores are two kinds, ballistospores and ascospores.

Ballistospores. Two genera, *Sporobolomyces* (7 species) and *Burella* (2 species) form *ballistospores*. These two genera in the family of *Sporobolomycetaceae* (having 4 genera, 2 of which are not yeasts) form a separate group among the yeasts. The yeasts in this family, reproducing mainly by budding, in surface cultures develop an aerial sterigma, a little stem. On the end of the aerial sterigma, a kidney-, circle- or spherical-shaped cell, smaller than the parent cell, forms. When ripe, this spore is thrown into the air. A drop of liquid is excreted at the neck of the spore; as it increases in size, it exerts a pressure and discharges the spore. The curve made by a ballistospore in the air is called sporabola. Under favorable conditions ballistospores either germinate and form conventional cells or develop again aerial sterigmata with new spores at the end.

Ballistospores are demonstrated by turning the surface culture upside-down above a sterile bed of agar medium. The thrown-out spores caught on the sterile agar surface germinate and grow into a colony showing the mirror image of the original colony.

Ascospores. Ascospores, or endospores, are the true sexual spores, and are far more significant than the asexually formed ballistospores. All yeasts capable of forming ascospores, the ascosporogeneous yeasts, are now classified in 19 genera (comprising about 75 species) and are grouped in one family, the *Endomyceta-*

ceae. For many years, taxonomists considered only the ascosporogeneous species as true yeasts. They placed them in the class *Ascomycetes* commonly called the sac fungi, (ascus meaning sac). Many higher fungi, including some mushrooms, form special sac-like cells called asci, where the sexual spores, or ascospores, develop. For yeast spores the yeast cell itself is the ascus.

The sexual origin of the ascospores in many yeasts is evidenced by the fusion of two cells immediately before the spores are formed, but in some yeasts it can be proven only by indirect methods. The cells of *S. cerevisiae* form ascospores without apparent sexual activity by that cell. Earlier reports on sexuality in this species met with skepticism until Winge and Laustsen (1938) demonstrated and Lindegren and Lindegren (1943) confirmed that diploidization of the haploid cells via conjugation of two cells is compulsory for ascospore formation, but that it may happen many generations earlier.

For each strain, variant, species, or genus the following characteristics are specific in varying degrees: The method used for spore formation; the number, shape and size of the spores; the surface appearance and the nature of the spore wall; the time required for spore formation; and the method of spore germination.

From point of morphology, four methods are distinguished by which yeasts produce ascospores: By parthenogenesis, immediately after izogamic conjugation, immediately after heterogamic conjugation, and after unsuccessful attempt of conjugation.

Parthenogenesis does not require conjugation of two cells immediately before sporulation; one vegetative cell performs the process alone. When exposed to proper conditions, its nucleus divides, usually twice, into four nuclei. Each surrounds itself with a portion of the cytoplasm (plasma sporogenic) and gradually grows at the expense of the original cell plasma until the newly formed spores reach maturation and develop a cell wall. The spores fill in the largest portion of the original cell. The left-over cytoplasm without a nucleus, often referred to as "free cell," usually is consumed as nutrient by the spores before or during germination. *S. cerevisiae* is the type species forming spores by parthenogenesis ("parthenon," virgin plus "genesis," origin).

In *izogamic conjugation* two morphologically identical cells produced by vegetative reproduction fuse to form a zygote, which then turns into an ascus where the ascospores are formed. In this process, when environmental conditions prompt the yeast to sporulate, each of two cells lying adjacent puts out a tube-like projection. These projections meet end to end, the connecting cell walls quickly dissolve, and the nuclei of the two cells unite, usually in the copulation canal. The two original cells may fuse completely, forming one elliptical or cylindrical zygote (stomatogeneous copulation), or the zygote and later the ascus may retain the outlines of the original cells connected with the canal. The combined nucleus surrounds itself with a dense protoplasm. In this stage the zygote is considered equivalent to a zygospore, but it quickly turns into an ascus by the division of the nucleus and the formation of the ascospores. The nucleus may divide once, twice or thrice, giving rise to the nuclei of two, four or eight ascospores. The gametes (the copulating cells) which form a zygote are frequently only one generation apart, the two cells derived from a common parent cell. If the original cells do not fuse completely, the spores divide half and half in the two bags connected by the canal.

There may be some deviation from this simplified scheme. Sometimes the two gametes attached to each other do not absorb the separating cell walls but form the ascospores without true conjugation. Infrequently, some cells of a species sporulating normally by izogamic conjugation may form spores by the process of parthenogenesis. Species of *Schizosaccharomyces* and *Zygosaccharomyces* are some of the yeasts sporulating mainly by izogamic conjugation.

In *heterogamic conjugation* two cells, different in size and age, conjugate before sporulation. Two main schemes of the heterogamic copulation are followed by yeasts. In one, the content of a not fully developed bud passes into the mother cell, which thus being fertilized turns into an ascus and forms one or two ascospores. Species of *Debaryomyces* follow this scheme.

In the other scheme, used by species of *Nadsonia*, the process is more elaborate. The content of the daughter cell moves into the mother cell where plasmogamy, the fusion of the protoplasm of the two cells, takes place and turns the mother cell into a zygote. This cell develops a new bud, opposite to the first one, and the contents of the zygote move into the new bud turning that into an ascus. In this ascus usually one spore is formed, seldom two.

Unsuccessfully attempted copulation before spore formation is observed in *Schwanniomyces*. The cells, when induced to sporulate, develop one or more projections, pseudocopulation canals, which try to make contact with another cell. The natural forces of affinity, however, seem to repulse rather than attract the projections. Attachment of these projections seldom occur, the separating cell walls never dissolve completely. Finally the cell turns into an ascus forming one or two spores by parthenogenesis.

The spore formation by *Candida pulcherrima* (*Torula* or *Torulopsis pulcherrima*) may constitute the fifth way of ascospore formation. This organism, still classified among the nonspore forming yeasts in genus *Candida*, propagates by budding. The regular yeast cells give rise to large, round, so-called pulcherrima

cells. These form one or several buds where ascospores are formed by parthenogenesis. A more thorough study is needed before all details are unfolded.

The maximum number of spores per ascus is specific within a species. As maximum, four spores are formed by *S. cerevisiae*, eight by *Schizosaccharomyces octosporus*, 16 or 32 by *Eremothecium ashbyii*, and one spore per ascus by the two species of genus Monosporella and so on.

In as much as spores develop around the new nuclei formed by the division of the parent nucleus, the normal number of spores would be 1, 2, 4, 8, 16, 32, and so on. Still the ascus of a strain which typically forms a maximum of four spores, frequently develops one, two or three spores. An earlier theory was based on the suggestion that some of the originally started four spores degenerate and only the stronger ones develop to full maturity. Geneticists claimed hereditary causes for the number of spores less than the maximum. Quite recently nutritionists have produced evidence showing that the number of spores is highly influenced by the type of sporulation or presporulation media used.

Shapes of spores are described as spherical, hemispherical, ellipsoidal, oval, walnut shaped, kidney shaped, sickle shaped, derby hat shaped, Saturn shaped (spherical with a ring around the equator), distorted Saturn shaped (sphere with an eccentrical ring), spindle shaped without flagellum or with an immotile flagellum on one end, helmet shaped, cornered, irregular. Surface characteristics and the nature of the spore wall are described as smooth, warty, rough, double walled, and thick walled. The spore wall of *Schizosaccharomyces* usually contains a starch-like substance.

Most *Saccharomyces*, *Zygosaccharomyces*, *Saccharomycodes*, *Schizosaccharomyces*, and *Torulaspora* form spherical spores with smooth surface; *Debaryomyces* spherical with warty surface; *Endomyces* spherical to hat shaped with smooth surface; *Endomycopsis vernalis* at first spherical, later hat shaped with smooth surface; most *Hansenula* have Saturn-shaped to derby-hat-shaped smooth spores with brim; *Schwanniomyces* form walnut-shaped spores with an equatorial Saturn brim and warty surface; *Monosporella*, *Nematospora*, *Coccidiascus*, *Eremothecium*, and *Ashbya* form spindle-shaped spores, and so on.

Wickerham and Burton (1954) demonstrated that in the species of *Hansenula* which they studied, all yeasts having hat-shaped ascospores were heterothallic and all having Saturn-shaped ascospores were homothallic.

Liberation of the spores from the asci may vary greatly with the species. *Saccharomyces fragilis* and *Hansenula* species with hat-shaped spores, liberate, dehisce, spores as soon as they are formed; others like *Hansenula saturnus* and *Pichia* liberate somewhat later, while *Saccharomycodes ludwigii*, most *Saccharomyces*

and *Zygosaccharomyces* having strong, walled asci do not liberate their spores until just before or during germination. Usually the swelling or germinating spores rupture the ascus. Spores of *Saccharomycodes ludwigii* may conjugate in the ascus before germination.

Liberated spores may swell to the appearance of small vegetative cells before germination; half-sphere or hat-shaped spores may swell into spheres. Liberated spores of certain species frequently clump together into masses, while the spores of other species stay free from each other. Many germinating spores assimilate the residual cytoplasm of the ascus and frequently dissolve the ruptured fragments of the asci.

Spore germination. Spores, when in proper environment, will germinate and produce vegetative cells. The germinating power or vitality of the spores varies. The more active the strain, closer to its natural (wild) habitat, the easier it sporulates and a larger percentage of spores are viable—they will germinate. Many times inbred strains sporulate more reluctantly and a smaller percentage of the spores germinate. Spores formed following direct fertilization (the nucleus divides, then fuses inside the cell), although looking normal, will not germinate. The spores of *Nadsonia richteri* maintain germinating power better if kept at the low temperatures of 0 to 5 C.

In order to observe under the microscope the slow process of spore germination, the fast propagating vegetative cells are killed in the mixture of spores and vegetative cells by exposing them to 50 per cent alcohol for 1 minute or to heat, 56 C for 5 minutes.

The spores of *Endomycopsis* germinate by a projection, called a germ tube or promycelium, growing out of the spore. The germ tube then produces vegetative cells. Two spores of *Saccharomycodes ludwigii* fuse before projecting a germ tube. Most spores germinate by beginning to bud or by developing arthrospores, germ tubes which become separated from the spores by fission. Some spores may fuse (conjugate) with their newly formed buds.

The time for maximum sporulation, depending on the conditions, may vary from less than 48 hours to several weeks. The optimum conditions as well as the minimum time may vary from strain to strain. The time requirement, using identical conditions, is frequently used in breweries to diagnose the presence of wild yeast in culture yeast, as wild yeasts sporulate much faster.

The optimum sporulation temperature for most yeasts is around 25 C. But sporulation may occur at temperatures as low as 2 C and as high as 48 C. The minimum sporulation time and the optimum temperature are more characteristic to a strain than to a species or genus.

When and why are spores formed? Spore formation in yeast seems to be a device to preserve the life of the

race when exposed to adverse conditions. It also is a mechanism to improve the qualities of progeny to meet life best equipped with the experiences of its ancestors.

The race preservation motive is evidenced by the fact that spores are frequently produced when conditions do not favor vegetative reproduction. Spores are better equipped to survive adverse conditions than are the vegetative cells. Improving the qualities of the progeny is evidenced by the fact that spore formation is one of the means by which ancestral qualities may be changed. Some abilities may be lost, others gained through the faculty of spores or their progeny of one strain to conjugate with spores or their progeny of another strain. Spores will form when spore formation is induced.

Early workers in the field specified the starvation of well developed, relatively young cells in the presence of plenty of air and moisture, and for most yeasts, a temperature of 25 C as the conditions to induce sporulation. It became evident that yeast will sporulate when the succession of vegetative reproduction is blocked by environmental conditions, principally by the lack of available sugar, during a time when special nutritional stimulants are present in the environment. In nature, vegetative reproduction is obviously blocked by the exhaustion of the sugar supply, for example, when the cells are washed by rain to the soil, and the special nutrients are supplied by autolyzed cells.

The essentials of sporulation technics will be reviewed here briefly.

De Seynes (1868) reported that yeast cells sporulate on the surface of water. Reese (1869) reported that yeast cells sporulate in six days on the surface of carrots and potatoes, cooked or raw.

Engel (1872) devised the classical gypsum block method. He placed washed yeast cells on the surface of wet plaster of paris blocks or ceramic blocks where they sporulated at 25 C within several days or weeks. Graham and Hastings (1941) modified this method by sterilizing the gypsum slants in test tubes. The method is still in use up to date. Klöcker (1924) moistened the gypsum blocks by dilute malt extract and Saito (1923) by manit-dikalium phosphate solution. Beijerinck (1898) obtained spores by placing washed yeast cells on purified wet agar surface. Both Beijerinck and Hansen (1883), who studied sporulation intensively, believed in starving the yeasts to force sporulation.

Gorodkowa (1908) obtained good sporulation on the surface of her famous Gorodkowa medium, 1 per cent peptone, 0.25 per cent glucose, and 2 per cent agar. When the sugar content was increased in this medium, no sporulation occurred, proving that the starvation theory with regard to carbohydrates is correct.

Welten (1914/15) demonstrated good sporulation on the surface and the interior of prune extract agar, also small amounts of $MgSO_4$ aided sporulation. He believed an acidic medium was essential.

Baltatu (1939) sporulated cells of Mycoderma (a non-sporeformer) in grape juice, but the spores germinated immediately.

Mrak, Phaff and Douglas (1942) observed that cells of Zygosaccharomyces conjugate and sporulate in abundance in the presence of dead yeast cells.

The more advanced methods recommend growth of the yeast on presporulation medium, then sporulation of the washed or unwashed cells on gypsum or ceramic blocks saturated with water and usually containing nutritional stimulants.

Lindegren's (1949) presporulation medium contains extracts of beet leaves and roots, juices of apricot and grape, dried yeast, glycerol agar, and $CaCO_3$. From this presporulation medium, cells taken to potato agar slants sporulate in abundance, but form predominantly two spores per asci. He obtained a large percentage of four spored asci when he transferred the cells from his presporulation medium to sterile plaster of paris kept moistened with water acidified with acetic acid to pH 4.0. At 23 C spores appeared in 12 to 40 hours compared to 3 to 14 days using the old method.

After a short period (24 hr) of vegetative reproduction, good sporulation is obtained in direct sporulation media. Henrici (1930) recommended V8 agar. Wickerham, Flickinger, and Burton (1946) improved Henrici's medium as follows: In 18 ounces of V8 vegetable juice, after adjusting its pH to 6.8, they dispersed ½ lb bakers' yeast, steamed the mixture for 10 minutes, readjusted the pH to 6.8, mixed with equal amount of distilled water, added 4 per cent agar, and sterilized the mixture. They obtained good sporulation of Hansenula, Zygohansenula, Pichia and Zygopichia in 3 days, of Saccharomyces and Zygosaccharomyces in 5 to 20 days at 24 to 25 C.

Phaff and Mrak (1949), in their excellent review, discuss the effect of factors influencing sporulation such as temperature, pH, humidity, presence of oxygen, types and concentrations of carbohydrates, nitrogenous compounds, metabolites and extracts of microorganisms, trace elements, and genetic factors. They emphasize that no universal sporulation medium has yet been developed beneficial to all types of yeast.

Kleyn's (1954) sporulation medium designed for S. cerevisiae contains Bacto-tryptose, sodium chloride, sodium acetate, Bacto agar, glucose (0.062 per cent); its initial pH is 6.9 to 7.1. On this medium, the yeasts reproduce by budding for 24 hours, then they begin to sporulate. His most interesting conclusions are these: Using his sporulation medium, no presporulation medium or plaster of paris blocks are necessary; acidic environment turns alkaline during sporulation; highest percentage of 3–4 spores per ascus (92 per cent) is obtained at pH 8.1 to 8.7 using phosphate or glycine

sodium hydroxide buffers; sodium acetate stimulates the formation of a high percentage of 3–4 spores per ascus, while magnesium acetate promotes 1–2 spores per ascus; in the presence of aspartic acid 1–2 spores are formed per ascus, while in the presence of glycine 3–4 spores are formed per ascus; sodium glutamate among the tested N sources induces the highest rate of sporulation in a semi-solid medium.

Kleyn's study, in agreement with some previous observations, suggests that the number of cells forming spores, the number of spores per ascus, and the sporulation time within the frame of genetic limitations, are primarily governed by nutritional and environmental factors.

In their classical taxonomic works, the Holland workers, Stelling-Dekker (1931) and Lodder and Kreger Van Rij (1952) when testing many hundreds of yeast strains in the culture collection of the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands, used all contemporary sporulating methods to determine the sporulating characteristics so important in the classification of yeasts. The various methods stimulated sporulation in the individual strains to a varying degree.

The study of ascospore formation by yeasts is further complicated by the fact that several ascosporogenous yeasts, after a few years' cultivation on laboratory media, lose their sporulating ability or become extremely difficult to sporulate. Lindner reported the loss of sporulating ability by several strains in relatively short periods of time. Stelling-Dekker (1931), while confirming Lindner's (1930) observation, found many yeasts sporulate with relative ease after being cultivated for 50 years on laboratory media. Also, Lodder and Kreger Van Rij (1952), trying a large variety of methods, were able to induce sporulation in several yeasts which did not sporulate under the conditions used by previous investigators.

The appearance of a yeast culture in liquid or on solid media is a part of yeast morphology. It is sufficient to note here, that the groups of yeast species exhibiting certain similarities in their cultural appearances quite frequently are apart in their philogenetic relations. A strain under identical conditions usually form surface colonies with the same size, shape, consistency, topography, color and other visible characteristics. They may vary for the same strain, with the ploidy, the composition of the medium, the pH, temperature, humidity of the atmosphere, and so on. If mutation occurred in a cell, the offspring of that cell will form a sector or a portion of the colony different in appearance from the rest. Frequently, the mutated section is so different that it appears as if it were an infection.

The presence of the maximum number of morphological characteristics defines a yeast into a certain group; the absence of one or another particular characteristic places the yeast into other groups; and the absence of more than one of the maximum morphological characteristics will classify a yeast again into other groups. The permanent loss or gain of characteristics may suggest the pathways of how one type of yeast might have developed from another type.

For instance, yeasts of *Endomycopsis* form mycelia as well as budding cells, and they also form ascospores. If a yeast of *Endomycopsis* loses the power to form ascospores, the remaining characteristics will place it into the unascosporogenic genus *Candida*, which forms mycelia as well as budding single cells, but no spores. But if a yeast of *Endomycopsis* loses its ability to form mycelia but retains its ability to form ascospores, it will fit into one of the genera of *Saccharomyces*, *Zygosaccharomyces*, *Hansenula*, and so on. If any of the budding and ascospore forming yeasts which form no mycelia like *Saccharomyces*, *Zygosaccharomyces*, *Hansenula*, and so on lose their ability to sporulate, they will become similar to *Cryptococcus* and other asporogenic genera reproducing by budding. The same *Cryptococcus* type of yeast may derive from *Candida* if this loses its ability to form mycelia.

In spite of the variety in morphological characteristics of yeasts and in spite of the vaguely specified position the various yeasts occupy among microorganisms, with some experience, based on the macroscopical and microscopical morphology, the about 167 yeast species are easily distinguished from the more than few hundred thousand species of molds and bacteria. Although future systems obviously will rely more on physiological characteristics, the present classification of yeasts is mainly built on its morphology.

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