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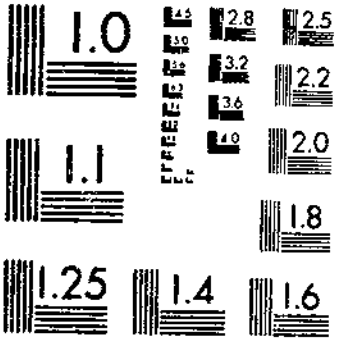

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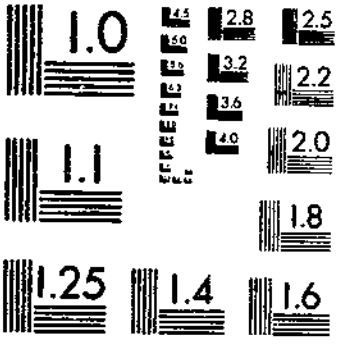

MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS-1963-A

Technical Bulletin No. 1029 *May, 1951*

UNITED STATES
DEPARTMENT OF AGRICULTURE
WASHINGTON, D. C.

Taxonomy of Yeasts[1]

1. TECHNIQUES OF CLASSIFICATION

2. A CLASSIFICATION OF THE GENUS HANSENULA

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INTRODUCTION

The ultimate objective of this bulletin is to present a method of classification that will lead to the defining of each genus of ascosporogenous yeasts so that it will contain only a single group of species which appear to be phylogenetically related. A similar definition,

[1] Received for publication May 15, 1950. Report of a study in which certain phases were carried on under the Research and Marketing Act of 1946.

covering essentially the same range of characteristics except the ability to form ascospores, will be used to define the corresponding nonsporogenous genus if such genus exists. This procedure necessitates some marked changes in the currently accepted classifications, but it should clarify relationships between certain sporogenous genera and their previously unestablished generic counterparts among the nonsporogenous yeasts.

Phylogenetic lines are recognizable through the use of common laboratory techniques. The separation of species is accomplished predominantly by biochemical tests, since several workers, among them Wickerham and Rettger (*113*)[2] and Skinner (*91*), have reported that physiological characteristics are less variable than morphological characteristics in the filamentous yeasts. This bulletin describes the techniques used by the author, and gives some techniques used currently or in the past by other yeast taxonomists. Additional techniques of limited application will be described in connection with the genera to which they apply.

Because variation occurs commonly within species of yeasts, due consideration is given the application of morphological and physiological characteristics in the identification of individual strains. Therefore, a brief discussion is presented to bring the reader's attention to the more common types of variation among the yeasts.

Each report in the series contains a table of the salient diagnostic characteristics by which species may be readily identified.

Part I

TECHNIQUES OF CLASSIFICATION

Isolation and Maintenance

The isolation of yeasts from substrates that contain many yeasts and relatively few bacteria and molds may generally be effected by streaking the material directly on a rich organic medium which contains 1 percent or more of glucose and has a moderately acid reaction. At the Northern Regional Research Laboratory, malt extract-yeast extract agar is used for isolating yeasts, preparing inocula for biochemical and other tests, and for the maintenance of cultures. This medium contains the following constituents in grams per liter of distilled water: 3 gm. malt extract, 3 gm. yeast extract, 5 gm. peptone, 10 gm. glucose, 20 gm. agar. The pH, which is not adjusted, is between 5 and 6, depending upon the particular batch of ingredients.

By lowering the pH between 3 and 4, malt extract-yeast extract broth may be used as an enrichment medium for the isolation of yeasts from populations consisting mainly of bacteria. If molds are present also, their growth may be restricted by obstructing the entrance of air to the culture. To accomplish this, a layer of sterile paraffin oil 1 centimeter deep is carefully placed on the surface of the inoculated enrichment medium, and the culture is incubated until growth has occurred and is then streaked on ordinary malt extract-yeast extract agar. This technique favors the development of fermen-

[2] Italic numbers in parentheses refer to Literature Cited, p. 50.

PREFACE

In early attempts at yeast classification, the characteristics employed for separating the larger groups of yeasts were the ability to grow as filaments, the ability to produce asci which consist of two cells fused together, the shape of the ascospores, the ability to produce a gaseous fermentation of one or more sugars, and the ability to produce pellicles on liquid media.

Evidence obtained in the present study indicates that the use of the foregoing characteristics led to the unintentional cleavage of natural, or phylogenetic groups into sections. Moreover, sections of different phylogenetic lines which had in common only one or two of the characteristics just mentioned were often assembled into the same genus.

These characteristics, applied by Hansen, Barker, and Klöcker during the early 1900's, continue in use as the basis for separating genera and subgenera in the presently accepted system of yeast classification. As a result, all of the larger genera of both the ascosporogenous and nonascosporogenous yeasts contain dissimilar types. The mixing has been thorough—in many cases the true relationships which exist among species of the different genera have been concealed.

Correction of this situation by the arrangement of species in what appear to be phylogenetic lines of related species is believed to be a major advance. The species of a given line are presumed to have been derived from a common ancestor because they share certain biochemical or sporulation characteristics. The evolutionary sequence in the origin of the species is believed to be revealed by the state of the nucleus in the vegetative culture. The species which consist exclusively of ascigerous haploid cells are considered the most ancient in origin, and those which consist exclusively of ascigerous diploid cells are considered the most recent. Species between these extremes are arranged in the order of increasing numbers of the diploid cells and of decreasing numbers of the haploid cells which exist together in growing cultures. A study of the change in properties from species to species of the yeasts thus arranged is believed to show step by step the acquisition of new properties during the evolution of the genus. The generic description is then made to include all of the species in this phylogenetic line and to exclude species of all other phylogenetic lines.

A noteworthy feature of the present classification is that it is based largely on studies of recent isolates from nature, collected by contemporary workers and by the author. An isolation program has led to the discovery of species of *Hanseniella* which are entirely haploid and very different from those species placed in the genus by Stelling-Dekker. Study of the new species has resulted in a clearer, more comprehensive conception of the entire group. The search for new species not only fills in many missing links in the phylogenetic chains of this and other genera, but it adds to the number of yeasts with special industrial and academic potentialities. The isolation and study of large numbers of strains of different species is necessary to determine which taxonomic characteristics are least variable, and therefore the most valuable, for the separation of species within the genus.

Probably the most important advance made in the classification presented herein is the marked increase in the number of physiological characteristics used for recognizing the individual species. With morphology so variable and biochemical tests so limited in number, it often was difficult to decide whether an isolate which differed in one or two characteristics from the description of the species that it most closely resembled, should be placed in that species or regarded as a new species. Under this new system, if an isolate belongs in a certain species, its similarities to that species are multiplied by the results of the numerous biochemical tests; if the isolate does not belong in that species, the differences are multiplied. Thus identification of isolates is made less subjective.

The increased number of biochemical tests here proposed does not increase inordinately the effort involved in the identification of yeasts. The chemically defined media on which these tests are based have recently been placed on the market in the form of powders. Their use greatly simplifies the preparation of media. Further, the author determines which of the 37 carbon sources he regularly employs give the most consistent and useful reactions for the separation of species. Then a limited number of these are selected for separating the species of a genus; in this case, the genus *Hansenula*.

This bulletin is divided into two parts. Part 1 describes the techniques used for the classification of yeasts. These techniques comprise procedures for isolating and preserving cultures, for conducting sporulation and morphological studies, and for performing assimilation, fermentation, vitamin deficiency, and other tests. The techniques dealt with in this paper are to be applied to the study of the yeasts generally; those which are applicable only to the study of one or two genera must await description in later reports on those genera. The principal types of variation occurring in yeasts are discussed. Part 2 presents a classification of the genus *Hansenula* based on the described techniques and on the phylogenetic conceptions of the author.

It is hoped that investigators who have described new species and those who have isolates which they believe may represent new or rarely isolated species, will send them to the Northern Regional Research Laboratory, Peoria, Ill., for inclusion in these studies.

Acknowledgment is made to Ernst A. Bessey for supplying the Latin descriptions and helping with the selection of names for the new species; to Kermit A. Burton and Richard J. Gill for their technical assistance; and Kenneth B. Raper, principal microbiologist, and George F. Jordan, editor of this Laboratory, for their many valuable suggestions and the hours of work they have contributed to the preparation of the manuscript.

Work in the laboratory has been greatly facilitated by the powdered media used in the carbon and nitrogen assimilation tests, vitamin deficiency tests, and morphology studies. These media were supplied by the Difco Co. of Detroit, Mich., and the splendid cooperation extended by H. W. Schoenlein of that company is greatly appreciated.

Thanks are extended to those persons in charge of the Centraalbureau voor Schimmelcultures, Delft, Holland, and of the American Type Culture Collection, and to all others who supplied cultures or frass from various species of trees.

tative species. In another procedure, a flask of an acidified liquid medium is inoculated and placed on a rotary shaker for 1 or 2 days. The molds will be prevented from sporulating, and the number of yeast cells will so greatly outnumber the mold pellets that further separation of the cells may be readily achieved by streaking. This method favors the development of both fermentative and oxidative yeasts.

Hertz and Levine (*39*) recommended media in which sodium propionate and diphenyl were incorporated as fungistatic agents to eliminate molds and permit the enumeration of yeasts in mixed populations.

Occasionally sirups, fondants, salad dressings, and other substrates of high osmotic pressure undergo fermentation by yeasts which cannot be isolated on media containing only 1 percent glucose. Depending upon the nature of the sample from which the yeasts are to be isolated, the following media may be used: 500 or 300 gm. glucose (the smaller amount is usually sufficient), 3 gm. yeast extract, 3 gm. malt extract, 5 gm. peptone, 30 gm. agar, in 1 liter distilled water. The pH is not adjusted.

The medium is streaked and the cultures are incubated at least a week. In general, yeasts grow slowly and produce small colonies on such media. Almost always, osmophilic yeasts can be successively subcultured on 30, 10, and finally on 1 percent glucose medium, and growth on the latter is usually more typical in appearance. Rarely will an osmophilic species be found which cannot become adapted to growth on ordinary media.

General information regarding yeasts capable of growth at high osmotic pressures may be obtained from papers dealing with species isolated from various substrates, including: Honey (*28, 57*); raw and refined sugars, sirups, and molasses (*20, 26, 36*); pickle brines and cucumber fermentations (*23, 24, 70*); fermenting musts of especially high sugar concentrations (*44, 45, 46, 47*); sweetened condensed milk (*40*); dried fruits (*4, 73, 74*); and insects (*16, 81*).

The yeasts associated with the animal body, with the exception of the genus *Pityrosporum*, may be isolated on media such as malt extract-yeast extract agar, tomato juice agar as used by Weinstein and Wickerham (*104*), malt agar as used by Stovall and Bubolz (*97, 98*), or Sabouraud's agar. Benham (*11, 12*) and Emmons (*22*) have given considerable attention to the development of media suitable for cultivation of the members of the small genus *Pityrosporum*.

Purification of isolates is accomplished by restreaking until the colonies are as homogeneous in appearance as space relationships between the colonies will allow. Where two or more forms reappear on subculture, it is possible that they represent variants of a single species. If they prove to possess the same physiological properties, the belief that they may be variants, rather than distinct species, is strengthened. Each variant is studied and its description is included in that species to which it is believed to belong.

All isolates are lyophilized according to the procedure described by Wickerham and Flickinger (*109*). The yeasts are grown on slants, and as soon as growth becomes abundant it is dispersed in sterile blood serum. The mixture is then frozen at approximately −40° C. and kept under vacuum near 5 until visibly dry. The

drying is continued at room temperature for 30 minutes. Then the tubes are sealed by means of a cross-fire torch while the vacuum pump is still running. The desiccated preparations are kept in a refrigerator until time is available for classifying them. Then the tubes are opened, the pellets suspended in liquid malt extract-yeast extract medium, and a loopful streaked on an agar plate. The resulting colonies provide starting material for sporulation and morphological and physiological studies. Lyophil preservation reduces loss of the ability to sporulate and provides a way of keeping indefinitely for future observation the sporulation process in all its stages. It greatly reduces loss of otherwise short-lived species, such as members of the genus *Brettanomyces*.

Atkin, Moses, and Gray (*3*) reported that lyophil preservation of brewers' yeasts resulted in the recovery of substrains differing from the parent strain in their ability to synthesize vitamins. Generally, variation consisted of a lessened capacity, although sometimes an increased capacity, to produce particular vitamins. Such changes, while disturbing, are not regarded by the author as serious, since Schultz and Atkin (*89*) found that strains of *Saccharomyces cerevisiae* could be separated into 13 types on the basis of their vitamin requirements. Should future investigations prove that qualitative differences in the more stable physiological characteristics, such as fermentation reactions, are induced by lyophilization, this procedure for the maintenance of stock cultures will have to be re-evaluated.

Morphology

Sporulation

Many media have been devised for the study of sporulation in yeasts. Mrak and McClung (*71*) reported finding yeast which sporulated on cucumber wedges but not on the other media they used. Later Mrak, Phaff, and Douglas (*72*) recommended an agar medium containing a mixture of extracts of cucumber, beet, potato, and carrot for maintenance of stock cultures so that ascospores might be available whenever needed. The Lindegrens (*54*) prepared a presporulation medium from fruit juices, vegetable extracts, dried yeast, glycerin, calcium carbonate, agar, and water. They reported that spores were formed on this medium if the yeasts were allowed to grow for a few weeks. If spores were needed sooner, some of the cells were transferred from the presporulation medium to gypsum slants, as devised by Graham and Hastings (*30*).

Stantial (*94*) obtained the best sporulation of *Saccharomyces cerevisiae* by continuously shaking cells in a solution containing 1 to 3 mg. of glucose and 10 to 30 mg. of potassium acetate per milliliter. Stovall and Bubolz (*97, 98*) obtained spores by growing yeasts on malt agar plates and permitting the cultures to dry completely. The colonies and agar were then rehydrated and the cells were observed for ascospores. Liver media, such as used by Etchells and Jones (*25*), sometimes favor sporulation. Nickerson and Thimann (*78*) found that dead yeast cells and filtrates of culture media in which *Aspergillus niger* had been grown would stimulate conjugation in *Zygosaccharomyces acidifaciens*. Later, these authors (*79*) found

that riboflavin and sodium glutarate had the same effect. Phaff and Mrak (*82, 83*) have recently published a review in which various sporulation procedures are compared and discussed.

The author routinely employs one or more of the following media for obtaining ascospores:

HENRICI'S VEGETABLE JUICE[3, 4] AGAR (*110*).—To 500 ml. of juice are added 500 ml. of distilled water. Ten grams of moist yeast (taken from a cake of bakers' yeast) is dispersed in the diluted juice. The mixture is adjusted to a pH of 7.0 with potassium hydroxide and 2 percent of agar is added. The medium is immediately heated to melt the agar and preclude fermentation, then tubed or bottled and autoclaved the usual 20 minutes. Excessive heating is avoided.

MALT EXTRACT AGAR SLANTS.—This is one of the best sporulation media. Twelve grams of agar and 400 ml. of distilled water are heated to melt the agar. Then 20 gm. of powdered malt extract is dissolved in the hot solution. The medium is dispersed in test tubes, autoclaved at 15 to 18 pounds for 15 minutes, then all tubes are slanted. The slants are kept in the refrigerator until used. The medium is acidic and therefore should not be remelted.

MALT EXTRACT-YEAST EXTRACT AGAR SLANTS.—This medium is described in the previous section on Isolation and Maintenance (p. 2).

CARROT WEDGES.—Fresh young carrots are washed and cut longitudinally in quarters. Each wedge is placed in a large (25 mm.) test tube with 3 ml. of distilled water. The wedges are sterilized 20 minutes with steam at 15 pounds pressure.

GYPSUM BLOCKS.—Although widely used for sporulation, gypsum blocks do not yield material suitable for lyophilization and are resorted to rarely.

The yeast to be studied is grown for 48 hours on a slant of malt extract-yeast extract agar, then transferred to a second slant which is incubated for 24 hours. Cells from the second culture are transferred by loop to the entire surface of slants and to the cut surfaces of plugs. The inoculation is light. After 2 weeks of incubation at 25° C., and again at 6 weeks if necessary, cells from the center of the culture are stained to aid in the detection of ascospores. The films are lightly fixed by heat, then stained in hot (80° C.) 5-percent aqueous solution of malachite green for 3 to 5 minutes. Decolorization is accomplished by washing in running water for 30 seconds. Counterstaining is done with 0.5-percent aqueous safranin for 10 seconds. The spores are stained blue and the vegetative cells red. The author has found that mature Saturn-shaped spores which bear thick rings resist both stains, and it should be borne in mind, as shown by Beaurerie (*5*), that members of the genus *Schizosaccharomyces* produce spores which retain the counterstain.

Gray (*31*) and McClung (*68*) have proposed modifications of the malachite green spore stain for yeasts and other fungi. Gray recommends staining for 1 minute over steam with a solution containing 0.5 percent malachite green and 0.05 percent basic fuchsin. After being washed and dried, the spores appear greenish blue and the vegetative

[3] A commercial preparation known as V-8.

[4] The use of names of commercial products or equipment in this bulletin does not imply either endorsement or recommendation by the Department of Agriculture over others of a similar nature not used.

cells are violet or pink. McClung prescribes the use of 1-percent malachite green dissolved without heating in 1 percent phenol solution. The steaming period is 2 minutes. The counterstain is 0.5-percent aqueous safranin.

Another spore stain is one commonly used to demonstrate spores in bacteria. The film is fixed with heat, flooded with carbol fuchsin, and steamed gently for about 2 minutes. Decolorizing is done with 2-percent lactic acid, or with 95-percent alcohol containing 1 percent concentrated hydrochloric acid. The film is washed in water, then counterstained 2 or 3 minutes with 1-percent aqueous thionin or with methylene blue. The vegetative cells are blue, the spores red. As mentioned previously, the spores in species of *Schizosaccharomyces* are not acid-fast, and will not retain the carbol fuchsin stain.

Langeron and Guerra (*48*) describe a more elaborate method of using the preceding stain by combining it with Nile blue and India ink. In successful stains the ascospore walls are colored red, the asci are white, the vegetative cells are blue, and the background is black. Several modifications of ascospore stains have been described recently by Phaff and Mrak (*82*).

After a staining procedure has demonstrated the presence of ascospores, cells from the various media are examined in a water mount. If the spores are still within the asci, the shape, size, and number of spores are recorded, along with a description of the type or types of asci, that is, whether conjugation is isogamic or heterogamic, whether it occurs between independent or between mother and daughter cells, or whether conjugation is lacking. Spores that are quite small or that have appendages that are difficult to see may be studied without interference from Brownian movement by carefully placing them on a glass slide, the surface of which is covered by a very thin coat of agar.

It is usually desirable to determine whether ascosporogenous yeasts are haploid or diploid. The type, or types, of asci in sporulated cultures will usually indicate in what proportions the ascigerous vegetative cells are haploid or diploid, since all asci showing conjugation presumably arise from haploid cells and those without conjugation arise from diploid cells.

CELLULAR MORPHOLOGY

Morphological studies of the yeasts are of limited use in taxonomy because of the extreme variability of these micro-organisms when grown in laboratory culture. Much of this variability results from differences among strains which comprise the individual species. Also important, however, is variability resulting from differences in the character and composition of media employed by different investigators. Whereas variability arising from the first of these causes must be accepted and evaluated, that arising from the second can be substantially reduced, as can also the variability resulting from differences in time and temperature of incubation.

The availability of pure vitamins and amino acids has permitted the development of chemically defined media that support the growth of practically all yeasts. In earlier investigations the author employed stock solutions combined in various ways to produce media suitable for different biochemical tests and morphological studies.

The reader is referred to papers reporting these investigations (*105*, *106*) for detailed information regarding the preparation of the media. Dehydrated media now are commercially available in the form of powders.[5]

Composition of the media used by the author in conducting the taxonomic studies herein is shown in table 1.

TABLE 1.—*Composition of the chemically defined media employed for determining morphological and physiological characteristics of yeasts*

Ingredient	Morphology agar	Nitrogen base for carbon assimilation tests	Carbon base for nitrate assimilation test	Vitamin-free base
	Micrograms	*Micrograms*	*Micrograms*	*Micrograms*
Compounds supplying trace elements:				
H_3BO_3	500	500	500	500
$CuSO_4 \cdot 5H_2O$	40	40	40	40
KI	100	100	100	100
$FeCl_3 \cdot 6H_2O$	200	200	200	200
$MnSO_4 \cdot 1H_2O$	400	400	400	400
$Na_2MoO_4 \cdot 2H_2O$	200	200	200	200
$ZnSO_4 \cdot 7H_2O$	400	400	400	400
Vitamins:				
Biotin	2	2	2	None
Calcium pantothenate	400	400	400	None
Folic acid	2	2	2	None
Inositol	2000	2000	2000	None
Niacin	400	400	400	None
Para-aminobenzoic acid	200	200	200	None
Pyridoxine hydrochloride	400	400	400	None
Riboflavin	200	200	200	None
Thiamine hydrochloride	400	400	400	None
	Milligrams	*Milligrams*	*Milligrams*	*Milligrams*
Amino acids:				
L-histidine monohydrochloride $\cdot 1H_2O$	10	10	1.0	10
DL-methionine	20	20	2.0	20
DL-tryptophan	20	20	2.0	20
Salts:	*Grams*	*Grams*	*Grams*	*Grams*
KH_2PO_4	1.00	1.00	1.00	1.00
$MgSO_4 \cdot 7H_2O$	.50	.50	.50	.50
NaCl	.10	.10	.10	.10
$CaCl_2 \cdot 2H_2O$	.10	.10	.10	.10
Carbon source: Glucose	10.00	None	10.00	10.00
Nitrogen sources:				
$(NH_4)_2SO_4$	3.50	5.00	None	5.00
Asparagine	1.50	None	None	None
Agar	18.00	None	None	None
	Milliliters	*Milliliters*	*Milliliters*	*Milliliters*
Distilled water	1,000	1,000	1,000	1,000

[5] One of the companies making dehydrated media (Difco) has successfully prepared these media in powdered form and as sterile liquids in tenfold concentration. One medium is for morphological studies and others are for carbon and nitrate assimilation and vitamin deficiency tests. The media for morphological studies and vitamin deficiency tests require only the addition of water to make them complete, whereas different carbon sources and nitrate must be added to the carbon and nitrate assimilation media, respectively.

Two nitrogen sources are used as a result of Thorne's work (*101, 102, 103*) which revealed the generally beneficial effect on growth of binary mixtures of nitrogenous compounds.

The medium is prepared by combining the ingredients listed in column 2 of table 1 or by adding the appropriate amount of the powdered medium (Difco) to distilled water. The medium is autoclaved at 15 pounds pressure for 15 minutes, then cooled and poured into plates to a depth about 1.5 mm. for the preparation of spot and streak cultures.

Luz Marie Dalmau (*19*) perhaps should be credited with originating the use of coverglasses to permit the microscopic observation of fungus cultures growing in petri dishes, hence such cultures are designated here as "dalmau plates" in honor of that mycologist. Benham (*10*) cut a slit with the inoculating needle through a deep layer of cornmeal agar in a petri dish. After the culture had matured, a coverglass was placed over it and the yeast was observed. Slide cultures for observation and photomicrographic purposes may be prepared by the procedures of Wickerham and Rettger (*112*), Diddens and Lodder (*31*) or Rivalier and Seydel (*87*). For routine identification of yeasts, dalmau plate cultures are preferred to slide cultures because their preparation requires less time, and no special precautions need be taken regarding humidity.

The author's present technique of preparing dalmau plates is similar to Benham's procedure, but differs in one very important respect: The coverglass is applied immediately after inoculation. This procedure leads to the formation of hyphae by many yeasts that would not form them if the coverglass were not applied at the time the medium is inoculated. Freshly poured dishes are set aside at room temperature for 24 to 48 hours to allow the surface of the agar to dry. Then the yeast to be studied is streaked across the plate near one side. The amount of inoculum used is small, and enough pressure is exerted on the needle to scarify, but not to split, the agar. Two point inoculations, again using small amounts of inoculum, are made on the same plate. A coverglass, 22 mm. square, is removed by forceps from absolute alcohol, drained, flamed, and cooled for a few seconds and then placed across the streak. Another coverglass is centered over one of the point inoculations. The plates are incubated at 25° C. for 6 to 8 days. The general appearance of the resulting cultures is shown in plate 1.

A description of the aerobic colony (pl. 1, *B*) is recorded, and cells from its edge are suspended in water and observed for arrangement, shape, and size. The smallest (exclusive of buds) and the largest normal healthy blastospores found in one or two fields, depending upon the number present, are measured. The presence or absence of hyphae on the aerobic portion of the streak is noted (pl. 1, *C*). Turning then to the anaerobic growth, if long hyphae are present in the covered section of the streak (dalmau streak), their length is determined with a millimeter scale. This section is then examined with a 4-mm. dry objective and the presence of true hyphae, pseudohyphae, or intermediate types is recorded.

If blastospores (budding yeast cells which differ from the hyphal cells in size or shape) are present on the hyphae, their arrangement and shapes are recorded also. Next, the covered growth which originated from the point inoculation (dalmau spot, pl. 1, *A*) is observed

microscopically. Generally, the number of hyphae developing from the point inoculation is much greater, and the number of blastospores on the hyphae is much smaller than obtains with the dalmau streak. In strains which form no hyphae, the blastospores in the dalmau spot are commonly larger and of a different shape from those in the dalmau streak.

Most hyphae can be readily recognized as either true hyphae or pseudohyphae, but some strains show developments midway between. The basic difference between the extremes is that pseudohyphae grow by budding, with each successive cell smaller than that which gave rise to it; true hyphae, on the contrary, elongate by continuous growth of the hyphal tip, which is followed by the formation of septa. The formation of septa lags behind the growth of the hyphal tip to such an extent that the tip cell, as measured from the tip to the first septum, is longer than the preceding cell, which is measured as the distance between the first and second septa, as shown in plate 2.

In practice, the following rules may be used in determining whether hyphae are true, pseudo, or intermediate. The tips of the longest hyphae should be observed in this determination.

1. True hyphae have refractive, straight septa which generally can be differentiated from the edges of vacuoles by their greater thickness and refractivity. Pseudohyphae have no discernible septa, and the ends of the intercalary cells are curved and not refractive. Intermediate hyphae usually show only a small percentage of the cells separated by septa.

2. In true hyphae, the longest tip cells are considerably longer than the cells immediately preceding them. In pseudohyphae, the tip cell is shorter than, or approximately equal to, the adjacent cell. In intermediate hyphae, only a very few will be found in which the tip cell is decidedly longer than the adjacent cell.

3. In general, the longer the individual hyphal cells, the greater is the development of the hyphae.

4. True hyphae usually show little or no constriction at the septa, or at the points where the septa will later be formed. Pseudohyphae show marked constrictions at the junctions of the cells. Intermediate hyphae usually show marked constrictions.

5. In general, hyphae which bear blastospores are more highly developed than those which bear no blastospores.

By application of rules 1 and 2, hyphae can be recognized as true, intermediate, or pseudohyphae.

Physiology

Carbon Assimilation Tests

Carbon assimilation tests have been used since 1889 when Beijerinck (*8*) introduced the auxanographic plate technique, a procedure which the Dutch taxonomists (*21, 60, 95*) still apply to the classification of yeasts. Diddens and Lodder's (*21*) method consists of placing small amounts of dry sugars on the surface of a heavily inoculated synthetic medium. Growth develops in the areas where assimilable compounds are placed. Strains that show poor growth are retested in the same medium to which yeast extract has been added. The results are usually read after 1 or 2 days' incubation.

The principal limiting factors of the plate procedure are the inadequate basal medium and the short incubation period. The yeast extract which is added to supply vitamins in the event no growth occurred on the basal medium also supplies carbon for growth of some species, and thus complicates the reading of results. A 2-day incubation period is much too short to permit successful use of pentoses, polyhydric alcohols, and organic acids as test materials. Petri plates permit too much dehydration of the medium during the long period of incubation required for the use of those compounds.

Wickerham and Burton (*107*) have reemphasized the importance of carbon assimilation tests in yeast taxonomy. They have included a number of compounds not previously employed in major taxonomic work and have introduced standardized methods with resultant improvements in the significance and reproducibility of such tests. The medium used by them for carbon assimilation tests is similar to that used for morphological studies (see p. 5) except that it contains no glucose, asparagine, or agar. Five grams of ammonium sulfate per liter is used as a nitrogen source. A tenfold concentration stock medium is prepared, based upon the formula given in table 1, or by dissolving 3.4 gm. of the powdered carbon assimilation medium (Difco) and the appropriate carbon source in 50 ml. of distilled water. If the latter is an acid or acid salt, the pH is adjusted to 5.2 by the addition of sodium hydroxide pellets. The solution is sterilized by passage through a Seitz filter and is kept in the refrigerator until needed. Five-tenths-milliliter portions are pipetted into clear 16-mm. tubes containing 4.5 ml. of sterile distilled water. The tubes, after shaking, are ready for inoculation. The final concentration of hydrocarbon is equivalent in assimilable carbon to a 0.5-percent glucose solution; however, raffinose is used in twice the usual concentration.

Soluble starch and inulin, which might be lost to a considerable extent in filters, are solubilized in distilled water by heating over a flame. The solution, containing 0.5 percent of the carbohydrate, is pipetted in 4.5 ml. amounts into 16-mm. tubes. The tubes are then plugged and sterilized for 15 minutes in the autoclave. Each tube receives 0.5 ml. of sterile basal medium of tenfold concentration, is shaken, and then is ready to be inoculated.

An inoculation medium is prepared which contains the usual concentration of other ingredients but only 0.1 percent of glucose. Each tube contains 10 ml. of inoculation medium. One milliliter is removed by pipette and used to suspend the growth from a 24- or 48-hour slant of the yeast to be studied. Two- to four-tenths milliliter of the suspension, depending upon the abundance of the growth on the slants, is returned to the inoculation medium and the culture is incubated at 25° C. for 48 hours. The inoculum is then diluted with the base medium which contains no glucose. With the aid of a photometer the density is made equal to that imparted by approximately 10 million cells per ml. of *Saccharomyces cerevisiae*, NRRL Y–567,[6] or 15 million cells per ml. of *Torulopsis utilis*, NRRL

[6] The abbreviation "NRRL" followed by a number indicates the designation given a strain in the Culture Collection of the Northern Regional Research Laboratory.

Y–900. Those concentrations of cells contained in 18-mm. (outside diameter) test tubes transmit about 55 percent of the light from the blue filter (420 Å) in a Lumitron photometer. If a photometer or other means for determining density is not available, 5 ml. of inoculum in 16-mm. tubes should be diluted until black lines drawn on a white card become visible through the tubes as dark bands (equivalent to a 2+ reaction as given below). Generally, about two volumes of diluent are required per volume of culture. Each tube in the set of test media receives 0.1 ml. of the diluted inoculum.

The tubes containing the various carbon sources are then incubated at 25° C. and read at approximately 7 days and again at 20 to 24 days. The cultures have the general appearance shown in plate 3. They are observed for pellicles and the presence of visible amounts of riboflavin (practically the only soluble yellow compound produced by yeasts), then are shaken and placed against a white card bearing lines approximately ¾ mm. wide, drawn with India ink. If the growth in the tubes completely obliterates the lines, it is recorded as 3+; if the lines appear as a diffuse band, the growth is recorded as 2+; and if the lines are distinguishable as such but have indistinct edges it is recorded as 1+. The absence of growth is recorded as −. A 3+ or 2+ reaction at 24 days is considered as positive, and a 1+ reaction as weak.

The following compounds are used in the carbon assimilation tests: glucose, galactose, L-sorbose, maltose, sucrose, cellobiose, trehalose, lactose, melibiose, raffinose, melezitose, inulin, soluble starch, D-xylose, L-arabinose, D-arabinose, D-ribose, L-rhamnose, D-glucosamine hydrochloride, ethyl alcohol, glycerol, *i*-erythritol, adonitol, dulcitol, D-mannitol, D-sorbitol, alpha-methyl-D-glucoside, salicin, potassium D-gluconate, calcium 2-keto-D-gluconate, potassium 5-keto-D-gluconate, potassium sodium saccharate, pyruvic acid, DL-lactic acid, succinic acid, citric acid, ethyl acetoacetate, and *i*-inositol.

Only those reactions obtained with the most important and especially reliable carbon compounds will be included in the tables showing the salient characteristics of each genus, or group of related genera (Part 2). Carbon assimilation tests are more sensitive in detecting enzyme systems than are fermentation tests, and the number of compounds of value in the assimilation tests is much greater than the numbers of sugars that have value in fermentation reactions. It is the author's opinion that the assimilation tests will prove to be the most valuable of all the biochemical procedures used in the classification of yeasts.

NITRATE ASSIMILATION TEST

In 1892 Beijerinck (*9*) reported that *Saccharomyces acetaethylicus* (*Hansenula anomala?*) assimilated nitrate but not nitrite. But Takahashi (*100*) found that nitrite could be assimilated by this yeast if the medium did not contain an amount of acid sufficient to make the nitrite excessively toxic. Stelling-Dekker (*95*) separated the genera *Pichia* and *Hansenula* on the difference in their ability to assimilate nitrate.

Castelli (*17*) reported that some common species of *Saccharomyces* and *Zygosaccharomyces* assimilate nitrate, and Nickerson (*75*) made the same claim for *Zygosaccharomyces acidifaciens*. These observa-

tions are at variance with those of Stelling-Dekker (*95*) who reported all species of the two subgenera as incapable of assimilating nitrate. This difference of opinion may be explained by the fact that no completely adequate procedure had been evolved for the assimilation test. A principal source of error is that young cells used as inoculum usually contain a high percentage of protein. When inoculated into a medium containing only nitrate as a source of nitrogen, many yeasts which are incapable of utilizing nitrate are capable of excreting much of their nitrogen (*103*). The excreted nitrogen is then used to form many more cells having a much lower protein content (*80*), and the final amount of growth may give a strongly, but falsely, positive test. Nickerson (*76*) and Skinner (*91*), while admitting the difficulties involved in the test for nitrite, regard the chemical test as superior to the assimilation test.

We have found that Stelling-Dekker's results with the auxonographic procedure correlates very well with the chemical test for nitrite and with our results from the assimilation test. She did, however, erroneously report some species, such as *Endomycopsis bisporus* Beck and *Endomycopsis vernalis*, as negative, probably because they failed to grow in the short incubation period used. Both of these species grow well, though latently, with nitrate as the sole source of nitrogen.

The ability of a yeast to assimilate nitrate is more easily determined than is its ability to produce nitrite, mainly because the color test for nitrite is complicated by the action of many yeasts in assimilating nitrite about as fast as it is liberated from nitrate. On the contrary, if the assimilation test is properly conducted, growth occurs only if the yeast is capable of assimilating nitrate, and once the test becomes positive it remains positive.

The test medium used for the present studies on nitrate assimilation is similar to that for tests on carbon assimilation, except that it contains when complete with water, 10 gm. of glucose and 0.78 gm. of potassium nitrate, per liter, and smaller amounts of each of the three amino acids (see table 1). It contains no asparagine or other source of nitrogen in amounts large enough to interfere seriously with the test. A tube of nitrate medium receives 0.1 ml. of the same inoculum used for the carbon assimilation tests. After the nitrate culture has been incubated about 7 days, even though nitrate has not been assimilated, it usually shows growth, probably due both to the soluble nitrogen compounds excreted by the cells of the inoculum and to the small amount of ammonium sulfate in the inoculation medium. Hence, a second nitrate tube is inoculated with one loop from the first. After a similar period of incubation the second tube is observed. If it shows a 2+ or 3+ reaction, the yeast is capable of assimilating nitrate; if there is no growth or a 1+ reaction, the yeast is considered incapable of assimilating nitrate.

Doubtful nitrate reactions are seldom encountered, but when met, they may be confirmed by the chemical test for nitrite. Four tubes of nitrate assimilation medium are inoculated by needle from a slant and tested at approximately 3, 5, 10, and 15 days. If the yeast under test assimilates nitrate, one or more of the tubes usually give a positive reaction.

The nitrite test employed is one of those recommended in the Manual of Methods for Pure Culture Study of Bacteria (*93*). The

reagents are (*a*) 0.8 gm. sulphanilic acid in 100 ml. of 5 *N* acetic acid, and (*b*) 0.6 gm. of dimethyl alpha-naphthylamine in 100 ml. of 5 *N* acetic acid. Five-tenths-milliliter quantities of each are added to each culture to be tested. A pink to red color indicates the presence of nitrite. If no color develops within approximately 10 minutes, a minute amount of zinc dust is added to reduce any nitrate which may be present. If no color follows the addition of zinc, the yeast under test has completely assimilated both the nitrate and the nitrite.

VITAMIN DEFICIENCY TEST

A number of investigators have studied vitamin deficiency patterns in yeasts. Perhaps Burkholder *et al.* (*14, 15*) have studied the greatest number of species. Lockhead (*50*) and Lockhead and Landerkin (*58*) have applied vitamin deficiency data to the classification of osmophilic species of *Zygosaccharomyces*. Leonian and Lilly (*51, 52, 53*) have reported that strains of *Saccharomyces cerevisiae* vary considerably from one another in the number of essential vitamins which they are incapable of synthesizing. By using massive inoculations and long periods of incubation, and successively transferring cells from a medium lacking only one required vitamin to a second medium lacking another required vitamin, etc., these investigators found that a strain originally deficient for five vitamins gradually developed the ability to synthesize all of them, and thereafter would grow rapidly in a medium devoid of all five.

Schultz and Atkin (*89*) separated strains of *Saccharomyces cerevisiae* into 13 distinct types on the basis of their vitamin requirements. These investigators believe that the determination of vitamin deficiency patterns should be included among the techniques used in the classification of yeasts. Over a 10-year period, they found the deficiency patterns to be relatively constant, though variations in growth rate occurred as a result of the condition of the yeast inoculum.

The writer believes that in many cases the inability of a given species to grow in the absence of a specific vitamin or combination of vitamins will be found to hold for all strains of that species. Until such facts are clearly demonstrated, however, he intends to limit his taxonomic use of deficiencies to a determination of whether a yeast can grow in the absence of all added vitamins.

The vitamin deficiency medium is similar to that used in the carbon assimilation tests except that 10 grams of glucose per liter is included, and the vitamins are excluded. The test is run in exactly the same manner as the nitrate assimilation tests. The first tube usually shows growth at 7 days, owing to the carry-over of vitamins in the inoculum. The yeasts which produce a 2+ or 3+ reaction in the second tube are capable of synthesizing all the vitamins they require for growth; those which produce no growth or a 1+ reaction are considered to be incapable of synthesizing all of the vitamins required for growth. In the author's experience, this test is proving of considerable taxonomic importance.

FERMENTATION TESTS

Guerra (*32*), Martin *et al.* (*67*), and Langeron and Guerra (*48*) used petroleum jelly or paraffin seals over the surface of media in fermentation tubes to reduce the amount of air entering the cultures,

thus increasing the intensity of the fermentation. Dr. Morrison Rogosa [7] suggested the use of rubber stoppers placed in the lips of fermentation tubes as a similar but simpler procedure.

Bouthilet, Neilson, Mrak, and Phaff (*13*) have stated that yeast extract made from brewers' yeast, or yeast autolyzate made from bakers' yeast are free from the fermentable sugar trehalose. Yeast extract prepared from bakers' yeast may contain sufficient trehalose, however, to interfere with the accuracy of fermentation tests in which it is used as the basal medium.

Fermentation tests had been run on hundreds of yeast strains in the Northern Regional Research Laboratory before a chemically defined medium was developed, and the original procedure is being maintained. The medium still in use contains 4.5 gm. of powdered yeast extract (a batch should be used which will support growth of the markedly vitamin-deficient apiculate yeasts, such as *Kloeckera brevis*), 7.5 gm. of peptone, 1,000 ml. of distilled water and enough bromthymol blue to give a sufficiently dense green color. Two-ml. quantities are placed in 150×12 mm. tubes carrying inserts measuring 50×6 mm. After sterilization, 1-ml. quantities of the various sugar solutions are added to the tubes. Aqueous solutions of glucose, galactose, maltose, sucrose, and lactose are prepared in 6 percent concentration, and solutions of raffinose are in 12 percent concentration. The solutions are sterilized by Seitz filtration and kept in the refrigerator. No seals or rubber stoppers are used.

The fermentation media are inoculated with 0.1 ml. of a suspension of cells made by suspending the growth of a 24- or 48-hour malt extract-yeast extract agar slant in 4.5 ml. of sterile distilled water. The cultures are incubated at 28° C. The tubes are shaken and observed for the amount of gas in the inserts or color of the indicator after 1, 2, 4, 6, 8, 12, 16, 20, and 24 days of incubation.

The raffinose fermentation is given special consideration. If no gas is formed and the indicator shows a neutral or alkaline reaction, the yeast under test does not attack raffinose. If gas is produced, the yeast may be either splitting fructose off the raffinose molecule and leaving the remaining disaccharide, melibiose, intact, or fermenting both the fructose and melibiose. To determine whether the raffinose has been fermented completely or for only one-third part, the raffinose tube is inoculated after 20 days of incubation with a second yeast, *Saccharomyces carlsbergensis*, NRRL Y–379, which ferments melibiose strongly. If evolution of gas follows the addition of *S. carlsbergensis*, the original yeast did not ferment melibiose; if no gas is produced, melibiose was fermented by the yeast under test. This procedure was described by Wickerham (*105*) in 1943.

Skinner and Bouthilet (*92*) are of the opinion that their procedure is preferable to the one just described. They ferment a raffinose medium with *Saccharomyces cerevisiae*. This yeast ferments only one-third of the sugar, leaving intact the resulting melibiose. This medium is then used for testing the ability of yeasts to ferment melibiose. The technique used by Skinner and Bouthilet requires two fermentation tubes, one raffinose and one melibiose, if the user is to obtain the highly desirable information as to whether raffinose is *not attacked at all*, fermented for one-third part, or fermented com-

[7] Verbal communication, 1948.

pletely. The author's technique requires only the raffinose tube in order to obtain all of this information.

Yeasts showing no gaseous fermentation of glucose at 8 or 12 days are probably nonfermentative. Information regarding the ability of such yeasts to hydrolyze disaccharides or polysaccharides may be obtained by inoculating the 8- or 12-day fermentation cultures with a yeast such as *Saccharomyces globosus* NRRL Y–109, which ferments glucose and and galactose strongly but has no action on maltose, sucrose, lactose, or raffinose. The yeast under test is capable of hydrolyzing those di- and polysaccharides from which it is capable of producing gas when acting in association with *Saccharomyces globosus*. The success of this test depends upon the fact that nonfermentative species commonly hydrolyze disaccharides faster than they assimilate the resulting monosaccharides.

GROWTH AT MODERATE OSMOTIC PRESSURE

Some yeasts cannot grow in media having high osmotic pressure, others tolerate them, and some prefer them. The ability of a yeast to grow in a medium of moderately high osmotic pressure may indicate the liklihood of finding such a yeast in osmotically active substances in nature. The test is of moderate taxonomic value.

The medium used in the test is prepared by dissolving 10 gm. of sodium chloride and 5 gm. of glucose in 100 ml. of distilled water. The solution is pipetted into 16-mm. tubes in 4.5 ml. quantities. The tubes are stoppered with cotton and autoclaved for 20 minutes. Each tube receives 0.5 ml. of glucose-free base medium of tenfold concentration and 0.1 ml. of the same inoculum as used in all the assimilation tests. A 1+ amount of growth after 20 to 24 days of incubation is recorded as weak, a 2+ amount of growth is recorded as moderate, and 3+ as good. A negative or 1+ reaction at 7 days, followed by 2+ or 3+ reaction at 20 to 24 days, is recorded as latent.

ABILITY TO LIQUEFY GELATIN

The ability of yeasts to liquefy gelatin is of definitely limited taxonomic value because very few yeasts attack proteins strongly.

Ten grams of gelatin are dissolved in 90 ml. of hot distilled water. The solution is pipetted in 1.5-ml. quantities into 16-mm. test tubes stoppered with cotton, and autoclaved for 15 minutes. After cooling to somewhat above room temperature, 0.5-ml. quantities of tenfold glucose concentrate, as used in the assimilation tests, are added. The final medium contains approximately 10 percent gelatin, 0.5 percent glucose, and 0.5 percent ammonium sulphate in addition to the usual vitamins, trace elements, and salts. Each tube receives the usual inoculum as employed in the assimilation tests. The inoculum is spread over the solid surface of the medium, then incubated at 25° C., at which temperature it remains solid unless acted upon by the yeasts. At approximately 7 and 24 days the depth of the liquid layer, if any, is measured in millimeters.

ABILITY TO GROW AT 37° C.

It is of interest taxonomically to determine if a yeast will grow at 37° C., because possible inferences regarding ability to grow in

association with warm-blooded animals may be obtained. A tube of the usual glucose assimilation medium, inoculated in the usual way, is incubated at 37° C. It is read in the usual manner at approximately 7 and 24 days. The results are recorded in the same manner as in the test for the ability to grow in the osmotic pressure medium.

PRODUCTION OF STARCH

Aschner, Mager, and Leibowitz (*1*) reported that under certain conditions, species of yeasts, mainly in the genus *Torulopsis*, synthesize starch which gives a typical blue color reaction with iodine. In 1946 Mager and Aschner (*65*) described a biochemical test based on this reaction. Plates containing a synthetic medium were inoculated and incubated for several days at 37° C., then flooded with Lugol's iodine solution. If the yeast under test grew at 37° C. and gave a positive starch test, the yeast was considered to be *Torulopsis neoformans*, a species capable of causing fatal infections of the central nervous system. A general discussion of starch synthesis from the biological aspect was published by Mager and Aschner (*66*), and another paper by Mager (*67*) on the chemistry of this starch also appeared in 1947.

The 24-day carbon assimilation test cultures which contained sugars or polyhydric alcohols are examined for the presence of starch. One drop of approximately 0.02 *N* iodine solution is added per tube and the tube shaken. Those strains producing starch will show reactions varying in intensity from blue to purple to green; the color may reside in the medium, in the cells, or in both.

In some genera the relatively large amounts of glycogen synthesized may interfere with the test, especially if stronger iodine solutions are used. The brown color typical of glycogen may mask a weak starch reaction. Where doubt arises, the tubes may be allowed to stand at room temperature for a few hours or overnight. The brown color indicating glycogen disappears but the blue color indicating starch remains.

The ability to produce starch has interesting applications in the taxonomy of yeasts.

PRODUCTION OF ESTERS

The yeast is streaked on malt extract-yeast extract agar in a petri dish and the culture is tested organoleptically after 24 or 48 hours of incubation. Except with a few genera, this test is of relatively little taxonomic value.

VARIATION

Morphological characters, and to a less extent physiological characters, may vary considerably from strain to strain within a given species. The principal types of variation which may be encountered in studies relating to the classification of yeasts will be considered briefly.

Variation is found in practically all species where several to numerous strains are available for study. Morphologically, variation consists of minor differences in color and texture of colonies, and in the size, shape, and arrangement of cells.[8] Physiologically, it is revealed

[8] See Wickerham and Rettger (*112*) on morphological variations in *Candida albicans*.

principally by variations in the ability to assimilate or ferment carbohydrates. Such normal variation is found in both freshly isolated and old stock cultures, and it has, in the past, led to the naming of many unwarranted species. An example is Langeron and Talice's (*50*) classification of the nonascosporogenous, filamentous yeasts—six genera being recognized on the basis of morphology. Later, Langeron and Guerra (*48*) combined five genera into the single genus *Candida*. It is now generally known that a single strain, or even a single culture, depending upon its age and conditions of cultivation, may reveal the main characteristics of two or three of Langeron and Talice's genera.

The author does not favor the current trend toward naming varieties merely on differences in size and shape of cells, because wide ranges of morphological variations are found in species which are frequently isolated.[9] Bedford (*7, p. 64-5*) has reported that some strains of *Hansenula anomala*, when grown on synthetic medium and on wort, have cells so different in shape and size that they would be classified in different varieties, depending on the medium used. The trend toward naming numerous varieties on morphological variations, if continued, will soon clutter the taxonomy of yeasts. The author believes it is better and simpler to set the limits of the species wide enough to include minor variations, varieties being created only for those strains which are almost different enough to be set aside as separate species. This concept explains the wide range of cell sizes and morphological appearances which will be given in the descriptions (Part 2) of the more commonly isolated species.

Physiological characters also vary from strain to strain of either freshly isolated or old stock cultures. It is necessary to determine, therefore, the physiological reactions of a large number of strains of each species, in order to ascertain which reactions are common to all strains and which are variable. As far as possible, variable reactions are not used in the separation of species. The number of strains studied by the author will be given in the description of each species, so that the reader may judge the author's knowledge of the range of variation of each species.

Strains of ascosporogenous yeasts generally, but not always, sporulate readily when freshly isolated from nature, though this character may be diminished or lost after a period of laboratory cultivation. Occasionally, nonascosporogenic variants show a slightly different shade or even a markedly different color than colonies of the same strain containing sporogenic cells. Strains of the genus *Nadsonia* represent an example of the species which may show a marked difference in color. When cultured on malt extract-yeast extract agar at 10° to 15° C., some strains may produce not only white, nonsporogenous colonies but also dark-brown, sporogenous colonies.

If an occasional isolate is identical to a given sporogenous species except that it does not produce spores, it is considered to be merely a nonsporogenous variant of that species. However, a few species may be isolated from nature as the ascosporogenous or as the nonascosporogenous form with about equal frequency. For the benefit of the user

[9] See Stelling-Dekker (*95*) on *Saccharomyces cerevisiae*, and Bedford (*7*) on *Hansenula anomala*.

of the forthcoming classifications, such species will be described in both the sporogenous and nonsporogenous genera under the name applied to each form, and a note will be included to this effect in both of the appropriate genera.

Another form of variation is characterized by the ability of strains to produce two types of colonies, one type having a mat surface and the other a glistening surface. Freshly isolated strains as well as old stock cultures may show this form of variation. It is found much more commonly in some genera, such as *Hansenula*, *Debaryomyces*, *Pichia*, *Mycoderma*, and *Candida*, than in others, such as *Saccharomyces* and *Schizosaccharomyces*, where the mat type is perhaps nonexistent. Genera in which these two forms of variations are found include mainly oxidative species; genera in which only glistening colonies are formed are composed predominantly or exclusively of fermentative species. Fabian and Wickerham (*39*) have shown that the mat forms produce less alcohol than the corresponding glistening forms of the same strain and also that they produce heavier pellicles on liquid media than the glistening forms. The author believes that the mat form is adapted to an aerobic type of metabolism, whereas the glistening form is better suited to an anaerobic existence.

The difference in culture appearance between the two types is generally due to a difference in the nature of the cells on the surface of the colonies. The mat colonies are commonly covered with somewhat water-resistant cells, often in long chains, which may be floated free from the remainder of the colony by gently pouring water into the petri dish. These detached cells may conceivably be the most primitive type of aerial blastoconidia produced by yeasts. They probably correspond to the blastoconidia formed by species of *Endomycopsis*, but differ by lacking aerial conidiophores. Usually the mat covering extends completely across the colonies, but sometimes covers only the center, leaving a broad glistening band at the edge. Highly glistening colonies are devoid of blastoconidia.

In some strains all degrees of transition exist between the extremes of white, powdery mat, and highly glistening surfaces. The medium greatly influences the formation of the "primitive" blastoconidia. For example, in the species *Hansenula anomala*, nearly all strains which produce glistening colonies on malt extract-yeast extract agar form mat colonies on the synthetic morphology medium. A morphological study of the mat-glistening type of variation in *H. anomala* (under the synonym of *Saccharomyces acris-sacchari*) has been reported by Wickerham and Fabian (*108*).

Another form of variation, which probably is not related to the type just discussed, occurs in cultures that have been maintained on laboratory media for a long time. Strains which produce smooth, glistening colonies when first isolated may in time give rise to colonies that are lumpy, convoluted, crateriform, or membranous. They are usually mat but often more or less moist in appearance. If the species is filamentous, tiny spicules or coremia may be produced on the surface of the colony. Morphologically, filamentous yeasts undergoing this change produce more hyphae and fewer blastospores than when the cultures were first isolated; usually the blastospores become elongated and their arrangement on the hyphae may be altered. Variation of this type has been described by Mackinnon (*62*) and by Mickle and

Jones (*69*) for *Candida albicans*. Little, if any, qualitative change in physiology accompanies this change in morphology. The change is usually reversible if the yeast has not existed too many years in its altered form. This form of variation may occur in oxidative or fermentative, filamentous, or nonfilamentous genera. Ascosporogenous yeasts which have undergone the change generally produce few or no ascospores.

Even before a change occurs in colony appearance, a marked reduction may be noted in the number of blastospores borne on the hyphae. Consequently, morphological characteristics of old stock strains of *Candida* and *Endomycopsis* may vary considerably from original descriptions which were based on freshly isolated cultures (*111, p. 416*). The tendency for arthrosporic fungi related to the yeasts to produce more mycelium and fewer arthrospores after continued cultivation on laboratory media is stressed by Ciferri, Verona, and Saggese (*18*).

Finally, a form of variation that should be mentioned concerns the change which some mucoid species show on continued cultivation in the laboratory. Such species may give rise to smooth, glistening, butyrous colonies or even to mat, crateriform, or convoluted colonies.

Many other forms of variation occur in yeasts, but those described here should serve to alert the person interested in yeast taxonomy to the principal morphological forms that he may expect to encounter in different strains or in a single strain of a given species. In addition to the literature already cited, information regarding variation in yeasts may be obtained from publications of Punkari and Henrici (*84, 85*), Fabian and McCullough (*27*), Langeron and Guerra (*49*) and Mackinnon (*63*).

Part 2

A CLASSIFICATION OF THE GENUS HANSENULA

History

The genealogy of several of the currently recognized genera of yeasts may be traced back to the genus *Saccharomyces* (Meyen) Reess of 1870. As defined by Reess (*86*) at that early date, the boundaries of this genus were extensive—any species that reproduced by budding and formed endospores could qualify, provided it did not produce a true mycelium. The analogous forms which produced hyphae were placed in *Endomyces* Reess.

Reess' definition appeared to be adequate up to the time when Hansen began his extensive study of yeasts, the results of which indicated the trends that taxonomy was to follow. Previous to the time of Hansen's (*38*) major cleavage of the genus *Saccharomyces* in 1904, it contained such currently recognized unrelated species as *Hansenula anomala* Hansen, *Pichia membranaefaciens* Hansen, *Kloeckera apiculata* Reess, and *Saccharomycodes ludwigii* Hansen.

The family Saccharomycetaceae, as described by Hansen at that time, corresponded roughly in its limits with Reess' *Saccharomyces*. It consisted of species which reproduced asexually by budding and formed endospores. Only a few of the species formed mycelium profusely.

Those species which rapidly produced prominent, dry, mat pellicles on liquid media were placed in two new genera which Hansen designated *Willia* and *Pichia*. The former was characterized by hat-shaped or Saturn-shaped ascospores, the latter by hemispheroidal or angular spores. Most of the species of *Willia* produced gaseous fermentations and esters, but none of *Pichia* produced fermentation. The latter was capable, however, of forming mycelia. Hansen selected his previously described *Saccharomyces membranae faciens* as the type species of the genus *Pichia* and his *Saccharomyces anomalus* as the type species of *Willia*. A seemingly intermediate form was *Saccharomyces anomalus* var. *belgicus* Lindner (*55*). The ascospores had the typical hat-shape, but glucose was only slightly fermented, and esters were not produced. Hansen placed this species in *Willia* also. He included Steuber's (*96*) isolate, known as *Saccharomyces anomala* var. III, a strain very similar to Lindner's var. *belgicus*, the principal difference being its inability to produce any gas from glucose.

Zikes (*115*) isolated from soil a yeast which formed hat-shaped spores, produced esters, fermented no sugars, formed pellicles slowly, and produced mucoid or submucoid colonies. He named it *Willia wichmannii*.

Klöcker (*71*) reviewed previous classifications of yeasts in 1906 in an attempt to provide an inclusive and up-to-date classification of the sporogenous yeasts. His definitions of *Willia* and *Pichia* were the same as Hansen's, but he gave descriptions of species which Hansen had listed but not described. In 1912, without offering a revised definition of the genera, Klöcker (*73*) placed in *Pichia* some new pellicle-forming species which did not conform to Hansen's definition. One was *Pichia polymorpha* which Klöcker described as having spherical ascospores and as fermenting glucose and sucrose; whether it formed esters was not stated. Another species was *Pichia suaveolens* Klöcker, which was reported as having predominantly spherical spores though some were observed to be somewhat flattened on one side. It fermented glucose and sucrose strongly and produced esters.

The Sydows (*99*) in 1919 gave only one sentence to the fact that a genus *Willia* C. Müll. (1889) in the Pottiaceae had priority over Hansen's genus. These investigators designated the latter as *Hansenula* Syd., a usage which has become generally recognized.

Guilliermond (*34*) in 1912 and Guilliermond and Tanner (*35*) in 1920 retained almost intact the names and descriptions given by Hansen in 1904 to *Willia*, but *Pichia* was expanded to conform with Klöcker's conception which included species having spheroidal ascospores and producing strong fermentation.

In 1925 Zender (*114*) produced a classification of the ascosporogenous yeasts which, had it been accepted, would have altered profoundly the taxonomy of yeasts. He desired a sharper separation of the genera comprising the family Saccharomycetaceae and the genus *Endomyces* of the Endomycetaceae. Those families, at the time Zender began his studies, were distinguished by the ability of the Endomycetaceae to form true mycelia and to form asci exclusively through the action of hypal cells. Zender cited, as examples of the poor separation of the two families, the species *Saccharomyces mara-*

inus Hansen which on gelatin forms typical septate, branched mycelium, and in liquid medium forms pseudohyphae consisting of chains of budding cells. On the other hand, he cited *Endomyces javanensis* Klöcker, which lacks a typical mycelium and produces ascospores in blastospores (budding yeast cells). Zender also wished to eliminate from family and generic descriptions fermentation reactions, pellicle formation, and such variable characteristics as cell size and shape, and colony appearances.

Zender proposed that ascospore shape be made the main characteristic by which families and genera should be determined. Accordingly, *Endomyces* was reserved for those species possessing hat-shaped ascospores, and to this genus he added a heterogeneous assortment of species, such as *Hansenia spora valbyensis* Klöcker, *Zygosaccharomyces pastori* Guilliermond, *Hansenula anomala* Hansen, and *Endomyces fibuliger* Lindner, all of which have widely different physiological characteristics. *Endomyces capsularis* Schionning and *Hansenula saturnus* Klöcker were moved to the new genus *Williopsis* created for those species possessing Saturn-shaped spores, the walls of which were smooth and the rings equatorial. *Pichia* was elevated to the rank of a family.

The complete disregard of physiological properties in the descriptions of genera made impossible the acceptance of Zender's classification by his successors in the field of yeast taxonomy. Had it been accepted and expanded, however, it might have developed into a more workable classification, despite its arbitrariness, than the currently accepted natural system. Its principal disadvantage would have been the lack of a foundation for an analogous scheme of the nonascosporogenous yeasts.

Shrewsbury (*190*) in 1930 presented a detailed morphological study of the genus *Willia* along with some results on pathogenicity tests. He concluded that the species which he had studied were not pathogenic for laboratory animals.

Stelling-Dekker (*197*) presented a carefully executed classification of the sporogenous yeasts in 1931. Her definition of *Hansenula* was similar to that given by Hansen to *Willia* except that it was limited to species which assimilated nitrate, fermented sugars strongly, and hydrolyzed esculin. Spore shapes assigned to the genus were hat-shaped, Saturn-shaped or flattened spheres. *Pichia* was limited to species which could not assimilate nitrate, ferment sugars strongly, or hydrolyze esculin strongly. Hat-shaped spores were added to the usual hemispheroidal and angular shapes. Such modifications of Hansen's descriptions gave a sharper separation of *Hansenula* and *Pichia*. Some changes were made in the membership of the two genera; notably, *Pichia suaveolens* Klöcker was transferred to *Hansenula*, and *Willia belgica* (Lindner) Hansen was transfered to *Pichia*. The genus *Pichia*, according to Klöcker's (*93*) suggestion, was divided into two subgenera. One, *Pichia*, was limited to species which always formed ascospores "parthenogenetically"; the other, *Zygopichia*, was limited to species in which some of the spores formed following conjugation of the vegetative cells. Stelling-Dekker created four varieties of *Hansenula anomala* based primarily on minor morphological differences.

Next, Lodder (*59*) divided the genus *Hansenula* into two subgenera. The name *Hansenula* was applied to the more common subgenus which included the species recognized by Stelling-Dekker, and a separate subgenus *Zygohansenula* was created to include the new species *Z. californica* which Lodder believed should be differentiated from the other species because some of the vegetative cells conjugated just before ascospores were produced. Lodder also described a new species, *Hansenula cifferii*.

Bedford (*7*) decided that Stelling-Dekker's *Hansenula javanica* (Groenewege) Dekker, and *H. anomala* vars. *robusta* Dekker and *productiva* (Beijerinck) Dekker were not sufficiently distinct from *H. anomala* and *H. anomala* vars. *sphaerica* (Naegeli) Dekker and *longa* Dekker to continue their separation as species or varieties. *H. anomala* var. *sphaerica* and *H. anomala* var. *longa* were retained but redefined on the basis of cell size in liquid synthetic medium. Bedford added the new variety, *H. anomala* var. *heteromorpha*. He reported that *H. anomala* and its varieties could be separated only on the basis of their cell size. This was not entirely satisfactory, as his cultures showed considerable variability in size in the same medium and in different media. He added the new species *H. subpelliculosa*.

Mrak *et al.* (*74*) stated that the distinctions between *Hansenula* and *Pichia* were rapidly fading. They pointed out that it was no longer possible to separate those genera on spore shape. Lodder's (*59*) *Pichia fermentans* removed the fermentation difference, and Bedford's (*7*) *Pichia kluyveri* produced esters and split esculin. This left the utilization of nitrate as the only point of difference because, morphologically, the two genera are very similar. Bedford (*7*) and Mrak *et al.* (*74*) found that, in contrast to all other *Hansenula* species, *H. subpelliculosa* did not attack nitrate in a completely synthetic medium. This was because *H. subpelliculosa* required vitamins and Bedford's synthetic medium lacked them. Had the essential vitamins been present, nitrate would have been attacked.

Mrak *et al.* (*74*) believed it unsound to differentiate two genera on a single characteristic, such as the ability to assimilate nitrate. They believed it might be desirable to form one genus, containing subgenera of the nature of *Pichia*, *Zygopichia*, *Hansenula*, and *Zygohansenula*. Nickerson (*77*), on the other hand, believed that the difference in action on nitrate indicates a fundamental difference between *Hansenula* and *Pichia*.

In summary, it may be stated that ever since the creation of *Hansenula* and *Pichia* nearly a half century ago, taxonomists have been attempting to more clearly define and to separate them. Yet the number of distinguishing characteristics has been reduced rather than multiplied. One by one new species have been isolated which possess at least one characteristic that formerly was believed to be characteristic for *Hansenula*, and at least one other that was believed to be characteristic of *Pichia*. Reduction in the number of distinguishing characteristics has now reached the point where Mrak *et al.* (*74*) advocate merger of the two genera. Further considerations regarding the relationship of *Pichia* and *Hansenula* are presented on page 142.

Description of the Genus

Hansenula Sydow, Ann. Mycol. 17:44. 1919. Type species,

H. anomala (Hansen) Sydow. *Ibid.*

Synonyms: *Willia* Hansen, Zentbl. f. Bakt. Parasitenk. Abt. II, 12: 538. 1904.

Hansenula (Hansen) Sydow emend. Stelling-Dekker, Die sporogenen Hefen. p. 436. 1931.

Zygohansenula Lodder, Zentbl. f. Bakt. Parasitenk. Abt. II. 86: 246. 1932.

Named for Emil Christian Hansen, the father of yeast taxonomy.

Yeasts which reproduce ascogenously exclusively by budding, form 1 to 4 ascospores per ascus, and assimilate nitrate. Heterogamic or isogamic conjugation of cells may occur just previous to sporulation; or conjugation may occur during the time ascospores are germinating, thus giving rise to diploid zygotes which remain in the vegetative stage until conditions favor sporulation, whereupon they are converted directly into asci without further conjugation. Ascospores are hat-shaped, hemispheroidal, Saturn-shaped or spheroidal, one to four per ascus. True hyphae, pseudohyphae, or no hyphae may be formed. Pellicles may or may not be formed, and sugars may or may not be fermented.

Species of *Hansenula* are commonly found on or in trees, in soil, as contaminants in industrial fermentations, and on fruit.

Keys to the Species

Some of the salient characteristics by which the species of the genus are recognized are presented in table 2. The order of appearance in the table is believed to correlate closely with phylogenetic development within the genus. For this reason, the descriptions of the species will follow in the same order as the names in the table. Supplementing the table is the usual dichotomous key for the convenience of the user. The key is based on the ability or inability of species to assimilate five selected carbon sources, grow in the absence of added vitamins, and to produce true or pseudohyphae.

TABLE 2.—*Salient diagnostic characters of the species of Hansenula*[1]

Species	Spore shape	Assimilation									Fermentation					Growth without added vitamins	Nuclei	Highest development of hyphae
		Nitrate	Galactose	Maltose	Sucrose	Trehalose	Raffinose	Rhamnose	Erythritol	α-m'glucoside	Glucose	Galactose	Maltose	Sucrose	Raffinose ⅔			
H. capsulata	Hat	+	−	+	−	+	−	+	+	+	+	−	−	−	−	−	Haploid	None.
H. beckii	do	+	−	+	+	+	−	+	−	+	−	−	−	−	−	−	Diploid	True.
H. canadensis	do	+	−	+	+	+	−	+	−	+	−	−	−	−	−	−	do	Pseudo.
H. minuta	do	+	−	−	−	+	−	−	−	−	+	−	−	−	−	−	Haploid	None.
H. silvicola	do	+	+	+	+	+	−	+	−	+	+	+	−	−	−	−	Mainly haploid.	Intermediate.
H. angusta	do	+	−	+	+	+	−	−	+	+	+	−	−	−	−	−	do	None.
H. subpelliculosa	do	+	V	+	+	+	+	−	+	+	+	−	V	+	+	−	Diploid	True.
H. jadinii	do	+	−	+	+	+	+	−	−	+	+	−	+	+	+	+	do	Pseudo.
H. schneggii	do	+	+	+	+	+	−	−	+	+	+	−	+	+	−	+	do. (?)	Do.
H. anomala	do	+	V	+	+	+	+	−	+	+	+	V	V	+	+	+	do	Do.
H. ciferrii	do	+	+	+	+	+	+	+	+	+	+	+	V	+	+	+	do	True.
H. californica	Saturn	+	−	V	+	V	−	+	−	+	+	−	−	−	−	−	Mainly haploid.	None.
H. suaveolens	do	+	−	−	+	−	+	−	−	−	+	−	−	+	+	+	Mainly diploid.	Pseudo.
H. mrakii	do	+	−	−	−	−	−	+	−	−	+	−	−	−	−	+	do	Do.
H. saturnus	do	+	−	−	+	−	+	+	−	−	+	−	−	+	+	+	do	Do.

[1] + Means growth where it refers to assimilation, gas where it refers to fermentation; V means variable; — means no growth where reference is to assimilation, no gas where reference is to fermentation.

A DICHOTOMOUS KEY TO THE SPECIES

[Based primarily on shape of *ascospores*, ability to assimilate carbohydrates, and ability to grow in a chemically defined medium to which no vitamins have been added.]

I. Ascospores hat-shaped or hemispheroidal;
  A. Raffinose, rhamnose and alpha-methylglucoside not assimilated.
    Hansenula minuta nov. sp.
  B. Alpha-methylglucoside assimilated; raffinose and rhamnose not assimilated.
    1. Galactose assimilated..... *Hansenula schneggii* (Weber) Dekker.
    2. Galactose not assimilated..... *Hansenula angusta* nov. sp.
  C. Raffinose and alpha-methylglucoside assimilated; rhamnose not assimilated.
    1. External source of vitamins not required.
      a. Erythritol assimilated..... *Hansenula anomala* (Hansen) Sydow.
      b. Erythritol not assimilated..... *Hansenula jadinii* nov. comb.
    2. External source of vitamins required.. *Hansenula subpelliculosa* Bedford.
  D. Rhamnose and alpha-methylglucoside assimilated; raffinose not assimilated.[1]
    1. Galactose assimilated..... *Hansenula silvicola* nov. sp.
    2. Galactose not assimilated.
      a. Erythritol assimilated..... *Hansenula capsulata* nov. sp.
      b. Erythritol not assimilated.
        (1) True hyphae produced..... *Hansenula beckii* nov. comb.
        (2) True hyphae not produced..... *Hansenula canadensis* nov. sp.
  E. Raffinose, rhamnose, and alpha-methylglucoside assimilated.
    Hansenula ciferrii Lodder.

II. Ascospores Saturn-shaped, or spheroidal without ring;
  A. Raffinose assimilated; rhamnose and alpha-methylglucoside not assimilated.
    Hansenula suaveolens (Klöcker) Dekker.
  B. Rhamnose assimilated; raffinose and alpha-methylglucoside not assimilated.
    Hansenula mrakii nov. sp.
  C. Raffinose and rhamnose assimilated; alpha-methylglucoside not assimilated.
    Hansenula saturnus (Klöcker) Sydow.
  D. Rhamnose and alpha-methylglucoside assimilated; raffinose not assimilated.
    Hansenula californica nov. comb.

DESCRIPTIONS OF THE SPECIES

Hansenula capsulata nov. sp.

Conjugatio heterogamica inter cellulas maternas et earum gemmas. Pileiformae ascosporae plerumque 2 in quoque asco. Cellulae vegetativae ellipsoideae vel sphaeroideae, 0.9μ×1.4μ ad 3.4μ×3.8μ. Pseudomycelia et vera mycelia deficiunt. Formantur coloniae nitidae et mucidae. Nitras, maltosum, trehalosum, rhamnosum, erythritolum et alpha-methylglucosidum assimilantur, at non galactosum, sucrosum, nec raffinosum. Glucosum fermentatur at non galactosum, maltosum, sucrosum, lactosum, nec raffinosum. Vitamina extranea sunt necessaria ad cresentiam. Isolata ex cuniculi larvarum sub cortice *Piceae*, *Pini divaricatae* (Ait.) Gord., *Pini echinatae* Mill., *Pini laricae* L., *Pini resinosae* Ait., et *Pini ponderosae* Douglas.

From the Latin, *capsula*, a little case, and referring to the capsular material surrounding the cells.

Conjugation is exclusively heterogamic and with few exceptions occurs between mother cells and their buds. The mother cells are usually spheroidal but may be ellipsoidal; daughter cells are spheroidal. Asci contain a maximum of two spores, and the asci rupture soon after the spores are produced. The spores at time of rupture are approximately 2.0μ to 2.6μ broad and 1.7μ high. They are hat-shaped with a thin, often downward turning brim. After liberation they tend to remain characteristically brim-to-brim. The pairs agglutinate in

[1] The assimilation of rhamnose and alpha-methylglucoside by *Hansenula capsulata* is sometimes weak.

large masses when produced abundantly. While still in the ascus, the young spores are not especially refractive; have small, if any, oil drops, and stain a uniform blue with malachite green. After the spores are liberated they become larger, reaching diameters of 3.5μ to 3.8μ. The spores appear more refractive probably because of stored fat, and the oil drops become larger. The hat shape is maintained, but the spores lose their ability to retain the malachite green stain. Best sporulation is obtained on malt extract agar and malt extract-yeast extract agar.

Colonies on dalmau plates are mucoid, glistening, large, white or gray-white, convex, circular, and entire. Colonies sometimes flow slowly over the surface of the agar when the plate is held vertically. Butyrous colony variants may occur after isolates have been cultivated for some time on laboratory media.

Cells from the edge of the dalmau colony occur singly and in pairs, rarely also in small clusters. Cells are spheroidal to ellipsoidal, 0.9μ×1.4μ to 3.4μ×3.8μ.

No hyphae are formed in dalmau streak or spot cultures.

No pellicles are formed on assimilation media.

This species always assimilates nitrate, glucose, maltose, cellobiose, trehalose, melezitose, soluble starch, xylose, L-arabinose, ribose, rhamnose, glucosamine hydrochloride, ethyl alcohol, glycerol, erythritol, adonitol, mannitol, sorbitol, alpha-methylglucoside, salicin, calcium 2-ketogluconate. It sometimes weakly assimilates nitrate, alpha-methylglucoside, and rhamnose. A positive color test for nitrite is readily obtained. Pyruvate and D-arabinose give variable reactions. Galactose, sorbose, sucrose, lactose, melibiose, raffinose, inulin, dulcitol, potassium 5-ketogluconate, potassium sodium saccharate, lactate, succinate, citrate, ethyl acetoacetate and inositol are not assimilated.

Only glucose is fermented, and the fermentation is slow. The insert is usually filled with gas at 6 or 8 days, but bubbles of gas are seldom observed rising through the medium.

Added vitamins are required.

Esters are not produced.

Gelatin is liquefied to a maximum depth of 5 mm.

There is no growth or very little growth in the osmotic pressure medium.

All strains isolated from trees located in Mississippi and California grow at 37° C., but only one of the strains from Canada grows at that temperature. This strain is exceptional in other characteristics which are mentioned subsequently.

Six strains were isolated from the tunnels of larvae in jack pine (*Pinus divaricata*) and spruce (*Picea* sp.) at Wabatongushi Lake approximately 16 kilometers north of Franz, Ontario, Can., during the fall of 1949. Five strains were isolated from similar material in short leaf (*Pinus echinata*) and loblolly (*Pinus taeda*) pines at Piney Woods, Mississippi, during the winter of 1949–50. Three strains were isolated from yellow (*Pinus ponderosa*) pines in the mountains of Santa Barbara County, Calif., in 1950. Strain NRRL Y–1842, isolated from Canadian frass, is designated as the type.

One strain of *Hansenula capsulata* NRRL Y–1889 merits special interest. It was isolated from frass of a red pine, the same tree which yielded the new species *canadensis*. It is different from all other

Canadian strains of the species—growing at 37° C., assimilating lactose latently (the only *Hansenula* now known which attacks this sugar), and having an interesting mode of conjugation between independent cells. About half of the conjugations are the usual mother-daughter type, but the remainder are between large spheroidal cells which conjugate with smaller, usually ellipsoidal cells, each bearing a very small bud on one end. The bud fuses directly with the large cell or with a short conjugation tube produced by the large cell. The nucleus of the smaller cell passes through its bud and on into the large cell where the ascospores are produced.

Except for the properties noted above, strain NRRL Y–1889 is characteristic of the species.

Hansenula beckii (Beck) nov. comb.

Synonyms:
Endomyces bisporus Beck, Ann. Mycol. 20: 219. 1922.
Endomycopsis bisporus (Beck) Dekker, Die sporogenen Hefen. p. 254, 1931.

The transfer of this species to *Hansenula* necessitates a change in name because Nannizzi (Repertorio sistematico dei miceti dell 'uomo e degli animali, Tratt. micopatol. umana, 4, p. 134, 1934) applied the name *Hansenula bisporus* to a species previously described by Matthet (Ann. soc. belge méd. trop. 6: 32. 1926.) under the name *Willia bispora*.

This yeast is renamed for Olga Beck, discoverer of the species.

No ascospores were obtained on any of the media used in sporulation tests. Beck (*6*) observed hat-shaped ascospores. She reported that all of the asci are formed "parthenogenetically," which is interpreted to mean that the vegetative cells of the species are diploid.

Colonies on dalmau plates are weakly glistening; flatly conical; smooth near the edge but rugose, convoluted, or papillate in the center; and filamentous. Cells at edge of the dalmau colony are ellipsoidal to cylindroidal, with a few cells in the shape of peanut hulls. Hyphal segments are present. Cells generally occur singly or in pairs, $1.7\mu \times 2.6\mu$ to $4.3\mu \times 13.6\mu$.

There are abundant aerobic and anaerobic hyphae in the dalmau streaks. True hyphae predominate and are commonly 5 to 8 mm. long in the anaerobic portion, with average ratio of $169\mu : 98\mu$ for four of the longest tip cells to the corresponding adjacent cells. These measurements indicate highly developed true hyphae. The hyphae branch to a slight degree and the few spheroidal and ellipsoidal blastospores they bear are situated mainly in small groups at the tips of short side branches. Sometimes numerous vegetative cells in the shape of peanut hulls are found. The dalmau spot shows abundant true hyphae and many peanut-shaped cells.

No pellicles are formed on assimilation media.

Test compounds assimilated are nitrate, glucose, maltose, sucrose, cellobiose, trehalose, melezitose, xylose, L-arabinose, D-ribose, rhamnose, ethyl alcohol, glycerol, adonitol, mannitol, sorbitol, alpha-methylglucoside, salicin, potassium gluconate, and pyruvic, lactic, succinic, and citric acids; test compounds not assimilated are galactose, sorbose, lactose, melibiose, raffinose, inulin, soluble starch, D-arabinose, glucosamine, erythritol, dulcitol, calcium 2-ketogluconate, potassium 5-ketogluconate, potassium sodium saccharate, ethyl acetoacetate, and inositol.

Added vitamins are required.

A faint, sweet odor may be present, but typical esters are not produced.

Gelatin is not liquefied.

There is no growth in the osmotic pressure medium.

Growth does not occur at 37° C.

Strain NRRL Y–1482, originally isolated by Beck from a species of wood beetle inhabiting spruce bark, is the only strain of this species in the NRRL collection.

Hansenula canadensis nov. sp.

Formantur asci ex transformatione cellularum vegetativarum diploidearum. Pileiformae ascosporae ad 2 in quoque asco. Cellulae vegetativae cylindroideae, 1.7μ×3.4μ ad 3.1μ×6.8μ. Vera mycetia deficiunt. Formantur coloniae glabrae, nec nucidae. Nitras, maltosum, sucrosum, trehalosum, rhamnosum, et alpha-methylglucosidum assimilantur, at non galactosum, raffinosum, nec erythritolum. Glucosum, galactosum, maltosum, sucrosum, lactosum et raffinosum non sunt fermentatur. Formantur esterae. Necessariae ad crescentiam sunt vitaminae externae. Isolata ex cuticulis larvarum sub cortice *Pini resinosae* Ait.

This species is named for Canada, the country from which the first strain was obtained.

Asci are formed exclusively from diploid vegetative cells. Each ascus contains a maximum of two hat-shaped ascospores which are liberated from the ascus when they are mature. The majority of spores are hat-shaped with a prominent and nearly flat brim. However, some spores are broadly ellipsoidal to spheroidal, having a subsequatorial ring that is thin and turns downward. The ascospores vary from 3.0μ to 3.5μ in diameter. Free spores occur singly and in small groups, and the almost exclusively brim-to-brim pairing, so characteristic of *Hansenula capsulata*, is not found in *H. canadensis*. The ascospores remain acid-fast after they escape from the ascus. Good sporulation occurs on malt extract agar.

Colonies on dalmau plates are smooth, weakly glistening, flatly conical, and without hyphae. Cells from the edge of the colony occur singly, in pairs, and in small groups. The cells are predominantly ellipsoidal, 1.7μ×3.4μ to 3.1μ×6.8μ. Some show budding on a broad base.

No hyphae are produced in the aerobic section of the dalmau streak, but under the coverglass many hyphae are formed. They are approximately ¾ mm. long, are exclusively pseudohyphae, and bear chains of blastospores. The blastospores are ellipsoidal to slightly clavate in shape and are about 5μ long. The tip cells of the hyphae and their adjacent cells vary from 9μ to 12μ in length.

Blastospores are so abundantly produced in the spot dalmau cultures that they are packed solidly around the pseudohyphae.

Very thin mat pellicles are formed on media used in the assimilation tests.

This strain assimilates nitrate, maltose, sucrose, cellobiose trehalose, melezitose, xylose, rhamnose, ethyl alcohol, glycerol, mannitol, sorbitol, alpha-methylglucoside, salicin, potassium gluconate, pyruvate, lactate, succinate, and citrate. Its action on raffinose, adonitol, and ethyl acetoacetate is very weak. The other compounds used in the assimilation tests are not assimilated.

No sugars are fermented.

Added vitamins are required.

Esters are produced.

Gelatin is not liquefied.
There is no growth in the osmotic pressure medium.
Growth occurs at 37° C.

Hansenula canadensis is more closely related to *H. beckii* than to any other species described, yet these two may be distinguished by the following characteristics. *H. beckii* does not grow at 37° C., neither does it produce esters, but *H. canadensis* does both. The greatest difference is in the type of hyphae they produce. *H. beckii* forms the longest hyphae of any species in the genus, and the tip cells vary from 140μ to 200μ in length, whereas the tip cells of *H. canadensis*, even in spot cultures, where they are longest, do not exceed 20μ.

Strain NRRL Y-1888 is at present the only strain in the collection. It was isolated from frass of a red pine, *Pinus resinosa* Ait., located in the Algoma District of Ontario. The tree had been killed by fire in June 1948, and was subsequently heavily attacked by bark beetles and wood borers. *Hansenula canadensis* was isolated in 1950 from frass made available by Drs. J. W. Cameron and M. L. Prebble of the Forest Insect Laboratory, Sault Ste. Marie, Ontario.

Hansenula minuta nov. sp.

Conjugatio heterogamica inter cellulas maternas et earum gemmas. Pileiformiae ascosporae ad 4 in quoque aseo. Cellulae vegetativae sphaeroideae, 1.4μ ad 1.3μ diam. Pseudomycelia et vera mycelia deficiunt. Formantur coloniae glabrae at nitidae, interdum submucidae. Trehalosum et nitras assimilantur at non galactosum, maltosum, sucrosum, raffinosum, rhamnosum, erythritolum, nec alpha-methylglucosidium. Glucosum fermentatur at non galactosum, maltosum sucrosum, lactosum, nec raffinosum. Necessariae ad crescentiam sunt vitamina externae. Isolata ex fermentantibus fungis, *Mycena Pura* Fries.

From the Latin, *minuta*, meaning small, and referring to the dimensions of the cells.

Heterogamic conjugation occurs, apparently exclusively between mother cells and their buds. The daughter cells are spheroidal, unusually small, measuring 1.0μ to 1.2μ, whereas the mother cells range from 3.4μ to 3.9μ. The connection between the conjugants remains narrow. Asci usually contain two spores per ascus, sometimes three or four, and they rupture upon the maturation of spores. Spores are predominantly hat-shaped with thin, narrow, downward-turning brims; some are apparently hemispheroidal without apparent brims. Spores vary from 1.2μ×1.6μ to 1.8μ×2.4μ. Spores are stained to moderate intensity by malachite green. Best sporulation occurs on malt extract and on malt extract-yeast extract agar slants.

Colonies on dalmau plates are small, smooth, highly glistening, submucoid, convex, and without hyphae. Colonies producing a pink soluble pigment are occasionally encountered. There is also a mucoid variant of this strain. All variants have the same sporulation and biochemical properties as the parent strain.

Cells from the edge of the dalmau colony are spheroidal, arranged singly and in pairs, with the wall somewhat indistinct owing to the presence of capsular material. Cells range from 1.4μ to 4.3μ in diameter.

In streak and spot dalmau cultures no hyphae are formed.

No rings or pellicles are formed on assimilation media.

This strain always assimilates nitrate, glucose, cellobiose, trehalose, xylose, ribose, glycerol, adonitol, mannitol, sorbitol, salicin, and pyru-

vic acid. It is variable or very weak in its assimilation of sorbose, ethyl alcohol, potassium gluconate, and lactic, succinic, and citric acids. The remaining test substances are not utilized.

Only glucose is fermented. The fermentation is rather slow in starting but always fills the insert with gas in 6 to 12 days.

Added vitamins are required.

Esters are not produced.

There is no growth in the osmotic pressure medium, nor at 37° C.

Gelatin is softened to a depth of 5 mm. so that it flows slowly when the tube is tipped.

Strain NRRL Y–411 is the only representative of this species in the NRRL collection. It was obtained from A. T. Henrici who isolated it from fermenting mushrooms (*Hypeonia pura* Fries.) It was received under the designation "*Saccharomyces* 39ff."

Zikes (*115*) in 1906 isolated from soil a yeast, *Willia wichmanni*, which is known only from the original culture, now lost. The description indicates that it may be closely related to *Hansenula minuta* because of the reported mucoid character of the colonies and the restricted ability to assimilate sugars. Its stated inability to ferment sugars presumably would indicate that it is more primitive than *H. capsulata*.

Zikes further described this species as having hat-shaped spores, and as assimilating glucose but not galactose, maltose, sucrose, lactose, raffinose, inulin, or dextrin. Ethyl acetate was produced. Pellicles were formed on liquid media. Cells from the sediment in wort were polymorphic, 3μ to 5μ long; in the pellicle the cells were 6μ to 40μ long. Its action on nitrate was not determined.

Willia wichmanni is not transferred to the genus nor included in the key or in the table of salient characteristics because the complete reactions cannot be given, and because reasonable doubt regarding its generic placement must remain until it has been reisolated from nature and its action on nitrate determined.

Hansenula silvicola nov. sp.

Conjugatio heterogamica. Pileiformae ascosporae ad 4 in quoque asco. Blastosporae ellipsoideae vel cylindroideae, plerumque catenulatae, ex cellulis pseudomycelii aut veri mycelii gemmantes. Vegetativae cellulae ellipsoideae vel cylindricae, 1.4μ × 2.1μ ad 3.5μ × 8.6μ. Formantur coloniae glabrae, nitidae, sine hyphis. Pellicula tenuissima formatur super media liquida. Galactosum, maltosum, sucrosum, trehalosum, rhamnosum, alpha-methylglucosidum et nitras assimilantur, at non raffinosum nec erythritolum. Glucosum et galactosum fermentantur at non maltosum, sucrosum, lactosum, nec raffinosum. Necessariae ad crescentiam sunt vitamina externae. Isolata ex gummi *Pruni serotinae* Ehrh.

The name is taken from the Latin, *silvicola*, an inhabitant of the woods.

Almost all asci result from heterogamic conjugation. The smaller cell of the pair is nearly always a daughter cell. The channel between the two cells usually becomes quite wide. A very few asci are formed directly from diploid cells and therefore show no evidence of conjugation. The asci rupture at maturity, freeing up to four hat-shaped spores each. The brim is thin, sometimes turned downward, and occasionally appears to be missing. The spores stain moderately strong with malachite green. Sporulation is best on V–S slants and carrot.

Colonies on dalmau plates are glistening, smooth, circular, flat to conical, and without hyphae. Growth on malt extract-yeast extract agar is somewhat mucoid in appearance.

Cells from the edge of the dalmau colony are ellipsoidal and cylindroidal, occurring singly, in pairs, and threes. There is occasional budding on a broad base. Hyphal segments are seldom seen. Cells range from 1.4μ × 2.1μ to 3.5μ × 8.6μ.

Hansenula silvicola usually produces no hyphae aerobically, and only a few under the coverglass in the dalmau streaks. These range up to 900μ. Occasionally, true hyphae are present, as shown by ratios of the length of the tip cells to the adjacent cells in three hyphae; 31μ:24μ, 38μ:28μ, and 38μ:22μ. At other times, the hyphae are almost exclusively pseudohyphae. Most of the hyphae produce large numbers of short cylindrical to ellipsoidal blastospores measuring 4μ to 7μ long. The blastospores are arranged in chains which form clusters at the septa, or they may be densely packed all along the hypha. The chains of blastospores vary in length from about 3 to 20 cells. Some hyphae have few or no blastospores.

Occasionally no hyphae are formed in dalmau spots. When they are formed, they appear in larger numbers and bear fewer blastospores than in the dalmau streak.

Very thin pellicles are formed on assimilation media.

Hansenula silvicola assimilates nitrate, glucose, galactose, sorbose, maltose, sucrose, cellobiose, trehalose, xylose, L-arabinose, ribose, rhamnose, ethyl alcohol, glycerol, adonital, sorbitol, alpha-methyl-glucoside, salicin, potassium gluconate, and pyruvic, succinic, and citric acid. Variable reactions occur in melezitose, mannitol, and lactic acid. It attacks none of the other compounds.

Glucose is immediately and strongly fermented. Galactose is latently fermented, and the insert is completely filled with gas within 12 to 16 days after inoculation.

Vitamins must be added to synthetic media to obtain growth.

A sweet, fruity odor is produced, but not the typical ester odor usually associated with the genus *Hansenula*.

Gelatin is not liquefied.

There is no growth in the osmotic pressure medium. Either rapid or latent growth may occur at 37° C.

Three strains have been isolated from gums of wild cherry, *Prunus serotina* Ehrh. The trees were located a maximum of 15 kilometers apart at Peoria, Ill. Strain NRRL Y–1678 is designated as the type.

Hansenula angusta nov. sp.

The Latin description of this species will be published by H. H. Hall and Dorothea J. Tennisson.

From the Latin, *angustus*, meaning narrow, and referring to the brim on the hat-shaped ascospores.

Conjugation is predominantly between mother cells and their buds. The mother cell is usually spheroidal but sometimes ellipsoidal, and the daughter cells are spheroidal. Heterogamic conjugation between unrelated cells is found very infrequently, and isogamic conjugation is encountered even more rarely. The direct conversion of diploid vegetative cells to asci is common. The diploid cells sporulate more rapidly than the haploid cells; thus, on malt extract-yeast extract

agar after 4 days of incubation about half of the intact asci show no conjugation, but at 24 days nearly all of the intact asci show conjugation. The asci formed from diploid cells sometimes develop a thick double wall before the spores are produced, and the outer wall occasionally may be seen in the process of being shed. Most asci rupture as soon as the spores are mature. The spores are hemispheroidal. Occasionally a spore with a fairly broad brim is found, but usually the brim is so reduced that the spores are suggestive of those found in the genus *Pichia* Hansen. Spores vary in size from 1.7μ × 1.4μ to 2.6μ × 1.7μ. They stain a uniform light blue with malachite green. Sporulation is most abundant on malt extract agar.

Colonies on dalmau plates are rather small, butyrous, glistening, and show faint radial striations. Frequently older colonies on yeast extract-malt extract agar have a pink tint.

Cells from the edge of the dalmau colony occur mainly as single cells, are spheroidal, and the larger ones have thicker cell walls. They vary in size from 2.2μ to 5.2μ.

No hyphae are formed in dalmau streak or spot cultures.

No pellicles are formed on assimilation media.

This species always assimilates nitrate, glucose, maltose, sucrose, trehalose, melezitose, xylose, D-arabinose, ribose, ethyl alcohol, glycerol, erythritol, adonitol, mannitol, sorbitol, alpha-methylglucoside, pyruvate, succinate, and citrate. Alpha-methylglucoside is assimilated latently. Sorbose is latently assimilated or not assimilated at all. Compounds which are not assimilated are galactose, cellobiose, lactose, melibiose, raffinose, inulin, soluble starch, L-arabinose, rhamnose, glucosamine hydrochloride, dulcitol, salicin, potassium gluconate, calcium 2-ketogluconate, potassium 5-ketogluconate, potassium sodium saccharate, sodium lactate, ethyl acetoacetate, and inositol.

Only glucose is fermented, and the fermentation is rather weak.

Added vitamins are required.

Esters are not produced.

Gelatin is softened to a depth of 1 to 2 mm. and flows slowly.

Growth is good at 37° C. and there is moderate growth in the osmotic pressure medium.

Only one strain of *Hansenula angusta*, NRRL Y-1798, is now in the collection, and it is designated as the type. This species was isolated in 1944 by Harlow H. Hall and Dorothea J. Teunisson from spoiled concentrated orange juice made from oranges grown in Florida.

Hansenula subpelliculosa Bedford, Mycologia 34: 636, 1942.

From the Latin, *sub*, less; *pellicula*, a thin skin.

The ascigerous vegetative cells of *H. subpelliculosa* are considered as being diploid, although extremely rare cases of heterogamic conjugation are observed. The ascospores for the most part are hat-shaped with a thick, prominent, and usually flat brim. In some strains spherical and broadly elliptical ascospores are also seen. They possess a ring which may be thin and downward curving, attached subequatorially; or the ring may be thick and attached subequatorially to tangentially. One to four spores are formed in asci which commonly rupture at maturity. Best sporulation is on carrot plugs and malt extract agar.

Colony on dalmau plate is smooth, glistening, and usually without hyphae. Cells at the edge of the colony occur singly and in pairs, are ellipsoidal, and range from 2.0μ × 3.1μ to 4.2μ × 7.0μ.

In the dalmau streak, hyphae may be present or absent both aerobically and anaerobically. The hyphae underneath the coverglass reach a maximum length of 1.2 mm. in some strains. Usually true hyphae of a low degree of development are present, and these show constrictions in the tip cells previous to the formation of septa. Such hyphae are broad and the septa distinct. The tip cells average about 50μ and the corresponding adjacent cells average about 32μ. The tip cells contain dense protoplasm but the remainder of the hyphae are highly vacuolated. Some strains produce pseudohyphae exclusively. Strains which produce hyphae abundantly show relatively few blastospores because of the low oxygen tension caused by the heavy growth. Strains in which hyphae are more isolated usually form blastospores in abundance. The blastospores are ellipsoidal and short cylindroidal, and occur singly and in chains up to eight or more cells in length. In the dalmau spot the number of hyphae varies from none to many.

Pellicles are thin or entirely lacking.

All of the strains studied assimilate nitrate, glucose, maltose, sucrose, trehalose, raffinose, ethyl alcohol, glycerol, erythritol, mannitol, sorbitol, alpha-methylglucoside, salicin, potassium gluconate, and pyruvic, lactic, succinic, and citric acids. They usually assimilate galactose, xylose, ribose, and adonitol. Approximately half of the strains assimilate cellobiose, melezitose, and soluble starch; and generally, but not always, a strain which utilizes one of these three compounds also utilizes the other two. L-arabinose is seldom assimilated. No strain assimilates sorbose, lactose, melibiose, inulin, D-arabinose, rhamnose, dulcitol, calcium 2-ketogluconate, potassium 5-ketogluconate, potassium sodium saccharate, ethyl acetoacetate, or inositol.

Glucose, sucrose, and one-third part of raffinose are fermented. Maltose is either not fermented or fermented very weakly. Galactose and lactose are not fermented.

Hansenula subpelliculosa cannot always be differentiated from the glistening colony type of *H. anomala* by assimilation or fermentation reactions, but the two species can always be readily differentiated by vitamin requirements. *H. subpelliculosa* will not grow in a vitamin-deficient medium whereas *H. anomala* does. Usually *H. subpelliculosa* produces true hyphae which can be readily distinguished from the pseudohyphae produced by *H. anomala*.

Esters are produced.

Gelatin is liquefied by some strains, to a maximum depth of about 10 mm.

Exceptionally good growth occurs in the osmotic pressure medium. Growth occurs at 37° C.

Bedford (*7*) isolated several strains from sources having high osmotic pressure, such as concentrated sugar-egg mixture, dried prunes, and grape juice. Etchells and Bell (*24*) obtained many strains from cucumber brines which contained 10 to 18 percent of salt by weight.

Thirty-five strains have been studied. Strain NRRL Y–1683 is typical.

Nonsporogenous strains of this species are occasionally encountered. An example is NRRL Y–1096, a strain used for the preparation of soy sauce.

Hansenula jadinii nov. comb.

Synonym:
Saccharomyces jadini Sartory *et al.*, Compt. rend. acad. sci., 194:1688. 1932.

Named for Professor Jadin, of the faculty of pharmacy, Strasbourg.

Ascigerous vegetative cells may be entirely diploid, as indicated by the absence of conjugation in the asci. Ascospores are hat-shaped with a broad and fairly thick brim. Usually there are four spores per ascus, and the asci may not rupture on maturation. Spores have been obtained only on cucumber and carrot wedges, and only in small numbers. If sporulation were more abundant, some haploid cells might be found which would conjugate.

Colony on dalmau plate is large, smooth, glistening, and without hyphae. Cells at the edge of the colony occur singly, in pairs, and a few are in chains; they are ellipsoidal and clavate, $3.4\mu \times 3.8\mu$ to $3.4\mu \times 8.6\mu$.

The dalmau streak shows in some areas pseudohyphae up to 270μ in length. The hyphae branch profusely and bear only a few chains of ellipsoidal and cylindroidal blastospores. The blastospores are rather poorly differentiated from the hyphal cells. Tip cells of the hyphae average about 10μ, the adjacent cells average about 11μ. There are no true hyphae. In some areas there are no pseudohyphae, only chains of blastospores.

No pellicles are formed on the assimilation test media.

This species assimilates nitrate, glucose, maltose, sucrose, cellobiose, trehalose, raffinose, melezitose, inulin, xylose, ethyl alcohol, glycerol, mannitol, sorbitol, alpha-methylglucoside, salicin, potassium gluconate, and pyruvic, lactic, succinic, and citric acids, and ethyl acetoacetate. It does not assimilate the other compounds.

Glucose, sucrose, and one-third part of raffinose are fermented, but the fermentations are rather weak.

Added vitamins are not required.

Esters are produced.

Gelatin is not liquefied.

There is no growth in the osmotic pressure medium. Good growth occurs at 37° C.

Hansenula jadinii, strain NRRL Y–1542, was isolated along with a staphylococcus from pus from a human abscess. Sartory *et al.* (*88*) reported that intercardiac inoculation of this yeast into guinea pigs led to their death in 5 to 7 months. The yeast was recovered from numerous lesions it had caused. This strain was obtained from the Centraalbureau in 1947; the Centraalbureau received it from Sartory.

Hansenula schneggii (Weber) Dekker, Die sporogenen Hefen., p. 432. 1931.

Synonym:
Willia schneggii Weber, Biochem. Z. 129: 208. 1922.

Named for the late Hans Schnegg who is known best for his publications on the technical aspects of brewing.

Weber observed only a very few hat-shaped ascospores, and these were found only on media containing sucrose. Neither Stelling-Dek-

ker (*95*) nor Bedford (*7*) obtained ascospores. The author has attempted to obtain ascospores on sucrose and raffinose media and in filter-sterilized beer, as well as on the usual sporulation media, but without success.

The dalman colony is mat, white, powdery, radially rugose, and without hyphae. Cells from the edge of the colony occur singly, in pairs, and in short chains. They are cylindroidal and ellipsoidal, 3.1μ × 3.4μ to 5.2μ × 8.6μ.

Underneath the coverglass in the dalman streak, abundant, highly branched pseudohyphae are produced which bear numerous spheroidal and ellipsoidal blastospores. The hyphae are about ½ mm. long, with the first and second cells of the hyphal tips about 10μ long.

White, folded pellicles are formed on assimilation media.

Hansenula schneggii assimilates nitrate, glucose, galactose, maltose, sucrose, cellobiose, trehalose, melezitose, soluble starch, xylose, ribose, ethyl alcohol, glycerol, erythritol, mannitol, sorbitol, alpha-methylglucoside, salicin, gluconate, pyruvate, lactate, succinate and citrate. Adonitol is assimilated weakly or not at all. The other compounds used in the tests are not assimilated.

This species ferments glucose and maltose strongly, and sucrose latently, but does not ferment galactose or raffinose. Bedford (*7*) found it to produce a weak fermentation of galactose.

Added vitamins are not required.

Esters are produced.

Gelatin is not liquefied.

There is weak or moderate growth in the osmotic pressure medium but no growth at 37° C.

Hansenula schneggii is represented by a single strain, Y-993.

Weber did not state the source from which *H. schneggii* was isolated.

Hansenula anomala (Hansen) Sydow, Ann. Mycol. 17: 44. 1919.

Synonyms:

Saccharomyces anomalus Hansen, Ann. de micrographie 3: 467. 1891.
Willia anomala Hansen, Zentbl. f. Bakt. Parasitenk. Abt. II, 12: 538. 1904.
Willia odessa Weber, Biochem. Z. 129: 208. 1922.
Willia producta (Beijerinck) Berkhout, De schimmelgeslachten *Monilia*, *Oidium*, *Oospora*, en *Torula*. Dissertation, Utrecht, p. 43. 1923.
Endomyces margaritae Zender, Bull. soc. botan. Genève 17: 258. 1925.
Hansenula anomala var. *longa* Dekker, Die sporogenen Hefen, p. 415. 1931.
Hansenula anomala var. *producta* (Beijerinck) Dekker, *Ibid.*, p. 427.
Hansenula anomala var. *robusta* Dekker, *Ibid.*, p. 419.
Hansenula javanica (Groenewege) Dekker, *Ibid.*, p. 420.
Hansenula nivea Castelli, Arch. Mikrobiol. 4: 528. 1933.
Hansenula panis Castelli, *Ibid.*, p. 522.
Saccharomyces acris-sacchari Fabian and Hall, Zentbl. f. Bakt. Parasitenk. Abt. II, 89: 34. 1933.
Hansenula anomala var. *heteromorpha* Bedford, Mycologia 34: 636. 1942.
Hansenula lambica (Kufferath) Dekker, as described by Bedford in Mycologia 34: 639. 1942.

Probable synonyms:

Saccharomyces acetaethylicus Beijerinck, Zentbl. f. Bakt. Parasitenk. Abt. I, 11: 70. 1892.
Willia bispora Mattfeld, Ann. soc. belge med. trop. 6: 32. 1926.
Hansenula bispora (Mattfeld) Nannizzi, Tratt. micopatol. umana 4: 134. 1934.

From the Greek, *anomalus*, different from the ordinary.

The species name was particularly appropriate at the time Hansen placed this yeast in the genus *Saccharomyces*, but when it was made

the type for his new genus *Willia*, the epithet became definitely inappropriate. However, on logical as well as on historical grounds, *Hansenula anomala* appears to be the best species to represent the genus.

Conjugation occurs at the time ascospores germinate, and no conjugation occurs immediately preceding the formation of asci. Asci rupture as the ascospores mature, except in some strains having cells large enough to easily accommodate the mature spores. Ascospores are hat-shaped, usually with a thick brim. Spores stain moderately strong with malachite green. Best sporulation is obtained on V–8 slants and carrot plugs.

Colonies of the different strains vary greatly in appearance when cultivated on malt extract-yeast extract agar and on other solid media commonly used in the isolation of yeasts. Not infrequently, isolates from substrates undergoing fermentation produce highly glistening, smooth colonies. Strains isolated from soil usually form mat, powdery white colonies which may be smooth or rugose. All degrees of transition are found between highly glistening and powdery mat extremes. In addition, crateriform, coarsely convoluted, and other rare shapes may appear.

Homologous mat and glistening forms usually have approximately equivalent physiological activities from a qualitative, though not from a quantitative standpoint. Fabian and Wickerham (*29*) found that the glistening types produced decidedly more alcohol than the corresponding mat types. The author believes the mat type represents an adaptation to aerobic conditions, the glistening type an adaptation to fermentative conditions. Both forms, if recently isolated from nature, will sporulate; but of two homologous pairs which the author has had in culture for 15 years, the two glistening forms still sporulate abundantly, whereas the two mat forms from which they were derived sporulate very poorly.

The range of variation in colony appearances encountered on the synthetic medium used for the dalmau preparations is much less than occurs on malt extract-yeast extract agar. Strains which form crateriform or convoluted mat colonies on the latter medium usually form the typical conical mat colonies on the synthetic medium, and the strains which produce highly glistening colonies on malt extract-yeast extract agar produce mat or, at most, only slightly glistening colonies on dalmau plates.

Cells from the edge of the dalmau colony vary greatly in size and shape within specific strains, and also from strain to strain. Some form spheroidal and broadly ellipsoidal cells varying from $2.0\mu \times 2.6\mu$ to $4.8\mu \times 5.2\mu$. Others produce predominantly cylindroidal cells varying from $2.0\mu \times 4.3\mu$ to $3.4\mu \times 33\mu$. These dimensions show a wide range of cell shape and size. The cells occur singly, in pairs, or in chains.

No true hyphae are formed in dalmau streak and spot cultures, but most strains produce from a few to abundant pseudohyphae. Rarely, strains are found which produce a very few septa. Forms characterized by glistening colonies produce fewer hyphae than the mat forms, and the difference is generally very marked. Hyphae usually vary in length from 0.5 to 1.0 mm., but may reach a length of 3 mm. Tip and adjacent cells may reach approximately equal lengths varying

from 8μ to 20μ but occasionally reach 30μ in those strains with the greatest hyphal development. The hyphae are often highly branched and bear few or many blastospores. These may be short cylindroidal, ellipsoidal, spheroidal, or ovoidal, with one or more shapes present in a single strain.

Pellicle formation ranges from very thin or lacking in some strains which produce glistening colonies on agar media, to very prominent, white, mat, folded pellicles in strains producing powdery, white, mat colonies under similar conditions. The powdery, white surface layer of such mat colonies consist of cells which resist wetting, and may be floated free of the colonies by gently pouring water into the petri dish. Throughout the genus, it is generally true that the thicker the layer which can be floated from the colony, the thicker will be the pellicle on liquid media.

All strains assimilate nitrate, glucose, maltose, sucrose, cellobiose, trehalose, raffinose, melezitose, soluble starch, ethyl alcohol, glycerol, erythritol, mannitol, sorbitol, alpha methylglucoside, salicin, and pyruvic, lactic, succinic, and citric acids. Almost always assimilated are galactose and xylose. Usually assimilated are ribose, adonitol and potassium gluconate. L-arabinose usually is not assimilated. Compounds not assimilated are sorbose, lactose, melibiose, inulin, D-arabinose, rhamnose, glucosamine, dulcitol, calcium 2-ketogluconate, potassium 5-ketogluconate, potassium sodium saccharate, ethyl acetoacetate, and inositol.

Glucose and sucrose are always fermented. Maltose is almost always fermented, though often weakly, and on extremely rare occasions it is not fermented to a detectable degree. Galactose may be fermented strongly, weakly, or not at all, depending upon the individual strain. Raffinose is fermented one-third.

No added vitamins are required for growth.

Esters are produced.

Gelatin is not liquefied.

Most strains grow well in the osmotic pressure medium. Growth is variable at 37° C.

Seventy-three strains have been studied. The forms producing mat colonies on malt extract-yeast extract agar are represented by strain NRRL Y-365, and the forms producing glistening colonies on this medium are represented by strain NRRL Y-366. The latter was derived from the former by treatment with lithium chloride (*1088*).

Hansenula anomala is the commonest and most widely distributed member of the genus. It occurs in soil; saps and gums of trees; on fresh, dried, and sugared fruits; syrups, and as an occasional contaminant in industrial food and fermentation processes. Hansen (*37*) originally isolated this species from brewery yeast and made it the type of the genus.

Hansenula ciferrii Lodder, Zentrbl. f. Bakt. Parasitenk. Abt. II,86: 245. 1932.

Named for Raffaele Ciferri who first isolated this yeast.

Asci show no evidence of conjugation, therefore all ascogenous cells of this yeast are diploid. The spores are hat-shape. Sporulation is poor, with carrot wedges yielding the most spores.

The strain is somewhat unstable morphologically. A series of variants have arisen spontaneously, but only the two extremes will be

described here. One gives rise to no hyphae, or to very primitive pseudohyphae, whereas the other gives rise to well-developed true hyphae.

NONFILAMENTOUS FORM.—Colonies on malt extract-yeast extract agar are smooth to slightly rugose, and slightly glistening. The dalman colony is weakly glistening, highly raised, with a lumpy and granular surface, irregular edge, and no hyphae. Cells from the colony edge occur singly, in pairs, or as irregular clusters numbering up to 30 or 40 cells. Cells are usually spheroidal, and measure from 2.6μ to 7.0μ. Some very large aberrantly-shaped cylindroidal and clavate cells occur singly and in groups. No hyphae are formed in either the aerobic or the anaerobic portions of the dalman streak, nor in the dalman spot. The large, aberrantly-shaped cells are fairly numerous under the coverglasses; those in the streak at 7 days are for the most part ruptured and dead, while those in the spot are still alive. The normal cells are small, spheroidal, and occur in chains of two, three, or four cells.

FILAMENTOUS FORM.—The colonies on malt extract-yeast extract agar are convoluted or crateriform. The dalman colony is mat, smooth, and fails to develop hyphae. The cells at the edge of the colony generally occur singly, and are spheroidal, cylindroidal, or ellipsoidal. They vary from 3.5μ in diameter for the smallest spheroidal cells to approximately 2.7μ × 23μ for the largest cylindroidal cells. There are no aberrant, clavate cells. Hyphae are abundant both aerobically and anaerobically in the dalman streak, reaching a length of 2 mm. under the coverglass. Ratios of length of tip cells to their adjacent cells are as follows for three pairs: 86μ : 50μ, 63μ : 41μ, and 115μ : 47μ. Those ratios indicate well developed true hyphae. Ovoidal and spheroidal blastospores are borne on the hyphae, sometimes singly and sometimes in chains, depending upon the degree to which the hyphae are crowded, and on the availability of dissolved atmospheric oxygen. Blastoconidia are also formed on the hyphae. These are specialized blastospores which have much reserve fat, a more refractive and perhaps thicker cell wall and, in this species, they seldom produce buds.

Thin mat pellicles form on some of the assimilation media, and only islets of pellicles form on others.

All variants of this species assimilate nitrate, glucose, galactose, maltose, sucrose, cellobiose, trehalose, raffinose, melezitose, soluble starch, L-arabinose, ribose, rhamnose, ethyl alcohol, glycerol, erythritol, adonitol, mannitol, sorbitol, alpha-methylglucoside, salicin, potassium gluconate, and pyruvic, lactic, succinic, and citric acids. Xylose is attacked weakly or not at all. None of the other compounds are assimilated.

Glucose, galactose, and sucrose are fermented, also raffinose for one-third part. Maltose is fermented very weakly or not at all.

Added vitamins are not required.

Esters are produced.

Gelatin is liquefied to a depth of 10 to 17 mm. *H. ciferrii* liquefies gelatin more actively than any other species in the genus.

Growth is generally weak, but sometimes moderate, in the osmotic pressure medium. Growth at 37° C. is variable.

Physiologically, *H. ciferrii* is very similar to *H. anomala*, but can be separated from it on rhamnose utilization. All forms of *H. ciferrii*

assimilate this methyl pentose, whereas assimilation has not been accomplished by any of the 73 strains of *H. anomala* yet studied.

Hansenula ciferrii has been isolated only once from nature, to the author's knowledge. It was found by Ciferri in abundance on the rind of the fruit of *Coumarouna punctata* Blake in San Domingo. The nonfilamentous type is represented by strain NRRL Y–1031; the filamentous type by NRRL Y–1322.

Hansenula californica (Lodder) nov. comb.

Synonym:
Zygohansenula californica Lodder, Zentbl. f. Bakt. Parasitenk, abt. II. 86: 246, 1932.

First isolated by E. M. Muller, in the State for which it is named.

In most strains, the majority of vegetative cells are haploid; thus the asci which they form show conjugation. Conjugation is predominantly heterogamic, but the ratio of heterogamic asci to isogamic asci is quite variable. In some strains the majority of the cells are presumably diploid because most of the asci show no evidence of conjugation.

Spores of all strains are Saturn shaped with thin rings. Many are found free of the asci. The best sporulation media are V–8, malt extract-yeast extract, malt extract, and carrot. Spores stain moderately with malachite green.

Colony on dalmau plate is smooth, glistening, moderately large, flat, and with hyphae. Cells from the edge of the colony are ellipsoidal, occur singly or in pairs, and measure from $2.2\mu \times 3.4\mu$ to $6.0\mu \times 8.6\mu$ Sometimes a few of the cells bud on a broad base.

There is no vestige of hyphae in either dalmau streak or dalmau spot cultures.

Pellicles are very thin or lacking on assimilation media.

All strains assimilate nitrate, glucose, sorbose, sucrose, cellobiose, xylose, rhamnose, ethyl alcohol, glycerol, mannitol sorbitol, alpha-methylglucoside, salicin, potassium gluconate, and pyruvic lactic, and succinic acids. Variations occur in maltose, trehalose, calcium 2-ketogluconate, citrate, and ethyl acetoacetate. None of the other carbon sources employed are attacked by any of the strains.

Only glucose is fermented.

Added vitamins are required.

Esters are produced.

Gelatin may be liquefied to a depth of 6 mm.

There is no growth in the osmotic pressure medium, nor at 37° C.

Of the 15 strains studied, NRRL Y–1680 sporulates strongly and is typical also in other characteristics. It was isolated from soil from southern Australia. Other strains have been isolated from soils from Belgium, Sweden, Minnesota, and California.

Hansenula suaveolens (Klöcker) Dekker, Die sporogenen Hefen, p. 399. 1931.

Synonyms:
Pichia suaveolens Klöcker, Zentbl. f. Bakt. Parasitenk. Abt. II. 35: 371. 1912.

From the Latin, *suaveolens*, of a sweet odor.

Diploid cells predominate in the vegetative stage, yet strains which sporulate heavily produce a small number of heterogamic and iso-

gamic asci among the predominant type which shows no evidence of conjugation. Asci contain one to four Saturn-shaped or spheroidal spores per ascus. The rings are so thin that they are barely visible, even with the oil-immersion objective. The rings impart a slightly angular appearance to the spores. Spores free from the ascus are observed frequently. Spores stain moderately with malachite green. Best sporulation is on malt extract and malt extract-yeast extract slants.

Colony on dalmau plate is mat, powdery, verrucose, without hyphae. Cells at edge of colony are spherodial, ellipsoidal, and cylindroidal, $1.7\mu \times 2.6\mu$ to $3.5\mu \times 10\mu$. Strains which produce crateriform colonies on malt extract-yeast extract agar produce cells up to 20μ long at the edge of dalmau colonies.

In the dalmau streak the pseudohyphae may be rather scant or abundant. They measure up to 1 mm. and often are highly branched. These branches gives rise to well-defined spheroidal or cylindroidal blastospores, or to cells that are cylindroidal like the hyphal cells except that they are shorter; or blastospores may be entirely lacking. Septa are observed infrequently. Tip and adjacent cells are approximately of equal length and vary from 6μ to 11μ.

Thin, or prominent mat, white, folded pellicles are formed on assimilation media.

Compounds assimilated are nitrate, glucose, sucrose, cellobiose, raffinose, inulin, xylose, ethyl alcohol, glycerol, mannitol, sorbitol, salicin, potassium gluconate, and pyruvic, lactic, and succinic acids. Succinic acid is utilized weakly. None of the other compounds employed in the tests are assimilated.

Sugars fermented are glucose, sucrose, and one-third part of raffinose.

Growth is obtained in synthetic media without the addition of vitamins.

Esters are produced.

Gelatin is not liquefied.

There is no growth in the osmotic pressure medium, or at 37° C.

H. suaveolens was isolated from Danish soil by Klöcker (*42*) and from Minnesota soils by Robert Bouthilet. The Minnesota isolates were supplied by E. M. Mrak. Of the four strains in the collection, strain NRRL Y–1725 sporulates abundantly and is typical of the species.

Wise and Appling (*113*) used *Hansenula suaveolens* NRRL Y–838 for the quantitative determination of D-xylose in hydrolyzates because of its ability to assimilate this pentose rapidly without assimilating L-arabinose. *Saccharomyces carlsbergensis* Hansen, NRRL Y–379, was used in conjunction with *H. suaveolens* to remove hexoses. Auernheimer, Wickerham, and Schniepp (*3*) have used the two yeasts just mentioned, plus *Candida guilliermondii* (A. Cast.) Langeron and Guerra, strain NRRL Y–488, to determine quantitatively the amounts of D-xylose and L-arabinose in hydrolyzates of agricultural residues.

Hansenula mrakii nov. sp.

Plerumque formantur asci ex transformatione cellularum vegetativarum diploidearum, at pauci asci ex conjugatione cellularum haploidearum. Ad 4 saturniformae ascosporae in quoque asco. Annuli ascosporarum crassi. Cellulae vegetativae sphaeroideae, ellipsoideae vel cylindroideae, $1.7\mu \times 2.6\mu$ ad $5.2\mu \times 7.2\mu$. Ex pseudohyphis oriuntur male differentiatae blastosporae. Coloniae non

nitidae, pulverulentae, radianter rugosae, sine hyphis. Super media liquida formantur plicatae, non nitidae, albae pellicolae. Rhamnosum et nitras assimilantur at non glactosum, maltosum, sucrosum, trehalosum, raffinosum, erythritolum, nec alpha-methylglucosidum. Glucosum fermentatur at non galactosum, maltosum, saccharum, lactosum, nec raffinosum. Vitamina externa non sunt necessaria. Isolata ex solo in vicinitate Dobodurae, in Nova Guinea.

This species is named for E. M. Mrak, in recognition of his work on the classification of yeasts and his generosity in supplying the writer with cultures and translations.

Vegetative cells are predominantly diploid, as indicated by the absence of conjugation in almost all of the asci. However, a few asci are formed by isogamic and heterogamic conjugation of haploid cells on malt extract-yeast extract agar in 2 to 3 weeks. Spores vary from one to four, and the asci rupture as soon as the spores are mature. The spores are Saturn-shaped with a rather thick ring which may be situated equatorially, subequatorially, or even tangentially. The capsule, of which the ring is a part, covers the entire spore and reduces staining with malachite green. Best sporulation is on V–8 malt extract-yeast extract agar slants, and on carrot wedges. V–8 slants may show approximately 98 percent of the cells converted to asci after 5 to 8 days of incubation.

Colony on dalmau plate is mat, slightly powdery, verrucose, radially rugose, conical, and without hyphae. Cells at the edge of the dalmau colony are spheroidal, ellipsoidal, cylindroidal, arranged singly and in pairs but some large clumps are made up of chains of cells which are the water-resisting cells from the powdery surface of the colony. Cells are $1.7\mu \times 2.6\mu$ to $5.2\mu \times 7.2\mu$.

The dalmau streak shows many pseudohyphae up to 700μ in length. They are highly branched, but the branches are short. They may bear blastospores which are ellipsoidal to cylindroidal, and poorly differentiated from the hyphal cells. The tip cells of the pseudohyphae and their adjacent cells are approximately of equal length, and reach 8.5μ to 10.3μ in length. There are no hyphae in the dalmau spot.

Folded, white mat pellicles are formed on assimilation media.

Hansenula mrakii assimilates nitrate, glucose, cellobiose, xylose, rhamnose, ethyl alcohol, glycerol, mannitol, sorbitol, salicin, potassium gluconate, and pyruvic, lactic, and succinic acids. The other compounds are not assimilated.

Only glucose is fermented.

Added vitamins are not required.

Esters are produced.

Gelatin is liquefied to a depth of some 7 mm.

There is no growth in the osmotic pressure medium, nor at 37° C.

H. mrakii, strain NRRL Y–1361, was isolated by the author from soil sent from the Dobodura area, New Guinea.

Hansenula saturnus (Klöcker) Sydow. Ann. Mycol. 17: 44. 1919.

Synonyms:

Saccharomyces saturnus Klöcker, Zentbl. f. Bakt. Parasitenk. Abt. II. 8: 129. 1902.

Willia saturnus (Klöcker) Hansen, Zentbl. f. Bakt. Parasitenk. Abt. II. 12: 538. 1904.

Williopsis saturnus (Klöcker) Zender, Bull. soc. botan. Genève 17: 272. 1925.

Named for the planet Saturn because of the shape of the ascospores.

In some strains conjugation may be limited to the period during which the ascospores are germinating, but most strains which sporu-

late abundantly show infrequent instances of isogamic conjugation after 1 to 3 weeks of incubation on malt extract-yeast extract agar. Some conjugations do not lead to spore formation, others do. Other sporulation media, such as V–8, is much less conducive to sporulation by haploid cells though the diploid cells may sporulate abundantly on it. Asci seldom rupture during or after maturation of the spores. The spores are Saturn-shaped with thick, usually equatorial rings, and resist staining with malachite green. Best sporulation is on malt extract-yeast extract, V–8 agar slants, and carrot plugs.

Colonies on dalmau plates vary from slightly glistening and nearly smooth to mat, white, rugose and verrucose; none develop hyphae.

Cells from the edge of the dalmau colony vary considerably in size and shape from strain to strain. The strain having the smallest cells, ranging from $2.0\mu \times 3.1\mu$ to $5.2\mu \times 5.5\mu$, possesses spheroidal, ellipsoidal, and ovoidal cells occurring singly or in rather large compact clusters (water-resistant cells from surface of colony) containing up to 30 or 40 cells. The strain having the largest cells, ranging from $8.6\mu \times 3.4\mu$ to $3.0\mu \times 15\mu$ has ellipsoidal and cylindroidal shapes occurring for the most part singly but also in pairs and infrequently in short chains.

In dalmau streak cultures some strains form no hyphae whatever, some form very primitive pseudohyphae consisting of highly branching chains of short (5μ) ellipsoidal cells, and some form rather highly branched pseudohyphae, composed of cylindroidal cells which reach a total length of 500μ. In the latter, tip and adjacent cells reach approximately equal lengths between 12μ and 17μ. No blastospores are produced on the hyphae. Very few strains form hyphae in dalmau spot preparations.

Smooth or folded pellicles are formed on assimilation media.

All strains assimilate nitrate, glucose, sucrose, cellobiose, raffinose, inulin, xylose, rhamnose, ethyl alcohol, glycerol, salicin, potassium gluconate, and pyruvic, lactic, and succinic acids. Only rarely are strains encountered which fail to assimilate mannitol, sorbitol, or citric acid. None of the other compounds used in the tests are assimilated.

All strains ferment glucose, sucrose, and one-third part of raffinose.

Added vitamins are not required.

Esters are produced.

Gelatin is not liquefied.

There is no growth in the osmotic pressure medium, or at 37° C.

Hansenula saturnus has been isolated from soil from the Himalaya mountains, Denmark, Italy, Egypt, Minnesota, Delaware, and Louisiana. Nineteen strains have been studied. Strain NRRL Y–1304 is a typical specimen, and shows conjugation of haploid cells on sporulation media.

Discussion

It is the opinion of the author than *Hansenula* Sydow and *Pichia* Hansen should be maintained as separate genera. The species of *Hansenula* are physiologically more active than the species of *Pichia*, not only in nitrate utilization, but also in the number of carbon sources which they are able to assimilate. The difference in action on nitrate appears to be a fundamental characteristic which does not vary from strain to strain in species of either *Hansenula* or *Pichia*.

Pertinent to the following discussion, plates 4 and 5 show ascospores of various shapes. They vary from hat-shape to Saturn-shape, with intermediate transitional forms. Occasionally, spheroidal spores without rings and hemispheroidal spores without brims are seen.

The subgenera *Hansenula* (Sydow) Lodder and *Zygohansenula* Lodder (*59*) have been combined because the author believes it is impractical to separate their component species into two groups, one having ascigerous vegetative cells which are exclusively diploid (*Hansenula*) and the other having ascigerous cells some of which are haploid and some diploid (*Zygohansenula*). Division into two subgenera is impractical as indicated by the following illustrations. Those strains of *H. saturnus* which sporulate heavily form asci promptly without conjugation by a direct transformation of the diploid cells. About 2 weeks later the few haploid cells in the culture start to conjugate and give rise to dumb-bell shaped asci containing typical spores. Such conjugation of cells at the end of the vegetative period has never before been reported for this well-known species. Conjugation, on the contrary, has not been observed by the author in those strains which sporulate poorly.

Thus, strains which sporulate heavily would qualify for *Zygohansenula* and the others would qualify for *Hansenula* were these subgenera to be maintained. *H. mrakii* and *H. suaveolens* also exist predominantly in the diploid state, yet a small number of haploid cells are present and conjugate after the diploid cells have produced spores. *H. californica* is composed of strains some of which are predominantly diploid and some predominantly haploid: hence, even in a single species, rather marked differences in evolutionary development are evident. Lodder (*61*) observed a somewhat similar situation with *Saccharomyces marxianus* Hansen and *Zygosaccharomyces marxianus* (Hansen) Guilliermond. Some strains possess only diploid vegetative cells whereas others possess exclusively haploid or a mixture of haploid and diploid cells. As a result, Lodder recommended the invalidation of the subgenus *Zygosaccharomyces*.

Stelling-Dekker (*95*) excluded from *Hansenula* species which formed true septate mycelia. However, Lodder (*59*) added to this genus *Hansenula ciferrii* which mutates into substrains with all degrees of variation between one extreme which does not produce even pseudohyphae to the other extreme which produces highly developed true hyphae. Bedford (*7*) described *H. subpelliculosa*, a species in which most strains produce short but undoubtedly true hyphae. The septa are broad, refractive, and very easily observed.

Endomyces bispora was described by Beck (*6*) in 1922 and placed in Reess' genus *Endomyces* because it produces true hyphae and ascospores. Stelling-Dekker (*95*), using the auxanographic technique, found this species incapable of utilizing nitrate, and probably for this reason transferred *Endomyces bispora* to her new genus *Endomycopsis* rather than to *Hansenula*. A culture of this species, obtained from the Centraalbureau voor Schimmelcultures, does assimilate nitrate and possesses other characteristics relating it to the genus *Hansenula*. We have included Beck's species, therefore, in the genus *Hansenula*.

Klöcker (*42*) was unable to obtain good sporulation in his original study of *H. suaveolens*, but he believed the spores were flattened on one side. Accordingly, he placed the species in *Pichia* Hansen,

Later workers (*7, 95*) have recognized its rightful placement in the genus *Hansenula* because of its physiological properties, even though they were unable to obtain spores. The author, using Klöcker's strain, has observed hundreds of spores, all of which were spheroidal. Extremely few of these appeared to have rings, and they were so thin as to be near the limit of visibility. The great majority appeared to have no ring. Therefore, the genus description has been expanded to include species having spheroidal spores without rings. Some strains of *H. suaveolens*, isolated from Minnesota soils by Robert Bouthilet, show spores which unquestionably possess very thin rings.

The hat-shaped ascospores of some of the entirely or partially haploid species have brims which are so reduced that the spores simulate the hemispheroidal spores found in certain species of *Pichia*. This is particularly true of *Hansenula angusta*.

Some new species added by the author produce no pellicles on liquid media, and the description of the genus has been amended in this respect.

Two of the new additions to the genus do not ferment any of the sugars, and some others do not produce esters. The description of the genus has been changed accordingly.

Consideration of the preceding paragraphs will indicate that redefining the genus has involved major changes in all except the two most basic characteristics—the ability to form ascospores and to assimilate nitrate.

Phylogenetic Relationships

Until 1942, when Bedford (*7*) studied the genus *Hansenula*, no member of the subgenus *Hansenula* Ludder had been reported as showing conjugation immediately preceding sporulation. The colonies were typically mat but never mucoid, and the cells were large. All species formed pellicles, produced esters, and fermented three or more sugars strongly. None required the addition of vitamins to obtain growth in synthetic medium. These yeasts are rather widespread in nature, occurring in soil, as contaminants in industrial processes, and on fruit. Bedford added another diploid species to the genus, *H. subpelliculosa*, which produced glistening, butyrous colonies and formed only a very thin, smooth pellicle, and required an external source of vitamins.

In the classification presented here, the author has added to the genus a group of species some of which are entirely haploid, and others predominantly haploid. Conjugation in this group is mainly heterogamic, often occurring between a cell and its bud. The colonies are glistening, sometimes mucoid or submucoid, but not mat or rugose. The cells are smaller than those of the diploid species. The species of the haploid group generally produce no pellicles or very thin pellicles on liquid media. Esters are produced only in traces if at all. Two sugars, at most, are fermented, and the fermentations are slow. All require the addition of vitamins to a synthetic medium for growth.

The completely haploid species of *Hansenula* are usually found in association with trees. The nature of this association is believed to protect these physiologically weaker yeasts by eliminating more

vigorous and, therefore, more competitive micro-organisms from the environment. *Hansenula capsulata* is found in the tunnels of larvae underneath the bark of certain conifers. The author is of the opinion that this yeast has become adapted to growth in the presence of pitch which is toxic to competitors, or that the association of this species with the pine bark beetles or their larvae may in some way be responsible for its survival. *Hansenula silvicola* is found in the gummy exudate of the wild cherry tree. This exudate is produced most abundantly in response to injury to the tree. *Hansenula capsulata* and, to a less extent, *H. minuta* and *H. silvicola* produce capsules which may represent one of the phases of the process of adaptation to a toxic and, therefore, protective environment.

It may be that these ancestral haploid types owe their existence to their ability in adapting themselves to surroundings which inhibit the growth of the great majority of micro-organisms. Of the diploid species thus far isolated from coniferous trees, none possess capsules, and it may be assumed they are so well adapted to their environment that this protective device is no longer needed.

The preceding paragraph indicates the author's opinion that the haploid species represent the earlier forms of the genus and the diploid species represent species more recently evolved. However, he maintains an open mind concerning this matter, which he believes will become further clarified as additional genera are carefully investigated. The reader may conclude that the haploids stemmed from the diploids by degenerative evolutionary processes, or he may agree with the author's opinion that the yeasts have shown a remarkable evolutionary accomplishment in progressing from entirely haploid species to entirely diploid species within the span of individual genera.

The present membership of the genus appears to consist of three lines, as shown by the phylogenetic diagram, figure 1. The lower line shows a loss in physiological activities as the nucleus became diploid. This line consists of three species, *H. capsulata*, *H. beckii*, and *H. canadensis*, isolated exclusively from coniferous trees. *Hansenula capsulata* is entirely haploid and ferments glucose slowly; the other two are diploid yet ferment no sugars. *Hansenula canadensis* is the only one of the three which produces esters. All three require an external supply of vitamins. The ability of *H. beckii* to produce true hyphae, and its inability to produce esters would indicate that its phylogenetic position and the position of *H. canadensis* should be reversed. The reason for the presently assigned positions is that Beck's (6) drawing of a sporulated culture suggests that *H. beckii* is partially haploid. Her statements, however, indicate that this species is entirely diploid. It is hoped that a sporogenous strain of this species will be isolated so that the positions of these two yeasts may be clarified.

There is some possibility, perhaps, that the loss of fermentative capacity and failure to become independent of the medium for vitamins are due to a well-established commensalism or symbiosis with the larvae and trees in which the three species are found. Progressive biochemical mutations might be detrimental to the established relationship. Or the relationship may be viewed as parasitism on the part of the yeast, with each succeeding species becoming increasingly more dependent upon its hosts.

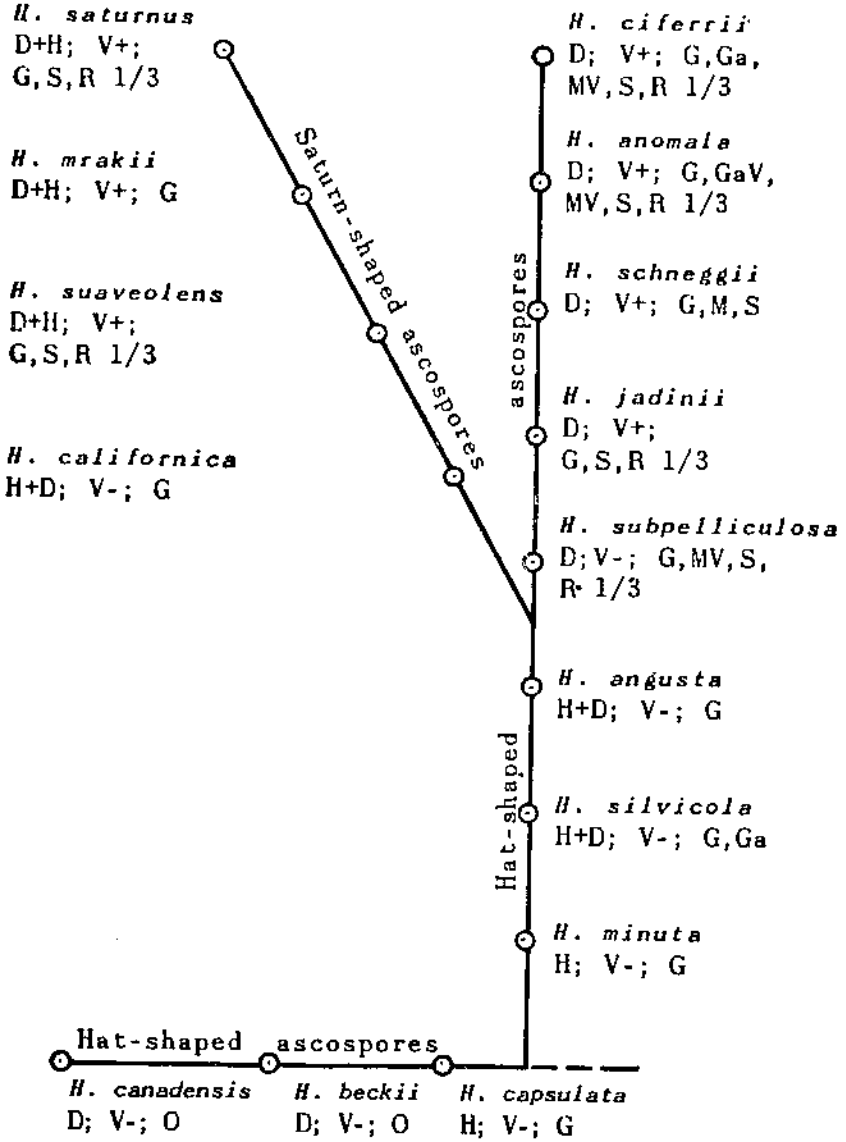

FIGURE 1.—Postulated phylogeny of the genus *Hansenula*. Key: *H*, haploid; *D*, diploid; *H+D*, more haploid than diploid cells; *D+H*, more diploid than haploid cells; *V—*, added vitamins required; *V+*, no added vitamins required; *O*, no fermentation; *G*, *Ga*, *M*, *S*, *R* ⅓ means glucose, galactose, maltose, sucrose, and raffinose 1/3 fermented, respectively; and *V*, variable.

The second branch of the phylogenetic line consists of species that likewise produce hat-shaped ascospores, but none of them were isolated from conifers. As the ratio of diploid to haploid cells increased from species to species, there was less dependence upon the medium for vitamins. There was an increase in the number of sugars fer-

mented, and an increase in the intensity of fermentation. *Hansenula minuta*, *H. silvicola*, and *H. angusta* produce ascigerous vegetative cells which are exclusively or predominantly haploid. *Hansenula minuta* produces asci exclusively by the conjugation of a cell with its own small bud. The asci of *H. silvicola* are produced, for the most part, by conjugation of a cell with its bud, but some are formed by conjugation of independent cells, and a small percentage of asci result from the direct transformation of diploid vegetative cells. These diploids are descendents of vegetative zygotes which were formed by conjugation of germinating ascospores, or by the conjugation of the immediate progeny of germinating ascospores. *Hansenula angusta* produces most asci by conjugation of cells with their buds, but many asci are produced by direct conversion of diploid cells and therefore show no conjugation.

In these three species, the brims of the hat-shaped ascospores are reduced in width and thickness, often turn sharply downward, and even may be missing. The colonies are mucoid to butyrous, and glistening. Pellicles are extremely thin or missing. A maximum of two sugars are fermented, and these are fermented weakly. Inasmuch as *H. angusta* produces relatively more diploid vegetative cells than does *H. silvicola*, it is assumed that the former species would ferment galactose if the necessary enzymes for the assimilation of this sugar were present. The strong odor of esters, as produced by the diploid species, is not produced by these three species. All three require an external source of vitamins.

The remaining species in this same line with hat-shaped spores are *H. subpelliculosa*, *H. jadinii*, *H. schneggii*, *H. anomala*, and *H. efferrii*. They are exclusively diploid. The brims of the spores are generally thick, wide and flat. None of the species, except *H. subpelliculosa*, requires an external source of vitamins. All produce esters.

Increasing numbers of sugars are fermented. In this connection it is of interest that such haploid or predominantly haploid species as *H. capsulata*, *H. silvicola*, *H. angusta*, and *H. californica* are capable of assimilating either maltose or sucrose, or both. All of the species produce a gaseous fermentation of glucose but none produces gas from either of the disaccharides. This may be due to slowness of hydrolysis. The alpha glucosidase produced by the species of the genus apparently is more specific for alpha-methylglucoside than for maltose. Thus, some strains of *H. californica* assimilate maltose and others do not, yet all assimilate alpha-methylglucoside. This same lack of specificity apparently persists among the diploid species, resulting in variable or latent fermentation of maltose.

Intensity of fermentation reaches a maximum in *H. subpelliculosa*, not only as judged by the evolution of gas but in amount of alcohol formed under anaerobic conditions. Mechanisms have been originated and adopted for the utilization of these greater amounts of alcohol and organic acids by species higher up on both branches of the fermentative line. *Hansenula schneggii*, *H. anomala*, *H. efferrii*, *H. suaveolens*, *H. wickii*, and *H. saturnus* all produce prominent pellicles composed of cells well situated for the oxidation of these compounds.

Hansenula anomala occupies an interesting intermediary position. It exists in nature as two forms. When grown on ordinary solid organic media, one form produces glistening colonies, and when

grown in similar liquid media it makes very thin pellicles and relatively large amounts of alcohol. The other form produces mat colonies, heavy pellicles, and only a small amount of alcohol accumulates in the medium. Thus, in *H. anomala*, the glistening colony form is similar to the type of colony found in the more primitive species, and the mat colony form is similar to the predominant colony type found in the more recently evolved species, namely *H. ciferrii*, *H. suaveolens*, *H. mrakii*, and *H. saturnus*.

Both forms in *H. anomala* are quite stable; the glistening colony forms in the other species just mentioned are less stable and are infrequently encountered. Thus, it is believed that the development of prominent pellicles is the principal mechanism developed by the diploid species to use the metabolites resulting from intensified fermentation processes. This oxidative capacity is revealed by the mat appearance of colonies. It may be noted that the surface layers of mat colonies can be floated free from the colonies by gently pouring water into the petri dish. The floating property of such cells is probably responsible in large degree for the formation of pellicles on liquid media. This property is not limited to highly fermentative species, for in the genera *Pichia* Hansen and *Debaryomyces* Klöcker there are nonfermentative, highly oxidative species which produce pellicles and mat colonies. In all cases, however, the production of pellicles would appear to increase the rate of oxidation in liquid cultures.

The third phylogenetic branch (fig. 1) consists of species having Saturn-shaped spores. They presumably cover a recent, short period of evolution, for none of them are either completely haploid or completely diploid. They show the same general trends in physiology as do the species with hat-shaped spores just discussed. Most strains of *H. californica* are predominantly haploid, others are predominantly diploid. The remaining species, *H. suaveolens*, *H. mrakii*, and *H. saturnus*, are haploid to a very small extent. Conjugation of adult vegetative cells occurs in all of these species, and it is mainly isogamic. *Hansenula mrakii* and *H. saturnus* occasionally produce glistening colonies, but the author has not yet obtained such variants as stable forms. *Hansenula californica* forms thin pellicles and glistening colonies while, on the contrary, the more strongly diploid species of this group form prominent pellicles and mat, rugose colonies. All produce esters. *Hansenula mrakii* does not assimilate disaccharides, and therefore it cannot ferment them, but the fermentation of glucose is strong. Because it produces thick rings on the spores, it is believed to be nearly as recent in origin as *H. saturnus*. Of the species with Saturn-shaped spores, only *H. californica* requires the addition of vitamins to the synthetic medium used in this study.

The factors used in fixing the approximate time of emergence of the first of the species having Saturn-shaped ascospores were: (1) The relative number of diploid to haploid cells in some strains of *H. californica* is greater than the ratio in *H. angusta*, but less than in *H. subpellicul*osa; (2) esters are produced by *H. californica* and *H. subpellicul*osa but not by *H. angusta;* (3) thin rings exist on the spores of *H. californica* and thin brims on the spores of *H. angusta*,

but thick brims are generally present on the spores of *H. subpelliculosa*. Fermentation reactions are of little help in placing the site of branching because galactose is not assimilated by either *H. californica* or *H. angusta* and, therefore, could not be expected to ferment. It is possible, of course, that species more primitive may be discovered in the line of species with Saturn-shaped spores, necessitating a downward shift of the branching point.

Having considered the characteristics upon which the phylogenetic relationships within the genus are based, the author believes that *the most fundamental trend is the progressive change from the haploid nucleus of the most primitive yeasts to the diploid condition of some of the most recently evolved species of yeasts.* The second trend is a lessening of the dependence on an external supply of vitamins. As may be noted in figure 1, this second characteristic has apparently been more recently acquired than either fermentative ability or the diploid state. A third trend is toward the development of the capacity to cause gaseous fermentation of an increasing number of sugars. This characteristic, however, is of little value in placing certain species which do not assimilate, and therefore cannot be expected to ferment, some of the sugars.

Presumably the most primitive type of conjugation in the genus *Hansenula* involves (1) a large cell and its small bud. Successively higher levels of development are indicated by (2) heterogamic conjugation of independent cells, (3) isogamic conjugation of independent cells, and (4) conjugation of germinating ascospores, conjugation of their immediate progeny, or nuclear fusion within either a single germinating ascospore or one of its immediate descendents. Types (1) through (3) occur at the end of the vegetative stage of growth of haploid cells; type (4) occurs at the beginning of the vegetative stage of those cells which go through the vegetative phase as diploid cells.

It is of interest that the ability to form hyphae (table 2) generally increases as the species become more diploid. The reverse is to be expected if one follows the common belief that ascomycetous yeasts are descendants of a filamentous, haploid progenitor such as *Eremascus fertilis* Stoppel. It may be noted, however, that all diploid species of *Hansenula* produce hyphae, whereas less than half of the haploid species do. It may be noted also that the two species with the most highly developed hyphae occupy positions at or near the diploid ends of two phylogenetic branches. One of these, *H. ciferrii*, presents an interesting anomaly in the ability to form hyphae. The original isolate produces neither pseudohyphae nor true hyphae, but it does form some aberrantly shaped large cells which indicate an abortive attempt to produce hyphae, as shown by the occasional presence of a septum in such abortive cells. This strain gives rise spontaneously to variants capable of forming an abundance of true hyphae. All these variants have the same unique assimilation reactions. The ability of a strain to exist in both filamentous and nonfilamentous forms may indicate that too much taxonomic importance has been given this characteristic in the past.

It is obvious that the author does not agree with Guilliermond's (*34*) belief that haploid and diploid yeasts represent two distinct phyloge-

netic lines, with the haploid yeasts stemming originally from a form similar to *Eremascus* and the diploid yeasts stemming from *Taphrina* Fries.

SUMMARY

1. The genera *Hansenula* and *Pichia* are maintained as separate entities.

2. The limits of the genus *Hansenula* as redefined in this study are considerably broadened as compared with previous definitions. The genus includes species which form one to four ascospores, assimilate nitrate, and reproduce asexually by budding, or by budding accompanied by the formation of pseudohyphae or true hyphae. The ascigerous vegetative cells may be exclusively haploid, exclusively diploid, or composed of rather definite proportions of haploid and diploid cells. The ascospores are hat-shaped, Saturn-shaped, hemispheroidal, or spheroidal. Pellicles may or may not be formed; sugars may or may not be fermented with the evolution of gas; esters may or may not be produced. Colonies may be mat or glistening, butyrous, submucoid, or mucoid. A moderately large number of carbon sources are assimilated.

3. Of the 15 species which are placed in the genus, 6 are new species and 3 are new combinations. New species are *Hansenula capsulata*, *H. canadensis*, *H. minuta*, *H. silvicola*, *H. angusta*, and *H. mrakii*. New combinations are *H. beckii*, *H. californica*, and *H. jadinii*, previously known as *Endomycopsis bispora*, *Zygohansenula californica*, and *Saccharomyces jadinii*, respectively. The change in species epithet of *Endomycopsis bispora* was necessitated by the previous use of the name *Hansenula bisporus* by Nannizzi.

4. Previously described varieties of *Hansenula anomala* have been eliminated as being inconsequential.

5. Possible phylogenetic relationships among the species of *Hansenula* are indicated. The ratio of ascigerous haploid to diploid cells is the primary characteristic on which the phylogeny is based. Secondary characteristics are variations in vitamin requirements, the number of sugars fermented, differences in the structure of the ascospores, the production of esters, the formation of pellicles, the type of colony, and size of the vegetative cells.

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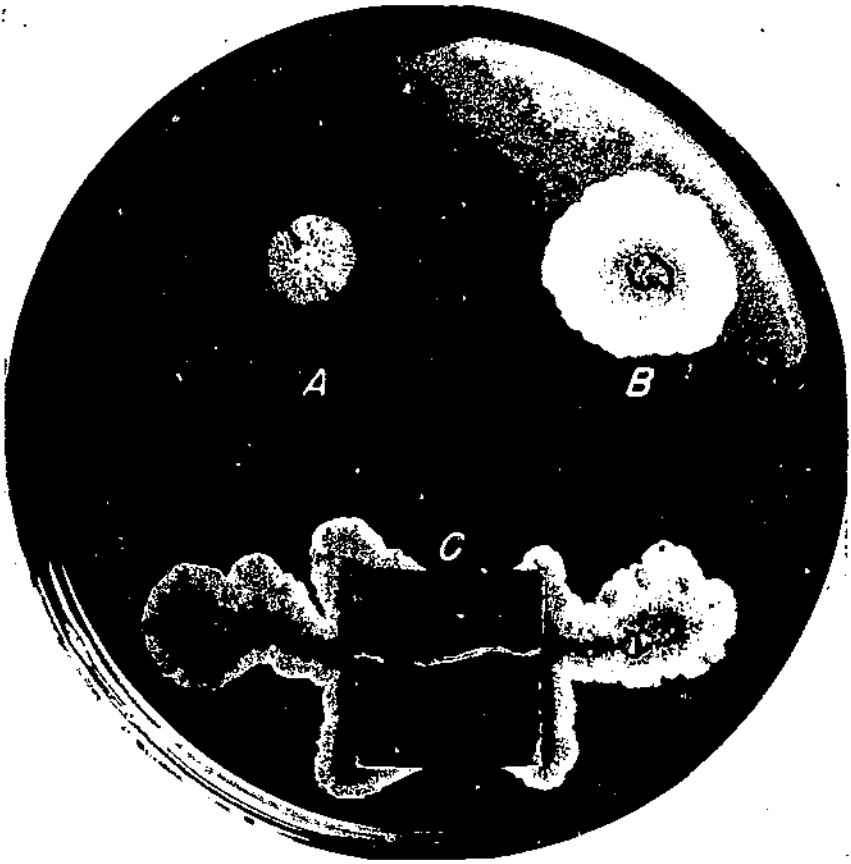
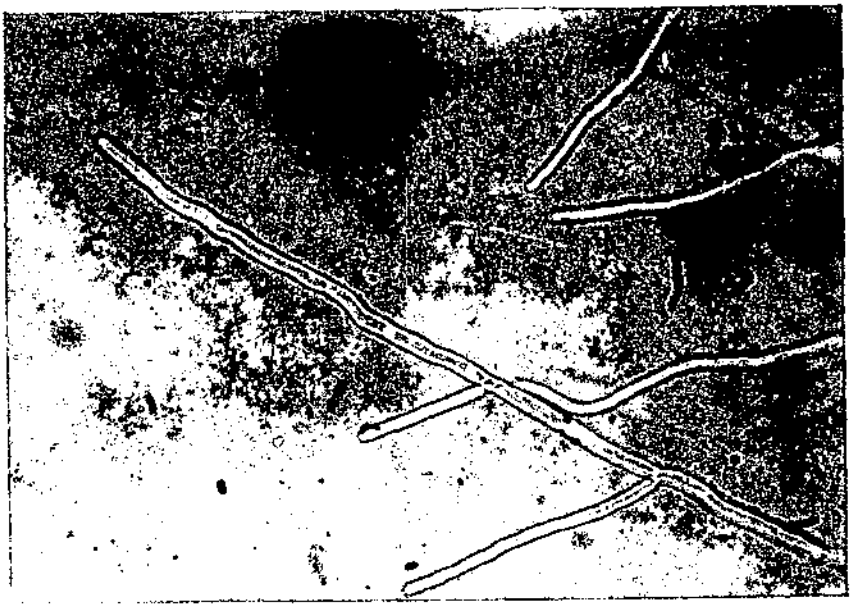
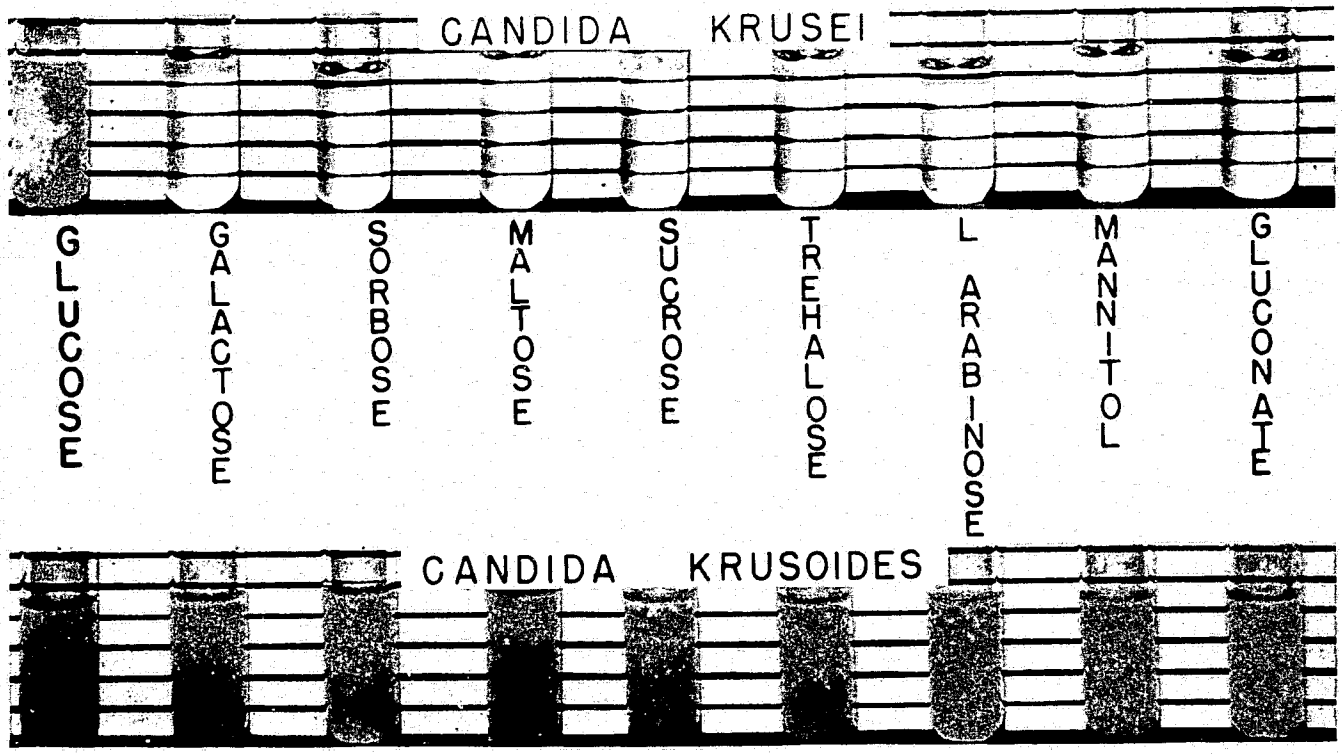


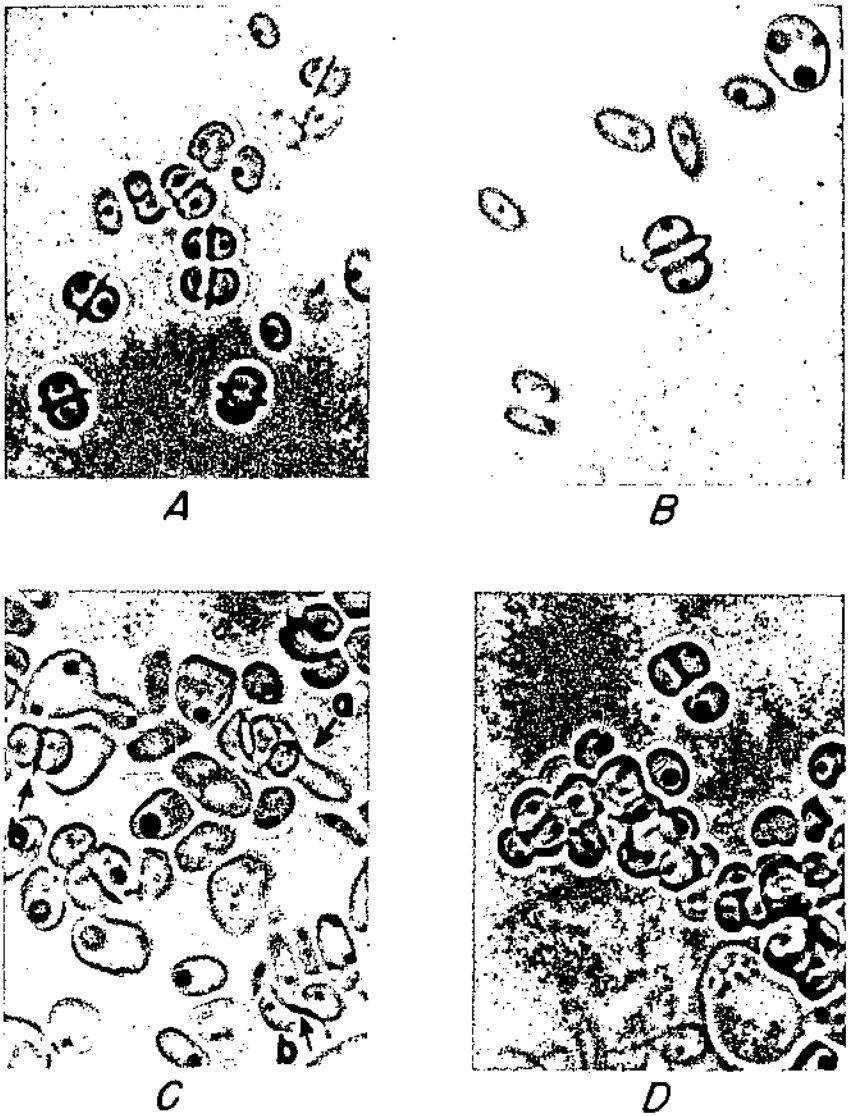
A dalmau plate of a species of *Candida*. *A*, dalmau spot, anaerobic, grown underneath a coverglass from a point inoculation. *B*, colony, aerobic, grown from a point inoculation. *C*, dalmau streaks consisting of aerobic and anaerobic areas. Dalmau preparations are used for the morphological study of all yeasts, filamentous and nonfilamentous.



Example of true hypha. The septa are characteristically straight and refractive. The distance between the tip of the hypha and the first septum is substantially greater than the distance between the first and second septa. × 440.



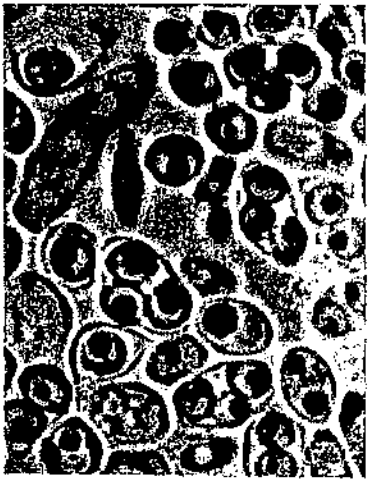
Assimilation of various carbon sources by two species of *Candida*, both of which produce an active gaseous fermentation of glucose, but neither of which ferment the common disaccharides. Assimilation is indicated by the degree to which the dark lines behind the cultures are obscured.



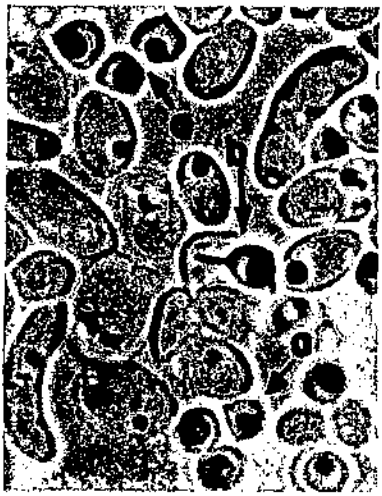
Ascospores representative of the genus *Hansenula*: *A*, *Hansenula capsulata* Dekker, refractive, hat shaped ascospores in typical brim to brim arrangements characteristic of this species. The spores increase markedly in size after liberation from the asci. *B*, *Hansenula etandonis*, although the ascospores of this species generally are hat shape, some of the spores, like the two shown here, are ellipsoidal with nearly tangential rings, thus resembling ascospores of the Saturn type. *C*, *Hansenula silvicola*. The wide opening between the two cells forming the asci (*a*), when accompanied by hat shaped ascospores (*b*), permits recognition of this species. Connections, which are much wider than the one shown here, are frequently found. *D*, *Hansenula augusta*—only a few of the ascospores of this yeast are distinctly hat shaped; most have very reduced brims.

The photomicrographs in both plates 4 and 5 are of strains grown on malt extract agar. The magnification is 2245 times.

A



B



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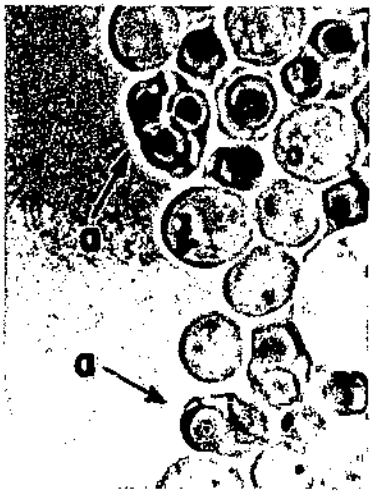

D

Ascospores representative of the genus *Hansenula*. A, *Hansenula anomala*: the free, hat shaped ascospores of this species, in common with ascospores of most of the other species of the genus, tend to agglutinate in clusters. B, *Hansenula saturnus*: the rings on the Saturn shaped ascospores are so thin that they are difficult to observe with the oil immersion objective, but the apiculate appearance imparted by the rings is a clue to their presence. C, *Hansenula minuta*: the angular appearance of the spores (*a*) indicate the presence of rings; rarely ascospores are observed which possess thick, eccentric appendages (*b*). D, *Hansenula saturnus*: ascospores of this species have the thickest rings of the species having Saturn shaped ascospores.

END