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CLASSIFICATION OF YEASTS FROM THE FERMENTATION OF COMMERCIALLY BRINED CUCUMBERS [1,2]

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During the fermentation of cucumbers in salt brines, a part of the microbiological activity is due to yeasts. Their portion of the fermentation proper is considered gaseous in nature and covers a wide range with respect to brine concentration, the maximum salt tolerance observed under commercial conditions being upwards of 20 to 22 percent by weight. Since a vigorous gaseous fermentation by yeasts in cucumber brines is associated with the production of "bloaters" or hollow cucumber stock, their behavior is of economic importance to the industry. Previous work on the subject has dealt chiefly with population studies. Specifically, these reports have been concerned with the influence of brine concentration (Etchells, 1941; Etchells and Jones, 1943); the addition of organic acids (Jones, *et al*, 1940); the addition of sugar (Veldhuis, *et al*, 1941); and more recently in connection with the amount of fermentation gas evolved and its composition (Etchells, Fabian, and Jones, 1945).

While the above work established that yeasts were associated with cucumber fermentations under conditions typical of the industry, it gave no information as to the species that might be growing in the brines. The present investigation was undertaken with a two-fold purpose; (1) to obtain more specific information on the principal types of yeasts occurring during the gaseous fermentation of salt-stock cucumbers, and (2) to determine whether a definite sequence of yeast types occurred during the period of general yeast activity. Basic information of this nature is of prime importance since only through such studies can suitable brining procedures or control measures be properly developed, with the ultimate objective of placing the cucumber pickling industry in the group of controlled fermentation industries.

Studies of the chiefly oxidative *surface* yeasts, associated with luxuriant scum formation, constitute the bulk of the information known concerning the yeast types present in connection with brined, salted, and pickled vegetables. The latter types are commonly present on the surface of brines exposed to air but sheltered from direct sunlight. At the outset in the present work a clear-cut distinction should be made between the film-forming yeasts and the fermentative *subsurface* yeasts in commercial cucumber brines which are responsible for the gaseous fermentation.

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[2] This study was carried out under a cooperative project with the Department of Horticulture of the North Carolina Agricultural Experiment Station.

It has been pointed out (Etchells and Jones, 1946; Etchells, Jones, and Lewis, 1947) that conditions permitting active growth of film-yeasts, such as afforded by open containers sheltered from direct sunlight, are not generally favorable for obtaining the best information as to the predominating subsurface yeast flora contributing to the gaseous fermentation. In the present study, 40 of the 42 commercial fermentations in large vats were under outside conditions. Hence, growth of surface scum was controlled by direct sunlight. In the case of the two sheltered vats, film-yeasts were present on the brines but the regular sampling technique (from approximately 6 feet beneath the brine surface) reduced contamination of the subsurface brine samples with film-yeasts to the point where it was no problem. Thus, species of *Debaryomyces*, *Pichia* and *Mycoderma* such as found by Mrak and Bonar (1939) from surface films of various brined foods would not ordinarily be expected in any numbers. This point is substantiated in the data to follow.

INTRODUCTORY STUDIES

During the 1946 brining season a beginning was made on the yeast classification work with 218 isolates which were obtained from 22 unsheltered fermenting vats, the latter being divided equally between two commercial plants (A and B) located in eastern North Carolina. The brining treatments were similar in that the initial concentration employed by both plants to cover the green cucumbers was designed to equalize at about 10 percent. They differed chiefly in the rate of increase of brine strength; plant A increased the brine strength to 15 percent in about 6 weeks, whereas plant B took about one-half that time to reach the same strength. At both plants, the fermentations selected were about 18 to 21 days old and all showed active gas evolution. These vats were sampled only three to four times each during the period from the middle of July until the first week of September. It was intended that this introductory study should give information on how many generic groups might be involved and the possible influence of the two brining treatments on these groups.

It developed that the 218 isolates could be divided into 213 that were asporogenous and 5 that were sporogenous. The asporogenous group was divided into 138 isolates which had small to tiny cells, fermented 3 sugars (glucose, sucrose, and raffinose ⅓), and were strongly nitrate positive; and 75 isolates that were round to oval, fermented both lactose and maltose, and were moderately to weakly nitrate positive. The first group was classified as *Torulopsis* and predominated during the first part of the fermentation; the second group was classified as *Brettanomyces*, and predominated during the latter part. Further, the two groups had one characteristic in common; both were short-lived on slant cultures of ordinary dextrose agar. This fact nearly resulted in loss of the collection of these yeasts. It was first discovered in connection with the tiny-celled type, and a large proportion of the isolates obtained from the brine samples taken during July and

August were dead by the latter part of September. The 75 lactose and maltose fermenters were then inspected and found to be in a very poor state of viability. These were finally revived by adding lactose broth directly to the slant culture. Thereafter, they were kept in 4 percent lactose broth at room temperature with excellent results for periods up to 9 months.

In October 1946, an effort was made to re-isolate the tiny-type *Torulopsis* from the original brine samples of the 22 vats taken during the two samplings of July and August and which had been refrigerated (3–4°C.). This was only partially successful. A possible explanation for this appeared to lie in the fact that 74 of the 75 *Torulopsis* isolates obtained came from the 11 fermentations followed at plant B, where the brines, as compared to those from plant A, had very little acidity. The rapid increase in brine salinity employed at plant B afforded very little opportunity for a fermentation by the lactic acid bacteria. This would indicate that the tiny-cell type *Torulopsis* was sensitive to the brine acid of the plant A samples during storage in the refrigerator.

It was indicated that the sporogenous isolates, classified as *Hansenula* and *Torulaspora*, were obtained less frequently since only 3 cultures of the former genus and 2 of the latter were found.

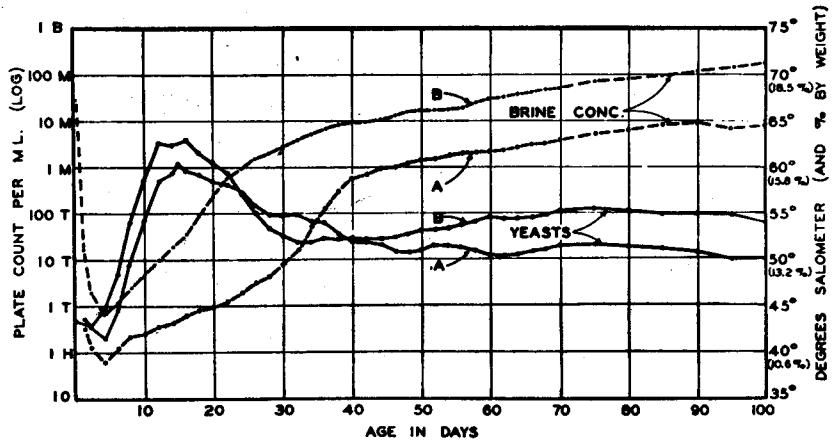

Figure 1. Yeast populations and the increase of brine concentration at two commercial plants (A and B).

EXPERIMENTAL

With the above points in mind, the work for the 1947 season was continued at the two plants mentioned in the 1946 study and included 10 fermentations at each plant. Sampling started shortly after the vats were filled and brined and continued at frequent intervals for approximately 100 days. Individual vats were chosen at random and represented different periods of the season during which cucumbers were being brined. Fer-

mentations occurred for the most part over a period from July until mid-October with brine temperatures within the range of 25 to 27°C., except for the period from mid-September to mid-October when they were 20 to 25°. Eighteen vats were located outside and were unsheltered; two at plant B were sheltered. The brine temperature of the latter two vats averaged about 3° lower than those that were outside. The rate of increase in brine strength employed by each plant, and the resulting yeast populations ob-

TABLE 1. ORIGIN OF 1226 YEAST ISOLATES FROM 20 FERMENTATIONS AT 2 COMMERCIAL PLANTS (1947 SEASON)

Vat Number (and Date Filled)	Vat Capacity (and cucumber size) [1]	Number of Isolates Obtained Using Glucose Agar		Fermentation Period Covered by Isolations
		Without Salt	With Salt	
PLANT A				
	bu.	*(no.)*		*days*
F–161 (7–3)	415	(3's)	40	27	11th to 104th; (16,14) [2]
F–163 (7–3)	425	(3's)	37	26	7th to 76th; (15,15)
F–164 (7–3)	450	(3's)	45	30	7th to 104th; (17,16)
F–167 (7–3)	725	(3's)	40	31	7th to 104th; (15,15)
F–221 (7–6)	475	(3's)	35	28	8th to 101st; (15,14)
F–236 (7–6)	495	(3's)	39	28	8th to 101st; (16,15)
F–287 (7–13)	580	(2's)	25	22	1st to 94th; (14,12)
F–288 (7–13)	320	(3's)	22	20	1st to 94th; (12,10)
F–291 (7–20)	600	(2's,1's)	37	21	1st to 87th; (12,11)
F–10 [3] (7–20)	390	(1's)	21	15	8th to 59th; (9,8)
Sub-total			341	248	
Total			589		

[1] Plant A: No. 1's, small cucumbers up to 1" in dia.; No. 2's, medium, up to 1½" dia.; No. 3's large, above 1½" dia.

Plant B: FR refers to field run cucumbers, ungraded as to size.

Plant A used the National variety; plant B, the Earliest of All.

[2] Figures in parentheses in column represent the number of brine platings on glucose agar without salt and with salt, respectively, from which isolations were made.

[3] Vat emptied by plant before observations were completed.

[4] Vats sheltered from weather; all others in the open.

Vat Number (and Date Filled)	Vat Capacity (and cucumber size) [1]		Number of Isolates Obtained Using Glucose Agar		Fermentation Period Covered by Isolations
			Without Salt	With Salt	
	bu.	(*no.*)			*days*
PLANT B					
21–20 (7–2)	650	(FR)	41	32	8th to 105th; (15,15) [2]
21–21 (7–3)	650	(FR)	36	25	11th to 104th; (15,14)
22–21 (7–3)	610	(FR)	41	24	11th to 104th; (15,12)
1–7 [3] (7–3)	625	(FR)	40	30	4th to 76th; (15,14)
23–3 (7–5)	850	(FR)	35	28	2nd to 102nd; (15,14)
23–4 (7–5)	775	(FR)	42	26	2nd to 102nd; (16,13)
14–10 [4] (7–9)	750	(FR)	42	23	5th to 98th; (16,12)
15–10 [4] (7–9)	1000	(FR)	31	26	8th to 98th; (13,13)
B1–1 (7–19)	620	(FR)	42	20	9th to 88th; (12,10)
B2–13 (7–19)	500	(FR)	31	22	9th to 88th; (12,11)
Sub-total			381	256	
Total			637		

tained, are shown in figure 1. The curves represent mean values for 10 fermentations at each plant.

The brine samples were collected, plated, and counted according to methods previously described by Etchells (1941) and later revised by Etchells and Jones (1946). Two collections of isolates (1,226 in all) were obtained; one from acidified dextrose agar, the other from the same medium containing 8 percent salt by weight. The purpose of the salted medium was to determine whether any types were present that required salt in relatively high concentrations for growth. None, however, was found and the plate counts for the fermentations were essentially the same on either medium. At each plating interval the plates from the high dilutions were set aside for picking representative colonies.

The details concerning the origin of the isolates obtained during the 1947 season are shown in table 1. Of the total number of isolates picked, approximately 48 percent came from the fermentations at plant A, and 52 percent from plant B. In both cases there were fewer isolates picked

from salt agar, since it had originally been intended only to supplement those from the unsalted media as a check against missing any of the halophilic types mentioned. In all but two cases the cultures isolated covered the fermentation period adequately from the standpoint of yeast activity. The exceptions were vat F–10 at plant A, and vat 1–7 at plant B; both were emptied before the last sample could be obtained.

The methods employed and classification systems used were essentially those outlined in the monographs by the Dutch workers, Stelling-Dekker (1931), Lodder (1934), Diddens and Lodder (1942), and Custers (1940). The work of Bedford (1942) was used for the genus *Hansenula*. Also, the excellent review by Henrici (1941) was most helpful, as was the generous assistance of Dr. L. J. Wickerham of the Northern Regional Research Laboratory, U. S. Department of Agriculture.

Certain modifications and additions were made in connection with the taxonomic tests of the Dutch workers. The basal broth for sugar fermentations consisted of peptone 0.5 percent, yeast extract 0.25 percent, and 3 to 4 percent solutions of the test sugars in 15 x 150 mm.-tubes containing 10 x 45 mm.-inserts. In order to determine the status of the raffinose fermentation (⅓ or complete fermentation), both raffinose and melibiose (in 4 percent solutions) were used. Here, owing to cost of these compounds, small 8 x 75 mm.-tubes were used either with a petroleum jelly seal or one of heavy mineral oil. The latter seal was preferred for the slow fermentation characteristic of some of the yeasts encountered. The basal medium used for sugars was also employed for determinations on the utilization of ethyl alcohol (3%). Inoculated and uninoculated basal medium controls were run in all cases.

For the nitrate test, the synthetic medium of Stelling-Dekker was used, but in liquid form fortified with an addition of 0.1 percent of yeast extract, which was necessary as a supplementary source of growth accessory substance for one yeast group (*Torulopsis*). Wickerham (1946) has shown that certain species of yeasts previously recorded as negative for utilization of ammonium sulfate, urea, and asparagine were found to be positive when required amounts of pure vitamins were added. In the present work the test for nitrate reduction was not by growth differential but by the common test for nitrites used by bacteriologists (S. A. B. Manual, 1937). In our opinion, the test as we use it is more satisfactory than the growth test on slants particularly for species where slant growth is ordinarily scant and for those which require a supplementary source of vitamins. The chemical test is not infallible, however, and the precautions outlined in the Manual should be followed, particularly in case of negative results. The test, according to Wickerham (1947), should be repeated at about 2-day intervals of growth to establish maximum nitrite accumulation and to avoid false negatives. The use of both slant and broth cultures for testing has proved very helpful in our hands when nitrate reduction is weak, or nitrite accumulation is of small magnitude.

For the sporulation tests a vegetable juice medium prepared from a

commercially canned product was used.[3] This medium was essentially the same as that originally described by Wickerham, Flickinger, and Burton (1946). We prepare the medium as follows: Equal parts of juice and water are adjusted to pH 6.0 by addition of 10 percent sodium hydroxide. Then, 2 percent agar and 0.4 percent calcium sulfate are added, the mixture is heated to dissolve the agar, tubed, and sterilized at 15 lbs. pressure for 15 minutes. The final pH is usually about 5.8. The medium has a tendency to foam and wet the plugs, but this can be corrected for the most part by placing the racks of tubed media in a boiling water bath for 10 minutes and then autoclaving carefully. The principal difference in the two media is the higher pH (6.8) preferred by Wickerham *et al.* and the addition of a yeast cake. Also, they prefer the use of fresh slants not over 8 hours old.

Early in the present study a test was made using the modified vegetable juice medium and seven other sporulating media, i.e., potato plugs, potato agar, dilute carrot juice agar (McKelvey, 1926), carrot plugs, Gorodkowa's agar, dilute cucumber juice agar, and gypsum slants (Lindegren, 1945). Two *Torulaspora* isloates and 4 *Hansenula* isolates from brines, together with 3 known species of *Saccharomyces* and 2 of *Hansenula*, were tested on each medium. The media were observed at intervals for a period of several weeks. Considering all genera as a group, the vegetable juice medium was considered superior for indicating spore formation; Gorodkowa's was next best. Carrot agar was the best individual medium for the *Hansenula*, but very poor to negative sporulation for these yeasts was obtained on the 2 potato media. However, the potato media proved superior for the *Torulaspora* isolates. The results for the known species were essentially the same in that the vegetable juice was the best single medium. One strain of *S. ellipsoideus* gave poor results on all media used. As a result of this and subsequent tests with known species from the Northern Regional Research Laboratory (Peoria), the vegetable juice agar was adopted as a screening medium for dividing all the brine isolates into sporogenous and asporogenous types. Representatives of the latter types were thoroughly tested, however, on several other media before recording negative results.

The test for mycelium production was made using point inoculations on poured plates of cornmeal agar to which centered cover glasses were applied (Wickerham and Rettger, 1939). The potato medium of Lodder was also tried but the above procedure gave better results. Modifications of Custers' "standard test" were used in detecting acid production in aerobic culture. Several concentrations of suspended chalk in slants and poured plates of both regular glucose agar and a synthetic agar with glucose were tried. The regular glucose agar (used in this and other tests where mentioned) consisted of the following ingredients; Glucose, 2 to 5 percent; yeast extract, 0.25 percent; $K_2H\ PO_4$, 0.1 percent; salt, 0.5 percent; peptone, 0.5

[3] Manufactured by Campbell Soup Co. under the name "V-8." It is probable that other products of essentially the same composition would prove satisfactory.

TABLE 2. SUMMARIZED MORPHOLOGICAL, CULTURAL, AND BIOCHEMICAL CHARACTERISTICS OF 1424 YEAST ISOLATES FROM 42 COMMERCIAL CUCUMBER FERMENTATIONS.

Generic group, species allocation, number of isolates.[1]	Vegetative Cells	Glucose Agar Growth	Glucose Broth Growth
I. *Torulopsis* Berlese			
A. *T. caroliniana* sp. nov. 717 isolates 49.6%	From veg. juice agar and glucose agar, cells small to tiny, av. 1.5–2 × 3–4μ. Short-oval to egg-shaped and in clusters; some elongated.	White, moist, glistening; secondary colonies along streak. Scant amount of growth. Cells short-lived; death in approx. 3–6 wks. on slants or in broth.	No ring; no film; broth clears rapidly.
B. *T. holmii* (Jörgensen) Lodder 4 isolates 0.3%	Cells predominately short-oval, 3–4 × 4–5.5μ.	Light buff, glistening, practically smooth.	No ring; no film.
II. *Brettanomyces* Kuff. et van Laer			
A. *B. versatilis* sp. nov. 561 isolates 38.8%	From broth 3–4 × 4–5μ, round to oval, some elongated. On agar slants, generally the same size. Characteristic cell colonies in broth. Ogive cells in old cultures.	Young growth white, glistening, smooth; old cultures develop light pink to lavender color from center. Cells short-lived in slant cultures. Abundant growth.	Ring; no film.
B. *B. sphaericus* sp. nov. 27 isolates 1.9%	Cells predominately round, but also may be slight oval; 3–5μ dia., some slightly larger; clusters of many cells. Ogive cells in old cultures.	White, smooth, glistening, abundant growth.	Ring; no film.
III. *Saccharomyces* (Meyen) Reess Subgenus *Zygosaccharomyces* Barker			
A. *Z.* sp 50 isolates 3.5%	From broth, cells generally large, mostly oval and in cell colonies. Av. 4–5 × 4.5–7μ; some larger; elongated chains of cells on old media.	White to light cream; dull; irregular; surface gently folded; moderate to abundant growth.	Ring; no film.
B. *Z.* sp. 9 isolates[5] 0.6%			
IV. *Hansenula* Sydow *H. subpelliculosa* Bedford 49 isolates 3.4%	Cells large, round, oval, sausage-shape, irregular; 6 × 8μ, some elongated. Clusters of several cells.	White, smooth, moist, glistening, abundant growth; discolors with age.	Ring; very slight film.
V. *Torulaspora* Lindner *T. rosei* Guillermond 6 isolates 0.4%	Cells predominately round to slight oval, 3–6μ dia., mostly 4.5μ. Clusters of three to four cells.	White, smooth, glistening, abundant growth. Some strains with granular surface.	Ring; no film.
VI. *Kloeckera* Janke *K. magna* (De 'Rossi) Janke 1 isolate 0.1%	Young cells, oval or apiculate, 3–6 × 5–12μ singly or pairs.	Grey, thin, moist, glistening, smooth; transparent wavey edges.	No ring; no film.

Growth in Ethyl Alcohol	Nitrate Assimilation	Action on Sugars		Ascospore Production	Outstanding Characteristics
		Fermented	Not Fermented		
Absent	Strongly positive	Glucose (2)[2] Sucrose (3) Raffinose (2)	Maltose Galactose A [3] Lactose Melibiose	Not found on 8 sporulation media observed over period of several months.	Small cell size; short-lived nature; strong nitrate test; rapid fermentation; scant amount of growth; characteristic colony formation.
Moderate	Negative	Glucose (2) Sucrose (2) Galactose (2) Raffinose (2)	Maltose Lactose Melibiose	Not found on sporulation media observed over period of several months.	Rapid fermentation of glucose, sucrose, galactose, and raffinose (1/3); short, oval cells.
Good growth with ring; no film.	Positive	Glucose (4) Sucrose (6) Maltose (5) Galactose (8) Lactose (8) Raffinose (12) Melibiose (12)		Not found on 8 sporulation media observed over period of several months.	Fermentation of maltose, lactose, and melibiose; usually a slow, prolonged fermentation behavior; death of cells in aerobic culture due to acid production; high salt tolerance.
Moderate growth with ring; no film.	Positive (latent)	Glucose (7) Maltose (12)	Sucrose A Galactose A Lactose Raffinose Melibiose	Not found on sporulation media observed over period of several months.	Predominately round cells; slow, prolonged fermentation of glucose and maltose; vigorous assimilation of galactose; high salt tolerance; death of cells in aerobic culture.
Moderate growth with ring after one month.	Negative	Glucose (3)	Sucrose [4] A Maltose [5] Galactose A Lactose Raffinose A Melibiose	Positive; isogamic or heterogamic conjugation; 1–3 spores per ascus. Av. spore 3 × 4.5μ; round to oval.	Limited fermentation power; variable action on sucrose in low concentrations; typical sporulation; high salt tolerance.
Moderate growth with ring; no film.	Positive	Glucose (3) Sucrose (3) Raffinose (7)	Maltose [6] A Galactose A Lactose Melibiose	Positive; usually hat-shaped spores, 1–4 per ascus; spores mostly 2 × 3μ; occasionally round spores, 3μ dia.	Lack of significant film formation; vigorous galactose assimilation; variable action on maltose; typical sporulation.
Moderate growth with very slight ring; no film.	Negative	Glucose (1.5) Sucrose (1.5) Raffinose (1.5)	Maltose Galactose Lactose Melibiose	Positive; conjugation tubes formed prior to spore formation, but no conjugation. Spores smooth, round, 1–4 per ascus, 3–4μ dia.	Formation of long conjugation tubes and absence of distinct evidence of conjugation; 1–4 characteristic spores, with oil drop.
Negative	Negative	Glucose	Sucrose Maltose Galactose Lactose Raffinose Melibiose	Not found on several sporulation media.	Apiculate cells and very strong acid production, exceeding the *Brettanomyces*.

[1] Percentages shown on basis of total isolations made (1444); 20 isolates (1.4%) remain not fully classified but include 3 isolates of *Candida*, 4 of *Endomycopsis*, and 1 atypical *Torula spora*. Also, totals include 218 cultures from 1946 season in the following groups: IA, 138; IIA, 75; IV, 3; V, 2.

[2] Numbers in parentheses refer to approximate age in days for maximum gas in insert.

[3] Assimilation of compound.

[4] Action on sucrose variable ranging from none to a latent fermentation.

[5] Nine of 59 isolates ferment maltose strongly in 3 days and apparently belong to a different species.

[6] Action on maltose variable, ranging from no fermentation to a latent, weak fermentation.

percent; and agar, 1.5 percent. The synthetic medium was the same as that used by Stelling-Dekker except it contained 0.01 percent yeast extract.

The most clear-cut results were obtained with poured plates of chalk glucose agar receiving a 2 mm. spot inoculation of the test yeast. At least two levels of chalk suspension, i.e., about 0.5 and 0.8 percent, were found to be helpful in determining the degree of acid production indicated by the cleared zone around the yeast growth. In preparation, the finely ground chalk should be kept in suspension and the plates poured just short of the agar solidification point.

RESULTS

The characteristics of the genera found during the 2-year study are shown in table 2. Column 1 of this table gives summarized data on the predominance of individual species.

Predominating Genera

The predominant genera found fall into the following groups: (1) genus *Torulopsis*; (2) genus *Brettanomyces*; (3) genus *Saccharomyces*, subgenus *Zygosaccharomyces*; (4) genus *Hansenula*; (5) genus *Torulaspora*; (6) genus *Kloeckera*; plus an initial group, genera *Rhodotorula* and *Debaryomyces*, and an unclassified group of 20 isolates.

Group I, Genus *Torulopsis*: Of the 721 cultures isolated in this group, four were identical to *T. holmii* (Jörgensen) Lodder and require no further discussion. The remaining isolates were a homogenous group having small to tiny cells, which ferment glucose, sucrose, and raffinose (⅓) rapidly, are short-lived, strongly nitrate positive, and apparently sensitive to the acid they produce. On chalk-containing media definite acid production is observed. They are rather tolerant of solutions of high osmotic pressure as indicated by rapid growth and gas production in broth containing 40 and 60 percent by weight of sucrose. In laboratory tests their tolerance to salt is > 15 but < 20 percent by weight. Their tolerance in cucumber brines is probably slightly above 15 percent.

These yeasts have certain characteristics in common with the three species isolated by Kroemer and Krumbholz (1931) and described in detail by Krumbholz (1931) as the "small-celled osmophilic *Saccharomyces*" which were identified as *S. stellatus*, *S. bacillaris*, and *S. granulatus*. Later, Lodder (1934) reclassified the first two as *Torulopsis stellata* and *T. bacillaris*, but did not mention the status of *S. granulatus*. We have been unable to locate a culture of this species either in this country or abroad.[4] Krumbholz describes *S. granulatus* as very small and short-lived; but it cannot be definitely identified with our group of isolates since he did not report on response to the nitrate test; nor did he find growth in raffinose, although dextrose and sucrose were fermented. Both *T. stellata* and *T.*

[4] Later, in June 1948 Miss N. J. W. van Rij of the CBS advised us that they had requested this yeast from Krumbholz shortly after his paper was published but the culture had died.

bacillaris ferment the same sugars as our isolates but are nitrate negative as recorded by Lodder (and confirmed by us in repeated tests) and would be eliminated on this count alone.

Although it appears that a definite relationship exists between the two small-celled osmophilic species of Kroemer and Krumbholz and the small-celled brine isolates, the relationship is not sufficient to prohibit species separation. Hence, we propose to place our isolates as *Torulopsis caroliniana* sp. nov.[5]

Group II, Genus *Brettanomyces*: The isolates placed in this genus consisted of 561 that fermented all seven test sugars (including lactose and maltose) and 27 that fermented only two, glucose and maltose. This second group are nearly spherical in shape (with a tendency for ogive shape in old cultures) and the fermentation of the two sugars is very slow. Also, they are inclined to be short-lived on slant cultures and death is presumably due to acid production as indicated by clearing of chalk agar. The nitrate test, however, is generally weak and latent, appearing after about 10 days on slant cultures. This point, plus the absence of any pseudomycelium, may appear to raise a question as to the allocation of this group to the *Brettanomyces*; but neither of these characteristics is considered critical by Custers (1940) in his thorough study of the genus. The fact also that they were obtained during the latter stage of fermentation (from six vats at plant B only, 1947) when the other *Brettanomyces* species was present, is an additional strong point in favor of their inclusion in this genus. They will be therefore considered as such for the present under the proposed name of *Brettanomyces sphaericus* sp. nov.

The group of 561 cultures that fermented all seven sugars was similar to *B. claussenii* Custers; differing in that they fermented raffinose and melibiose (equivalent to complete raffinose). They differed also, in that the cells were round to oval as compared to the more elongated cells of

[5] Since completion of the present investigation, a culture labelled *Torulopsis lactis-condensi* (Hammer) was received from the "Centraalbureau." It was isolated by Hammer in 1919, from gassy sweetened condensed milk. Our study of this yeast showed, in part, that it was inclined to be small-celled, and fermented glucose, sucrose, and raffinose rapidly (1 to 2 days). Furthermore, it reduced nitrate very strongly. The fermentation of raffinose was somewhat surprising in view of the negative test reported by Olson and Hammer (1935) in a redescription of the original characteristics (Hammer, 1919) for *Torula lactis-condensi*. The nitrate reduction test had not been used in either study. Thus a close relationship undoubtedly exists between this yeast and our *Torulopsis caroliniana* sp. nov. However, the cells of *T. lactis-condensi* are distinctly larger than those of our isolates and have a somewhat different shape and internal appearance. Furthermore, *T. lactis-condensi* resembles *T. caroliniana* only to a limited degree in respect to being short-lived on both liquid and solid media (including chalk agar); nor is *T. lactis-condensi* critical in this property with respect to usual cultivation methods. These differences seem sufficient to separate the two yeasts, and this would be in keeping with accepted yeast classification methods in practice today (Stelling-Dekker, Lodder, Diddens and Lodder, Custers). Possibly further study of other isolates of *T. lactis-condensi* from condensed milk (other than the CBS culture) would indicate the exact extent of the similarity between the two yeasts. However, additional strains do not appear to be available here or abroad (Wickerham, 1948).

B. claussenii. The *Brettanomyces* from brine origin were extremely salt-tolerant, as might be expected from their appearance in the fermentation at a time of high brine strength. In laboratory tests they grew on solid media containing 24 percent salt by weight. Fermentation in a 60 percent sucrose solution was also better than in a 40 percent solution, although there was much more cell growth in the latter. Tests on cornmeal agar did not reveal the presence of pseudomycelium for the isolates tested, and essentially the same results were obtained when *B. claussenii* (NRRL 1414) was used. The pseudomycelium for *B. claussenii* in the illustration by Custers shows it to be extremely poorly developed. Yet the brine isolates showed even less. Strong acid production by the *Brettanomyces* on slant cultures is given considerable taxonomic weight by Custers. This characteristic was thoroughly studied on glucose media with different concentrations of chalk suspended in the agar. All the yeast genera found in brines as well as the four known species of *Brettanomyces* were tested for acid production. In brief, it was observed that the known *Brettanomyces* (*B. bruxellensis*, *B. lambicus*, *B. anomalus*, and *B. claussenii*) species as reported by Custers are very strong acid producers; however, this characteristic is not restricted to them alone. The lone *Kloeckera* species from brine was more active. Also, other genera from brines (*Torulopsis* and *Hansenula*) were good chalk-clearers at the 0.5 to 0.8 percent suspension level. The *Brettanomyces* isolates from brine likewise produced acid as indicated by the zone of chalk cleared, but were not equal to the four known species.

To our knowledge this is the first time that yeasts of the genus *Brettanomyces* have been obtained and described from sources other than European breweries. In his thorough study of the genus *Brettanomyces*, Custers found that all but one of the 17 strains collected were of brewery origin. The exception was an isolate obtained from fermenting grape must, reidentified by him as *B. bruxellensis*. There was no indication that the genus had been isolated outside of Europe. There is the possible exception of a single isolate from 31° Balling sirup reported by Bedford (1942) as *B. bruxellensis*. Bedford did not describe the isolate which was incidental to a thorough study of the *Hansenula* genus, and attached no significance to its occurrence. A study of his thesis (Bedford, 1941) shows several characteristics that make his placing of the isolate as *B. bruxellensis* questionable. Since Bedford's culture was presumably no longer living (Mrak, 1948) no comparison could be made with our CBS culture of *B. bruxellensis*.[6]

The fermentation reactions and different morphology of the *Brettanomyces* isolates from high-salt brines during the latter portion of the fermentation is considered sufficient basis for species separation from *B.*

[6] Later, however, we were informed by Wickerham (1948), who was one of the reviewers of this paper, that Bedford's isolate was in his collection. He stated that it does not belong in *Brettanomyces*, but is a species closely related to *Torulopsis utilis*. We are inclined to accept Wickerham's classification of Bedford's isolate as being correct.

claussenii. The name *Brettanomyces versatilis* sp. nov. is suggested. Two *Brettanomyces* isolates, Y-207 and Y-232, were found during the 1946 study that were characterized by growth but no fermentation of sucrose in low concentrations; however, both raffinose and melibiose (complete raffinose) were fermented and in other respects they were similar to the main group. For the present they are considered as variations of *B. versatilis* sp. nov.

Group III, Genus *Saccharomyces*, subgenus *Zygosaccharomyces*: The 59 isolates in this genus were divided into two groups; A, 50 that fermented glucose, but gave a variable action on sucrose; and B, 9 that fermented glucose, sucrose, and maltose. Growth in sucrose media prior to making the test did not appear to influence the results for the Group A isolates; nor did the use of 10 percent sucrose solution instead of the 4 percent solution regularly used. Whereas the 10 and 20 percent solutions of sucrose gave good growth but no gas, the 40 and 60 percent solutions were fermented. This indicated an osmophilic relationship for the action on sucrose. Growth in salt media was within the range of 20 to 24 percent by weight. Group B yeasts were not tested for salt tolerance on laboratory media, but their growth in the fermenting brines at about 18 percent is ample proof of their ability to withstand high osmotic pressures. The *Zygosaccharomyces* are well known for their tolerance toward solutions of high osmotic pressure, particularly those containing sugar, such as honey, maple syrup, and concentrated grape musts. This subject has been adequately reviewed by Henrici (1941). Their tolerance to high salt brines, however, does not appear to be so well established. (Cf. Addendum.)

In an attempt to place the yeasts of Group A as to species, a compilation of the detailed characteristics of some 25 known species was prepared. About one-half of these fell into the "weakly fermentative" class. Species separation appeared to be based on such minor differences that it appeared that continued attempts would further complicate an already confused picture. For this reason, our *Zygosaccharomyces* will be listed merely as species A and B until a satisfactory taxonomic examination of the described species is made by workers in this field. The new culture list by the "Centraalbureau" (CBS, 1947) names 50 species, 8 of which have synonyms; undoubtedly there are more than this conservative number.

Group IV, Genus *Hansenula*: The 49 isolates placed in this genus were characterized by the fermentation of glucose, sucrose, and raffinose (⅓); a variable maltose fermentation; a vigorous galactose utilization; and either extremely thin film production or no film at all. These characteristics (supplemented with the rest shown in table 2) are similar, but not identical, to the species *H. subpelliculosa* Bedford. The variable fermentation of maltose is not entirely consistent; but out of 11 cultures studied by Bedford, one did not ferment this sugar. In a test of an additional 136 cultures by us obtained from refrigerated brine samples (which are not included in our present total of *Hansenula* isolates), 58 fermented maltose in varied degrees while 78 did not. As to utilization of galactose, Bedford had 6 out

of 11 cultures that gave a negative test in a synthetic medium. We are inclined to believe that the fermentation of maltose could well be considered a weak characteristic for the species, providing a sufficient number of cultures are studied. It is obvious that galactose utilization is variable for Bedford's isolates; hence, we believe that for the present no sound basis exists for separating our isolates from the species *Hansenula subpelliculosa* Bedford. For this reason, the 49 isolates listed in table 2 and the 136 obtained from refrigerated brine samples are placed in that species. Representative isolates fermented 60 percent sucrose, and on laboratory media tolerated between 20 and 24 percent salt by weight. When compared to the pseudomycelium produced by *H. anomala*, that produced by the brine *Hansenula* appeared insignificant.

Group V, Genus *Torulaspora*: The 6 cultures of this genus were considered identical with *T. rosei* Guillermond. Four characteristic spores per ascus were frequently found on vegetable juice agar although this important characteristic is not mentioned for the species by Stelling-Dekker. Any redescription of this yeast should include this observation on sporulation. A single culture of *T. rosei* was isolated from grapes by Mrak and McClung (1940), but otherwise it appears not to have been found in this country. The salt tolerance for the brine isolates was between 15 and 20 percent. Rapid growth and fermentation of 40 and 60 percent sugar solutions was obtained.

Group VI, Genus *Kloeckera*: A single culture similar to *Kloeckera magna* (De 'Rossi) Janke was obtained. As previously mentioned, this yeast produced more acid in aerobic culture, using a chalk agar, than any of the four known species of *Brettanomyces*. This was found to be true at different levels of chalk suspension (approx. 0.5, 0.8, and 1.6 percent). Mrak *et al.* (1942) demonstrated that an isolate of *Kloeckera africana* (and one of *Hansenula anomala* var. *sphaerica*) clarified slants of Custers' chalk media more readily than did species of other genera (*Pichia*, *Hanseniaspora*, *Candida*, *Torulopsis*) isolated from figs.

Initial Group, Genera *Rhodotorula* and *Debaryomyces*: Earlier work (Etchells, 1941; and Etchells and Jones, 1943) has shown that the beginning of active yeast development in cucumber brines of about 10 percent strength starts in about 1 week. It was also shown that the initial yeast populations present at the time the vats are filled and covered with brine drop to extremely low numbers during the first few days prior to active development. In the present study, it had been planned to investigate the initial flora at the time the vats were filled and brined. This would be termed the "initial" plating at "0" days. This portion of the study was not fully realized for two reasons; (1) such vats were not always available at both plants, and (2), more important, there was a high incidence of molds in most of the initial brines sampled, making the plates useless for picking colonies, although after the first few days the molds are no longer found. Of the 7 vats that were sampled initially, only 3 had plates (salt

agar only) that were at all suitable for picking. Twelve isolates were picked. Of the 9 placed generically, 4 were classified as *Rhodotorula* and 5 as *Debaryomyces*. Representatives of these genera were not obtained again throughout the study of the 20 fermentations. (The above yeasts have not been included in the total number of isolates since they represent initial values for only 3 of the 20 vats studied and were obtained from only one plating medium.)

Unclassified Group: Twenty isolates were obtained which have not yet been fully classified. Although they have been studied rather thoroughly, some do not fit into known species descriptions, for one reason or another. Some segregation of the group, however, can be made. Seven of the isolates are film yeasts, four of which were taken from the heavy surface film of the two sheltered vats followed at plant B and properly should be excluded from this study. All of the film yeasts produced well developed pseudomycelium and four appear to have a mixture of both true and pseudomycelium. The latter 4 have been classified as *Endomycopsis*. The remaining three film yeasts, which came from 1- to 2-day-old samples, appear to fall in the genus *Candida*. The finding of four isolates classified as *Endomycopsis* is significant because heretofore yeasts of this genus have not been associated with surface films present on brined material (Mrak and Bonar; Henrici).

Distribution of Isolates According to Source

For this discussion, cultures from both media used for isolations will be grouped together since no outstanding floral differences arose through the use of the glucose agar with salt. The summarized results (table 3) show that two yeasts, *T. caroliniana* sp. nov. and *B. versatilis* sp. nov., were consistently found in all 20 fermentations and represented the bulk (88 percent) of all cultures identified. It appears that the individual brining treatment may have exerted an influence on the frequency with which certain of the minor species were obtained. For example, all of the isolates of *Torulopsis holmii*, *Torulaspora rosei*, 45 of the 46 *Hansenula subpelliculosa* cultures, and 50 of the 59 *Zygosaccharomyces* isolates came from plant A. Also, none of the 27 cultures of the new species, *B. sphaericus* was obtained at plant A. As mentioned before, generally this plant employed a much slower rate of increase in brine strength (from 10 to 16 percent) than plant B, and considerably lower total yeast populations were obtained (Cf. figure 1). In connection with the 45 isolates of *H. subpelliculosa* obtained at plant A, 28, or 62 percent, came from one fermentation (vat 167). This was the only case of what amounted to an active fermentation by this yeast. The 50 *Zygosaccharomyces* (species A) cultures came from 8 fermentations at plant A, whereas the 9 cultures of species B all came from one fermentation (sheltered vat 15–10) at plant B.

TABLE 3. DISTRIBUTION OF BRINE YEASTS ACCORDING TO SOURCE OF ISOLATION (1947 SEASON).

Vat No.	I *Torulopsis*				II *Brettanomyces*				III *Zygosaccharomyces*				IV *Hansenula*		V *Torulaspora*		Unclassified		Total Isolations per Vat	
	T. caroliniana		*T. holmii*		*B. versatilis*		*B. sphaericus*		*Z.* sp. A		*Z.* sp. B		*H. subpelliculosa*		*T. rosei*					
Plant A	GA[1]	GS[2]	GA	GS	GA	GS	GA	GS	GA	GS	GA	GS	GA	GS	GA	GS	GA	GS	GA	GS
F–161	18	12	0	0	15	13	0	0	3	1	0	0	4	1	0	0	0	0	40	27
F–163	19	13	1	1	10	9	0	0	4	1	0	0	2	2	0	0	1	0	37	26
F–164	20	14	1	0	14	12	0	0	8	2	0	0	2	2	0	0	0	0	45	30
F–167	12	9	0	0	11	11	0	0	0	0	0	0	17	11	0	0	0	0	40	31
F–221	18	16	0	0	9	12	0	0	5	0	0	0	1	0	1	0	1	0	35	28
F–236	17	11	1	0	13	14	0	0	6	2	0	0	1	1	1	0	0	0	39	28
F–287	7	9	0	0	12	9	0	0	2	3	0	0	0	0	1	1	2	0	25[3]	22
F–288	9	10	0	0	12	10	0	0	0	0	0	0	1	0	0	0	0	0	22	20
F–291	22	8	0	0	7	9	0	0	8	4	0	0	0	0	0	0	0	0	37	21
F–10	6	5	0	0	13	10	0	0	1	0	0	0	0	0	0	0	1	0	21	15
Sub-total	148	107	3	1	116	109	0	0	37	13	0	0	28	17	3	1	5	0	341	248
Plant total	255(43.3%)		4(0.7%)		225(38.2%)		0		50(8.6%)		0		45(7.7%)		4(0.7%)		5(0.8%)		589	

Plant B	GA	GS	GA	GS	GA	GS	GA	GS	GA	GS	GA	GS	GA	GS	GA	GS	GA	GS
21–20	23	11	0	0	18	19	0	1	0	0	0	0	0	0	0	0	0	1	41	32
21–21	20	13	0	0	15	12	0	0	0	0	0	0	0	0	0	0	1	0	36	25
22–21	22	10	0	0	19	14	0	0	0	0	0	0	0	0	0	0	0	0	41	24
1–7	17	14	0	0	23	16	0	0	0	0	0	0	0	0	0	0	0	0	40	30
23–3	18	11	0	0	14	17	0	0	0	0	0	0	1	0	0	0	2	0	35	28
23–4	25	13	0	0	17	11	0	2	0	0	0	0	0	0	0	0	0	0	42	26
14–10[4]	27	13	0	0	7	4	5	6	0	0	0	0	0	0	0	0	3	0	42	23
15–10[4]	21	11	0	0	2	2	1	7	0	0	7	2	0	0	0	0	0	4	31	26
B1–1	24	9	0	0	16	9	0	2	0	0	0	0	0	0	0	0	2	0	42	20
B2–13	14	8	0	0	15	11	0	3	0	0	0	0	0	0	0	0	2	0	31	22
Sub-total	211	113	0	0	146	115	6	21	0	0	7	2	1	0	0	0	10	5	381	256
Plant total	324(50.9%)		0		261(41.0%)		27(4.2%)		0		9(1.4%)		1(0.2%)		0		15(2.3%)		637	
GRAND TOTAL 20 Vats	579(47.3%)		4(0.3%)		486(39.7%)		27(2.2%)		50(4.1%)		9(0.7%)		46(3.8%)		4(0.3%)		20(1.6%)		1226	

[1] Isolates from glucose agar.
[2] Isolates from glucose agar with salt.
[3] Total includes 1 *Kloeckera magna* obtained at the first sampling, 24 hrs. after filling and brining.
[4] Sheltered vats; all others in open.

Predominance of Individual Species

The results for the 2-year study with respect to the individual number of isolates assigned to individual species, and their percentage of the total isolations made (column 1 of table 2), are given for the six generic groups

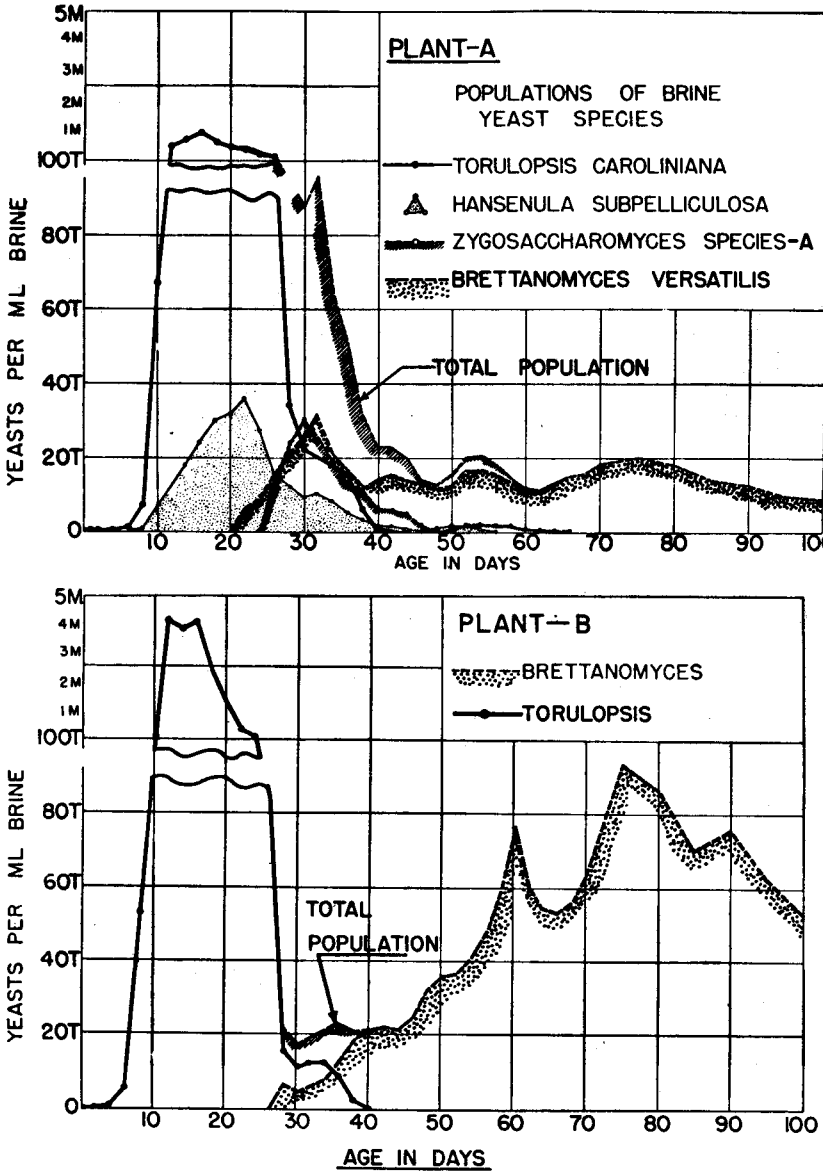

Figure 2. Yeast populations at two commercial plants according to sequence of species. Upper part, plant A; lower part, plant B.

encountered, in the decreasing order of frequency with which they were found.

The *Torulopsis* and *Brettanomyces* genera represented 1,309 cultures out of 1,444, slightly over 90 percent of all yeasts isolated, were obviously by far the types most frequently obtained. The remaining four genera (*Hansenula*, *Zygosaccharomyces*, *Torulaspora*, and *Kloeckera*), constituted but 115 cultures, about 8 percent of the total isolates. A breakdown of the two principal genera found shows that two individual species within *Torulopsis* and *Brettanomyces*, i.e., the tiny-celled yeast *T. caroliniana* sp. nov. and the fermentative yeast *B. versatillis* sp. nov., predominated, accounting for 1,278 cultures, 88.4 percent of the total cultures. The former species represented almost 50 percent of all cultures, and the latter nearly 40 percent.

Before it can be stated whether a definite sequence of species existed during the general fermentation, a few remarks concerning their respective periods of activity are needed. At both plants the tiny-celled yeast, *T. caroliniana* sp. nov., after starting growth within about 7 days, represented the bulk of the total populations, until about the 30th day, when their numbers declined. At plant B, these yeasts reached much higher numbers than at plant A. Between the 25th to 40th day, the fermentative yeast, *B. versatilis* sp. nov., appeared and thereafter was consistently found throughout the remainder of the 100-day period of observation. In the case of plant B this yeast appeared later but reached definitely higher numbers (five-to ten-fold) than for plant A. The less frequently obtained species from plant A were found as follows: The cultures of *Torulopsis holmii* and of *Torulaspora rosei* appeared at the end of the fermentation by *Torulopsis caroliniana*. The *Zygosaccharomyces* (Type A) appeared between the 32d to the 55th day. The *Hansenula subpelliculosa* cultures generally were found near the end of the *T. caroliniana* fermentation; in the case of vat 167 they were consistently found between the 18th and 49th days. The less frequently obtained yeasts at plant B were the Type B *Zygosaccharomyces* found between the 43rd to 70th day (in one vat only); and the poorly fermentative *Brettanomyces* species found between the 23rd to 70th day.

A general picture as to yeast sequence in the fermentations at plants A and B, based on the glucose agar isolates from the 1947 season, is shown in figure 2. In the preparation of these diagrams, only data for four principal yeasts that showed definite individual population trends were used. Data for the two sheltered vats from plant B (vats 14–10 and 15–10) are omitted because the brine strengths in these two cases, after reaching 15 percent, were not increased as were the 8 outside vats.

Description of New Species

Torulopsis caroliniana sp. nov.

Cellulae parvae vel perpusillae, breve ovales vel ovoideae, plerumque aggregatae, in agaro glucoso in 24–48 horis 1.5–2 x 3–4 μ; in mediis liquidis multo saccharosis leniter

majores; fermentationem glucosii, sucrosii, raffinosii (⅓) in diebus 2–3 inducens, jure glucoso cito claro, annulo et pellicula absentibus; fermentatio maltosii, galactosii, lactosii, et melibiosii non visa, set assimilatio galactosii producta; auctus in alcoholi ethylico carens; reductio valida nitrati ad nitritum in diebus 3–4; pseudomycelium non visum in agaro solanaceo nec maltoso in 3 hebdomadibus; in jure solanaceo solo coloniae cellularum elongatarum praesentes; ascosporae non visae in mediis 8 sporulantibus menses plures observatis; cellulae caducae (3–6 hebdomades) in mediis vulgaribus; productio definita acidi in mediis cretaceis; culturae in mediis glucosis albae, madidae, coruscae, auctu moderato; colonia gigantea in agaro glucoso parva, circa 1 cm mense una, typice margine lobato et annulis concentricis praedita, centro in aetate ferruginosa; fermentationem rapidam solutionum 40–60% saccharium inducens; toleratio salis $>$ 15 sed $<$ 20% in culturis, in fermentationibus salsimentorum naturalibus supra 15%.

In fermentationibus salsimentorum cucumeris (10–15% salis) in diebus 7–30.

Cells, small to tiny, short-oval to egg-shaped, usually in clusters. On glucose agar slants and broth at 24 to 48 hours, cells average 1.5–2 x 3–4 μ; cells slightly larger in liquid media with higher sugar concentrations. Rapid fermentation of glucose, sucrose, raffinose (⅓) within 2 to 3 days. Glucose broth clears rapidly; no ring, no film. No fermentation observed for maltose, galactose, lactose, and melibiose; but, moderate assimilation of galactose is obtained. No growth in ethyl alcohol. Strong reduction of nitrate (KNO_3) to nitrite after 3 to 4 days (chemical test). Pseudomycelium not found on cornmeal or malt extract agar in 3 weeks; none found in potato broth, only cell colonies with more elongated cells. Ascospores not found on 8 sporulating media observed over several months. Cells short-lived on slants of ordinary media (3 to 6 weeks). Definite acid production on chalk containing media. Glucose slant cultures white, moist, glistening with scant to moderate amount of growth. Giant colony on glucose agar small, approximately 1 cm. at 1 month, characteristically lobed edge with concentric rings. Slight rust color starting at the center with age. Rapid fermentation of high sugar solutions (40 and 60 percent by weight). Tolerance to salt is $>$ 15 but $<$ 20 percent by weight in cultural tests; tolerance in natural brine fermentations slightly above 15 percent. Source: During the active fermentation of cucumber brines (10 to 15 percent salt), starting on about the 7th day, and continuing until about the 30th day.

Brettanomyces versatilis sp. nov.

Cellulae plerumque ovales, interdum rotundae; in agaro et jure glucosis in 48–72 horis 3–4 x 4–5 μ; cellulae ogivae in culturis vetustis; fermentationem glucosii, sucrosii, maltosii, galactosii, lactosii, raffinosii et melibiosii inducens; fermentatio mediorum saccharinorum tarda et menses plures protracta; auctus in alcoholi ethylico bonus, annulo praesenti sed pellicula carenti, etiam in jure glucoso; reductio nitrati in nitritum moderata; pseudomycelium non visum in agaro farinae zeae in 3 hebdomadibus nec in jure solanaceo in 2 mensibus; ascosporae non visae in mediis 8 sporulantibus in mensibus pluribus; cellulae in mediis vulgaribus caducis (4–6 hebdomades) cultures circa 9 menses in jure 4% lactoso in temperatura vulgari persistantes; productio valida acidi in medio cretaceo in culturis aerobicis; culturae juveniles in agaro glucoso albae, coruscae, leves, demum in culturis vetustis e centro roseae usque lavendulae; colonia gigantea in agaro glucoso alba, levis, annulis concentricis inconspicuis praedita, demum

infra e centro rosea usque lavendula; fermentationem solutionum sucrosium (40–60%) inducens; toleratio salis extrema (20–24%) in fermentationibus artifactis et naturalibus.

In fermentationibus salsimentorum cucumeris in diebus 30 usque 100 longe et tarde continuanti.

Cells predominately oval but some round. On glucose agar slants and broth at 48 to 72 hours, cells average 3–4 x 4–5 μ. Characteristic cell colonies from broth; typical ogive cells in old cultures. Fermentation of glucose, sucrose, maltose, galactose, lactose, raffinose, and melibiose. Characteristic slow, prolonged fermentation of sugar media over a period of months. Good growth in ethyl alcohol; ring but no film. Ring but no film in glucose broth. Moderate reduction of nitrate (KNO_3) to nitrite (chemical test). Pseudomycelium not found on cornmeal agar in three weeks, or in potato broth after 2 months. Ascospores not found on 8 sporulating media observed over several months. Cells short-lived on slants of ordinary media (4 to 6 weeks). Cultures can be maintained for approximately 9 months in 4 percent lactose broth at room temperature. Strong acid production on chalk media in aerobic culture. Young, glucose slant cultures white, glistening and smooth. Latent development of a pink to lavender color in old cultures starting from center area. Giant colony on glucose agar dull white, smooth, with slight concentric rings. Latent development from center (bottom) of pink to lavender color. Fermentation of high sucrose solutions (40 and 60 percent by weight). Extremely salt-tolerant in both laboratory tests and natural fermentations (20 to 24 percent by weight). Source: During the latter part of the fermentation of cucumber brines, starting at about 30 days and continuing in a slow, prolonged manner until at least the 100th day.

Brettanomyces sphaericus sp. nov.

Cellulae plerumque rotundae, in agaro glucoso in 48–72 horis 3–5 μ in diam., vel interdum paulo majores, in jure aggregatae; cellulae ogivae in culturis vetustis; fermentationem tardam glucosii maltosiique inducens, non sucrosii, galactosii, lactosii, raffinosii nec melibiosii; sucrosium et galactosium utuntur; auctus moderatus in alcoholi ethylico, annulo praesenti sed pellicula carenti, etiam in jure glucoso; reductio nitrati ad nitritum latens; pseudomycelium non visum in agaro farinae zeae in 3 hebdomadibus; ascosporae non visae in mediis variis sporulantibus menses plures observatis; cellulae caducae in mediis vulgaribus; productio acidi moderata in mediis cretaceis in culturis aerobicis; culturae juveniles in medio glucoso albae, leves, coruscae, integrae, auctu abundanti; fermentatio solutionum 40% glucosium auctu flocculento; fermentatio tarda solutionum 60% sucrosium, ad 40% debilis, ad 20, 10 et 5% absens; toleratio salis magna in mediis artifactis et fermentationibus naturalibus 18–20%.

In statibus ultimis fermentationis salsimentorum cucumeris in concentrationibus magnis (15–20%) salis.

Cells predominately round; those from glucose agar slants and broth at 48 to 72 hours, 3–5 μ in diameter; some slightly larger. Clusters of many cells in broth; a tendency for ogive cells in old cultures. Slow fermentation of glucose and maltose. Sucrose, galactose, lactose, raffinose, and melibiose not fermented, but sucrose and galactose utilized. Moderate growth in

ethyl alcohol; ring but no film. Ring but no film in glucose broth. Latent reduction of nitrate (KNO_3) to nitrite. Pseudomycelium not found in cornmeal agar in three weeks. Ascospores not found on several sporulating media observed over several months. Cells somewhat short-lived on slants of ordinary media. Moderate acid production on chalk media in aerobic culture. Young, glucose slant cultures white, smooth, glistening, entire, with abundant growth. Fermentation of 40 percent glucose solutions with flocculent growth. Slow fermentation of 60 percent sucrose solutions; feeble fermentation of 40 percent; no fermentation at lower concentrations (20, 10, and 5 percent). Very salt-tolerant in artificial media and in natural brine fermentations (18 to 20 percent by weight). Source: During latter part of the fermentation of certain cucumber brines at high salt concentration (15 to 20 percent).

TABLE 4. EXAMINATION OF SALT-STOCK CUCUMBERS FROM SEVEN FERMENTATIONS (PLANT A) FOR BLOATERS OR HOLLOW CUCUMBERS.

Vat Number (and Date Opened–1948)	Amount of Salt Stock Examined [1]	Bloaters Found	
	bushels	*bushels*	*percent*
F–161 (4–20)	265	144	54
F–163 (4–24)	282	66	23
F–164 (4–27)	274	106	39
F–167 (5–1)	391	176	45
F–221 (5–28)	302	114	38
F–236 (5–27)	305	159	52
F–288 (5–27)	177	81	46

[1] Vats contained large sized cucumbers (1½ inches in diameter or larger).

Bloater Formation During Fermentation

After the completion of the curing period, the salt-stock from the 10 vats at one of the plants was graded out for percentage of bloaters (hollow cucumbers). This was done according to regular plant procedure. The results for seven vats, those that contained large sized cucumbers (1½ inches in diameter and larger), are shown in table 4. The proportion of bloaters found ranged from 23 to 54 percent of the total amount of stock in individual vats. Bloater formation was attributed to the gaseous yeast fermentation, and *Torulopsis caroliniana* was considered to be the principal

species involved. The results on the number of bloaters formed are comparable to those reported by Jones, Etchells, Veerhoff, and Veldhuis (1941) for large sized cucumbers cured in barrels and 85-bushel vats at 10.5 percent brine strength.

Three lots, vats F-287, F-291 and F-10, not shown in table 2, contained small sized stock (less than 1½ inches in diameter). These graded out less than 10 percent bloaters. The relationship of less bloaters being formed during gaseous fermentation when smaller sized cucumbers are brined is the same as that shown earlier by the above authors.

DISCUSSION

The tiny yeast, *T. caroliniana* sp. nov., and the new *Brettanomyces* species, *B. versatilis*, were in consistently higher numbers in the brines of plant B as compared to plant A. This is attributed to the influence of the more rapid increase in brine strength employed by plant B which markedly reduced the growth of the less salt-tolerant lactic acid bacteria, thereby leaving more fermentable material from the cucumbers for the yeasts. This relationship was readily demonstrated by definitely lower brine acidities for plant B, as compared to plant A, and lower numbers of lactic acid bacteria as demonstrated by microscopic examination and plate counts for these organisms. Previous studies (Jones and Etchells, 1943) have shown that only a very small amount of fermentable sugar remains in the brines by the end of the first 30 days. Hence, the *Brettanomyces* fermentation evidently was supported by another carbon source; probably the ethyl alcohol produced earlier by the *Torulopsis* species, *T. caroliniana*, or the organic acids contributed chiefly by the lactobacilli.

The two species, *T. caroliniana* and *B. versatilis*, dominated the yeast flora at plant B; they were also the types most frequently isolated at plant A. There was evidence, however, of fermentation trends of two additional yeasts, *Hansenula subpelliculosa* and *Zygosaccharomyces* (species A) at the latter plant. Although neither of these yeasts was found in all ten fermentations, they were, however, found with sufficient frequency to indicate at least a minor role in the average fermentation. The well-developed fermentation by *H. subpelliculosa* in vat 167 would be sufficient evidence to warrant inclusion of this yeast in any general consideration of the yeast flora. Since both of these yeasts made their appearance near the end of active development of the tiny-celled *Torulopsis*, it seems plausible that lack of a readily fermentable carbon source was the major factor which limited their continued development. Neither type grows particularly well in ethyl alcohol as compared to the yeast that succeeded them in the brines, namely, *B. versatilis*. The latter species at plant A did not reach populations much above 10,000 to 15,000 per ml. from about the 35th day on, which is further evidence of lack of nutrients in these brines. It is doubtful if the brine acidity had much of an inhibitory influence on the species of *Hansenula*, *Zygosaccharomyces*, and *Brettanomyces* in plant A brines. Such an effect would have first been noticeable on the *Torulopsis* fermenta-

tion, since these yeasts are much more sensitive to the brine acid than the other genera mentioned.

In an investigation of this kind, the principal limiting factor on the extent of species separation is the number of isolates than can be picked and subsequently identified. Even with an unlimited number of isolates from the high dilution plates there is always the question of what minor species might be present at lower population levels. In this connection, it is conceivable that the *Brettanomyces* started growth at about the same time as the tiny-celled *Torulopsis* (about 7 days); but due to lower population levels did not become "predominating" until the tiny yeasts were reduced to a level so that the former group appeared on the plates. The same is probably partly true for the *Hansenula* and *Zygosaccharomyces*. Better separation could no doubt be effected by development of differential media for detection of specific yeast groups, based on their particular biochemical properties.

SUMMARY

A study of the yeasts predominating during the fermentation of cucumbers under commercial conditions is presented. During the 1946 and 1947 seasons, 1,444 isolates were obtained by frequent sampling of 42 fermenting vats at two commercial pickle plants in eastern North Carolina. During the period of observation, the brine strength of the fermentations was within the range of 10 to 18 percent by weight.

The 1,444 isolates were reduced to the following six genera in the order of frequency of isolation; *Torulopsis* (721); *Brettanomyces* (588); *Zygosaccharomyces* (59); *Hansenula* (49); *Torulaspora* (6); *Kloeckera* (1); plus 20 isolates not fully classified. The first two genera named represented a total of 1,309 cultures or slightly over 90 percent of all yeasts isolated. Furthermore, two new species within these genera, the tiny yeast *Torulopsis caroliniana* sp. nov., and the very fermentative yeast, *Brettanomyces versatilis* sp. nov., predominated, accounting for 1,278, or slightly over 88 percent of the total cultures. The first species dominated the early part of the fermentation and was then followed by the second in a slow fermentation which continued up to the end of the observation period (about 100 days). This was the most clear-cut yeast sequence obtained for all 20 fermentations at both plants. In addition, 4 cultures of *Torulopsis holmii* (Jörgensen) Lodder were found, as well as 27 cultures that have been placed in the *Brettanomyces* genus as *B. sphaericus* sp. nov.

The remaining four genera (*Zygosaccharomyces, Hansenula, Torulaspora*, and *Kloeckera*) obtained less frequently consisted of 115 cultures, or about 8 percent of the total isolates. These cultures were placed as follows: The 59 *Zygosaccharomyces* (50, type A; 9, type B) were not identified as to species due to the confused state of the genus; the *Hansenula* were considered to be *H. subpelliculosa* Bedford; the *Torulaspora* isolates were identical to *T. rosei* Guillermond; the single isolate of *Kloeckera* was similar to *K. magna* (De 'Rossi) Janke. All the above 115 cultures, with the exception of 9 maltose fermenting *Zygosaccharomyces* cultures and 1

culture of *H. subpelliculosa* were obtained from one plant, as were the 4 cultures of *T. holmii*. This was attributed to the difference in brining treatment.

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ADDENDUM

The 9 yeast isolates referred to in this report as species B of the genus *Zygosaccharomyces* (Cf. table 2, column 1, Group III, and table 3, column 7) are considered to be the same as *Z. halomembranis* sp. nov. recently obtained from films on commercial cucumber brines. (Cf. Etchells, J. L., & T. A. Bell. 1950. Film yeasts on commercial cucumber brines. Food Tech., 4:77–83.)

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