MICROBIOLOGICAL STUDIES OF HONEY

I. HONEY FERMENTATION AND ITS CAUSE
II. INFECTION OF HONEY BY SUGAR-TOLERANT YEASTS

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PART I.—HONEY FERMENTATION AND ITS CAUSE

INTRODUCTION

Spoilage of extracted honey due to fermentation and souring has become, particularly in recent years, a matter of grave concern to Canadian bee-keepers and others engaged in the handling and marketing of this product. In former years, when honey production had not attained its present-day dimensions, and when honey in general was not held in storage for any great length of time before consumption, fermentation, though not unknown, was generally assumed to be the result of extracting honey in an insufficiently ripened state. In the trade, however, the menace of fermentation did not assume the serious proportions it now presents as long as relatively little of the honey crop was held over from one year to the next.

The relationship of fermentation to the honey industry, however, has changed in late years. The post-war period has seen in Canada an increased production of honey, and particularly of extracted honey, our export trade has widened, home consumption has increased and become less seasonal in nature. These developments in the production and marketing of honey have all tended to increase the period of storage of honey before it reaches the table of the consumer, and as a consequence of this lengthened period of exposure of what is by nature a perishable product, spoilage by fermentation has assumed much more serious proportions, and may now be said to be one of the chief difficulties which those engaged in honey production and marketing have to face and the source of serious financial losses in the industry.

Formerly cases of spoilage by fermentation were usually regarded as being simply due to improper processing on the part of the bee-keeper on account of its being extracted before thoroughly ripened. Such unripe honey is generally found to be high in moisture which would naturally favour the development of microorganisms capable of inducing fermentation. Many instances of fermentation occur, however, where the honey is apparently thoroughly ripened and where the moisture content of the honey is within limits usually considered satisfactory. That various factors are concerned with the phenomenon of fermentation seemed most probable in the light of observation of those in the trade. With the object of contributing towards a better understanding of honey fermentation investigational work has been undertaken by the Division of Bacteriology, Central Experimental Farm, and the material presented in this bulletin, dealing with one phase of the subject, is based on data obtained during the past two years.[1]

[1] We wish to acknowledge with thanks the co-operation and helpful advice accorded us at all times by the Dominion Apiarist, Mr. C. B. Gooderham, and staff of the Bee Division.

PREVIOUS INVESTIGATIONS OF HONEY FERMENTATION

So far comparatively few investigations have been made dealing with the question of fermentation of honey, and the literature in this connection is, in consequence, small. The first important work appears to be that of Nussbaumer (10)[2] in Switzerland to whom credit is due for having demonstrated that sugar-tolerant yeasts are a factor in causing honey fermentation. Investigating a sample of fermented honey said to have been Canadian in origin, Nussbaumer succeeded in isolating and describing two types of yeast, both *Zygosaccharomyces*. One species (Type A) consisted mainly of elliptical cells, from 5-8 μ long and 3·5-5 μ wide, though capable of forming long cylindrical cells in old wort-gelatine cultures. Type A was found capable of fermenting dextrose, levulose maltose, mannose, saccharose, dextrin and galactose readily and xylose and raffinose with difficulty. Lactose, mannite and arabinose were not fermented. The other species (Type B), also mainly elliptical cells from 5-8 μ long and 4-6 μ wide, was able to ferment dextrose, levulose, mannose, saccharose, dextrose and galactose readily, xylose with difficulty. Lactose, maltose, mannite, arabinose and raffinose were not fermented. Neither of the yeasts was found to be identical with *Zygosaccharomyces priorianus* isolated by Klöcker from the bodies of honey bees.

Nussbaumer further established that the yeasts isolated were able to ferment honey solutions of high concentration. In testing three honeys of 15·35, 15·90 and 22·35 per cent water respectively, he found the yeasts capable of fermenting only the last in the undiluted state, though all fermented in various dilutions including 90 per cent honey. The author concludes that fermentation occurs only as a result of a high moisture content, the bare presence of yeast cells being insufficient to cause its spoilage. The effect of heating honey as a means of preventing fermentation was also first reported by Nussbaumer. Tests conducted on honey inoculated with spore material of Type B led the author to conclude that heating to 70° C. (158° F.) for 30 minutes was sufficient to prevent further fermentation.

Investigating an outbreak of fermentation in Russia, Richter (14) succeeded in isolating a yeast as the causal agent. Fermentation was first noted in extracted honey including samples which had already crystallized. Further investigation of the apiaries of the district in question revealed, unexpectedly, that an alcoholic fermentation was also taking place in the comb and that, with abundant gas formation, the contents of the cells were bursting out and running in streams over the hives. The yeast responsible, named *Zygosaccharomyces mellis acidi,* was found to consist of round to slightly elliptical cells of an average diameter of 3-4 μ and proved to be a highly sugar-tolerant type. It was found to be capable of fermenting dextrose, levulose and saccharose actively, galactose feebly, but incapable of fermenting maltose, lactose, dextrin and raffinose. The author furthermore compared the yeast with *Z. barkeri* and *Z. priorianus* and found it to differ in fermentative properties from these two species.

For a period of fifteen years following the work of the two European investigators little or no research appears to have been carried on in connection with honey fermentation. Latterly, however, the increasing losses which honey producers and exporters have experienced have given an impetus to investigational work with the result that recent contributions to the subject have been made from two laboratories while the investigations reported in the present bulletin were in progress.

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[2] For references to the literature see p. 35.

The first work reported in America is that of Marvin (8, 9), who described the isolation of five strains of yeasts from samples of fermented honey. All five strains were capable of growing in concentrated solutions of honey and were believed to be the cause of the spoilage. Apart from a description of their fermentative properties no further characterization of the yeasts appears to have been yet made. The author, however, makes an important contribution to the question of the relationship of crystallization to fermentation, which helps to explain why fermentation, commonly considered as taking place chiefly in unripe honey, may occur in "normal" honeys which are, to all intents and purposes, ripe. When honey crystallizes, the less soluble dextrose separates out while the levulose is left in solution, though necessarily in less concentrated solution than the sugars in the original honey. In a sample of honey of 16·3 per cent moisture, crystallization of the dextrose left the liquid portion of the honey, containing the levulose, with 20·8 per cent moisture, providing thus more favourable conditions for yeast growth than the honey previous to crystallization. In further studies on the effect of heat, the same author reports that honey heated to 160° F. will not ferment.

Shortly after the publication of the work of Marvin appeared a contribution to the subject of honey fermentation by Fabian and Quinet (2), though in view of the previous work of the investigators already referred to, it is impossible to subscribe to the belief expressed in the preface as to the first account of the cause of honey spoilage. These workers isolated from samples of fermented honey five species of yeast, four of which were classed as *Zygosaccharomyces*, the fifth as a species of *Torula*. Of these types, three were identified with previously described *Zygosaccharomyces*, namely, *Z. japonicus*, *Z. barkeri*, and *Z. priorianus*, while two were regarded as new species and called respectively *Zygosaccharomyces mellis* and *Torula mellis*. The authors also studied the relationship of moisture to honey fermentation, confirming the earlier work of Nussbaumer (to whom no reference is made) as to the importance of a sufficiently high moisture content in honey as a fundamental factor in fermentation. The critical moisture point is regarded as being approximately 21 per cent. As a result of studies on the effect of heat on yeast growth the authors report that heating honey to 62·5° C. (145° F.) for thirty minutes is sufficient to prevent fermentation.

INVESTIGATION OF FERMENTED HONEY

In this work a microbiological study was made of 13 samples of fermented honey, kindly procured for us by the Bee Division, Central Experimental Farm, a description of the samples appearing in table I.

TABLE I.—DESCRIPTION OF FERMENTED HONEY SAMPLES[1]

Sample	Designation	Description
A	Bruce 29	Obtained at Lucknow, Ont., Sept., 1926; originally light amber in colour and granulated solid, found to have fermented Feb., 1927; when examined July, 1927, was soft and fluid, dark amber in colour, with a fermented though not unpleasant odour.
B	Bruce 34	Obtained at Pinkerton, Ont., Oct., 1926; originally light golden in colour and granulated solid; found to have fermented Feb., 1927; when examined July, 1927, softly granulated with a frothy layer on surface, light golden in colour.
C	Bruce 35	Obtained at Pinkerton, Ont., Oct., 1926; originally light amber in colour and granulated solid; found to have fermented Feb., 1927; when examined was granulated yet fluid, light amber in colour with an unpleasant aroma.
D	Bruce 39	Obtained at Kincardine, Ont., Feb., 1927; originally light amber in colour and granulated solid; found to have fermented March, 1927; when examined, was semi-granulated yet fluid, dark amber in colour, with an unpleasant aroma.

[1] We are indebted to Mr. W. G. LeMaistre, B.S.A., of the staff of the Bee Division for the information contained in Table I.

Table I.—Description of Fermented Honey Samples—*Concluded*

Sample	Designation	Description
E	Dufferin 15	Obtained at Amaranth Station, Ont., Nov., 1926; originally light amber in colour and granulated solid; found to have fermented, Dec., 1926; when examined was granulated yet soft and fluid, especially towards surface, dark amber in colour, with fermented though not unpleasant odour.
F	Durham 2	Obtained at Orono, Ont., Aug., 1926; originally light golden in colour and granulated solid; found to have fermented Feb., 1927; when examined was mostly granulated, a layer on top of loose, coarsely granulated honey, an intermediate layer of liquid and a lower layer of coarsely granulated honey, light golden in colour.
G	Durham 11	Obtained in Port Hope, Ont., Sept., 1926, originally light amber in colour and granulated solid; found to have fermented Feb., 1927; when examined was granulated yet soft, with a layer of foam on surface, dark amber in colour, except a thin light amber bottom layer.
H	Durham 21	Obtained at Port Hope, Ont., Oct., 1926; originally dark in colour and granulated; found to have fermented Feb., 1927; when examined was half liquid and half semi-solid, very dark in colour.
I	Essex 7	Obtained at South Woodslee, Ont., Aug., 1926; originally light white in colour and granulated solid; found to have fermented Nov., 1926; when examined was soft and frothy throughout with an unpleasant aroma, light golden in colour.
J	Peterboro 43	Obtained at Peterboro, Ont., Sept., 1926; originally dark amber in colour and granulated solid; found to have fermented Feb., 1927; when examined was granulated yet soft and fluid, with unpleasant aroma, dark amber in colour.
K	Prescott	Obtained in Prescott County; when examined was dark amber in colour, partly granulated with top layer of liquid.
L	Prince Edward 1	Obtained at Consecon, Ont., Aug., 1926; originally light amber in colour and granulated solid; found to have fermented Nov., 1926; when examined mostly solid and full of bubbles, with a liquid layer on surface, dark amber in colour with an unpleasant odour.
M	No. 101	Obtained from warehouse at Montreal, Que.; dark grade honey produced in Ontario, semi-solid, with much froth.

Preliminary tests for the purpose of isolating organisms capable of fermenting honey were made by making plate cultures from the various honey samples, using as media wort agar (Bacto), nutrient agar containing 10 per cent honey, and two special agar media containing 10 per cent honey with the addition of peptone, K_2HPO_4 and $MgSO_4$, of pH of 4·5 and 7·0 respectively. These media proved to be unsuccessful for the isolation of the causal organisms, the plates showing in most cases no growth whatever or at most a rare colony of mold or yeast incapable of fermenting honey.

EFFECT OF HONEY CONCENTRATION ON YEAST DEVELOPMENT

The unsuitability of the media employed in the preliminary tests suggested the presence of osmophilic organisms in fermented honey, incapable of developing in media of low honey concentrations. Accordingly a series of tests was conducted to note the effect of the concentration of honey in otherwise identical nutrient solutions upon fermentation by organisms transferred from fermented honey. Table II shows the influence of honey concentration in tests with 15 media of concentrations varying from 0 to 67 per cent honey. Inoculations were made from 5 different samples of fermented honey, and as a comparison similar inoculations were made with cultures of two non-osmophilic yeasts representing "normal" types.

TABLE II.—EFFECT OF HONEY CONCENTRATION UPON FERMENTATION, 10 DAYS AT 37° C.

Inoculation	Solution containing peptone 0·1%, K^2HPO_4 0·02, $MgSO_4$ 0·01, $CaCl_2$ 0·01 and honey as below:														
	0 p.c.	1 p.c.	5 p.c.	9 p.c.	18 p.c.	26 p.c.	32 p.c.	38 p.c.	43 p.c.	48 p.c.	52 p.c.	56 p.c.	60 p.c.	64 p.c.	67 p.c.
FERMENTED HONEY—															
Bruce 34	—	—	—	—	—	sl.	+	sl.	+	+	+	++	++	++	+++
Bruce 35	—	—	—	—	—	—	+	sl.	sl.	+	++	++	++	+++	+++
Dufferin 15	—	—	—	—	—	—	—	tr.	sl.	+	+	+	++	+++	+++
Essex 7	—	—	—	—	—	+	sl.	+	+	++	++	++	+++	+++	+++
Peterboro 43	—	—	—	—	—	—	+	+	+	+	+	+	+++	+++	+++
NON-OSMOPHILIC YEASTS—															
S. ellipsoideus	—	+	+	+	+	++	++	++	+	+	+	tr.	—	—	—
Yeast 148 (from canned apples)	—	++	++	++	++	++	+++	++	++	+	+	+	—	—	—

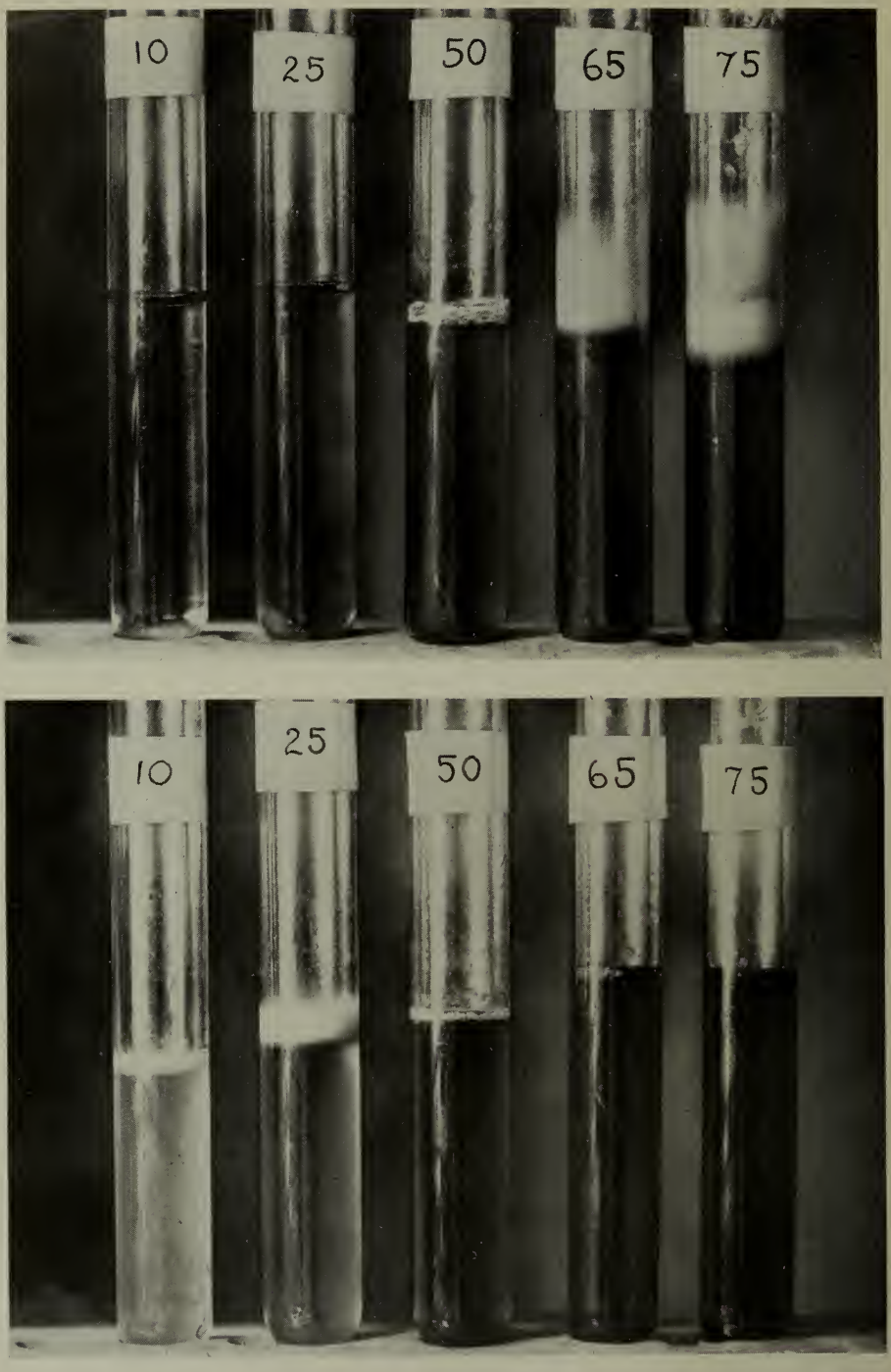

FIG. 1.—Showing difference in the effect of honey concentration upon growth of organisms from fermented honey and "normal" yeasts. In solutions containing 10, 25, 50, 65 and 75 per cent honey respectively, organisms from fermented honey (above) are able to cause fermentation in the higher concentrations only, while "normal" yeasts (e.g. *S. cerevisiæ*), shown in the lower series of tubes, are able to ferment less concentrated solutions but are incapable of developing in the honey solutions of higher density. (1 week at 37°C.)

In Table III are shown the results of a similar test in which, however, the basic solution, to which the different concentrations of honey were added, was somewhat altered. The results, however, were quite similar, and illustrate in striking fashion (see fig. 1) the difference between the organisms responsible for honey fermentation and "normal" yeasts with respect to their relationship to osmotic pressure.

TABLE III.—EFFECT OF HONEY CONCENTRATION ON FERMENTATION. ORGANISMS FROM FERMENTED HONEY COMPARED WITH NON-OSMOPHILIC YEASTS.
10 days 37° C.

Inoculum	Solution containing peptone 0·1 p.c., K_2HPO_4 0·02, NaCl 0·05, $MgSO_4$ 0·02, $CaCl_2$ 0·02 and honey as below.				
	10%	25%	50%	65%	75%
FERMENTED HONEY—					
Bruce 35	—	tr.	+ + +	+ + +	+ + +
Dufferin 15	—	—	+ + +	+ + +	+ + +
Durham 11	—	—	+ +	+ + +	+ + +
Prince Edward 1	—	—	+ + +	+ + +	+ + +
No. 101	—	—	+ +	+ + +	+ + +
NON-OSOMOPHILIC YEASTS—					
S. cerevisiae	+ + +	+ + +	+ +	—	—
S. ellipsoideus	+ +	+ +	+ +	—	—
Yeast No. 148 (canned apples)	+ + +	+ + +	+ + +	—	—
Yeast No. 149 " "	+ +	+ + +	+ + +	—	—
Yeast No. 1 0 " "	+	+	+	—	—

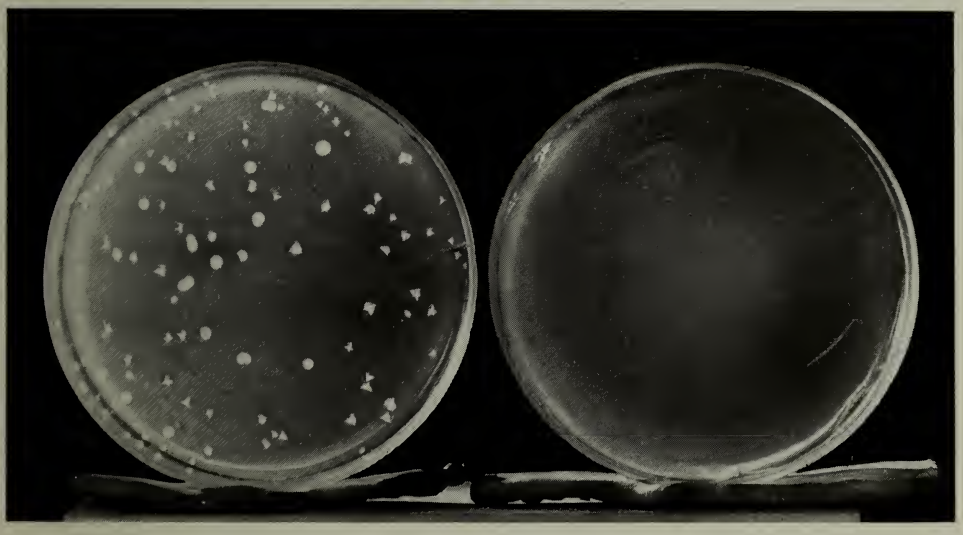

FIG. 2.—Showing effect of honey concentration upon development of yeast colonies from fermented honey (37°C.). A medium containing 60 per cent honey permits of good colony development (left), while in an otherwise similar medium containing 25 per cent honey no growth is to be seen (right).

ANALYSIS OF FERMENTED HONEY

The necessity of a substrate of suitably high osmotic index having been demonstrated, an agar medium containing 60 per cent honey was prepared and adopted as standard for the enumeration and the isolation of the organisms from fermented honey, the composition being as follows:—

Honey	600·0	gram per liter.
Peptone	1·0	" "
K_2HPO_4	1·0	" "
$MgSO_4$	0·5	" "
Ammonium tartrate	0·5	" "
NaCl	0·1	" "
$CaCl_2$	0·1	" "
Agar	25·0	" "

In the preparation of the medium, the agar is dissolved separately in 500 c.c. water by autoclaving, after which the honey, warmed, is added together with the other ingredients, water being gradually added with stirring to make a volume of 1,000 c.c. The medium is tubed and sterilized as usual.

To note whether the effect of honey concentration on colony development in plate cultures is similar to that shown in solution (table III), quantitative tests were made on a number of fermented honeys in which the development of colonies at 37° was compared on honey agar containing 60, 25 and 5 per cent of honey respectively. The results shown in table IV and fig. 2 demonstrate clearly the value of a high honey concentration in obtaining maximum colony development.

TABLE IV.—EFFECT OF HONEY CONCENTRATION OF AGAR MEDIUM UPON DEVELOPMENT OF COLONIES FROM FERMENTED HONEY (37° C.)

Sample	Dilution	Colonies on plates of honey agar containing per cent honey as below.		
		60 p.c.	25 p.c.	5 p.c.
Bruce 29	1/1000	221	1	0
		236	0	0
		186	0	0
		186	0	0
		215	0	0
Durham 2	1/250	39	1	0
		36	0	0
		44	0	0
		27	1	0
		29	0	0
Durham 21	1/250	100	1	0
		99	0	0
		105	1	1
		108	0	0
		100	0	0
No. 101	1/1000	77	1	1
		104	0	0
		65	0	0
		98	0	1
		90	0	0

In addition to a suitable plating medium, the concentration of the dilution blanks used in quantitative estimations suggested itself as another possible factor affecting the development of organisms accustomed to living in solutions of high density. To test this, plate cultures were made with two fermented honeys using 60 per cent honey agar as medium. Three sets of quintuplicate plates were poured in each case of similar dilutions, in which 50 per cent honey,

20 per cent honey and tap water respectively were employed for the dilution blanks, dilutions being left for fifteen minutes before plating. The results, given in table V, point to a disturbing effect of exposure of organisms from honey to solutions of lower osmotic pressure, and consequently dilution blanks of 50 per cent honey were employed for the quantitative tests.

Table V.—Effect of Concentration of Dilution Blanks upon Colonies Developing on Plates of 60% Honey Agar

—	Sample 1	Sample 2	Sample 3
Average number of colonies on plates—			
Dilution blank—50% honey	21·6	53·2	8·0
20% honey	16·6	30·2	5·0
Tap water	2·0	2·8	0·0

Using the procedure described, determinations of the number of colonies of organisms from thirteen samples of fermented honey were made. Plates were made in quintuplicate, incubated at 37° for six days and then left several days at room temperature before being counted, the results being shown in table VI.

Table VI.—Quantitative Analysis of Fermented Honey

Sample	Yeast colonies per gram (average of 5 plates)
Bruce 29	52,200
Bruce 34	25,900
Bruce 35	68,600
Bruce 39	18,100
Dufferin 15	24,000
Durham 2	8,750
Durham 11	24,400
Durham 21	25,600
Essex 7	381,000
Peterboro 43	122,300
Prescott	6,070
Prince Edward 1	17,850
No. 101	86,800

The colonies developing from fermented honey were in all cases those of yeasts, bacteria not having been observed on any of the plates. Macroscopic examination of the cultures did not reveal any marked difference between the forms developing from the various samples analyzed. For the purpose of studying more closely the types of yeasts encountered, ten colonies from each sample of honey were selected and subcultures made on 60 per cent honey agar slants, making in all 130 yeast cultures.

A study of the cultures isolated soon revealed the fact that they represented but a small number of different types. For comparison, inoculations were made of each to honey solution, honey agar slants, and to yeast water honey agar and standard 60 per cent honey agar for giant colony formation. Comparison of the type of growth, both at 37 degrees and 20 degrees, aided by microscopic observations, resulted in the elimination of large numbers of the original cultures as being of identical types. Further cultural and morphological study, together with the employment of additional media, such as potato, carrot, honey gelatine and various carbohydrates permitted of further elimina-

tion of those kept, which had been subjected to repeated plating out and retesting to insure purity of type. The original 130 cultures were in this way finally reduced to 4 different types, all representatives of the genus *Zygosaccharomyces.*

DESCRIPTION OF YEASTS ISOLATED FROM FERMENTED HONEY

In the preliminary tests on the relationship of honey concentration to yeast development an incubation temperature of 37 degrees was employed. At this temperature media of high osmotic pressure permit of abundant development of honey yeasts which do not grow, however, on substrates of lower concentration. In studying the action of such yeasts on various carbohydrates it was found impossible to prepare with those of less solubility, solutions of sufficiently high concentration to permit of yeast growth. An incubator of 30° C. having become available, it was found that at the lower temperature honey yeasts were able to develop in lower concentrations of honey and sugars, than at 37° C. The interesting observation was also made that there is a definite correlation between the temperature and the minimum honey concentration at which honey fermenting yeasts are able to develop. This phenomenon will be discussed later (see p. 33).

For the determination of the characteristics of yeasts from fermented honey, also of those from other sources described later, observations were made of the growth on honey agar 70 per cent, honey agar 15 per cent, honey broth per cent, honey gelatine 15 per cent, and in various carbohydrates in 10 per cent solution. For the last named test a basic medium of yeast extract was employed to which the carbohydrates were added, Durham fermentation tubes being employed. The following were employed for fermentation studies: arabinose, xylose, dextrose, levulose, mannose, galactose, saccharose, maltose, lactose, raffinose, dextrin, mannite, dulcite and salicin. In addition, giant colony formation was observed on honey agar 15 per cent using 200 c.c. Erlenmeyer flasks containing 50 c.c. of medium. Observation was also made of growth at 37 degrees on honey agar of 15 per cent and 70 per cent respectively, this being considered an important point of characterization, particularly in classifying nectar yeasts. Examination for spore formation was made on such media as honey agar, carrot and potato while, in addition, gypsum blocks were employed in many cases.

Culture J7 (*See* fig. 3)

In young cultures on 15 per cent honey agar cells elliptical to round, occurring generally in pairs or short chains. Reproduce by budding. The great majority of the cells are 4-6 μ in length and 3-4 μ in width; seldom longer than 7 μ. On 70 per cent honey agar cells slightly smaller. Isogamic copulation may be observed on such media as carrot, 70 per cent honey agar, yeast-honey agar, etc., with spore formation. Generally two to four spores observed in ascus, spores being round and approx. 3 μ in diameter.

Honey Agar 15 Per Cent.—Growth abundant, filiform, spreading slightly, cream coloured, becoming somewhat darker in shade with age; also becoming raised, very folded and wrinkled; glistening in young cultures but soon becoming dull; slightly cheesy in consistency, medium unchanged.

Honey Agar 70 Per Cent.—Growth abundant, filiform, light brown in colour, becoming darker; raised and irregularly folded and wrinkled, dull, softly brittle in consistency.

Honey Broth 15 Per Cent.—Vigorous alcoholic fermentation with gas; moderate clouding, abundant light brown ring growth, abundant flocculent sediment.

Honey Broth 70 Per Cent.—Vigorous alcoholic fermentation with gas; abundant brownish ring growth, with tendency to form pellicle.

Giant Colony, Honey Agar 15 Per Cent.—Abundant growth, colony tending to heap up into irregular folds and wrinkles; cream coloured becoming darker in shade; edge lobate and somewhat irregular in outline; at first glistening, later becoming dull.

Carrot.—Growth abundant, cream coloured, raised; surface finely wrinkled and folded, edge irregular forming fine fringe; dull; medium unchanged.

Potato.—Growth moderate, lighter in colour than on carrot being whitish-grey; surface verricose and irregular; dull, soft cheesy consistency, medium unchanged.

Milk.—Moderate brownish-yellow surface ring of finely dotted growth on side of tube; no change in liquid after 6 weeks other than a very faint acidity.

Gelatine.—No liquefaction in 15 per cent or 70 per cent honey gelatine after 6 weeks.

Growth at 37°C.—Grows well on 70 per cent honey agar, feebly on 15 per cent.

Fermentations.—Ferments dextrose, levulose, mannose, saccharose and maltose with acid and gas; some acidity but no gas with galactose and mannite; no acid or gas with arabinose, xylose, lactose, raffinose, dextrin, salicin or dulcite.

This yeast appears to have the some fermentative properties as the fourth type described by Fabian and Quinet, who consider the yeast isolated by them to correspond to *Zygosaccharomyces priorianus* Klöcker, isolated from the bodies of bees. This hardly appears justified since Klöcker himself (7), not as reported in Guilliermond (3), states that *Z. priorianus* ferments dextrose and maltose, but not saccharose and lactose. It appears, therefore, that our yeast J7 is not identical with this species, nor does it, from the description of the type isolated by Fabian and Quinet, correspond with that of the last named authors. Our yeast was the least abundant of the four types isolated and stood out prominently from the other three by reason of its characteristic growth on solid media. As it does not, therefore, appear to have been previously described the name of *Zygosaccharomyces nussbaumeri* n.sp. is suggested for this yeast.

Culture E6 (*See* fig. 4)

In young cultures on 15 per cent honey agar cells mostly elliptical, though some appear almost round. Occur mostly in pairs, short chains or small irregular clusters. May reproduce asexually by budding. Average size of cells 5 μ long by 3·5 μ wide, few being larger than 6 μ by 4 μ. In old honey broth cultures there is a tendency to form distinctly elongated cells. Copulation may be observed on such media as honey agar, potato and carrot, resulting in the formation of ascospores, usually 2 to 4 in number, and generally about 3 μ in diameter.

Honey Agar 15 Per Cent.—Growth abundant, filiform, cream coloured at first, becoming darker in shade with age; surface covered with fine folds with a pronounced striated fringe, later tending to become verrucose; dull lustre, butyrous consistency, medium unchanged.

Honey Agar 70 Per Cent.—Moderate, filiform growth, brownish, becoming gradually darker in shade, slightly raised; surface mostly smooth, with tendency to become slightly verrucose; at first glistening, later becoming dull; butyrous consistency.

Honey Broth 15 Per Cent.—Vigorous alcoholic fermentation with gas; slight light brown surface ring, slight clouding, moderate flocculent sediment.

Honey Broth 70 Per Cent.—Vigorous fermentation with gas; fairly heavy brownish surface ring.

Giant Colony, Honey Agar 15 Per Cent.—Spreading, irregularly round, somewhat raised, at first cream coloured, becoming darker with age; surface finely wrinkled and granular; irregular lobed and striated fringe round colony; rather dull in lustre.

Carrot.—Growth moderate, irregular, raised, cream coloured, with contoured surface; dull in lustre, butyrous consistency medium unchanged.

Potato.—Growth moderate, irregular, at first cream coloured, becoming later pale yellowish-green in shade; raised and rugose, generally glistening at first, becoming later dull in lustre; soft cheesy consistency.

Milk.—Faint whitish to pale yellow dotted growth on side of tube; no change in milk after 6 weeks.

Gelatine.—No liquefaction in 15 per cent or 70 per cent honey gelatine after 6 weeks.

Growth at 37°C.—Grows well on 70 per cent honey agar; no growth on 15 per cent.

Fermentations.—Dextrose, levulose, mannose and saccharose are fermented with acid and gas, the last less readily; no fermentation of arabinose, xylose, galactose, maltose, lactose, raffinose, dextrin, mannite, salicin or dulcite.

This yeast has the same fermentative properties as Marvin's strain A and Type II of Fabian and Quinet, who consider the latter to correspond with Z. *barkeri* (Barker) Saccardo et Sydow. Although comparison of the cultural characteristics of our yeast with those of Fabian and Quinet's Type II is rendered somewhat difficult by their use of such terms as "butyrous, brittle" to denote consistency and "glistening, dull" to describe lustre, we are likewise inclined to regard our culture E6 as corresponding to *Zygosaccharomyces barkeri* (Barker) Saccardo et Sydow.

Culture D1 (*See* fig. 5)

Young cultures on honey agar 15 per cent show mostly elliptical cells, although some are noticeably elongated and more irregular in shape than J7 or E6. Cells occur singly, in pairs, or in short chains and groups. The majority of the cells are 4·5-6 μ long and 3-4·5 μ wide, the longer cells attaining a length of 8 or 9 μ. On 70 per cent honey agar cells may frequently be round to lemon-shaped. May reproduce asexually by budding, and also by means of ascospores formed as a result of isogamic copulation. Copulation may be seen on such media as potato, gelatine, carrot, yeast-honey agar, though this yeast (also M1), does not form spores as readily as J7. Spores, however, may be best obtained on old 70 per cent honey gelatine cultures, 2 to 4 generally being noted in the ascus. The spores usually measure 3 to 3·5 μ in diameter.

Honey Agar 15 Per Cent.—Growth abundant, filiform, cream coloured, becoming later darker in shade; growth somewhat raised, surface finely rugose, edge lobate; slightly glistening at first but becoming dull in lustre later; butyrous consistency.

Honey Agar 70 Per Cent.—Growth abundant, filiform, brown in colour, becoming darker with age; raised with finely wrinkled surface, at first glistening but later dull in lustre; consistency butyrous.

Honey Broth 15 Per Cent.—Vigorous alcoholic fermentation with gas; light brownish surface ring, no pellicle; liquid clouding with moderate flocculent sediment.

Honey Broth 70 Per Cent.—Vigorous alcoholic fermentation with gas; brownish ring at surface.

Giant Colony, Honey Agar 15 Per Cent.—Round colony with irregular lobate edge, somewhat raised, cream coloured, becoming darker; surface finely granular and verrucose, glistening at first, becoming later dull.

Carrot.—Moderate growth, cream coloured, somewhat raised, dull, butyrous, medium unchanged.

Potato.—Growth abundant, irregular, raised, rugose, cream coloured becoming later brownish; dull lustre, soft cheesy consistency, medium unchanged.

Milk.—Slight, pale yellowish, dotted growth on side of tube; medium unchanged after 6 weeks.

Gelatine.—No liquefaction noted after 6 weeks in 15 per cent or 70 per cent honey gelatine.

Growth at 37° C.—Grows well on 70 per cent honey agar, no growth on 15 per cent.

Fermentations.—Dextrose, levulose and mannose are fermented with acid and gas production. Other carbohydrates tested were not fermented.

This yeast has the same fermentative properties as Marvin's strains I, IA and B, and Type III of Fabian and Quinet, and as far as may be judged by a comparison of their characteristics, our culture D1 may be classed as *Zygosaccharomyces mellis* Fabian et Quinet.

Culture M1 (*See* fig. 6)

In young cultures on 15 per cent honey agar cells are mostly round to elliptical with considerable variation in size. Cells may be seen in pairs or singly, but there is a tendency to adhere together in clumps of various sizes. Cells vary in length from 3 to 8 μ and in width from 2·5 to 6 μ, the average size being 5 μ by 3·5 μ. Reproduces asexually by budding. Spores are formed by isogamic copulation which may be noted on such media as carrot, honey agar and honey gelatine. Of the substrates tried by us, 70 per cent honey gelatine is best adapted for the observation of spores which occur generally two in number in the ascus. More seldom, 3 may be noted. The spores, which are round to oval, generally measure 2·5 to 3·5 μ in diameter.

Honey Agar 15 Per Cent.—Growth abundant, filiform, at first light cream in colour, turning darker with age; surface becoming covered with dotted growth, giving a verrucose appearance; lustre dull, opaque, consistency butyrous.

Honey Agar 70 Per Cent.—Moderate filiform growth, greyish-brown in colour, surface somewhat wrinkled near edges and somewhat dotted; glistening at first, becoming later dull in lustre; butyrous consistency.

Honey Broth 15 Per Cent.—Vigorous fermentation with gas, liquid becoming clear, with abundant flocculent sediment; surface ring, dark cream coloured.

Honey Broth 70 Per Cent.—Vigorous fermentation with gas; abundant dark brown surface ring.

Giant Colony, Honey Agar 15 Per Cent.—Growth spreading, slightly raised and convex, cream coloured to slightly brownish, surface becoming dotted in older cultures; edge even or slightly lobate; at first slightly glistening but becoming later dull in lustre.

Carrot.—Growth abundant, spreading slightly, cream coloured, somewhat raised toward centre; edge lobate, surface contoured, slightly glistening, medium unchanged.

Potato.—Growth less abundant than on carrot, filiform to beaded, raised, surface rough, at first dull, cream coloured later becoming very faintly greenish; cheesy consistency, medium unchanged.

Milk.—Slight, pale yellowish, dotted growth on side of tube; medium unchanged after 6 weeks.

Gelatine.—Liquefaction in 15 per cent gelatine, none in 70 per cent after 6 weeks.

Growth at 37° C.—Grows well on 70 per cent honey agar, no growth on 15 per cent.

Fermentations.—Dextrose, levulose and mannose are fermented with acid and gas formation. Other carbohydrates tested were not fermented.

Although culture M1 has the same fermentative reactions as culture D1 yet its different morphological and cultural characteristics on various media, together with its power of liquefying 15 per cent honey gelatine, entitle it to separate classification. As we have not yet encountered any similarly described organism, the name *Zygosaccharomyces richteri* n. sp. is suggested.

PART II.—INFECTION OF HONEY BY SUGAR-TOLERANT YEASTS

On the basis of investigations already made it appears to be quite definitely established that sugar-tolerant yeasts are the active agents in causing fermentation of honey. Accordingly, measures to combat this spoilage of honey will be two-fold in scope, embracing, not only a consideration of means to prevent the development of yeasts already present in honey, but also a study of possible measures to reduce the initial infection of honey by such yeasts to a minimum. It was, therefore, considered a matter of importance to secure information regarding possible sources of contamination by sugar-tolerant yeasts.

A. INVESTIGATION OF FLOWERS VISITED BY BEES

NECTAR YEASTS

Several studies have already been recorded by investigators in Europe and Japan dealing with the occurrence of yeasts in the nectar of various flowers, although, as far as we are aware, these investigations have not been concerned with the examination of nectar for the presence of yeasts capable of fermenting honey, nor have tests been made apparently to determine whether types isolated were in any degree sugar-tolerant.

Reukauf (13) and Stoltz (18) isolated yeasts from the flowers of various plants, the former advancing the view that, in general, particular yeast types are associated with particular flowers on account of variations in the composition of the nectar. Stoltz recognized a relationship between the visiting of flowers by insects and the occurrence of yeasts in the flower. Hilkenbach (4) described 12 forms of yeasts isolated from a variety of flowers. This

author also reports the presence of yeasts in 60 per cent of the flowers of plants examined in summer and in 20 per cent of those examined in the spring. Furthermore insects play the chief rôle in the infection of flowers by yeasts although the wind is also a factor. Unlike Reukauf, the author noted no special relationship between yeast types and particular groups of flowers.

Schuster and Ulehla (17) investigated some 32 species of plants, finding infection by yeasts in 23 cases. In all, 10 forms of yeasts were isolated by these authors, two being quite widely distributed in various plants, while other types were found only in particular species. The same investigators furthermore noticed that infection was brought about chiefly through the agency of insects visiting the plants.

Schoellhorn (16) made investigations of nectar yeasts from different varieties of winter plants, and found that the nectar of flowers of the same species collected in different localities at the same season contained frequently the same kind of yeast. The infection, moreover, may recur the following year at the same time. Less infection was found in alpine plants than in those plants from the plains, while greenhouse flowers were almost always sterile. The authors found the yeasts isolated to be chiefly *Torulae* capable of fermenting monosaccharides only.

More recently Jimbo (6) has reported from Japan investigations of yeasts isolated from flower nectar. During the period from April 20 to June 27, 273 flowers of 23 species of plants were examined for yeast infection, positive findings being obtained in the case of 120 flowers (44 per cent), representing almost all the species under investigation. The author distinguished in all 22 forms of yeast, finding, however, no definite relationship between the species of plant and the type of yeast present. The rate of infection was stated to increase with the seasonal rise in temperature. The yeasts isolated were all incapable of causing an alcoholic fermentation with dextrose, saccharose, maltose and lactose while in no case was ascospore formation observed.

Summing up the work already done, it may be said that yeasts are frequently found in various plants and that insects play an important part in their distribution, the wind being a less important means of furthering their spreading. Whether definite yeast types are associated with definite plants is as yet an unsettled question in view of the conflicting evidence on this point. Of the yeasts so far isolated, spore forming types have apparently not been described. The relationship of nectar yeasts to honey fermentation has not been considered in the works referred to, and no data are available regarding the sugar-tolerance of yeasts hitherto isolated from flowers nor their possible rôle as active agents in honey fermentation.

PRESENT INVESTIGATIONS

To study the presence in various flowers of sugar-tolerant yeasts which might conceivably be carried by bees to infect the nectar in the hives, a series of experiments was conducted during the season 1928 in which examinations were made of flowers commonly visited by bees during the course of the honey flow.

During the period June 5 to September 14, examinations were made of the flowers in the vicinity of the Experimental Farm which were being visited by bees at the time, the tests embracing 34 varieties of flowers. In making the examination it was not considered essential, from the standpoint of the experiment, to differentiate between the nectar and other parts of the flower with which the bee might come in contact. Suitable portions of the flower, depending upon the type, were transferred, using aseptic precautions, to sterile tubes of 80 per cent honey broth, which medium permitted the development of sugar-tolerant types only.

During the course of the tests 44 separate examinations were made, in each instance 12 tubes being inoculated from different individual flowers, giving in all 528 tubes.

For the sake of comparison an equal number of tubes was inoculated from the same sources using, instead, a 15 per cent honey broth. The tubes were incubated at 30°C. to observe the development of fermentation, tubes being held, if necessary, as long as 6 weeks before finally recording negative results. In Table VII are recorded the inoculations made throughout the season and the findings observed.

TABLE VII.—FERMENTATION IN TUBES INOCULATED FROM FLOWERS VISITED BY BEES

Lab. No.	Date	Flowers examined	Percentage of tubes showing fermentation	
			80 p.c. honey	15 p.c. honey
1	June 5	Dandelion (*Taraxacum officiniale*, Weber)	41·7	50·0
2	" 5	Apple blossom (*Pyrus* sp.)	58·3	33·3
3	" 5	Purple lilac (*Syringa* sp.)	0·0	50·0
4	" 5	White honeysuckle (*Lonicera*, L.)	75·0	50·0
5	" 5	Siberian pea (*Caragana arborescens*, Lam.)	58·3	41·7
6	" 5	Cherry blossom (*Prunus* sp.)	8·3	0·0
7	" 7	Crab-apple blossom (*Pyrus* sp.)	25·0	16·7
8	" 7	Rowan-tree (*Pyrus Aucuparia* (L.) Ehrh.)	16·7	8·3
9	" 7	Japanese barberry (*Berberis Thunbergii*, D.C.)	25·0	0
10	" 7	Dogwood (*Cornus pubescens*, Willd.)	50·0	41·7
11	" 7	White lilac (*Syringa* sp.)	8·3	8·3
12	" 7	Spiræ (*Spiræa* sp.)	0	0
13	" 13	Pink honeysuckle (*Lonicera* sp.)	41·7	8·3
14	" 14	Pine (*Pinus*, L.)	33·3	0
15	" 14	Wild mustard (*Brassica arvensis* (L). Ktze.)	50·0	8·3
16	" 27	Red clover (*Trifolium praetene*, L.)	16·7	25·0
17	" 27	White daisy (*Chrysanthemum Leucanthemum*, L.)	8·3	8·3
18	" 27	Fleabane (*Erigeron philadelphicus*, L.)	58·3	8·3
19	" 27	Raspberry (*Rubus idaeus*, L.)	91·7	0
20	" 28	Dutch clover (*Trifolium repens*, L.)	33·3	16·7
21	" 28	Alsike clover (*Trifolium hybridum*, L.)	8·3	0
22	" 28	Bladder campion (*Silene latifolia*, Mill.)	83·3	8·3
23	" 29	Hound's tongue (*Cynoglossum officiniale*, L.)	8·3	50·0
24	July 4	Mock orange (*Philadelphus* (Riv.) L.)	25·0	0
25	" 4	Yellow wood (*Cladrastis*, Raf.)	25·0	8·3
26	" 11	Red clover (*Trifolium praetense*, L.)	16·7	16·7
27	" 11	Alsike clover (*Trifolium hybridum*, L.)	58·3	25·0
28	" 11	Dutch clover (*Trifolium repens*, L.)	75·0	25·0
29	" 11	Yellow trefoil (*Medicago lupulina*, L.)	33·3	25·0
30	" 11	White sweet clover (*Melilotus alba*, Desr.)	58·3	25·0
31	" 20	Alfalfa (*Medicago sativa*, L.)	0	8·3
32	" 26	White Sweet clover (*Melilotus alba*, Desr.)	50·0	25·0
33	" 26	Basswood (*Tilia* sp.)	58·3	0
34	" 26	Canada thistle (*Cnicus arvensis* (L.), Scop.)	16·7	0
35	Aug. 10	White sweet clover (*Melilotus alba*, Desr.)	83·3	0
36	" 10	Golden rod (*Solidago* sp.)	66·7	16·7
37	" 17	Buckwheat (*Fagopyrum esculentum*, Moench.)	58·3	0
38	" 20	White sweet colver (*Melilotis alba*, Desr.)	66·7	0
39	" 29	Buckwheat (*Fagopyrum esculentum*, Mcench.)	16·7	0
40	" 29	Dutch clover (*Trifolium repens*, L.)	41·7	0
41	" 29	Red clover (*Trifolium praetense*, L.)	75·0	66·7
42	" 29	Butter and eggs (*Linaria vulgaris*, Hill)	75·0	25·0
43	" 31	Sunflower (*Helianthus*, L.)	25·0	0
44	Sept. 14	Sunflower (*H. lianthus*, L.)	100·0	0

Microscopic examination of the cultures in 15 per cent honey broth showed in many cases the presence of bacteria as well as yeasts, although in no case were bacteria found in the cultures in 80 per cent honey broth, yeasts being the only microorganisms encountered apart from occasional mold contamination which latter had no part in the actual fermentation.

ISOLATION OF CULTURES

As we were interested primarily in establishing the presence of sugar-tolerant yeasts only in flowers, the 80 per cent honey cultures alone were used for isolation of yeasts for more detailed study. From one or more tubes, depending upon the number fermented, plate cultures were made using 80 per cent honey agar and incubated at 30°C. Yeasts alone were found to grow and isolations were made of the different types developing for further replating and examination in pure culture.

The cultures isolated, seventy-one in number, were subjected to the same process of comparison and elimination as previously described for those from fermented honey, and in this way the number of types was reduced to eleven, the frequency of their occurrence being summarized in table VIII.

TABLE VIII.—FREQUENCY OF OCCURRENCE OF TYPES OF SUGAR-TOLERANT YEASTS ISOLATED FROM FLOWERS

Yeast Type	Isolated from Flower Examination (for number reference see Table VII)
N 4	1, 2, 4, 5, 7, 13, 14, 15, 17, 20, 22, 24, 25, 34, 37.
N 8	8, 43
N 11	9, 10, 11, 19, 44
N 18b	17, 18, 19, 26, 27, 28
N 23	16, 23, 28, 33
N 24	1, 2, 4, 5, 6, 8, 10, 13, 18, 19, 20, 21, 24, 25, 32, 33, 37, 38, 43, 44
N 32b	30, 32, 35, 36, 43
N 34b	34
N 38a	18, 27, 38
N 39a	39, 40
N 41	33, 41, 42, 44

It will be observed from the data presented in tables VII and VIII that almost all the species of flowers examined contained sugar-tolerant yeasts, negative results in all of the twelve tubes being recorded in only three instances, namely, purple lilac June 5, spirea June 7 and alfalfa July 20. Our findings do not lend support to the hypothesis that certain yeast types are associated with special flower types. There does appear, however, to be some indication of seasonal influence upon certain species of yeasts. For instance, type N 18b was found only from June 27 to July 11, type N 32b from July 11 to August 31, and type N 41 from July 26 to September 14. Although in some cases yeast types were restricted to one or very few species of flowers, yet others, such as N4 and N24, were encountered in a wide range of flowers recurring in cultures thoroughout a comparatively long period.

Of the eleven types of yeasts isolated from flowers, two proved to be specifically the same as two of those isolated by us from fermented honey. Type N8, found in the rowan-tree blossom June 7 and in the sunflower August 31, proved to be identical with culture M1 from fermented honey, white type N 41, isolated from basswood July 26, red clover August 29, butter and eggs August 29 and sunflower September 14, could be identified with culture J7, likewise isolated first from fermented honey. In addition, type N 24, found in a variety of flowers including dandelion, apple blossom, honeysuckle, cherry blossom, rowan-tree blossom, Dutch and alsike clover, sweet clover, buckwheat and sunflower, appears, as far as we are able to judge, to be very closely related to, if not identical with, *Torula mellis*, found by Fabian and Quinet in fermented honey.

DESCRIPTION OF YEASTS ISOLATED[1]

Culture N4 (*Zygosaccharomyces* sp.) (*See* fig. 7)

In young cultures on honey agar 15 per cent, cells are round to oval, occurring generally singly, in pairs or in small groups. Average size of cells is 4·5 μ in diameter, varying in most cases from 3 to 5·5 μ. Reproduces asexually by budding. Spores are formed resulting from copulation and may be readily observed on various media such as honey agar, honey gelatine and in the ring growth at the surface of honey broth cultures. Usually one to four ascospores observed in the ascus, the spores being generally unevenly divided, three being the most common number observed (honey broth). Spores are round to oval, usually 3 to 3·5 μ in diameter, some attaining a length of 4·5 μ.

Honey Agar 15 Per Cent.—Growth slow and generally scanty to moderate, at first beaded along line of inoculation; cream coloured becoming somewhat darker with age; raised and contoured, dull; butyrous consistency.

Honey Agar 70 Per Cent.—Scanty to moderate growth, filiform, light brown in colour; edge lobate, dull lustre, soft butyrous consistency.

Honey Broth 15 Per Cent.—Weak fermentation with gas, liquid slightly cloudy with flocculent suspension; dark cream coloured ring at surface; fairly abundant flocculent sediment.

Honey Broth 70 Per Cent.—Active alcoholic fermentation; brownish surface growth with scanty sediment.

Giant Colony, Honey Agar 15 Per Cent.—Growth slow, tending to pile up with comparatively little spreading; cream coloured, rugose surface, dull lustre and butyrous consistency.

Carrot.—Growth scanty; dull, cream coloured, raised and beaded at first; medium unchanged.

Potato.—Growth absent or very scanty; when it appears finely dotted, cream coloured; medium unchanged.

Milk.—After six weeks no change apart from a very slight ring growth at surface of milk on sides of tube.

Gelatine.—No liquefaction observed after eight weeks with 70 per cent or 15 per cent honey gelatine.

Growth at 37°C.—Grows on 70 per cent honey agar, no growth on 15 per cent honey agar.

Fermentations.—Dextrose and levulose are the only sugars fermented of those tried. No fermentation observed with arabinose, xylose, galactose, mannose, saccharose, maltose, lactose, raffinose, dextrin, mannite, salicin or dulcite.

Culture N8 (*Zygosaccharomyces richteri* n.sp.)

Found to be identical with Culture M1 isolated from fermented honey and described on p. 15.

Culture N11 (*Torula* sp.) (*See* fig. 8)

Young cultures on honey agar 15 per cent show mostly oval to round cells which vary considerably in size. On other media the appearance is much the same. The cells are seen generally in pairs, in short chains of three or four

[1] For sources and frequency of occurrence of yeasts see Tables VII and VIII.

members or in groups of several cells due to multiple bud formation from a single mother cell. The cells usually measure from 2 to 4·5 μ in length and from 1·5 to 4 μ in width, the average size being approximately 2·5 by 2 μ. Reproduces asexually by budding. Ascospore formation has not been observed.

Honey Agar 15 Per Cent.—Growth abundant, at first filiform, then spreading, light cream coloured; surface at first smooth, becoming later verrucose in central portion; glistening, soft butyrous consistency.

Honey Agar 70 Per Cent.—Growth abundant, filiform at first, then spreading and flat; surface smooth; brown in colour, lustre at first glistening but later becoming dull; butyrous consistency.

Honey Broth 15 Per Cent.—Alcoholic fermentation with gas, liquid cloudy; yellowish-brown ring at surface; abundant, flocculent sediment.

Honey Broth 70 Per Cent.—Alcoholic fermentation with gas; a heavy brown ring at surface of liquid.

Giant Colony, Honey Agar 15 Per Cent.—Round, spreading, cream coloured, convex colony, with somewhat irregular, lobate edge; surface smooth and glistening, showing in older cultures rugose central portion; butyrous.

Carrot.—Abundant growth, dark cream coloured, raised and verrucose, glistening, butyrous; medium unchanged.

Potato.—Abundant growth, light brown in colour; raised, contoured surface; dull lustre, stiffly butyrous consistency; medium unchanged.

Milk.—After eight weeks no noticeable change in medium; yellowish surface ring growth.

Gelatine.—No liquefaction observed in 15 per cent or 70 per cent honey gelatine after eight weeks.

Growth at 37° C.—Grows well on 70 per cent honey agar, slightly on 15 per cent.

Fermentations.—Dextrose, levulose, mannose, saccharose and raffinose are fermented with acid and gas. No fermentation observed with arabinose, xylose, galactose, maltose, lactose, dextrin, mannite, salicin or dulcite.

Culture N18b (*Torula* sp.) (*See* fig. 9)

Young cultures on 15 per cent honey agar show small oval to round cells which may occur singly, in pairs, in short chains or in small groups. The average size is 3 by 2 μ, the cells varying in length from 2·5 μ to 4 μ and in width from 1·5 to 2 μ. On 70 per cent honey agar cells somewhat larger, the average size being 3·5 by 2·5 μ. Surface ring growth in 70 per cent honey broth shows the cells more variable in size, some attaining a diameter of 5·0 μ. There is likewise tendency to multiple bud formation. In old cultures on yeast honey agar, cells contain highly refractile bodies. Reproduces asexually by budding. Spore formation has not been observed.

Honey Agar 15 Per Cent.—Growth abundant, at first filiform, greyish-cream in colour, then spreading and becoming light brown; surface with fine network markings, glistening with soft butyrous consistency.

Honey Agar 70 Per Cent.—Moderate growth, filiform, brown, slightly raised, smooth, glistening, butyrous, becoming somewhat viscous in old cultures.

Honey Broth 15 Per Cent.—Active alcoholic fermentation, liquid cloudy with fine suspension; moderate to heavy finely flocculent sediment; cream coloured ring at surface.

Honey Broth 70 Per Cent.—Vigorous fermentation; light brown ring at surface of liquid with slight pellicle.

Giant Colony, Honey Agar 15 Per Cent.—Round, slightly convex colony with irregular edge, light brown in colour; central portion becomes covered with network and dotted growth; lustre glistening.

Carrot.—Growth abundant and irregular in outline, fairly flat, with irregular surface, slightly glistening; soft butyrous consistency.

Potato.—Growth abundant and irregular, fawn coloured, surface verrucose and glistening; consistency stiffly butyrous.

Milk.—At first light yellowish surface ring; later, after 6 weeks, coagulation with soft curd, the reaction being alkaline.

Gelatine.—Liquefaction in 15 per cent honey gelatine, none in 70 per cent after 2 months.

Growth at 37°C.—Slight growth on 70 per cent honey agar; grows well on 15 per cent, the growth being slightly salmon coloured.

Fermentations.—Ferments dextrose, levulose, mannose, saccharose, and raffinose with acid and gas. No fermentation was observed with maltose, lactose, dextrin, galactose, mannite, arabinose, salicin, or dulcite.

Culture N23 (*Torula* sp.) (*See* fig. 10)

Young cultures on honey agar 15 per cent show round to oval cells, generally found singly or in pairs with occasional short chains. The majority of cells are 3 to 4·5 μ long and 2·5 to 4 μ wide, variations in width from 2 to 5 μ being seen. In old cultures on this and on carrot the cells are mostly elongated and very irregular in shape, with irregular budding giving in some instances the appearance of rudimentary copulation. Spore formation, however, has not been observed. On honey agar 70 per cent the cells are less uniform in size, showing more larger cells than on 15 per cent honey which may attain a diameter of 10 μ. There is also much multiple bud formation. Many cells on this medium, in the ring growth on honey broth 70 per cent and on potato are elongated, and in older cultures are frequently joined end to end in short chains to give the appearance of mycelium.

Honey Agar 15 Per Cent.—Growth abundant, at first cream coloured and smooth, becoming later more coffee coloured with tendency to spread; older cultures normally become verrucose; at first glistening, becoming dull in older cultures; butyrous consistency.

Honey Agar 70 Per Cent.—Growth moderate, filiform, at first flat, smooth and glistening with surface finely verrucose; becoming dark brown, dull and viscous with age.

Honey Broth 15 Per Cent.—Fermentation with gas, liquid moderately cloudy with abundant brownish-yellow ring at surface; abundant, finely flocculent sediment.

Honey Broth 70 Per Cent.—Alcoholic fermentation with gas; abundant brownish-yellow surface ring.

Giant Colony, Honey Agar 15 Per Cent.—Round, with irregular lobate edge, convex, light brown in colour; smooth at first, becoming finely verrucose, faintly glistening; butyrous consistency.

Carrot.—Growth moderate, slightly pinkish; surface raised and rugose; glistening, butyrous; medium unchanged.

Potato.—Scanty to moderate growth, irregular in outline, raised; at first light cream coloured, becoming brown and later chalky; dull lustre, stiffly butyrous consistency; medium discoloured brownish.

Milk.—Action slow, resulting after six to eight weeks in an acid coagulation with soft curd.

Gelatine.—Slight liquefaction of 15 per cent honey gelatine in six to eight weeks; no liquefaction observed in 70 per cent gelatine after eight weeks.

Growth at 37°C.—Grows at this temperature on both 70 per cent and 15 per cent honey agar, showing a slightly pinkish colouration on the latter medium.

Fermentations.—Dextrose, levulose, mannose, saccharose and raffinose fermented with acid and gas. In lactose an acidity is produced but no fermentation in this or in arabinose, xylose, galactose, maltose, dextrin, mannite, salicin or dulcite.

Culture N24 (*Torula (Mycotorula) mellis*) (*See* fig. 11)

In young cultures on honey agar 15 per cent, cells are fairly large, round to oval in shape, and may occur often in short chains. There is a frequent tendency for cells to germinate into promycelia of varying lengths. Cells are thick walled and vary for the most part from 4·5 to 9μ in length and from 3 to 7·5 μ in width, the average size being 7 by 5·5 μ. On honey agar 70 per cent majority of cells round or slightly oval, varying in diameter from 4 to 10 μ, although frequently elongated, cylindrical cells are seen from 10 to 25 μ in length, often joined together in chains to form mycelia. Most of the cells contain one or several round refractile bodies which microchemical tests reveal as oil or fat globules. The yeast reproduces asexually by budding. Spore formation has not been observed on any of the media employed.

Honey Agar 15 Per Cent.—Growth abundant, at first filiform, raised and much wrinkled, dirty white in colour with dull lustre. As culture ages the colour changes to yellowish, greenish and finally as it spreads and increases in volume becomes dark brown to black, the surface being very folded and wrinkled with occasional white or grey outgrowths having a fungoid appearance; medium shows dirty greenish discoloration.

Honey Agar 70 Per Cent.—Growth abundant, spreading, greenish-brown in colour, surface folded and wrinkled; in very old cultures colour changes to dark brown to black.

Honey Broth 15 Per Cent.—Moderate alcoholic fermentation with gas; a heavy thick pellicle, brownish-green in colour, forms readily on surface; at first a flocculent, yellowish growth in liquid which sinks leaving the liquid clear.

Honey Broth 70 Per Cent.—Active alcoholic fermentation; a yellowish surface growth readily forms, which turns later to brownish-yellow in colour; abundant growth on side of tube.

Giant Colony, Honey Agar 15 Per Cent.—General form of colony, round; spreading with edge rather indefinite owing to its growth into the medium; at

first brownish-yellow with the outer portion greyish; surface much raised and covered with minute globular outcroppings; in older cultures colour turns darker, finally becoming black, and heaped up in lumps, dirty grey or white patches appearing giving a fungoid appearance.

Carrot.—Moderate growth, at first pale yellowish in colour, raised and lumpy, soon becoming darker in shade as it changes through green and brown and finally becomes black; dull in lustre, with somewhat dry, flaky consistency; medium slightly greenish.

Potato.—Growth moderate, raised and folded; at first light coloured but becoming darker, changing gradually to dark brown; dull lustre with consistency rather tough.

Milk.—Dark greenish to brown growth at surface of liquid on sides of tube, after some weeks coagulation without much change of reaction.

Gelatine.—After eight weeks, some liquefaction of 15 per cent honey gelatine, none observed in 70 per cent.

Growth at 37° C.—The organism grows on honey agar 70 per cent, though no growth was obtained on 15 per cent.

Fermentations.—Dextrose, levulose, galactose, mannose, and maltose are fermented with acid and gas. No fermentation observed with arabinose, xylose, saccharose, lactose, raffinose, dextrin, mannite, salicin or dulcite.

This organism appears to be very closely related to, if not identical with, a yeast found by Fabian and Quinet in fermented honey and named by them *Torula mellis*. Following Guilliermond's classification this type would fall into the genus *Torula*. Although it shows some relationship to *Mycoderma*, yet its fermentative properties prevent its inclusion in that genus. On the other hand, its capacity for rapid scum formation, which in *Torula*, according to Guilliermond only occurs after fermentation, together with its power of forming mycelium out of elongated cells and pronounced fungoid appearance on certain media, suggest the advisability of its inclusion in a separate genus from *Torula*. We are inclined to regard the classification of Janke (5) most suitable, by which this type of yeast would be placed in the genus *Mycotorula*, first proposed by Will (20) in 1916 in his classification of non-sporulating yeasts.

Culture N32b (*Torula* sp.) (*See* fig. 12)

In young cultures on honey agar 15 per cent, the majority of the cells are distinctly elongated, being usually twice as long as wide, although oval and round forms may be met with occasionally. Cells occur generally singly or in pairs. Length of cells varies from 2 to 6 μ, the width seldom exceeding 2·5 μ. Average size of cell 4·5 by 2 μ. On honey agar 70 per cent there is greater variation in shape, frequent ellipsoidal and round cells being seen in addition to the more elongated forms. In older cultures large, round, thick walled cells, 7 to 8 μ in diameter, may be found, usually budding off elongated cells. On honey gelatine 70 per cent the cells are not so distinctly elongated as on honey agar 15 per cent or on carrot, being mostly oval to round and forming groups of several members due to budding. The yeast reproduces asexually by budding. Ascospore formation has not been observed on any of the media tested.

Honey Agar 15 Per Cent.—Growth abundant, spreading, fawn coloured, becoming darker with age; surface at first smooth, becoming later verrucose; glistening; soft butyrous consistency.

Honey Agar 70 Per Cent.—Growth moderate, filiform, dark brown in colour, surface smooth and glistening; consistency butyrous becoming later slightly viscous.

Honey Broth 15 Per Cent.—Active alcoholic fermentation with gas; liquid moderately cloudy with fine suspension; brownish-yellow ring at surface; abundant, finely divided sediment.

Honey Broth 70 Per Cent.—Active alcoholic fermentation with gas; brownish-yellow surface ring.

Giant Colony, Honey Agar 15 Per Cent.—Round, slightly raised, dark cream coloured colony; surface smooth towards edges, becoming verrucose towards central portion; edge entire and slightly lobate; glistening.

Carrot.—Growth abundant, light cream coloured, somewhat raised and rugose; slightly glistening, with soft butyrous consistency; medium unchanged.

Potato.—Abundant growth, cream coloured and somewhat spreading; surface rather rugose and slightly glistening; consistency butyrous at first, becoming stiffer.

Milk.—Light brownish ring at surface of liquid; slow coagulation of milk in six to eight weeks, giving a soft curd; reaction alkaline.

Geltatine.—Liquefaction observed after eight weeks with honey gelatine 15 per cent and 70 per cent.

Growth at 37°C.—Absent with both 15 per cent and 70 per cent honey agar.

Fermentations.—Dextrose, levulose and mannose are fermented with acid and gas. No fermentation observed with arabinose, xylose, galactose, saccharose, maltose, lactose, raffinose, dextrin, mannite, salicin or dulcite.

Culture N34b (*Torula* sp.) (*See* fig. 13)

In young cultures on honey agar 15 per cent, cells mostly oval in shape, generally found singly or in pairs, occasionally in small clusters. On this medium cells fairly uniform in size, being usually 2 to 3 μ in length and 1·5 to 2 μ in width, some attaining, however, a length of 3·5 to 4 μ. On honey agar 70 per cent the cells are generally larger, with greater variation in size. Cells are round to oval and may vary from 2·5 to 6·5 μ in diameter. Ring growth in concentrated honey broth shows round or oval cells which exhibit a greater tendency to multiple bud formation. Reproduction is asexual by budding. Formation of ascospores has never been observed on any of the media used.

Honey Agar 15 Per Cent.—Growth abundant, with tendency to spread, light coffee coloured; surface smooth becoming somewhat verrucose in older cultures; glistening; soft butyrous consistency.

Honey Agar 70 Per Cent.—Moderate filiform growth, brown in colour, with smooth surface, becoming later somewhat verrucose; glistening, with butyrous consistency becoming slightly viscous.

Honey Broth 15 Per Cent.—Moderate fermentation with gas; liquid cloudy; heavy brownish ring at surface; abundant sediment.

Honey Broth 70 Per Cent.—Active fermentation with gas; moderate ring, light brown in colour with tendency to weak pellicle formation.

Giant Colony, Honey Agar 15 Per Cent.—Round, slightly raised, dark cream coloured; surface smooth towards edge, becoming verrucose towards central portion, edge entire; glistening.

Carrot.—Growth abundant, irregular and spreading, somewhat raised and rugose, cream coloured, glistening with soft butyrous consistency; medium unchanged.

Potato.—Growth abundant, irregular, raised and dull; at first pinkish but later becoming light brownish and finally assuming a purplish tinge; cheesy consistency; medium slightly darkened.

Milk.—Pale yellow surface ring growth; slow coagulation with soft curd, reaction being alkaline after 8 weeks.

Gelatine.—Liquefaction in honey gelatine 15 per cent, none observed in 70 per cent after 8 weeks.

Growth at 37°C.—Grows on honey agar 15 per cent, not on 70 per cent medium.

Fermentations.—Dextrose, levulose, mannose, saccharose, and raffinose. are fermented with acid and gas. No fermentation observed with arabinose, xylose, galactose, maltose, lactose, dextrin, mannite, silicin or dulcite.

Culture N38a (*Torula (Mycotorula)* sp.) (*See* fig. 14)

Young cultures on honey agar 15 per cent show large, thick walled, oval cells frequently occurring singly or in pairs. As in culture N 24 cells may give rise to promycelium of irregular shape and varying length. Cells may vary in length from 5 to 10 μ and in width from 4 to 6·5 μ, the average size being approximately 7 μ by 5 μ . In old cultures on this medium the whitish, fuzzy growth appearing on the surface may be seen to be composed of mycelium threads, 2 to 3 μ in diameter. Beneath the surface, the growth consists of oval or elongated cells, generally found singly or in pairs or small groups, with occasional long cells, containing many refractile bodies. On honey agar 70 per cent the growth, which does not have, even in old cultures, the fungoid appearance which is seen on the less concentrated honey medium, is found to consist chiefly of elongated cells, of very irregular outline, frequently joined in short irregular mycelium, often branched. Thick walled oval cells may also be seen. Spore formation has not been observed on the media tried by us.

Honey Agar 15 Per Cent.—Growth abundant, at first filiform, yellowish, wrinkled with fine fringe, later becoming much raised with fungoid appearance; as culture ages the colour darkens, culture becoming dark brown, with white or greyish fuzzy growth appearing on surface, first in patches, and then covering most of the surface; consistency tough; medium discoloured dirty brownish.

Honey Agar 70 Per Cent.—Abundant growth, spreading over surface, brown in colour, slightly raised; surface fairly even, covered with very fine dots giving the whole a dull leathery appearance; slightly gummy consistency; on this medium culture has not fungoid appearance typical of growth on less concentrated honey substrate.

Honey Broth 15 Per Cent.—Fermentation with gas; whitish pellicle on surface and abundant ring growth round side of tube, yellowish to greenish-brown in colour; considerable sediment may be formed due partly to sinking of surface growth.

Honey Broth 70 Per Cent.—Alcoholic fermentation with gas; light brownish pellicle on surface and abundant ring growth of a deeper brown shade.

Giant Colony, Honey Agar 15 Per Cent.—General form of colony round, spreading, with edge rather indefinite owing to growth into the medium; at first yellowish with surface finely wrinkled and showing radial folds; in older cultures the colour changes to dark brown, the surface finally being covered with grey or whitish fuzzy growth, giving a fungoid appearance.

Carrot.—Growth abundant, at first dull white and dry looking, later becoming a dirty greyish fuzzy covering, very tough and tenacious; medium shows dirty greenish discoloration.

Potato.—Abundant growth, becoming black, with white or greyish fuzzy outgrowths; medium shows dirty brownish discoloration.

Milk.—At first a heavy greenish to black surface ring growth; later coagulation takes places with slightly acid reaction, followed by the peptonization of the curd, the reaction changing to alkaline.

Gelatine.—After eight weeks liquefaction slight in honey gelatine 70 per cent, more marked in the 15 per cent medium.

Growth at 37° C.—No growth observed on honey agar 15 per cent or 70 per cent.

Fermentations.—Dextrose, levulose, galactose, mannose, saccharose, maltose, lactose, raffinose and dextrin are fermented. No fermentation observed in the case of arabinose, xylose, mannite, salicin or dulcite.

Culture N39a (*Torula* sp.) (*See* fig. 15)

Young cultures on honey agar 15 per cent show small oval cells, some noticeably elongated, occurring singly, in pairs, in short chains or small clusters. Average size of cell 3 μ long by 2 μ in width, the majority being 2·5 to 4 μ in length and 2 to 2·5 μ in width, though occasional cells may attain a length of 5 μ. In older cultures on this medium and on carrot the cells are more cylindrical in shape. Carrot cultures nine weeks old show the cells to be noticeably elongated forms, generally quite narrow, the average dimensions being 3·5 μ by 1·5 μ, the majority varying in length from 3 to 6 μ and in width from 1·25 to 2 μ. Rare long forms up to 12 μ in length may be seen. On honey agar 70 per cent the cells are oval and quite uniform in size, the great majority ranging from 2·5 to 4 μ in length and from 1·5 to 2·5 μ in width. Reproduces asexually by budding. Formation of ascospores has not been observed in any of the media used.

Honey Agar 15 Per Cent.—Growth rather slow and scanty, narrow filiform or beaded, chocolate coloured, raised and glistening; consistency butyrous.

Honey Agar 70 Per Cent.—Growth slow and scanty, filiform or beaded, dark brown in colour, glistening, butyrous.

Honey Broth 15 Per Cent.—Active fermentation with gas; liquid becomes clear with dark brownish sediment at bottom; no surface growth.

Honey Broth 70 Per Cent.—Active fermentation with gas; no appreciable surface growth.

Giant Colony, Honey Agar 15 Per Cent.—Growth less than average, spreading to a less extent from the point of inoculation; irregular in outline,

raised, with edge lobate and furrowed edge; surface contoured but mostly smooth, glistening; colony chocolate-brown in colour.

Carrot.—Growth rather scanty, light cream coloured, becoming yellowish, raised and glistening; slimy consistency; medium slowly decolorized.

Potato.—Scanty growth, light cream coloured, smooth and glistening; slimy consistency; medium unchanged.

Milk.—No change observed after eight weeks.

Gelatine.—No liquefaction observed in eight weeks in honey gelatine 15 per cent or 70 per cent.

Growth at 37° C.—None observed on honey agar 70 per cent or 15 per cent.

Fermentations.—Dextrose, levulose, mannose, saccharose, and raffinose are fermented. No fermentation observed in the case of arabinose, xylose, galactose, maltose, lactose, dextrin, mannite, salicin or dulcite.

Culture N 41 *(Zygosaccharomyces nussbaumeri* n. sp.)

This culture was found to correspond with culture J7, found in fermented honey and previously described on p. 12.

B. INVESTIGATION OF HIVE NECTAR DURING THE HONEY FLOW

Coincident with the experiments described in Part IIA on the examination of flowers visited by bees, a study was made of the yeast types occurring in hive nectar at the apiary of the Central Experimental Farm. During the period June 1 to September 14, weekly examinations were made of nectar, samples being taken each time from frames chosen from four different colonies selected at random. The nectar samples, collected in sterile containers, were taken to the laboratory and inoculations made as follows:—

80 per cent honey broth
15 per cent honey broth
lactose broth (standard 0·5 per cent) } for fermentation

80 per cent honey agar plates
15 per cent honey agar plates
dextrose agar (1·0 per cent)
nutrient agar } for colony growth

All cultures were incubated at 30° C. observations of fermentation being made in the liquid cultures, while the plate cultures were examined to note the types of organisms developing, the results being shown in table IX.

Table IX.—Inoculation from Hive Nectar to Various Media. Samples collected from June 1 to Sept. 14

Lab. No.	Date	Colony No.	Fermentation			Growth on Solid Media			
			80 p.c. honey broth	15 p.c. honey broth	Lactose broth	80 p.c. honey agar	15 p.c. honey agar	Dextrose agar	Nutrient agar
								–	–
1	June 1....	.....	+	–	–	Y	Y	–	–
2	" 2...	.....	+	–	–	Y	Y	–	–
3	" 14...	.....	+	–	–	Y	Y B	–	–
4	" 14 ..		+	–	–	Y	Y B	–	–
5	" 22 ..	213	+	–	–	Y	B	B	B
6	" 22 ..	246	+	–	–	Y	Y B	B	B
7	" 22 ..	239	+	–	–	Y	B	B	B
8	" 22 ..	108	+	–	–	Y	B	B	B

TABLE IX.—INOCULATION FROM HIVE NECTAR TO VARIOUS MEDIA. SAMPLES COLLECTED FROM JUNE 1 TO SEPT. 14—*Concluded*

Lab. No.	Date	Colony No.	Fermentation			Grown on Solid Media			
			80 p.c. honey broth	15 p.c. honey broth	Lactose broth	80 p.c. honey agar	15 p.c. honey agar	Dextrose agar	Nutrient agar
9	" 28	266	+	—	—	Y	B	Y B	B
10	" 28	217	+	—	—	Y	B	Y B	Y B
11	" 28	214	+	—	—	Y	B	B	B
12	" 28	IX	+	—	—	Y	Y B	Y B	B
13	July 6	277	+	—	—	Y	Y B	Y B	Y B
14	" 6	262	+	—	—	Y	B	Y B	Y B
15	" 6	247	+	—	—	Y	B	B	B
16	" 6	227	+	—	—	Y	Y B	Y B	B
17	" 12	237	+	—	—	Y	Y B	Y B	Y B
18	" 12	218	+	—	—	Y	Y B	Y B	Y B
19	" 12	268	+	—	—	Y	Y B	Y B	Y B
20	" 12	296	+	—	—	Y	Y B	Y B	B
21	" 19	228	+	—	—	Y	B	Y B	B
22	" 19	223	+	—	—	Y	Y B	B	B
23	" 19	245	+	—	—	Y	Y B	B	Y
24	" 19	218	+	—	—	Y	B	B	B
25	" 26	228	+	—	—	Y	Y	B	—
26	" 26	262	+	—	—	Y	Y B	Y B	Y B
27	" 26	237	+	—	—	Y	B	Y	B
28	" 26	218	+	—	—	Y	Y B	Y	Y
29	Aug. 2	299	+	—	—	Y	B	B	Y B
30	" 2	217	+	—	—	Y	B	B	B
31	" 2	IX	+	—	—	Y	B	B	B
32	" 2	246	+	—	—	Y	B	Y B	B
33	" 9	272	+	—	—	Y	Y	Y	B
34	" 9	258	+	—	—	Y	—	Y	B
35	" 9	275	+	—	—	Y	—	Y	B
36	" 9	120	+	+	—	Y	B	Y	—
37	" 16	246	+	—	—	Y	B	B	B
38	" 16	205	+	—	—	Y	B	B	B
39	" 16	299	+	—	—	Y	B	B	B
40	" 16	211	+	—	—	Y	Y	Y	—
41	" 16	....	+	—	—	Y	Y	B	B
42	" 23	263	+	—	—	Y	Y	B	—
43	" 23	296	+	—	—	Y	B	B	B
44	" 23	223	+	—	—	Y	B	B	B
45	" 23	219	+	—	—	Y	B	B	B
46	" 31	217	+	+	—	Y	Y B	B	B
47	" 31	246	+	—	—	Y	B	B	—
48	" 31	211	+	—	—	Y	Y B	B	B
49	" 31	299	+	—	—	Y	B	B	B
50	Sept. 7	....	+	+	—	Y	Y	—	Y B
51	" 7	.....	+	—	—	Y	Y	—	—
52	" 7	.....	+	+	—	Y	Y	—	—
53	" 7	.....	+	+	—	Y	Y	—	B
54	" 14	269	+	+	—	Y	Y	—	B
55	" 14	228	+	—	—	Y	Y	—	—
56	" 14	279	+	—	—	Y	Y	B	B
57	" 14	266	+	+	—	Y	Y	—	B

+ = fermentation
− = no fermentation

Y = Yeasts
B = Bacteria
− = No growth

It will be noted from table IX that in every sample of nectar examined evidence was found of the presence of sugar-tolerant yeasts capable of fermenting concentrated solutions of honey. Positive results were obtained in all cases with the fermentation tests in 80 per cent honey broth while yeasts, and yeasts only, were found on 80 per cent honey agar plates. In comparatively few instances was fermentation noted with the weaker concentration of honey. Growth, however, was observed, in most cases, which upon microscopic examination, was found to be due to bacteria, occasionally associated with yeasts. The tests in lactose broth yielded in all cases negative results, pointing to a probable absence in the hive nectar of bacteria of the *colon-aerogenes* group. That

organisms other than osmophilic yeasts may be found in nectar, however, may be seen from the results of the plate cultures, which, apart from those with 80 per cent honey agar, showed frequent colonies of bacteria and yeasts. These were, however, not investigated further since our chief interest lay with those appearing on the concentrated medium.

From the 80 per cent agar plates, subcultures were made as in the case of yeasts from fermented honey and from flowers. Sixty cultures in all were isolated and, as before, compared with a view to determining the number of types. In all only 4 types were found, two occurring but once, one twice, while a fourth type embraced all the other cultures isolated representing thus a much more frequent type.

The most frequent yeast, H27, was found in hive nectar in every sample examined with but one exception, and under the conditions prevailing must be considered as having been a constant inhabitant of the apiary during the season. This yeast, moreover, was found to be identical with culture M1 from fermented honey and with N8, isolated as already indicated from rowan-tree blossom and sunflower. Type H9 was found to be similar to N11 occurring in the flowers of white lilac, dogwood, raspberry, Japanese barberry and sunflower. Type H52b, found twice towards the end of the season, namely on August 31 and September 7, proved to be identical with J7 from fermented honey, which yeast was likewise isolated from flowers at approximately the same period of the year. The remaining type from hive nectar, H38a, was found but once and did not coincide with any yeasts previously studied by us.

DESCRIPTION OF YEASTS ISOLATED FROM HIVE NECTAR

Culture H9 (*Torula* sp.)

This culture was found to correspond with Culture N11, isolated from flowers and previously described on p. 20.

Culture H27 (*Zygosaccharomyces richteri*)

This culture was found to correspond with Culture M1 from fermented honey and with Culture N8, obtained from flowers and previously described on p. 15.

Culture H38a (*Saccharomyces* sp.) (*See* fig. 16)

Young cultures on honey agar 15 per cent show large oval or round cells generally occurring singly or in pairs formed by budding. Average size of cell, 8 μ in length by 6 μ in width with variations in length from 6 to 10 μ and in width from 3·5 to 8 μ . On honey agar 70 per cent, cells of approximately the same shape and size are seen. Asexual reproduction by budding occurs and also by means of ascospore formation not preceded by any copulation. Ascospore formation may be readily observed on gypsum blocks and on potato. The number of spores in the ascus ranges from 1 to 4, the spore being slightly oval in shape, more seldom round, and measuring 5 by 4 μ when fully developed.

Honey Agar 15 Per Cent.—Growth abundant, filiform, light yellowish to cream coloured; surface smooth and rather dull; consistency butyrous.

Honey Agar 70 Per Cent.—Growth slow, scanty to moderate in amount, filiform, dark brown in colour, surface smooth and glistening; consistency butyrous.

Honey Broth 15 Per Cent.—Alcoholic fermentation with gas, liquid slightly cloudy; abundant brownish ring growth; abundant sediment.

Honey Broth 70 Per Cent.—Alcoholic fermentation with gas, rather slow; dark brown surface ring growth.

Giant Colony, Honey Agar 15 Per Cent.—General form of colony round; raised with irregular lobate edge; surface contoured with tendency to concentric markings; dull lustre.

Carrot.—Abundant growth, spreading irregularly, white to light cream coloured; surface raised and rugose, glistening; consistency soft butyrous; medium unchanged.

Potato.—Moderate growth, much raised, chalky in appearance with slightly yellowish tinge; dull lustre and of dry flaky consistency.

Milk.—Slight, yellowish ring growth at surface; no change in medium after 8 weeks.

Gelatine.—Liquefaction in honey gelatine 15 per cent; none observed in 70 per cent after 8 weeks.

Growth at 37°C.—Present on honey agar 15 per cent; none on 70 per cent at this temperature.

Fermentations.—Dextrose, levulose, mannose, saccharose, maltose, and raffinose are fermented. No fermentation observed with arabinose, xylose, galactose, lactose, dextrin, mannite, salicin or dulcite.

Culture H52b *(Zygosaccharomyces nussbaumeri)*

This culture was found to be identical with culture J7 from fermented honey and culture N41, isolated from flowers and has been previously described on p. 12.

C. SUGAR-TOLERANT YEASTS IN THE EXTRACTING HOUSE

In considering the question of infection of honey by yeasts capable of inducing fermentation, the possibility of contamination at the time of extraction was thought worthy of study. A short series of tests was accordingly arranged at the time an extraction of honey was being made at the extracting house of the Bee Division, Central Experimental Farm.

In making the examination swabs were taken of the interior of the tanks used for handling the honey, immediately before they had been cleaned out for use. Swabs were taken from the centrifuge tank, the holding tanks and also from the interior of the pipe line from the former to the latter.

Inoculations were made directly into 80 per cent honey broth. In addition to the swabs, poured plates of 80 per cent honey agar were exposed for a few minutes to the air of the extracting house, the machinery being in motion at the time to encourage the distribution of organisms in the air.

YEASTS ISOLATED

In all, five types of sugar-tolerant yeasts were isolated from the cultures prepared in the extracting house, which were all identical with, or closely related to, yeasts already isolated from honey, flowers or from hive nectar. From the swabs made from the honey tanks 4 types were obtained and from the air one type as indicated in table X.

Table X.—Yeast Types Found in Extracting House

Culture	Source	Description
Ex 1.....	Honey tanks.....	Identical with N4, found in various flowers.
Ex 2.....	"	Identical with N11, found in various flowers and H9, isolated from hive nectar.
Ex 3.....	"	Same as M1 from fermented honey, N8 from flowers and H27 from hive nectar.
Ex 4.....	"	Considered to be a variety of J7 from fermented honey, N41 from flower nectar and H52b from hive nectar. (See Fig. 17)
Ex 5.....	Air.....	Identical with N24, found in nectar of various flowers.

GENERAL DISCUSSION

From the data already presented, it is evident that honey as ordinarily produced is open to serious contamination by sugar-tolerant yeasts capable of setting up fermentation when conditions suitable for their development are met. That extracted honeys may be considered, in a large proportion of cases, to harbour organisms of fermentation has been already indicated by some workers. Nussbaumer (10) found evidence of the presence of yeasts in a large percentage of samples of apparently normal Swiss and "foreign" honeys examined, while Marvin (9) expressed the belief that yeast cells are found in almost all honeys. From our experiments already outlined it would appear that, in the case of extracted honey at least, yeast contamination is partly preventable by the beekeeper and partly beyond his control. There is no doubt that by the exercise of careful precautions to maintain strict cleanliness at the time of extraction, and to insure that his containers and utensils are as nearly sterile as possible, a portion of the yeast contamination of his extracted honey may be eliminated. Such precautions a beekeeper should be willing to take, not only in his own interests, but in the interests of the honey industry.

Honey, however, may be contaminated in the comb and for this the chief blame may be laid, at least tentatively, upon the bee. As early as 1906 White (19) stated that honey from a healthy hive is, as a rule, sterile, while more recently Sackett (15) as a result of examinations of comb honey states, in confirming the findings of White, that it is uniformly sterile. We believe it is just possible that had these authors employed a medium of high honey concentration their findings would have been otherwise. In the course of some tests made on a number of samples of normal comb honey, inoculation made by transferring a loopful of honey removed aseptically from the cell yielded positive results in 50 per cent of the tubes containing 80 per cent honey medium and in but 16 per cent of the tubes containing 15 per cent honey medium.

Our studies of yeasts found in flowers commonly visited by bees during the honey flow and of those present in the hive nectar itself (parts II, A and B) indicate the presence of fewer types of yeast in the nectar of the hive than may presumably be transported by the bees from the flowers. This gives occasion for the interesting speculation as to the existence of an antiseptic action in hive nectar which might exert a selective inhibitory effect upon a miscellany of yeasts types brought to the hive. This question appears to be worthy of special investigation, for the furtherance of any natural zymocidal action, should such be established, would be the first step in the production of yeast-free honey or honey of low contamination, which condition should be reflected in a lessening of fermentation of the marketed product.

There is, indeed, a more or less widespread belief that minute quantities of formic acid are introduced into the honey by the bees just previous to the capping of the cells, and Browne (1) suggests that the presence of this acid acts as

a preservative. According to Phillips (12), however, no such action on the part of the bees has been definitely observed, the author regarding the supposed introduction of formic acid as but a fantastic explanation of the origin of the acid of honey, now considered by him to consist chiefly of malic acid.

In further considering hive nectar it might pertinently be asked why unripe honey in the hive, contaminated as it may be by sugar-tolerant yeasts, does not readily ferment in the comb. Apart from the question of any inhibitory antiseptic action, it is suggested, from consideration of a phenomenon observed in the study of yeasts from fermented honey, that the temperature of the hive, in the neighbourhood of blood heat, 37·5°C., is a favourable factor in helping to suppress fermentation, particularly with nectar of high moisture content. It was observed, with all 4 species of yeasts isolated from fermented honey, that within limits, the temperature bore a distinct relationship to the minimum honey concentration in which the yeast was capable of growing.

Table XI.—Growth of Honey Fermenting Yeasts on Honey Agar of Different Concentrations as Related to Temperature

Temperature	Honey concentration per cent by weight						
	7	14	28	42	56	70	84
42° C.	−	−	−	−	−	−	−
40°	−	−	−	−	−	+ +	+ +
38°	−	−	−	±	+ +	+ + +	+ + +
36°	−	−	+ +	+ + +	+ + +	+ + +	+ + +
34°	+	+ +	+ + +	+ + +	+ + +	+ + +	+ +

From table XI, presenting data from tests with cultures E6, D1 and M1, it will be noted that within the temperature range 34-42°C. the minimum concentration which permits of yeast growth rises with the temperature, showing that a hive temperature of blood heat prevents growth in honey concentrations which at lower temperatures would show yeast development.

FACTORS CONCERNED WITH FERMENTATION OF EXTRACTED HONEY

As first pointed out by Nussbaumer (10), the mere presence of yeast cells in a sample of honey is not sufficient to induce fermentation. Conditions favouring the development of the yeasts must be met, and although moisture and temperature have generally been considered, and doubtless are, the chief factors, other influences are concerned which may, under conditions, assume the rôle of limiting factors. Possible influences upon fermentation may be briefly indicated:—

(*a*) Temperature.—Little or no growth of fermenting yeasts occurs above 40° C. (104° F.). On the other hand, Marvin (9) reports that fermentation can be prevented by storing honey at 52° F. Tests made by us at a temperature of 50° F. confirm these findings, no yeast growth having been obtained in our cultures.

(*b*) Moisture.—This is doubtless the chief factor affecting honey fermentation under ordinary conditions and its importance has long been emphasized (Nussbaumer, l.c.). Other things being equal, a honey of high moisture content will ferment more readily than one of a low percentage of water, Fabian and Quinet (2) noting a critical moisture point of approximately 21 per cent. Quite apart from the case of the honey of originally high moisture content must be considered, as the above authors point out, the well-known

property of honey to absorb water, which process may continue, if no means to check it are taken, until conditions permit of yeast growth with consequent fermentation.

(*c*) Crystallization.—The relationship of crystallization to fermentation, pointed out by Marvin (8), has already been referred to (see p. 5). Crystallization acts indirectly by increasing the moisture content of the levulose portion of the honey remaining in solution, and may thus present favourable growth conditions for yeasts, which in the uncrystallized honey, would be unable to develop. This helps to explain why fermentation, often considered a fault of unripe honey, may occur in honeys which have been well ripened.

(*d*) Degree of Inversion of Sugars.—Although by far the greater portion of the sugars present in honey are in the form of invert sugar, yet approximately 2 per cent of the average honey is composed of saccharose, the content of which sugar may vary, according to Woodman (21) from 0 to 10 per cent. In otherwise similar honeys, then, those showing a greater proportion of invert sugar to saccharose will have greater osmotic pressure and will consequently offer greater resistance to the action of yeast. In cases, therefore, where the moisture content is very close to the critical point, the sugar ratio may well be a factor in helping to aid or prevent fermentation. In this connection Paine, Birckner and Hamilton (11), have shown that in the case of chocolate fondant candy, the addition of invertase to the fondant, by causing an inversion of a portion of the saccharose, causes sufficient increase in the density and osmotic pressure of the syrup phase of the fondant as to render it resistant to fermentation by sugar-tolerant yeasts.

(*e*) Content of Soluble Nitrogen.—As is the case with all micro-organisms, the growth of yeast is influenced by the chemical composition of the medium in which it lives and the supply of material serving as a source of food and energy. Apart from the question of fermentable sugars, the readiness with which yeast will ferment will be affected by the presence of nitrogen compounds in the honey. To illustrate this point, a test was conducted in which 60 per cent solutions of honey were prepared containing added nitrogen in different form. Five series of tubes were arranged, each being inoculated, in duplicate, from a sample of fermented honey. In Table XII, the ranking is given indicating the readiness with which fermentation took place.

Table XII.—Effect of Different Nitrogenous Compounds upon Readiness with which Honey Ferments

Inoculum	Added Nitrogen (0·1 p.c.)					Check No. added N.
	Difco Peptone	Proteose Peptone	Asparagin	$(NH_4)_2SO_4$	KNO_3	
Bruce 35	3	2	4	1	5	6
Dufferin 15	3	1	2	3	5	6
Durham 11	2	1	2	5	2	6
Prince Edward 1	3	1	5	2	4	6
No. 101	4	1	2	3	5	6
All	3	1	3	2	5	6

The hypothesis that the composition of honey, in so far as its nitrogenous constituents are concerned, may have a practical bearing upon fermentation is supported by the interesting observation made by Richter (14) in investi-

gations already referred to (see p. 4). This author found that the outbreak of fermentation was concurrent with the appearance, in the district in question, of an abnormal secretion of honey-dew, particularly on lime trees *(Tilia)*, which is stated to contain from 2·78 to 3·40 per cent of protein. As a result the honey in the locality was abnormally rich in nitrogenous compounds to which condition the outbreak of fermentation was, in the author's opinion, to be attributed.

SUMMARY

A study of fermented honey obtained from different sources has been made and in all cases yeasts have been found to be the organisms responsible for the spoilage. From the samples investigated four types of yeasts have been isolated and studied, which were found to be specially sugar-tolerant types capable of developing in high concentrations of sugar which inhibit the growth of ordinary yeasts.

The four types isolated from fermented honey were studied morphologically and physiologically, and were found to be all representatives of the genus *Zygosaccharomyces*. Two of the types were identified with previously described yeasts, namely *Z. barkeri* (Barker) Saccardo et Sydow and *Z. mellis* Fabian et Quinet. The remaining two types were considered to be species not previously described and were designated *Zygosaccharomyces nussbaumeri* n. sp. and *Zygosaccharomyces richteri* n. sp. respectively.

Examinations were made throughout the season of 34 kinds of flowers commonly visited by bees during the honey flow, for the presence of sugar-tolerant yeasts. Eleven different yeasts capable of fermenting high concentrations of honey have been isolated and described, two of which proved to be identical with two of the types found in fermented honey. Another type was also found to be similar to *Torula mellis* Fabian et Quinet, likewise isolated from fermented honey. Thus the identity of certain yeast types found in fermented honey with those occurring in floral nectar has been established.

A study was made of nectar from the bee hives throughout the season for sugar-tolerant yeasts, the results indicating an early and constant infection although the yeasts types found were less numerous than those isolated from the flowers examined. Four yeast types were isolated from the hive nectar and studied. Three of these yeasts were found to be similar to types found in flowers, while of these, two were also identical with yeasts from fermented honey.

From the honey tanks and air of the honey extracting house five sugar-tolerant yeasts have been isolated and studied, all of which were recognized as types also found in flowers. Three were, in addition, identified with yeasts found in fermented honey. In view of this, the importance of thorough cleaning and sterilization of equipment at the time of extraction is emphasized.

Factors influencing honey fermentation are discussed in the light of the data obtained.

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FIG. 3.—Culture J7

A. Vegetative cells, 15 per cent honey agar, 3 days.

B. Old culture (4 months), 70 per cent honey agar, showing asci and ascospores.

C. Old culture (4 months), yeast-honey agar, showing spore formation.

Photo. Giant colony, 15 per cent honey agar, 58 days.

FIG. 4.—Culture E6

A. Young culture, 15 per cent honey agar, 3 days, showing budding and also copulation.

B. Three month culture, 15 per cent honey agar, showing ascospores.

C. Culture on potato, 8 weeks, showing ascospores.

Photo. Giant colony, 15 per cent agar, 42 days.

FIG. 5.—Culture D1

A. Vegetative cells, 15 per cent honey agar, 3 days.

B. Culture on potato, 2 weeks, showing copulation.

C. Ten week culture, 70 per cent honey gelatine, showing asci with ascospores.

Photo. Giant colony, 15 per cent honey agar, 42 days.

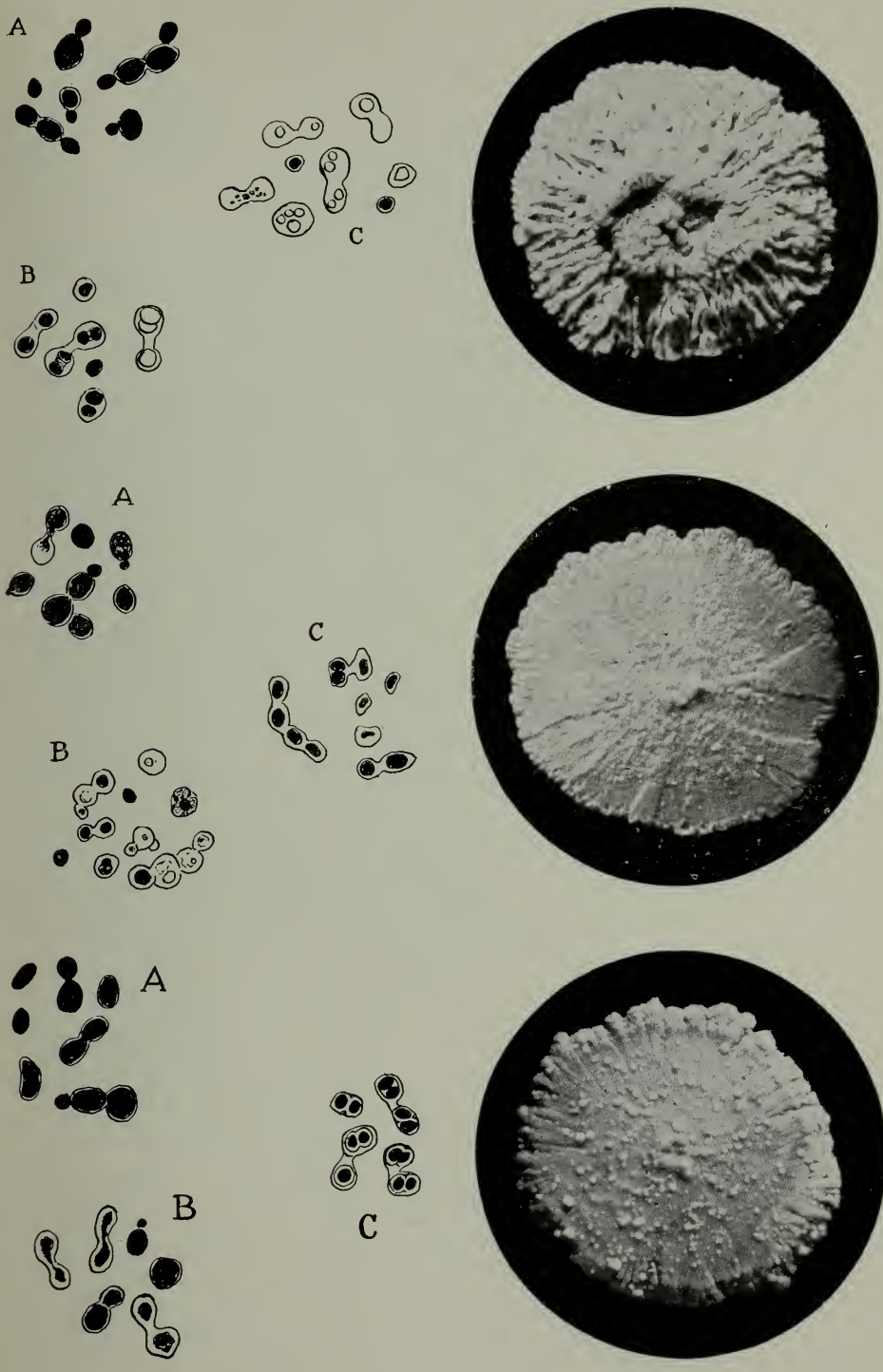
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Fig. 6.—Culture M1

A. Young culture, 15 per cent honey agar, 3 days.

B. Three week culture, 15 per cent honey agar, showing copulation.

C. Ten week culture, 70 per cent honey gelatine, showing asci with ascospores.

Photo. Giant colony, 15 per cent honey agar, 45 days.

Fig. 7.—Culture N4

A. Young culture, 15 per cent honey agar, 3 days, vegetative cells, also copulation.

B. From ring growth, 15 per cent honey broth, 8 weeks, showing ascospores.

C. Four month culture, 70 per cent honey gelatine, showing ascospores.

Photo. Giant colony, 15 per cent honey agar, 30 days.

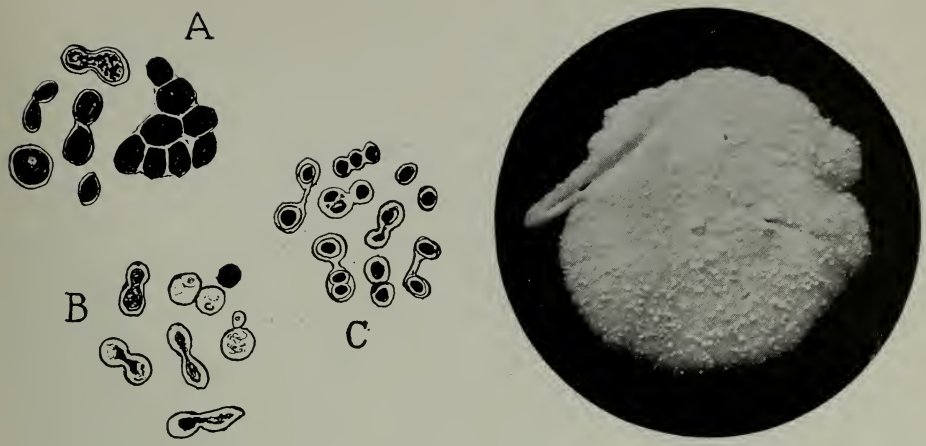
Fig. 8.—Culture N11

A. Four day culture, 15 per cent honey agar, showing budding.

B. Old culture (3 months), 15 per cent honey agar.

C. Culture on 70 per cent honey agar, 8 weeks.

Photo. Giant colony, 15 per cent honey agar, 35 days.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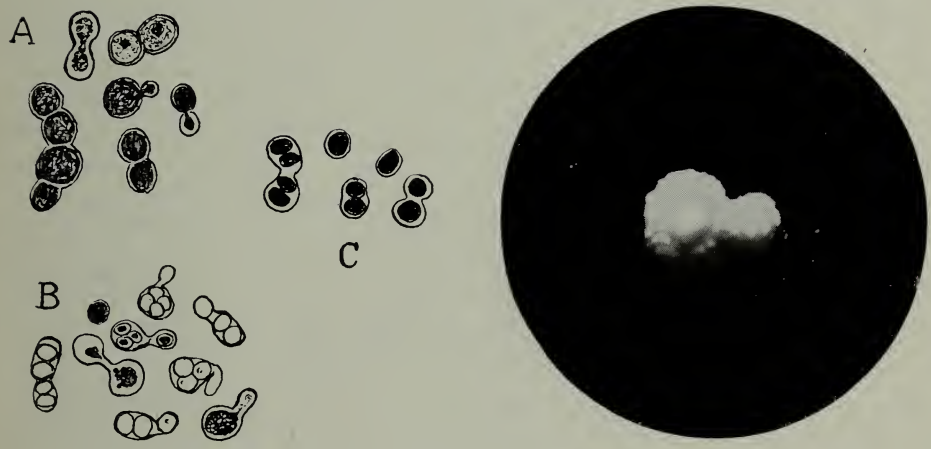
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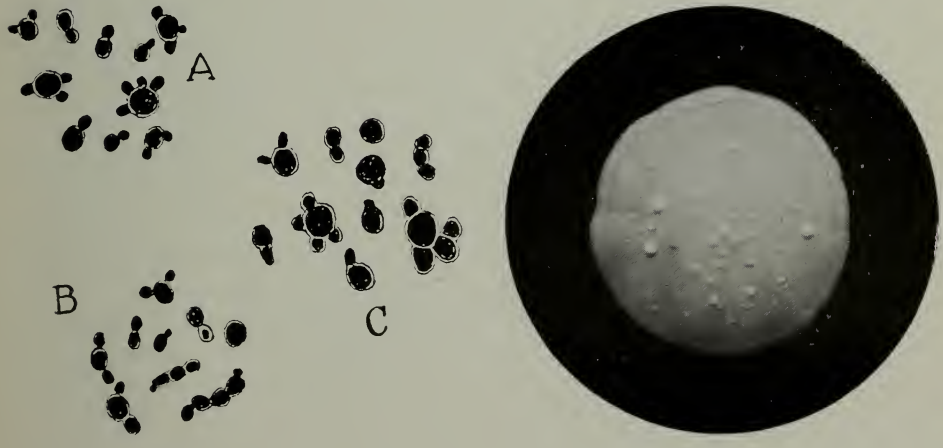
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FIG. 9.—Culture N18b

A. Young culture, 3 days, 15 per cent honey agar.

B. From ring growth, 70 per cent honey broth, 2 weeks.

C. Yeast-honey agar culture, 2 months.

Photo. Giant colony, 15 per cent honey agar, 54 days.

FIG. 10.—Culture N23

A. Young culture, 15 per cent honey agar, 3 days.

B. Old culture, 15 per cent honey agar, 2 months, showing irregular cells.

C. Four month culture, 70 per cent honey agar, showing enlarged, elongated cells forming mycelial growth.

Photo. Giant colony, 15 per cent honey agar, 60 days.

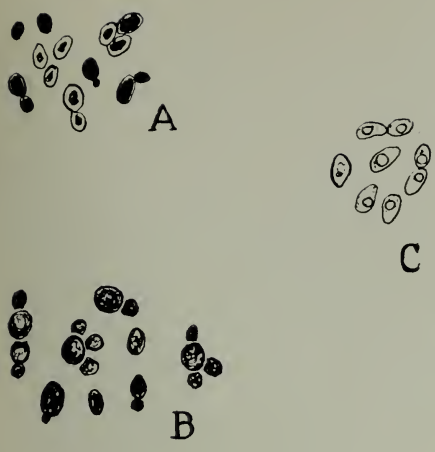
FIG. 11.—Culture N24

A. One day culture, 15 per cent honey agar, showing thick-walled vegetative cells, and cells germinating to form promycelia.

B. Culture on 70 per cent honey broth, 3 weeks, showing large, elongated cells.

C. Four week culture, 70 per cent honey agar, showing oval and elongated cells, also mycelial growth.

Photo. Giant colony, 15 per cent honey agar, 35 days.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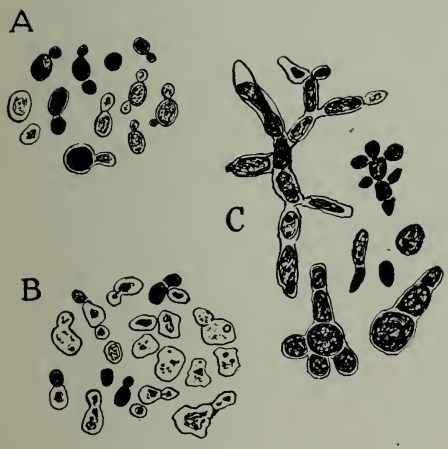
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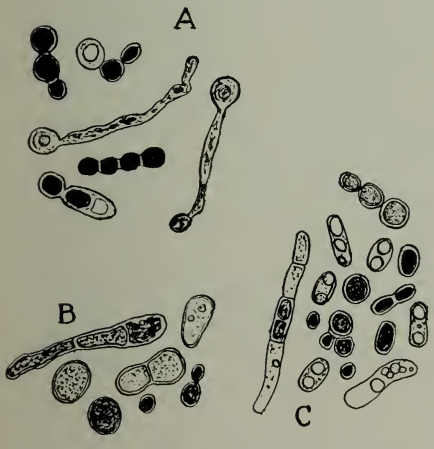
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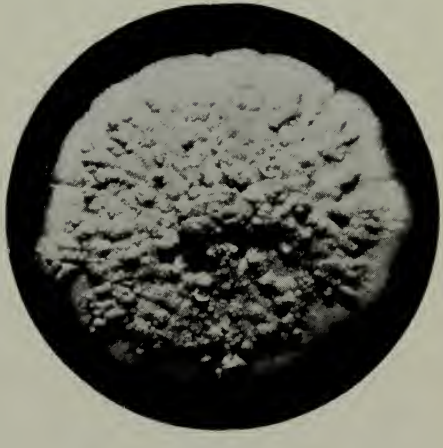

FIG. 12.—Culture N32b

A. Young culture, 15 per cent honey agar, 3 days, showing budding.

B. Old culture (3 months), 70 per cent honey agar, showing large, round cells budding.

C. Two month culture, 70 per cent gelatine.

Photo. Giant colony, 15 per cent honey agar, 35 days.

FIG. 13.—Culture N34b.

A. Young culture, 15 per cent honey agar, 3 days.

B. Old culture, 70 per cent honey agar, 8 weeks.

C. From ring growth, 70 per cent honey broth, 3 months, showing budding.

Photo. Giant colony, 15 per cent honey agar, 54 days.

FIG. 14.—Culture N38a

A. Young culture, 15 per cent honey agar, 3 days, showing thick-walled oval cells and promycelium.

B. Old culture, 15 per cent honey agar, 10 weeks, showing cells budding and large, elongated cells.

C. From ring growth, 10 per cent honey broth, 8 weeks, showing elongated cells giving mycelial growth.

Photo. Giant colony, 15 per cent honey agar, 16 days.

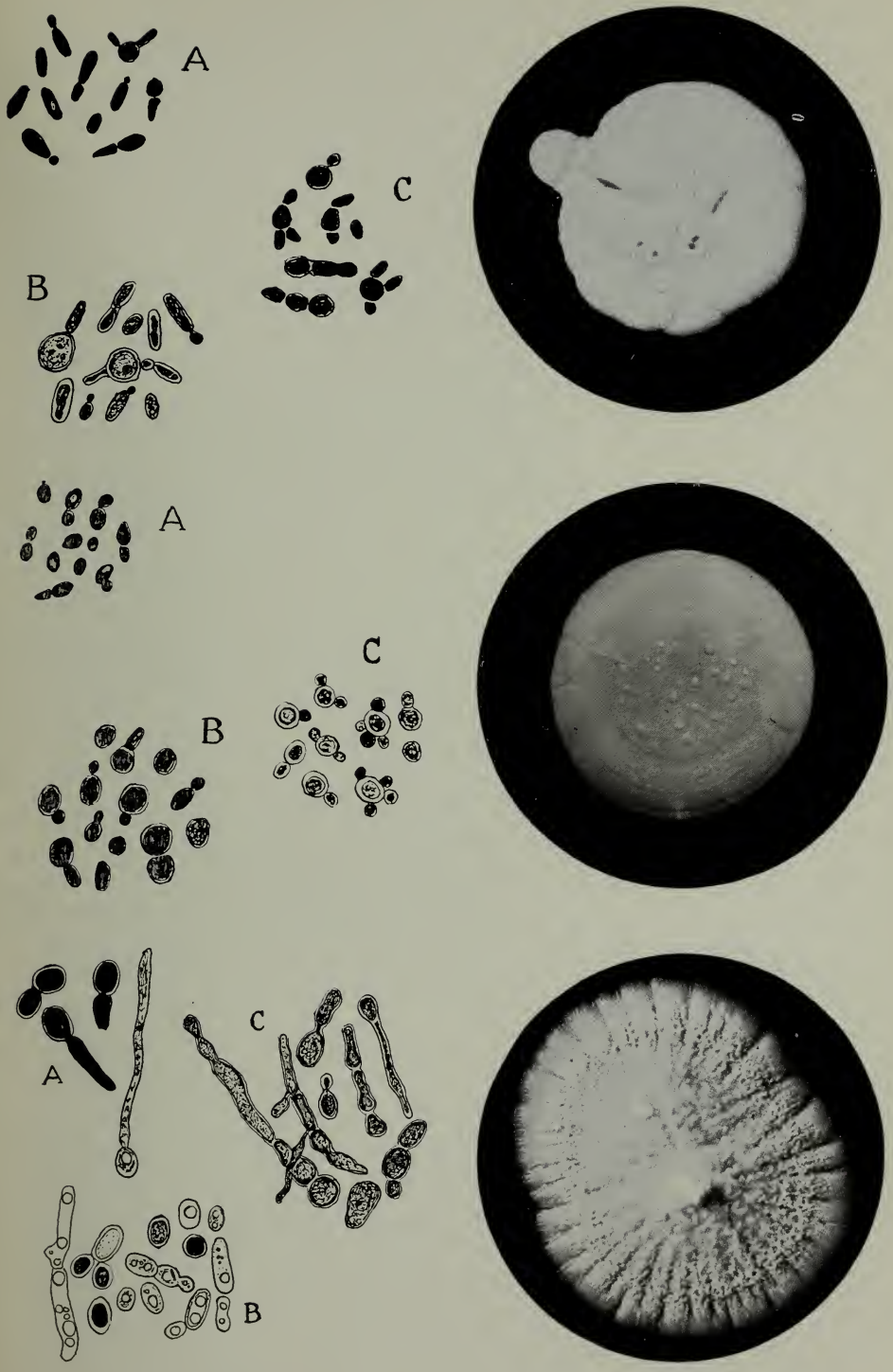
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FIG. 15.—Culture N39a

A. Ten day culture, 15 per cent honey agar, showing budding and chain formation.

B. Old culture, 70 per cent honey agar, 9 weeks.

C. Carrot culture, 9 weeks, showing elongated cells.

Photo. Giant colony, 15 per cent honey agar, 54 days.

FIG. 16.—Culture H38a

A. Four day culture, 15 per cent honey agar, showing vegetative cells and budding.

B. Five day culture on gypsum block, showing asci with ascospores.

C. Potato culture, 10 days, showing ascospores.

Photo. Giant colony, 15 per cent honey agar, 39 days.

FIG. 17.—Culture Ex4

A. Vegetative cells, 15 per cent honey agar, 3 days.

B. Old culture (8 weeks), on potato, showing copulation and ascospore formation.

C. Ten week culture, 15 per cent honey agar, showing asci and ascospores.

Photo. Giant colony, 15 per cent honey agar, 55 days.


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