STUDIES ON ASCOSPORE FORMATION

IN

SACCHAROMYCES MEYEN

by

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A thesis submitted to the Department of Botany
in partial fulfilment of the
requirements for the degree
Doctor of Philosophy

Hamilton College
McMaster University

May 1952

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Scope and Contents of Thesis:

Studies of certain ecological, chemical, and physical factors as they affect ascospore formation in bakers' yeast. Especial attention is given to the effect of carbon dioxide, oxygen, temperature, and cell concentration. The ability of 43 yeast cultures of differing types and geographical sources to form ascospores on three sporulation media is compared. Variations in the numbers of ascospores formed by isolates from a commercial yeast package as well as in the progeny of a single isolate were observed. A practical hybridization method is described. Based on the findings of these studies a technique for the preservation of yeast cultures is suggested.
With 20 tables and 5 figures.

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INTRODUCTION

The following studies were undertaken primarily to investigate the effects of certain environmental factors upon ascospore formation in yeasts. Aside from the purely academic interest involved, such studies have a considerable practical bearing on taxonomy, hybridization, and the maintenance of stock cultures.

The ability to form ascospores and the characteristics of these spores are major criteria in the classification of yeasts according to the Stelling-Dekker system (37). Yeasts capable of forming ascospores are placed in the order Endomycetales of the Ascomycetes, while the non-sporogenous yeasts are included in the order Moniliales of the Fungi Imperfecti. This primary separation of the sporogenous and non-sporogenous yeasts is common to most other classifications, a very recent example being that of Wickerham (42). Because of the difficulty of inducing ascospore formation in some yeasts, irregularities in taxonomic placement have occurred. For example, Diddens and Lodder (6) found 9 yeasts that were able to form asci in a group of about 300 cultures previously classified as "non-sporogenous" by Stelling-Dekker. It seems evident that further knowledge of the conditions that govern ascospore formation would assist materially in taxonomic studies.

Recent advances in yeast genetics have stimulated interest in the phenomenon usually referred to in the literature as "sporulation", but more precisely termed ascospore formation, in yeast. A supply of

ascospores, or of haploid cells resulting from the germination of single ascospores, is necessary in hybridization studies. This topic will be dealt with more fully elsewhere in this thesis.

Several methods of preserving yeast cultures are commonly practiced. These methods include the storage of vegetative cells on solid and liquid media of varying nutrient composition at different temperatures, as well as the preservation of cells in vacuo in a dried state. An example of this latter method is the lyophil process described by Rogers (31) in 1914. These methods have certain disadvantages in that the recovery of active cultures may be difficult, or that ascospore formation may occur rendering the cultures genetically less stable. Atkins, Moses and Gray (3) have recently reported that approximately 99.9% of the yeast cells in cultures prepared by the lyophil technique described by Wickerham and Andreason (40) failed to survive the treatment. Furthermore they noted a high incidence of variance in behaviour of revitalized cultures. It would seem that a definite need exists for a method whereby stock cultures of yeast might be maintained without genetic change or loss of viability. A study of the conditions affecting ascospore formation could contribute to solving this problem since it might suggest ways of preventing ascospore formation and thus render cultures more stable.

Phaff and Mrak (27, 28) have recently prepared a comprehensive and critical review of the literature dealing with the phenomenon of sporulation in the family Endomycetaceae. This makes an extensive review of the subject unnecessary here, and only studies bearing directly on work to follow will be cited.

DeSeynes (33) and Rees (30) described the morphology of ascospores and noted some of the environmental conditions necessary for their formation. DeSeynes found that when a wine yeast was transferred from grape must to a solution containing only a small amount of sugar, ascospore formation took place; Rees showed that a substrate of vegetable tissue supported the development of ascospores. It was, however, Engel (11) in 1872 who introduced a method of forming ascospores in vegetative cells upon a gypsum block which, with some modifications, is still a commonly employed method. The greater portion of the literature dealing with ascospore formation is concerned mainly with the development of more reliable methods of obtaining ascospores and follows, in general, the same approach to the problem as was adopted by these three early investigators. In 1898, Beijerinck (4) described a technique in which he induced ascospore formation upon a washed agar medium. Later, in 1908, Gorodkowa (12), recognizing the disadvantages of the plaster block method, recommended a medium composed of a small amount of glucose, beef extract, and sodium chloride as a sporulation medium. McKelvey (25) induced ascospore formation with a weak carrot extract agar, while more recently Mrak, Phaff and Douglas (26) recommended a vegetable extract medium, prepared from carrots, beets, potatoes and cucumbers, as a sporulation medium. Wickerham et al. (41) and Lindegren and Lindegren (22) have investigated the values of commercial mixed vegetable juices and several fruit and vegetable extracts respectively, Wickerham and his co-workers recommended the addition of bakers' yeast to Henrici's V-8 juice agar (41) prepared from the juice of eight vegetables. The pH was adjusted to 6.8. The Lindegrens suggested the use of a pre-

sporulation medium made by mixing extracts of beet leaves and roots with canned apricot juice and grape juice. A liquid medium containing sodium acetate and dextrose was developed by Stantial (35) in which bakers' yeast readily formed spores. In further studies Stantial (36) and later Elder (8, 9, 10), found that a relationship seemed to exist between the numbers of cells and the amount of acetate in a liquid culture. For example, when 2 mgm. of dextrose and 20 mgm. of sodium acetate were present in a given volume of medium with approximately 20 million cells, high yields of asci were obtained. That is, the actual volume of liquid is not as important as the ratio of the concentration of these chemicals to the number of cells. A medium suggested by Elder and employed in the work reported here contained 0.04% dextrose and 0.14% anhydrous sodium acetate in distilled water.

In previous work (1, 2) the writer compared sporulation media containing acetate with certain other sporulation media and concluded that the former were superior. It was found possible to simplify the Stantial-Elder method by substituting solid media in place of the liquid pre-sporulation and sporulation media. This obviated the necessity of filtration to free the cells from the culture medium and at the same time allowed the direct transfer of cells to sporulation medium without the adjustment of cell numbers as recommended by Stantial and Elder. The solid sporulation medium contained 0.04% dextrose, 0.14% anhydrous sodium acetate, and 2% agar.

During the forementioned studies certain observations were made regarding the effect of environment on ascospore formation on acetate-dextrose media. The findings of Stantial and Elder that the concentration

of cells in a given volume of liquid sporulation medium influences the yield of ascospores was confirmed. The highest yields were obtained with concentrations of about 1 million cells per ml. of medium. The depth of liquid medium was found to affect the yield of asci, very few being found when the depth of the medium exceeded 1 cm. The factors responsible for these effects were not determined at that time. It was felt that a more fundamental approach through the study of the effects of gaseous environment, temperature, light, cell concentration and strain variability might contribute to the solution of this problem and provide a better understanding of the factors governing ascospore formation in acetate-dextrose media.

In the literature comparatively little attention has been paid to the effect upon ascospore formation of such factors as the concentration of cells, aeration, and temperature of incubation. Hansen (13, 14) stated that "air" was required if ascospore formation was to occur. This statement has seemed sufficient definition of this factor for most workers and is found repeatedly in the literature. Slator (34) showed that only a small amount of air was necessary for sporulation when he introduced 1 cm. of air pressure to "carefully exhausted" tubes containing vegetable cells in "slightly acidified water (0.2% phosphoric acid neutralized with potassium hydroxide to give a pH value of 5-6)", and subsequently observed ascospores in the solution. No asci were produced in a similar solution containing cells incubated without air. Stantial found that cells in test tubes containing liquid sporulation medium yielded higher percentages of ascospores when the cultures were aerated by rocking. Elder (10) later showed that

a small volume of culture suspension in an Erlenmeyer flask (where a considerable surface area existed) gave comparable yields of asci to those obtained in a rocked tube culture. Maneval (24), and Lindegren and Hamilton (21) found ascospores in the outer parts of yeast cakes and yeast colonies respectively, where better aeration of cells occurs. It is interesting to note that mixtures of air and ethyl alcohol vapors, and air and carbon dioxide exert an inhibitory effect upon ascus formation, according to Bright, Dixon and Whymper (5). The results of Maneval and Lindegren and Hamilton might possibly be explained on the basis of inhibition by carbon dioxide of sporulation within the yeast cakes and colonies.

The effect of temperature as a factor in ascospore formation has also been studied, particularly with respect to the maximum and minimum temperatures at which sporulation occurs. Several workers have determined the lower ranges of temperature at which ascospores are formed by different yeast strains. Lafar (18), describing the characteristics of ascospores in a general treatise on yeasts, lists the results of these studies by different workers none of whom observed ascospore formation below 3°C.

Knowledge of the effects of environmental factors upon ascospore formation permits of wider control over their production. This is important in breeding or developing new strains of yeast since haploid cells are required for crossings--a fact that has been demonstrated by Winge and his coworkers (45). Up to 1935, the formation of ascospores in the genus Saccharomyces was considered to occur parthenogenetically. Winge (43), however, showed that haplophase and diplophase occurred in this genus and that ascospores were of haploid nature. Two other workers, Kruis and Satava

(17) and Satava (32), had described the process as early as 1918, but since their work was published in Czech it went unnoticed until the time of Winge's publication, when the two descriptions were brought before the Sixth International Botanical Congress in Amsterdam in 1935. Winge showed that in ascospore formation a diploid cell undergoes meiosis, and the resultant ascospores are haploid. Further, it was shown that these haploid spores, or vegetative cells arising from them, by copulation or fusion initiate the diploid state or the vegetative phase in which the cells are again able to sporulate. This demonstration of alternation of generations through the existence of haplophase and diplophase indicated that the Mendelian principles of genetics as established from the study of higher plants might also be applicable to yeasts. Winge and Lausten (44) showed that segregation takes place in bakers' yeast and concluded that only cultures obtained from single ascospores can be considered to be pure cultures. Barring mutations, such cultures theoretically will remain genetically unchanged. For these studies Winge and his co-workers, employing a micromanipulator (which allows handling of individual ascospores), went on to demonstrate artificial species hybridization by crossing a baking yeast with S. validus (45) and later, on the basis of their former studies, to then produce 14 new hybrids, some of which were inter-varietal, some inter-specific, and one inter-generic. Some of these possessed improved qualities for industrial purposes.

Lindegren and his associates in the United States have made notable contributions to the biology of yeast, certain of which are at variance with the concepts held by Winge. The interpretations by Lindegren for certain of his findings have recently been discussed by Winge and Roberts (46).

Lindegren's discovery in 1943 of two mating types in Saccharomyces is one of his most important contributions (19). The method employed in the hybridization experiments involved the mixing of vegetative haploid cell suspensions derived from two ascospores of opposite mating types to allow every opportunity for fusions between haploid cells to occur. This procedure was termed "mass mating" by Lindegren. A review of his work on mating types and of other related studies (20), as well as a later summary of his more recent work (23), has been published by Lindegren.

From the studies made by both the Winge and Lindegren groups it has been established conclusively that inheritance and genetic behaviour in yeast is similar to that observed in higher plants. Further, it has been clearly demonstrated that yeast breeding work can be conducted in a manner alike in principles to that now practiced with higher plants. Hybridization is conditioned by the availability of gametes. In the case of yeasts these may be either ascospores or haploid cells arising from germinating ascospores. In the resultant fusions the production of types differing from the parent or parents follows. Knowledge of the factors governing ascospore formation should provide means of obtaining greater yields of ascospores than heretofore. This might lead to the development of a method for rapidly obtaining improved industrial strains. Such a method would involve the following:

1. The mixture of large numbers of ascospores obtained from one or several strains.

2. After allowing time for the fusions to occur the mixture would be exposed to a selective environment in which the growth of improved types will be favoured. Such a method may be valuable, for instance, in

the development of strains with increased tolerance to sulphur dioxide, or other toxic substances in industrial fermentations. In these studies, the possibility of employing such a method is investigated.

METHODS AND MATERIALS

The terms "sporulation medium" and "pre-sporulation medium" are found extensively throughout the text. The former means any special medium employed to induce ascospore formation, and the latter refers to a medium on which cells are multiplied before being transferred to a sporulation medium.

Sporulation media employed were (1) a liquid sporulation medium, prepared by dissolving 0.04% dextrose and 0.14% anhydrous sodium acetate in distilled water. It will be referred to herein as acetate-dextrose solution. This medium is essentially the same as that employed by Elder; (2) a solid sporulation medium, (to be referred to as acetate-dextrose agar), prepared by adding 2% agar to (1); (3) water agar, prepared by the addition of 2% agar (Difco) to distilled water. Beijerinck (4) used a similar agar and this latter is presumed to be comparable to it. All media were sterilized in an autoclave under 15 pounds steam pressure for 20 minutes.

The pre-sporulation media were (a) Nutrient Agar (Difco), to which was added 5% dextrose and 0.5% tartaric acid. The tartaric acid was added as 10% sterile solution to the dextrose nutrient agar after sterilization and just prior to its use either in petri dishes or test tubes; (b) a medium composed of 2% agar and commercial grape juice, the latter containing approximately 5% grape sugar.

Stock cultures of the yeasts employed in these studies were prepared and maintained on grape juice agar slants in the following manner: transfers

of each strain were made in duplicate to grape juice agar slants, and after 48 hours at room temperature sterile paraffin oil was added to one group and all were stored at 5°C. Henry (15) has shown that such "oil cultures" remain viable for 10 years apparently without any change in physiological behaviour.

Unless otherwise stated the percentage of cells containing ascospores in a culture on acetate-dextrose agar was estimated by examining microscopically a minimum of 400 cells in an aqueous suspension prepared by adding sterile water to the culture slant and with a wire loop freeing the cells from the agar surface.

Commercial yeast packages are rarely free from bacterial or fungus contaminants and even with the dilution-plate method the possibility still exists of obtaining a single cell culture that is contaminated. In the early stages of this work, besides using acidified agar to inhibit the development of bacterial contaminants in the pre-sporulation cultivation of the yeasts, cultures were often washed repeatedly in 5% tartaric acid, and replated on acidified glucose nutrient agar or grape-juice agar.

It was later found simpler to purify cultures by sub-culturing on a medium containing streptomycin, (dihydro-streptomycin sulphate, Lily and Co., available in ampoules, 1 gm. streptomycin equivalent to 1,000,000 units). A stock solution was prepared by adding sufficient of the salt to distilled water so that 1 ml. was equivalent to 1,000 international units. The stock solution was added to the culture medium as required to give a concentration of 50 units per ml. of medium.

Vegetative cells employed in the preparation of sporulation cultures were subcultured at least twice on pre-sporulation medium. When

cultures in acetate-dextrose solution were prepared the cell numbers per ml. were adjusted by means of a haemocytometer. The ratio of cells to sporulation medium was that recommended by Elder and stated in the Introduction. Acetate-dextrose agar cultures were prepared according to the method described by the writer (2). In that method the vegetative cells were subcultured twice on a pre-sporulation medium and then transferred from 24-48 hr. old cultures directly, by means of a wire loop, to the surface of the acetate-dextrose agar slants. In experiments where the number of cells per unit volume of medium was to be determined, acetate-dextrose agar was dispensed in 5 ml. volumes in test tubes, and 20 ml. volumes in petri dishes. When using test tube cultures a known volume of sterile water was added to the tubes at the conclusion of the experiment. The cells were freed from the agar surface with a wire loop and the concentration of the cells in the resulting suspension was determined by means of a haemocytometer. When using petri dish cultures the following method was adopted for estimating the number of cells that would remain on the agar surface:

Acetate-dextrose agar plates were flooded with 5 ml. of cell suspensions of known densities. After ten minutes the excess liquid was drained off by inverting the plates over filter paper. In preliminary experiments it was found that approximately one-fifth of the cells flooded on a petri dish would remain on the agar surface after draining. This conclusion was arrived at in the following manner: Haemocytometer cell counts were made of suspensions before and after flooding, as were plate counts on plates flooded with dilute suspensions. Direct microscopic counts of cells on a representative number of fields of plates flooded

with heavy suspensions were also made and the estimated total number of cells per plate was arrived at by calculation. The results thus obtained were comparable and indicated that approximately one-fifth of the cells flooded upon a petri dish would remain on the agar surface after flooding with 5 ml. of suspension.

Throughout these studies frequent use is made of the haemocytometer for determining the concentration of cell suspensions. It is important to realize that in estimating cell counts by this method an error of 5% may be expected (38), the error obviously being dependent upon the number of cells involved.

The procedures followed in certain experiments relating to the effects of pure gases and gas mixtures on ascospore formation are described in the text with these particular experiments.

EXPERIMENTAL

1. EFFECT OF ENVIRONMENT ON ASCOSPORE FORMATION.

(a) Effect of Cell Concentration

The effect of 6 cell concentrations on ascospore formation by two bakers' yeast isolates on acetate-dextrose agar was investigated.

Petri dishes containing 20 ml. of agar were flooded with 6 different concentrations of vegetative cell suspensions in the manner described in Methods and Materials. Petri dishes containing 20 ml. of water agar were also flooded with the same suspensions. The estimated numbers of cells remaining on the plates after the excess liquid was drained off are listed in Table 1, with the yields of asci after 14 days of incubation at room temperature. The experiment was conducted in duplicate on acetate-dextrose

agar. One series of plates using water agar was also included.

Table I

Effect of Cell Concentration on Ascospore Formation

Strain 1						
Estimated Millions of Cells per 20 ml. of medium	20	2.	.2	.02	.002	.0002
Percent Yield Asci after 14 Days at Room Temperature						
Acetate-Dextrose Agar	35, 30	38, 39	33, 37	33, 25	20, 18	16, 24
Water Agar	52	45	30	17	24	25
Strain 2						
Estimated Millions of Cells per 20 ml. of Medium	20	2.	.2	.02	.002	.0002
Percent Yield Asci after 14 Days at Room Temperature						
Acetate-Dextrose Agar	50,55	31,33	16,25	26,30	12,18	10,14
Water Agar	25	11	8	10	10	4

After 4 days incubation, the multiplication of single cells on the surface of the agar plates containing relatively few cells had occurred to such an extent that small colonies were noted microscopically by both reflected and transmitted light. Microscopic examination of the three series of plates containing relatively greater numbers of cells showed

that very little multiplication had taken place. Higher yields of ascospores were obtained from both strains on plates containing the higher cell concentrations. It would seem that the number of ascospores formed is related to the number of cells per unit volume of medium.

An experiment similar to the foregoing was conducted in which 5 cell concentrations of two other yeast strains were employed. In this experiment a No. 1 cover glass (22 mm. x 50 mm.) was placed on the surface of each petri dish after the excess cell suspension had been drained off, so that by microscopic observation an immediate comparison of ascospore formation could be made between cells exposed to the air and those under increasing degrees of anaerobiosis.

As shown in Table II high yields of ascospores were obtained with strain 1, where the concentration of cells was 28 million or more per 20 ml. of medium, and with the strain 2, high yields were obtained in all concentrations, though these were somewhat higher in the heavier concentrations. Whereas strain 1 failed to form ascospores to any extent on water agar, strain 2 behaved very differently, giving higher yields as the concentration of cells upon the medium diminished. The foregoing two experiments agree in that a relationship is indicated between the number of cells per unit volume of medium and the yield of ascospores. From the tables it is apparent also that the relationship is not of the same order for each yeast strain tested.

Observing directly the cells beneath the cover glasses it was seen that in plates containing high cell concentrations very few, if any, ascospores were formed; but at the lower concentrations ascospore formation did occur, though less than where the cells were exposed to the air. When

Table II

Effect of Cell Concentration on Ascospore Formation

Strain 1					
Estimated Millions of Cells per 20 ml. of Medium	115	57	28	14	7
Percent Yield Asci after 14 Days at Room Temperature					
Acetate-Dextrose Agar	31,29 28	30,28 25	35,35 30	20,17 12	7,5 4
Water Agar	0	1	1	1	1
Strain 2					
Estimated Millions of Cells per 20 ml. of Medium	100	50	25	12	6
Percent Yield Asci after 14 Days at Room Temperature					
Acetate-Dextrose Agar	98,95 98	95,98 61	81,65 64	61,62 55	66,60 65
Water Agar	14	10	27	75	85

ascospores did develop beneath the cover glasses they were more abundant near the edges. This suggests that the availability of air may govern ascospore formation in the cells beneath the cover glasses.

(b) Effect of Gaseous Environment

In the preceding experiment evidence was obtained to show that aeration has a direct bearing on ascus formation. In an effort to explain this effect, further experiments were conducted.

(i) Ascospore Formation in Acetate-Dextrose Solution under Varying Conditions of Aeration

An experiment was conducted to investigate the degree to which the presence or absence of air affected ascospore formation in a depth of medium previously shown to give high yields of ascospores. Employing approximately one million cells per ml. of solution, liquid sporulation cultures were prepared in vials (4 ml. capacity, 15 mm. x 45 mm.) to a uniform depth of 5 mm. Ten vials fitted with screw caps were allowed to stand at room temperature (not tightly sealed so as to allow gaseous exchange). Ten similar vials were tightly closed to restrict aeration to the air contained in the vials. Sterile paraffin oil was layered on the medium in 10 additional vials to prevent aeration of the cultures.

Table III

The Comparative Effect on Ascospore Formation of Varying Conditions of Aeration

Series	Time of Incubation	Individual Vial Yields of Asci in Percent					Mean	Stand. Dev.	Stand. Error
I									
Not	7 days	49	44	48	52	45	47.6	3.2	1.5
Airtight	14 days	66	70	77	57	70	68.0	7.3	3.3
II									
Airtight	7 days	33	24	10	24	13	20.8	9.3	4.1
	14 days	34	25	28	25	15	25.4	6.9	3.1
III									
Airtight plus Paraffin Oil	7 days	10	11	10	9	12	10.4	1.1	0.5
	14 days	23	29	15	30	17	22.8	6.7	3.1

Five cultures of each series were examined after 7 days incubation and the remaining five vials of each series were examined after 14 days uninterrupted incubation. The yield of ascospores from the 3 series of vials is shown in Table III. Higher yields were obtained from those vials not tightly capped. When the data were subjected to statistical analysis it was seen that no real difference existed between the percent asci after 14 days in Series II and Series III. However, a significant difference did exist between Series I and both other series. It is evident that when aeration of sporulation cultures was interfered with, ascospore formation was decreased. This may be because one or more of the components of air is required for ascospore formation. Or it may be that some substance produced by the cells and prevented from escaping by the paraffin oil and the tightly screwed caps, inhibited sporulation in this experiment.

(ii) Effect of Four Gaseous Environments on Ascospore Formation by Three Yeast Strains in Acetate-Dextrose Solution

To investigate further the effect observed in the preceding experiment sporulation cultures were incubated in four gaseous environments. Cell suspensions in vials containing acetate-dextrose solution, as in the foregoing experiment, were placed in sealed cans of approximately 1 litre capacity, provided with inlet and outlet valves. The gases employed were oxygen, nitrogen, carbon dioxide and air. The oxygen and nitrogen used were obtained from commercial cylinders, while the carbon dioxide was generated by adding hydrochloric acid to sodium carbonate. The nitrogen contained 0.1 - 0.3% oxygen as an impurity, while the oxygen contained 0.5% nitrogen. The gaseous environments were renewed daily, by pressure, with the respective gases. This procedure is often -- (page 18)

referred to as "sweeping out". Three yeast strains were included, and for each combination of yeast strain and gaseous environment there were four cultures in vials.

Table IV

Effect of Four Gaseous Environments on Ascospore Formation by Three Yeast Strains in Acetate-Dextrose Solution After 14 Days at Room Temperature

Yeast	Incubation Atmosphere	Percent Asci				Mean	Stand. Dev.	Stand. Error
Strain I	Oxygen	34	30	25	23	28.0	5.0	2.5
	Nitrogen	18	16	12	8	13.5	4.4	2.2
	Carbon Dioxide	0	0	0	0	0.0	0.0	0.0
	Air	43	41	37	40	40.2	2.5	1.3
Strain II	Oxygen	42	38	32	26	34.5	7.0	3.5
	Nitrogen	24	20	15	10	17.3	5.9	3.0
	Carbon Dioxide	0	0	0	0	0.0	0.0	0.0
	Air	49	45	41	38	43.3	4.8	2.4
Strain III	Oxygen	48	41	33	30	38.0	8.1	4.1
	Nitrogen	18	12	10	5	11.3	5.4	2.7
	Carbon Dioxide	0	0	0	0	0.0	0.0	0.0
	Air	55	50	80	60	61.3	13.2	6.6

The results of this experiment are summarized in Table IV. From the table it would appear that the difference between treatments is significant. The highest yields of ascospores were obtained from cultures

incubated in air. Cultures incubated in the presence of oxygen alone formed asci in greater numbers than those incubated in a nitrogen environment. No ascospores were found in any culture incubated in an atmosphere of carbon dioxide.

(iii) Effect of Four Gaseous Environments on Ascospore Formation by Three Yeast Strains on Acetate-Dextrose Agar

To determine whether these gases would have the same effect on ascospore formation by cells on a solid medium an experiment similar in design to the foregoing was conducted.

Petri dishes containing acetate-dextrose agar were inoculated with vegetative cells in two ways--by flooding and by streaking. The former method required the preparation of an aqueous cell suspension and the latter the direct transfer of cells from glucose nutrient pre-sporulation agar to the sporulation medium. Fairly heavy but uniform streak inoculations were made on the sporulation medium plates and the aqueous cell suspensions were prepared so as to leave approximately 20 million cells resting on the surface of the medium after the plates were drained of the excess suspension. The cultures were incubated in bell jars at room temperature. In the bell jars the plates, less the tops, were stacked so that each plate surface was freely exposed to the particular gaseous environment. This was accomplished by placing two glass rods between each plate. The gaseous environments were renewed by pressure, daily, with the respective gases.

As in the preceding experiment, no ascospores were produced in any cultures incubated in an atmosphere of carbon dioxide (Table V). All three yeast strains behaved more or less in a similar manner. Ascospore

yields on acetate-dextrose sporulation medium were again consistently higher in air. But, while in the preceding experiment yields of ascospores were higher in oxygen than in nitrogen, this was not always so here.

Table V

Effect of Four Gaseous Environments on Ascospore Formation by Three Yeast Strains on Acetate-Dextrose Agar

Percent Yield of Asci on Flooded Plates after 14 Days at Room Temperature

Yeast	Incubation Atmosphere	Percent Asci				Mean	Stand. Dev.	Stand. Error
Strain I	Oxygen	12	12	8	10	10.5	1.9	1.0
	Nitrogen	18	26	23	25	23.0	3.6	1.8
	Carbon Dioxide	0	0	0	0	0.0	0.0	0.0
	Air	41	40	33	38	38.0	3.6	1.8
Strain II	Oxygen	22	31	26	28	26.8	3.8	1.9
	Nitrogen	24	30	25	22	25.3	3.0	1.5
	Carbon Dioxide	0	0	0	0	0.0	0.0	0.0
	Air	36	35	30	-	33.7	3.2	1.9
Strain III	Oxygen	24	29	33	40	31.5	6.8	3.4
	Nitrogen	22	25	23	30	25.0	3.6	1.8
	Carbon Dioxide	0	0	0	0	0.0	0.0	0.0
	Air	45	45	37	42	42.3	3.8	1.9

Table V (cont'd)

Percent Yield of Asci on Streaked Plates after 14 Days at Room Temperature

Yeast	Incubation Atmosphere	Percent Asci				Mean	Stand. Dev.	Stand. Error
Strain I	Oxygen	33	37	36	43	37.3	4.1	2.1
	Nitrogen	22	22	23	26	23.3	1.9	1.0
	Carbon Dioxide	0	0	0	0	0.0	0.0	0.0
	Air	40	43	44	48	43.8	3.3	1.7
Strain II	Oxygen	33	36	37	41	36.8	3.3	1.7
	Nitrogen	43	43	38	41	41.3	2.4	1.2
	Carbon Dioxide	0	0	0	0	0.0	0.0	0.0
	Air	50	50	43	48	47.8	3.3	1.7
Strain III	Oxygen	52	54	56	57	54.8	4.9	2.5
	Nitrogen	30	30	35	38	33.3	4.0	2.0
	Carbon Dioxide	0	0	0	0	0.0	0.0	0.0
	Air	64	57	65	66	63.0	4.1	2.0

In two instances out of 6 the mean values of ascospore yields were higher in nitrogen. The practice of streaking cells to inoculate sporulation medium was seen to support as high yields of ascospores as the other method described (flooding petri plates with a cell suspension).

(iv) Effect of varying concentrations of Oxygen, Carbon Dioxide, and Nitrogen on Ascospore Formation

The obvious implications of the results obtained in the preceding experiments were investigated in a more intensive study of the effects of the three gases,--oxygen, carbon dioxide, and nitrogen--in varying concentrations, on ascospore formation by one yeast isolate.

For this work a gas mixing panel designed primarily for other work was generously made available by the Horticultural Experiment Station, Vineland Station, Ontario. A detailed description of this apparatus will not be given here, since it will be described elsewhere by the designer, Dr. J. H. L. Truscott. The apparatus makes possible the mixing of two or three gases in varying concentrations with a reasonable degree of facility and accuracy. Nitrogen was employed as the diluting gas with both oxygen and carbon dioxide. In addition, employing an Orsat apparatus, gas analyses were made before, during, and at the end of the experiments to check the composition of the gases passing over the cultures. Streams of pure gas or mixed gases were passed continuously for a 7-day period over vegetative cells on the surface of acetate-dextrose agar slants. In Figure 1, the procedure followed to provide the particular gases is outlined. The cylinders of compressed gases (1) are connected to reduction valves (2) whereby the gas pressure is reduced to 0.5 lbs. per sq. in. The gases then flow both to the mixing panel (3) where the different mixtures are made, and into separate lines as pure gases. The gases are then passed through hydrators (4) to the cultures (7), the rate of flow being regulated by control valves (6). To provide pure nitrogen free of oxygen, the nitrogen gas was passed through a pyrogallic acid absorber (5).

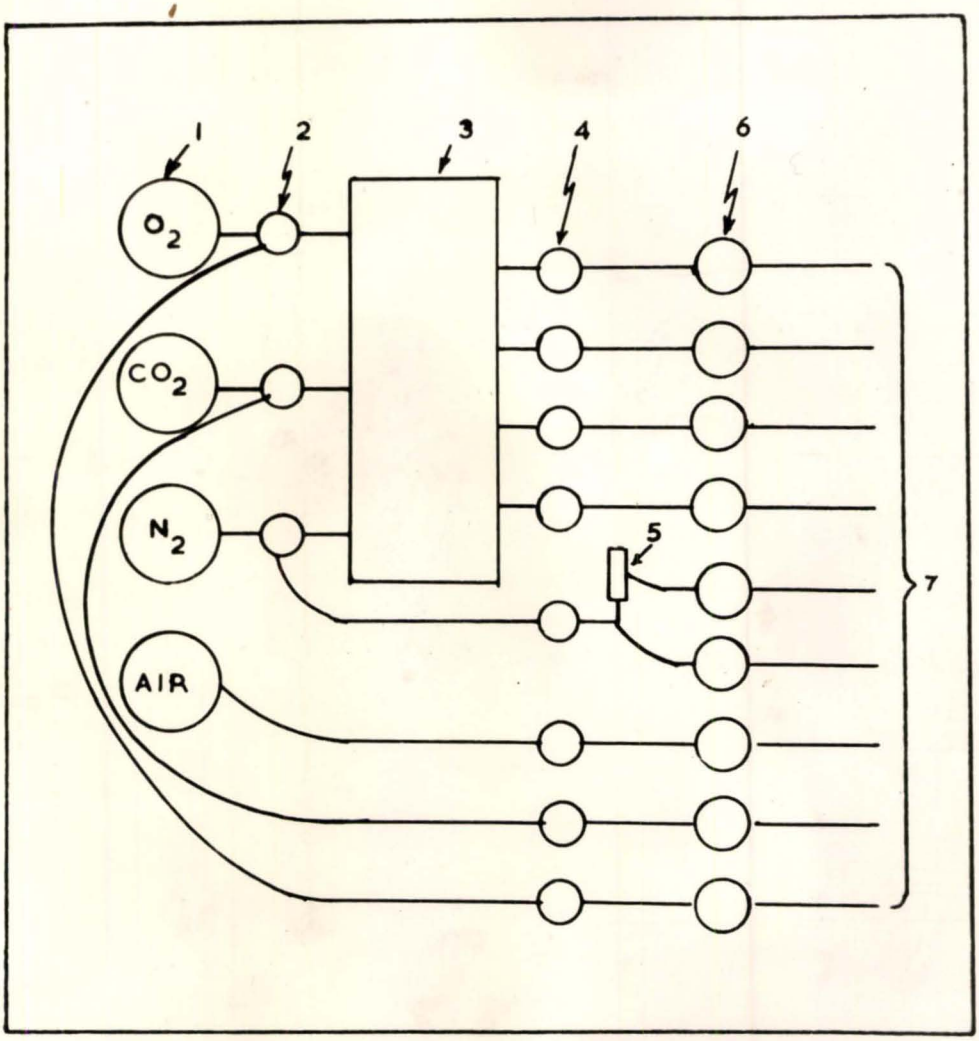

Figure 1. A schematic diagram showing the manner in which pure gases and gas mixtures are provided as gaseous environments. For explanation see text.

The composition of the compressed gases in the cylinders was: Oxygen:- at least 99.5% oxygen, and up to 0.5% nitrogen;

Carbon Dioxide:- at least 99.9% carbon dioxide with a trace of air and moisture;

Nitrogen:- 99.9% nitrogen, 0.1% oxygen. (The foregoing data were supplied through the courtesy of the manufacturer, Canadian Liquid Air Co., Toronto.)

Although fluctuations in temperature were within the range 18.3-23°C., the temperature remained for the most part close to 21-22°C.

In determining the percentage of ascospores formed, units of 100 cells were examined, at least five such units for each tube. With the apparatus, it was possible to employ at one time 7 gases or mixtures of gases, in addition to compressed air. The data listed in Table VI were obtained from five consecutive runs of the apparatus. These data are presented in graphic form in Figure 2.

As in the preceding experiments on the effect of gaseous environment, carbon dioxide inhibited ascospore formation. At carbon dioxide concentrations above 25% very few ascospores were found. The number of ascospores formed varied inversely with the concentration of carbon dioxide more especially below 25%. When carbon dioxide was mixed with air (50% carbon dioxide + 50% air) instead of nitrogen, ascospore formation was likewise prevented. In the presence of a rather low concentration of carbon dioxide, 3.6-4.2%, ascospore formation was reduced from 24.4% ± 1.8, (the yield in nitrogen from cylinders which contained 0.1% oxygen) to 19.7% ± 1.5. In nitrogen from which the oxygen had been removed by passing the gas through a pyrogallic acid absorber, the yield of ascospores was

Table VI

Effect of Varying Concentrations of Pure Gases and Gas Mixtures upon Ascospore Formation after 7 Days at Room Temperature

Gaseous Environments	Mean Gas Concentration	Number of Units of 100 Cells Counted	Mean Percent Yield of Asci	Standard Error
Still Air*		50	39.4	1.1
Flowing Air		50	42.5	2.6
Air plus 50% Carbon Dioxide		20	00.0	0.0
Carbon Dioxide	100%	50	00.0	0.0
	72% ± 6.0	10	00.0	0.0
	42.9% ± 6.0	10	00.0	0.0
	40.3% ± 3.4	10	0.5	0.3
	31.6% ± 1.2	10	1.0	0.3
	29.9% ± 3.0	10	11.0	0.4
	26.4% ± 0.1	10	1.9	0.5
	24.4% ± 0.1	10	4.5	0.8
	14.0% ± 0.8	10	8.1	1.0
	6.1% ± 0.1	10	16.0	1.5
	3.9% ± 0.3	10	19.7	1.5
Oxygen	100%	50	37.7	1.1
	68.8% ± 1.6	20	48.1	1.9
	41.1% ± 1.3	15	65.8	1.8
	12.0% ± 0.8	10	70.8	1.0
	2.6% ± 0.1	20	67.7	1.4
	0.1% **	50	24.4	1.8
	0. % ***	45	16.9	1.7

* Slants not attached to the apparatus
** Oxygen impurity in nitrogen cylinders
*** Cylinder nitrogen gas after absorption of oxygen by pyrogallic acid.

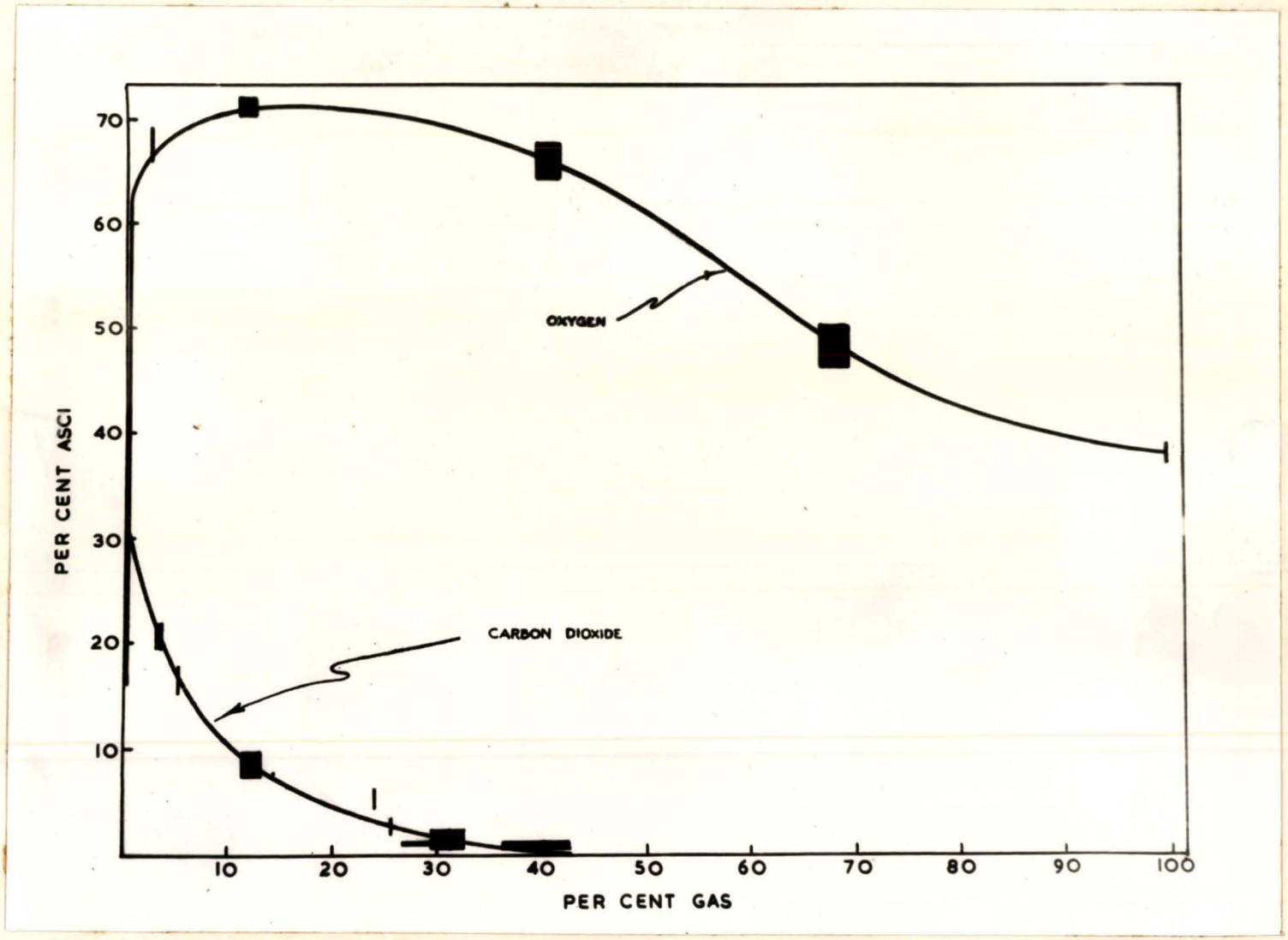

Figure 2. The effect of varying concentrations of oxygen and of carbon dioxide on ascospore formation. The horizontal dimensions of the points in the graph represent the range of variation in the gas concentration observed during the experiments. The vertical dimensions represent the standard error of the determinations of the percent asci.

16.9% ± 1.7, indicating that oxygen may not be essential for ascospore formation. However, where a very small amount of oxygen was present (0.1% as in nitrogen direct from the cylinder) ascospore formation increased to 24.4% ± 1.8. As the concentration of oxygen increased, the yield of ascospores was also increased until at 11.2-12.8% a yield of 70.8% ± 1.0 was observed. But at higher concentrations of oxygen ascospore formation was depressed.

It may be concluded from these experiments that "aeration" favours sporulation in yeast in two ways: (1) oxygen exerts a stimulating effect, although this gas seems not essential, and (2) aeration removes carbon dioxide produced by the cells before it reaches an inhibitory concentration. The latter is evidently the more important, since in air plus 50% carbon dioxide sporulation was not observed; yet this mixture would contain about 10% oxygen, an amount that, according to the graph, would be very stimulating.

(v) Effect of Reduced Atmospheric Pressure on Ascospore Formation

Vegetative cells of three bakers' yeast strains cultivated for 48 hours on grape juice agar were transferred to acetate-dextrose agar slants and incubated at 23°C. for 14 days under normal atmospheric pressure, and in two dessicator jars in which the air pressure was reduced from 760 mm. to (a) 580 mm. and (b) 200 mm. Both conditions of reduced pressure were obtained by the use of a Nash HYTOR vacuum pump operated by a 3 H.P. electric motor. The appearance of condensate on the inner surface of the jars indicated that they were saturated with respect to water vapour.

The yields of ascospores formed under the three conditions of pressure are shown in Table VII. In these environments of lowered oxygen tension, large numbers of ascospores were formed. The yields, however, were less in the partially evacuated jars than those obtained from cultures incubated under normal air pressure. A considerable number of cells incubated under reduced pressure exhibited a tendency to develop into elongated cells, thereby losing their characteristic elliptical shape.

Table VII

Effect of Reduced Atmospheric Pressure on Ascospore Formation by Three Yeast Strains on Acetate-Dextrose Agar

Yeast Strain	Percent Yield of Ascospores after 14 Days at 21°-23°C.								
	Normal Air Pressure of 780 mm.			Air Pressure Reduced to 580 mm.			Air Pressure Reduced to 200 mm.		
1	77	78	80	60	60	62	38	44	48
2	75	77	80	45	50	57	42	45	46
3	75	83	85	56	58	63	36	42	47

This condition was more noticeable in cells held under 200 mm. of pressure. It is conceivable that the normal physiological behaviour of the cells may similarly be modified, interfering with ascospore formation in the cells. In many cells where ascospore formation occurred, the ascospores were observed to be in a linear arrangement-- a condition that is not common in S. cerevisiae. This point is of some taxonomic importance. Pomper (29) observed that the Lindegren sporulation procedure gave two types of asci,

elongated and round to oval, while with the Stantial method only the latter type was observed.

The behaviour of the cells in this environment is in conformity with that observed by Slator (34) who found that 1 cm. of air pressure permitted ascospore formation. These results are also in agreement with those of the experiments dealing with varying concentrations of oxygen, which showed that low concentrations of oxygen stimulated sporulation, though not as high yields were obtained as in air.

(vi) Effect of Ethyl Alcohol Vapour on Ascospore Formation

Cells were flooded onto acetate-dextrose agar plates. Inside the inverted tops of these were placed small aluminum containers holding 5 ml. of ethyl alcohol solutions of the following concentrations: 0, 1, 2, 5, 10, 20, 40, and 95% in distilled water. After 7 days at 22-23°C. these plates were examined for ascospore formation. No ascospores were observed in cells exposed to vapours from 10, 20, 40, and 95% alcohol, whereas in the absence of alcohol 50-60% sporulation occurred. At 1% alcohol 2-5% ascospores were observed, while at 5% they were very rare. The viability of the cells was tested by plating on glucose nutrient agar. At 20% alcohol a marked decline in viability was evident, while but a few cells from 40% and none from 95% gave rise to colonies. Obviously, sporulation is inhibited at a concentration below that which is toxic to the cells. Somewhat similar observations were made by Stantial (35), who found that 2% alcohol in a liquid medium prevented sporulation, and more recently Bright et al. (5) noted that in the presence of alcohol vapours sporulation was reduced.

(c) Effect of Light on Ascospore Formation

The possibility that light might affect ascospore formation seemed worthy of investigation since it would indicate whether sporulation cul-

tures should be kept in the dark or not. To this end, two experiments were conducted--one employing two isolates of two different yeast strains, and the other these same isolates plus one isolate of a third yeast strain. The experiments were also designed to compare the yields of asci from cultures heavily seeded with vegetative cells with those lightly seeded.

One series of sporulation cultures was incubated for 14 days in the dark, while another series, maintained under similar conditions of temperature and humidity, was exposed to daylight transmitted through a glass brick window.

The yields of asci obtained in the two experiments are summarized in Tables VIII and IX. In Table VIII the data are analysed to assess the effect of light where it is evident that a statistically significant difference existed with strain 1. Higher yields were obtained in the dark with this strain. However, with strains 2 and 3 no significant difference in yields occurred. In Table IX the same data are analysed to compare the effect of light and heavy inoculation. With the exception of cultures of strain 3 incubated in the dark, yields from heavily seeded cultures were significantly higher than those from lightly seeded cultures. This is in agreement with the observations made in experiments relating to the effect of cell concentration on ascospore formation.

Table VIII

Yield of Ascospores from Cells of Three Yeast Strains Streaked on Acetate-Dextrose Agar and Incubated in Diffused Daylight and in the Dark

Yeast Strain	Type of Streak Inoculation of Plates	Percent Asci After 14 Days at Room Temperature					
		In Diffused Daylight			In the Dark		
		Counts	Mean	Stand. Error	Counts	Mean	Stand. Error
1	Lightly	57,60,62 57,50,55,51	56.0	1.7	64,71,82 59,61,64,63	66.7	3.1
	Heavily	67,68,70 68,67,77,62	68.4	1.7	83,85,85 67,71,72,82	77.6	2.9
2	Lightly	52,60,64 35,38,45,44	48.3*	4.2	68,71,77 47,52,47,67	61.3*	4.7
	Heavily	68,67,72 63,64,61,60	65.0	1.6	85,75,88 64,65,69,82	75.4	3.7
3	Lightly	40,49,54,57	50.0	3.7	58,67,69,74	67.0	3.3
	Heavily	60,61,70,74	66.3**	3.4	66,68,76,84	73.5**	4.1

* Not significantly different
** Not significantly different

Table IX

Effect on Ascospore Formation of Light and Heavy Inoculations

Yeast Strain	Held in:	Percent Yield of Ascospores			
		Inoculated Lightly		Inoculated Heavily	
		Mean	Stand. Error	Mean	Stand. Error
1	Daylight	56.0	1.7	68.4	1.7
	Dark	66.7	3.1	77.6	2.9
2	Daylight	48.3	4.2	65.0	1.6
	Dark	61.3	4.7	75.4	3.7
3	Daylight	50.0	3.7	66.3	3.4
	Dark	67.0*	3.3	73.5*	4.1

* Not significantly different.

(d) Effect of Temperature on Ascespore Formation

(i) Pre-Sporulation Temperature

Vegetative cells of two bakers' yeast isolates were transferred to dextrose nutrient agar slants and after 48 hours' incubation at 27.5°C., 30-31°C., and 37.5°C. respectively, were transferred to slants of acetate-dextrose agar and kept at room temperature. The concentration of vegetative cells on the sporulation medium was between 10 million and 14 million per ml. of medium in all culture tubes. This was determined at the end of the experiment by the method described in the Methods and Materials.

The percent yields of asci after 14 days are listed in Table X. It will be noted that a pre-sporulation temperature of 37.5°C. seemed less favourable for subsequent ascospore formation than temperatures of 30-31°C., or 27.5°C.

Table X

Effect of Pre-Sporulation Temperature on Subsequent Ascospore Formation at Room Temperature after 14 Days.

Presporulation Temperature	Percent Yield of Asci					
	Strain 1			Strain 2		
27.5°C.	48	55	47	42	42	55
30.0°C.	45	45	49	54	56	58
37.5°C.	13	15	18	6	7	10

Vegetative cells did not multiply on the pre-sporulation medium at 37.5°C. as rapidly as did the cells growing at the other temperatures, nor did they have the general morphological appearance of the latter cells, being generally somewhat smaller and in many instances more elongated.

(ii) Effect of Chilling Sporulation Cultures on Ascospore Formation

Acetate-dextrose slant cultures of three bakers' yeast isolates were chilled at -18°C., -3.5°C., 2.5°C., and 4.5°C. for 14 days, then held for a further 14 days at room temperature. The results were summarized in Table XI.

Table XI

Effect of Chilling for 14 Days on Subsequent Ascospore Formation on Acetate-Dextrose Agar

Temperature of Chilling (°C.)	Percent Asci After 14 Days at Room Temperature								
	Strain 1			Strain 2			Strain 3		
4.5	51	58	52	60	53	55	50	56	67
2.5	44	50	55	52	48	35	45	56	52
-3.5	38	40	31	39	44	33	55	48	40
-18	13	12	10	23	31	34	38	34	30
Control at Room Temperature for 28 Days	68	60	65	68	70	63	74	72	80

Cultures which were exposed to the lower temperature did not yield as many ascospores as did those chilled to 2.5°C., suggesting a relationship between the degree of chilling and subsequent ascospore formation. The table includes the control cultures kept at room temperature for 28 days.

From microscopic examination of cultures chilled below 0°C., it appeared that a certain amount of cell injury had occurred due to the effect of low temperature. Some evidence of plasmolysis was observed in the cultures incubated at -18°C. Ice-crystals within the medium, at the low temperatures, were noted macroscopically. Since at the lower temperatures

of chilling ascus yields were less, a possible effect of low temperature chilling is that cells are killed, thus reducing the number of cells that could form ascospores.

(iii) Effect of Varying Temperatures on Ascospore Formation

Frequent reference in the literature has been made to a temperature range (20-30°C.) in which attempts at inducing ascospore formation are more likely to succeed. However, with a few exceptions to be dealt with later, no critical studies have been made of the temperature limits within which ascospore formation occurs, or the numbers formed at those temperatures for any particular type of yeast.

Twelve bakers' yeast isolates previously subcultured on presporulation medium of grape juice agar for 48 hours were transferred to acetate-dextrose agar slants for incubation at temperatures ranging from -23.3°C. to 37.5°C. The concentration of vegetative cells on the sporulation media was approximately 10-20 million cells per ml. of medium--a concentration that has, in another experiment, been shown to give satisfactory yields of ascospores. Maintenance of uniform conditions of temperature and high humidity throughout the 14-day period was assured in the following manner. Cultures at temperatures below 21°C. were incubated in cabinets specially designed for storage purposes, respiration experiments, and related studies. The relative humidity in these cabinets was maintained at 90% or higher. Cultures at temperatures above 21°C. were kept in incubators. Throughout this experiment, the temperatures did not vary more than ±0.5°C.

In Table XII the yields of ascospores are given for the 12 cultures employed in this experiment. No ascospores were observed in cultures incubated at a temperature of 34.4°C. and only an occasional ascus was

observed at 32.2°C. while no asci were observed at 6.7°C. and only rarely at 8.9°C. With these strains of yeast the optimum temperature for sporulation seems to be from 15.6°C. to 27.5°C.

Table XII

Effect of Temperature on Ascospore Formation on Acetate-Dextrose Agar

Temperature of Incubation °C.	Percent Asci after 14 Days											
	Yeast Isolate Number											
	71	81	91	72	82	92	73	83	93	74	84	94
-23.3	0	0	0	0	0	0						
6.7	0	0	0	0	0	0						
8.9	1	1	1	1	1	1				12	14	3
10.0	1	5	4									
11.1	10	12	7	1	10	5				27	26	23
12.8	17	12	20									
15.6	40	57	25	59	52	61				23	42	41
18.3	44	76	43				27	25	22	64	60	49
21.0	75	80	66	69	70	76						
24.0										60	65	85
27.5							52	55	44			
30.0							28	27	26			
32.2				2	0	1	rare	rare	rare			
34.4				0	0	0	0	0	0			
37.5				0	0	0	0	0	0			

(iv) Effect of Temperature on the Rate of Ascospore Formation

Two other bakers' yeast isolates were tested in a similar manner to the foregoing to determine whether ascospore formation would occur at temperatures above and below those at which sporulation took place in the preceding experiment, should the incubation period be extended to 28 days. One culture of each strain at each temperature was examined after 7 days and 18 days. After 28 days all the cultures were examined. In this way the rate of sporulation at different temperatures could be compared.

The results of these examinations are listed in Table XIII, and are plotted in Figures 3 and 4. Evidently ascospore formation was delayed at the lower temperatures. At 7 days at 8.9°C. or less no asci were observed in either strain, but after further incubation ascospores were formed, as low as 4.4°C. with Strain 1, though not below 8.9°C. in Strain 2. However, at 8.9°C. this latter strain yielded about 20% ascospores after 28 days. This gradual increase in ascospore numbers did not obtain at 34.4°C., at which temperature no ascospores were observed in either strain after 28 days. It would appear that the upper limit for sporulation is very definite, since fairly high numbers of ascospores were noted at 33.3°C. even after 7 days. From the data the optimum range for sporulation would seem to be about 20-28°C.

Comparing these results with those of the preceding experiment, a gradual decrease in the numbers of ascospores with lower temperatures and a sharp suppression of ascospore formation near the maximum temperature were common to both. However, the maximum temperature seemed slightly lower in the preceding experiment. Possibly strain difference is responsible.

Table XIII

Effect of Temperature on Ascospore Formation in Two Bakers' Yeast Isolates on Acetate-Dextrose Agar

Incubation Temperature	Percent Asci After											
	7 Days				18 Days				28 Days			
°C.	Strain 1											
4.4	0	0	0	0	0	0	0	0	4	4	3	1
									0	0	0	0
									0	0	0	0
6.7	0	0	0	0	0	0	0	0	4	3	1	0
									0	0	0	0
									0	0	0	0
8.9	0	0	0	0	3	1	1	0	13	12	11	9
									16	12	11	8
									15	10	9	5
16.1	25	24	19	19	47	42	40	34	47	45	40	39
									38	36	35	32
									48	47	44	42
24.0	67	66	63	60	63	63	61	56	70	70	65	59
									61	61	60	55
									70	58	57	56
27.5	59	56	55	52	73	71	65	64	77	71	70	66
									68	64	64	61
									77	75	75	66
33.3	40	39	35	30	29	23	21	18	24	23	22	22
									22	21	16	15
									30	26	26	24
34.4	0	0	0	0	0	0	0	0	0	0	0	0
									0	0	0	0
									00	0	0	0

Continued on next page.

Table XIII (Cont'd)

Effect of Temperature on Ascospore Formation in Two Bakers' Yeast Isolates on Acetate-Dextrose Agar

Incubation Temperature °C.	Percent Asci After 7 Days				18 Days				28 Days			
	Strain 2											
4.4	0	0	0	0	0	0	0	0	0	0	0	0
									0	0	0	0
									0	0	0	0
6.7	0	0	0	0	0	0	0	0	0	0	0	0
									0	0	0	0
									0	0	0	0
8.9	0	0	0	0	14	13	9	8	28	27	27	25
									20	20	16	13
									18	15	13	10
16.1	24	22	22	20	40	40	40	39	55	53	52	51
									54	53	50	46
									52	52	44	43
24.0	43	39	37	35	77	75	74	71	89	85	85	80
									83	82	77	74
									77	72	72	68
27.5	39	32	32	31	54	53	49	47	60	57	56	54
									64	63	61	59
									66	64	64	60
33.3	33	30	26	26	25	25	25	23	24	22	19	18
									28	25	25	23
									26	23	21	21
34.4	0	0	0	0	0	0	0	0	0	0	0	0
									0	0	0	0
									0	0	0	0

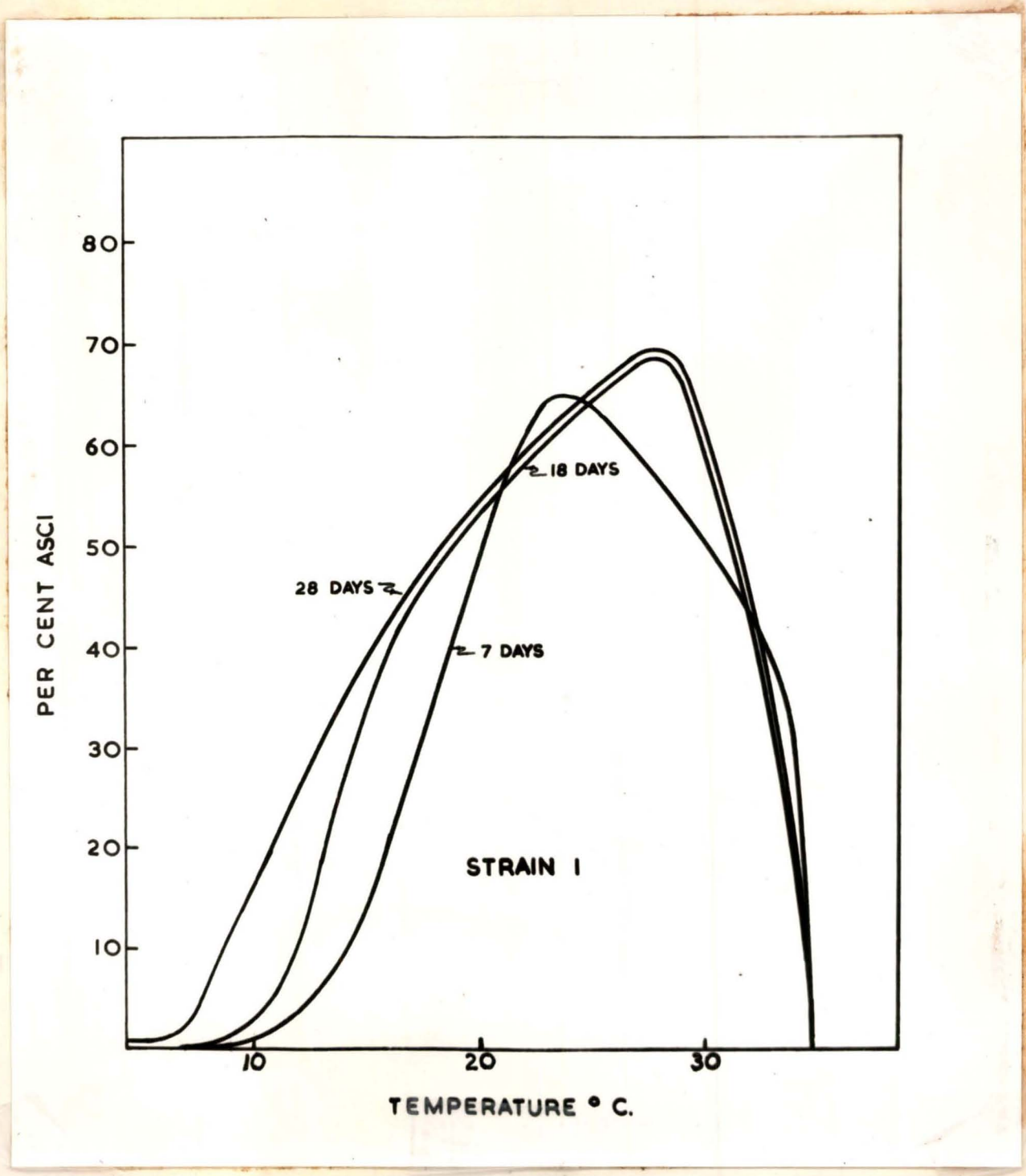

Figure 3. The effect of temperature and time of incubation upon ascospore formation in a bakers' yeast isolate (Strain 1).

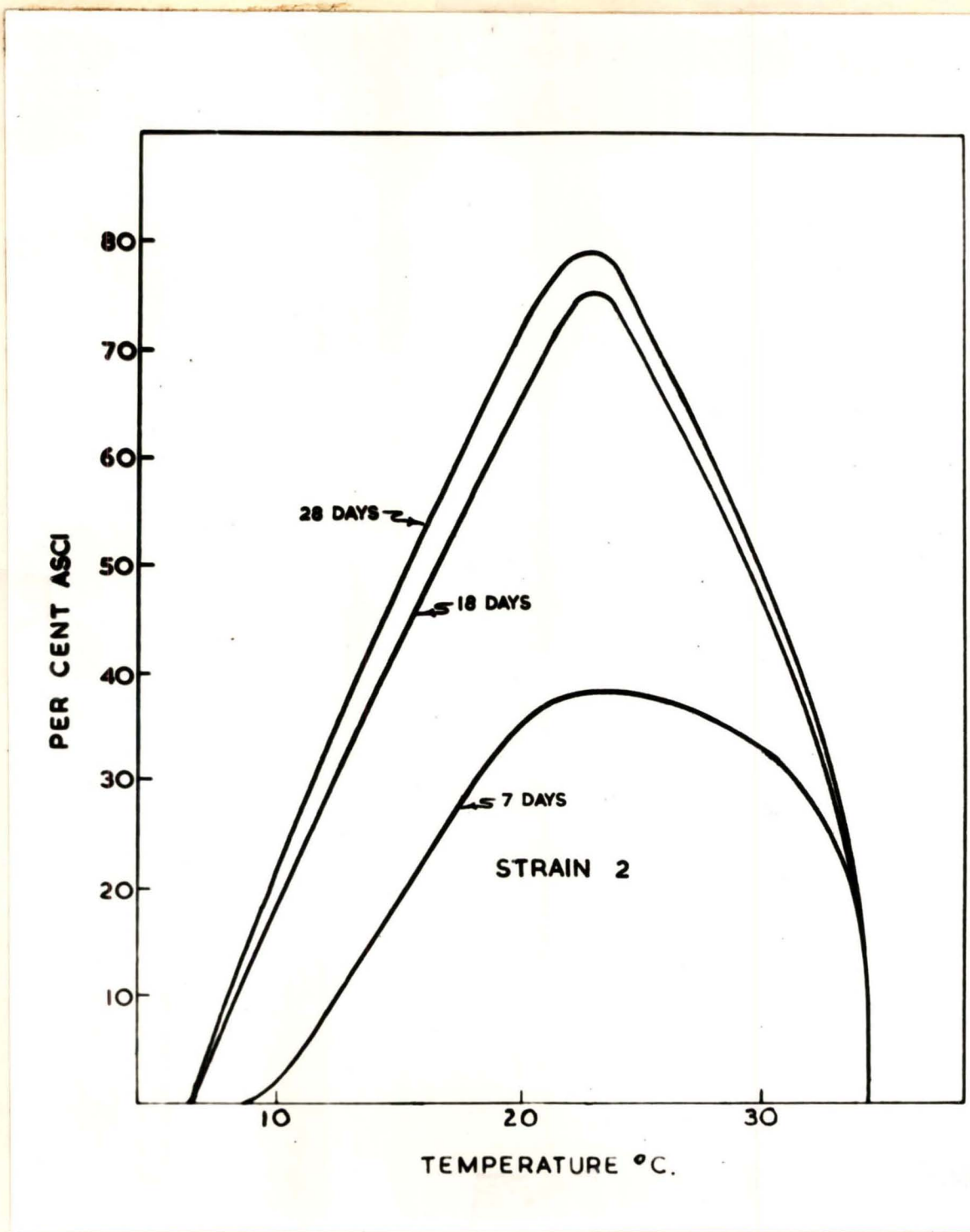

Figure 4. The effect of temperature and time of incubation upon ascospore formation in a bakers' yeast isolate (Strain 2).

(v) Effect of Refrigeration on Ascospore Formation Rate

In the preceding experiment it was seen that with Strain 1 in a temperature of 6.7°C. or lower, ascospore formation did not occur in 18 days, but did occur within a further 10 day period. This effect of low temperature on the rate of sporulation was considered worthy of further investigation. A comparison was made of the number of ascospores formed by three yeast strains after 7 days, 14 days and 28 days under varying temperature conditions. One set of acetate-dextrose agar cultures was held at 3°C., while a second set was held at room temperature. One group was stored 7 days at 3°C., then held at room temperature for a further 7 days to determine if storage at a low temperature such as 3°C. would effect sporulation.

The percent asci observed in these cultures at the above mentioned time intervals is listed in Table XIV and Figure 5. Three conclusions are suggested by these data, namely, (1) a seven day period is not sufficient for maximum yields of ascospores at room temperature; (2) Holding cultures beyond 14 days at this temperature does not seem to increase the yield,, and (3) Holding cultures for a week at 3°C. does not materially retard subsequent sporulation at room temperature.

The amount of sporulation at the low temperature employed (3°C.) was similar to that of the previous experiment in that here also very few ascospores were formed, and strain variations were apparent. The effect of low temperature, prior to a 7-day period at room temperature, on ascospore formation corresponded closely to that observed in the chilling experiment (p. 33) in which certain cultures were stored for 14 days at 2.5°C. Whereas in the chilling experiment a slight suppression of ascospore yields was noted after exposure to temperatures of 4.5°C. and 2.5°C. for

14 days, no such effect was observed in the present experiment in which cells were exposed to 3°C. for 7 days.

Table XIV

A Comparison of Ascospore Formation by Three Yeast Isolates at Room Temperature (RT) and 3.0°C.

Yeast Strain	Incubation Temperature	Percent Asci After 7 days					P	14 days					28 days				
		Counts				Mean		Counts				Mean		Counts			Mean
1	3.0°C.	0	0	0	0	0.0		1	0	0	0			1	0	0	0	
		0	0	0	0			1	0	0	0			1	1	0	0	
		0	0	0	0			1	0	0	0	<1		1	1	0	0	<1
	RT.	34	36	37	40			55	59	61	63			50	58	60	60	
		34	35	39	43			75	77	78	82			63	61	68	64	
		42	42	44	45			75	74	75	81	71		67	70	72	58	
		46	52	44		39								59	61	61		63
	7 days 3.0°C. 7 days at RT.	51	55	56	58													
		45	49	52	59													
		51	54	55	61	54												
2	3.0°C.	0	0	0	0	0.0		0	0	0	0	0.0		0	0	0	0	0.0
		0	0	0	0			0	0	0	0			0	0	0	0	
		0	0	0	0			0	0	0	0			0	0	0	0	
	RT.	19	24	25	25			71	75	75	77			80	83	85	89	
		27	31	31	33			61	66	74	76			91	74	77	82	
		33	36	35	37			65	66	68	69			83	84			
		39	43	47		36						70						83
	7 days 3.0°C. 7 days at RT.	43	46	47	51													
		30	35	37	37													
		34	39	46	48	42												
3	3.0°C.	0	1	2	2			13	14	16	16			15	18	20	22	
		2	4	5	8			7	8	11	11			13	15	17	18	
		1	3	3	6	3		6	7	8	9	11		9	9	10	11	15
	RT.	47	48	50	50			80	81	86	89			72	73	74	75	
		51	53	54	61			74	76	81	83			79	76	78	81	
		62	65	52	53			77	78	80	84			84	86	67	72	
		56	57	58		54						81		73	77	80		77
	7 days 3.0°C + 7 days at RT.	43	45	48	50													
		42	42	47	50													
		37	38	44	48	44												

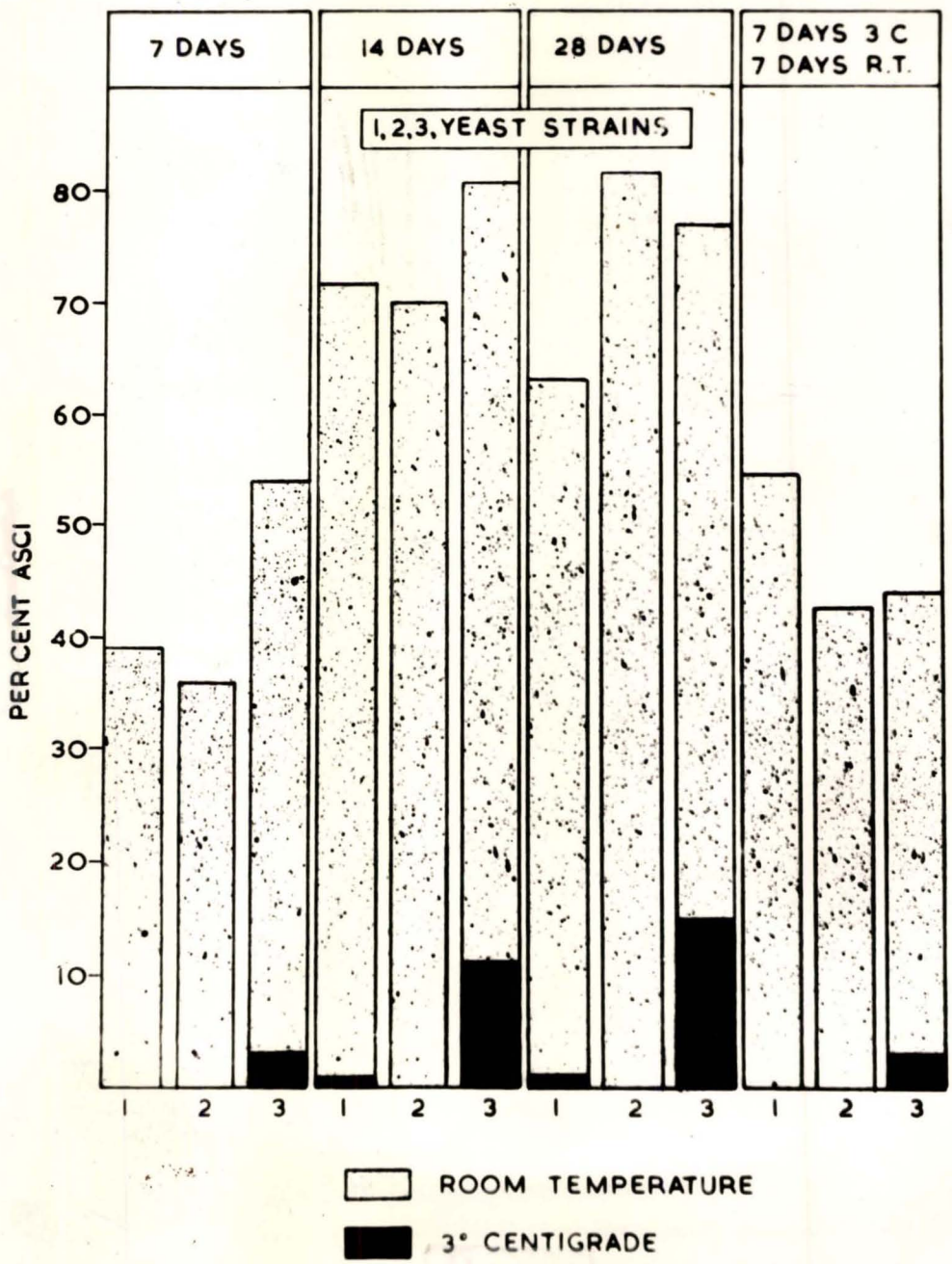

Figure 5. Ascospore formation in three bakers' yeast strains at Room Temperature and at 3°C. and in the same strains after 7 days at 3°C. + 7 days at Room Temperature.

2. VARIATION IN ASCOSPORE YIELDS AMONG CULTURES.

(i) A Comparison of Ascospore Formation by 43 Yeast Cultures

It is generally accepted that yeasts vary in their ability to sporulate on any of the commonly employed media. Such variation has been mentioned on several occasions in the preceding experiments. It was therefore considered of interest to compare the ability of different yeast cultures to form ascospores on acetate-dextrose agar and on two other media.

An experiment, in two parts---A and B, was designed to determine the range of variation in ascospore formation of yeasts from widely separated geographical localities. With the exception of 6 strains isolated from plant tissues, these yeasts were of some known industrial importance.

Four strains had been identified previously by other workers. The remainder of the cultures were studied taxonomically and grouped into genera (Table XV), on the basis of morphological and biochemical characteristics according to the classifications of Stelling-Dekker (37) and Diddens and Lodder (7).

Classification of the cultures was not always carried beyond the genus. It was felt that further separation of the strains would in many cases be unjustified, since in the existing classification so many contradictions and ambiguities in descriptions are found that it is virtually impossible to separate one "species" from another. Although these classifications are accepted as currently the most complete, it was not possible to separate several strains into definite species for it was found that though an identification might be made on a morphological basis the biochemical characteristics observed were found to vary with the descriptions in the keys. With the possible exception of Saccharomyces the writer

Table XV

Classification of the 43 Yeast Cultures Studied in This Experiment

Reference Number	Industrial Designation	Botanical Designation
1	Bakers' Yeast (Fleischmann)	Saccharomyces cerevisiae
2	Bakers' Yeast (Lallemand)	" "
3.	Bakers' Yeast (Best)	" "
4	Tokay 1	" cerevisiae var. ellipsoideus
5	Tokay 2	" "
6	Tokay 3	" "
7	Burgundy	" "
8	Moselle (1)	" "
9	Jerez No.5 (1)	" " sp. (?)
10	Flor	Mycoderma sp. (?)
11	Moselle (2)	Saccharomyces cerevisiae var. ellipsoideus
12	Port (1)	" "
13	Port (2)	" "
14	Port (3)	" "
15	Burgundy	" "
16	Jerez No. 5 (2)	" sp. (?)
17	Tokay 4	" cerevisiae var. ellipsoideus
18	Davey A	" cerevisiae
19	Davey B	" "
20	Davey C	" "
21	Davey D	" "
22	Roja Tempanillo	" cerevisiae var. ellipsoideus
23	German "5"	" cerevisiae
24	Meyer	" "
25	Castrucci	" "
26	Steinberg Champagne	" "
27	H. A. Yeast	" "
28	White Wine	" "
29	Red Wine	" "
30	Wild Yeast	Rhodotorula sp.
31	Wild Yeast	Hanseniaspora sp.
32	Wild Yeast	Saccharomyces sp.
33	Wild Yeast	Hansensiaspora sp.
34-35	Wild Yeasts	Rhodotorula sp.
36-39	Wild Yeasts	Cryptococcus sp.
40	High protein strain	Torula utilis
41	High protein strain	Torula berlese
42	High fat strain	Nectaromyces reukaufii
43	High fat strain	Nectaromyces reukaufii

feels that definite identification of the species of many genera on the basis of existing classification cannot be made. Tanner (39), discussing yeast classification, believes this confusing situation is due to the lack of suitable techniques by which to demonstrate many characteristics. Jorgensen (16) is of the opinion that a reliable species identification of an isolate can only be accomplished by a comparison with cells from a subculture of a species or strain on which the original description was made. This would, at best, be a formidable task, and quite impractical for most workers.

For the experiment all strains were subcultured on pre-sporulation medium twice before being transferred to sporulation media. The two pre-sporulation media employed for the multiplication of vegetative cells were those described in the Methods and Materials. Vegetative cells placed upon sporulation media were from actively growing 24-hour cultures. To facilitate comparison of the ability to form ascospores by these different strains, three sporulation media were employed. Namely:

(1) Acetate-dextrose agar

(2) Gorodkowa's medium

(3) Water agar.

Details of the preparation of these media are given in the section on Methods and Materials.

Sporulation media for Part A were dispensed in test tubes as slants, and for Part B in petri dishes.

As shown in Table XVI, ascospore formation occurred most frequently and to a greater degree in cultures upon acetate-dextrose agar. Of the 30 cultures forming ascospores in this medium, 13 cultures yielded 50% or

Table XVI

Ascospore Production on Three Sporulation Media
by 43 Yeast Cultures Multiplied on Two Pre-Sporulation Media

Culture Identification Number	Part A		Part B			
	Acetate-Dextrose Agar	Gorodkowa Medium	Acetate-Dextrose Agar	Gorodkowa Medium	Water Agar	Number of Ascospores Per Ascus
1	90	20	90	20	5	1 - 4
2	90	2	90	10	5	1 - 4
3	90	20	90	20	5	1 - 4
4	5	rare	5	rare	0	1 - 2
5	7	rare	5	rare	0	1 - 2
6	21	rare	10	rare	0	1 - 2
7	65	10	70	5	40	1 - 2
8	15	2	20	2	1	1 - 2
9	5	rare	15	rare	0	1 - 2
10	0	0	0	rare	0	
11	20	2	25	1	1	1 - 2
12	50	4	40	2	5	1 - 2
13	40	12	35	8	5	1 - 2
14	60	14	40	5	10	1 - 2
15	45	20	50	10	10	1 - 4
16	25	0	20	0	0	1 - 2
17	rare	rare	rare	rare	0	1
18	70	40	65	35	65	1 - 3
19	1	1	1	1	0	1 - 2
20	45	25	50	25	35	1 - 3
21	1	0	1	0	0	1
22	0	0	0	0	0	
23	45	10	35	5	20	1 - 3
24	25	0	25	0	0	1 - 3
25	60	40	55	25	45	1 - 4
26	10	0	10	0	0	1 - 2
27	35	1	20	rare	0	1 - 2
28	35	10	45	5	30	1 - 3
29	55	20	55	20	45	1 - 3
30	0	0	0	0	0	
31	25	0	25	0	0	1 - 3
32	70	0	65	0	0	1 - 2
33	60	0	75	0	0	1 - 2
34-43	0	0	0	0	0	

The pre-sporulation media employed were grape juice agar in A, and glucose nutrient agar in B.

more asci, whereas no yield of more than 40% was observed from cultures on Gorodkowa's medium and seven of the same cultures failed to form ascospores on water agar. One culture (No. 10) formed asci only on Gorodkowa's medium, while 7 formed ascospores only on the acetate-dextrose medium. The three bakers' yeast isolates readily formed asci with 4 spores, but only two of the other strains were able to do so. Obviously wide differences in the ability to form ascospores exist between the various strains employed in this experiment. All cultures developed dense cell populations on Gorodkowa's medium. In acetate-dextrose agar the vegetative cells became more granular, as ascospore formation proceeded, losing the typical appearance of actively growing yeast cells. Cells upon Gorodkowa's medium became less refractile and appeared paler than those on the pre-sporulation media, while cells upon water agar more closely retained their original appearance though exhibiting a tendency to swell, probably owing to the imbibition of water from the medium by the cells.

(ii) Variation with Respect to Ascospore Formation Among Isolates from a Single Package of Yeast

It is obvious from the preceding experiment that variations in the yield of ascospores are very great among yeasts from different sources. It seemed of interest to investigate in this respect the range of variation that might occur within a single package of commercial yeast.

Pellets from a package of Fleischmann's Yeast were added to a water blank. A series of dilutions of this suspension was prepared and plated with glucose nutrient agar containing 50 International Units of Streptomycin per ml. Twenty isolations were made to glucose nutrient agar slants

from individual colonies present on the plates containing the higher dilutions. After 48 hours at 27.5°C., vegetative cells from these cultures were transferred, by streaking, to acetate-dextrose agar slants and held at room temperature for 14 days. The percent ascospore formation in these sporulation cultures is given in Table XVII. Ascospore formation varied among cultures from 10% to 50%. These results suggest that isolates from a package of yeast are not all equally capable of ascus formation.

At a later date this experiment was repeated with eleven other isolates obtained from a package of "Best" yeast. The yields of asci obtained are tabulated in Table XVIII.

These results were analysed statistically to determine whether the differences, as shown in the table, were significant. Yields from individual isolates were compared one with the other. Since no statistical difference was apparent between the following isolates, 91, 93, 95, 97, 99, and 101, they were grouped together. The mean for these isolates was determined as being 73.7 ± 1.0. The remaining isolates in the experiment were compared with this mean, four of which gave yields significantly different from this mean.

(iii) Variation with Respect to Ascospore Formation Among Ten Single Cell Isolates from a Single Isolate Culture

The experimental procedure employed closely paralleled that of the previous experiment. The ten isolates were obtained by plating cells from a presumably single cell isolate and picking off portions of ten discrete colonies. The yields of ascospores on acetate-dextrose agar are given in Table XIX. Four of the ten isolates tested gave yields of asci significantly different from the mean yield of the remaining six yeast isolates.

Table XVII

Variation in Ascospore Formation by Twenty Isolates From a Commercial Yeast After 14 Days on Acetate-Dextrose Agar

Isolate No.	Millions of Cells per ml. of Medium	Percent Asci
1	12.0	10*
	12.6	33*
2	11.2	59
	10.6	53
3	9.3	22
	18.7	21
4	14.7	31
	18.0	35
5	14.6	36
	8.1	22**
6	17.4	18
	17.7	13
7	14.1	27
	15.6	26
8	14.8	25
	18.1	30
9	16.8	43
	15.2	28**
10	12.3	47
	13.2	49
11	12.1	28
	10.9	28
12	10.1	39
	9.1	34
13	11.8	37
	10.1	14**

(continued on page 51)

Table XVII (continued)

Variation in Ascospore Formation by Twenty Isolates
From a Commercial Yeast After 14 Days on Acetate-Dextrose Agar

Isolate No.	Millions of Cells per ml. of Medium	Percent Asci
14	42.4	21
	23.5	38
15	18.1	24
16	8.5	21
	10.3	23
17	14.6	43
	16.7	30
18	13.8	40
	18.4	30
19	13.3	38
	10.2	27
20	12.8	55
	13.3	55

* Replicates

** Culture contaminated or excessive condensate in which cells from the slant had collected, resulting in a spoiled sporulation culture.

Table XVIII

Variation in Ascospore Formation by Eleven Yeast Isolates from a Package of Commercial Yeast after 14 Days on Acetate-Dextrose Agar

Isolate No.	Percent Asci*	Mean	Standard Error
91	74,81,77,75,70,60, 78,79,78,72,73,61,	73.2	1.9
92**	60,65,58,57,59,59, 56,66,53,51,	58.4	1.4
93	70,60,66,68,65,71, 72,73,80,75,74,72,	70.5	1.5
94	59,60,66,67,73,70, 73,64,79,75,74,77,	69.8	1.7
95	85,79,85,83,80,82, 71,75,68,69,79,78,	77.8	1.7
96**	66,65,56,63,81,81, 76,77,46,54,49,50,	63.7	3.7
97	71,73,60,71,73,73, 77,74,72,73,75,71,	72.3	1.3
98**	49,51,63,51,65,68, 52,65,53,52,53,56,	56.5	2.1
99	73,71,79,78,77,77, 72,72,73,67,77,79,	74.6	1.1
100**	80,81,77,77,75,82, 85,82,82,84,80,82,	80.6	1.0
101	73,71,79,81,71,68, 69,73,75,78,72,73,	73.6	1.2

* Each figure based on the examination of 100 cells.

** Isolates giving statistically different yields from the mean yield of the remaining isolates.

The yields of the latter six isolates did not differ significantly from that of the parent culture.

Table XIX

Variation in Ascospore Formation by Ten Yeast Isolates from a Single Isolate after 14 Days on Acetate-Dextrose Agar

Isolate Number	Percent Asci with Statistical Analysis		
	Percent Asci*	Mean	Standard Error
Control 99		74.6	1.1
99-1	81,83,81,84,77, 69,75,69,73,75,	76.7	1.7
99-2	78,68,81,75,74, 80,75,72,69,73,	74.5	1.3
99-3	70,75,80,81,77, 74,63,72,73,70,	73.5	1.7
99-4**	56,57,48,58,45, 46,52,55,51,52,	52.0	1.4
99-5	70,64,67,67,62, 79,78,73,84,83,	72.7	2.5
99-6	77,77,76,72,68, 78,68,62,71,66,	71.5	1.6
99-7**	70,71,65,70,66, 67,75,75,73,64,	69.6	1.3
99-8**	83,90,79,85,83, 82,87,79,92,84,	84.4	1.4
99-9**	78,82,77,83,85, 81,73,79,80,82,	80.0	1.1
99-10	75,73,76,77,75, 80,77,72,77,81,	76.3	1.0

* Each figure based on examination of 100 cells.

** Isolates giving statistically different yields from Control 99.

The results of these experiments strongly suggest that considerable variation in the ability to form ascospores may be expected, not only among isolates from a single package of yeast, but among the progeny of a presumably single cell isolate. However, it should be noted that the extent of the variations observed in the latter two experiments was not nearly as great as that observed among the 43 different yeast cultures from various sources.

3. EMPLOYMENT OF ASCOSPORES IN HYBRIDIZATION STUDIES

The main object of a hybridization method is to bring about a fusion of gametes. With yeasts, two methods designed for this purpose have been dealt with in the Introduction. Employing a micromanipulator, Winge made crossing between single isolated cells, while Lindegren mated suspensions of haploid cells derived from two different ascospores. As mentioned previously, this latter procedure has been termed "mass mating" by Lindegren (20), partially, no doubt, to differentiate the method from that in which single cells are crossed, and also to indicate that ample opportunity exists for many fusions to occur. These methods are of value where careful genetical studies are being pursued.

It seemed worth investigating whether improved strains for industrial purposes could be obtained from fusions between large numbers of ascospores from one or several yeast strains. Although this method would not permit careful genetic analysis, it might possibly be of some practical importance. The success of this method would depend upon the availability of large numbers of ascospores. These may be obtained, as has been shown in these studies and in previous work (1, 2), from the use of acetate-dextrose agar

as the sporulation medium. Such a hybridization method would give practical application to the researches described here on the environmental factors influencing ascospore formation.

To test the value in hybridization work of ascospores produced by the method previously described by the writer (2), and to investigate the possibility of obtaining improved strains of yeast by the hybridization procedure just described (in which haploid cells from several strains are mixed together), several experiments were conducted with varying degrees of success. One of these is described below and can be regarded, so far as procedure is concerned, as typical of the other experiments.

(i) The Development of Increased Tolerance to Ethyl Alcohol by Yeast Strains through Hybridization.

From the yeast collection acquired concurrently with this work, fourteen cultures were selected that in other studies had been shown to grow in the presence of 13% but not 16% by volume of ethyl alcohol. This characteristic was again checked for each culture and sporulation cultures were then prepared employing acetate-dextrose agar slants. After 14 days the cells from individual cultures were transferred to tubes of the medium recommended by Lindegren (20) for encouraging fusions. Cells from all of the fourteen sporulation cultures were added to the 11 additional tubes of Lindegren's medium to allow fusions between strains. After 36 hours the cells were separated from the mating medium by centrifuging. Culture tubes containing 5% dextrose nutrient broth to which had been added 16% ethyl alcohol were inoculated with the centrifuged cells and incubated at room temperature. These cultures were observed for evidence of growth

employing the following criteria: photoelectric turbidity measurements, pH values determined colorimetrically (changes in Brom-cresol purple indicator showing formation of acid), and with a Beckman Potentiometer, refractometer readings (indicating decrease in sugar content of medium). After 48 hours one of the tubes containing cells from mixed matings showed marked changes indicative of cell growth and multiplication, while after 96 hours 3 others showed changes suggestive of cellular activity. It is noteworthy that no tubes containing "selfed" cells of the fourteen cultures showed evidence of growth. Cells from the four cultures were transferred to sterile grape juice and fermented to the limit of ethyl alcohol tolerance with the periodic addition of sugar.

Table XX

Fermentative Capacity of Four Yeast Cultures Following Hybridization

Culture	Growth in 16% alcohol in glucose nutrient broth tubes after: 48 hours	96 hours	% alcohol obtained by fermentation of grape juice*	
M_1b	-	?	15.90	15.90
M_1c	yes	yes	16.04	16.10
M_2c	-	?	15.90	15.82
M_3b	-	?	15.00	15.01

* Determined by a TAG Twin Ebulliometer (C. J. Tagliabue Corp., Newark, N. J.)

The amount of alcohol produced by cells from the four cultures is shown in Table XX. Of these, three cultures produced just less than 16% ethyl alcohol, while one culture produced over 16% alcohol. Thus it has been possible to develop at least one yeast strain capable of growth

in the presence of 16% by volume of ethyl alcohol from strains of lower alcohol tolerance.

This experiment indicates that ascospores obtained by the convenient method developed by the writer may be successfully employed in hybridization experiments entailing the mixing of ascospores of several yeast strains.

DISCUSSION

From the results obtained in this work it is apparent that ascospore formation on acetate-dextrose agar is conditioned to a very considerable degree by environmental factors.

Consider first the effect of cell concentration. It was seen that ascospore formation occurred over a wide range of cell concentrations, with no clear optimum, though it was less frequent at the lower concentrations tried. Stantial (36), however, found an optimum concentration of cells for a given concentration of acetate. In previous studies (2) the writer observed an optimum concentration of 1-2 million cells per ml. of medium. In both of these former studies a liquid medium was employed, whereas in the present work the cells rested upon an agar surface,which may explain the differences noted. Strain difference might also be a factor here. This is suggested by the writer's previous work (2) in which at low cell concentrations ascospore formation was reduced in one strain but not in another.

From the studies relating to gaseous environment, it is apparent that both oxygen and carbon dioxide affect ascospore formation, the former being stimulating and the latter inhibitory. A very small amount of oxygen markedly raised the ascospore yields. For example, when the small oxygen impurity (o.1%) was removed from bottled nitrogen the yield of ascospores was reduced about 30% from that obtained in unpurified nitrogen. Again,

when the concentration of oxygen was 2.6%,a fourfold increase in sporulation resulted as compared with purified nitrogen. Above 2.6% oxygen concentration no marked increase in yields occurred. On the contrary, an inhibitory effect of oxygen is indicated at high concentrations. In a 100% oxygen environment ascospore yield was about half that obtained at 12%. Low concentrations of carbon dioxide mixed with nitrogen are not so effective in suppressing, as are similar concentrations of oxygen in stimulating sporulation. However, at a concentration of about 40% carbon dioxide almost total suppression of ascospore formation was observed. It is noteworthy that this inhibitory effect of carbon dioxide is capable of nullifying the stimulating effect of oxygen. This is apparent from the fact that in air plus 50% carbon dioxide no ascospores were formed.

Ascospore yields in flowing air were approximately 43%, which is considerably less than what would be expected from the graph, in view of the fact that air contains about 21% oxygen. It could be that this is due to the presence of carbon dioxide in the air exerting an inhibitory effect on sporulation.

The results of the work dealing with the effect of varying concentrations of oxygen and carbon dioxide allow an interpretation of the observations made in previous experiments in which the aeration of cells was interfered with. In the writer's experience yields of ascospores were for the most part not so high in a liquid as on a solid medium. This behaviour of the cells may be related not only to the availability of oxygen, but also to the production of carbon dioxide by the cells. In a liquid medium the cells sink to the bottom and the liquid would retard the diffusion of the carbon dioxide out into the air. With a solid medium this gas

would pass directly into the atmosphere and thus have less effect upon sporulation. The passage of oxygen to the cells from the atmosphere would be similarly affected. The writer observed in previous work (1) that an increase in the depth of liquid medium was found to depress sporulation, but an explanation of the condition was lacking at that time. In view of the rôle played by carbon dioxide as outlined in these present studies, the condition could be accounted for in the following way. The larger the volume of suspension added to the vials, the larger would be the number of cells present, and thus more carbon dioxide would be produced. It seems reasonable to assume that diffusion of this carbon dioxide from the cells to the air would be less rapid as the depth of liquid increased. Thus, in vials containing the larger volumes, more carbon dioxide would be present and ascospore formation would be depressed accordingly. Here again the depression of sporulation could be explained by the barrier to oxygen diffusion presented by the liquid.

When paraffin oil was layered on liquid cultures an added barrier to the diffusion of carbon dioxide and oxygen was present. This could account for the further reduction of ascospore yields in liquid medium covered with paraffin oil. Moreover, when an area of agar surface was covered with a cover glass, ascospore formation diminished from the edge of the glass in toward the centre. The foregoing explanation appears applicable here as well.

At this point it is of interest to recall the observations of Maneval (24), who noted the presence of ascospores in yeast cakes only on the outside layers of cells, as well as those of Lindegren and Hamilton (21) that when cross-sections of yeast colonies were made, ascospores were found

only in the outermost layer of cells. Maneval concluded that oxygen was required for ascospore formation. The studies reported here are in accord with Maneval's view, but suggest the additional possibility that carbon dioxide (formed by the cells) attained a sufficient concentration within the yeast cakes or colonies to inhibit ascospore formation.

Some volatile product or products of metabolism other than carbon dioxide could also be responsible for the suppression of sporulation observed in deep liquid media and under coverslips. This possibility should not be overlooked, since it was noted that alcohol vapour inhibited sporulation.

Reduced atmospheric pressure was seen to lower the yields of asci. Since the composition of the air was not altered so far as the ratio of the gases to one another was concerned, even at the most reduced pressure (200 mm.), a supply of oxygen was not lacking to the cultures. Ascospore formation occurred,though not to the same extent as in air under normal atmospheric pressure. Slator (34) found no ascospores in tubes from which the air had been exhausted, but stated also that traces of air were sufficient to induce sporulation. He found that 1 cm. of air pressure was sufficient to cause the production of spores. The latter observation is in accordance with the writer's finding that a small amount of oxygen stimulated sporulation.

Hansen (13) in 1883 observed spores in S. pastorianus II Hansen, at 3°C., but with a S. cerevisiae strain not below 8°C., while with an ellipsoideus strain not below 7.5°C. Lafar (18) reported the work of several investigators who determined the minimum temperature of ascospore formation in different yeasts. This temperature was usually above 13°C. for bottom fermentation species. According to Lafar, however, Will observed

ascospores in a Saccharomyces strain at 8°C. and at 3°C. after 28 days in a haze forming yeast. Guilliermond, according to Phaff and Mrak (27), reported in 1928 a minimum temperature of 0.5 - 3.0°C. for S. pastorianus with a maximum of 30.5 - 31.5°C. and an optimum of 27.5°C.

In the present experiments the writer has investigated the minimum, optimum, and maximum temperatures for ascospore formation by a number of bakers' yeast isolates. With respect to the minimum temperature, this was conditioned by strain difference and time of incubation. These additional factors could well explain the discrepancies between the observations of the other workers referred to above. The writer observed that the minimum temperature for one strain was 4.4°C. (after 28 days) and for another, after 7 days, 3°C. This is lower than that reported previously for S. cerevisiae. Furthermore, in connection with the studies reported here and other work, a collection of yeasts has been maintained by the writer for several years, some of them under oil. The collection has been stored at a temperature slightly below 5°C. Over a period of from 6 months to 1 year ascospore formation occurred in certain cultures under one or both of the storage conditions (with or without oil). However, possibly because the carbon dioxide evolved by the culture was more readily diffused to the air, more ascospores were observed in cultures to which no oil had been added.

The maximum temperature for ascospore formation was seen to be sharper than the minimum. No ascospores were found at 34.4°C. or higher, but liberal numbers were found at 30.0°C. and frequently at 33.3°C. This temperature limit is higher than that reported by Guilliermond for Saccharomyces pastorianus. It is worth noting that Stantial (34) obtained good sporulation at 35°C. when the yeast was multiplied in pre-sporulation medium

at that temperature.

Considering the optimum temperature for sporulation, Guilliermond's report of an optimum at 27.5°C. is supported by the present studies, but in addition it has been shown that the optimum range is quite broad, being from about 15 - 30°C., though strain variation is noted.

Diffused light had but a slight effect on ascospore formation. Statistical analysis of the results obtained in these experiments suggest that more ascospores may be formed in the absence of light.

Variations in the number of ascospores formed on sporulation media were observed in the work comparing 43 yeast strains on three media. Here acetate-dextrose agar gave yields of asci more often, and frequently in greater numbers, than the other two. Thus, from these studies and previous work (1) it is evident that the acetate-dextrose agar medium is a very effective substrate for inducing ascospore formation in many yeast strains.

In view of the fact that this method is very convenient to use, it may prove to be of considerable practical value. Phaff and Mrak (28) found that the Stantial-Elder method gave a greater number of ascospores than did other methods in about 75% of the Saccharomyces cultures studied, as well as giving good results with yeasts of other genera. However, they recommend fresh carrot wedges and, as an alternative, Lindegren's pre-sporulation medium followed by transfer to gypsum blocks, while stating that "the Stantial method also gives excellent results, but it is tedious and therefore only recommended in cases of doubt and when other methods fail". Gorodkowa (12), discussing the plaster block, stated that "this method requires a special carefulness and pedantry in work, and what is important, it is capricious and sometimes does not justify the trouble and

loss of time spent with it".* In fairness to the gypsum block method it should be understood that not all the effects of pre-sporulation media upon ascospore formation had at that time been recognized. The criticism of Phaff and Mrak of the Stantial-Elder Technique is not applicable to the acetate-dextrose agar, since it is a simple agar medium, easily prepared and practical. This convenient method of inducing ascospore formation has been successfully applied repeatedly in instructional work. Further, Pomper (29) has recently suggested that this method would have "a great advantage over procedures involving complex natural media, since the nutritional environment can be exactly controlled. This might be of considerable importance, for example, in the experiments on the inheritance of adaptive enzymes where the experimenter might wish to control rigidly the substrates to which the yeast was exposed during sporulation".

The suitability of this method of producing ascospores from yeast for use in hybridization is evident from the example cited here. Though, as stated previously, this method of hybridization does not allow careful genetic analysis, it does permit almost an unlimited number of fusions to occuf. The chances, therefore, of developing improved strains are much increased over those where the single crossing technique is employed, or where haploid cells from two ascospores are mixed, since success will be conditioned to a large measure by the number of ascospores.

The problem of inhibiting rather than inducing sporulation in yeast is also of some importance. The need for a new method of maintaining cultures in a viable and more stable genetic state has been pointed out in the Introduction. As mentioned in the discussion of minimum temperature above, even refrigerated cultures developed ascospores in 6 months to a

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* Translated from the Russian.

year. The results of the experiments on gaseous environment and temperature suggest a promising method of preventing ascospore formation in stored cultures. It will be recalled that a 50:50 mixture of air and carbon dioxide completely inhibited sporulation. From comparative tests made on cells stored in air, in pure carbon dioxide, and in 50% carbon dioxide no toxic effect due to carbon dioxide was detected.

Hence, the writer suggests the following method for maintaining stock cultures of yeast. To young slant cultures at least 50% carbon dioxide gas is introduced. The slants are then sealed, and stored at a low temperature. This interesting possibility is now under investigation. Obviously, critical judgment of it must be withheld for some time--in all probability for several years.

SUMMARY

The effects on ascospore formation of certain environmental factors were studied. These factors included cell concentration, gaseous environment, pressure, light, and temperature.

Ascospore formation occurred over a wide range of cell concentrations. While no upper limit for sporulation was evident from these studies, ascospore formation occurred more profusely on acetate-dextrose agar when moderately heavy cell concentrations were employed.

Carbon dioxide was found to suppress ascospore formation. In a concentration of 50% or more, no ascospores were obtained. Small amounts of oxygen greatly stimulated ascospore formation while very high concentrations suppressed it to some extent. Oxygen was not able to overcome the inhibitive effect of carbon dioxide.

Under reduced pressure yields of ascospores were decreased.

Alcohol vapours suppressed sporulation possibly through toxicity.

Diffused light was seen to exert only a minor depressing effect on ascospore formation.

Extremes of temperature were seen to depress the yield of ascospores. A relatively sharp maximum temperature seems to exist, while the minimum temperature is less precise. The latter is dependent to some extent upon the time of incubation and the particular strain of yeast under study.

A wide variation in yields of ascospores was observed among 43 yeast cultures from various sources. Variation was also noted among isolates from a commercial package of yeast as well as from isolates obtained from a single isolate culture. In the latter two instances this variation was less marked. With the 43 cultures mentioned above, the superiority of acetate-dextrose agar over two other sporulation media was demonstrated.

A method for hybridization which may prove of some value industrially in the development of new or improved strains of yeast is described.

In addition, applying the findings of these studies on gaseous environment and temperature, a method of maintaining stock cultures in a non-segregating condition is proposed.

ACKNOWLEDGMENTS

The writer wishes to thank Dr. J. J. Miller for many helpful discussions and advice, and also Dr. N. W. Radforth, for his interest throughout the course of these studies. Thanks

are due also to Dr. M. L. Elder, Canadian Canners Ltd., Hamilton, Ontario, who loaned manuscripts and publications relating to her work and that of Dr. Stantial. Financial support and laboratory facilities made available by the Horticultural Experiment Station, Vineland Station, Ontario, for a portion of this work are gratefully acknowledged.

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